TRAUMATICALLY-INDUCED DEGENERATION AND REACTIVE ASTROGLIOSIS IN THREE-DIMENSIONAL NEURAL CO-CULTURES: FACTORS INFLUENCING NEURAL STEM CELL SURVIVAL AND INTEGRATION

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TRAUMATICALLY-INDUCED DEGENERATION AND REACTIVE ASTROGLIOSIS IN THREE-DIMENSIONAL NEURAL CO-CULTURES: FACTORS INFLUENCING NEURAL STEM CELL SURVIVAL AND INTEGRATION

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SUMMARY

Traumatic brain injury (TBI) results from a physical insult to the head and often results in temporary or permanent brain dysfunction; however, the cellular pathology remains poorly understood and there are currently no clinically effective treatments. Properly designed and characterized laboratory models that adequately recapitulate the biomechanical and cellular pathophysiology of neural trauma provide valuable experimental platforms for the elucidation of the cellular response to injury as well as serving as test-beds for potential therapeutic interventions. A primary goal of this work was to develop and characterize a novel three-dimensional (3-D) \textit{in vitro} paradigm of neural trauma integrating a robust 3-D neural co-culture system and a well-defined biomechanical input representative of clinical TBI. Mechanically injured 3-D co-cultures were then interfaced with neural stem cells (NSCs) to serve as an \textit{in vitro} test-bed for the evaluation of factors influencing NSC survival and integration.

Specifically, a novel 3-D neuronal-astrocytic co-cultures system was developed, establishing parameters resulting in the growth and vitality of mature 3-D networks, potentially providing enhanced physiological relevance and providing a robust platform for the mechanistic study of neurobiological phenomena. Furthermore, an electromechanical device was developed and characterized that is capable of subjecting 3-D cell-containing matrices to a defined mechanical insult, with a predicted strain manifestation at the cellular level - the first model to date capable of correlating cellular outcome with specific 3-D biomechanics. Following independent development and validation, these novel 3-D neural cell and mechanical trauma paradigms were used in
combination to develop a mechanically-induced model of neural degeneration and reactive astrogliosis. This *vitro* surrogate model of a degenerating and reactive astrogliotic environment was then exploited to assess factors influencing NSC fate upon delivery to this environment, revealing that specific factors in a degenerating environment were detrimental to NSC survival. This work has developed enabling technologies for the *in vitro* study of neurobiological phenomena and responses to neural injury, and may aid in elucidating the complex biochemical and molecular cascades that occur after a traumatic insult. Furthermore, the novel paradigm here developed may provide a powerful experimental framework for improving treatment strategies for therapeutic implementation following CNS injury, and therefore serve as a valid pre-animal test-bed.
CHAPTER I

INTRODUCTION AND BACKGROUND

Traumatic Brain Injury

Traumatic brain injury (TBI) is caused by a mechanical insult to the head and often results in temporary or permanent brain dysfunction. TBI represents a major health and socioeconomic problem as each year 1.4 million Americans are affected, causing over 50,000 deaths (Langlois, et al., 2004). At least 5.3 million Americans, close to two percent of the U.S. population, are chronically affected by TBI-related disorders (Thurman, et al., 1999). Over 33% of the annual injury-related deaths in the U.S. are attributed to TBI (Sosin, et al., 1995), primarily from firearm, motor vehicle, and fall-related incidents (Langlois, et al., 2004). The primary causations of TBI vary greatly based on age, with motor vehicle-related incidents and assaults most prevalent in adults (15 - 34 years old) and falls most frequent in children and the elderly (0 - 7 and > 65 years old, respectively) (Langlois, et al., 2004). However, the overall highest death rate attributed to TBI occurs from motor vehicle-related incidents, most prevalent with individuals 15 - 24 years old, with twice as many fatalities in males compared to females (Langlois, et al., 2004). For the survivors of TBI, functional impairment includes deficiencies in cognitive ability (memory, concentration, judgment, mood, managing emotions), motor abilities (balance, coordination, strength), and sensory perception (tactile perception, vision, olfaction) (Smith, et al., 1994; Adelson, et al., 2000; Corrigan, et al., 2004; Povlishock and Katz, 2005). TBI can cause seizure disorders and increase
the risk for Alzheimer’s disease, Parkinson’s disease, and other neurological disorders that become more prevalent with age (NINDS, 2002). The cumulative medical costs and indirect costs (e.g., wage loss from recovery time and inability to resume pre-injury employment) associated with TBI totaled an estimated $56.3 billion in the United States in 1995 (Thurman, 2001).

**Biomechanics of Traumatic Brain Injury**

Correlation of the physical parameters inducing brain injury and the extent of the resulting tissue loss and functional impairment is crucial to accurately diagnosing and treating patients as well as developing accurate experimental models. A traumatic insult is caused by a physical deformation of the brain resulting from 1) inertial forces caused by high acceleration of the head that causes diffuse brain movement, and/or 2) impact forces resulting from direct head contact with a blunt or penetrating object that causes focal brain deformation (McLean and Anderson, 1997). For inertial injuries, high angular acceleration and deceleration of the head about the cranio-cervical junction imparts deformation to brain tissue (e.g., restrained occupant in a motor vehicle collision) (Gennarelli, 1993). Inertial loading has been correlated with severe, diffuse brain injury, potentially causing concussion and coma (Margulies and Thibault, 1992; Gennarelli, 1993). Furthermore, the angular acceleration of the head and injury severity have been causally related, indicating a dependency on strain rate as well as a strain magnitude in eliciting tissue damage (Margulies, et al., 1990; Margulies and Thibault, 1992; Gennarelli, 1993). Impact injuries can result from the accelerating head striking an object with a much greater mass (e.g., concrete in the case of a fall, an automobile
dashboard in the case of a motor vehicle collision) where skull deformation is translated directly to brain tissue, with or without fracture of the skull (Ommaya, et al., 1994). In addition, impact injuries can occur due to an object delivering a high force over a small surface area that can penetrate the skull and damage brain tissue with or without serious skull deformation (e.g., a bullet). For non-penetrating injuries, damage can occur due to the impact and/or acceleration of the brain as well as due to the deceleration and/or impact as the brain returns to a resting position (McLean and Anderson, 1997). Further aspects of the tissue deformation are critical to outcome as the physical properties of brain tissue make shear the dominant mode of deformation upon mechanical loading since brain tissue has a high compressive modulus and a low shear modulus; thus failure in neural tissue is primarily attributed to shear (Holbourn, 1943; Shuck and Advani, 1972; Sahay, et al., 1992). Thus, TBI is initiated by the transfer of kinetic energy to the head (and brain), and governing parameters involve the acceleration/deceleration of the head (e.g., magnitude, angular versus linear, and the plane of translation), the time increment of acceleration/deceleration, the mass of the head, and the properties of the contacting material (e.g., stiffness and surface area, when applicable) (Gennarelli, et al., 1982; Margulies, et al., 1990; McLean and Anderson, 1997). Injury levels surpassing cellular/tissue thresholds for damage are thus complicated and are governed by the interrelations of these parameters.

**Primary and Secondary Responses to Injury**

Primary cell death occurs during and immediately following a mechanical insult when local cellular stresses surpass physical thresholds. This type of damage is a direct
result of the mechanical insult and the cells cease to function through necrosis, a passive cell death mechanism. The events surrounding the acute insult set into motion a series of secondary complications which may induce subsequent cell death (McIntosh, 1994; Raghupