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# Hybrid Adult Neuron Culture Systems for Use in Pharmacological Testing

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# HYBRID ADULT NEURON CULTURE SYSTEMS FOR USE IN PHARMACOLOGICAL TESTING

by

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# ABSTRACT

Neuronal culture systems have many applications, such as basic research into neuronal structure, function, and connectivity as well as research into diseases, conditions, and injuries affecting the brain and its components. In vitro dissociated neuronal systems have typically been derived from embryonic brain tissue, most commonly from the hippocampus of E18 rats. This practice has been motivated by difficulties in supporting regeneration, functional recovery and long-term survival of adult neurons in vitro. The overall focus of this dissertation research was to develop a dissociated neuronal culture system from human and animal adult brain tissue, one more functionally and developmentally correlative to the mature brain. To that end, this work was divided into five interrelated topics: development of an adult in vitro neuronal culture system comprised of electrically functional, mitotically stable, developmentally mature neurons from the hippocampus of adult rats; creation of stable two-cell neuronal networks for the study of synaptic communication in vitro; coupling of electrically active adult neurons to microelectrode arrays for high-throughput data collection and analysis; identification of inadequacies in embryonic neuronal culture systems and proving that adult neuronal culture systems were not deficient in similar areas; augmentation of the rat hippocampal culture system to allow for the culture and maintenance of electrically active human neurons for months in vitro. The overall hypothesis for this dissertation project was that tissue engineered in vitro systems comprised of neurons dissociated from mature adult brain tissue could be developed using microfabrication, defined medium formulations, optimized culture and maintenance parameters, and cell-cycle control.

Mature differentiated glutamatergic neurons were extracted from hippocampal brain tissue and processed to purify neurons and remove tissue debris. Terminally differentiated rat hippocampal neurons recovered in vitro and displayed mature neuronal morphology. Extracellular glutamate in the culture medium promoted neuronal recovery of electrical function and activity. After recovery, essential growth factors in the culture medium caused adult neurons to reenter the cell cycle and divide multiple times. Only after reaching confluence did some neurons stop dividing. Strategies for inhibition of neuronal mitotic division were investigated, and manipulation of the cdk5 pathway was ultimately found to prevent division in vitro. Prevention of mitotic division as well as optimization of culture and maintenance parameters resulted in a neuronal culture system derived from adult rats in which the neuronal morphology, cytoskeleton and surface protein expression patterns, and electrical activity closely mirrored mature, terminally differentiated adult neurons in vivo. Improvements were also made to the growth surfaces on which neurons attached, regenerated, and survived long-term. Culture surfaces, in this case glass cover slips, were modified with the chemical substrate N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine (DETA) to create a covalently modified interface with exposed cell-adhesive triamine groups. DETA chemical surfaces were also further modified to create high-resolution patterns, useful in creating engineered two-cell networks of adult hippocampal neurons. Adult hippocampal neurons were also coupled to microelectrode array systems (MEAs) and recovered functionally, fired spontaneously, and reacted to synaptic antagonists in a manner consistent to adult neurons in vivo. Last, neurons from the brains of deceased Alzheimer's disease (AD) patients and from brain tissue excised during surgery for

Parkinson's disease (PD), Essential Tremor (ET), and brain tumor were isolated and cultured, with these neurons morphological regenerating and electrically recovering in vitro.

# ACKNOWLEDGMENTS

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# CHAPTER 1 - GENERAL INTRODUCTION

The field of neuroscience is an interdisciplinary endeavor that is devoted to the scientific study of the nervous system. The nervous system is composed of networks of neurons, supporting cells (i.e. glial cells), and other tissue types. The formation of functional *in vitro* circuits from neurons allows studies to be performed at both the systems and cognitive level as well as at the molecular and cellular level [1], both of which are still understood poorly. Overall, the goals of biologists, chemists, physicists, psychologists, medical doctors, computer scientists, engineers, and mathematicians that make up the field fall into three main categories: "To describe the human brain and how it functions, to determine how the nervous system develops, matures, and maintains itself through life, and to find ways to prevent or cure many devastating neurological and psychiatric conditions [2]." Eric Kandel stated the task of neural science "is to explain behavior in terms of the activities of the brain. How does the brain marshal its millions of individual nerve cells to produce behavior, and how are these cells influenced by the environment...? The last frontier of the biological sciences—their ultimate challenge—is to understand the biological basis of consciousness and the mental processes by which we perceive, act, learn, and remember" [1].

At the molecular, cellular, and systems level, neuroscience research investigations are reliant upon the existence of accurate systems that mimic the human brain and/or conditions found in the human brain. Commonly, however, researchers have been forced to rely upon abstract models that have flaws or inadequacies due to either the complexity of the brain or the complex nature of most neurological conditions, injuries, or diseases. Human-based model systems for neurodegenerative diseases

such as AD and for central nervous system (CNS) injury such as traumatic brain injury (TBI) are limited, and this limitation has been a major bottleneck in translating basic science into clinical applications and therapeutic discoveries. The ideal research model, from a purely experimental standpoint, would be to utilize human volunteers in experiments in order to understand various diseases and injuries [3]. However, from an ethical standpoint this is impossible in any situation where damage would knowingly or potentially occur to the human subject. Thus, over most of the last century drug development and research aimed at solving clinical neuropathologies has been limited to observational studies or studies utilizing live animals, brain slices, or single cells from animal/human systems. The main problems with these approaches are that in most cases research using animal models does not translate well to the human condition and more applicable functional *in vitro* model systems derived from adult and/or human tissue are limited.

Rat and mouse disease models mimicking pathological symptoms of human neurological and neurodegenerative conditions are widely used in research to understand their onset and progression as well as in testing of drugs and therapies in their reversal [4-6]. Hippocampal slice cultures, where thin slices of intact hippocampal tissue are removed from adult brain tissue, are most widely used for electrophysiological studies into synaptogenesis, neuronal communication, long-term potentiation (LTP) and plasticity, and pathophysiology of brain disease [7-10]. During the last decade several functional *in vitro* systems have been developed to study CNS and peripheral nervous system (PNS) disorders using embryonic and fetal rat and mouse tissues, with studies of communication in dissociated cultures typically relying

upon hippocampal neurons extracted from the brains of embryonic rat / mouse embryos [11-22]. Some work has been performed on the refinement of *in vitro* dissociated neuronal systems to use adult brain tissue rather than embryonic brain tissue [11, 12]. While these systems supported the morphological recovery of adult hippocampal neurons *in vitro*, issues with support of long term survival and full recovery of electrical activity of neurons in these culture systems has prevented its widespread use as a research tool [4, 23, 24]. Additional studies have been conducted to develop short-term culture systems using human neurons, which while partially successful in supporting short-term survival of dissociated human neurons or neural progenitors, however the neurons were not evaluated for basic electrical functionality [25-27].

The creation of functional *in vitro* systems has become a great priority not just for neuroscientists but for researchers in general. The economics of scientific research has driven the need for more efficient research mediums, and the simplicity of *in vitro* systems allowed quick gathering and interpretation of results [28]. Recent problems with drugs or therapies that have shown potential in rat or mice studies, before failing in human trials, highlights the need for more correlative research systems. Finding and creating research models that more closely relate to the adult human brain has the potential to lower research costs, decrease the duration of research projects, and increase the chances of finding beneficial treatments and therapies for neurological and neurodegenerative conditions [29].

This dissertation research focuses on the development of a dissociated neuronal culture system from mature brain tissue of adult rats, improving upon existing embryonic and adult neuronal culture systems [11-22, 30] to create an *in vitro* model that is more

functionally and developmentally correlative to neurons in the mature adult brain. To that end, this work has been divided into four interrelated topics: development of an adult *in vitro* neuronal culture system comprised of electrically functional, mitotically stable, developmentally mature neurons from the hippocampus of adult rats; creation of stable two-cell neuronal networks for the study of synaptic communication *in vitro*; coupling of electrically active adult neurons to microelectrode arrays for high-throughput data collection and analysis; extending the rat hippocampal culture system to allow for the culture and maintenance of electrically active human neurons for months *in vitro*. The overall hypothesis for this dissertation project is that tissue engineered *in vitro* systems comprised of neurons dissociated from mature adult brain tissue can be developed using microfabrication, defined medium formulations and optimized culture and maintenance parameters.

The importance of the hippocampus in both memory formation and in the manifestation and progression of neurodegenerative diseases points toward its usefulness as a research tool into both basic brain research as well as neurodegenerative conditions. The hippocampus is considered a major component of the brain of mammals, playing important roles in the consolidation of information from short-term to long-term memory and spatial navigation [7, 31-33]. During AD, the damage suffered by neurons in the hippocampus manifests in the memory problems and disorientation exhibited by sufferers of the disease [34]. Memory formation in the hippocampus is a function of electrical transmission of signals from one neuron to another, in the process forming new synaptic connections between neurons, strengthening existing synaptic connections, and modifying synaptic parameters

through LTP or long-term depression (LTD). Synaptic connections between neurons in the hippocampus can be considered either inhibitory or excitatory. In excitatory synapses, the neurotransmitter glutamate conveys fast excitatory neurotransmission, primarily acting via the activation of ionotropic and metabotropic receptors [35]. Excitatory neurons in the hippocampus release the neurotransmitter glutamate into the synaptic cleft which binds to ligand-gated ion channels in the postsynaptic membrane, producing an influx of ions to create an excitatory postsynaptic potential (EPSP) [36-38]. If the EPSP is strong enough to reach the threshold potential, an action potential will be triggered in the postsynaptic cell [39]. In addition, activation of these receptors plays a major role in neuronal differentiation, CNS development, LTP, and memory formation *in vivo* [35, 40, 41]. At very high levels, glutamate causes excitotoxicity, where activation of ion channels leads to the influx of toxic levels of calcium into the neuron [11, 42, 43].

*In vitro* dissociated neuronal culture can model the various functions and capacities of the hippocampus and hippocampal neurons in the brain. The goal of developing functional *in vitro* neuronal systems is to produce long-term culture systems that support recovery of electrical activity of neurons in a controlled environment that is also capable of reproducing disease states. Most commonly, embryonic hippocampal neurons extracted from the brains of embryonic rat or mouse embryos have been used to model adult brain systems [11-22]. Embryonic neurons have been used because of a number of factors, but mainly because of the following:

1. Extraction and processing of E18 rat hippocampal tissue yields a homogeneous neuronal culture system.

2. Lack of tissue debris after the dissociation of the neuronal tissue allows for cell counting and deposition of specific densities of neurons onto culture surfaces.

3. Embryonic neurons are electrically active *in vitro.*

4. Synaptic connections form between embryonic neurons *in vitro.*

5. Embryonic neurons survive *in vitro* for periods of time long enough for the completion of scientific investigations.

Conversely, utilization of adult tissue as the source for neurons for dissociated culture systems, while most accurate in reflecting the properties of neurons *in vivo*, has been limited [11, 12, 14, 42, 44, 45]. Many issues exist in the creation of adult neuronal culture systems:

1. Difficulty in limiting damage to neurons during the dissociation of adult brain tissue

2. Presence of cell debris after dissociation of adult neuronal tissue, debris which has previously been shown to be inhibitory to neuronal viability and neurite regeneration [46-49]. This debris contains nerve-growth inhibiting factors that act as inhibitors of axonal growth and cell survival. Three distinct CNS myelin proteins inhibit axon regeneration by binding to the Nogo66 receptor (NgR): Myelin-associated glycoprotein (MAG), Nogo-A, and Oligodendrocyte-myelin glycoprotein (Omgp). Chondrotin sulfate proteoglycan (CSPG), arretin, and tenascin have also been associated with the inhibition of regeneration

3. Presence of tissue debris after the dissociation of the neuronal tissue prevents cell counting and deposition of specific densities of neurons onto culture surfaces.

4. Difficulty in supporting *in vitro* survival and physical regeneration of adult neurons

5. Difficulty in achieving *in vitro* functional electrical recovery of adult neurons [14, 50]

6. *In vitro*, post mitotic adult neurons revert to a proliferative state due to the action of the essential culture medium factor basic fibroblast growth factor (bFGF) [51]. The factor bFGF promotes GABA-negative neurons to survive by influencing both glucosyceramide synthesis and the voltage-dependent calcium channels (VDCCs) [51, 52].  It also up-regulates cyclin-dependent kinase 5 (cdk5) expression, triggering neuronal re-entry into the cell cycle [51, 53].  Cdk5, while generally expressed in neurons in the cdk5/p35 complex, is normally expressed by neurons at levels that exert an influence on neurite outgrowth and migration but not proliferation [53-55].  Removal of bFGF from culture medium causes death of adult neurons *in vitro.*

7. Synaptic connectivity of adult neurons has not been achieved *in vitro*.

8. Medium formulations, while providing trophic support for adult neurons for 1-2 weeks *in vitro*, have not consistently supported long-term survival of these neurons.

In the hippocampus of embryonic rats, neural progenitors differentiate into neurons between E15-E18 [56-58].  While these are differentiated neurons, they are developmentally immature, with transcriptional profiling identify two-thirds of genes are only expressed postnatal and with >95% of expressed genes showing highly significant changes during postnatal development [57].  When examining the machinery responsible for synaptic transmission and EPSP, gene expression for the NMDA

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channel subunits NR2A and NR2B is not detected until near birth on E21-22, with expression not peaking until P20 [59]. *In vitro*, NR2A/B channels are detected at only very low levels until after 2 weeks in embryonically derived neurons [60]. AMPA channel expression is also limited at birth, only increasing postnatal [61]. Gene expression for the axonal sodium transporter subunit 1 begins around P15, increasing until P30 [62, 63]. *In vitro* and *in vivo*, expression patterns for all genes, specifically axonal and synaptic channels responsible for signal transmission, show significant changes over the course of the first few weeks after birth [58-60, 62, 64, 65]. The usefulness of these developmentally immature neurons for studies of neuronal electrical activity and synaptic transmission is limited by this lack of or limited expression of the neuronal machinery responsible for electrical transmission in the adult brain. In addition, using immature neurons in studies of neurodegenerative diseases or drug discovery can yield results that at times cannot be correlated to the function or action of mature neurons in adult brain tissue. Conversely, an adult hippocampal culture system could be used to study the function of neurons, neuronal interactions, aging and neurodegenerative disease from a new perspective where the essential ion channels, receptors and other cellular components had matured in these neurons *in vivo* [66].

This dissertation research has attempted to solve some of the difficulties associated with culturing adult neurons in dissociated cell culture conditions. The dissection and dissociation of the tissue, the culture process, the cellular attachment substrate DETA, the use of anti-apoptotic and oxidative molecules Trolox ® and cerium oxide nanoparticles [67, 68], and the defined serum-free media used during dissection, dissociation, plating, and long-term maintenance of adult hippocampal neurons were all

optimized to try to maximize the number of cells that attached, survived, and regenerated *in vitro.* Mature differentiated glutamatergic neurons were extracted from hippocampal brain tissue and processed to purify the neurons and remove tissue debris. Terminally differentiated rat hippocampal neurons recovered *in vitro* and displayed mature neuronal morphology. After recovery, adult neurons returned to the cell cycle and divided multiple times. During each mitotic division, the neuron retracted their neurites and divided, and each post-mitotic neuron quickly re-extended their axons and dendrites. This neuronal cell division cycle was repeated every 24-48 hours, a period also marked by limited neuronal electrical activity [69]. Only after the neurons reached confluence did some neurons stop dividing, and neurons were stimulated to recover *in vivo*-like electrical activity through the introduction of glutamate to the maintenance medium of the culture. Strategies for inhibition of neuronal mitotic division were investigated, and roscovitine, a purine analog that is a potent and selective inhibitor of cdk5 [33, 70], was identified as an effective agent in the prevention of bFGF triggered division *in vitro.* Prevention of mitotic division as well as optimization of culture and maintenance parameters resulted in a neuronal culture system derived from adult rats in which the morphology, cytoskeleton and surface protein expression patterns, and electrical activity closely mirror neurons *in vivo.* Maturity of neurons in dissociated cell culture is not only a function of the maturity of the original tissue source but also in the expression patterns of receptors, receptor subunits, and structural proteins. Neurons expressed synaptophysin, the presynaptic vesicle glycoprotein used to quantify synapses [71]. The NMDA and AMPA channel subunits NR2A, NR2B, and GluR2/3, the distinctive postsynaptic ligand-gated ion channels that control EPSP [72-75], were

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all present in adult hippocampal neurons in this culture system. These and other improvements have allowed electrically active, developmentally mature adult neurons to survive for several months *in vitro*, providing a stable system with potential for a wide range of applications.

Improvements were also made to the growth surfaces on which neurons attach, regenerate, and survive long-term. Culture surfaces, in this case glass cover slips, were modified with the chemical substrate DETA to create a covalently modified interface with exposed cell-adhesive amine groups that has been shown to be stable for long periods in culture. This stability *in vitro* contrasts to poly-D-lysine (PDL) and poly-ornithine which are physisorbed. DETA has been previously shown to be superior in the promotion of attachment, regeneration and long-term survival of embryonic neurons *in vitro* [30, 44, 76-78]. In addition, DETA, with its triamine functional group exposed at the surface, strongly attaches to neurons and allows for all non-neuronal debris such as extracellular matrix (ECM) proteins, myelin debris, and cell fragments to be washed from the cover slip surface. DETA chemical surfaces were also further modified to create high-resolution patterns, which could be useful for the creation of engineered two-cell networks of these adult hippocampal neurons [78, 79]. Adult hippocampal neurons were also able to be coupled to MEAs, recovering functionally, firing spontaneously, and reacting to synaptic antagonists in a manner consistent to connected neurons *in vivo*. Last, we were also able to isolate and culture neurons from the brains of deceased Alzheimer's disease patients as well as from brain tissue excised during surgery for Parkinson's disease, Essential Tremor, and brain tumor, with these neurons morphological regenerating and electrically recovering *in vitro*.

This novel neuronal culture system of synaptically connected excitatory neurons derived from adult brain tissue has many potential uses.  Such a system could be used to study the function of neurons, neuronal interactions, aging and neurodegenerative disease from a new perspective where the different ion channels, receptors and other cellular components matured *in vivo* instead of *in vitro* [66]. This system provides a unique tool that can be used for studies into LTP [66, 80, 81].  It also would be useful in high-throughput drug studies, neurocomputing and biorobotics [5, 6, 23, 24], and has a unique application to studying regeneration of injured adult neurons, especially in TBI. Additionally, targeted and reversible induction of mitotic activity in neurons *in vivo* has great potential as a therapeutic intervention for late-stage neurodegenerative disease, such as Alzheimer's disease or TBI [33, 82, 83].

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# CHAPTER 2 - ADDITION OF GLUTAMATE TO SERUM FREE CULTURE PROMOTES RECOVERY OF ELECTRICAL ACTIVITY IN ADULT HIPPOCAMPAL NEURONS IN VITRO

## Introduction

As people live longer, neurological disorders and diseases that affect the aging brain have become more common, however success to date for reversing these diseases has been limited. One of the reasons this research has been hindered has been the flawed, limited, or non-existent research models of aged or diseased human brain tissue. Neuronal cell culture, one of the most commonly used research models, is generally derived from embryonic rat or mouse tissue. Although this system has been used effectively in many studies, a neuronal culture system derived from adult brain tissue could be a much more relevant and effective model system. Such a system could be used to study the function of neurons, neuronal interactions, aging and neurodegenerative disease but from a new perspective where the different ion channels, receptors and other cellular components matured in vivo instead of in vitro [1]. It also would be useful in drug studies, neuroprosthetic devices, neurocomputing and biorobotics for the same rational [2-4], and has a unique application to studying regeneration of injured adult neurons, especially in traumatic brain injury (TBI). These applications were the goal behind an earlier attempt at creating an adult hippocampal culture system, developed using a serum-free culture media with a biological surface [5]. While this system supported the morphological recovery of adult hippocampal neurons *in vitro*, issues with the support of both long-term survival and full recovery of electrical activity of neurons in this culture system appears to have prevented its widespread use as a research tool [3, 4, 6].

To overcome these issues, modifications were made to a defined neuronal culture system previously developed for embryonic hippocampal neurons [7]. First, culture surfaces were modified with the chemical substrate DETA, creating a covalently modified interface with exposed cell-adhesive amine groups, which has previously been shown to promote the attachment, regeneration and long-term survival of embryonic neurons *in vitro* [7-11]. Second, a neurotransmitter was added to the adult hippocampal culture media, an approach found to successfully trigger electrophysiological recovery in cultured adult spinal cord neurons [12]. This neurotransmitter, glutamate, conveys fast excitatory neurotransmission *in vivo*, primarily acting via the activation of ionotropic and metabotropic receptors [13]. In addition, activation of these receptors plays a major role in neuronal differentiation, CNS development, long-term potentiation and memory formation *in vivo* [13-15]. In most embryonic hippocampal cultures, microMolar concentrations of glutamate are incorporated into the culture media in order to replicate these effects *in vitro* [16-18]. However, issues with excitotoxicity led to the removal of glutamate from the media gradually beginning at day 4 in earlier attempts to culture adult hippocampal neurons [5, 19, 20]. Our hypothesis was that, lacking this vital neurotransmitter, the adult neurons *in vitro* could not fully recover the characteristic electrical activity found for neurons *in vivo* in the previous system.

In this study, the earlier adult hippocampal cell culture technique was modified to include silane-modified DETA surfaces and the application of 25 $\mu$M glutamate for 1 to 7 days, which was introduced after 21 days *in vitro (div)* to minimize excitotoxicity. These changes promoted long-term neuronal survival and full recovery of electrical activit*y*,

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providing a system for studying neurodegenerative diseases and disorders such as Alzheimer's disease and especially neuroregeneration of injured adult tissue.

**Materials and Methods**

*Surface Modification of the Cover Slips*

Glass cover slips (Thomas Scientific 6661F52, 22 x 22 mm$^2$ no. 1) were cleaned by acid washing using a 50/50 mixture of concentrated hydrochloric acid and methanol. The cover slips were washed three times, 30 minutes per wash, and were rinsed in distilled de-ionized water between each washing. The DETA (N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine, United Chemical Technologies Inc., Bristol, PA, T2910KG) monolayer was formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904) [9]. The DETA-coated cover slips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. The DETA formed a reaction site limited monolayer on the surface of the cover slip [9].

*Surface Characterization of the Cover Clips after DETA Monolayer Formation*

The DETA cover slips were characterized to authenticate the monolayer formation. First, contact angle measurements were taken using an optical contact angle goniometer (KSV Instruments, Monroe, CT, Cam 200). The contact angle for the DETA-coated cover slips was 54.2 +/- 0.2, which was previously shown to be acceptable for neuronal hippocampal culture [9]. Second, X-ray Photoelectron Spectroscopy (XPS) (FISONS ESCALab 220i-XL) was used to characterize the elemental and chemical state of the DETA-coated cover slip surfaces. The XPS survey

scans as well as high-resolution N 1s and C 1s scans, using monochromatic Al K$\alpha$ excitation, were obtained, similar to previously reported results [9, 21, 22].


*Isolation and Culture of Adult Hippocampal Neurons*

Hippocampi were removed from 3-6 month-old Sprague Dawley rats, which were purchased from Charles River. Hippocampal cells were extracted and isolated based upon a previously described method [5]. In brief, adult rats were euthanized by exposure to $CO_2$ according to practices that adhered to IACUC policies and the hippocampal regions of the brain were removed. The hippocampi were sliced into small pieces, collected in a mixture of Hibernate A (http://www.brainbitsllc.com) and an antibiotic/antimycotic (Ab/Am, Invitrogen, 15240-062), and enzymatically digested in a papain solution (2 mg/ml Hibernate A) (Worthington, LS003119). Next, the tissue was triturated in 6 ml of fresh Hibernate A - Ab/Am to dissociate the tissue into a cell suspension. This 6 ml cell suspension was then layered over a 4 ml step gradient (Optiprep diluted 0.505:0.495 [v/v] with Hibernate A-Ab/Am and made to 15, 20, 25 and 35% [v/v] in Hibernate A-Ab/Am) and centrifuged at 800 $\times$ g for 15 minutes, 4ºC. Hippocampal neurons were collected from the second and third layers within the gradient. These layers were collected, diluted with 5 ml of fresh Hibernate A-Ab/Am and centrifuged at 500 x g for 7 minutes. The supernatant was removed and the cell pellet resuspended in culture media, composed of Neurobasal A (Gibco, 10888), B27 supplement (Invitrogen, 17504-044), glutamax (Invitrogen, 35050-061), antibiotic/antimycotic (Invitrogen, 15240-062) and basic FGF (5 ng/ml, Invitrogen, 13256-029). 500 $\mu$l of the cell suspension was applied to each cover slip for 1 hour, the

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cells adhered during this time, and then an additional 2 ml of media was added to each cover slip.   After four days, the existing media was replaced by fresh culture media. Thereafter, every four days half the media was removed and replaced with fresh media. Remaining cultures were discarded after 90 days.

*Application of Glutamate*

This study utilized the excitatory neurotransmitter glutamate, *N*-Acetyl-*L*-glutamic acid (Sigma, 855642), which is essential for normal brain function [23]. It was applied at different dosages, where the initial concentrations were derived from previous glutamate excitotoxicity studies [14, 17, 18].   Glutamate doses, 10 $\mu$M (*Group G21-10*), 25 $\mu$M (*Group G21-25*) and 100 $\mu$M (*Group G21-100*), were separately applied to adult hippocampal neurons after 21 *div.*  After incubating for different time periods, neuronal viability and electrical activity were evaluated and compared to cultured neurons not exposed to glutamate.   Short-, medium-, and long-term effects from the application of 25 µM glutamate to adult hippocampal neurons 21 *div* were evaluated after 1 hr (*Group G21-1h)*, 1 day (*Group G21-1d*), 7 days (*Group G21-7d*) and 14 days (*Group G21-14d*). The optimal parameter for *in vitro* glutamate application was used to comprehensively study the effect of this neurotransmitter on neurons cultured for different periods.  25 $\mu$M glutamate was added to neurons 14 *div* (*Group G14-25*), 21 *div* (*Group G21-25*), 31 *div* (*Group G31-25*) and 38 *div* (*Group G38-25*).  After 24 hours, neuronal electrical activity and viability were evaluated.  Control cultures, where glutamate was not applied, were also evaluated after 14 *div* (*Group C14*), 21 *div* (*Group C21*), and 31 *div* (*Group C31*). The neurotransmitter solution was freshly prepared for each experiment.    The

underlying mechanism behind the glutamate-mediated improvement in neuronal electrical properties was investigated through the use of cycloheximide (CHX, Sigma, C4859), which is a protein synthesis inhibitor shown to block preconditioning in neurons [24]. CHX was first applied at concentrations of 10 $\mu$g/ml (*Group CHX10*), 20 $\mu$g/ml (*Group CHX20*), and 80 $\mu$g/ml (*Group CHX80*) to neurons 21 *div*, with cell viability in each group assessed after 24 hours. Next, 20 $\mu$g/ml CHX was applied to neurons 21 *div* for 1 hour before 25 $\mu$M glutamate was introduced (*Group G21CHX20*). The neurons were evaluated for changes in viability and electrical properties after 1 day.

*Immunocytochemistry*

Cover slips were rinsed with 1X PBS two times. Cells on these cover slips were fixed with 10% glacial acetic acid and 90% ethanol for 20 minutes at room temperature. Cover slips were again rinsed 3 times with 1X PBS. Cells were permeabilized for 5 min with a permeabilizing solution (5 mM Lysine + 0.5% Triton X-100 + 100 ml of 1X PBS), and were then blocked for 2 hr (5% normal donkey serum in permeabilizing solution). Anti-neurofilament M polyclonal antibody (Chemicon, AB1981, diluted 1:1000), anti-MAP 2A/2B (MAB364, Chemicon, diluted 1:1000), anti-nestin (MAB5326, Chemicon, diluted 1:1000) and anti-GFAP monoclonal antibody (MAB360, Chemicon, diluted 1:400) were added in blocking solution for 12 hr at 4ºC. After 3X washes with 1X PBS, fluorescently labeled secondary antibodies were applied for 2 hours. Vectashield mounting medium (H1000, Vector Laboratories, Burlingame, CA) was used to mount the cover slips onto slides. The cover slips were observed and photographed with an Ultra VIEW$^{TM}$ LCI confocal imaging system (Perkin Elmer).

*Electrophysiology*

Whole-cell, patch-clamp recordings were performed in a recording chamber that was placed on the stage of a Zeiss Axioscope, 2 FS Plus, upright microscope in Neurobasal culture medium (pH was adjusted to 7.3 with *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid [HEPES]) at room temperature. Patch pipettes (6-8 M$\Omega$) were filled with intracellular solution (K-gluconate 140 mM, ethylene glycol-bis[aminoethylether]-tetraacetic acid 1 mM, MgCl$_2$ 2 mM, Na$_2$ATP 5 mM, HEPES 10 mM; pH 7.2). Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (Axon, Union City, CA) amplifier. Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recordings and analysis were performed with pClamp 8 (Axon) software. Inward currents that had the characteristics of fast sodium currents (ISCs), and outward currents that had the characteristics of potassium currents (OPCs) were measured in voltage clamp mode using voltage steps of 10 mV from a –70 mV holding potential. Whole-cell capacitance and series resistance were compensated and a p/6 protocol was used. The access resistance was less than 22 M$\Omega$ and tight seals were measured to be above 1 G$\Omega$. Action potentials (APs) were measured with 1 s depolarizing current injections from the – 70 mV holding potential.

Selection of cells for electrophysiological characterization was based upon morphology. Phase bright pyramidal neurons with large branching apical dendrites and small basal dendrites were selected. Cells with this morphology stained positive for neurofilament and negative for GFAP. Neurons cultured for 14, 21, 28 and 38 days

were electrically characterized for evaluation of glutamate-mediated electrical recovery. Additionally, neurons cultured between 1-14 *div* and 39-87 *div* were electrically characterized to evaluate recovery and maintenance of electrical activity.

*Cell Survival Study*

Survival of the cultured adult hippocampal neurons was evaluated following the addition of glutamate (10, 25, 100 $\mu$M), cycloheximide (10, 20, 80 $\mu$g/ml), or simultaneous addition of 25 $\mu$M glutamate and 20 $\mu$g cycloheximide. In the first method, the total number of neurons on each cover slip was approximated both before and after the application of each factor(s). Using a phase contrast microscope at 20X magnification, neuronal cell counts were made from 20 random spots on each cover slip, an area equal to 4% of the total area of the cover slip (22 $\times$ 22 mm). The total number of living neurons on each cover slip was mathematically determined. In the second method, the number of living versus dead neurons was quantified using a Live/Dead Assay kit (Molecular Probe, L-3224). The percentage of living neurons on each cover slips was calculated from total cells counted, both living and dead. Cell counts were made using a phase-contrast microscope with epiflourescent light source. Total cells were approximated after cells, both living and dead, were counted on 20 randomly selected spots at 20X magnification. Neurons *in vitro* were also morphologically evaluated throughout the culture period.

*Statistical Analysis*

Statistical analysis included performing a two-sample *t*-test on the morphological, immunocytochemical, live/dead assay and electrophysiological data.   Parameters obtained from the DETA-plated adult hippocampal neurons treated with glutamate ± cycloheximide were compared with DETA-plated hippocampal control neurons which were not treated.   Numerical summary results are reported as a mean, plus or minus the sample standard error of the mean (± SEM).   For two-group comparisons, the student's *t*-test was used.

**Results**

*Regeneration of Adult Hippocampal Neurons with Limited Recovery of Electrical Activity in a Defined System*

After deposition onto DETA surfaces, the adult hippocampal neurons adhered to the surface within 60 minutes (Figure 2-1 A).  Immediately following attachment, most neurons displayed characteristics indicative of recovery from the trauma of cell culture, with some regeneration occurring in all viable neurons by the end of the initial 24 hours.  After 2 *div*, recovery of axonal and dendritic processes was clearly visible (Figure 2-1 B, C) and by 14 *div* the vast majority of adult neurons had typical neuronal morphology (Figure 2-1 B, C), which was maintained up to 80 *div* (Figure 2-1, D-I).  These adult neurons exhibited phase-bright, smooth-appearing somas, with one or more small dendrites and a large apical dendrite with second-order dendritic branching.  While few non-neuronal cells were present after 14 *div*, a small population of glial cells (astrocytes, oligodendrocytes, microglia) remained for the duration of the cultures.  Terminally differentiated, mature neurons were distinguished from immature neurons and progenitor cells through immunocytochemical analysis with antibodies against MAP-2, a protein specific to mature neurons [25], and nestin, a protein found specifically in immature and progenitor cell populations [26].  During the first 14 *div*, recovering adult neurons displayed MAP2 as well as nestin expression, possibly pointing to the regression of mature neurons to a more immature phenotype during regeneration.  After 14 *div*, nestin expression was not observed. The majority of the cells after 21 *div* and 38 *div* (90% and 94% respectively) displayed expression of the neuronal structural proteins neurofilament and/or MAP-2, while the remaining cells expressed the glial-specific structural protein GFAP (Figure 2-1 J, K, L).  The low percentage of non-neuronal cells

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may be attributed to the poor support of glial growth and survival provided by the Neurobasal-A/B27 media [5].

In a previous study of *in vitro* adult neuronal electrical properties, both embryonic and adult hippocampal neurons were evaluated based upon recovery of electrical activity and the ability to fire action potentials [27]. The embryonic hippocampal neurons were able to fire action potentials in approximately 84% of the neurons studied at 14 *div* in this earlier work. However, the percentage of neurons obtained from the hippocampus of adult rats that were able to fire action potentials was much lower, between 25% and 55%. In the current study, whole-cell patch-clamp experiments were used to evaluate *in vitro* electrical activity of cultured adult hippocampal neurons using the same conditions and the results obtained were similar to those found in the previous study. Additionally, adult neurons were selected for 4 different periods for the initial electrical property study: 14 *div* (*Group C14*), 21 *div* (*Group C21*), 28 *div* (*Group C28*) and 38 *div* (*Group C38*). The current flow (inward and outward) in these control populations of neurons was limited or non-existent in many cases. The number of neurons with induced inward sodium currents ranged from 38.5% (*Group C14*) to 53.8% (*Group C38*), while the number with induced outward currents ranged from 38.5% (*Group C14*) to 76.9% (*Group C38*) (Table 2-1, rows 1-4). The percentage of these neurons that fired action potentials was similar to the previously reported results (Table 2-1, rows 1-4: 23.1% to 38.5%).

*Lower Concentrations of Glutamate Trigger Excitotoxicity in Recovering Neurons versus Morphologically Recovered Neurons in Vitro*

To test our theory that neurotransmitter treatment enables functional recovery of injured neuronal cells, adult hippocampal neurons were challenged with various concentrations of glutamate during the following periods: 1) recovery, during which the neurons recovered from the trauma of cell culture *(0-3 div)*, 2) regeneration, during which morphology characteristic of *in vivo* neurons, was recovered *(3-14 div)*, and 3) after long-term survival *(14-x div)*. 10 $\mu$M, 25 $\mu$M or 100 $\mu$M aliquots of glutamate were added to the adult neuronal culture medium during each of these culture periods, and the effect upon neuronal health and viability was assessed after 1 hr, 1 day, and 4 days. Excitotoxicity caused by each concentration of glutamate *(10 $\mu$M, 25 $\mu$M, 100 $\mu$M)* and application period *(1 h, 1 d, 4 d)* was measured, as well as on the *in vitro* neuronal viability during each culture period (*0-3 div, 3-14 div, 14-x div)*.

The different concentrations of glutamate evoked significantly different levels of excitotoxicity in the neurons for each culture period examined. When glutamate was added to the culture media after the initial 1 h plating period (*0-3 div)*, even low glutamate concentrations were strongly excitotoxic; damaging and killing the majority of the cultured neurons within 1 day. During the regeneration period *(3-14 div)*, although a longer incubation period was required, glutamate damaged or killed the majority of neurons within 4 days. However, in the third period *(14-x div),* minimal excitotoxicity was observed after the adult neurons were incubated with low concentrations of glutamate (10 $\mu$M, 25 $\mu$M). Higher concentrations of glutamate ($\geq$ 100 $\mu$M) remained significantly excitotoxic.

In the next step of the investigation, glutamate was applied after the cultured neurons appeared to be completely regenerated morphologically in the culture system.

First, adult hippocampal neurons 21 *div* were incubated with 25 $\mu$M glutamate for 1 h, 1 d, 7 d, and 14 d. Short-term application of glutamate was found to cause very little excitotoxicity in these neurons, while long-term incubation of glutamate provoked excitotoxicity to a greater degree (Table 2-1). Next, 21 *div* adult hippocampal neurons were incubated for 1 d with 10 $\mu$M, 25 $\mu$M or 100 $\mu$M glutamate. Glutamate at concentrations of 10 $\mu$M and 25 $\mu$M was minimally excitotoxic, causing very little damage and death in these neurons. However, at 100 $\mu$M, glutamate evoked significant neuronal death, with only 69% of the neurons surviving after the incubation period (Table 2-1). Finally, 14, 21, 31, and 38 *div* adult hippocampal neurons were incubated with 25 $\mu$M glutamate for 1 day. A small percentage of neurons in each of these cultures died in response to glutamate, with toxicity the same in each culture. Overall, glutamate at low $\mu$M concentrations, and with a short incubation time, was minimally excitotoxic to adult neurons that were morphologically recovered *in vitro*, and this provided the baseline culture system conditions for the electrophysiological characterization.

*Glutamate Applied for a Minimum of 24 Hours Increases the Number of Electrically Active Adult Hippocampal Neurons in Vitro*

After morphological recovery of the adult hippocampal neurons ($\geq$ 14 *div*), recovery of *in vitro* neuronal electrical activity was investigated to determine if the introduction of glutamate to the neurons through the culture medium had a positive effect on functional recovery. In order to determine the parameters that produced the best electrical recovery, glutamate was applied for periods of 1 h to 14 days at

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concentrations of 10, 25, or 100 $\mu$M to the hippocampal neurons at 14, 21, 31 or 38 *div*.

After the application of glutamate under each set of conditions, the electrical properties of these neurons were fully evaluated by whole cell patch-clamp electrophysiology and compared to the electrical activity of neurons without the glutamate treatment. In addition, the electrical activity of adult neurons up to 80 *div* was measured in cultures where 25 $\mu$M glutamate had been applied from 21 to 28 *div* as shown in Figure 2-1I and detailed in Figure 2-3*.*

Initially, the shortest and best incubation time that induced improved activity was evaluated. 25 $\mu$M glutamate was applied to 21-day-old adult, hippocampal neuronal cultures for periods of 1 h, 1 d, 7 d or 14 d, after which neuronal electrical activity was immediately analyzed. After an incubation period of 1 h with glutamate, the cultured neurons exhibited no improvement in activity, both in the number of neurons with current flow (ISCs, OPCs) and action potentials (APs) (Table 2-1, *Groups G21-1h* vs. *C21*). However, after glutamate was applied for longer durations (1 d, 7 d, 14 d), the number of neurons with ISCs and/or OPCs increased significantly, to 71% and 85% respectively in those neurons exposed for 24 hours (Table 2-1, *Group G21-1d)*. In addition, a significant increase in the number of neurons with the ability to fire APs was triggered by exposure to glutamate for 1 or more days. After 25 $\mu$M glutamate was applied for 1 d, 7 d, or 14 d, 70-80% of the adult neurons were able to fire APs, a 35-40% improvement versus untreated neurons (Table 2-1, *Groups G21-1d* vs. *C21; Groups G21-7d* vs. *C28; Groups G21-14d* vs. *C38*).

Efficacy versus excitotoxicity of glutamate at different concentrations was re-examined to find the least excitotoxic level of glutamate that still triggered the recovery

of neuronal electrical activity *in vitro*. 10 μM, 25 μM, or 100 μM glutamate was added to neurons at 21 *div* for 1 day. After incubation with 10 μM glutamate, ISCs, OPCs and APs were observed in 51%, 63% and 53% of patched neurons respectively, a very slight improvement in electrical activity versus untreated neurons. After incubation with 25 μM glutamate, only slightly excitotoxic under these *in vitro* conditions, 71% of neurons had ISCs, 85% had OPCs and 71% fired APs. This was an improvement of 24, 32 and 35% respectively versus untreated neurons. The 100 μM dose of glutamate indicated similar efficacy, but triggered a significant excitotoxic response (Table 2-1, *Groups G21-10; G21-25; G21-100; C21*). Finally, a dose of 25 μM glutamate was applied to the neurons at 14, 21, 31, and 38 *div* for 24 hours. When compared to untreated control cultures of similar age, a higher percentage of neurons in each treated culture exhibited ISCs (15-24%), OPCs (20-30%) and APs (24-36%). However, approximately 20% fewer neurons displayed electrical activity when glutamate treatment was initiated after 14 *div* versus 21, 31, and 38 *div*.

The best experimental conditions for improving adult neuronal cultures were found by minimizing glutamate excitotoxicity while maximizing glutamate-mediated recovery of neuronal electrical activity *in vitro*. Challenging cultured adult neurons at 21 *div* with 25 μM glutamate for 1 to 7 days caused manageable levels of excitotoxicity, while significantly improving the electrical potential of these cultured neurons. This combination of culture age, glutamate concentration and incubation time yielded significantly improved electrical activity with minimal neuronal cell loss. Representative voltage and current clamp traces (21 and 78 *div*) are displayed in Figure 2-2.

*Extracellular Glutamate Applied for a Minimum of 24 Hours to Fully Recovered Adult Hippocampal Neurons in vitro Resulted in Increased Inward and Outward Current Flow in Electrically Active Neurons*

The effect from glutamate at various concentrations and incubation times to neurons at different days *in vitro* was measured in relation to the changes in mean resting membrane potential, membrane resistance, membrane capacitance, peak inward current, peak outward current and action potential amplitude. These results are summarized as mean $\pm$ SE (Table 2-2). Statistical analysis performed on the results between the different group's resting membrane potential, membrane resistance, membrane capacitance and action potential height indicated no significant change due to adult neuronal exposure to glutamate. However, incubation with glutamate triggered significant changes in the peak inward and outward currents (Table 2-2). The amplitude of current flow into the cultured neurons after application of glutamate on average doubled as compared to the current flow observed in the control neurons, from $-$ 188.3 $\pm$ 72.4 (*Group C21*) to $-$ 403.6 $\pm$ 98.5 (*Group G21-25*). Likewise, the amplitude of current flowing out of the glutamate-treated cultured neurons increased on average 2.5 to 3 times versus the control neurons, from 304.2 $\pm$ 144.2 (*Group C21*) to 923.5 $\pm$ 201.2 (*Group G21-25*). In the different cultures incubated with 25 $\mu$M glutamate for at least 1 d (1 d, 7 d, 14 d), no significant amplitude differences in mean peak ISCs or OPCs were evident between these cultures.

In addition, cycloheximide (CHX), an inhibitor of protein synthesis that had previously been used to block preconditioning in neurons [24], was used to block all possible changes in protein synthesis induced by incubation of the neurons with glutamate. When hippocampal neurons (21 *div*) were pre-incubated for 1 h with CHX

(20 mg/ml) prior to the introduction of 25 mM glutamate, all improvements in neuronal electrical activity were completely blocked (Tables 2-1 & 2-2).

**Discussion**

In this study, a defined culture system for adult hippocampal neurons was developed that included a cell-adhesive silane surface and a serum-free media containing glutamate. This culture system allowed adult neurons to recover full electrical activity and survive long-term *in vitro* for more than 80 *div*. First, culture surfaces, in this case glass cover slips, were modified with the chemical substrate DETA to create stable surfaces with exposed cell-adhesive amine groups. These chemically modified surfaces not only promoted adult neuron attachment, regeneration and long-term [28], but were also stable, reproducible and can be further modified to create high-resolution patterns, which could be useful for the creation of engineered networks of these adult hippocampal neurons [9, 10, 29]. Second, the optimal glutamate concentration, culture age for glutamate application and duration of glutamate exposure were determined such that excitotoxicity was minimized and neuronal electrical improvements was maximized, which resulted in the long-term survival of electrophysiologically functional adult neurons. Based on the experimental results, challenging 21 *div* adult neurons with 25 $\mu$M glutamate for 1 to 7 d caused minimal excitotoxicity, while inducing recovery of full electrical activity *in vitro*. Together, these improvements allowed adult neurons to functionally recover and to survive for several months *in vitro*, providing a stable test-bed for long-term study of the mature mammalian brain.

In fast excitatory neurotransmission, the neurotransmitter glutamate primarily acts via the activation of ionotropic and metabotropic receptors. In addition to conveying fast excitatory neurotransmission, activation of these channels appears to

play a major role in neuronal differentiation and CNS development, as well as in processes that give rise to long-term potentiation and memory formation [13]. Recovery of the neuronal electrical activity in this culture system, induced by the application of glutamate, illustrates a role for glutamate beyond simple neurotransmission. The application of glutamate triggered this change either through transient or persistent cellular changes. Transient changes in activity could have occurred as a direct result from the presence of glutamate, triggering changes in passive membrane properties (capacitance and resistance), alteration in the magnitude of the voltage-activated sodium, potassium and calcium currents [30-33], or alteration in the activity levels of the ion channels. Persistent changes to neuronal excitability would be dependent upon the induction of gene expression to cause long-lasting changes in the adult neurons. Results from this study point to the induction of persistent changes in these cultured adult neurons as improved electrical activity was not evident in the adult neurons after only one hour of glutamate application.

In this culture system, glutamate appears to activate gene transcription through the same mechanism found *in vivo*. Through activation of ionotropic and metabotropic receptors *in vivo*, glutamate has been shown to trigger gene activity in immature, newborn and mature hippocampal neurons. In immature neurons, glutamate regulates cell proliferation, neuronal differentiation and survival responses primarily through the N-methyl-D-aspartate receptor (NMDAR) channels activation [34-36]. However, in mature neurons glutamate regulates the expression of adaptive response genes as well as genes that regulate more complex neural functions, such as learning and memory, though the activation of L-type voltage gated calcium channels (L-VGCC) rather than

NMDAR channels [36]. Activation of these channels triggers calcium influx, thus activating a number of signaling molecules. These molecules potentiate the signals through the activation of downstream signaling proteins such as protein kinase A (PKA), $Ca^{2+}$/calmodulin dependent protein kinase type IV (CaMK-IV), regulated S6 protein kinase (Rsk) and other pathways to amplify the calcium signal and carry it to the nucleus. In the nucleus these kinases phosphorylate transcription factors, such as cyclic AMP response element binding protein (CREB) or myocyte enhancer factor 2 (MEF2), make them competent to mediate gene transcription [37]. Although glutamate activates transcription factors like CREB in both mature and immature neurons, different genes are activated in each case. Overall, different signaling pathways trigger the expression of genes that regulate the survival, differentiation and function of immature neurons (cAMP-CREB pathway) [14, 38], neurite outgrowth [13, 17, 18] or activity-dependent synaptic plasticity and trophic factor-dependent neuronal survival [37, 39]. In this adult neuronal culture system, glutamate appeared to induce gene activation, resulting in increased numbers of neurons firing APs and in significantly increased sodium and potassium current amplitude. The initiation of protein synthesis by the glutamate treatment is supported by the experiments where CHX addition blocked any functional recovery of electrical activity due to glutamate addition. These differences in the activated signaling pathways in the adult neurons from that observed in development may give insight to pathways necessary for neuroregeneration, specifically for recovery in TBI.

In this study, silane-modified DETA surfaces and the transient application of glutamate have been incorporated into an adult, hippocampal cell culture system in

order to sustain long-term neuronal survival as well as to promote full recovery of electrical activity *in vitro*. Together, these improvements allowed electrically active adult neurons to survive for several months *in vitro*, providing a stable system with potential for a wide range of applications. Promising applications include the long-term study of the mature brain, neurological disorders, and diseases affecting the aging brain. In addition, because DETA monolayers can be applied not only to glass cover slips but also to electrodes, this system can be extended to integrate living and electronic systems. Overall, potential uses for such a system range from research into the function of neurons, neuronal interactions, aging, and neurodegenerative disease [1] as well as drug studies, neuroprosthetic devices, neurocomputing, and biorobotics [2-4], or most importantly, neuroregeneration, especially in TBI.

**Table 2-1**.  Sample groups (based upon the timing, duration, and dosages of glutamate application):

| Groups | Total number of cells patched | Number of cells with induced inward sodium current (ISC) | Number of cells with induced outward potassium current (OPC) | Number of cells which fired single action potentials (SAP) | Average fraction of cells alive (vs total number of cells, live + dead) |
|---|---|---|---|---|---|
| C14 | 13 | 5 (38.5%) | 5 (38.5%) | 3 (23.1%) | 94% |
| C21 | 17 | 8 (47.1%) | 9 (52.9%) | 6 (35.3%) | 96% |
| C28 | 14 | 7 (50.0%) | 8 (57.1%) | 5 (35.7%) | 95% |
| C38 | 13 | 7 (53.8%) | 10 (76.9%) | 5 (38.5%) | 95% |
| G21-10 | 19 | 10 (52.6%) | 12 (63.2%) | 10 (52.6%) | 88% |
| G21-25 | 34 | 24 (70.6%) | 29 (85.3%) | 24 (70.6%) | 84% |
| G21-100 | 17 | 15 (88.2%) | 15 (88.2%) | 12 (70.6%) | 69% |
| G21-1h | 15 | 7 (46.7%) | 10 (66.7%) | 4 (26.7%) | 93% |
| G21-1d | 34 | 24 (70.6%) | 29 (85.3%) | 24 (70.6%) | 84% |
| G28-7d | 15 | 10 (66.7%) | 12 (80.1%) | 11 (73.3%) | 78% |
| G35-14d | 14 | 11 (78.6%) | 11 (78.6%) | 11 (78.6%) | 65% |
| G14-25 | 13 | 7 (53.8%) | 9 (69.2%) | 7 (53.6%) | 86% |
| G21-25 | 34 | 24 (70.6%) | 29 (85.3%) | 24 (70.6%) | 84% |
| G31-25 | 18 | 12 (66.7%) | 14 (77.8%) | 13 (72.2%) | 82% |
| G38-25 | 16 | 11 (68.8%) | 11 (68.8%) | 10 (62.5%) | 83% |
| CHX10 | | | | | 89% |
| CHX20 | | | | | 84% |
| CHX80 | | | | | 52% |
| G21CX20 | 23 | 8 (34.8%) | 12 (52.2%) | 5 (21.7%) | 66% |

Comparison of the total number of neurons patched after exposure to different culture conditions. The groups were designed to find the optimal conditions under which glutamate induced maximal improvements to the electrical activity of the cultured, adult hippocampal neurons while causing the least amount of neuronal cell death.  These various conditions resulted in different groups which measured the effect of neuronal electrical activity and cell death of the following conditions:  no glutamate application – controls (day 14 (C14), day 21 (C21), day 28 (C28), day 38 (C38)); different concentrations of glutamate applied to day 21 neurons (10 $\mu$M (G21-10), 25 $\mu$M (G21-25), 100 $\mu$M (G21-100)); different durations for the application of 25 $\mu$M glutamate to day 21 neurons (1 hour (G21-1h), 1 day (G21-1d), 7 days (G21-7d), 14 days (G21-14d)), 25 $\mu$M glutamate applied for 24 hours to different aged cultures (day 14 (G14-25), day 21 (G21-25), day 31 (G31-25), day 38 (G38-25)), different concentrations of cycloheximide (CHX) (10 $\mu$g/ml (CHX10), 20 $\mu$g/ml (CHX20), 80 $\mu$g/ml (CHX80)), and inhibition that protein synthesis (20 $\mu$g CHX / ml media) has on glutamate (25 $\mu$M, 24 hour duration, day 21 culture) induced improvement (G21CX20).  Cell death was measured both through a live/dead assay and through cell counts.  The live neuron percentage value is the fraction of neurons that are live versus the total (live and dead).

**Table 2-2**. Comparison of the electrical properties of those neurons that exhibited action potentials.

| | C14 | C21 | C28 | C38 | G21-10 | G21-25 | G21-100 | G21-1h | G21-7d | G21-14d | G14-25 | G31-25 | G38-25 | G21CX20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days *in vitro* before addition of glutamate | n/a | n/a | n/a | n/a | 20 | 20 | 20 | 21 | 21 | 21 | 13 | 30 | 37 | 20 |
| Total days *in vitro* | 14 | 21 | 28 | 38 | 21 | 21 | 21 | 21+1 hour | 28 | 35 | 14 | 31 | 38 | 21 |
| Number of neurons (*n*) | 13 | 17 | 14 | 13 | 19 | 34 | 17 | 15 | 15 | 14 | 13 | 18 | 16 | 23 |
| Resting potential (mV) | -70 | -69 ±1.56 | -69.05 ±1.05 | -69.63 ±0.45 | -70.25 ±1.92 | -71.4 ±0.88 | -70.9 ±0.54 | -71.4 ±1.36 | -69 ±1.24 | -69.1 ±1.42 | -70.3 ±0.84 | -69.75 ±1.06 | -69.5 ±0.92 | -68.1 ±0.53 |
| Input resistance (m$\Omega$) | 23.1 | 23.1 ±9.62 | 23.1 ±2.34 | 22.9 ±1.74 | 23 ±14.15 | 25.74 ±10.06 | 19.11 ±5.64 | 23.1 ±11.49 | 22.75 ±2.21 | 23.1 ±6.3 | 26.86 ±9.51 | 22.3 ±11.15 | 27.44 ±6.06 | 16.11 ±8.34 |
| Capacitance (pF) | 15.41 | 15.77 ±8.1 | 14.57 ±6.5 | 14.67 ±4.8 | 12.86 ±11.69 | 10.55 ±4.96 | 8.9 ±3.88 | 13.14 ±9.28 | 11.22 ±2.7 | 10.63 ±5.76 | 11.13 ±6.75 | 12.13 ±3.65 | 10.96 ±7.62 | 9.35 ±7.45 |
| Peak inward current (pA) | -172.4 | -188.3 ±72.4 | -165.2 ±57.3 | -178.1 ±66.3 | -237.2 ±77.3 | -403.6 ±98.5 | -388.3 ±97.4 | -173.3 ±88.9 | -402.2 ±77.5 | -343.7 ±87.4 | -302.5 ±93.6 | -378.8 ±73.4 | -379.8 ±43.9 | -255.6 ±92.4 |
| Peak outward current (pA) | 285.4 | 304.2 ±144.2 | 298.6 ±74.9 | 285.6 ±71.4 | 479.3 ±177.7 | 923.5 ±201.2 | 788.3 ±243.6 | 276.3 ±81.4 | 802.4 ±344.5 | 734.0 ±133.2 | 723.4 ±174.3 | 893.2 ±202.4 | 933.1 ±355.3 | 402.5 ±166.3 |
| Action potential height (mV) | 28.2 | 29.7 ±2.1 | 31.8 ±3.1 | 30.3 ±2.8 | 34.2 ±4.2 | 30.6 ±2.8 | 27.1 ±4.3 | 34.2 ±4.4 | 31.7 ±1.9 | 32.6 ±3.2 | 26.4 ±2.1 | 29.1 ±3.2 | 28.4 ±1.1 | 31.2 ±2.3 |

The different glutamate application conditions resulted in different groups, which measured the effect of neuronal electrical properties in the following conditions: no glutamate application – controls (day 14 (C14), day 21 (C21), day 28 (C28), day 38 (C38)); different concentrations of glutamate applied to day 21 neurons (10 $\mu$M (G21-10), 25 $\mu$M (G21-25), 100 $\mu$M (G21-100)); different duration for the application of 25 $\mu$M glutamate to day 21 neurons (1 hour (G21-1h), 1 day (G21-1d), 7 days (G21-7d), 14 days (G21-14d)), 25 $\mu$M glutamate applied for 24 hours to different aged cultures (day 14 (G14-25), day 21 (G21-25), day 31 (G31-25), day 38 (G38-25)), and inhibition that protein synthesis (20 $\mu$g CHX / ml media) has on glutamate (25 $\mu$M, 24 hour duration, day 21 culture) induced improvement (G21CX20).
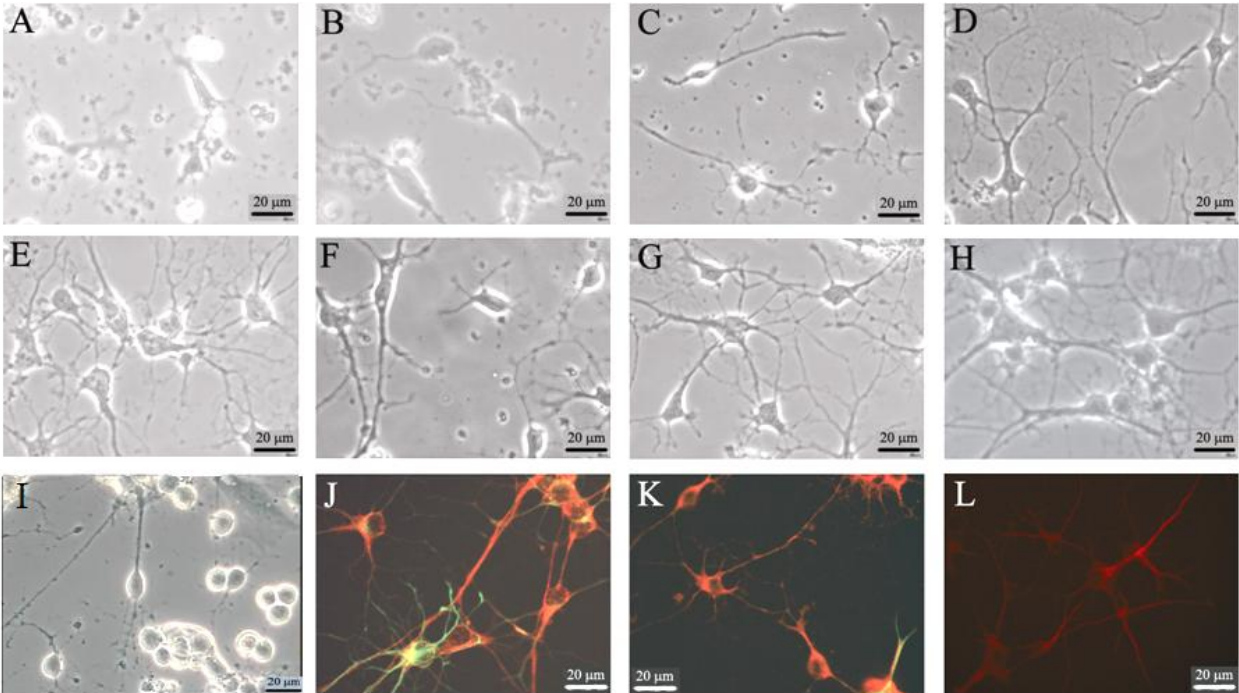
**Figure 2-1. Rat hippocampal neuron *in vitro*.** Representative phase-contrast and anti-neurofilament/anti-GFAP immunostained pictures of neurons cultured from adult hippocampal tissue. This figure ilustrates both the recovery of the hippocampal neurons in this defined *in vitro* system as well as the purity of the neuronal culture, neurons versus glial cells. A-I illustrates phase-contrast images of living cultures taken during different culture ages, immediately following cell culture through 28 days *in vitro*, 40x view. (A) Phase picture of neurons *in vitro* 1 hour following the attachement of neurons onto a silane-modified coverslip. (B) 6 hours post-attachment (C) 2 days post-attachment. Note the rapid recovery of axons as well as the phase bright cell soma. (D) Phase picture of the neurons after 7 days *in vitro*. (E) Phase picture of the neurons after 14 days *in vitro*. Morphologically these adult-derived hippocampal neurons are fully recovered. (F) Phase picture of neurons after 22 days *in vitro*. (G) Phase picture of neurons after 22 days *in vitro*, with a prior 1-day exposure to 25 $\mu$M glutamate added to the culture media on day 21. (H) Phase picture of neuron after 78 days *in vitro*. (I) Phase picture of neuron after 78 days *in vitro*, after incubation with 25 $\mu$M glutamate between days 21 to 28, further visualized after application of antibodies against neurofilament (red) and GFAP (green) antibodies (J, K) and (red) MAP-2 (L)
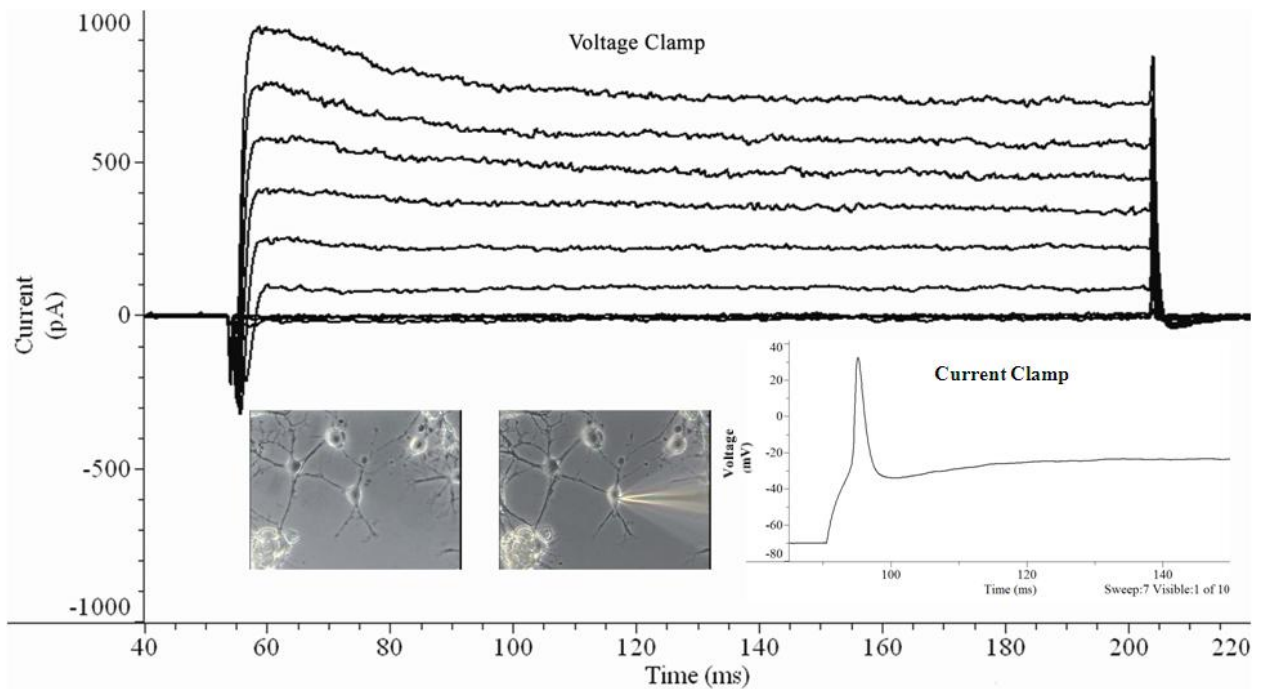
**Figure 2-2. Representative traces for voltage and current clamp of an adult neuron 21 *div*.** Neurons retain the ability to move current into and out of cells through the voltage-gated ion channels (voltage clamp trace) as well as to fire single action potentials after electrical stimulation (current clamp trace). These traces originated from adult hippocampal neurons after 21 days *in vitro*, where 25μM glutamate had been applied to the culture medium between days 20 and 21.
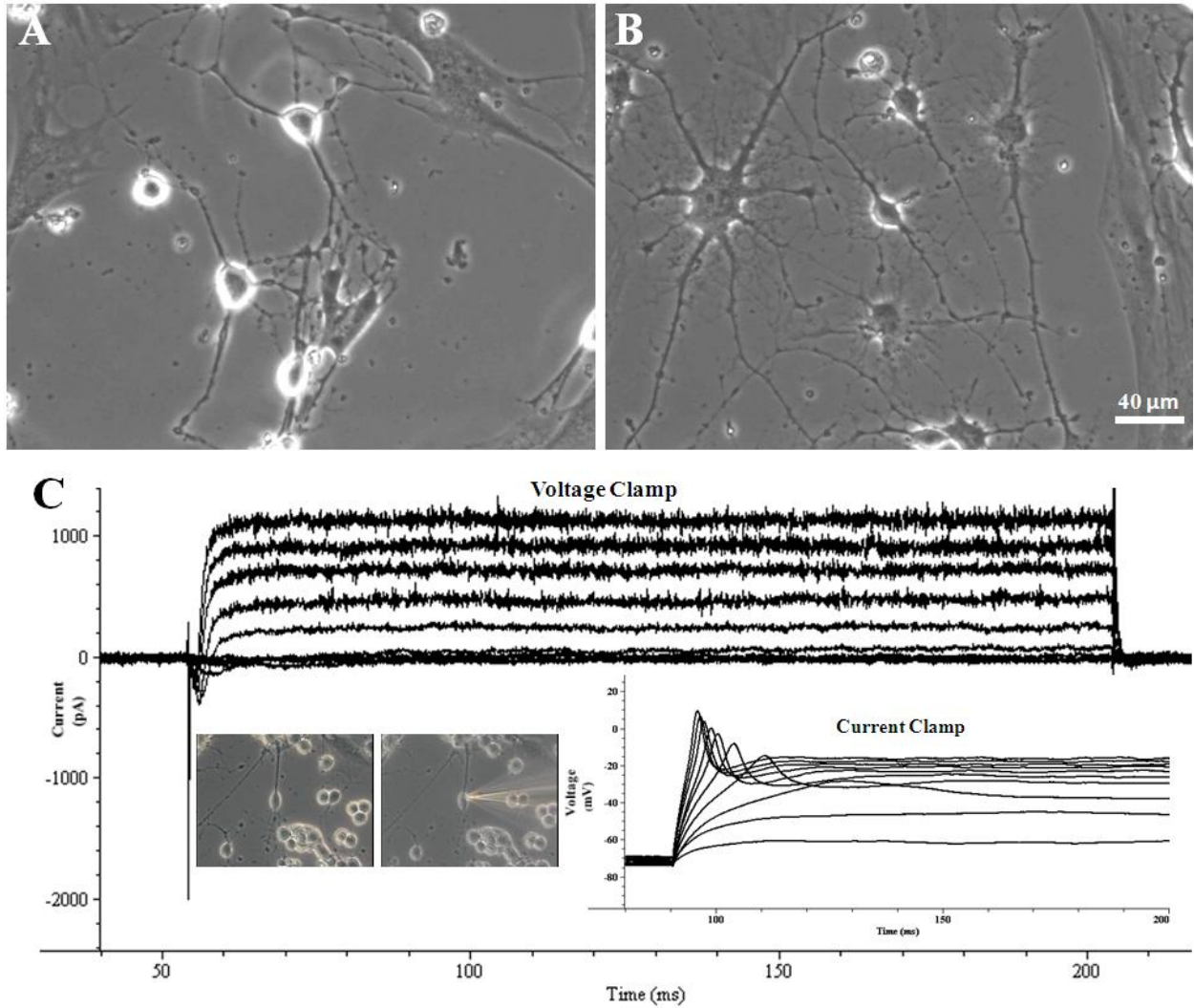
**Figure 2-3.   Representative phase-contrast pictures and electrophysiological recordings of adult hippocampal neurons after approximately 80 *div*.**  (A, B) Phase contrast pictures of neurons 80 *div*.  (C) Representative traces for voltage and current clamp of an adult neuron 78 *div*.  Neurons retain the ability to move current into and out of cells through the voltage-gated ion channels (voltage clamp trace) as well as to fire single action potentials after electrical stimulation (current clamp trace).  These traces originated from adult hippocampal neurons after 78 days *in vitro*, where 25 $\mu$M glutamate had been applied to the culture medium between days 21 and 28.

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# CHAPTER 3 - DERIVATION OF A POPULATION OF STABLE ELECTRICALLY ACTIVE NEURONS FROM THE ADULT RAT BRAIN THROUGH MANIPULATION OF CDK5 ACTIVITY

## Introduction

Neurodegenerative conditions as well as traumatic brain injury (TBI) are characterized by widespread loss of functional, active neurons [1, 2]. Investigations into treatments and therapies for these conditions are reliant upon the existence of accurate systems mimicking the conditions found in the human brain. Widely used systems include rat and mouse disease models mimicking pathological symptoms of the diseases, embryonic neuron cell culture systems including dissociated cell culture and brain slices, and later stage testing on human subjects [3-5]. Some work has been performed on the refinement of *in vitro* dissociated neuronal systems to use adult brain tissue rather than embryonic brain tissue [6, 7]. Problems exist with these current *in vitro* culture systems. Difficulty in limiting damage during the dissociation of adult brain tissue, mitigation of cellular trauma from the release of myelin inhibitory proteins, problems promoting both regeneration and long-term survival of adult brain-derived neurons, and proliferation of previously quiescent terminally differentiated neurons are among the most common problems encountered in creating such an *in vitro* system.

Adult central nervous system neurons have long been described as post mitotic, with neurons arrested in a G0 phase of the cell cycle. A new hypothesis has emerged, however, that this cell cycle arrest in neurons is not permanent, but rather mature differentiated neurons must constantly regulate themselves to keep from progressing through the cell cycle [8]. *In vitro*, post mitotic neurons revert to a proliferative state due to the action of the essential factor basic fibroblast growth factor (bFGF) [9]. The factor

bFGF also promotes GABA-negative neurons survival by influencing both glucosyceramide synthesis and the voltage-dependent calcium channels (VDCCs) [9, 10]. It also up-regulates cyclin-dependent kinases 5 (cdk5) expression, triggering neuronal re-entry into the cell cycle [9, 11]. Cdk5, while generally expressed in neurons in the cdk5/p35 complex, is normally expressed by neurons at levels that exert an influence on neurite outgrowth and migration but not proliferation [11-13]. Cyclin-dependent kinase inhibitors (CKIs), which includes the competitive cdk5/p35 inhibitor roscovitine, normally play an important role in regulatory decisions controlling progression through the cell cycle [13, 14].

In this study we have developed a dissociated neuronal cell culture system derived from the hippocampus of adult rats. Through the action of regeneration promoting growth factors, most importantly bFGF, and the dose-dependent application of novel anti-mitotic factors we are able to activate or deactivate cdk5 mediated cell cycle progression to promote or control the division of mature, terminally differentiated neurons. The application of this cell cycle control to an improved serum-free culture system has allowed for either the expansion of primary adult neuronal cells under controlled conditions or for the development of a stable population of primary neurons that both morphologically and functionally regenerate without expansion. These neurons are applied to culture surfaces coated with the chemical substrate N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine (DETA), a surface previously shown to be superior in the promotion of attachment, regeneration and long-term survival of neurons *in vitro* [15-20]. A stable population of electrically active neurons has been derived through this system from adult rat hippocampal tissue, neurons that have been

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maintained for over 80 days *in vitro* (*div*).  Overall, potential uses for such a system range from research into the function of neurons, neuronal interactions, aging, and neurodegenerative disease [21] as well as drug studies, neuroprosthetic devices, neurocomputing, biorobotics [4, 5, 22], and most importantly neuroregeneration, especially in TBI.

**Materials and Methods**

*DETA surface modification and characterization*

Glass cover slips (Thomas Scientific 6661F52, 22mm×22mm no. 1) were cleaned by acid washing using a 50/50 mixture of concentrated hydrochloric acid and methanol. The cover slips were washed three times, 30 min per wash, and were rinsed in distilled de-ionized water between each washing. The DETA (N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine, United Chemical Technologies Inc., Bristol, PA, T2910KG) monolayer was formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904) [17]. The DETA-coated cover slips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. The DETA formed a reaction site limited monolayer on the surface of the cover slip [17]. The DETA cover slips were characterized to authenticate the monolayer formation. First, contact angle measurements were taken using an optical contact angle goniometer (KSV Instruments, Monroe, CT, Cam 200). The contact angle for the DETA-coated cover slips was 54.2±0.2, which was previously shown to be acceptable for neuronal hippocampal culture [17]. Second, X-ray Photoelectron Spectroscopy (XPS) (FISONS ESCALab 220i-XL) was used to characterize the elemental and chemical state of the DETA-coated cover slip surfaces. The XPS survey scans as well as high-resolution N 1s and C 1s scans, using monochromatic Al Kα excitation, were obtained, similar to previously reported results [15, 17].

*Cell culture methodology and medium used for adult rat hippocampal culture*

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The hippocampus of adult rats (Charles River, age 6-12 months) were dissected and homogenized into small tissue fragments in cold medium (~4ºC) consisting of Hibernate-A, Glutamax, and antibiotic-antimycotic.  The tissue was then digested for 30 minutes at 37°C in calcium-free Hibernate-A (HA) containing 6 mg papain / 12 ml (HA).  Following digestion, the tissue was washed three times with cold Hibernate-A media to remove any active enzyme.  Next, the tissue was suspended in *Dissociation Media* (Table 3-1)*,* formulated for dissociation of rat and mouse adult brain tissue into individual cells, and dissociated into individual cells through mechanical dissociation with fire-polished Pasteur pipettes.  The cells were suspended in *Plating Media* (Table 3-1), used after tissue break-up to promote neuronal attachment and regeneration, and then deposited onto DETA-coated glass cover slips for 30-45 minutes.  DETA, with its triamine functional group exposed at the surface, strongly attaches to neurons and allows for all non-neuronal debris such as ECM proteins, myelin debris, and cell fragments to be washed from the cover slip surface [15, 16, 18, 19].  Following this washing step fresh Plating medium was applied and remained for the first 3 *div*.  On 3 *div* and every fourth subsequent day, ½ of the medium was removed and replaced with *Maintenance Media* (Figure 3-1, Table 3-1)*,* supporting the long-term maintenance of these neurons.  Each type of media was formulated to specifically meet the challenges presented during each stage of the cell culture process, allowing for significantly improved survival, regeneration, and long-term growth (Table 3-1, Figure 3-1).  The osmolarity of the media was adjusted to match the osmolarity of adult rat cerebrospinal fluid (295-305mOsm) [23, 24].  In the *Dissociation Medium* (Table 3-1), the use of caspase inhibitors and antioxidants [25] during the dissociation of the tissue minimized

both oxidative damage as well as the progression into apoptosis which normally results from cellular damage during this stage. Inclusion of these anti-oxidants as well as specific growth factors in the *Plating Media* (Table 3-1) was found to promote attachment and regeneration of neurons *in vitro.* For long-term survival, the anti-oxidants were removed from the *Maintenance Medium* (Table 3-1).

*Control of Neuronal Division in vitro*

bFGF (invitrogen, 13256-029, 5ng/ml) triggered neuronal cell proliferation until confluence, a period marked both my rapid neuronal division as well as limited or absent functional electrical activity. After neuronal confluence, 25µM glutamate ((N-Acetyl-L-glutamic acid, Aldrich, 855642) triggers induction of neuronal electrical recovery [20]. Control of the division of neurons in this culture system was investigated experimentally as follows: (1) bFGF removal from both the Plating and Maintenance medium. (2) Fluorodeoxyuridine (FudR, Sigma-Aldrich, F-0503) addition at 10 and 50 µM (3) arabinoside-D (ara-C, Sigma, C-6645) plus deoxycytidine addition. (4) Trolox (Sigma, 238813) addition at 40 and 100 µM. (5) Aphidicolin (Aph, Sigma, A0781) addition at 1.5 µM. (6) Roscovitine (Rosc, Sigma, R7772) addition at 1, 5, and 10 µM. All factors were added before the onset of neuronal division (2 *div*), some after the onset of division (after 3 *div*).

*Time-Lapse Microscopy*

Time-lapse recording was performed immediately after the cells were plated. Living cells were observed under an inverted microscope (Zeiss-Axiovert 100) equipped

with Plan-Neofluar 10x objective (Zeiss, Oberkochen, Germany) and a humidified incubation chamber for constant temperature at 37ºC and 5% $CO_2$. Pictures were captured with a Hamamatsu C8484-05G digital charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan). Experiments were run under the control of Okolab software (OKO-lab, Ottaviano, NA, ITALY). Pictures were taken under the control of the software every 5 minutes, and live cell image sequences were compiled to create videos, 12 images per second (video 3-1).

*Electrophysiology*

Whole-cell, patch-clamp recordings were performed at room temperature. PH was adjusted to 7.3 with HEPES acid and base, ion concentrations with NaCl to approximately 130 mM, and osmolarity of 290-300 mOsm. Cells were visualized on the stage with a Zeiss Axioscope, 2 FS Plus, upright microscope in maintenance culture medium. Patch pipettes (4-8 M$\Omega$) were filled with intracellular solution (K-gluconate 140 mM, EGTA 1 mM, $MgCl_2$ 2 mM, $Na_2ATP$ 5 mM, HEPES 10 mM; pH 7.2), osmolarity approximately 260 mOsm. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (MDS Analytical Devices) amplifier. Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recordings and analysis were performed with pClamp software. Whole-cell capacitance and series resistance were compensated electronically. Only cells with access resistance less than 22 M$\Omega$ were analyzed. Inward currents that had the characteristics of fast sodium currents, and outward currents that had the characteristics of potassium currents were measured in voltage clamp mode using voltage steps of 10 mV from a –

70 mV holding potential. Action potentials were measured with 1 s depolarizing current injections to elicit action potentials from a –70 mV holding potential. Cells were morphologically selected for electrophysiological characterization. Selected cells were phase bright pyramidal neurons with large branching apical dendrites and small basal dendrites. Cells with this morphology stained positive for anti-neurofilament-M (Nf-M) and/or MAP2. Neurons were electrically characterized after 6, 13, and 25 *div*.

*Immunocytochemistry*

To prepare cells for immunocytochemical characterization, cover slips were rinsed twice with Phosphate Buffered Saline (PBS). Cells were fixed with 4% paraformaldehyde for ten minutes at room temperature, and subsequently rinsed three times with PBS. Cells were permeabilized for five min in PBS with 5 mM Lysine and 0.5% Triton X-100, and were then blocked for two hours by adding 5% normal donkey serum. Anti-Neurofilament-M polyclonal antibody (Nf-M, intracellular filament found in mature neurons, Chemicon, AB1981, diluted 1:1000), anti-NeuN (nuclear marker in mature neurons, Chemicon, diluted 1:1000), anti-Ki-67 (nuclear marker in dividing cells, Chemicon, diluted 1:1000), anti-NR2A (NMDA channel subunit expressed in the post-synaptic cell membrane, AB1548, diluted 1:75), anti-NR2B (NMDA channel subunit expressed in the post-synaptic cell membrane, MAB5216, diluted 1:150) were added in blocking solution for 12 hr at 4ºC. After 3 washes with PBS, fluorescently labeled secondary antibodies (Invitrogen, A11011, A21449, and A11029) were applied for two hours. Vectashield mounting medium (H1000, Vector Laboratories, Burlingame, CA)

was used to mount the cover slips onto slides.  The cover slips were observed with an Ultra VIEWTM LCI confocal imaging system (Perkin Elmer).

*Statistical analysis*

Numerical summary results are reported as a mean, plus or minus the sample standard error of the mean ($\pm$ SEM).  Neuronal cell density immediately following plating onto cover slips was normalized to 100 to allow for comparisons of the effect of growth factor removal or mitotic factors intervention.  For two-group comparisons, statistical analysis included a two-sample student's t-test on electrophysiological data.

**Results**

In this study we have created an improved primary dissociated neuronal culture system derived from the hippocampus of adult rats. Previous attempts at this have yielded culture systems in which the neurons survive and eventually recover electrically, but unfortunately exhibit uncontrolled proliferation [6, 20, 26]. We discovered a means to control the proliferation of neurons *in vitro*, allowing for functionally active primary adult neurons to be maintained for long periods of time under dissociated cell culture conditions. Additionally, we were able to expand a population of primary mature neurons, arrest proliferation at any point, and promote functional electrical recovery as mediated by the addition of glutamate at 25 µM to the culture medium [20].

*Conditions for culturing neurons derived from the hippocampus of adult rats:*

All parameters of the adult culture system were optimized for the support of neuronal attachment, regeneration, and long-term survival *in vitro* (Figure 3-1, Table 3-1). Cellular trauma during the cell culture process was minimized by first lowering the temperature of the *Dissection medium* to 4ºC. Next, extracellular matrix proteins and connective tissue in the brain fragments were digested using papain (2 mg / ml HA minus $Ca^{2+}$, 37ºC shaking water bath, 80 revolutions per minute) followed by repeated rinses of the tissue to inactivate the enzyme. Oxidative damage from free radicals released during tissue dissociation was limited by inclusion of the powerful anti-oxidants Trolox [27] and dextrose-coated cerium oxide nanoparticles [25] in the *Dissociation and Plating medium*. Direct neuroprotection against apoptotic agents was achieved by adding caspase inhibitors (1, 3, and 6) to the *Dissociation medium*. The tissue was

dissociated with fire-polished glass Pasteur pipettes in order to maximize cell breakup while minimizing cellular trauma.  Last, applying the dissociated neurons onto glass cover slip(s) coated with the cell adhesive surface DETA in *Plating medium* for 45 minutes allowed adherent neurons to attach with minimal attachment of cellular debris. Following this plating period, a gentle wash of the cover slip(s) with warm HA (37ºC) caused the majority of cellular debris, including myelin inhibitory factors present after the breakup of myelin sheaths [28], to be removed. Improvements to these basic culture parameters optimized neuronal survival and regeneration (Figures 3-2 to 3-4), allowing for investigation into the electrical properties and expression patterns displayed by adult neurons in a controlled *in vitro* environment (Figure 3-5).

*Inhibition of cell cycle progression in mature neurons in vitro*

New media formulations (Table 3-1) along with the time- and dose-dependent application of growth factor and transcription factor mediators (Figure 3-1) were applied to promote neuronal recovery and long-term functional survival.  While these improved culture system parameters supported adult neurons, they also forced these terminally differentiated primary neurons to enter the cell cycle and divide (Figure 3-2).  While critical growth factors, specifically basic fibroblast growth factor (bFGF), and dissociated cell culture conditions supported the survival and regeneration of mature neurons derived from adult rat hippocampal tissue, they also triggered re-entry into the cell cycle and division through upregulation of cyclin and cyclin-dependent kinase (cdk) expression [9, 11, 29-31].  The mitotic division of these neurons was arrested *in vitro* after neurons reached confluence (Figure 3-3B, control) and these previously dividing

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neurons were shown to exhibit both morphological and electrically recovery after 25μM glutamate was added to the culture media for at least 24 hours [20]. Despite the utility of this system, for more relevant investigations into primary dissociated neurons the rapid proliferation of neurons must be eliminated.

Multiple strategies were investigated to prevent primary terminally differentiated adult hippocampal neurons from returning to the cell cycle and dividing. The ultimately successful strategy for preventing proliferation under cell culture conditions was to add roscovitine, a competitive cdk inhibitor. Roscovitine was introduced into the culture medium at 1, 5, and 10 μM both before the onset of neuronal division (2 *div*) and after the onset of neuronal division (7 *div*) (Figure 3-3 A, B). When 1 μM roscovitine was applied on day 2, the cell population increased more slowly than without roscovitine (Figure 3-3A). 10 μM roscovitine caused a decrease in the neuronal population when applied on day 2, and a sharp loss of neurons when introduced on day 7 (Figure 3-3B). A stable population of neurons was achieved through the application of 5 μM roscovitine on days 2-7 *in vitro* and 2 μM after day 7 as a long-term "booster" concentration (Figure 3-3B). In addition, the health and maturity of this stable neuronal population was evident in the rapid and lengthy extension of primary axons by these neurons, to as much as 4-5 times longer on average versus untreated control neurons (Figure 3-3C). A stable population of neurons derived from the hippocampus of adult rats was achieved using this cell culture system and roscovitine treatment. Where control neuron populations proliferated to confluence (Figure 3-3D, left column), roscovitine treated neuronal populations did not. These roscovitine treated, non-proliferating cultured neurons exhibited morphological characteristics (Figure 3-3D, right column) previously

seen exclusively in cultured embryonic neurons. Additionally, these non-proliferating neurons expressed the mature neuronal markers MAP2 and NeuN, but lacked expression for the nuclear marker of proliferating cells, Ki-67 (Figure 3-3E). After several weeks in culture, the population of neurons *in vitro* remained stable (Figure 3-3 A, B), with very few neurons detaching from the DETA surfaces.

Other strategies for mitotic elimination that ultimately proved ineffective in the prevention of neuronal proliferation *in vitro* were also investigated. First, bFGF was removed from the *Plating media* and/or the *Maintenance media*. Neurons exposed to medium lacking bFGF did not divide. Rather than remaining static, however, neuronal populations deprived of bFGF decreased rapidly *in vitro*, with almost complete loss of neurons evident after two weeks (Figure 3-3A: FGF-P AND FGF-M, 4A). During this two week period, the morphology of the neurons dramatically changed, with the axon and branched dendrites retracting toward the cell body and ultimately forming a branched ring of neurites that surrounded the cell soma (Figure 3-4B). The factor bFGF proved to be an essential component of the serum-free culture medium for support of neuronal survival, recovery, and regeneration.

Mitotic inhibitors were also investigated to eliminate the division of the cultured neurons. FudR (10 and 50 µM) and ara-C were added to the culture medium on 2 *div* (Figure 3-3A: F-10, F-50, and ara-C). This strategy was unsuccessful because all dividing cells were forced into apoptosis, eliminating the cultured primary neurons rather than preventing neuronal division. Additional new agents, Trolox and Aphidicolin, both with potential anti-mitotic activity, were tested to inhibit the division of neurons in this culture system. Trolox, an antioxidant with anti-mitotic properties toward cancerous

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human breast cancer cells [32], was not effective in preventing primary neurons from returning to cycle *in vitro* at both 40 and 100µM (Figure 3-3A: T-40, T-100). Its antioxidant activity was found, however, to improve neuronal survival (Figure 3-3A), and was incorporated into both the *Dissociation* and *Plating media* at 70nM (Table 3-1)*.* Aphidicolin, a cell cycle inhibitor previously effective against bone marrow cell division [33], also did not display effective antimitotic activity. At 1.5 µM, a concentration high enough to impede the return of the neurons to the cell cycle, aphidicolin caused most cultured neurons to either necrose or apoptose (Figure 3-3A: A-1.5), and limited regeneration of neurites and functional electrical properties of those surviving neurons.

*Electrical activity and membrane channel expression of adult hippocampal neurons in vitro after elimination of mitotic activity*

Neurons were evaluated for electrical recovery after 6, 13, and 25 *div* using whole-cell patch clamp electrophysiology to evaluate the electrical potential of neurons, defined by the ability to move current into and out of the cell and to fire and propagate action potentials. The recovery of electrical activity *in vitro* was found to be significantly different between populations of neurons allowed to divide unchecked and neurons where mitotic division was prevented through application of roscovitine. When allowed to mitotically divide unchecked *in vitro*, neurons did not fully recover electrically for up to 3 weeks *in vitro,* and then only after the neurons reached confluency and were stimulated with 25 µM glutamate for more than 24 hours [20]. Prevention of neuronal division *in vitro* through application of roscovitine (5 µM days 2-7 *in vitro* followed by 2 µM roscovitine after day 7) significantly changed the time-scale required for electrical recovery of neurons *in vitro.* Glutamate (25 µM) had been applied after 5 *div*. Neurons

recovered after 6 *div* and showed the ability to move current into and out of the cell as well as to fire action potentials (Table 3-2, column 1, Figure 3-5 A).  Further recovery of these neurons was found after 13 and 25 *div*, as seen in increased resting membrane potential (Vm), increased membrane resistance (Rm), increased current flow, both inward sodium and outward potassium, as well as increased action potential amplitude (Table 3-2, Figure 3-5 A).  Figure 3-5A shows example traces displaying current flow into and out the neuron and an action potential.

*Protein expression indicates maturity of adult hippocampal neurons in vitro*

The maturity of neurons cultured in this system was examined.  Neurons that were allowed to mitotically proliferate were probed for expression of neurofilament-M, Ki-67, and NeuN (Figure 3-2C).  While positive for the neuron-specific structural protein neurofilament-M, none of the neurons were positive for the mature neuronal nuclear marker NeuN.  Instead, all neurons were positive for Ki-67, expressed exclusively in dividing neurons active in the cell cycle.  After roscovitine was added to the cell culture medium, neurons no longer divided and the expression pattern changed.  Neurons still expressed mature structural proteins (MAP2), but instead of the proliferation marker Ki-67 they expressed the mature nuclear marker NeuN (Figure 3-3E).  These neurons were also probed for NMDA channel subunit expression. Neurons in this cell culture system were probed immunocytochemically for each of the two subunits, and NR2A subunits outnumbered NR2B subunits, with both subunits present (Figure 3-5B, 1-2).

*Expansion of a population of primary neurons in vitro*

The combination of bFGF and roscovitine has allowed for a culture system of terminally differentiated primary adult hippocampal neurons in which the neuronal population can be kept stable or can be expanded. When these neurons were expanded under the basic culture conditions described in Figure 3-1 and Table 3-1 without roscovitine, three distinct phases were observed. In phase 1 (0-3 *div*), mature neurons were able to recover from the trauma of cell culture and regenerated morphologically, as shown by the expression of neurofilamant-M, the 150 kDa structural protein uniquely found in neurons in the brain, and NeuN, a nuclear marker expressed exclusively in mature neurons [34]. The components of the *Plating medium* (Table 3-1)*,* specifically growth factors, anti-oxidants, and Neurobasal-A with adjusted osmolarity, were essential for this recovery and regeneration. In phase 2 (3-21 *div),* bFGF in the *Maintenance media* (Table 3-1) induced neurons to re-enter the cell cycle through its activity in up regulating cdk5 expression [2, 11, 35, 36]. Previously quiescent, terminally differentiated phase bright neurons with long axons and branched dendritic trees first retracted their neurites into the cell soma (Figure 3-2A: 18-32 minutes), divided (Figure 3-2A: 60 minutes), and finally re-extended their axons and dendrites (Figure 3-2A: 60-108 minutes, 2B), a period observed to occur on average 90 minutes from neurite retraction, division, and neurite extension. Neurons that have previously divided repeat this process of cell division and double in number on average every 24-36 hours. During these periods, neurons displayed expression patterns consistent with both neurons and dividing cells, with all cells displaying both neuronal morphology and expression of Ki-67, a nuclear marker for proliferating cells [37], and Nf-M (Figure 3-2, C). At the same time, however, these dividing neurons were not expressing NeuN.

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Based on the pattern of all neurons during phase 1, specifically with all cells expressing mature neuronal markers, as well as the universal expression of proliferation markers throughout phase 2, it was determined that mature neurons were dividing, not the subset of neural progenitors known to exist in the dentate gyrus of the hippocampus. If only amplification of the smaller progenitor neuronal population of neurons had been occurring, then only a subset of neurons present would have shown evidence of Ki-67 expression.

In phase 3 (21-90 *div),* neurons reached confluence and stopped proliferating, a point at which neuronal expression of Ki-67 ended and NeuN reappeared. However, a passage of the confluent neurons from one DETA cover slip to multiple DETA cover slips at a lower cell density allowed for the continued proliferation of neurons beyond that seen on one cover slip alone, extending phase 2. During phase 2 neuronal electrical activity, specifically the ability to move current into and out of the cell and to fire action potentials (APs) was limited or non-existent. As the cells moved into phase 3, neurons were stimulated to recover their electrical activity through the introduction of 25 µM glutamate to the Maintenance medium. When cycloheximide (CHX), an inhibitor of protein synthesis that had previously been used to block preconditioning in neurons [38], was used to block all possible changes in protein synthesis induced by incubation of the neurons with glutamate all improvements in neuronal electrical activity were completely blocked. The introduction of glutamate mediated the cellular transcription activity to mature the previously dividing neurons [20].

**Discussion**

In this study we developed a dissociated neuronal cell culture system derived from the hippocampus of adult rats. Through the action of regeneration promoting growth factors, most importantly bFGF, and the dose-dependent application of novel anti-mitotic factors we were able to activate or deactivate cdk5 mediated cell cycle progression to promote or control the division of mature, terminally differentiated neurons. The application of this cell cycle control to an improved serum-free culture system supported the expansion of primary adult neuronal cells under controlled conditions across multiple passages with the ability to arrest mitotic division at any time through the application of roscovitine. Functional recovery of these previously dividing neurons could then be triggered by glutamate. Cell cycle control also allowed for the development of a stable population of primary neurons that both morphologically and functionally regenerated without expansion.

In this system, the dissection and dissociation of the tissue, the culture process, and the medium used for the dissection, dissociation, plating, and long-term maintenance of the neurons were all optimized to maximize the number of neurons that attach, survive, and regenerate *in vitro.* Mature differentiated glutamatergic neurons were extracted from hippocampal brain tissue and processed to purify the neurons and remove tissue debris. Terminally differentiated rat hippocampal neurons recovered *in vitro* and displayed mature neuronal morphology. After recovery, adult neurons returned to the cell cycle and divided multiple times. During each mitotic division, the neuron retracted their neurites and divided, and each post-mitotic neuron quickly re-extended their axons and dendrites. This neuronal cell division cycle was repeated

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every 24-48 hours, a period also marked by limited neuronal electrical activity [20]. Only after the neurons reached confluence did some neurons stop dividing, and neurons were stimulated to recover *in vivo*-like electrical activity through the introduction of glutamate to the maintenance medium of the culture. Strategies for inhibition of neuronal mitotic division were investigated, and roscovitine was identified as an effective agent in the prevention of bFGF triggered division *in vitro*. Prevention of mitotic division as well as optimization of the culture and maintenance parameters has resulted in a neuronal culture system derived from adult rats in which the morphology, cytoskeleton and surface protein expression patterns, and electrical activity closely mirror neurons *in vivo*. Maturity of neurons in dissociated cell culture is not only a function of the maturity of originating source of tissue but also in the expression patterns of receptors, receptor subunits, and structural proteins. The NMDA channel subunits NR2A and NR2B are examples of differential subunit expression between immature neurons in the embryonic and postnatal brain and mature neurons in the adult brain. Neither NR2A nor NR2B channel subunits are expressed in embryonic neurons. Expression of these channels begins around postnatal day 7 [39], and may not be expressed in neurons derived from E18 embryos at all. Both NMDA channel subunits were expressed by neurons in this culture system. These and other improvements have allowed electrically active adult neurons to survive for several months *in vitro*, providing a stable system with potential for a wide range of applications.

Improvements were also made to the growth surfaces on which neurons attach, regenerate, and survive long-term. Culture surfaces, in this case glass cover slips, were modified with the chemical substrate DETA to create a covalently modified

interface with exposed cell-adhesive amine groups that has been shown to be stable for long periods in culture. This stability *in vitro* contrasts to PDL and poly-ornithine which are physisorbed. DETA has been previously shown to be superior in the promotion of attachment, regeneration and long-term survival of embryonic neurons *in vitro* [15-19]. In addition, DETA, with its triamine functional group exposed at the surface, strongly attaches to neurons and allows for all non-neuronal debris such as ECM proteins, myelin debris, and cell fragments to be washed from the cover slip surface. DETA chemical surfaces can also be further modified to create high-resolution patterns, which could be useful for the creation of engineered networks of these adult hippocampal neurons [19, 40].

Adult central nervous system neurons have long been described as post mitotic, with neurons arrested in a G0 phase of the cell cycle. A new hypothesis has emerged, however, that this cell cycle arrest in neurons is not permanent, but rather mature differentiated neurons must constantly regulate themselves to keep from progressing through the cell cycle [8]. Cyclin/cdk complexes normally play an important role in regulatory decisions controlling progression through the cell cycle [13]. In neurons, cyclin-dependent kinase inhibitors (CKIs), small peptides that block cyclin/cdk activity either by forming an inactive complex or by acting as a competitive ligand, seem to be naturally involved in the mechanism of cell cycle arrest [41, 42]. In fact, loss of CKIs *in vivo* results in alteration of neuronal cell cycle kinetics [41]. While this arrest of neuronal cell division is life-long, late-onset neurodegenerative diseases may be accompanied by loss of neuronal cell cycle control. Cell-cycle protein re-expression has been reported in neurons from patients with Alzheimer's disease, Parkinson's disease, stroke, TBI, and

other neurodegenerative conditions [1, 2, 43-45], and dysregulation of this enzyme has been implicated in multiple neurodegenerative conditions including Alzheimer's [12, 46].

The factor bFGF, a member of the family of heparin binding growth factors [47], has been shown to be an essential component of the adult neuronal maintenance medium as it promotes both the survival and regeneration of neurons *in vitro*. In fact, the promotion of neuronal survival by bFGF is almost exclusively exerted on GABA-negative neurons [9]. The factor bFGF induces glucosylceramide synthesis in cultured hippocampal neurons, stimulating axonal growth [10]. It also interacts with the L-type voltage dependent $Ca^{2+}$ channels (VDCCs) to stimulate both neuronal survival and neurite branch formation [9]. These actions make bFGF an essential factor in the culture and maintenance of mature neurons *in vitro*. The utility of this mature neuronal cell culture system was disrupted, however, by another action of bFGF. bFGF triggered neuronal re-entry into the cell cycle through the up regulation of cdk5 expression [11], which resulted in the uncontrolled proliferation of previously post mitotic neurons. Cdk5, which is generally expressed in neuronal cells and plays an important role in neurite outgrowth and migration [11], is expressed by neurons in the presence of bFGF *in vitro* at very low levels between 24-48 hours (lag phase), at high levels during 72-112 hours (proliferative phase) and remains constant after 112 hours (stationary phase) [11]. The level of cdk5 expression corresponded to the proliferative capability of neurons in this culture system, with mitotic division of neurons initiating after 3 *div* and continuing until the neurons reached confluence on the surface. Passage of these confluent neurons to a lower density culture resulted in continued proliferation.

In order to develop a culture system of primary neurons that both morphologically and functionally regenerate without expansion, the initiation and progression of neurons through the cell cycle had to be regulated. First, bFGF was removed from the culture and growth media. This resulted in massive changes in the cultured neurons, with evidence of reorganization of axons, loss of the dentritic trees, appearance of vesicular inclusions, and ultimate loss of neurons due to death. Mature differentiated neurons did not survive long-term or regenerate properly under serum-free culture conditions without the presence of bFGF in the culture medium, and bFGF was reintroduced to the culture medium. In initial attempts to modulate the proliferative effect from bFGF on neurons, commonly used mitotic inhibitors such as FudR and ara-C were used in an attempt to eliminate neuronal cell proliferation. However, because all mitotically active cells were eliminated, this approach proved to be ineffective [48]. As an alternative to these mitotic inhibitors, other agents previously found to have an effect against dividing populations of neurons were investigated. The alpha-tocopherol analog trolox [32, 49], aphidicolin [33], and roscovitine [11, 13, 29] were each shown to be effective in limiting or eliminating cancer and/or progenitor cell division. Of these three agents, roscovitine eliminated neuronal division in this culture system with limited impact on neuronal recovery, regeneration, and long-term survival. Roscovitine, a purine analog that is a potent and selective inhibitor of cdks, is particularly effective as a competitive inhibitor of cdk5/p35 [2, 50], and was introduced into the culture medium in a time- and dose-dependent manner. Roscovitine exerted a strong inhibitory effect upon proliferation and cell cycle progression triggered by the up regulation of cdk5 by bFGF. While cdk5/p35 is active in post mitotic neurons, normal physiological levels do not induce mitotic

division of neurons [11, 13]. The proliferative effect due to the over expression of cdk5 was inhibited by roscovitine, and a stable population of neurons was established. This stable, non-dividing population of mature neurons demonstrated more activity, greater mean axonal length, and more highly branched neurites when compared to neuronal populations where cell cycle re-entry had not been regulated with roscovitine.

This newly discovered ability to both culture primary adult hippocampal neurons under serum-free culture conditions, maintain them in a primary, non-dividing state, and utilize them for purposes of both basic research, drug discovery, and therapeutic testing represents a new and exciting breakthrough in the quest for faster and more targeted drug discovery. The described ability for these post mitotic neurons to re-enter and progress through the cell cycle *in vitro*, ultimately dividing multiple times, represents a new paradigm previously thought to be beyond the capability of primary neurons in the brains of higher vertebrates. The use of cdk inhibitors, specifically roscovitine, to prevent the induction of neuronal division and return neurons to a quiescent yet functionally and electrically active state, capable of forming complex network connections and communication, opens up possibilities into a whole new realm of research in disease mechanisms and potential therapeutics. While neurons in the mature brains of higher vertebrates had previously been thought to be terminally differentiated and incapable of cellular division, we have proven not only can they be induced to divide but to also to return to a non-dividing and functional state.

The ability of mature neurons to divide *in vitro* after bFGF was introduced to the culture medium has potential implications *in vivo*. Overexpression of bFGF or introduction of bFGF into the adult brain potentially could trigger mature neurons to

retract neurites, undergo mitotic division, regenerate mature morphology, and reconnect into the neuronal network. Similar mechanisms have previously been seen in the conversion of non-neuronal cells to functional neurons through the introduction of defined factors [51]. Targeted and reversible induction of mitotic activity in neurons *in vivo* has great potential as a therapeutic intervention for late-stage neurodegenerative disease, such as Alzheimer's disease, or TBI [2, 8, 46]. If the same conditions and environment were to be created *in vivo* as was present in this *in vitro* cell culture system, the remaining mature neurons present in the brains of patients ravaged by age, disease, or injury could potentially be induced to divide and integrate into the brain as a functional network. These neurons could replace those neurons previously lost to damage, age, and disease. In addition, there has been much conjecture about using neural stem cells in cell replacement therapies in various neurodegenerative disease [51, 52]. Using populations of the patients own neurons that have been expanded and then returned to a non-proliferative state with CKIs *in vitro* may be a more effective therapy. Last, because DETA monolayers can be applied not only to glass cover slips but also to electrodes, another potential use of this system lies in its ability to be extended to integrate living and electronic systems.

**Table 3-1: Media compositions**

| Component | Quantity | Vendor | Catalog # |
|---|---|---|---|
| **Dissection medium:** | | | |
| Hibernate-A, calcium free | 500ml | Brain Bits | HA-Ca |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| **Dissociation medium:** | | | |
| Hibernate-A | 500ml | Brain Bits | HA |
| B27 | 2% | Gibco | 17504-044 |
| Glutamax | 2mM | Gibco | 35050-061 |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone | 4μM | Sigma | C0480 |
| Z-Val-Ala-Asp fluoromethyl ketone | 5μM | Sigma | C2105 |
| Dextrose-coated Cerium Oxide Nanoparticles | 100nM | | |
| (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid | 70nM | Sigma | 238813 |
| *Osmolarity adjusted to 295mOsm with NaCl* | | | |
| **Plating medium:** | | | |
| Neurobasal-A | 500ml | Gibco | 10888 |
| B27 | 2% | Gibco | 17504-044 |
| Glutamax | 2mM | Gibco | 35050-061 |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| BDNF, recombinant human | 20ng/ml | Cell Sciences | CRB600B |
| NT-3, recombinant human | 20ng/ml | Cell Sciences | CRN500B |
| bFGF, recombinant human | 5ng/ml | Invitrogen | 13256-029 |
| Insulin-like Growth Factor-I (E3R) human | 20ng/ml | Sigma | I2656 |
| Dextrose-coated Cerium Oxide Nanoparticles | 100nM | | |
| (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid | 70nM | Sigma | 238813 |
| *Osmolarity adjusted to 295mOsm with NaCl* | | | |
| **Maintenance medium:** | | | |
| Neurobasal-A | 500ml | Gibco | 10888 |
| B27 | 2% | Gibco | 17504-044 |
| Glutamax | 2mM | Gibco | 35050-061 |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| BDNF, recombinant human | 20ng/ml | Cell Sciences | CRB600B |
| NT-3, recombinant human | 20ng/ml | Cell Sciences | CRN500B |
| bFGF, recombinant human | 5ng/ml | Invitrogen | 13256-029 |
| Insulin-like Growth Factor-I (E3R) human | 20ng/ml | Sigma | I2656 |
| *Osmolarity adjusted to 295mOsm with NaCl* | | | |

Culture periods for use of each medium: *Dissection* - dissection and enzymatic digestion of the tissue specimen. *Dissociation* – mechanical dissociation of the tissue specimen. *Plating* – attachment of individual cells to substrate through culture day 3. *Maintenance* – culture day 3 through the end of the culture

**Table 3-2: Electrical properties of adult rat hippocampal neurons after 6, 13, or 25 *div,* examined through whole-cell patch clamp electrophysiology**

|  | 6 *div* | 13 *div* | 25 *div* |
|---|---|---|---|
| Number of cells examined | 14 | 14 | 13 |
| Vm (mV) | -36.5 ± 2.8 | -45.6 ± 2.9 | -45.9 ± 4.3 |
| Rm (mΩ) | 185 ± 37.4 | 328 ± 32.1 | 337 ± 38.9 |
| Cm (pF) | 13.1 ± 2.9 | 18.2 ± 1.2 | 17.6 ± 2.1 |
| Rseries (mΩ) | 16.7 ± 2.8 | 17.2 ± 1.3 | 17.9 ± 2.0 |
| Inward Current, $Na^{2+}$ (pA) | -722 ± 47.0 | -868 ± 87.7 | -849 ± 122.8 |
| Outward Current, $K^+$ (pA) | 1110 ± 121.5 | 1332 ± 84.3 | 1412 ± 74.2 |
| Vthr (mV) | -41.5 | -42.7 | -41.9 |
| AP Amplitude (mV) | 87.6 ± 9.1 | 98.3 ± 8.2 | 96.4 ± 11.1 |
| AP Duration (ms) | 4.6 ± 0.9 | 5.3 ± 1.7 | 5.2 ± 1.8 |

*div*=culture days *in vitro*, Vm=resting membrane potential, Rm=membrane resistance, Cm=membrane capacitance, Rseries=series resistance, Vthr=action potential threshold voltage, AP=action potential.  Data is presented as mean +/- S.E.M.
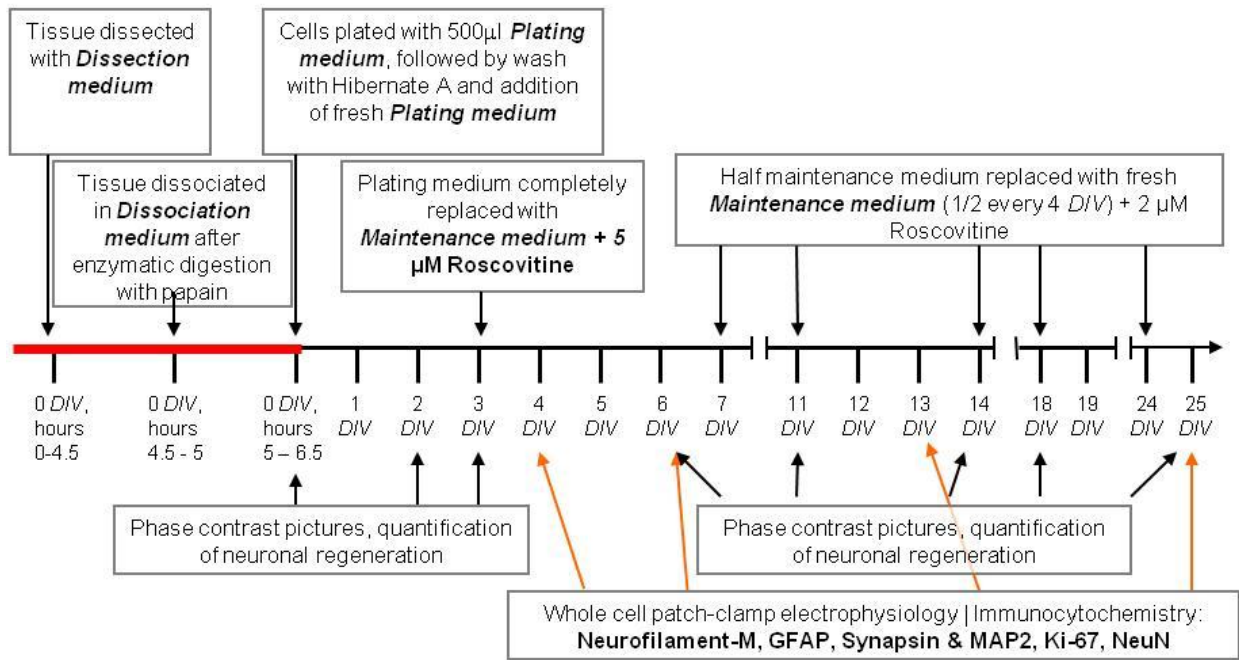
**Figure 3-1: Culture methodology for processing and dissociation of adult rat hippocampal tissue to create and maintain a dissociated neuronal cell culture.** The time scale of the cell culture shows timed application of growth factors and quantification of various neuronal parameters. The cells were examined immunocytochemically after 6, 14, and 25 days *in vitro* (Neurofilament-M, Ki-67, NeuN, MAP2, Synapsin, and GFAP). Electrical parameters of cells were examined after 6, 13, and 25 days *in vitro.* Cell division examined with and without applied Roscovitine between 1-80 days *in vitro*
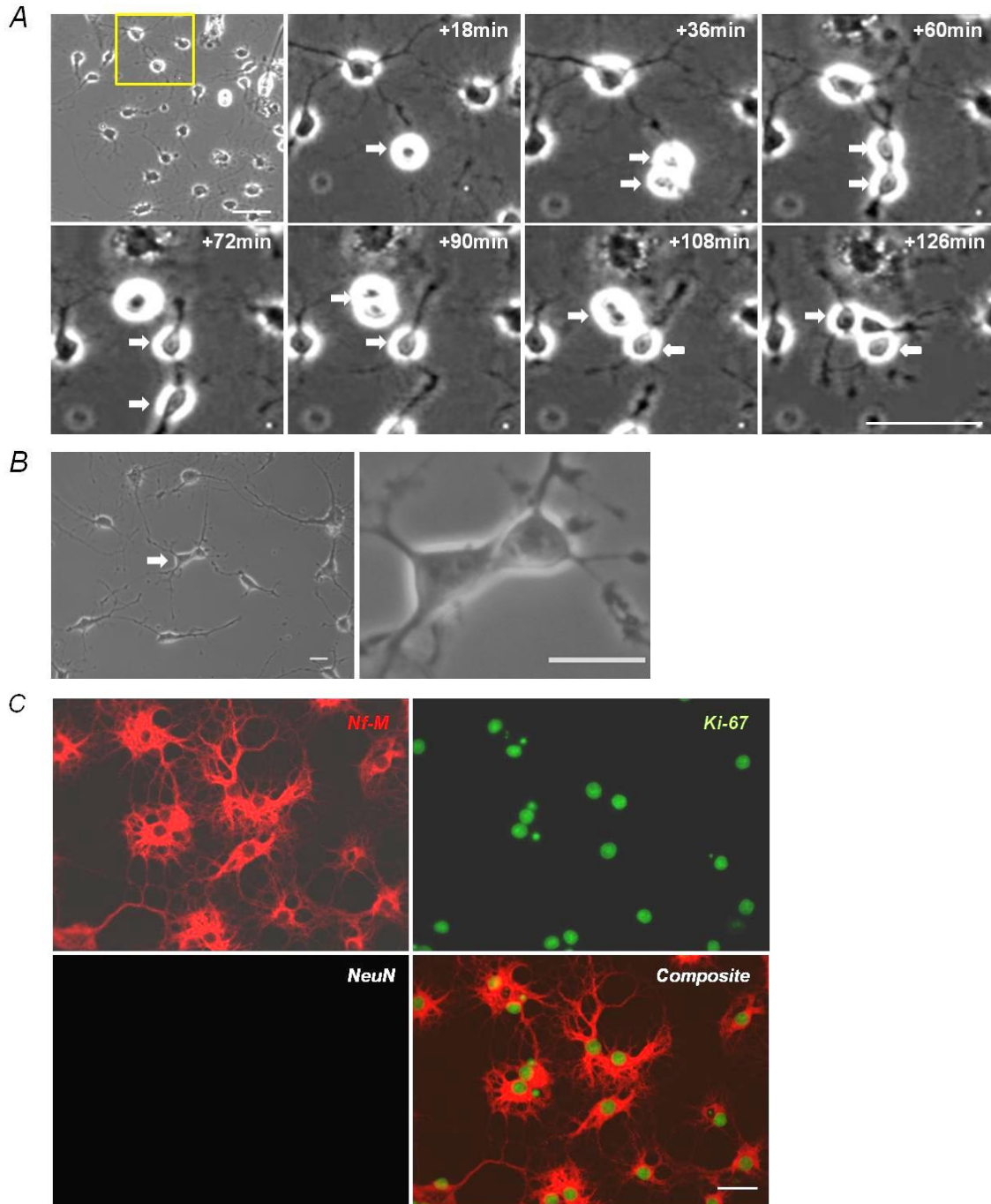
**Figure 3-2: bFGF induces adult hippocampal neurons to divide *in vitro*.** A: Screen shots from time-lapse video of neurons dividing *in vitro*. Scale bar 50 microns . B: Adult neuron(s), after mitotic division, reestablishing neurites and connections. Scale bar 20 microns . C: Dividing cells express Ki-67 (green) and neurofilament-M (red), do not express NeuN. Scale bar 20 microns

A

B

C

D

Control        5μM Rosc

11 DIV        11 DIV

17 DIV        17 DIV

20 DIV        20 DIV
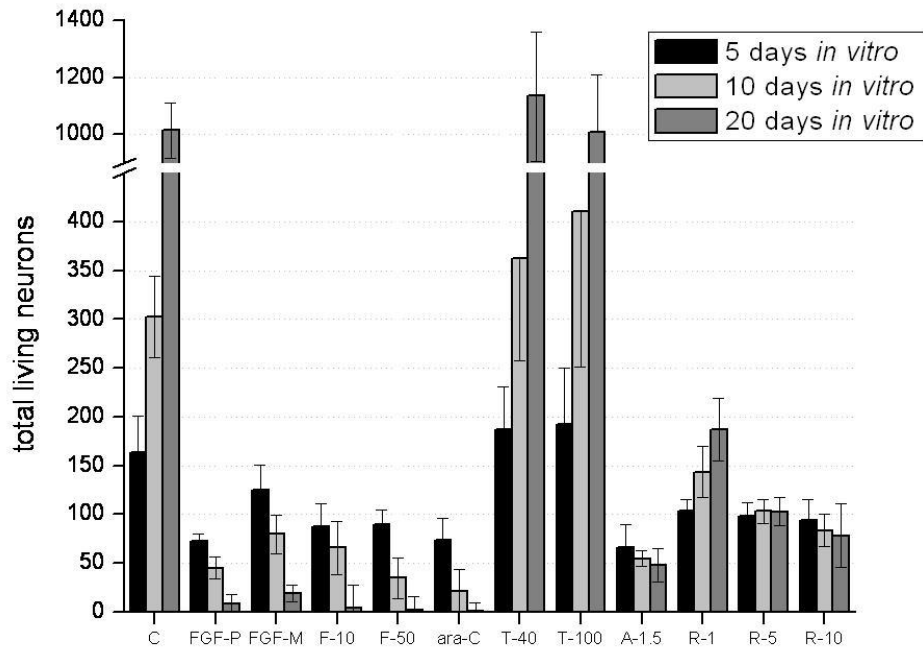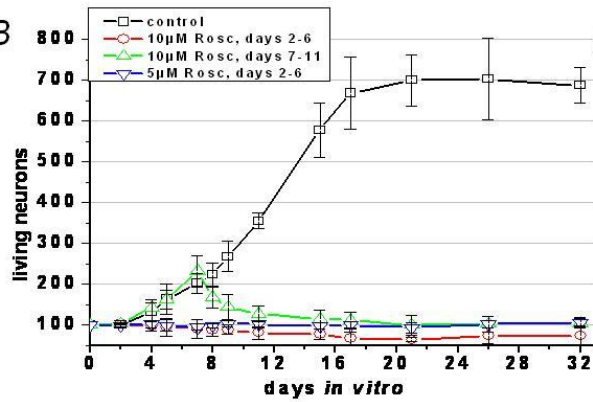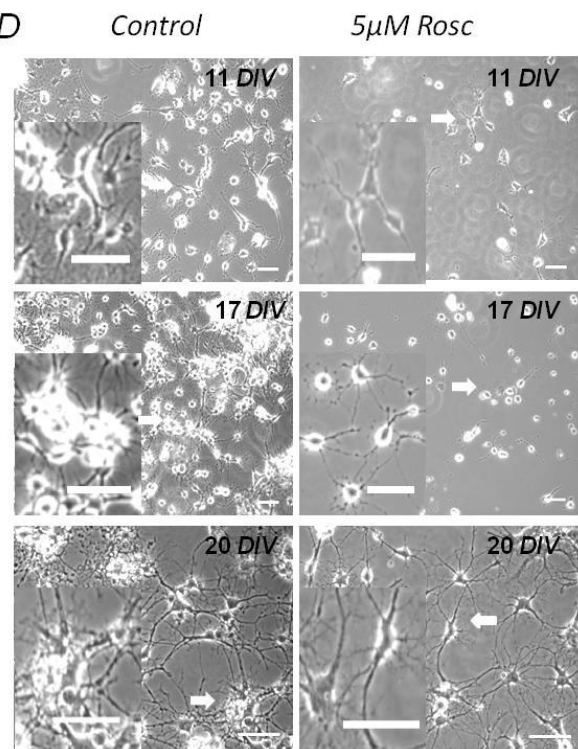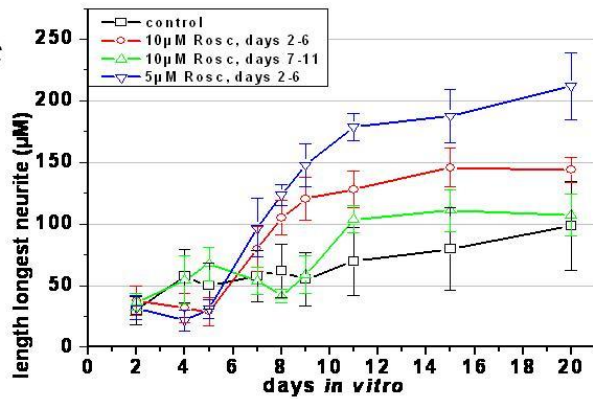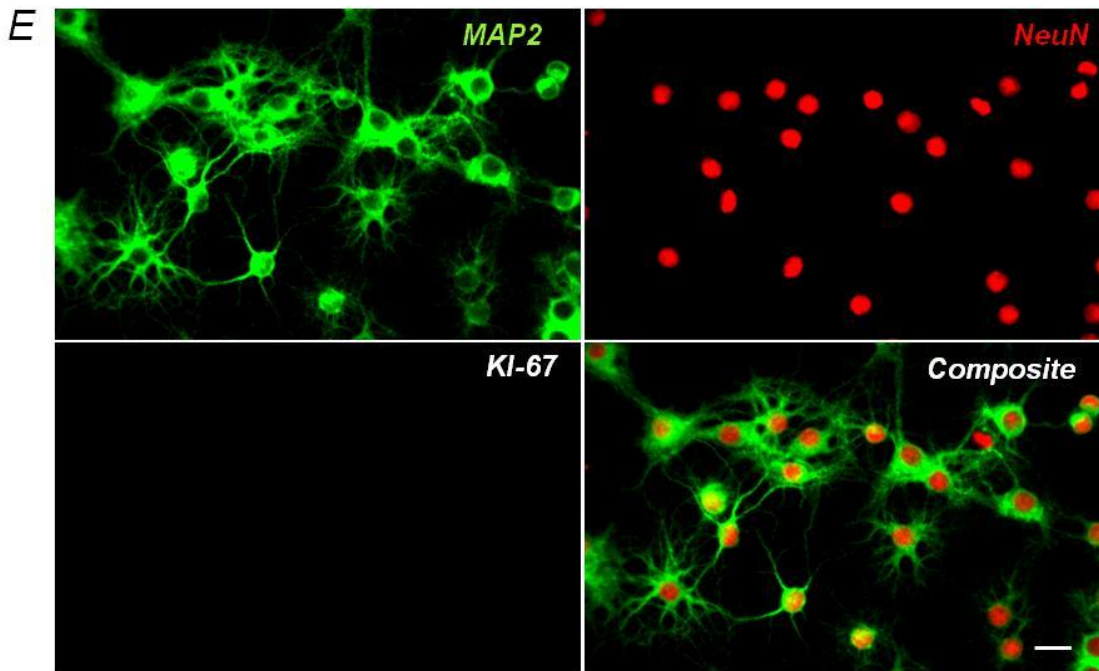
**Figure 3-3:** **Roscovitine prevents mature adult hippocampal neurons from returning to the cell cycle in the presence of bFGF and dividing *in vitro*.** A. Neuronal population after growth factor removal or addition of mitotic factors. All day 0 cell densities normalized to 100 to allow for comparison. C – control, standard medium and factors, FGF-P - bFGF removed from both the plating and maintenance medium, FGF-M - bFGF removed from the maintenance medium, F-10 – 10 µM FudR on 2 *div*, F-50 – 50 µM FudR on 2 *div*, ara-C – ara-C on 2 *div*, T-40 – 40 µM Trolox on 2 *div*, T-100 – 100 µM Trolox on 2 *div*, A-1.5 – 1.5 µM Aph on 2 *div*, R-1 – 1 µM Rosc on 2 *div*, R-5 – 5 µM Rosc on 2 *div*, R-10 – 10 µM Rosc on 2 *div*. B, C: Effect on neuronal cell division and neurite length from the addition of different concentrations of Rosc *in vitro*. All day 0 cell densities normalized to 100 to allow for comparison. Control - neurons were not treated. Group 1 – neurons treated with 10µM Rosc days 2-6, 2 µM Rosc after day 6. Group 2, neurons treated with 10 µM Rosc days 7-11, 2µM Rosc after day 11. Group 3, neurons treated with 5 µM Rosc days 2-6, 2 µM Rosc after day 6. D: Phase pictures of control, untreated neurons (bFGF, no Rosc) and neurons in the presence of both bFGF and Rosc, between 11-20 *div*. Adult neurons not treated with Rosc divide *in vitro* until confluent, while the population of neurons after treatment with 5 µM Rosc was stable *in vitro*. scale 50 microns. E: Neurons express MAP2, NeuN, do not express Ki-67 *in vitro* in the presence of 5 µM Rosc. Scale 20 microns
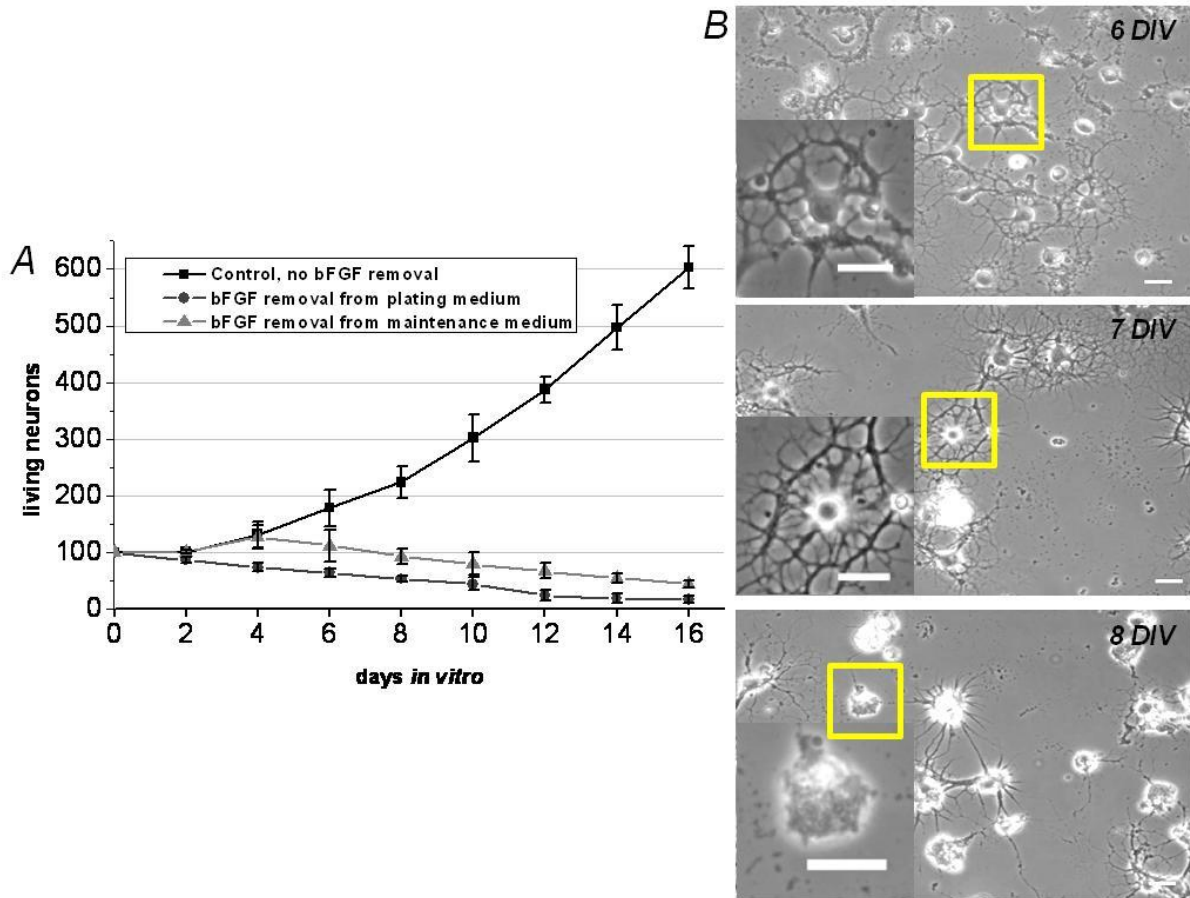
**Figure 3-4: Effect from bFGF removal on neuronal survival and proliferation.** A. bFGF in culture medium caused neurons to divide *in vitro,* while removal of bFGF caused neuronal loss through apoptosis and necrosis. All day 0 cell densities normalized to 100 to allow for comparison. B. Removal of bFGF from the culture media prevented neuronal division but triggered apoptosis. 6, 7, and 8 days *in vitro* in neuronal populations grown in media without bFGF. Scale, 20 microns
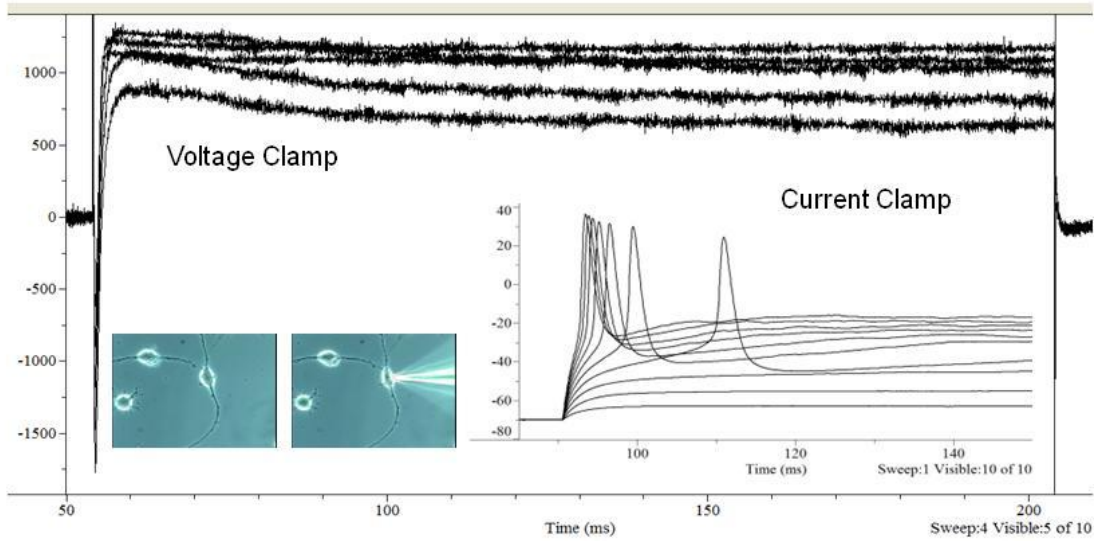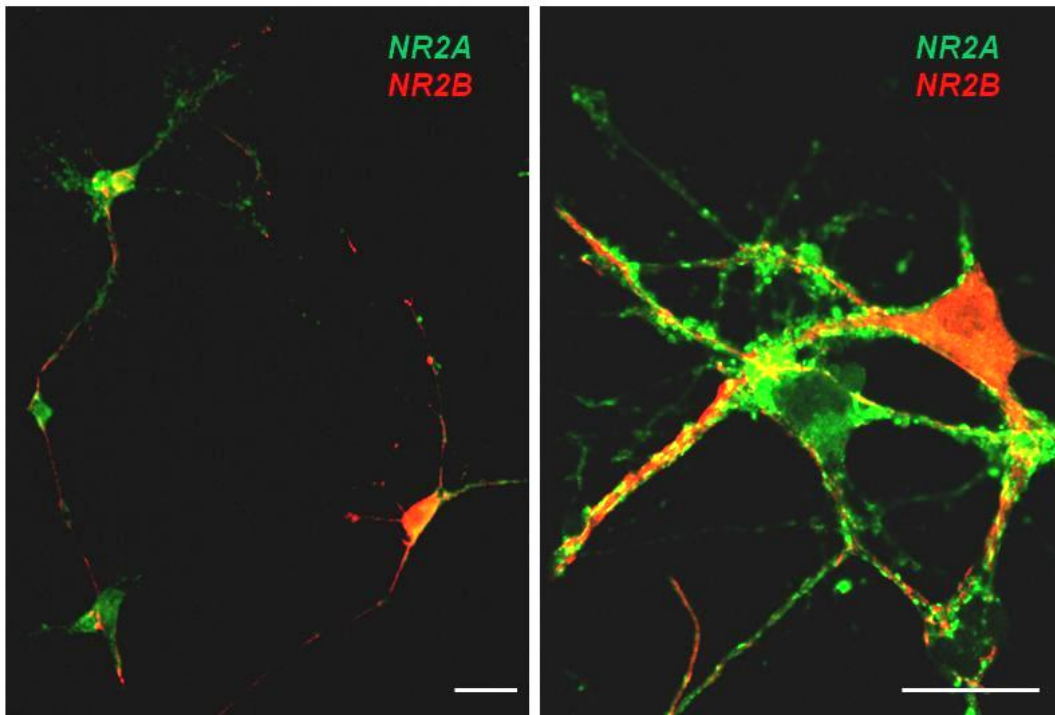
**Figure 3-5**: **Neuronal maturity identified through electrical activity and protein expression analysis:** A. Representative trace for voltage and current clamp of an adult neuron firing a single action potential as well as moving current into and out of the cell through voltage-gated ion channels. These traces originated from a adult rat hippocampal neurons after 13 *div*, B, NR2A and NR2B expression after 20 *div* in neurons in roscovitine treated cultures. Scale bars 20 microns.

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# CHAPTER 4 - TWO CELL NETWORKS OF ADULT HIPPOCAMPAL NEURONS ON SELF-ASSEMBLED MONOLAYERS FOR THE STUDY OF NEURONAL COMMUNICATION *IN VITRO*.

## Introduction

Chemical conduction of action potentials in the central nervous system (CNS) depends upon the formation of synapses between the axon of one neuron and the dendrite or soma of another [1, 2].  At a synapse, the plasma membrane of the presynaptic terminal, or synaptic bouton, comes in close contact with the membrane of the target postsynaptic cell, with extensive molecular machinery present in each to link the two membranes together [3].  Excitatory neurons in the hippocampus release the neurotransmitter glutamate into the synaptic cleft which binds to ligand-gated ion channels in the postsynaptic membrane, producing an influx of ions to create an excitatory postsynaptic potential (EPSP) [4-6].  If the EPSP is strong enough to reach the cell's activation threshold, an action potential will be triggered in the postsynaptic cell [7].

Synaptogenesis, synaptic communication, and synaptic plasticity have been extensively studied using hippocampal brain slice or dissociated cell cultures in a variety of serum containing or serum-free *in vitro* systems [8].  Studies of communication in dissociated cultures typically rely upon hippocampal neurons extracted from the brains of embryonic rat or mouse embryos [9].  Hippocampal slice cultures, where thin slices of intact hippocampal tissue are removed from adult brain tissue, are most widely used for electrophysiological studies into neuronal communication, long-term potentiation (LTP) , and pathophysiology of brain disease [10-12].

Previously, we developed an adult hippocampal culture system utilizing a novel culture method, novel serum-free media formulation, and the non-biological substrate N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine (DETA) [13, 14]. This substrate forms self assembled monolayers (SAMs) on any hydroxylated surface, is non-degradable by cells, promotes both the attachment and regeneration of neurons *in vitro*, and, through photolithographic patterning, can be used to control neuronal attachment and direct axonal outgrowth [15, 16]. Laser ablation photolithography has been used to generate patterned surfaces with regions that support cell adhesion and regeneration, and other regions that do not [17]. Poly(ethylene) glycol (PEG) SAMs prevent the adsorption of proteins on glass surfaces by the entropy/hydrated surface hypothesis [18-20]. Therefore, a surface composed of small connected regions of DETA surrounded by PEG would facilitate the adhesion of small numbers of neurons and direct regeneration to facilitate the formation of small networks of synaptically connected neurons.

In this study, we demonstrate the formation of small networks of electrically active, synaptically connected neurons derived from the hippocampus of adult rats. Limited neuronal attachment and directed neurite outgrowth to form these small networks was controlled using the photolithographic patterned substrates PEG-DETA. Serum-free media formulations, anti-mitotic factors, and the neurotransmitter glutamate supported the formation of small synaptically connected networks composed of mature, terminally differentiated glutamatergic neurons that were stable for long periods of time *in vitro*. These finding fill a void in the study of synaptogenesis, synaptic communication, synaptic plasticity, neuropharmacology, and brain disease pathophysiology in mature neurons by allowing for easy identification of connected

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neurons and by providing a stable culture system by which the same neurons can be studied over time.

**Materials and Methods**

*DETA surface modification and characterization*

Glass cover slips (VWR cat. nr. 48366067, 22×22 mm2 No. 1) were chemically cleaned by using serial acid baths. First, the surfaces were soaked in a 50:50 solution of concentrated hydrochloric acid (VWR cat. nr. EM1.00314.2503) in methanol (VWR cat. nr. BJLP230-4) for 2 hours. Second, the surfaces were immersed in concentrated $H_2SO_4$ for at least 2 hours. After each acid soak, the surfaces were carefully washed 3 times with DI water. The final water rinse was followed by boiling the glass slides in DI water for 30 minutes. The cleaned glass slides were placed in an oven set at 110 °C and allowed to dry overnight. The DETA (N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine, United Chemical Technologies Inc., Bristol, PA, T2910) monolayer was formed by the reaction of the cleaned and dried cover slips with a 0.1% (v/v) solution of the organosilane in dry toluene (VWR cat. nr. BDH1151) [21]. Dry toluene was prepared by distillation over metallic sodium to remove any water or other contaminants. The cover slips were heated to 100 °C in DETA-toluene solution, rinsed with toluene, reheated to 100 °C in toluene, and then oven dried.

*PEG surface modification*

Glass cover slips (VWR cat. nr. 48366067, 22×22 mm2 No. 1) were chemically cleaned and dried as mentioned above. The cover slips were then coated with a PEG-terminated silane by a modified protocol from Papra et al [22]. Grafting was done by incubation of the glass cover slips in 3 mM PEG-silane, 2-[Methoxypoly (ethyleneoxy)propyl]trimethoxysilane (Gelest, Tullytown, PA), solution in toluene with

37% HCl added to achieve a final concentration of 0.08% (0.8 ml HCl/l) for 45 minutes at room temperature. The glass cover slips were then rinse once in toluene, twice in ethanol and twice in DI water, and sonicated in DI water for 5 minutes to remove the non-grafted material. The surfaces were blown dry with nitrogen and stored in a dessicator until needed.

*Deep-UV photolithography of PEG-silane monolayers*

PEG-silane modified glass cover slips were patterned using a deep UV (193 nm) excimer LASER (Lambda Physik, Santa Clara, CA) at a pulse power of maximum 200 mJ and a frequency of 10 Hz for 45 seconds through a quartz photomask (Bandwidth Foundry, Eveleigh, Australia). The photomask was written in dark-field polarity such that the patterns were transparent and the area surrounding the patterns was opaque. The UV irradiation of PEG-silane slides through this mask resulted in the formation of glass patterns surrounded by a PEG-coated background. After patterning the samples were immediately derivatized with DETA (back-fill).

*Back-fill of patterned PEG-silane monolayers with DETA-silane*

The patterned PEG-silane substrates were backfilled with DETA following a protocol similar to the one mentioned above. The difference consisted in using mild reaction temperatures, in order to protect the PEG monolayer. A 0.1% (v/v) DETA-toluene solution was prepared inside the glove box. The DETA-toluene solution was removed from the glove box and transferred to the beaker containing the samples. The cover slips were gently heated to no more than 65 °C in DETA-toluene solution, rinsed

with toluene and reheated to 65 °C in toluene. After the final wash the samples were allowed to dry at room temperature for at least 48 h before use.   The values are reported as the mean ± SEM.

*Contact angle goniometry analysis*

The surface contact angle of un-modified (clean glass cover slips) and modified (PEG or DETA) substrates was measured by contact angle goniometry using a Ramé Hart (Netcong, NJ) contact angle goniometer. In all cases, the contact angle of a static sessile drop (5µl) of water placed on the sample was measured three times and averaged. A contact angle of less than 5 degrees was obtained for clean substrates.  An average of 37 ± 2o was measured for PEG substrates, and of 49 ± 2o for DETA control slides.

*X-Ray Photoelectron Spectroscopy*

In order to authenticate the monolayer formation, both PEG and DETA control surfaces were characterized by X-ray Photoelectron Spectroscopy (XPS) using a VG ESCALAB 220i-XL spectrometer equipped with an aluminum anode and a quartz monochromator. The spectrometer was calibrated against the reference binding energies of clean Cu, Ag and Au samples. XPS survey scans were recorded in order to determine the relevant elements (pass energy of 50 eV, step size of 1 eV). Si 2p, C 1s, N 1s, and O 1s high resolution spectra were recorded in order to determine the quality of the surfaces (pass energy of 20 eV, step size of 0.1 eV). The fitting of the peaks was performed with Avantage version 3.25 software provided by Thermo Electron

Corporation. The quality of the surfaces was in agreement with previously reported results [21-23].

*Palladium-catalyzed metallization of patterned silane monolayers*

Patterned samples were visualized using a palladium-catalyzed copper reduction reaction, modified from Kind et al [24]. In this reaction, copper is deposited in regions containing the amine terminated silane DETA.

*Adult rat hippocampal dissociated cell culture methodology*

Adult neurons are extracted, dissociated, cultured, and maintained using a protocol and medium described elsewhere [14]. Briefly, the hippocampus of adult rats (Charles River, age 6-12 months) were dissected and homogenized into small tissue fragments in cold medium (~4ºC) consisting of Hibernate-A, glutamax, and antibiotic-antimycotic. The tissue was digested for 30 minutes at 37°C in calcium-free Hibernate-A (HA) containing 6 mg papain / 12 ml (HA no calcium). Following digestion, the tissue was washed three times with cold HA media to remove any active enzyme. Next, the tissue was suspended in Dissociation Medium and broken apart into individual cells through mechanical dissociation with fire-polished Pasteur pipettes. The dissociated cells were suspended in Plating medium and then deposited onto DETA-coated glass cover slips for 30-45 minutes. The cover slips were washed with warm HA by gently swirling the medium to remove tissue debris. Following this washing step fresh Plating medium was applied and remained for the first 3 *div*. On 3 *div* the medium was removed and replaced by Maintenance Medium with 5 µM Roscovitine (Rosc, Sigma,

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R7772). All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines.

*Adult neuronal network formation on patterned PEG-DETA surfaces*

After 4 *div*, the adult hippocampal neurons on the DETA cover slips were passaged to PEG-DETA patterned cover slips. Briefly, neurons were dislodged from the DETA with trypsin (.05% trypsin / EDTA in HBSS, Gibco, 25200). Trypsin inhibitor (trypsin inhibitor, soybean, Gibco, 17075-029) in Dissociation medium at 0.5 mg per ml deactivated the trypsin. The dislodged neurons were collected and spun at 500 x g for 5 minutes. The supernatant of deactivated trypsin in HBSS was discarded, and the neuronal cell pellet was suspended in 1 ml Plating medium. The neurons in suspension were counted using a Bright-Line hemacytometer, and neurons were plated onto the PEG-DETA patterned cover slips at 50 cells / $mm^2$ in Plating medium supplemented with 5 µM Rosc. After two days post-plating (*dpp*), glutamate (N-Acetyl-L-glutamic acid, Aldrich, 855642) was added to the plating medium to a final concentration of 25 µM. On 3 *dpp* and again every 4 day after, ½ the medium was removed and replaced with fresh Maintenance medium supplemented with 2 µM Rosc.

*Time-Lapse Microscopy*

Time-lapse recording was performed immediately after the cells were plated onto PEG-DETA Fish patterns. Living cells were observed under an inverted microscope (Zeiss-Axiovert 100) equipped with Plan-Neofluar 40x objective (Zeiss, Oberkochen, Germany) and a humidified incubation chamber for constant temperature at 37ºC and

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5% CO$_2$. Pictures were captured with a Hamamatsu C8484-05G digital charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan). Experiments were run under the control of Okolab software (OKO-lab, Ottaviano, NA, ITALY). Pictures were taken under the control of the software every 5 minutes, and live cell image sequences were compiled to create videos, 12 images per second (video 4-1).

*Immunocytochemistry and Laser Scanning Confocal Microscopy*

To prepare cells for immunocytochemical characterization, cover slips were rinsed twice with Phosphate Buffered Saline (PBS). Cells were fixed with 4% paraformaldehyde for ten minutes at room temperature, and subsequently rinsed three times with PBS. Cells were permeabilized for five minutes with 0.5% Triton X-100 in PBS, and were then blocked for two hours in 5% normal goat serum in PBS. Anti-neurofilament-M (Chemicon, AB5735, 1:500), anti-synaptophysin (Chemicon, MAB368, 1:300), and either anti-NMDAR2A (Chemicon, AB1555P, 1:200), anti-NMDAR2B (Chemicon, AB15557P, 1:200), or anti-glutamate receptor 2 & 3 (Chemicon, AB1506, 1:50) were added in blocking solution for 12 hr at 4°C. After 3 washes with PBS, fluorescently labeled secondary antibodies (Invitrogen, A11011 (594nm), A21449 (647nm), and A11029 (488nm), 1:200) in blocking buffer were applied for two hours. Vectashield mounting medium with DAPI (H1200, Vector Laboratories, Burlingame, CA) was used to mount the cover slips onto slides. Fluorescent images were acquired with the UltraView spinning disc confocal system (PerkinElmer) with AxioObserver.Z1 (Carl Zeiss) stand, and a Plan-Apochromat 40x/1.4 Oil DIC plan-apochromat objective with

26 μm resolution. Z-stack projections of the scanned images were generated and modified within the Volocity image processing program (PerkinElmer).

*Dual whole-cell patch clamp electrophysiology*

Extracellular recording solution was comprised of Neurobasal-A medium. The solution contained 130 mM NaCl, 1.8 mM $CaCl_2$, 5.2 mM KCl, 1 mM $MgCl_2$, 2.2 mM $NaHCO_3$, and 10 mM HEPES (pH 7.3) (300 mOsm). Patch pipettes (4-8 MΩ) were filled with intracellular solution (K-gluconate 140 mM, EGTA 1 mM, $MgCl_2$ 2 mM, $Na_2ATP$ 5 mM, HEPES 10 mM; pH 7.2). Cells were visualized on the stage with a Zeiss Axioscope, 2 FS Plus, upright microscope in Maintenance culture medium. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (MDS Analytical Devices) amplifier. Signals were low-pass filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recordings and analysis were performed with Clampex software. Whole-cell capacitance and series resistance were compensated electronically. Only cells with access resistance less than 22 MΩ were analyzed. Inward currents that had the characteristics of fast sodium currents, and outward currents that had the characteristics of potassium currents, were measured in voltage clamp mode. Voltage step length was 50 ms, incremented 20 mV per step, 1 s between each step, with a holding potential of -70 mV. The action potential threshold was measured in current-clamp mode with increasing 1 s depolarizing current injections. The protocol for determining the presence and type of neuronal synapses has been described previously [24]. In brief, the presynaptic neuron was set in current clamp mode and the postsynaptic neuron in voltage clamp mode. The type of synapse

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is determined by holding the presynaptic membrane at 3 different holding potentials: -70 mV, -30 mV, 0 mV.

**Results**

*Surface modification to create cell-adhesive "Fish" patterns of DETA against a background of non-cell supportive PEG*

The DETA foreground surrounded by PEG background provided a pattern that supported the attachment of neurons and directed regeneration of dendrites and axons along surface cues in order to promote the formation of small networks of neurons (Figure 4-1). Control cover slips were used in order to test the quality of the PEG-DETA patterns: (1) one PEG cover slip was ablated without a photomask followed by DETA deposition, and (2) a DETA monolayer was deposited upon a second PEG without prior ablation. Laser irradiation and DETA deposition were done in the same conditions as for the PEG-DETA patterns.

The XPS measurements of the control cover slips show that PEG formed a SAM on glass cover slips (Figure 4-1 A). Additionally, DETA formed a SAM on ablated PEG, but was not incorporated (or only incorporated in traces amounts) in the unexposed PEG regions (Figure 4-1 B-C). Further, static water contact angle measurements of 92±2 validated the hydrophilicity of the laser exposed PEG after DETA rederivatization. However, the non-ablated PEG monolayer was not affected by the reaction with DETA, as was also revealed by the contact angle values of 45±3 on unexposed PEG control cover slip(s), values that are close to the ones for pure PEG.

The pattern uniformity was verified by copper reduction metallization (Figure 4-1 D), with the light regions representing the cell-adhesive DETA regions of the patterns. The "Fish" patterns, so named because of its similarity to a fish icon, have two somal adhesion sites (SAS) of 30 µm diameter approximately 150 µm apart connected by 5 µm lines of DETA. The dimensions of the patterns promoted the attachment of neurons

onto the SAS, regrowth of axons along the connecting lines, and dendritic branching along the dotted strips of DETA. Copper deposition results were consistent between Fish pattern batches, showing the reproducibility of these patterns.

*Attachment and regeneration of neurons on patterns*

Small patterns of adult hippocampal neurons were prepared using defined media formulations and a two step culture process that allowed the optimal number of neurons to be deposited for proper in vitro neuronal network formation (Figure 4-2). In the first step, the hippocampus of adult rats was processed to dissociate the neurons. These neurons were plated on DETA cover slips (Figure 4-3, 4 *div*). After 4 *div*, a period during which the neurons recover and regenerate, the neurons were passaged from the DETA cover slip(s), counted, and plated at 50 cells / $mm^2$ on PEG-DETA Fish patterned cover slips. Neuronal conformity to the Fish patterns was analyzed throughout the study, with the optimum conformity being 2 neurons per Fish pattern, each on opposite SAS, with 100% of neurons found on the DETA patterns versus the PEG background. As shown in Table 4-1, pattern conformity improves over time, increasing from 7.1 ± 1.2 neurons per pattern after 2 *dpp* to 3.5 ± 0.4 after 14 *dpp*. Similar improvements to pattern conformity were seen in neurons per SAS and the percentage of neurons on the patterns versus the background (72.3±6.7% on 14 *dpp*). Pattern conformity improved over time in part due to the non-adhesive nature of the PEG background, with neurons either migrating to the more adhesive DETA regions or washing off the cover slip(s). Additionally, adult neurons on the patterned regions continued to regenerate neurites and to migrate along the patterned area (video 4-1).

*Formation and maintenance of synaptic connections*

While the adult neurons physically connected their axons and dendrites along the guided DETA path of the Fish pattern, testing was needed to determine whether functional synaptic connections had formed. First, control cover slips with passaged neurons were examined immunocytochemically after 14 *dpp* to determine the expression of pre- and postsynaptic markers. The presynaptic marker synaptophysin and the postsynaptic receptor sub-units for AMPA and NMDA channels were all present (Figure 4-4 A). These pre- and postsynaptic proteins indicated the presence of mature synapses [24-28].

Dual-patch clamp electrophysiology was performed on neurons on Fish patterns after 14 *dpp* in order to measure the function of the synaptic connections between neurons. By varying the holding potential of the presynaptic neurons to -70 mV, -30 mV, or 0 mV, the type of synapse(s) present, either excitatory, inhibitory, or electrical, was able to be measured. Each type of synapse manifests in different responses in the postsynaptic neuronal current flow [24].

Two neurons on the Fish pattern were simultaneously patched (Figure 4-4 B-a), and electrophysiological recordings were performed to show the neuronal nature of these cells. The channel conductance of each of the cells was measured in voltage clamp mode, with both cells showing strong inward and outward sodium and potassium-dependent current flow consistent with neurons (Figure 4-4 B-b). The electrical ability of each cell, as a function of ability to fire action potentials, was measured in current clamp mode. Each cell generated a single action potential upon stimulation, consistent with

adult hippocampal neurons in dissociated cell culture (Figure 4-4 B-c). Next, neuron A, the presynaptic neuron, was held at -70 mV (Figure 4-4 B-d), -30 mV (Figure 4-4 B-e), or 0 mV (Figure 4-4 B-f), an action potential was evoked, and the postsynaptic currents were measured in neuron B. The large inward current response at -70 mV holding, lower response at -30 mV holding, and very small response at 0 mV holding were consistent with excitatory synaptic connections.

**Discussion**

The development of an *in vitro* system where attachment and regeneration of adult hippocampal neurons was guided using photolithography to form small synaptically connected neuronal networks represents a significant technological advancement. PEG-DETA surfaces prepared by laser ablation photolithography were sufficient to direct the attachment of cells specifically to the DETA "Fish" patterns while restricting attachment to the PEG background. Mature, terminally differentiated neurons derived from hippocampal brain tissue of adult rats attached, adhered, and regenerated functional neurites along the guided DETA cues of the Fish pattern. These neurons were found to be both electrically active and synaptically connected, and displayed synaptic connectivity characteristic of excitatory glutamatergic neurons.

In previous experiments we developed a culture system that supported the attachment, survival, and regeneration of electrically active neurons derived from the hippocampus of adult rats [13, 14]. Neurons cultured under these conditions were passaged from unpatterned DETA cover slips to PEG-DETA fish patterned cover slips. These cells reacted to the guidance cues provided by the DETA substrate to attach and regenerate along the DETA lines to form small engineered neuronal networks. Initially the majority of cells attached to the PEG background but over time fewer cells were found on the PEG background versus the DETA patterns (23.5 ± 13.2% on DETA patterns on 2 *dpp*, improving to 72.3 ± 6.7% on DETA patterns on 14 *dpp*). Additionally, pattern conformity improved over time, improving from 7.1 ± 1.2 neurons per DETA pattern on 2 *dpp* to 3.5 ± 0.4 on 14 *dpp*.

The electrical characteristics of individual adult hippocampal neurons were identified in earlier experiments [13, 14, 29]. Individual neurons on DETA fish patterns have distinctive adult neuronal sodium and potassium currents and fire action potentials when stimulated (Figure 4-5 B-b, c). Using dual whole cell patch-clamp electrophysiology, the function and properties of synaptic connections between neurons on these patterns were measured. Excitatory synapses, with large inward current response in the postsynaptic neuron in response to action potential in the presynaptic neurons at -70 mV holding, lower response at -30 mV holding, and very small response at 0 mV holding, were identified.

Chemical conduction of action potentials in the CNS depends upon the formation of synapses between the axon of one neuron and the dendrite or soma of another [1, 2]. At a synapse, the plasma membrane of the presynaptic terminal, or synaptic bouton, comes in close contact with the membrane of the target postsynaptic cell, with extensive arrays of molecular machinery present in each to link the two membranes together [3]. Excitatory neurons in the hippocampus release the neurotransmitter glutamate into the synaptic cleft which binds to ligand-gated ion channels in the postsynaptic membrane, producing an influx of ions to create an EPSP [4-6]. If the EPSP is strong enough to reach the threshold, an action potential will be triggered in the postsynaptic cell [7]. Expression of the machinery required for excitatory synaptic connections was evaluated in adult hippocampal neurons in this culture system. Neurons expressed synaptophysin, the presynaptic vesicle glycoprotein used to quantify synapses [28]. The NMDA and AMPA channel subunits NR2A, NR2B, and GluR2/3, the distinctive postsynaptic ligand-gated ion channels that control EPSP [24-27], were all present in

adult hippocampal neurons in this culture system. The presence of both presynaptic and postsynaptic EPSP proteins and characteristic excitatory synaptic electrophysiological parameters confirmed the presence of excitatory synapses between neurons on Fish patterned cover slips.

DETA's efficacy as a biological substrate for bioengineering applications is founded in its reproducible nature and its ability to be patterned using photolithography [21]. Its role in patterning applications as the cell-permissive substrate that is surrounded by non-cell permissive SAMs is further strengthened because it is not degraded by the cells plated on it [22, 23]. This characteristic of DETA allows it to form sharp patterns that do not blend with the non-cell permissive PEG background monolayer and promotes pattern stability under long-term culture conditions [15, 23]. PEG SAMs prevent the adsorption of proteins on glass surfaces by the entropy/hydrated surface hypothesis [18-20]. Therefore, a surface composed of small connected regions of DETA surrounded by PEG facilitates the adhesion of small numbers of neurons and directs regeneration form small networks of synaptically connected neurons. These SAMs can also be applied to any hydroxylated surface or material, meaning these fish patterns can be applied to micro-electrode array (MEA) devices for high throughput electrical studies of small networks of neurons.

In this study, the adhesion of adult hippocampal neurons and neurite outgrowth were managed using patterned PEG-DETA Fish patterns. Neurons in this system recovered to form small networks of synaptically connected excitatory neurons. This system provides a unique tool that can be used for studies into LTP [30-32]. In addition, this functional *in vitro* system would enable high-throughput neuropharmacology

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studies, facilitating drug development and furthering research into different neurological

disorders.

**Table 4-1: Neuronal conformity to PEG-DETA Fish Pattern(s).**

| Culture Day (post passage) | Neurons per Pattern | Neurons per SAS | % on Patterns |
|---|---|---|---|
| Day 2 | 7.1 ± 1.2 | 1.3 ± 1.1 | 23.5 ± 13.2 |
| Day 6 | 5.2 ± 0.9 | 2.2 ± 0.7 | 47.5 ± 11.2 |
| Day 10 | 3.7 ± 0.5 | 2.1 ± 0.6 | 65.3 ± 9.8 |
| Day 14 | 3.5 ± 0.4 | 2.0 ± 0.7 | 72.3 ± 6.7 |

Attachment and regeneration of neurons on the Fish patterns was quantified by counting the number of neurons (1) attached to any part of the two-cell network pattern and (2) specifically to the somal adhesion sites (SAS). The percentage of neurons attached to the DETA patterns versus the PEG background was quantified.
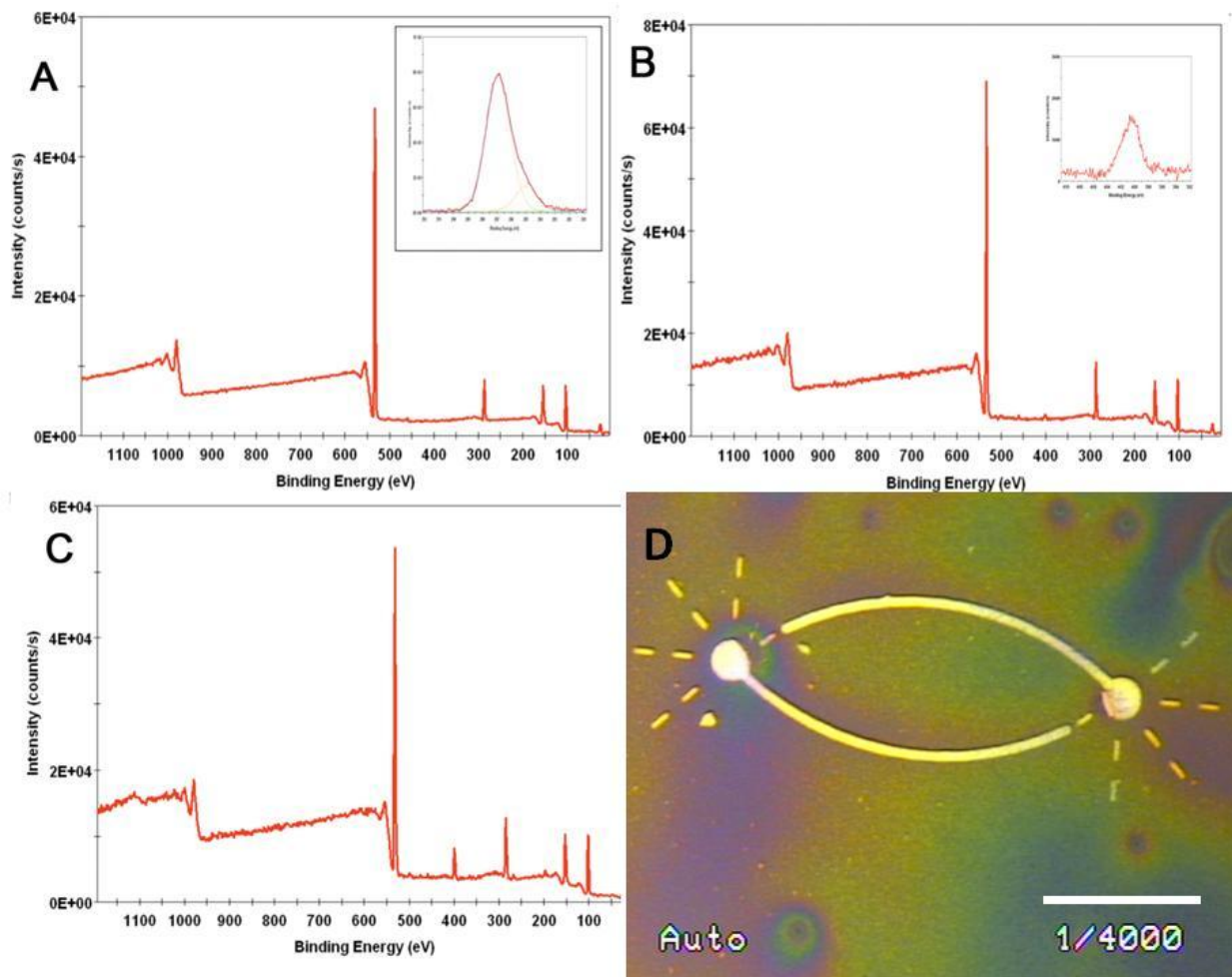
**Figure 4-1: XPS analysis and metallization reaction for PEG-DETA patterns.** A) XPS survey spectrum of PEG-coated glass cover slip (insert shows C1s spectrum), B) XPS survey spectrum of DETA on PEG-coated glass cover slip (insert shows N1s spectrum) analysis of the two layers, C) XPS survey spectrum of DETA on ablated PEG-coated glass cover slip, D) Image of the two-cell network Fish pattern visualized using palladium catalyzed copper reduction metallization (light lines indicate the DETA regions). Scale bar = 75 μm, line width 5 μm, somal adhesion site (SAS) = 30 μm
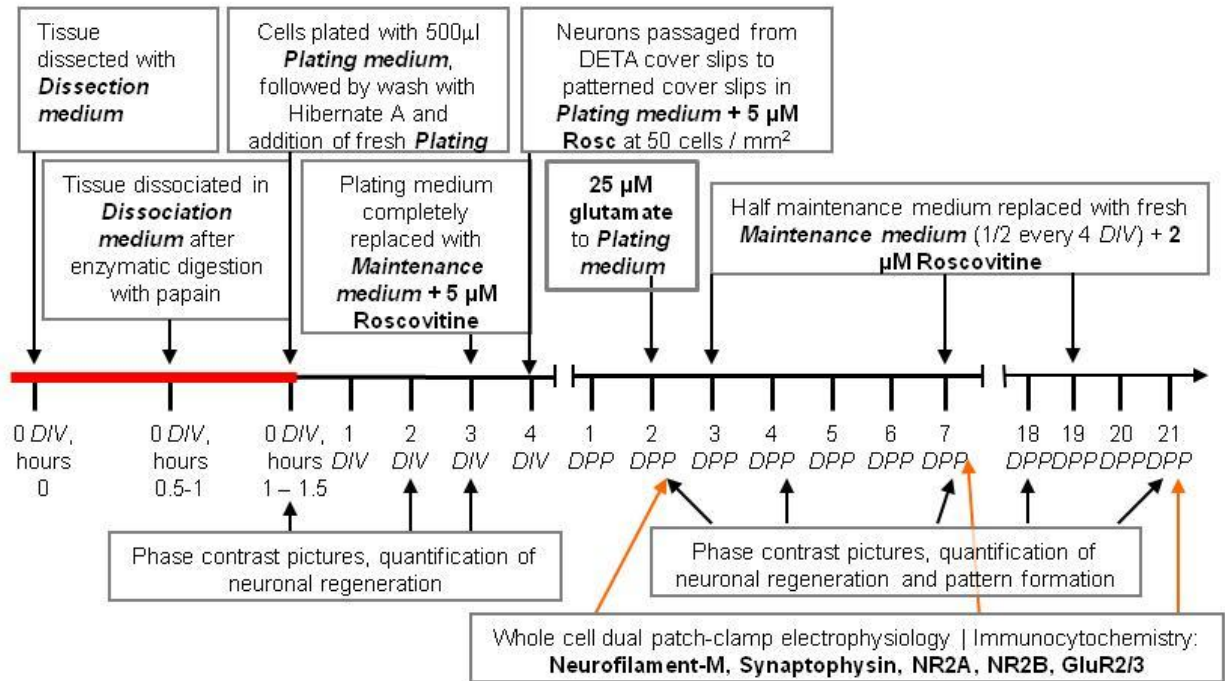
**Figure 4-2: Time-line of the adult hippocampal cell culture process and passage onto PEG-DETA Fish patterned cover slips.** *div* – days *in vitro*, *dpp* - days *post-passage*
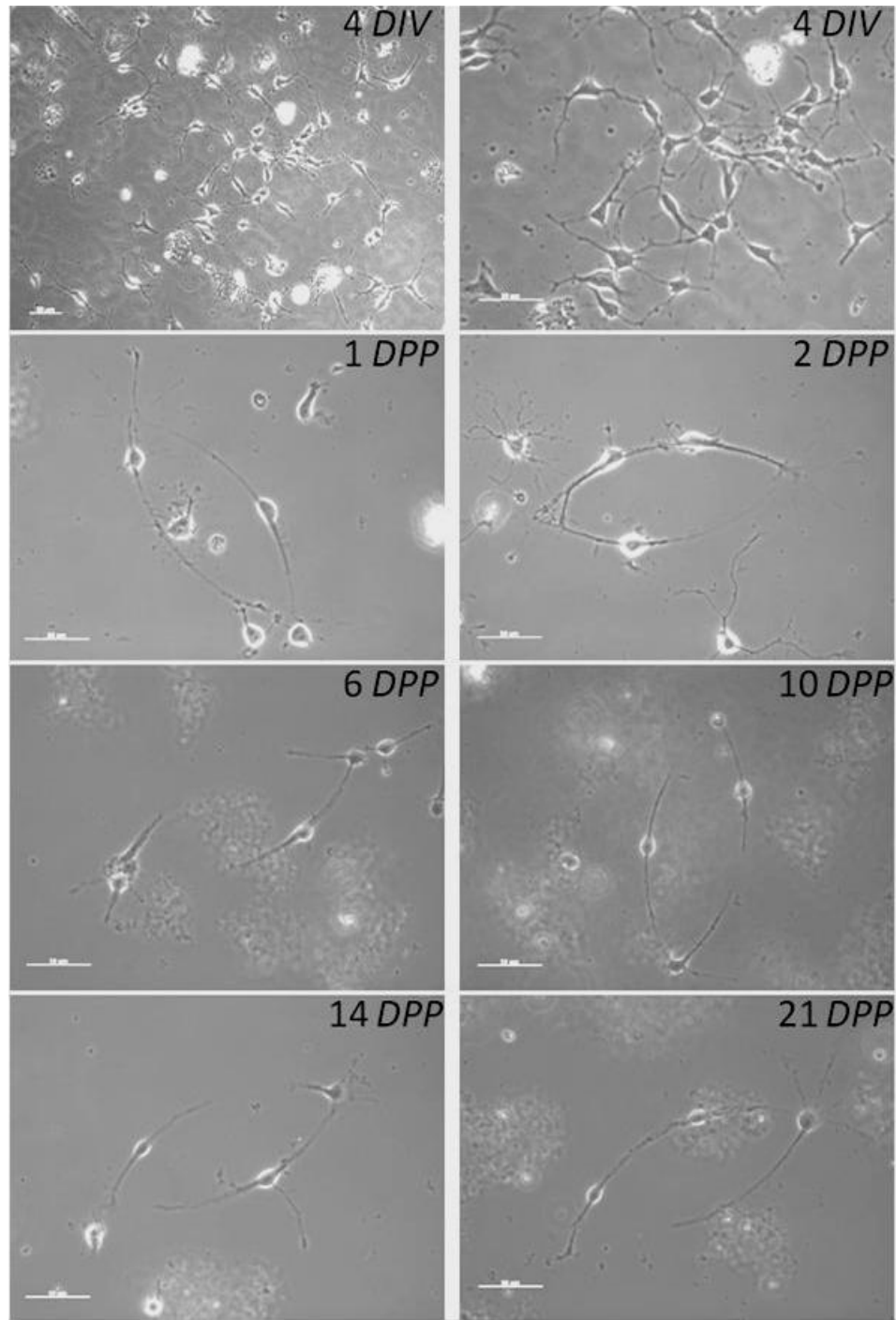
**Figure 4-3: Time-course pictures of neurons on culture after 4** *div*, **1** *dpp*, **2** *dpp*, **6** *dpp*, **10** *dpp*, **14** *dpp*, **21** *dpp*. Scale bar = 50 μm
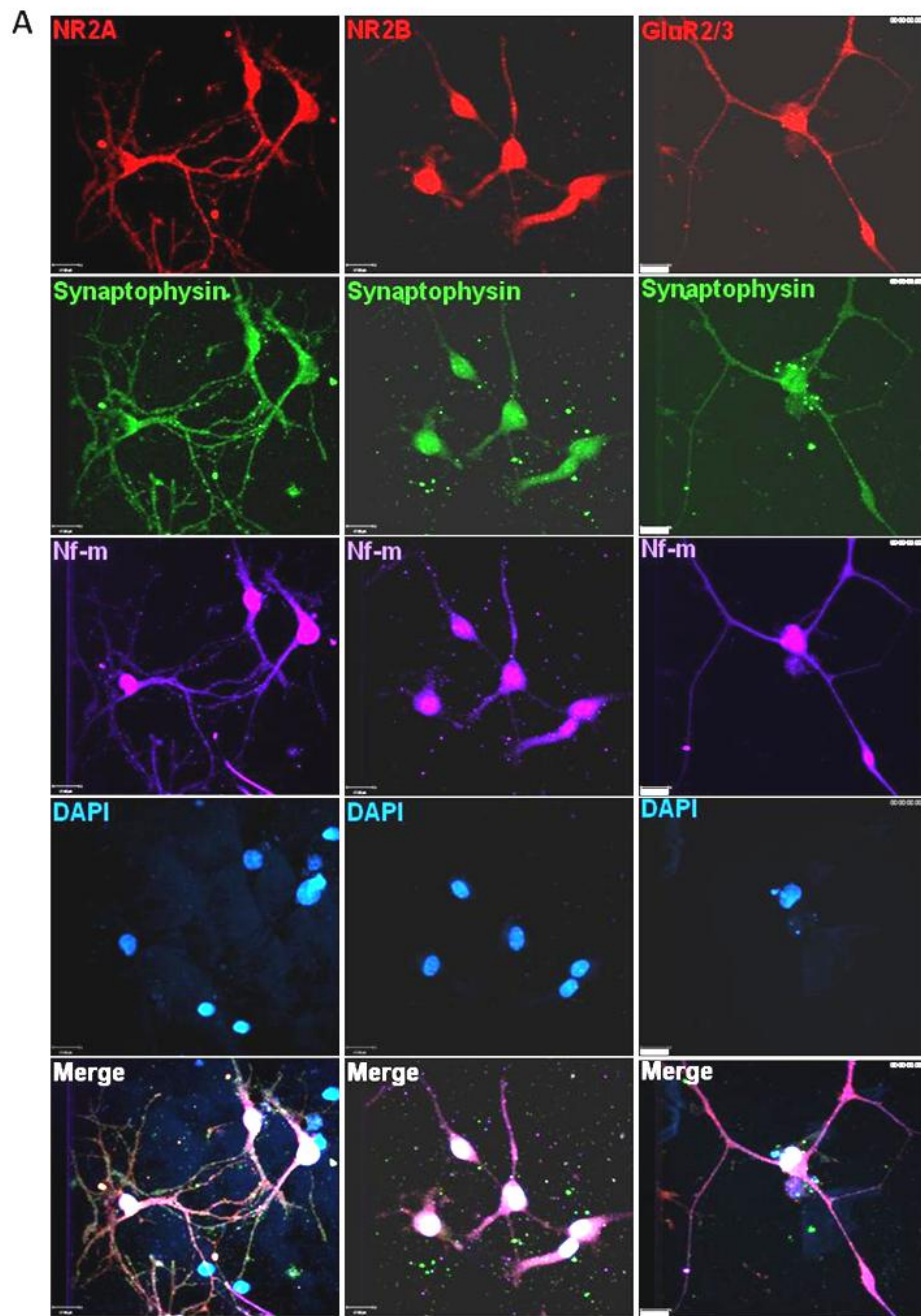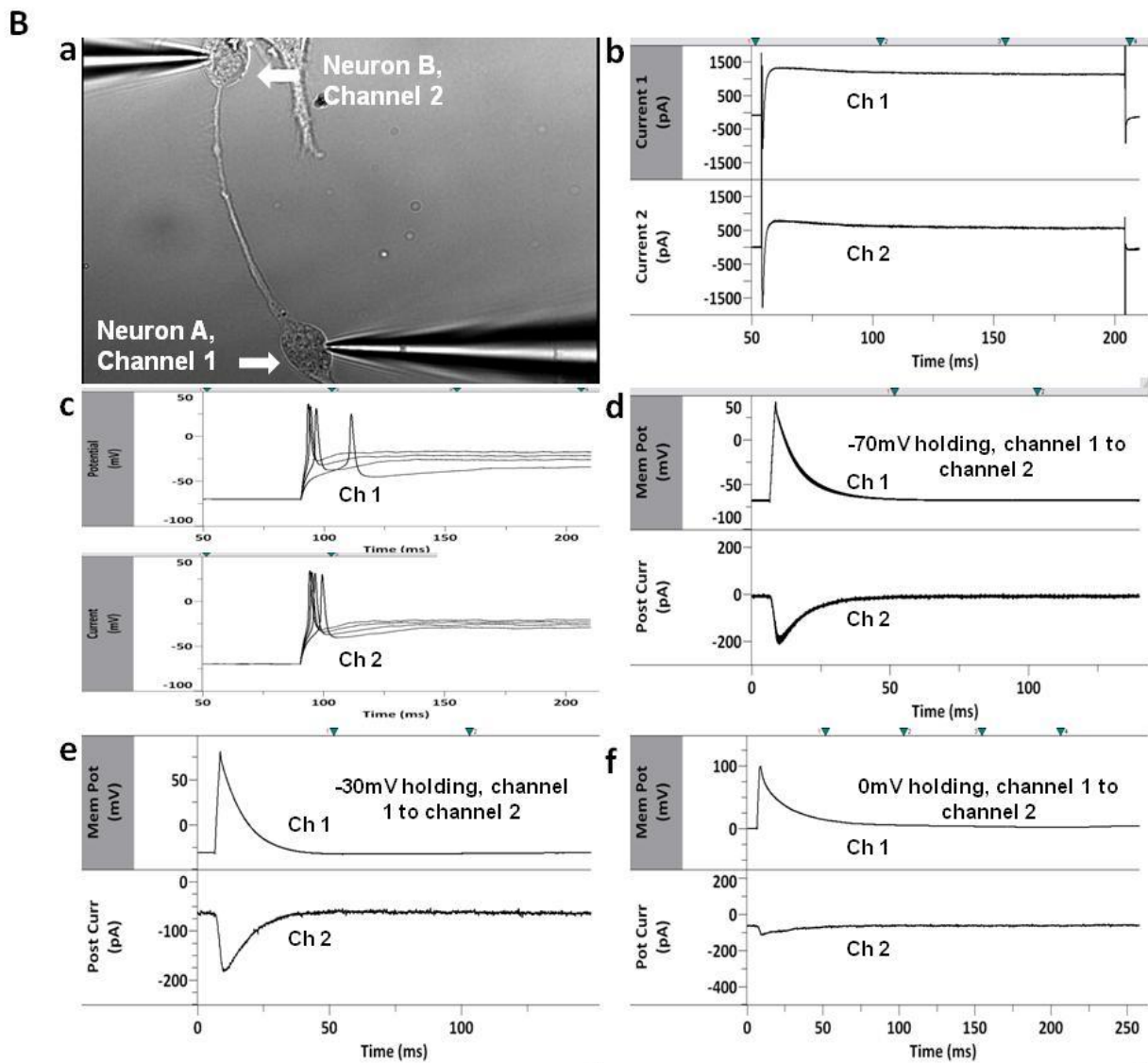
**Figure 4-4: Functional two-cell networks.** A, NR2A, NR2B, or GluR2/3 (red); synaptophysin (green); neurofilament-M (far-red); and DAPI (blue) expression after 14 *dpp* in adult neurons on DETA-coated control cover slips. Scale bars 17 μm. B. Dual patch clamp recordings were performed on neurons on Fish patterns (B-a). Electrophysiological recordings showed both cells were neurons (B-b: voltage-gated sodium and potassium channels in voltage-clamp experiments. B-c: action potentials generated upon stimulation in current clamp mode where the cells were held at -70 mV. Neuron A – Channel 1, Neuron B – Channel 2). Synaptic connections between the neurons and the type of synapse were measured. Pre-synaptic neurons (Channel 1) were held at -70 mV (B-d), -30 mV (B-e), or 0 mV (B-f) and action potentials were evoked. Postsynaptic Currents (Channel 2) were measured.

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## CHAPTER 5 – THE COMPARISON OF NMDA AND AMPA CHANNEL EXPRESSION AND FUNCTION BETWEEN EMBRYONIC AND ADULT NEURONS UTILIZING MICROELECTRODE ARRAY SYSTEMS.

**Introduction**

Electrophysiological studies into learning, memory formation, neurotoxic compounds and neurodegenerative conditions, and drug discovery most commonly rely upon patch-clamp electrophysiology [1, 2]. While this method provides detailed information, it is very labor-intensive, complicated, and has very low throughput in relation to non-invasive techniques for measuring electrical activity like MEAs. MEAs are innovative tools used to perform electrophysiological experiments for the study of neuronal activity and connectivity in populations of neurons from dissociated cultures [3, 4]. Where patch-clamp electrophysiology can only measure the activity of cells over the short-term (< few hours), MEA systems can measure the same population of neurons and the chronic effect of toxic compounds, drugs, etc on those neurons for long periods of time (days – months) [5-8]. Uses for these systems include studies into the mechanisms of learning and memory formation [9] and investigations into drug discovery, neurodegenerative diseases, and biosensor applications [5, 6, 10].

A common limitation of neuronal MEA systems has been the reliance upon neurons derived from embryonic tissue [3, 10, 11]. While these are differentiated neurons, they are developmentally immature, with transcriptional profiling showing two-thirds of genes are only expressed postnatal and >95% of expressed genes showing highly significant changes during postnatal development [12]. When looking at the machinery responsible for synaptic transmission, gene expression for the NMDA channel subunits NR2A and NR2B was not detected until near birth, with expression not

peaking until P20 [13].  *In vitro*, NR2A/B channels are detected at only very low levels until after 2 weeks in embryonically derived neurons [14].  AMPA channel expression is also limited at birth, only increasing postnataly [15].  Gene expression for the axonal sodium transporter subunit 1 begins around P15, increasing till P30 [16, 17].  *In vitro*, expression patterns for all genes,  specifically axonal and synaptic channels responsible for signal transmission, is similar to that seen *in vivo* at the same time point, with gene expression showing significant changes over the course of the first few weeks after birth [13, 14, 16, 18-20].  The usefulness of these developmentally immature neurons for studies of neuronal electrical activity and synaptic transmission is severely limited by this lack or limited expression of the neuronal machinery responsible for transmission in the adult brain.   In addition, using these immature neurons in studies of neurodegenerative diseases or drug discovery can yield results that cannot be correlated to the function or action of mature neurons in adult brain tissue.

We have developed a dissociated culture system of mature, terminally differentiated neurons derived from adult rat hippocampal tissue [21, 22].  Adult neurons recover and regenerate *in vitro*, display expression patterns consistent with mature neurons *in vivo*, are electrically active, and form functional synaptic connections [23].  These neurons were used to develop a dissociated neuronal culture system on MEAs.  Moving toward using a high-throughput hybrid *in vitro* system utilizing mature, adult neurons will expand and improve drug testing and basic research by providing a viable, easily manipulatable alternative to expensive, resource intensive *in vivo* testing.  Both embryonic and adult cultures were maintained for more than 90 days *in vitro* (*div*) on MEAs to obtain baseline recordings of spontaneous activity.  We probed the cultures

with synaptic transmission antagonists against NMDA, AMPA, and GABA$_A$ channels and found significant differences indicative of differing receptor profiles of adult and embryonic neurons *in vitro*. We then evaluated both embryonic and adult neurons for NMDA channel subunit expression and AMPA channel subunit expression over 36 *div.* Our results signify that neurons derived from embryonic tissue did not express mature synaptic channels for several weeks, and consequently their response to synaptic antagonists was significantly different than that of neurons derived from adult tissue sources. These results establish the usefulness of this unique hybrid system derived from adult hippocampal tissue for drug discovery and fundamental research.

## Materials and Methods

*Substrates and surface modification*

Glass cover slips (Thomas Scientific 6661F52, 22mm×22mm no. 1) were cleaned by acid washing using a 50/50 mixture of concentrated hydrochloric acid and methanol. The cover slips were washed three times, 30 min per wash, and were rinsed in distilled de-ionized water between each washing. The DETA (N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine, United Chemical Technologies Inc., Bristol, PA, T2910KG) monolayer was formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904) [24]. The DETA-coated cover slips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. The DETA formed a reaction site limited monolayer on the surface of the cover slip [24].  The DETA cover slips were characterized to authenticate the monolayer formation. First, contact angle measurements were taken using an optical contact angle goniometer (KSV Instruments, Monroe, CT, Cam 200). The contact angle for the DETA-coated cover slips was 54.2±0.2, which was previously shown to be acceptable for neuronal hippocampal culture [24]. Second, X-ray Photoelectron Spectroscopy (XPS) (FISONS ESCALab 220i-XL) was used to characterize the elemental and chemical state of the DETA-coated cover slip surfaces. The XPS survey scans as well as high-resolution N 1s and C 1s scans, using monochromatic Al Kα excitation, were obtained, similar to previously reported results [24, 25].

Clean gold MEAs (MUSE, Axion Biosystems, 8x8 electrodes) were sterilized with 70% alcohol and then incubated with 1 ml poly-L-lysine (100 µg/ml) for 30 minutes. An

area just large enough to cover all electrodes was additionally coated with 3 ml laminin (2 µg/ml) for 30 minutes.

*Embryonic rat hippocampal dissociated cell culture methodology*

Embryonic hippocampal neurons were cultured using a protocol described previously [26, 27]. Pregnant rats, 18 days in gestation, obtained from Charles River were euthanized with carbon dioxide and the fetuses were collected in ice-cold Hibernate E (BrainBits)/B27/Glutamax$^{tm}$/Antibiotic-antimycotic (Invitrogen) (*Dissecting Medium)*. Each fetus was decapitated and the whole brain was transferred to fresh ice cold dissecting medium. After isolation, the hippocampi were collected in a fresh tube of dissecting medium. The tissue was enzymatically digested at 37°C for 10 minutes with papain (Worthington 3119), 12 mg / 6 ml Hibernate-A (- calcium) (BrainBits) + 0.5 mM Glutamax (Invitrogen). Hippocampal neurons were obtained by triturating the tissue using a fire-polished Pasteur pipette. After centrifugation, the cells were resuspended in culture medium (Neurobasal/B27/Glutamax$^{tm}$/antibiotic-antimycotic) and plated on MEAs at 500 cells / mm$^2$. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines.

*Adult rat hippocampal dissociated cell culture methodology*

Adult neurons were extracted, dissociated, cultured, and maintained using a protocol and medium described elsewhere [22]. Briefly, the hippocampus of adult rats (Charles River, age 6-12 months) were dissected and homogenized into small tissue

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fragments in cold medium (~4ºC) consisting of Hibernate-A, glutamax, and antibiotic-antimycotic. The tissue was digested for 30 minutes at 37°C in calcium-free Hibernate-A (HA) containing 6 mg papain / 12 ml (HA no calcium). Following digestion, the tissue was washed three times with cold HA media to remove any active enzyme. Next, the tissue was suspended in Dissociation Medium and broken apart into individual cells through mechanical dissociation with fire-polished Pasteur pipettes. The dissociated cells were suspended in Plating medium and then deposited onto DETA-coated glass cover slips for 30-45 minutes. The cover slips were washed with warm HA by gently swirling the medium to remove tissue debris. Following this washing step fresh Plating medium was applied and remained for the first 3 *div*. On 3 *div* the medium was removed and replaced by Maintenance Medium with 5 µM Roscovitine (Rosc, Sigma, R7772). All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines.

After 4 *div*, the adult hippocampal neurons on the DETA cover slips were passaged to MEAs. Briefly, neurons were dislodged from the DETA with trypsin (.05% trypsin / EDTA in HBSS, Gibco, 25200). Trypsin inhibitor (trypsin inhibitor, soybean, Gibco, 17075-029) in Dissociation medium at 0.5 mg per ml deactivated the trypsin. The dislodged neurons were collected and spun at 500 x g for 5 minutes. The supernatant of deactivated trypsin in HBSS was discarded, and the neuronal cell pellet was suspended in 1 ml Plating medium. The neurons in suspension were counted using a Bright-Line hemacytometer, and neurons were plated onto the MEAs at 500-1000 cells / mm2 in Plating medium supplemented with 5 µM Rosc. After two days, glutamate (N-Acetyl-L-glutamic acid, Aldrich, 855642) was added to the plating medium

to a final concentration of 25 µM.  After 3 days and again every 4th day after, ½ the medium was removed and replaced with fresh Maintenance medium supplemented with 2 µM Rosc.

*Immunocytochemistry and Laser Scanning Confocal Microscopy*

To prepare cells for immunocytochemical characterization, cover slips were rinsed twice with Phosphate Buffered Saline (PBS).  Cells were fixed with 4% paraformaldehyde for ten minutes at room temperature, and subsequently rinsed three times with PBS.  Cells were permeabilized for five minutes with 0.5% Triton X-100 in PBS, and were then blocked for two hours in 5% normal goat serum in PBS.  Anti-neurofilament-M (Chemicon, AB5735, 1:500), anti-synaptophysin (Chemicon, MAB368, 1:300), and either anti-NMDAR2A (Chemicon, AB1555P, 1:200), anti-NMDAR2B (Chemicon, AB15557P, 1:200), or anti-glutamate receptor 2/3 (Chemicon, AB1506, 1:50) were added in blocking solution for 12 hr at 4°C.  After 3 washes with PBS, fluorescently labeled secondary antibodies (Invitrogen, A11011 (594nm), A21449 (647nm), and A11029 (488nm), 1:200) in blocking buffer were applied for two hours. Vectashield mounting medium with DAPI (H1200, Vector Laboratories, Burlingame, CA) was used to mount the cover slips onto slides.  Fluorescent images were acquired with the UltraView spinning disc confocal system (PerkinElmer) with AxioObserver.Z1 (Carl Zeiss) stand, and a Plan-Apochromat 40x/1.4 Oil DIC plan-apochromat objective with 26 µm resolution. Z-stack projections of the scanned images were generated and modified within the Volocity image processing program (PerkinElmer).

*Extracellular recordings*

The MEA chips (Axion Biosystems) provided 64 platinum-black coated gold-electrodes with a diameter of 30 µm, organized in an 8 by 8 array with 200 µm pitch. The head stage of the recording system (Axion Biosystems) was pre-heated to 37 °C before MEAs with adult or embryonic hippocampal cultures were investigated. Baseline activities of neuronal networks were recorded for 3 minutes with 25 kHz. Network activity was recorded five times per week for more than 90 *div* using the software *Axion's Integrated Studio* (AxIS). Signal amplitudes 6 times larger than the standard deviation of the base line were detected as action-potential spikes. The spike data was then imported into Matlab 2010b (The MathWorks) for further processing.

*Experimental Procedure*

Baseline spontaneous activity in adult and embryonic neurons was recorded for 3 minutes daily, starting on 7 *div* and continuing until either the MEAs lost activity or more than 90 days elapsed. Synaptic antagonists were administered to both adult and embryonic neurons on 14 *div* and at various time points between 30 and 60 *div*, and 3 minute recordings were made to quantify the effect from these antagonists on spontaneous activity. D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 25µM, Tocris Bioscience, 0106), 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 25µM, Tocris Bioscience, 1045), and (-)-Bicuculline methobromide (Bicuculline, 50µM, Tocris Bioscience, 0109) were separately administered. Each experiment was followed by a complete washout of the antagonist and replacement with fresh medium.

*Evaluation and statistics*

Data analysis was performed off-line using programs written in MATLAB to analyze "spike" files created during each MEA recording. Each 3 minute dataset was processed using a 3-step method to remove or exclude inactive and / or noisy channels. Channels with less than 7.5 action potentials (APs) per minute were treated as inactive. After processing, the following parameters were extrapolated: "active channels" – a number from 0 to 64. "AP Frequency" – number of total measured APs divided by recording time. The mean and standard deviation of that number was independent from active channels. "AP Activity" – 1 divided by the time between two subsequent APs, or frequency 1/s. "Burst" – where more than 1 AP showed up with 1 ms. "Average burst frequency" – 1 divided by the time-difference between two subsequent bursts. "In-burst frequency" – amount of APs within a burst divided by the duration of that burst. "Non-burst frequency" – like the activity, but only APs not associated with burst were included. "Burst duration" – time-interval from the first AP in a burst to the last AP in a burst. Effect from synaptic antagonists were measured as the percent of active channels or AP frequency of treated embryonic or adult cultures versus the baseline activity of the same cultures recorded before administration of the antagonist.

**Results**

Embryonic rat hippocampal neurons were plated directly on MEAs at a density of 500 cells / mm$^2$. Adult rat hippocampal neurons were first cultured on DETA coated cover slips. While basic fibroblast growth factor (bFGF) was necessary for survival of adult neurons in culture, bFGF caused rat neurons to divide *in vitro*; administration of Roscovitine at 3 *div* prevented neuronal mitotic activity [22]. After 4 days on DETA cover slips, the adult neurons were passaged onto MEAs at a density of 500-1000 cells / mm$^2$ (Figure 5-1). Supplementing the adult neurons with 25 µM glutamate on the 2 *div* increased the electrical activity of the adult neurons [21]. Sporadic spontaneous firing activity was detected in both the adult and embryonic neurons between 7–10 *div*, with stable, reliable recordings thereafter. Phase-contrast pictures of the neurons and the MEAs were taken daily to daily assess the condition of the cells as well as verify of lack of physical electrode degradation (Figure 5-2). Three-minute recordings of spontaneous activity were taken of each active MEA daily. Neurons, both adult and embryonic, were stable and electrically active on MEAs for more than 90 *div.* Movement of MEAs from the incubator to the recording stage and the subsequent 3 minute recording period did not significantly affect the pH or temperature of the medium, represented by consistent baseline activity. MEAs were incubated with their covers off to allow gas exchange but covered upon removal from the incubator to reduce contamination, media evaporation, and gas exchange while recordings were taken. No significant changes were observed in baseline neuronal activity, either adult or embryonic neurons, as a result of medium changes.

*Spontaneous activity of Adult and Embryonic Neurons*

Adult and embryonic neurons were cultured at similar cell densities on MEAs, and spontaneous activity was recorded for 3 minutes daily from 1 to 90 *div*. Each daily recording was processed using a 3-step method to exclude inactive or noisy channels (Figure 5-3). Channel by channel activity levels for each 180 second recording period were illustrated in the event map raster plot, activity map raster plot, and burst event histogram. Active channels and channel activity levels were also diagramed in the 2-D channel map, where color is the third dimension of data. The frequency range across the different active electrodes has been graphed in the frequency distribution plot. Finally, all the captured and processed parameters have been listed for each MEA recording, including active channels, AP frequency, AP activity, average burst frequency, and average in-burst frequency.

Over the more than 90 day culture period, embryonic MEAs consistently displayed a higher number of active channels, with an average of 40 channels active per MEA versus 20 channels in adult MEA cultures (Figure 5-4A). Action potential (AP) firing frequency, or APs per second, was consistent between the two cultures. A firing frequency of approximately 2 - 4 APs per second in embryonic and 1.5 - 4 APs per second in adult MEAs was measured (Figure 5-4B). The AP activity had a much greater variance and was consistently higher in adult neurons (Figure 5-4C). Adult neurons spent less time resting before subsequent firing events. Spontaneous bursting activity, where more than 1 AP was fired within 1 ms, was captured in both the adult and embryonic neurons. Both the burst frequency and the in-burst frequency were consistent between the systems (Figure 5-4 D, E).

*The effect on the activity of adult and embryonic neurons by synaptic antagonists*

Prior to the addition of synaptic channel antagonists, activity baselines were recorded for all embryonic and adult MEAs. In separate experiments, 25 µM D-AP5 (NMDA channel antagonist), 25 µM CNQX (AMPA channel antagonist), and 50 µM bicuculline (GABA$_A$ antagonist) were administered to embryonic and adult MEAs, and activity was recorded for 3 minutes.

D-AP5 (25 µM) caused a significant decrease in the number of active channels in both adult and embryonic MEA cultures (Figure 5-5). Adult cultures lost a greater percentage of active channels (-90.12 ± 5.9% on 14 *div,* -82.22 ± 5.9% on 30-60 *div*) versus embryonic cultures (-64.92 ± 3.85% on 14 *div,* -36.23 ± 7% on 30-60 *div*). Changes in the action potential frequency also varied with lower frequencies in adult cultures (-76.31 ± 8.3% on 14 *div*, -82.47 ± 16.6% on 30-60 *div*) which were significantly different from the measured effect on embryonic cultures. On 14 *div,* the firing rate of embryonic neurons increased +90.39 ± 6.1%, while in 30-60 day old cultures the firing rate decreased -69.5 ± 7%. The difference in NMDA channel expression (NR2A and NR2B subunits) in adult and embryonic neurons *in vitro* likely caused the contrasting reaction to D-AP5 in the two populations of neurons. While NMDA channel expression in 14 *div* embryonic neurons was low or non-existent, expression was high in adult neurons on 14 *div* (Figure 5-6 A,B)*.* Because adult neurons expressed a greater number of NMDA channels, their reaction to the competitive NMDA antagonist D-AP5 was much more pronounced.

Addition of CNQX (25 µM) caused the activity of far fewer channels to be lost in both adult and embryonic MEA cultures as compared to D-AP5 (Figure 5-5). Adult cultures lost a greater percentage of channel activity (-52.14 ± 2.5% on 14 *div,* -24.35 ± 5.0% on 30-60 *div*) versus embryonic cultures (-23.25 ± 4.5% on 14 *div,* -0.34 ± 6.5% on 30-60 *div*). Changes in the action potential frequency in both the adult and embryonic cultures was not affected by CNQX after 14 *div*. The activity of the neurons was only slightly lower in both embryonic and adult cultures between 30 to 60 *div* (-30.58 ± 5.5% drop in adult MEAs*,* -46.89 ± 4.5% drop in embryonic MEAs). While expression of the AMPA channel subunits GluR2/3 was not observed in embryonic neurons on 2 *div,* expression had increased to mirror adult levels by 14 *div*, so no differences were expected between adult and embryonic neurons to AMPA channel antagonists (Figure 5-6, C)*.*

After MEA measurements were recorded, the antagonists were washed from the neurons with an entire media change. 24 hours after, the activity of the neurons had returned to baseline levels.

**Discussion**

Our results demonstrated that adult neurons cultured from the hippocampus of rats recovered functionally and had the capacity to fire spontaneously on MEAs for more than 90 *div.* Additional culture conditions were employed to allow adult neurons to be densely deposited on the MEAs, to prevent these neurons from reentering the cell cycle and dividing, and to improve recovery of electrical activity *in vitro.* Adult hippocampal neurons were first cultured onto DETA cover slips. Dense debris that was present along with neurons and glia after dissociation of the hippocampal tissue prevented cell counting, and cells were placed on these cover slips at unknown concentrations. After the cells had recovered for 3 *div,* roscovitine, a cdk5 inhibitor, acted to prevent neuronal reentry into the cell cycle, an action mediated by bFGF [22]. Cells in this low-density cell culture were passaged and deposited onto MEAs at 500-1000 cells / $mm^2$, a density high enough to allow the formation of multiple synaptic connections. This along with the action of glutamate in the culture media promoted elevated electrical activity in the adult neurons [21]. After 7 to 10 *div*, sporadic spontaneous firing activity was detected, and stable reliable recordings were evident after this point.

Neuronal MEA systems have typically relied upon neurons derived from embryonic tissue [3, 5-7, 10, 11]. We compared this adult hippocampal MEA system to traditional embryonic MEA systems. Embryonic neurons were plated onto MEAs at a density of 500 cells / $mm^2$. As on adult MEAs, sporadic spontaneous firing was detected between 7-10 *div*, with more stable activity after 10 *div.* Recording from both adult and embryonic MEAs were made daily for more than 90 *div.* Embryonic MEAs

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consistently displayed higher numbers of active channels, with an average of 40 channels active versus 20 active on adult MEAs. The majority of other parameters, including AP frequency, activity, average burst frequency, and average in-burst frequency, all displayed similar characteristics between the adult and embryonic MEA systems. This new adult MEA system, at its core, exhibited similar firing characteristics to the more traditional embryonic MEA systems.

While embryonic neurons regenerate and fire APs on MEAs, the developmental maturity of these neurons had not been established. In the hippocampus of embryonic rats, neural progenitors differentiate into neurons between E15-E18 [12, 18, 28]. While these are differentiated neurons, they are developmentally immature, with transcriptional profiling showing two-thirds of genes are only expressed postnatal and >95% of expressed genes show highly significant changes during postnatal development [12]. When looking at the machinery responsible for synaptic transmission, gene expression for the NMDA channel subunits NR2A and NR2B was not detected until near birth on E21-22, with expression not peaking until P20 [13]. During this developmental period, NR2B was predominantly expressed in the early postnatal brain while NR2A expression increased to eventually outnumber NR2B, with each subunit lending different kinetics of excitotoxicity, neurotoxicity, and plasticity [29]. *In vitro*, NR2A/B channels are detected at only very low levels until after 2 weeks in embryonically neurons [14]. AMPA channel expression is also limited at birth, only increasing postnatal [15]. Gene expression for the axonal sodium transporter subunit 1 begins around P15, increasing till P30 [16, 17]. *In vitro*, expression patterns for all genes, specifically axonal and synaptic channels responsible for signal transmission, is

similar to that seen *in vivo*, with gene expression showing significant changes over the course of the first few weeks after neuronal differentiation [13, 14, 16, 18-20]. The importance of these dynamics of neuronal maturation highlights the need for gene and protein expression in study population of neurons to mirror that in mature adult neurons *in vivo*. If embryonic neurons do not express the same machinery responsible for electrical activity or signal propagation in adult neurons, then their response to neurotoxic agents or drug therapies may not be correlative to responses in the mature adult brain.

Our method of culturing MEAs with adult neurons resulted in a system where NMDA and AMPA channel subunits were expressed throughout the lifespan of the culture. NMDA channel subunits NR2A and NR2B as well as AMPA channel subunits GluR2/3 were expressed on and after 2 *div.* This contrasted greatly from neurons derived from embryonic tissue, with delayed NMDA channel expression until after 14 *div* and delayed AMPA channel expression until 14 *div.* Responses of these neurons to NMDA and AMPA channel antagonists were found to be significantly different in embryonic neurons as compared to adult neurons, with each antagonist decreasing activity in adult neurons to a greater degree than in embryonic neurons. Our results show that embryonic neurons in culture develop a mature profile of ion channel subunits after 3-4 weeks. Therefore, embryonic neurons should not be used until they have fully matured in culture especially in studying neurodegenerative diseases such as Alzheimer's where synaptic protein profiles may play a critical role in the process of synaptic failure.

In comparison to embryonic MEA systems, our method, measurements of neuronal activity using adult hippocampal neurons on MEAs, is more applicable to the adult brain. While preparation of these MEAs was slightly more complicated than embryonic neuronal MEAs, the end result yielded a high-throughput screen methodology that is directly correlative to the dynamics of learning and memory formation in the adult brain. Additionally, this system can facilitate quicker, more reliable, and more correlative investigations into drug discovery, neurotoxic agents, and neurodegeneration. Last, this method and be used in the future to allow for the generation of MEAs using adult human neurons.

In conclusion, we have demonstrated critical differences between adult and embryonic neurons and their respective synaptic connections which could be highly relevant in neurodegenerative disease research. By demonstrating the similarities and differences between adult and embryonic neurons and the response of each to synaptic antagonists, the usefulness of this adult neuron culture system has been established for application in neuronal regeneration and drug discovery studies. By incorporating adult neurons into an MEA system, a high-throughput system has been created to enable the screening of a large number of cells and the study of pathogen and drug effects on the same population of cells over an extended period of time. This screen could find important applications in pharmaceutical drug development by providing an *in vitro* high-throughput test platform for investigations into neurodegenerative disease, traumatic brain injury, stroke, drug discovery, and fundamental research.

**Figure 5-1: Adult and embryonic MEA culture and lifecycle.** Adult neurons: Mature terminally differentiated  adult neurons were extracted from the hippocampus of adult rats and plated onto DETA cover slips.  After 4 days the neurons were passaged from these cover slip(s) onto MEAs that had been coated with PDL / laminin (for cell adhesion).  Embryonic neurons:  Neurons from the hippocampus of embryonic day 18 rat fetuses were extracted and plated directly on MEAs that had been coated with PDL / laminin.  Electrical recordings of spontaneous neuronal activity were performed for up to 3 months.   In addition, ion channel receptor antagonists were introduced and their effects were measured against baseline electrical activity.  64-channel axion biosystems MEAs were used.
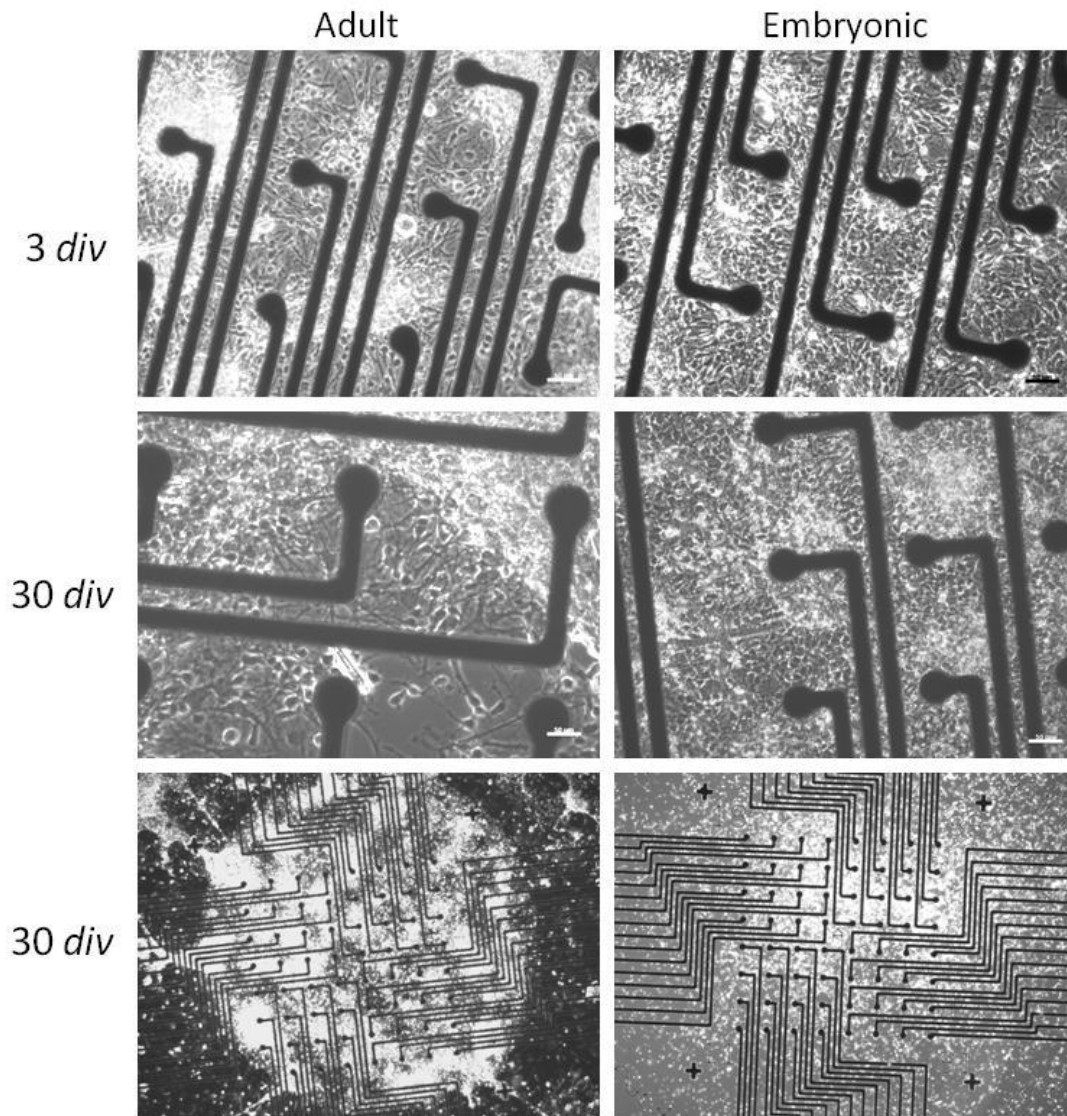
**Figure 5-2:  Phase contrast images of cultures on MEAs after 3 div and 30 *div*.** Neurons were applied between 500 to 1000 cells / mm$^2$.  Cells attached and regenerated on the PDL / laminin surface covering the MEAs, seen in the dense collection of cells covering the electrodes.  Each 64-Channel MEA is arranged in an 8 x 8 array of electrode 30 µM in diameter and spaced 200 µM apart. The MEA was sampled 25,000 times per second, at 16 bits of depth. Scale bar = 50 µm.
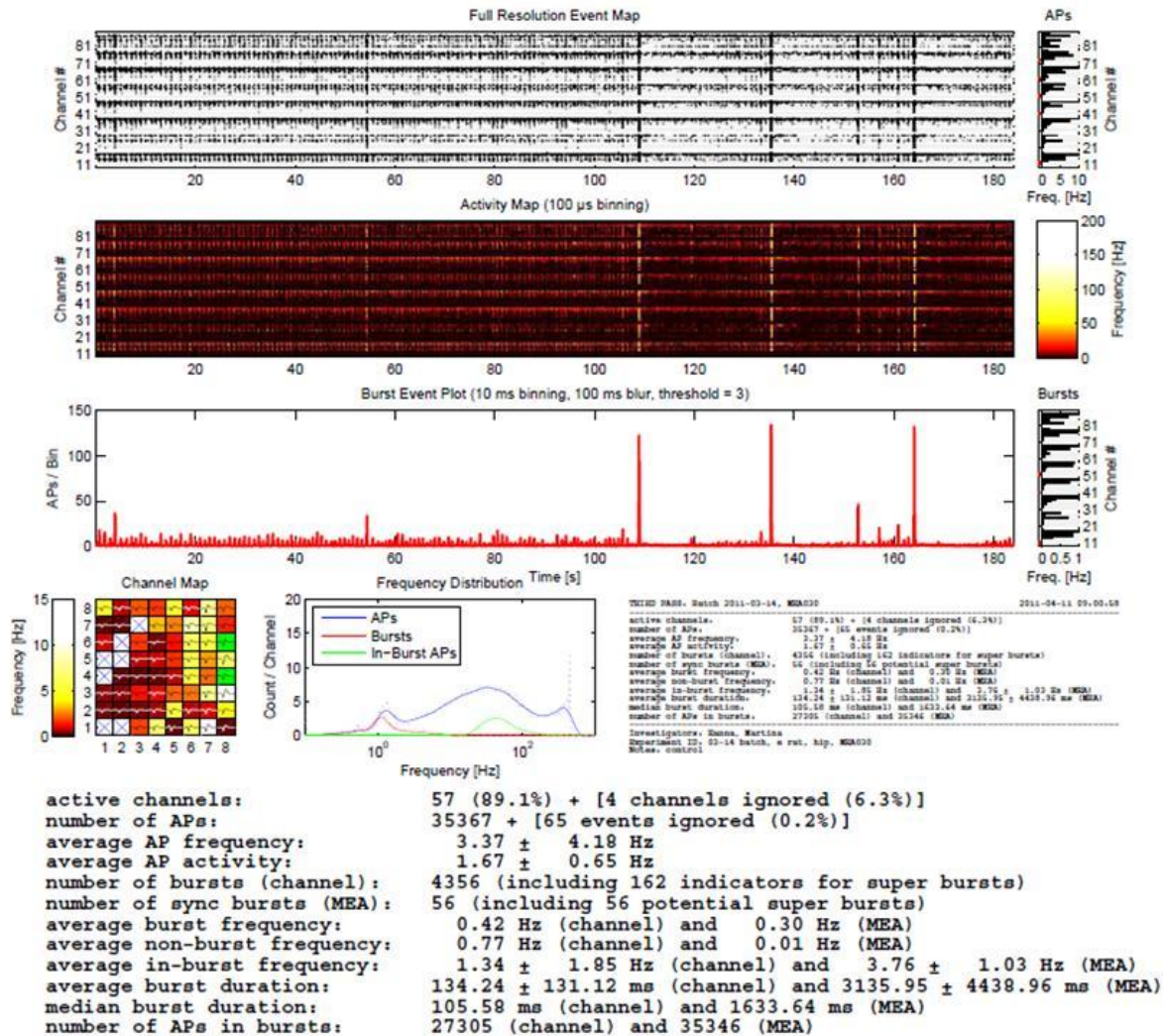
**Figure 5-3: MEA data processing:** The spontaneous activity of embryonic and adult neurons was recorded for 3 minutes each day over the study period. Each 3 minute dataset was processed in a 3-step method. This method allowed inactive or noisy channels to be excluded. After processing, the following parameters were extrapolated: "active channels" – a number from 0 to 64. Channels with less than 7.5 APs per minute were treated as inactive. "AP Frequency" – number of total measured APs divided by recording time. The mean and stdev of that number is independent from active channels. "AP Activity" – is 1 divided by the time between two subsequent APs, or frequency 1/s. "Burst" – where more than 1 AP shows up with 1 ms. "average burst frequency" – 1 divided by the time-difference between two subsequent bursts. "In-burst frequency" – amount of APs within a burst divided by the duration of that burst. "Non-burst frequency" – like the activity, but considers on APs that are not associated with bursts. "Burst duration" – time-interval from the first AP in a burst to the last AP in a burst.

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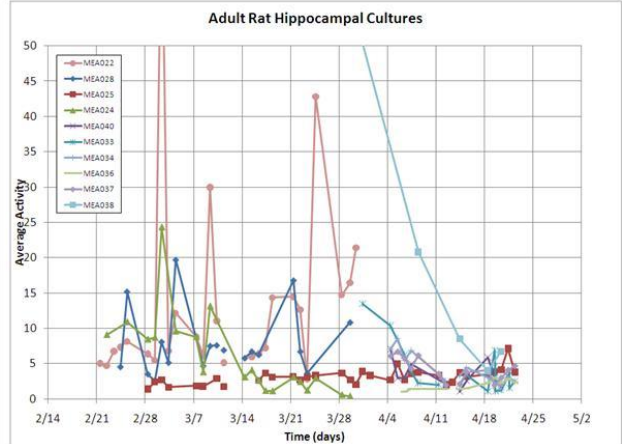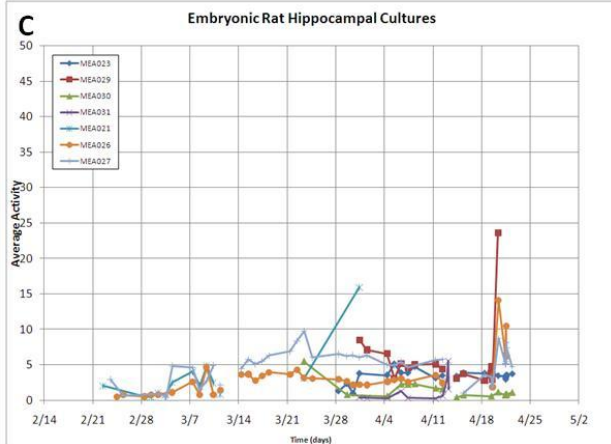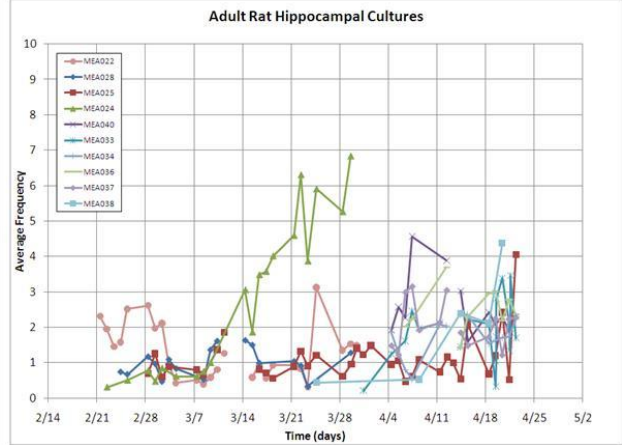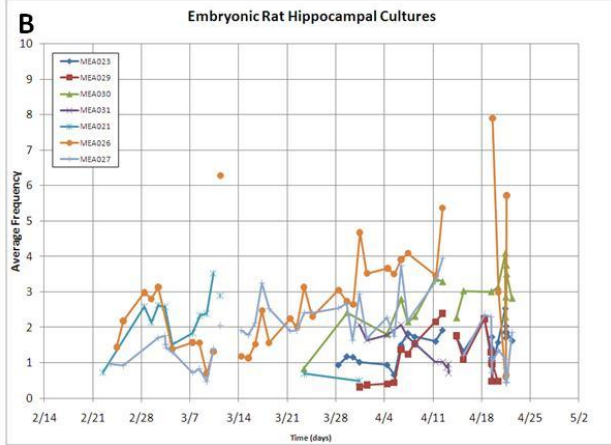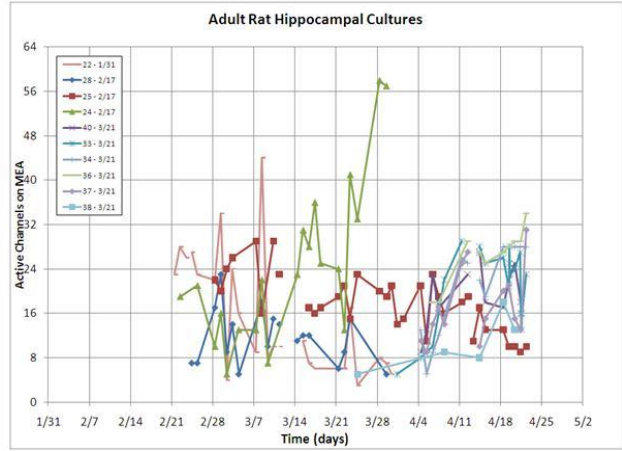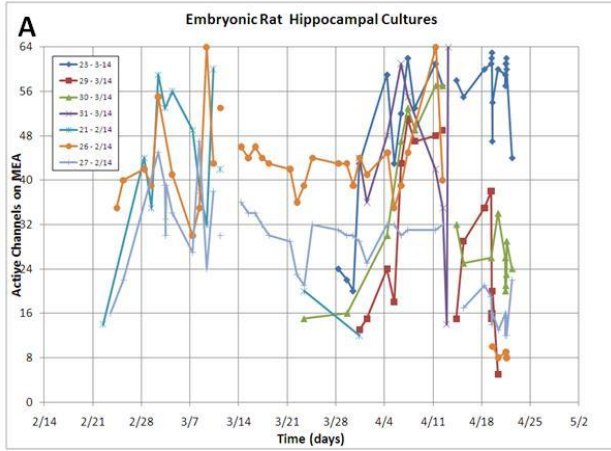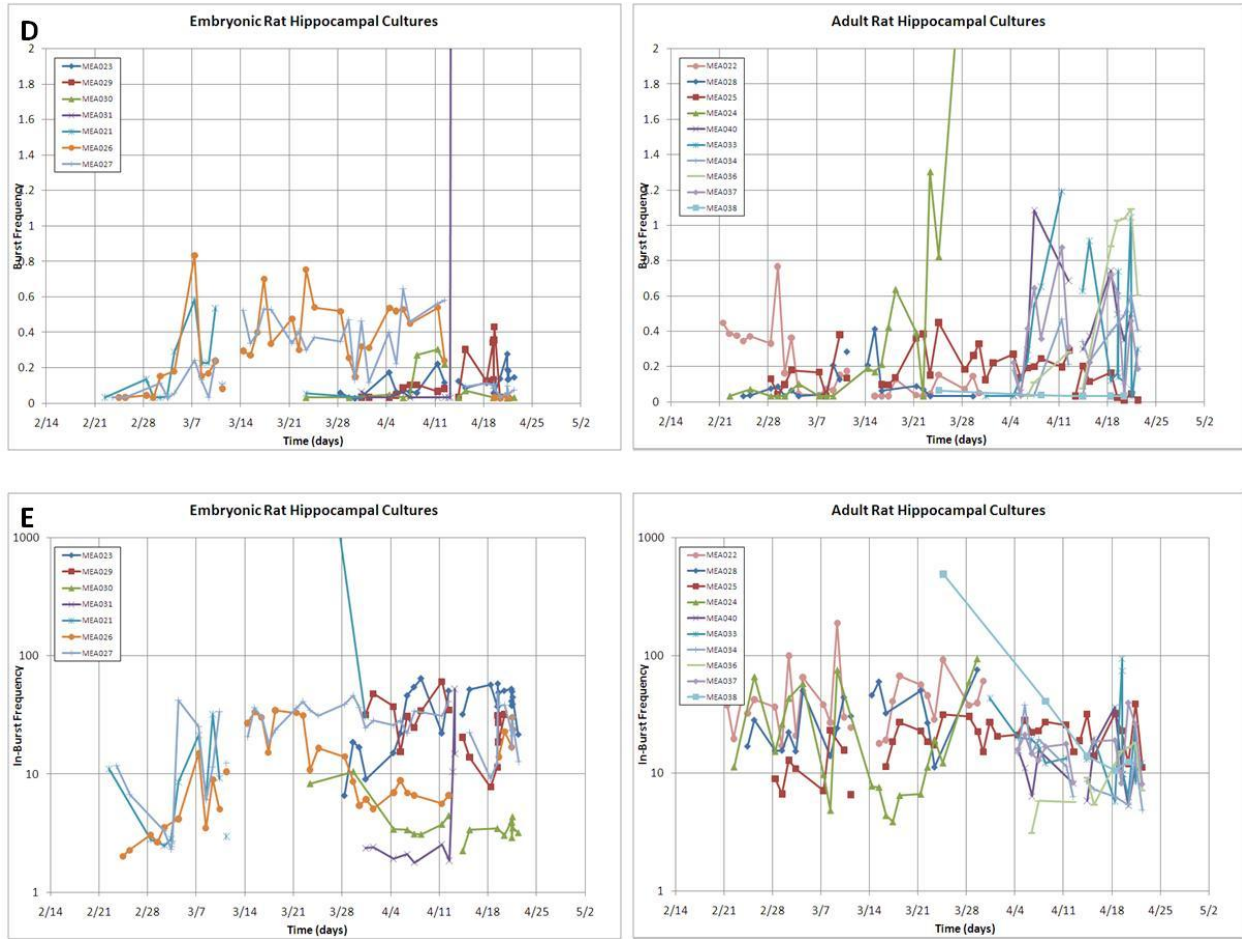
**Figure 5-4: Basic firing patterns of embryonic and adult hippocampal neurons on MEAs over time.** The spontaneous activity of embryonic and adult neurons was recorded for 3 minutes each day over the study period. The activity data from each day was processed to filter out inactive channels. (A) Number of active channels. (B) Frequency of APs. (C) Average activity, or 1 divided by the time between two subsequent APs (frequency 1/s). (D) Burst frequency. (E) In-burst frequency. MEA number to plating date: Embryonic: MEA 23 – 3/14, MEA 29 – 3/14, MEA 30 – 3/14, MEA 31 – 3/14, MEA 21 – 2/14, MEA 26 – 2/14, MEA 27 – 2/14. Adult: MEA 22 – 1/31, MEA 28 – 2/17, MEA 25 – 2/17, MEA 24 – 2/17, MEA 40 – 3/21, MEA 33 – 3/21, MEA 34 – 3/21, MEA 36 – 3/21, MEA 37 – 3/21, MEA 38 – 3/21. Gaps in graphs indicates activity was not recorded on that day.

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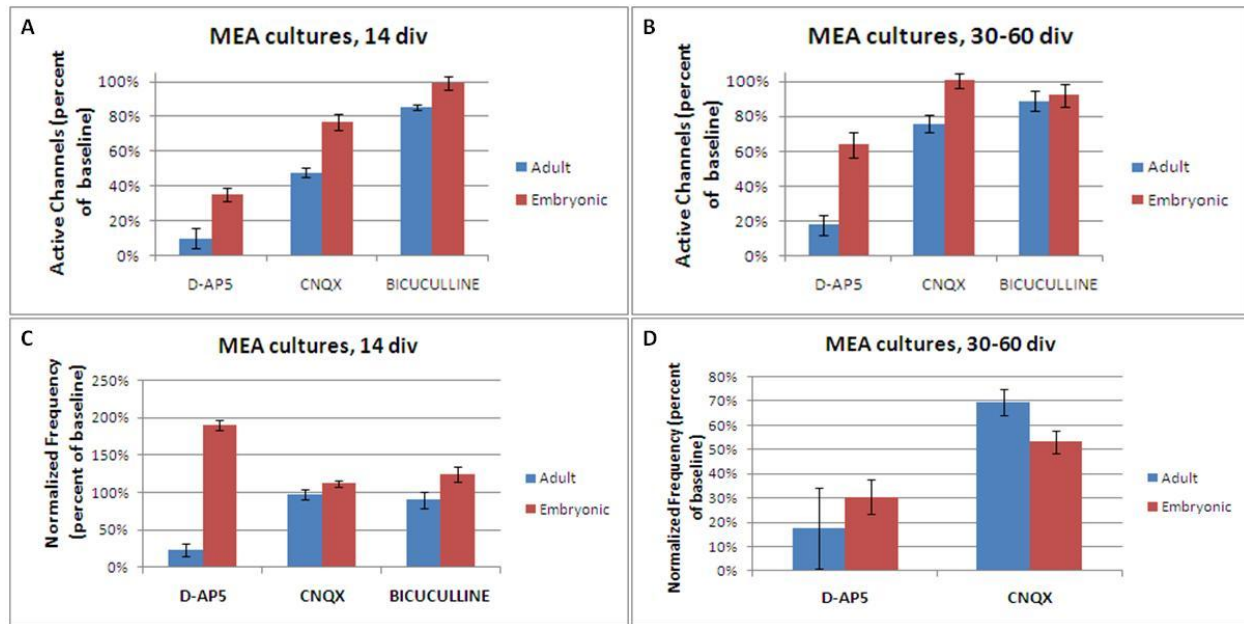
**Figure 5-5: Comparison of the impact on adult or embryonic spontaneous activity from addition of synaptic antagonists.** Active channels (A,B) or AP frequency (C,D) were evaluated in adult or embryonic hippocampal neuron MEA systems on either 14 or 30-60 *div* in the presence of D-AP5 (25 µM), CNQX (25 µM), or Bicuculline (50 µM) in culture medium. Adult neurons showed significantly decreased active channels and AP frequency due to D-AP5 in both early 14 *div* cultures as well as older 30-60 *div* cultures. This drop in activity was significantly different from embryonic 14 *div*, where fewer active channels were lost and activity increased in the remaining channels. The AMPA-channel antagonist CNQX also caused a decrease in spontaneous activity. The drop in activity between adult and embryonic cultures was, however, only reflected in the loss of more active channels in the adult system. AP frequency declines were consistent between the two culture systems. Bicuculline had limited effect on spontaneous activity in both embryonic and adult neurons.

**A**

Embryonic | Adult

| synaptophysin | NMDAR2A | synaptophysin | NMDAR2A |
| Nf-M | Merge | Nf-M | Merge | 2 *div*

| synaptophysin | NMDAR2A | synaptophysin | NMDAR2A |
| Nf-M | Merge | Nf-M | Merge | 14 *div*

| synaptophysin | NMDAR2A | synaptophysin | NMDAR2A |
| Nf-M | Merge | Nf-M | Merge | 36 *div*

**B**

Embryonic | Adult

synaptophysin | NMDAR2B | synaptophysin | NMDAR2B

Nf-M | Merge | Nf-M | Merge

2 *div*

synaptophysin | NMDAR2B | synaptophysin | NMDAR2B

Nf-M | Merge | Nf-M | Merge

14 *div*

synaptophysin | NMDAR2B | synaptophysin | NMDAR2B

Nf-M | Merge | Nf-M | Merge

36 *div*

**Figure 5-6:    Expression of presynaptic proteins and postsynaptic channel subunits in embryonic and adult neurons *in vitro*:**  NR2A, NR2B, or GluR2/3 (red); synaptophysin (green); neurofilament-M (far-red); and DAPI (blue) expression after 2, 14, and 36 *div.*  NMDAR2A (A) and NMDAR2B (B) were not expressed in embryonic neurons on 2 *div* and were not strongly expressed on 14 *div* (when compared to channel expression in adult neurons).  After 36 *div,* the channels were expressed by the embryonic neurons at similar levels to the adult neurons.  The AMPA receptor subunits GluR2/3 (C) were expressed by embryonic neurons by 14 *div*. These postsynaptic channel subunits were all found in adult neurons from 2 – 36 *div.*  Synaptophysin and Neurofilament-M expression grew stronger as both the embryonic and adult neurons recovered and regenerated *in vitro.*  Scale bars 17 μm.

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## CHAPTER 6 - A DEFINED, DISSOCIATED, AND FUNCTIONAL NEURONAL CULTURE SYSTEM DERIVED FROM HUMAN BRAIN TISSUE SAMPLES.

**Introduction**

Human-based model systems for neurodegenerative diseases such as AD and for CNS injury such as traumatic brain injury (TBI) are limited, and this limitation has been a major bottleneck in translating basic science into clinical applications and therapeutic discoveries. The ideal research model, from a purely experimental standpoint, would be to utilize human volunteers in experiments in order to understand various diseases and injuries.[1] However, from an ethical standpoint this is impossible in any situation where damage would knowingly or potentially occur to the human subject. Thus, over most of the last century drug development and research aimed at solving clinical neuropathologies has been limited to studies that have utilized live animals, animal slice cultures or single cells from animal / human systems. The main problems with these approaches are that in most cases research using animal models does not translate well to the human condition and more applicable functional *in vitro* model systems derived from human tissue are limited.

The goal of developing functional *in vitro* neuronal systems is to produce long-term culture systems that facilitate recovery of functional electrical properties of neurons in a controlled environment that is also capable of reproducing disease states. In the case of *in vitro* systems that seek to use neurons derived from adult tissue to recreate sub-systems from the central nervous system (CNS) and peripheral nervous system (PNS), the utilization of human tissue has been limited. Ideally, using primary cells

derived from both healthy and compromised adult humans for all of these model systems could more accurately reflect the disease state that exists in humans.[2]

This is especially true for Alzheimer's disease (AD) where there has been a multitude of factors postulated to be involved with disease pathogenesis, but there is still little consensus on a definitive causative factor or treatment of this debilitating disease.[3],[4] AD is one of the most common neurodegenerative disorders affecting approximately 5-10% of the human population over the age of 65 years, numbers expected to rise in the next decades.[5],[3]   Most of the current research focus in AD has been in trying to determine the cause of the disease, developing biomarkers for early detection, and treating the symptoms after the disease clinically manifests. However, very little research has been directed at restoring function in neurons damaged by Amyloid-β (Aβ), Tau, or other proposed causative agents. This gap in research is in large part due to larger difficulties currently existing in translational and clinical research as well as a current lack of good model systems that mimic the human disease conditions. The ability to observe the diseased neurons directly in a controlled model system could be used to effectively target the causative effects of AD.

During the last decade several functional *in vitro* systems have been developed to study CNS and PNS disorders using adult, fetal, and embryonic rat and mouse tissues.[6-16] Additional studies have been conducted to develop short-term culture systems using human neurons.[17, 18] [19] Though these culture systems were partially successful in supporting short-term survival of dissociated human neurons or neural progenitors, these neurons were not evaluated for basic electrical functionality.[17-19] Currently, no dissociated cell culture system of diseased primary neurons from the

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human brain has been reported. The creation of such a system to analyze the function of neurons under defined conditions combined with the ability to collect, manipulate, and observe diseased human neurons would be of great benefit in fundamental research, in the development of therapeutic interventions by clinicians, and in personalized medical diagnostics.

In this study, we have developed a serum-free *in vitro* culture system for neurons isolated from the brains of deceased AD patients as well as from brain tissue excised during surgery for Parkinson's disease (PD), Essential Tremor (ET), and brain tumor. Culture conditions and media formulations were developed to maximize morphological regeneration, electrical recovery, and long-term survival *in vitro*. The resulting model is comprised of a unique serum-free medium, auto-catalytic nanoparticles, a surface engineered substrate and a defined culture methodology. This novel system allowed functional electrical recovery and long-term survival of healthy human neurons and limited recovery of diseased neurons in culture. Further, dissociated neurons from AD samples expressed *in vitro* those features characteristic of neurons exhibiting AD pathology *in vivo*, including a lack of dendritic branching and spine formation.

**Materials and Methods**

The culture method has been modified over the three years of the project. Initial experiments were performed on brain specimens extracted from the hippocampus during autopsy of patients who had died from complications due to neurodegenerative disease. These specimens were processed using an earlier unrefined culture technique and media formulations. This technique and media was subsequently optimized and used to process all brain tissue specimens extracted from living patients with Parkinson's, Central Tremor, or brain tumor. Later cadaver brain tissue samples were processed with the new refined technique, as seen in Table 6-4.

*Patients and tissue samples*

Two types of brain tissue samples were obtained and processed. Initially, brain tissue specimen(s) were extracted during autopsy from the hippocampus of patients. The majority of these patients had died due to complications from neurodegenerative diseases (11 samples). However, one specimen was extracted from a patient with no known neurodegenerative condition (Table 6-4). Brain tissue slices, approximately 3 - 10 mm in width, were removed from the parahippocampal region of the brain during autopsy, two to six hours after death. The length of time between the death of the patient and the removal of the brain tissue was minimized when possible, as this period directly correlates to the subsequent culture results. This was seen in a supplemental study performed on rats. Rats were killed through exposure to $CO_2$ and periods of 0, 2, or 4 hours elapsed prior to dissection of the brain tissue. The number and quality of

living neurons after 1, 3, and 6 *div* was significantly lower the longer the period between death and dissection (Figure 6-8 A, B)

Second, brain tissue specimen(s) were removed from living patients undergoing two types of surgical intervention: electrode implantation for deep brain stimulation (DBS, 12 samples total) or cancerous brain tumor excision (TE, 3 samples total) (Table 6-2). For the treatment of non-Parkinsonian ET, electrodes were placed in the ventrointermedial nucleus (VIM) of the thalamus. For dystonia and symptoms associated with PD, the lead was placed in either the globus pallidus or subthalamic nucleus.[20] During both types of electrode implantation a small section of brain tissue was removed in order to make room for the electrode. Because the size of the tissue section removed was very small, the yield of cells from the subsequent dissociation and cell culture was also generally low (Table 6-2, between 300 – 13,800 total cells). During brain cancer surgery small sections of brain tissue were removed in close proximity to the tumor. These tissue sections were generally larger than those obtained during electrode implantation and were obtained from widely varying areas of the brain. Due to the larger specimen size, greater numbers of cells were evident following the cell culture (Table 6-2, between 27, 900 – 65,700 total cells). Specimen(s) were processed only when the time between surgical excision and cell culture was less than six hours. Longer periods of time between surgical excision and cell culture resulted in lower neuronal yield as well as shorter survival time for the neurons *in vitro* (Figure 6-8 C, D).

All procedures were approved by the International Review Boards (IRBs) from both the University of Central Florida (UCF) and from Orlando Regional Medical Center.

*Optimized cell culture methodology and medium used for surgical and later AD samples*

Brain samples were collected in cold medium (~4ºC) consisting of Hibernate-A, Glutamax, and antibiotic-antimycotic. The samples were digested for 30 minutes at 37°C in Hibernate-A (HA) containing 6 mg papain / 12 ml (HA). *Dissociation Media*, initially formulated for the dissociation of rat and mouse adult brain tissue into individual cells[21-23], was used during the mechanical dissociation of tissue. *Plating Media*, which followed the dissociation media, promoted attachment and then regeneration of cells on the DETA-coated surfaces. *Maintenance Media* promoted the long-term maintenance of dissociated surgical cells and was applied to cultured cadaver neurons and surgical neurons after four *days in vitro*.

Each type of media was formulated to meet the challenges present during a specific stage of the cell culture process, and allowed for significantly improved survival, regeneration, and long-term growth (Table 6-1, Figure 6-1). The osmolarity of the media was adjusted with NaCl to match the osmolarity of human cerebrospinal fluid (295-305 mOsm).[24, 25] In the *Dissociation Medium*, antioxidants, both cerium-oxide nanoparticles [26] and Trolox [27], were added during the dissociation of the tissue to minimize oxidative damage. In addition, the inhibitors of caspase 1, 3, and 6 (Sigma, C0480 & C2105) were added to limit apoptosis during the dissociation of the tissue. Antioxidants and growth factors (Table 6-1) in the *Plating Media* promoted the regeneration and survival of the cells. The cells survived in some cases for 48 *div* with ½ media replacement (*Maintenance medium*) every fourth day (Figure 6-1).

*Initial cell culture-methodology and medium used for AD samples*

In early experiments, AD samples were dissociated and the cells were plated in medium that consisted of Neurobasal A/ 2% B27/ Glutamax/ Antibiotic-Antimycotic/ G5 Supplement/ Recombinant Mouse PDGF-BB/ IGF/ anti-apoptotic/ nanoactive Cerium Oxide nanoparticles. Cerium-oxide nanoparticles were obtained and used as described by Das et al, 2007 [26]. After two days in culture, the complete plating medium was replaced with culture medium consisting of Neurobasal A/ 2% B27/ Glutamax / antibiotic-antimycotic / recombinant mouse basic FGF. Thereafter half of the medium was replaced every four days. Later AD tissue samples were processed using the protocol described above for the surgical samples. The conditions for each sample are listed in the Results.

*DETA surface modification*

Glass cover slips (VWR 48366067, 22×22 mm$^2$ No. 1) were cleaned using 1:1 HC l-methanol followed by a concentrated $H_2SO_4$ soak for two hours. The DETA (United Chemical Technologies Inc. T2910-KG) film was formed by the reaction of the cleaned surfaces with 0.1% (v/v) mixture of organosilane in freshly distilled toluene (VWR BDH1151). In brief, the cleaned surfaces were heated to about 100°C in organosilane mixture, rinsed with toluene, reheated to about 100°C in toluene, and then dried overnight (100°C). Surfaces were characterized by static water contact angle measurements using a Rame-Hart Model 250 goniometer, and by X-ray photoelectron spectroscopy (XPS) using an Escalab 200i spectrometer (VG Scientific) by monitoring the N 1s peak.[28-30]

*Electrophysiology*

Whole-cell, patch-clamp recordings were performed at room temperature. Extracellular recording solution was comprised of Neurobasal-A medium. The solution contained 130 mM NaCl, 1.8 mM $CaCl_2$, 5.2 mM KCl, 1 mM $MgCl_2$, 2.2 mM $NaHCO_3$, and 10 mM HEPES (pH 7.3) (300 mOsm). Patch pipettes (4-8 M$\Omega$) were filled with intracellular solution (K-gluconate 140 mM, EGTA 1 mM, $MgCl_2$ 2 mM, $Na_2ATP$ 5 mM, HEPES 10 mM; pH 7.2). Cells were visualized on the stage with a Zeiss Axioscope, 2 FS Plus, upright microscope in maintenance culture medium. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (MDS Analytical Devices) amplifier. Signals were low-pass filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recordings and analysis were performed with Clampex software. Whole-cell capacitance and series resistance were compensated electronically. Only cells with access resistance less than 22 M$\Omega$ were analyzed. Inward currents that had the characteristics of fast sodium currents, and outward currents that had the characteristics of potassium currents, were measured in voltage clamp mode. Voltage step length was 50 ms, incremented 20 mV per step, 1 s between each step, with a holding potential of -70 mV. The action potential threshold was measured in current-clamp mode with increasing 1 s depolarizing current injections. Cells were electrically characterized on Days 4, 13, and 15 *in vitro*.

*Immunocytochemistry*

After electrophysiological characterization, cover slips were rinsed twice with Phosphate Buffered Saline (PBS). Cells were fixed with 4% paraformaldehyde for ten

minutes at room temperature, and subsequently rinsed three times with PBS. Cells were permeabilized for five min with a permeabilizing solution (0.5% Triton X-100 in PBS), and were then blocked for two hours (5% normal donkey serum in PBS). Anti-neurofilament-M polyclonal antibody (intracellular filament found in mature neurons, Chemicon, AB1981, diluted 1:1000), anti-NeuN (nuclear marker in mature neurons, Chemicon, diluted 1:1000), anti-Ki67 (nuclear marker in dividing cells, Chemicon, diluted 1:1000) and anti-GFAP (cytoplasmic marker in glial cells, MAB360, Chemicon, diluted 1:400) antibodies were added in blocking solution for 12 hr at 4ºC. After three washes with PBS, fluorescently labeled secondary antibodies were applied for two hours. Vectashield mounting medium (H1000, Vector Laboratories, Burlingame, CA) with DAPI (nuclear stain) was used to mount the cover slips onto slides. The cover slips were observed with an Ultra VIEWTM LCI confocal imaging system (Perkin Elmer).

*Statistical analysis*

Numerical summary results are reported as a mean, plus or minus the sample standard error of the mean ($\pm$ SEM). Statistical analyses were performed using the student's t-test on electrophysiological data.

*Role of the Funding Source*

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**Results**

*General culture conditions for samples obtained from human subjects:*

The attachment, survival, and regeneration of the dissociated neurons after cell culture were maximized through careful control of the following parameters: temperature of the *Dissection* and *Transport* media (4ºC) and time between death or surgery and initiation of the culture (two to six hours). The tissue sample was processed first by enzymatic dissociation of tissue in Hibernate A (HA) (37ºC shaking water bath, 80 revolutions per minute), then repeated washes of the tissue solution to wash away the papain, gentle dissociation of the tissue into cells with *Dissociation Media* using a fire-polished glass Pasteur pipette. This was followed by the plating of the cells onto the cell adhesive surface (DETA). After 45 minutes, a gentle wash of the cover slip with warm HA (37ºC) removed the majority of cellular debris, including myelin and myelin inhibitor factors.[31] Maintenance of the culture occurred every four days with half media removal and replacement with fresh *Maintenance Media*. This regimen (Figure 6-1) supported neuronal survival and regeneration (Figure 6-2; Table 6-2, culture results), and allowed for the investigation of the electrical properties (Table 6-3) and expression patterns displayed by adult human neurons in a controlled *in vitro* environment (Figures 6-3, 6-7).

*Details of cell culture results from human brain tissue surgically removed during DBS or TE surgery:*

Neurons cultured from the 12 DBS and 3 TE surgical brain tissue specimens demonstrated similar regeneration and survival *in vitro* (Figure 6-2). These neurons exhibited morphological regeneration within two to four *div*, with the neurons displaying

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phase bright cell somas, multi-branched dendrites, and strong, polarized axons. Neurons retained this morphology over the life-span of the culture, which averaged 30 days and for up to 48 *div* (Figure 6-2). The small size of the brain tissue specimens available was reflected in the low cell yield for each sample (Table 6-2, total number of cells after 6 *div*). Because the TE samples were larger than the DBS samples, the cell yield was higher.

The time between surgical excision of the brain tissue and the initiation of the cell culture strongly influenced the culture results. Longer periods of time between surgical excision and cell culture resulted in lower neuronal yield as well as shorter survival time for the neurons *in vitro*. This was quantified using adult rat hippocampal tissue that was cultured after storage in *Transport medium* for 0, 3, or 6 hours on ice. The number of neurons living after 1, 3, and 6 *div* was quantified for each sample (Figure 6-8 C, D). The number of living neurons was significantly lower for those samples stored for 6 hours. The living cells in this batch also had shorter and less highly branched neurites.

*Immunocytochemical investigations of cells from surgical samples:*

Two TE samples (Table 6-2) previously investigated electrophysiologically were probed with neurofilament-M, NeuN, and Ki67, allowing for both the determination of the neuronal nature of these cells as well as the lack of cellular division. The cells expressed both the neuronal cytoskeletal protein neurofilament-M and the neuronal nuclear marker NeuN. The cells did not express Ki67, a nuclear marker for dividing cells (Figure 6-3). A dividing population of neurons derived from the hippocampus of

adult rats was used as a positive control, and the nuclei of these cells stained for Ki67 expression.

Four DBS cultures (Table 6-2) previously investigated electrophysiologically were probed with neurofilament-M and GFAP to determine the ratio of neurons to glial cells. Very few cells expressed GFAP (4.3 ± 2.7%), while neurofilament-M expression was seen in most cells (87.6 ± 6.8%)(Figure 6-3). An adult rat hippocampal culture (14 *div*) was used as a positive control, with 8% staining for GFAP and 88% staining for neurofilament-M.

*Electrical investigations of neurons from surgical samples:*

The surgical sample neurons were evaluated for functional electrophysiological recovery. Day 4 and day 15 DBS cultures and day 4 and day 13 TE cultures were evaluated (Table 6-2, survival / electrical activity). For DBS neurons, after four *div* the Na$^+$ current flow averaged -637 pA and after 15 *div* it was -758 pA. K$^+$ current flow after four *div* averaged 937 pA and after 15 *div* it was 1221 pA. For TE neurons, after four *div* Na$^+$ current flow averaged -432 pA and after 13 *div* it was -873 pA (Table 6-3, Inward Currents). K$^+$ current flow after four *div* averaged 637 pA and after 13 *div* it was 1231 pA (Table 6-3, Outward Currents). These properties were not found to be significantly different between sample types or culture age. This indicates that in both cases the cultured human neurons exhibited consistent electrical activity *in vitro* beginning just a few days *in vitro*, and this activity was maintained. The main electrical difference between DBS and TE neurons was in the firing frequency exhibited by neurons derived from the two sources. DBS neurons fired single action potentials, while the majority of

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TE neurons were able to repetitively fire action potentials (Table 6-3, Firing Frequency). Voltage and current clamp traces for both the DBS and TE samples show both the inward and outward sodium and potassium currents and the single or multiple action potentials generated after depolarizing current injections (Figure 6-4). Groups of individual time series have been averaged, with only the average displayed.

*Details of cell culture results from AD samples:*

Neurons were cultured from hippocampal slices removed from the brain of AD and dementia patients during autopsy between 2-6 hours after death. Shorter periods between death and the initiation of the culture resulted higher yields of healthy neurons (Figure 6-8 A, B). In five cases, four of advanced AD and one from vascular dementia, the cells did not survive or recover *in vitro*. In one experiment, where tissue was obtained from a patient suffering from Lewy body dementia (LBD), the cells survived for four to five days but exhibited poor regeneration and contained a large number of vesicular bodies throughout the cell somas. In five other cases, where deceased individuals were suffering from mid stage AD, the cells survived for 10-15 days. In each of these ten cases, lack of dendritic branching as well as large vesicles indicative of trauma and early to late stage apoptosis or autophagy were evident[32] (Figure 6-5, A-C). In one age matched patient with no known neurodegenerative condition (Table 6-4, NOR-110209) cells survived for 15 days with phase bright cell somas, branching apical dendrites, small basal dendrites, and limited vesicles in the cell soma. Nine neurons from this sample were patched. None was electrically active (Figure 6-5, D).

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*In vitro* culture of dissociated AD hippocampal brain tissue resulted in heterogeneous cellular morphologies indicative of the cell populations present in this region of the brain. Some cells had characteristic pyramidal morphologies, whereas others had bipolar morphologies. Additionally, there were large numbers of cells which had no distinct morphologies or were devoid of any distinct processes. Cell bodies were rough and filled with numerous vacuole-like structures, indicative of the initiation of apoptosis or autophagy. The dendritic processes were missing in most of the neuron-like cells. Beyond ten days *in vitro,* these vacuole like inclusions in the cell body became more spread out, resulting in detachment of the cells from the substrate and / or apoptotic cell death (Figure 6-5).[32] The results from the live / dead assay (Figure 6-6) show the viability of most attached neurons at five through seven days *in vitro*. After day ten, however, most of the cells began to detach or undergo apoptosis and die. In addition to the viability of the cells *in vitro,* the live / dead assay indicated the different morphologies (pyramidal, multi-polar, bipolar, irregular shaped) of the hippocampal cells present *in vitro*.

AD neurons were probed with Neurofilament-M and DAPI after 12 *days in vitro* (Figure 6-7). Most neurons lacked dendritic processes or branching, and several neurons were multinucleated (Figure 6-7, C–D).  23 neurons from 5 diseased brain samples were patched.  None was electrically active.

**Discussion**

Currently there is enormous interest in developing *in vitro* cell culture models for the study of human neurodegenerative diseases and traumatic brain injuries. This stems in part from the growing interest in moving away from animal model test. A straightforward technique to culture human neurons from both healthy and diseased patients, especially AD patients, could enable a new paradigm in personalized medicine and clinical diagnostics. In addition, traumatic brain injury (TBI) has been elevated to new prominence in the neurological field due to returning war veterans and a more focused effort to evaluate the consequences of sports injuries.[33, 34]

In this study we successfully developed a defined *in vitro* culture model for primary human neurons consisting of an engineered synthetic substrate, serum-free medium formulation, and novel culture methodology. This tissue was derived from deceased patients as well as DBS and TE surgical samples. This novel culture system can be used to study individual or small networks of dissociated neurons isolated from human brain tissue. The main component of this novel *in vitro* system was (1) the media formulations and (2) the culture methodology, each of which were adapted from previous studies.[21-23] Anti-apoptotic molecules, Trolox ® and cerium oxide nanoparticles were added to the dissociation medium in order to limit apoptotic pathways and oxidative cell damage.[22, 35] The osmolarity of all media was adjusted to more closely mirror that found in human cerebral spinal fluid.[24, 36] Growth factors known to be important in adult CNS regeneration and maintenance (BDNF, NT-3, bFGF, and IGF-1[37-39] (Table 6-1)) were present in the medium.

Another crucial component of this cell culture system was an engineered growth substrate consisting of a DETA monolayer self-assembled on a clean glass cover slip(s).[29, 30] There are three major rationales for using the synthetic DETA substrate in this study. First, the triamine moiety in DETA mimics the growth factor spermadine, which has been shown to prolong cell life.[40] Second, DETA substrates can be coupled with specific extracellular matrix molecules and different contact molecules to systematically study the role of ECM proteins during brain injury and disease. Third, these surfaces can be used to create high-resolution patterns of hippocampal neurons for circuit formation and analysis either on cover slips or MEAs.[29]

The neurons isolated from tissue removed during DBS or TE recovered a healthy morphology and survived in long-term culture for up to 48 *days in vitro* (Figure 6-2). Immunocytochemical characterization of the surgical samples was conducted using the mature neuronal marker NeuN and the proliferation marker Ki67, and showed that most cells were mature, non-proliferating neurons (Figure 6-3). Both DBS and TE neurons were electrically evaluated, and both types were electrically active (Figure 6-4, Table 6-3). Results from these cultures contrast to cultured cells isolated from AD cadaver brain tissue. While these cells were neurofilament-M positive, indicating they were neurons, they exhibited limited morphological recovery, a shortened survival time, and lack of electrical activity recovery *in vitro*. Additionally, among cultured neurons derived from AD tissue a sub-population of neurons with multiple nuclei was present. We believe that such multiple nuclei could be a signature of AD samples, as has been noted in rare instances where multinucleated giant cells were filled with dense Abeta42 and Abeta40.[41]

We hypothesize the observed limitations in recovery noted from the AD neurons compared to the surgically excised neurons *in vitro* were a function of two factors: (1) the diseased state of the AD neurons and (2) the amount of time between death and extraction of tissue from the deceased AD patients. While the time from tissue removal to culture could be somewhat controlled with the surgical samples, this was less so with the samples from the AD cadavers. However, during the three year time period of the study the coordination between the recovery pathology teams and our lab was improved and this time was reduced, leading to a more efficient process. Despite its limitations this system provides a novel test bed for drugs for studying the survival, morphological recovery, and promotion of electrical recovery in a controlled environment of human neurons. Additionally, this system provides a new modality to investigate neuronal pathology associated with AD disease separate from the surrounding brain tissue and support cells, can be used to further understanding of the mechanisms of disease onset, progression, and end stage, and most importantly could facilitate the quick evaluation of potential drug targets and treatments for both AD markers and AD therapies, potentially on a personalized basis.

As noted above, a primary obstacle identified in this study was time between patient death, autopsy and tissue collection, and elapsed time for cell culture (Table 6-2, 6-4). With the surgical samples, which were collected during controlled situations where tissue samples were immediately introduced to cold storage and transport media, limited cell trauma and death was encountered. However, in tissue samples received from AD cadaver patients, the issues were more complex and include inconsistencies in the time period between patient death, varying transport times to the medical facility for

autopsy, time of autopsy, the ischemic environment present in the brain after death, and most importantly the neurodegenerative brain condition itself. These issues manifested in limited cell survival and regeneration *in vitro*. However, hippocampal samples removed from an age matched patient with no known neurodegenerative condition exhibited improved survival, morphological recovery, and longer term survival despite the presence of all previously described obstacles with the exception of AD pathology (Table 6-4, NOR-110209). This suggests that the disease condition could be the dominant factor involved in neuronal recovery and suggests a clear target for investigating reversal of neurodegeneration in a controlled environment.

The effect on survival and regeneration from the previously described obstacles in AD cultures was investigated using rat brain tissue. This animal study measured the effects resulting from the amount of time between the death of the animal and the initiation of the cell culture process. The length of time between the death of the animal and the initiation of cell culture directly influenced the survival and regeneration of the cells (Figure 6-8 A, B). We hypothesize that the ischemic conditions in the brain after death was a primary factor influencing the difference in success observed between AD patient samples and surgical samples. Through closer coordination between the recovery team, pathologist, and culture effort this could be overcome to a certain degree in future efforts in our lab as well as for others attempting this culture process.

In drug-discovery research, there is an increasing demand for cost effective high-throughput *in vitro* test methods as a substitute for *in vivo* toxicity testing and drug candidate evaluations. With *in vitro* experiments, the development and validation of a complex set of methods is necessary to accomplish this substitution[42-44] and many

cell-culture based methods have been proven to possess predictive values for different aspects of a compound's effect (absorption[45], metabolism[46], neurotoxicity[47], cardiac toxicity[48], etc.). Thus, a new function-based assay focused on human primary neurons would be of use from the general field of drug discovery to basic neuroscience, but especially for evaluating causative effects of AD disease and its possible remedies. This would be especially useful in investigating methods for the restoration of function of damaged neurons, as little or no progress has been made in reversing the effects of this tragic disease or in neuronal damage due to TBI.

As compared to previous models,[17, 18] [19] this system would be more robust due to the long-term survival of the human neurons obtained from the surgical samples. Further it was demonstrated for the first time that these healthy neurons regain both neuronal morphology and electrical activity *in vitro*. The diseased neurons obtained from the AD patients did not survive as long in culture but exhibited the characteristic features of degenerating AD neurons. The hope is that future improvements to both the speed of tissue reclamation as well as improved culture processing will make a culture system derived from AD brain tissue a more viable research tool. This tissue was obtained from deceased AD patients as well as DBS and TE surgical samples, but could be extended to include tissue from any part of the CNS to provide models for diseases such as Parkinson's, Multiple Sclerosis, and other neurological disorders.

In summary, we have established a robust system to enable functional studies of healthy and diseased human neurons which could be used for understanding disease pathology in drug discovery through changing electrical characteristics in individual neurons from toxic disease agents and after drug therapy.

**Table 6-1: Media compositions**

| Component | Quantity | Vendor | Catalog # |
|---|---|---|---|
| *Dissection & Transport:* | | | |
| Hibernate-A | 500ml | Brain Bits | HA |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| *Dissociation medium:* | | | |
| Hibernate-A | 500ml | Brain Bits | HA |
| B27 | 2% | Gibco | 17504-044 |
| Glutamax | 2mM | Gibco | 35050-061 |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone | 4$\mu$M | Sigma | C0480 |
| Z-Val-Ala-Asp fluoromethyl ketone | 5$\mu$M | Sigma | C2105 |
| Dextrose-coated Cerium Oxide Nanoparticles | 100nM | | |
| ($\pm$)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid | 70nM | Sigma | 238813 |
| *Osmolarity adjusted to 294mOsm with NaCl* | | | |
| *Plating medium:* | | | |
| Neurobasal-A | 500ml | Gibco | 10888 |
| B27 | 2% | Gibco | 17504-044 |
| Glutamax | 2mM | Gibco | 35050-061 |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| BDNF, recombinant human | 20ng/ml | Cell Sciences | CRB600B |
| NT-3, recombinant human | 20ng/ml | Cell Sciences | CRN500B |
| bFGF, recombinant human | 5ng/ml | Invitrogen | 13256-029 |
| Insulin-like Growth Factor-I (E3R) human | 20ng/ml | Sigma | I2656 |
| Dextrose-coated Cerium Oxide Nanoparticles | 100nM | | |
| ($\pm$)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid | 70nM | Sigma | 238813 |
| *Osmolarity adjusted to 296mOsm with NaCl* | | | |
| *Maintenance medium:* | | | |
| Neurobasal-A | 500ml | Gibco | 10888 |
| B27 | 2% | Gibco | 17504-044 |
| Glutamax | 2mM | Gibco | 35050-061 |
| Antibiotic / Antimycotic | 1% | Gibco | 15240-096 |
| BDNF, recombinant human | 20ng/ml | Cell Sciences | CRB600B |
| NT-3, recombinant human | 20ng/ml | Cell Sciences | CRN500B |
| bFGF, recombinant human | 5ng/ml | Invitrogen | 13256-029 |
| Insulin-like Growth Factor-I (E3R) human | 20ng/ml | Sigma | I2656 |
| *Osmolarity adjusted to 297mOsm with NaCl* | | | |

Culture periods for use of each medium:  Dissection and Transport - dissection, transport, and enzymatic digestion of the tissue specimen.  Dissociation – mechanical dissociation of the tissue specimen.  Plating – attachment of individual cells to substrate through culture day 3.  Maintenance – culture day 3 through the end of the culture

**Table 6-2: Temporal Lobectomy samples and cell culture results per sample**

| Sample | Patient Age | Patient Sex | Patient Condition | Cause of Surgical Intervention | Brain Region | Time btwn Surgery & Culture (hrs) | Total Number of Cells (6 *div*) | Survival / Electrical Activity in Culture |
|---|---|---|---|---|---|---|---|---|
| DBS-0821 | 57 | M | P | DBS | TL | 5.02 | 1,400 | Survived 15-20 *div* |
| DBS-0828 | 57 | M | P | DBS | FL | | 300 | Survived 10 *div* |
| DBS-0902 | 62 | F | | | | | 3,700 | Survived 15-20 *div* |
| DBS-0925a | 79 | M | P | DBS | FL | 6.32 | 450 | Survived 8 *div* |
| DBS-0925b | 57 | M | ET | DBS | FL | 7.81 | | Survived 10 *div* |
| DBS-1003 | 68 | F | P | DBS | FL | 3.07 | 2,200 | Survived 35-40 *div* (EC day 15, IC day 15, 35) |
| DBS-1014 | 67 | F | P | DBS | FL | | 1,600 | Survived 15-20 *div* |
| DBS-1104 | 82 | M | P | DBS | FL | 4.01 | 1,700 | Survived 30-35 *div* (EC day 4, IC day 25) |
| DBS-1202 | 54 | M | P | DBS | FL | 3.50 | 4,200 | Survived 45-50 *div* (EC day 15, IC day 10) |
| DBS-1209 | 71 | F | ET | DBS | FL | 5.00 | 13,800 | Survived 45-50 *div* (EC day 4, IC day 18) |
| DBS-0127 | 58 | M | ET | DBS | FL | 8.23 | 2,900 | Survived 25-30 *div* |
| DBS-0210 | 63 | M | P | DBS | FL | 6.02 | 3,000 | Survived 10-15 *div* |
| TE-0828 | 67 | M | Tumor | TE | | 2.79 | 27,900 | Survived 45-50 *div* (EC day 4, 13, IC day 18) |
| TE-0916 | 79 | M | Tumor | TE | | 3.54 | 41,900 | Survived 45-50 *div* (EC day 4, 13, IC day 22) |
| TE-1202 | 44 | F | Tumor | TE | FL | 5.52 | 65,700 | Survived 30-35 *div* (EC day 13) |

TL=Temporal Lobe, FL=Frontal Lobe, DBS=Deep Brain Stimulation, insertion of electrode, TE=Tumor Excision, IC=Immunocytochemistry, EP=Electrophysiology. Specimen taken from patients with Parkinson's, Central Tremor, or brain tumors. Specimen usually from ventral infundibular nucleus. 12 total samples were recovered during DBS surgery, 3 total samples from TE surgery. Specimen put into media within 15 minutes. Culture conditions for all sample outlined in Figure 6-1. Average cell yield was higher from TE surgery. The maximum period cells were observed to survive *in vitro* was between 45-50 days.

**Table 6-3: Electrical properties of neurons after 4, 13, or 15 days *in vitro*, examined through single cell patch-clamp electrophysiology**

| | Surgical, DBS | | Surgical, TE | |
|---|---|---|---|---|
| | 4 *div* | 15 *div* | 4 *div* | 13 *div* |
| Number of cells examined | 13 | 15 | 16 | 15 |
| Vm (mV) | -38.7 ± 3.4 | -43.6 ± 2.1 | -25.7 ± 5.7 | -32.9 ± 8.9 |
| Rm (mΩ) | 255 ± 27.6 | 344 ± 22.6 | 177 ± 23.9 | 223 ± 18.7 |
| Cm (pF) | 14.1 ± 1.3 | 17.3 ± 2.1 | 19.6 ± 3.2 | 17.8 ± 2.7 |
| Rseries (mΩ) | 17.6 ± 2.0 | 18.1 ± 1.7 | 16.3 ± 1.7 | 18.2 ± 2.9 |
| Inward Current, Na$^+$ (pA) | -637 ± 57.6 | -758 ± 74.3 | -432 ± 233 | -873 ± 322 |
| Outward Current, K$^+$ (pA) | 937 ± 47.2 | 1221 ± 74.0 | 637 ± 111 | 1231 ± 288 |
| Vthr (mV) | -37.6 | -43.7 | -52.1 | -57.2 |
| Firing Frequency | 0.9 ± 0.05 | 1.0 ±.07 | 1.7 ± 0.6 | 2.67 ± 0.38 |
| AP Amplitude (mV) | 89.6 ± 11 | 97.2 ± 12.1 | 83.4 ± 13.2 | 92.3 ± 11.1 |
| AP Duration (ms) | 4.2 ± 1.2 | 5.1 ± 2.3 | 3.9 ± 1.2 | 5.4 ± 2.1 |
| AHP Amplitude (mV) | -5.3 | -5.47 | -7.9 | -8.92 |
| AHP Duration (ms) | 42.3 | 53.2 | 32.1 | 37.8 |

DBS = samples removed during Deep Brain Stimulation electrode implantation, TE = samples removed during Tumor Excision, *div* = culture days *in vitro*, Vm = resting membrane potential, Rm = membrane resistance, Cm = membrane capacitance, Rseries = series resistance, Vthr = action potential threshold voltage, AP = action potential. Firing frequency: within the 1 s depolarizing current injection, the firing frequency is the number of action potentials generated per voltage step after the threshold has been reached. Electrical properties of neurons *in vitro* derived from human neuronal tissue removed either during electrode implantation or tumor excision surgery. Data is presented as mean +/- S.E.M.

**Table 6-4: Human cadaver brain tissue samples and cell culture results per sample**

| Diseased Sample | Patient Age | Patient Sex | Patient Condition | Brain Region, weight (grams) | Tissue Collection Medium | Culture Dissociation Parameters | Culture Medium (per sample) | Survival in Culture |
|---|---|---|---|---|---|---|---|---|
| AD-91507 | 83 | F | AD | Hip, ND | HA+B27+ Ceria | Papain 30 min + Gradient | G5+PDGF and followed by Basic FGF | Survived in culture for 10-15 *div* (L-D Assay) |
| AD-92607 | 80 | M | AD | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | Survival in culture for 10 *div* (L-D Assay) |
| VD-113007 | 90 | M | VD | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | No Survival |
| VD-121707 | 86 | F | AD | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | Survival in culture for 20 *div* (IC on day 12) |
| AD-10708 | 74 | M | AD, Mid-Stage | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | Survival for 10 *div* |
| AD-10708 | 90 | M | AD, Advanced | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | No Survival |
| DLBD-42308 | 79 | M | DLBD | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | Survival for 4-5 *div* |
| AD-50508 | 80 | M | AD, Advanced | Hip, ND | HA+B27+ Ceria | Papain 30 min+ Gradient | G5+PDGF and followed by Basic FGF | No Survival |
| AD-52109 | 87 | M | AD, Advanced | Hip, 1020gr | HA+B27+ Ceria | Papain 30 min + Dissociation Media | See Figure 6-1 | No Survival |
| AD-60909 | 82 | M | AD, Advanced | Hip, 932gr | HA+B27+ Ceria | Papain 30 min + Dissociation Media | See Figure 6-1 | No Survival |
| AD-90409 | 87 | F | AD, Mid-Stage | Hip, ND | HA+B27+ Ceria | Papain 30 min + Dissociation Media | See Figure 6-1 | Survival 10 *div* |
| NOR-110209 | 91 | F | No neurological condition | Hip, 1320gr | HA+B27+ Ceria | Papain 30 min + Dissociation Media | See Figure 6-1 | Survival and regeneration 14 *div* |

AD = Alzheimer's Disease, VD = Vascular Dementia, DLBD = Lewy Body Dementia, NOR = no neurological condition, M = Male, F = Female, Hip = Hippocampus, ND = not determined, HA = Hibernate A, G5 = G5 Supplement, PDGF = Platelet Derived Growth Factor, L-D Assay = Live dead assay, Ceria = cerium oxide nanoparticles, *div* = days *in vitro*. Summary of hippocampal cultures from brain tissue removed during autopsy. All but one individual suffered from a neurodegenerative condition.
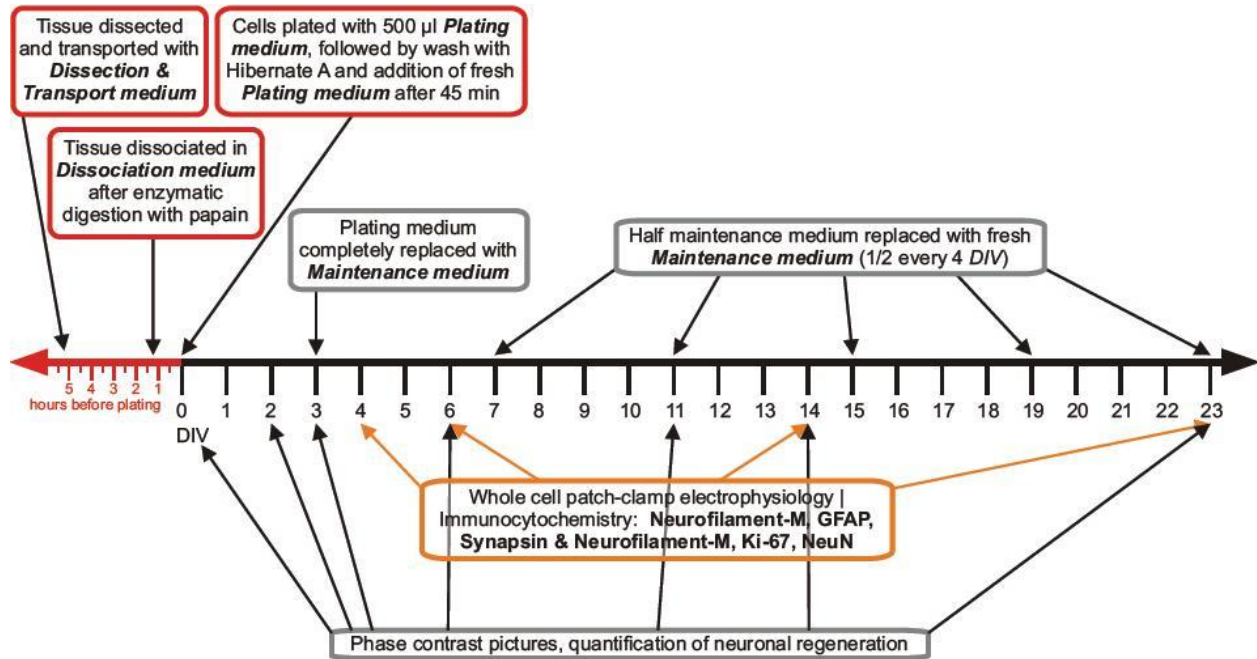
**Figure 6-1: Culture methodology for the processing and dissociation of human brain specimens to create and maintain a dissociated neuronal cell culture.** The time scale of the cell culture indicates timed application of growth factors and quantification of various neuronal parameters. The cells were examined immunocytochemically after 4, 6, 14, and 23 days *in vitro* (Neurofilament-M, Ki-67, NeuN, Synapsin, and GFAP). Electrical parameters of the cells were examined after 4, 13, and 15 days *in vitro.*
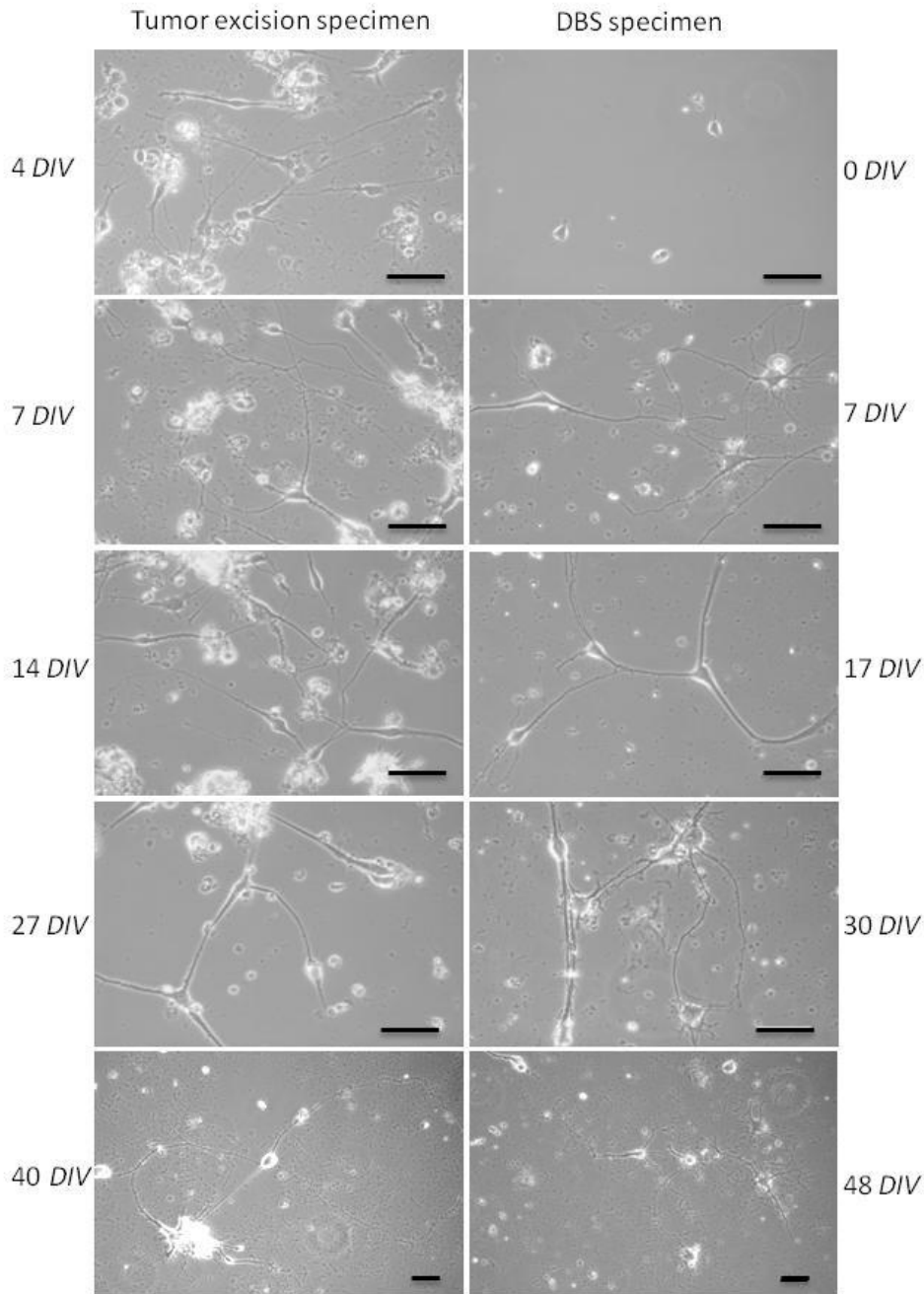
**Figure 6-2: Phase contrast pictures of dissociated cells *in vitro* derived from Temporal Lobectomy brain specimen.** Cells *in vitro* cultured from brain tissue extracted from patients with Parkinson's, Central Tremor, or brain tumors. Parkinson's or Central Tremor specimens were extracted during DBS electrode implantation. Brain tumor specimens were extracted during tumor excision surgery. Each sample was processed to dissociate the cells, and the cells regenerated and survived for up to 48 *div*. *div* = days *in vitro,* DBS = Deep Brain Stimulation*. Scale* bar = 40 µm.
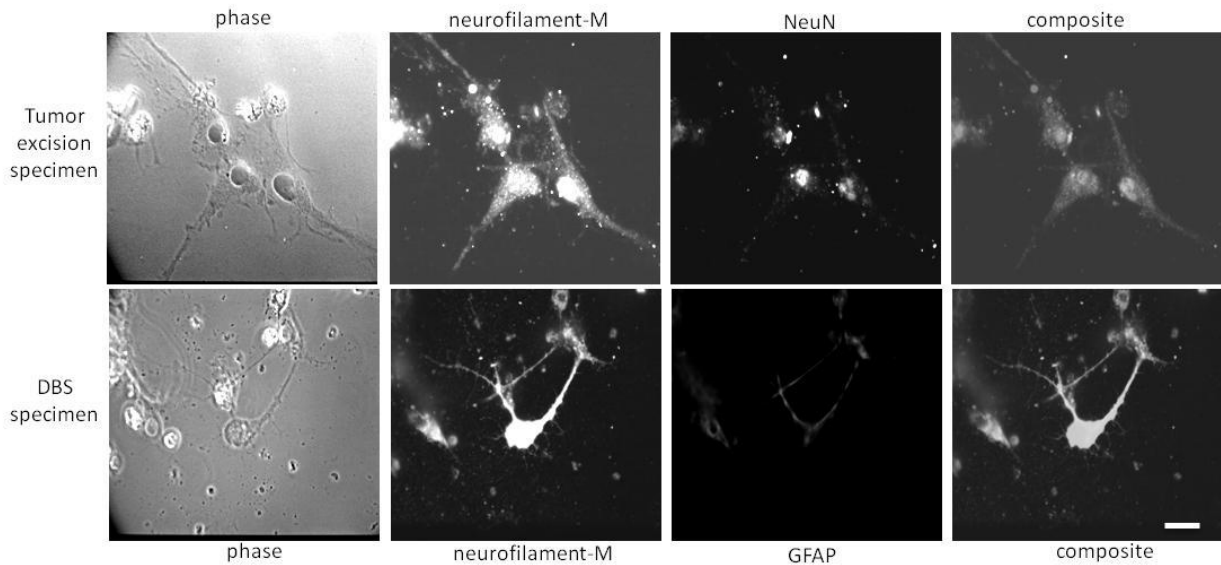
**Figure 6-3: Immunocytochemical characterization of dissociated cells *in vitro* derived from surgical brain samples obtained during deep brain stimulation surgery or tumor excision surgery.** From specimen removed during tumor excision: Phase, Neurofilament-M (red) , NeuN (blue), Ki67 (green, not expressed), composite . Expression of Nf-M and NeuN indicated maturity of neurons, lack of Ki67 expression indicated lack of cell division. From brain tissue extracted during DBS electrode implantation: Phase, Neurofilament-M (green), GFAP (red), composite. Cells positive for neuronal marker neurofilament-M, negative for glial marker GFAP. Scale bar = 25 µm
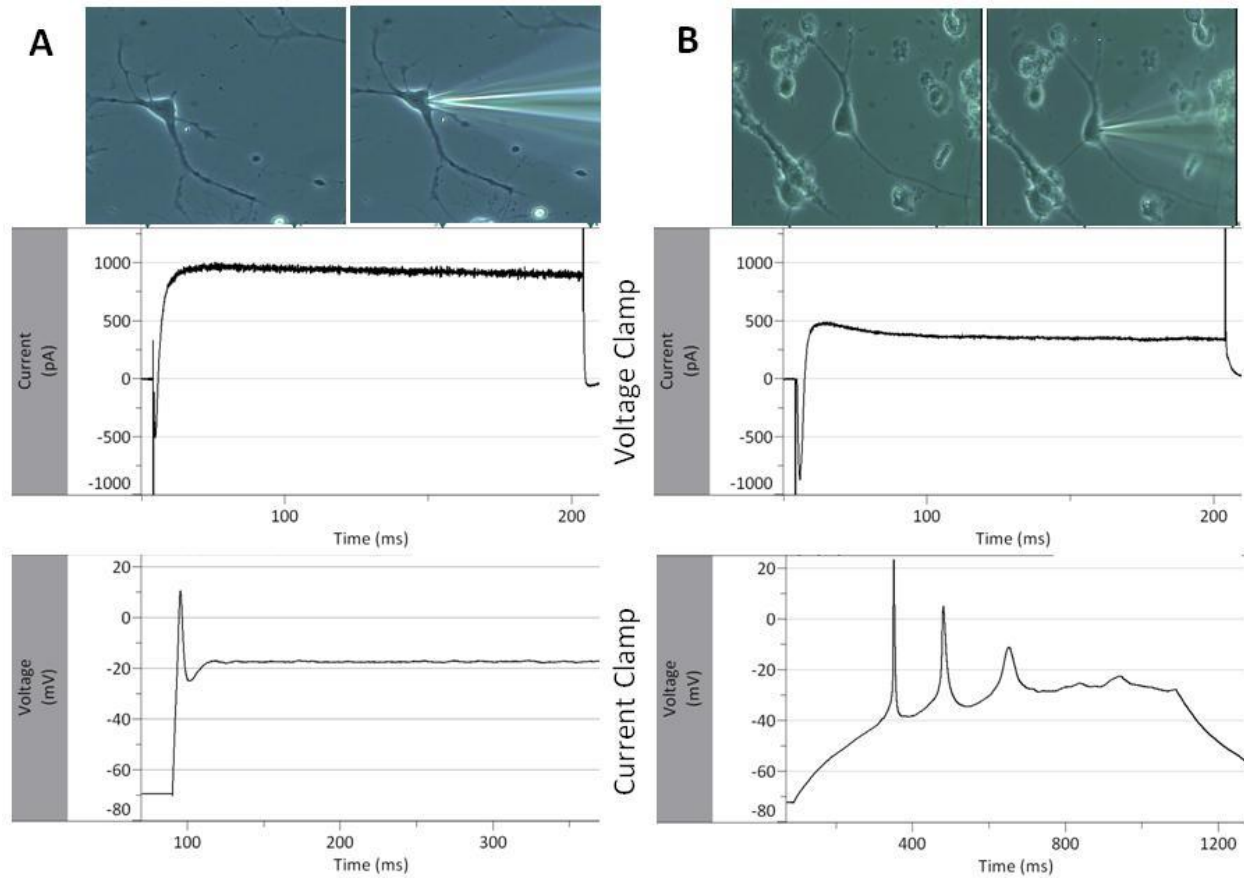
**Figure 6-4: Electrical properties of neurons *in vitro* derived from brain specimen(s) excised during (A) electrode implantation for DBS or (B) tumor excision surgery.** After neurites have reformed, neurons were probed electrically to determine their *in vitro* properties. Both current flow (voltage clamp) and AP generation (current clamp) were measured in cells extracted from surgical samples extracted during either electrode implantation (A) or tumor excision (B) surgery. Voltage-gated sodium and potassium channels were measured in voltage-clamp experiments, action potentials were measured in Current-clamp mode. Voltage clamp stimulation protocol: Voltage step length of 50 ms, increment of 20 mV per step, time between the starts of each step was 1 s, holding potential of -70 mV. Current clamp stimulation protocol: The action potential threshold was measured with increasing 1 s depolarizing current injections. Groups of individual time series have been averaged, with only the average displayed.
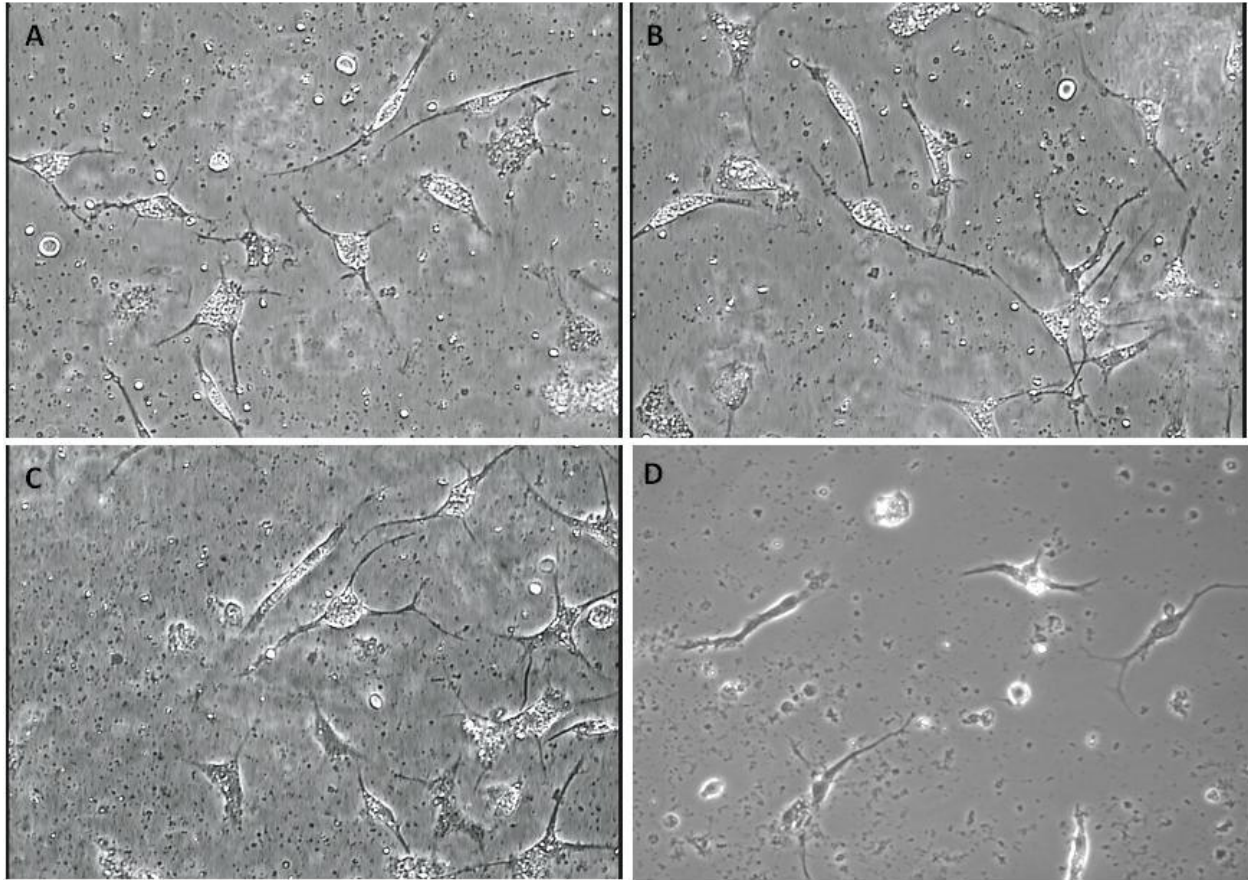
**Figure 6-5:  Pictures of dissociated cells *in vitro* derived from the hippocampus of deceased Alzheimer's patients during autopsy**.  (A-C) cells cultured from a brain specimen(s) removed during autopsy from a patient with a neurodegenerative disorder, 5-7 *div*.  Multiple vesicles were evident in the cell bodies, commonly found in neurons undergoing autophagy or apoptosis.  (D) cells cultured from a brain specimen(s) removed during autopsy from a patient without a neurodegenerative disorder (NOR-110209).  Vesicular exclusions not present.  Scale bar = 40 µm.
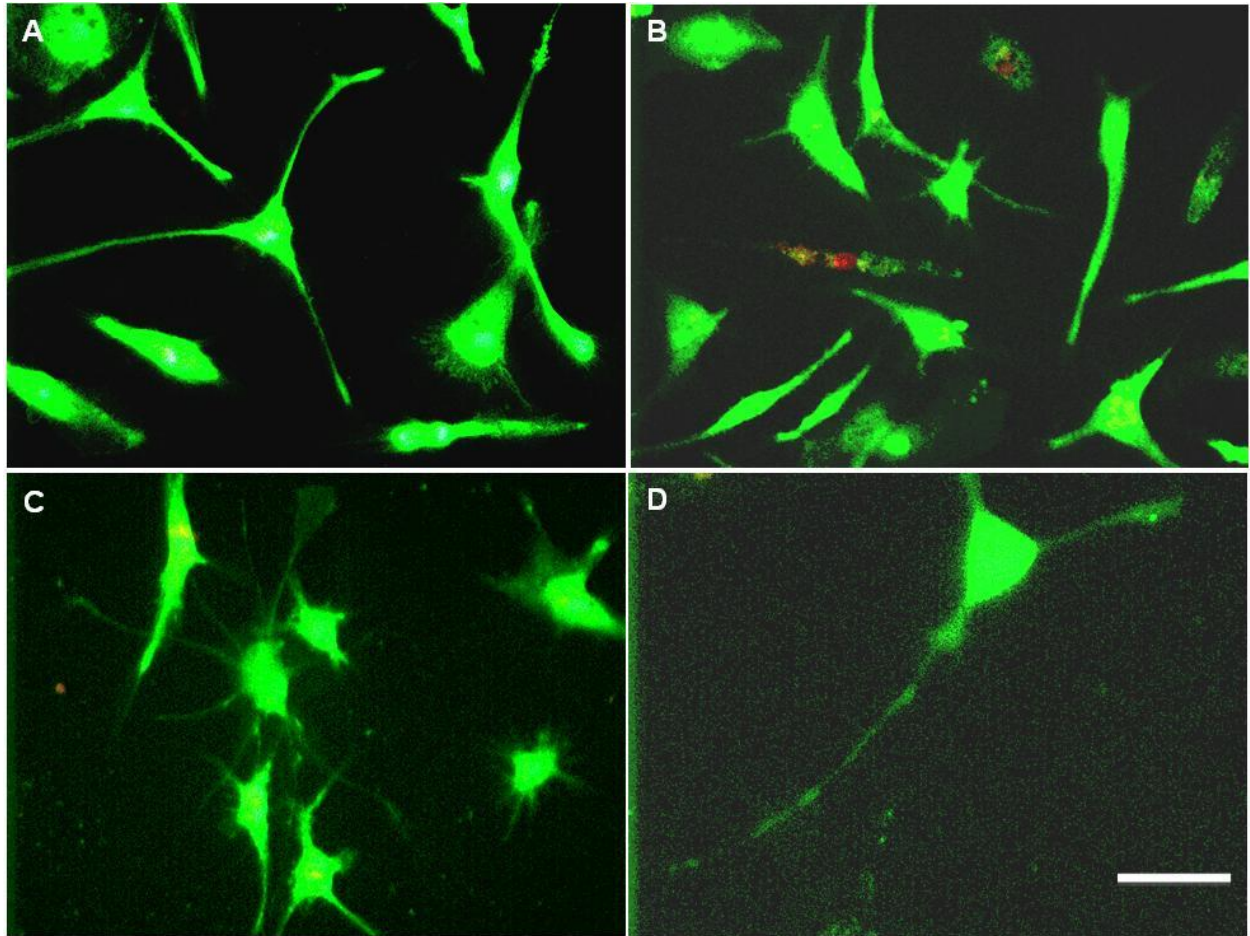
**Figure 6-6: Live Dead Assay of dissociated cells *in vitro* derived during autopsy from the hippocampus of deceased patients with a neurodegenerative disorder.** Cells between 5-7 *div*. Live cells – green, dead cells – red. Despite the presence of vesicles in the cell bodies, the majority of cells were alive between 5-7 *div*. *div* = days *in vitro*. Scale bar = 30 μm.
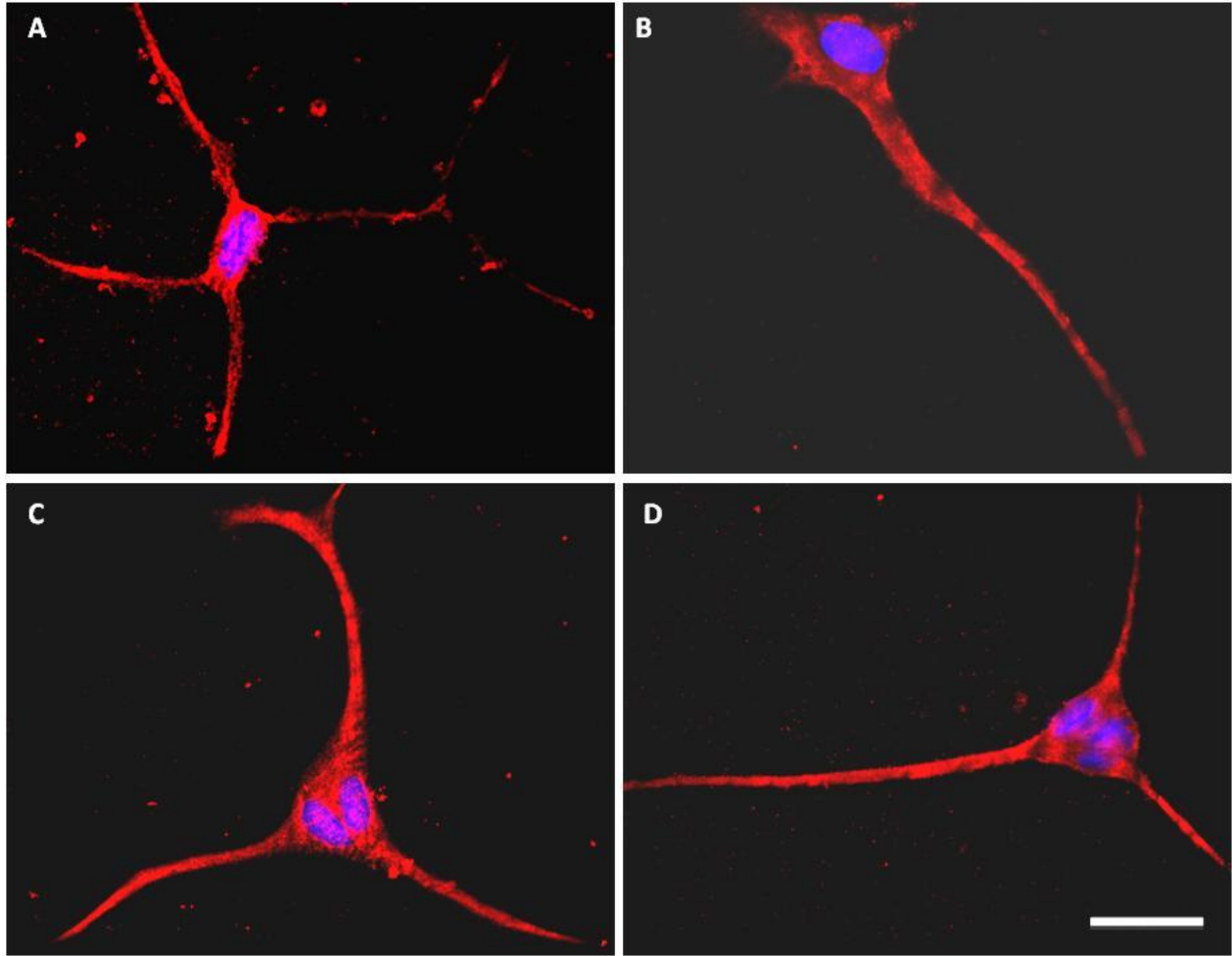
**Figure 6-7: Immunocytochemical characterization of dissociated cells *in vitro* derived from the hippocampus of deceased Alzheimer's patients.** Immunocytochemical evidence for the presence of neurons after 12 days *in vitro* (Neurofilament-M - red, DAPI - blue). (A) neuron with minimal branching, (B) neuron lacking branched processes, (C-D) multinucleate neurons with minimal branching. Scale bar = 30 µm.

**A** Dissection after 0, 2, or 4 hours after death

**B**

|  | Phase-contrast | Live / Dead |
| --- | --- | --- |
| 0 hour delay | | |
| 2 hour delay | | 6 *DIV* |
| 4 hour delay | | 6 *DIV* |
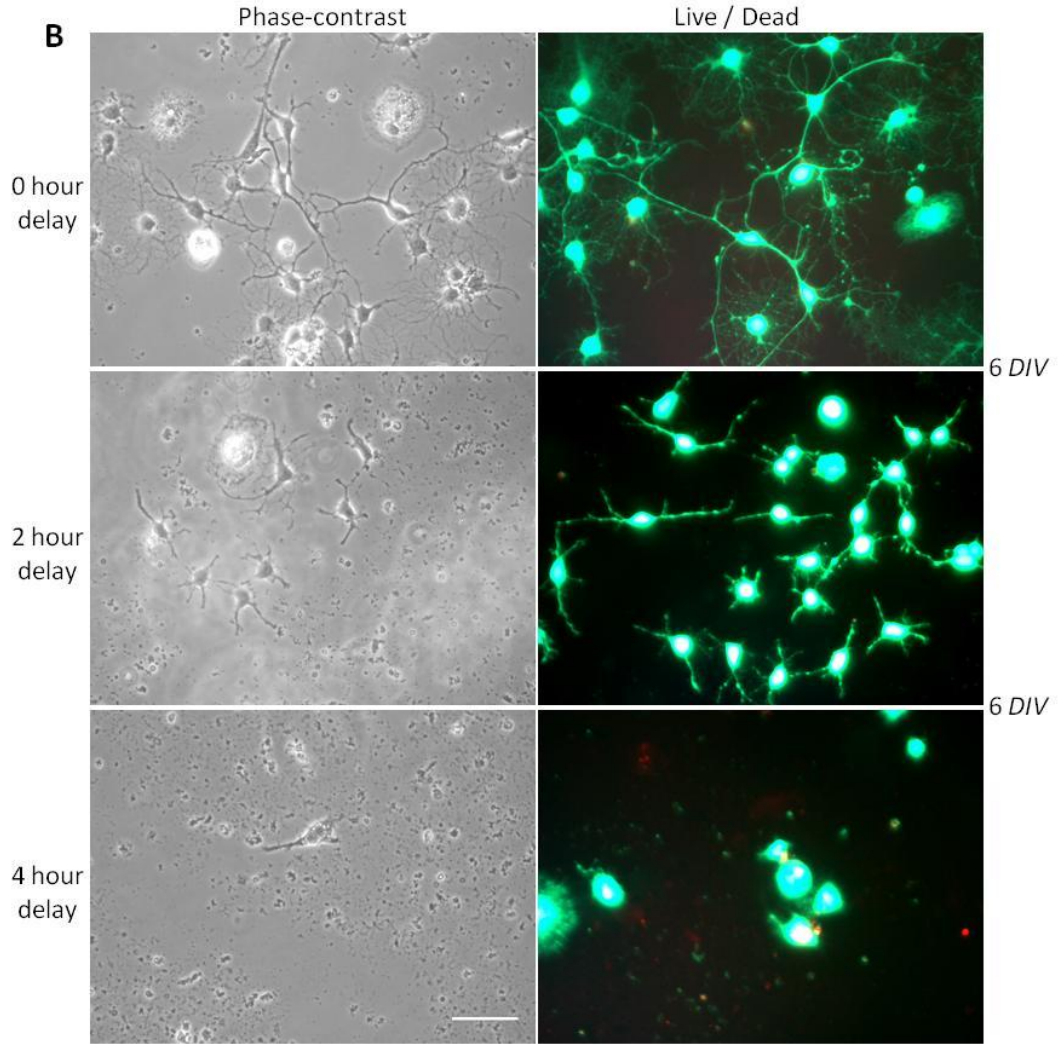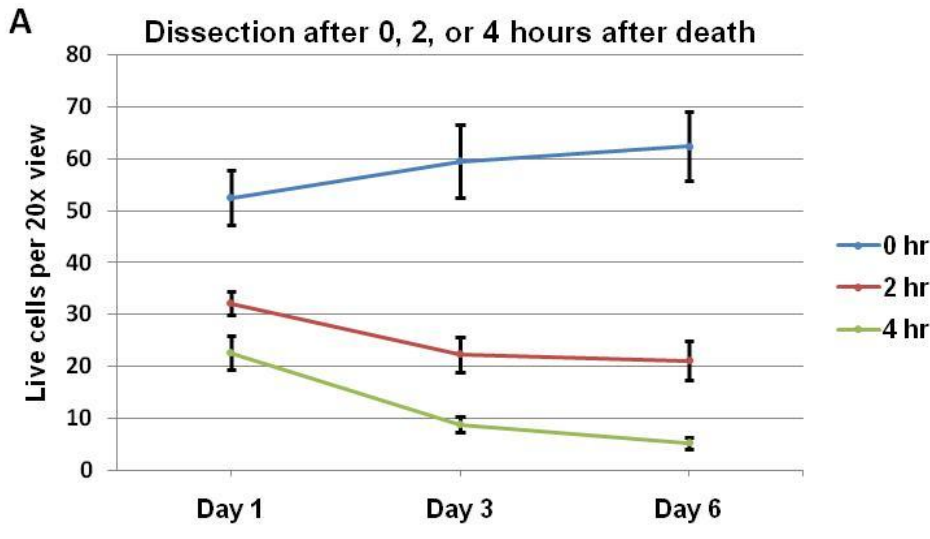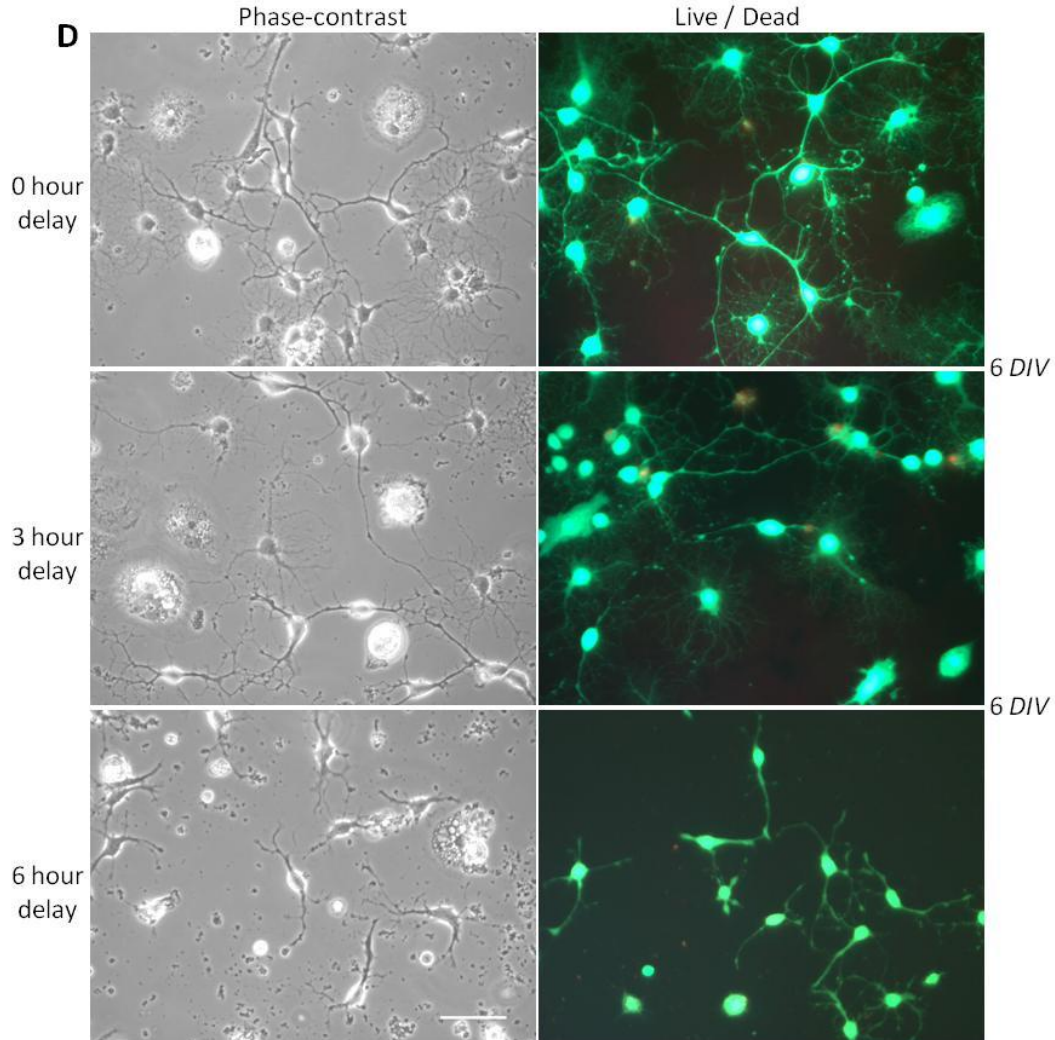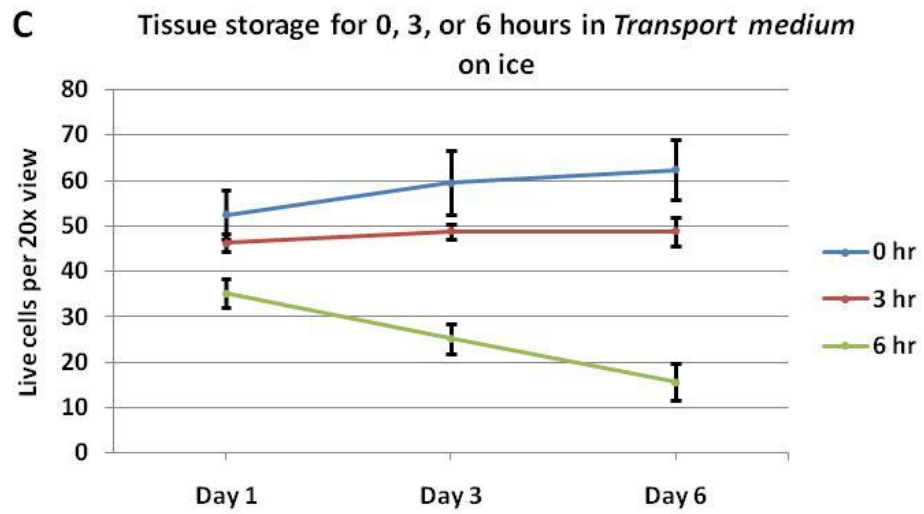
**Figure 6-8:** *In vitro* **effect from a dissection delay or culture delay on neuronal viability of rat hippocampal neurons**. (A, B) dissection of the brain tissue did not

occur for 0, 2, or 4 hours after the death of the rat. (A) Dissection after 0, 2, or 4 hours. Live / Dead assay for viable neurons cultured after dissection of the brain tissue was delayed for 0, 2, or 4 hours after the death of the rat. The number of living neurons after 1 *div* was significantly lower for the 2 and 4 hour samples versus the 0 hour sample. Further loss of neurons was seen in the 2 and 4 hour samples after 3 and 6 *div*. (B) 6 *div*, the neurites on those cells derived from brain tissue extracted without delay were longer and more highly branched than those cell derived from brain tissue extracted after a 2 or 4 hour delay. Scale bar = 50 μm. (C, D) dissection of the hippocampus occurred immediately, but the tissue was stored in *Transport medium* on ice for 0, 3, or 6 hours before the culture was initiated. (C) Culture after 0, 3, or 6 hour storage in *Transport medium*. Live / Dead assay for viable neurons cultured after rat hippocampal tissue had been stored in *Transport medium* on ice for 0, 3, or 6 hours. The number of living neurons after 1, 3, and 6 *div* was only slightly lower after 3 hours storage, but was significantly lower if the tissue had been stored for 6 hours before the initiation of the culture. Regeneration of the neurons was significantly less after 6 days for those samples stored for 6 hours. (D) 6 *div*, the neurites on those cells derived from brain tissue processed with a delay of 3 hours or less were longer and more highly branched than those cell derived from brain tissue processed after a 6 hour delay. Scale bar = 50 μm.

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# CHAPTER 7 - GENERAL DISCUSSION

Tissue engineering, an interdisciplinary field that applies the concepts and methodologies of engineering toward studying problems in the life sciences [1], is an emerging field with widespread applications into both basic and translational research in the field of neuroscience. At the molecular, cellular, and systems level, neuroscience research investigations are reliant upon the existence of accurate networks that mimic the human brain and/or functions of the human brain. Tissue engineered neuronal systems that mirror the function of individual or small networks of neurons in the brain can be used to determine basic neuronal function, to extrapolate disease processes, and to translate this knowledge into new drugs, devices, and treatment options for clinical patients [2].

Historically, researchers have been forced to rely upon *in vivo* or *in vitro* models that have flaws or inadequacies due to either the complexity of the brain or the complex nature of most neurological conditions, injuries, or diseases. Rat and mouse *in vivo* disease models have been created that mimic pathological symptoms of human neurological and neurodegenerative conditions. These models have been widely used in research to understand the onset and progression of these conditions as well as in testing of drugs and therapies in their reversal [3-5]. Hippocampal slice cultures, where thin slices of intact hippocampal tissue are removed from adult brain tissue, have been used for electrophysiological studies into synaptogenesis, neuronal communication, long-term potentiation, and pathophysiology of brain disease [6-9]. During the last decade several functional *in vitro* systems have been developed to study central and peripheral nervous system disorders using embryonic and fetal rat or mouse tissues,

typically with rat E18 hippocampal neurons [10-21].  This practice of using embryonic rather than mature brain tissue has been predicated upon difficulties in supporting regeneration, functional recovery and long-term survival of adult neurons in vitro. Some work has been performed on the refinement of *in vitro* dissociated neuronal systems using adult brain tissue rather than embryonic brain tissue [10, 11].  While these systems supported the morphological recovery of adult hippocampal neurons *in vitro*, issues with the support of both long-term survival and full recovery of electrical activity of neurons in these culture systems have prevented its widespread adoption as a research tool [3, 22, 23].  Additional studies have been conducted to develop culture systems using human neurons, which while partially successful in supporting short-term survival of dissociated human neurons or neural progenitors, were not evaluated for basic electrical functionality [24-26].

One of the main challenges in translational neuroscience research can be found in using the knowledge gained from abstract models of neuronal or neurological function in the development of human clinical treatments or therapies.  The main problems with using these systems:

1. Animal models of neurodegenerative or neurological conditions do not translate well to the human condition

2. More applicable functional *in vitro* model systems derived from adult and / or human tissue have been limited or unavailable

3. *In vitro* embryonic neuronal culture systems are not mature functionally nor do they display mature neuronal protein expression patterns [27-35].  The usefulness of these developmentally and functionally immature neurons for

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studies of neuronal electrical activity and synaptic transmission is severely limited by the lack or limited expression of the neuronal machinery responsible for electrical transmission in the adult brain. In addition, using these immature neurons in studies of neurodegenerative diseases or drug discovery can yield results that cannot be correlated to the function or action of mature neurons in adult brain tissue.

Conversely, an adult hippocampal culture system could be used to study the function of neurons, neuronal interactions, aging and neurodegenerative disease from a new perspective where the essential ion channels, receptors and other cellular components found in adult neurons had matured *in vivo* [36].

This dissertation research has sought to create a new, more relevant dissociated neuronal culture system derived from the hippocampus of adult rats. A few studies have shown that neurons derived from the hippocampal tissue of adult rats could survive and regenerate *in vitro* under serum-free conditions [3, 10, 11, 22, 23]. However, while the adult neurons regenerated morphologically under these conditions, both the electrical activity characteristic of *in vivo* neurons as well as long-term neuronal survival was not consistently recovered *in vitro*. In chapter 2, we demonstrated a defined culture system with the ability to support functional recovery and long-term survival of adult rat hippocampal neurons. In this system, the cell-adhesive substrate, N-1 [3-(trimethoxysilyl) propyl]-diethylenetriamine (DETA), supported neuronal attachment, regeneration, and long-term survival of adult neurons for more than 80 days *in vitro*. Additionally, the excitatory neurotransmitter glutamate, applied at 25 $\mu$M for 1 to 7 days after morphological neuronal regeneration *in vitro*, enabled full recovery of neuronal

electrical activity.   This low concentration of glutamate promoted the recovery of neuronal electrical activity but with minimal excitotoxicity.  These improvements allowed electrically active adult neurons to survive *in vitro* for several months, providing a stable test-bed for the long-term neuronal studies.

This newly developed neuronal culture system derived from adult rat hippocampal tissue simplified the culture process while demonstrating reliability, reproducibility, and relevancy.   In chapter 3, this dissociated neuronal cell culture system was modified.  Through the action of regeneration promoting growth factors, most importantly bFGF, and the dose-dependent application of the novel anti-mitotic factor roscovitine cdk5 mediated cell cycle progression was activated or deactivated to promote or control the division of mature, terminally differentiated neurons.   The application of this cell cycle control to an improved serum-free culture system supported the expansion of primary adult neuronal cells under controlled conditions across multiple passages with the ability to arrest mitotic division at any time.   Functional recovery of these previously dividing neurons could then be provoked by glutamate. Cell cycle control also allowed for the development of a stable population of primary neurons that both morphologically and functionally regenerated without expansion.

This newly discovered ability to both culture primary adult hippocampal neurons under serum-free culture conditions, maintain them in a primary, non-dividing state, and utilize them for purposes of basic research, drug discovery, and therapeutic testing represents a new and exciting breakthrough in the quest for faster and more targeted drug discovery.  Additionally, the described ability for these post mitotic neurons to re-enter and progress through the cell cycle *in vitro*, ultimately dividing multiple times,

represents a new paradigm previously thought to be beyond the capability of primary neurons in the brains of higher vertebrates.  The use of cdk inhibitors, specifically roscovitine, to prevent the induction of neuronal division and return neurons to a quiescent yet functionally and electrically active state capable of forming complex network connections and communication, opens up possibilities into a whole new realm of research in disease mechanisms and potential therapeutics.  While neurons in the mature brains of higher vertebrates had previously been thought to be terminally differentiated and incapable of cellular division, it has been proven not only that they can be induced to divide but to also to return to a non-dividing and functional state.

In chapter 4, the directed formation of small networks of electrically active, synaptically connected adult neurons was demonstrated through the use of engineered chemically modified culture surfaces.  Although synaptogenesis, synaptic communication, synaptic plasticity, and brain disease pathophysiology can be studied using brain slice or dissociated embryonic neuronal culture systems, the complex elements found in neuronal synapses makes specific studies more difficult to examine using these random cultures.  The study of synaptic transmission in mature adult neurons and factors affecting synaptic transmission are generally studied in organotypic cultures, in brain slices, or *in vivo*.  However, engineered neuronal networks can allow these studies to be performed instead on simple functional two-cell neuronal networks derived from adult brain tissue.  Photolithographic patterned self-assembled monolayers (SAMs) were used to create the two-cell "Fish" network pattern.  This pattern consisted of the cell permissive SAM DETA and was composed of two 30 µm somal adhesion sites connected with 5 µm lines acting as surface cues for guided axonal and dendritic

regeneration.  Surrounding the DETA pattern was a background of a different non-cell permissive SAM poly(ethylene glycol) (PEG).  Adult hippocampal neurons were first cultured on cover slips coated with DETA monolayers and were later passaged onto the PEG-DETA Fish patterns in serum-free medium.  These neurons followed surface cues, attaching and regenerating only along the DETA substrate to form small engineered neuronal networks.  These networks were stable for more than 21 days *in vitro*.

The development of an *in vitro* system where attachment and regeneration of adult hippocampal neurons was guided using photolithography to form small synaptically connected neuronal networks represents a significant technological advancement.  PEG-DETA surfaces prepared by laser ablation photolithography were sufficient to direct the attachment of cells specifically to the DETA "Fish" patterns while restricting attachment to the PEG background.  Mature, terminally differentiated neurons derived from hippocampal brain tissue of adult rats attached, adhered, and regenerated functional neurites along the guided DETA cues of the Fish pattern.  These neurons were found to be both electrically active and synaptically connected, and displayed synaptic connectivity characteristic of excitatory glutamatergic neurons.  This system provides a unique tool that can be used for studies into LTP [36, 38, 39].  In addition, this functional *in vitro* system would enable high-throughput neuropharmacology studies, facilitating drug development and furthering research into different neurological disorders.

Microelectrode arrays (MEAs) are innovative tools used to perform electrophysiological experiments for the study of neuronal activity and connectivity in populations of neurons from dissociated cultures.  A common limitation of neuronal

MEA systems has been the reliance upon neurons derived from embryonic tissue. The results described in chapter 5 demonstrate an adult dissociated neuronal culture system on MEAs. To characterize the type of synaptic connections between cell types, spontaneous network activity from both embryonic and adult neurons on MEAs were concurrently measured in the presence/absence of synaptic transmission antagonists against NMDA, AMPA, and GABA$_A$ channels for more than 90 days *in vitro* (*div*). In addition, both embryonic and adult neurons were evaluated for NMDA and AMPA channel subunit expression over 36 *div.* These results established that neurons derived from embryonic tissue did not express mature synapses for several weeks *in vitro,* and consequently their response to synaptic antagonists was significantly different than that of neurons derived from adult tissue sources. These results establish the utility of this unique hybrid system derived from adult hippocampal tissue for drug discovery and fundamental research including neuronal development and regeneration. Moving toward using this high-throughput hybrid *in vitro* system will expand and improve drug testing and basic research by providing a viable, easily manipulatable alternative to expensive, resource intensive *in vivo* testing.

We have demonstrated critical differences between adult and embryonic neurons and their respective synaptic connections which could be highly relevant in neurodegenerative disease research. By demonstrating the similarities and differences between adult and embryonic neurons and the response of each to synaptic antagonists, the usefulness of this adult neuron culture system has been established for application in neuronal regeneration and drug discovery studies. By incorporating adult neurons into an MEA system, a high-throughput system has been created to enable the

screening of a large number of cells and the study of pathogen and drug effects on the same population of cells over an extended period of time. This screen could find important applications in pharmaceutical drug development by providing an *in vitro* high-throughput test platform for investigations into neurodegenerative disease, traumatic brain injury, stroke, drug discovery, and fundamental research.

*In vitro* culture models of primary human neurons have enormous application in developing therapies for neurodegenerative diseases and traumatic brain injuries. Electrically functional primary human neurons had not been cultured *in vitro*. The research in chapter 6 describe the process to dissociate neurons from brain tissue samples of Alzheimer's disease (AD) cadavers and from brain tissue extracted from patients undergoing brain surgery. These neurons were cultured on silane-modified cover slips in a serum-free, defined system containing antioxidant nanoparticles. Neurons isolated from AD cadavers and surgical samples were cultured and regenerated *in vitro*. These neurons were electrically active, fired repetitive action potentials and survived in culture up to 48 days. Neurons from AD patient cadavers exhibited limited survival duration and many exhibited pathological properties found in AD such as limited process regeneration, the absence of dendritic spine formation and vesicular inclusions. This *in vitro* model facilitated the recovery and long-term culture of neurons obtained from surgical procedures as well as the limited regeneration of neurons from AD disease patients, where the AD neurons appeared to recapitulate the diseased phenotype. This *in vitro* model system could be applicable for studies in traumatic brain injury (TBI) as well as for personalized medicine evaluation in neurodegenerative diseases.

Overall, the novel elements of these new culture system that allowed adult neurons to survive, recover, regenerate, and become electrically active *in vitro* are:

1. Cell-adhesive DETA substrate which supports attachment, regeneration, and long-term survival of adult neurons.

2. Serum-free medium

3. Key growth factors for support of cell survival, regeneration, and recovery

4. Neurotransmitter (glutamate) mediated electrical activity recovery

5. Optimized cell culture process to remove neurotoxic myelin inhibitory proteins present in tissue debris released after tissue dissociation

6. Anti-apoptotics (caspase 1, 3, 6 inhibitors) in the cell dissociation medium.

7. Cerium oxide nanoparticles and Trolox©, used during the culture through 2 days *in vitro*

8. Control of neuronal cell division, prompted by bFGF and inhibited by roscovitine.

9. Photolithographic patterned self-assembled monolayers for guided attachment and regeneration of neurons. This allowed for the formation of small networks of neurons for easy evaluation of electrical connectivity between neurons *in vitro.*

10. Passage of neurons to allow the deposition of specific densities of adult neurons onto patterned surfaces, supporting 2-cell network formation, expansion of populations of adult neurons dividing due to the action of the cdk5 activator bFGF, or deposition of dense concentrations of neurons onto microelectrode array systems (MEAs)

11. High-throughput MEA systems.

These elements supported the survival, regeneration, electrical activity, synaptic connectivity, and long-term survival of mature adult neurons *in vitro*.

Overall, potential uses for such systems range from research into the function of neurons, neuronal interactions, aging, neurodegenerative disease [36], drug studies, neuroprosthetic devices, neurocomputing, biorobotics [40-42], and neuroregeneration. The ability of mature neurons to divide *in vitro* caused by bFGF has potential implications *in vivo*. Overexpression of bFGF or introduction of bFGF into the adult brain potentially could trigger mature neurons to retract neurites, undergo mitotic division, regenerate mature morphology, and reconnect into the neuronal network. Similar mechanisms have previously been seen in the conversion of non-neuronal cells to functional neurons through the introduction of defined factors [43]. Targeted and reversible induction of mitotic activity in neurons *in vivo* has great potential as a therapeutic intervention for late-stage neurodegenerative disease, such as Alzheimer's disease, or TBI [44-46]. If the same conditions and environment were to be created *in vivo* as was present in this *in vitro* cell culture system, the remaining mature neurons present in the brains of patients ravaged by age, disease, or injury could potentially be induced to divide and integrate into the brain as a functional network. These neurons could replace those neurons previously lost to damage, age, and disease. In addition, there has been much conjecture about using neural stem cells in cell replacement therapies in various neurodegenerative disease [43, 47]. Using populations of the patients own neurons that have been expanded and then returned to a non-proliferative state with cyclin-dependent kinase inhibitors (CKIs) *in vitro* may be a more effective therapy. Last, because DETA monolayers can be applied not only to glass cover slips

but also to electrodes, another potential use of this system lies in its ability to be extended to integrate living and electronic systems.

In chapter 4, the adhesion of adult hippocampal neurons and neurite outgrowth were managed using patterned PEG-DETA Fish patterns. Neurons in this system recovered to form small networks of synaptically connected excitatory neurons. This system provides a unique tool that can be used for studies into LTP [36, 38, 39]. In addition, this functional *in vitro* system would enable high-throughput neuropharmacology studies, facilitating drug development and furthering research into different neurological disorders. In chapter 5 we described the creation of a high-throughput adult MEA system that can evaluate drugs and neurotoxic agents very quickly. In comparison to embryonic MEA systems, this method, measurements of neuronal activity using adult hippocampal neurons on MEAs, is more applicable to the adult brain. While preparation of these MEAs was slightly more complicated than embryonic neuronal MEAs, the end result yielded a high-throughput screen methodology that is directly correlative to the dynamics of learning and memory formation in the adult brain. Additionally, this system can facilitate quicker, more reliable, and more correlative investigations into drug discovery, neurotoxic agents, and neurodegeneration. Last, this method can be used in the future to allow for the generation of MEAs using adult human neurons.

Last, in drug-discovery research, there is an increasing demand for cost effective high-throughput *in vitro* test methods as a substitute for *in vivo* toxicity testing and drug candidate evaluations. With *in vitro* experiments, the development and validation of a complex set of methods is necessary to accomplish this substitution[48-50] and many

cell-culture based methods have been proven to possess predictive values for different aspects of a compound's effect (absorption[51], metabolism[52], neurotoxicity[53], cardiac toxicity[54], etc.). Thus, a new function-based assay focused on human primary neurons would be of use from the general field of drug discovery to basic neuroscience, but especially for evaluating causative effects of AD disease and its possible remedies. This would be especially useful in investigating methods for the restoration of function of damaged neurons, as little or no progress has been made in reversing the effects of this tragic disease or in neuronal damage due to TBI.

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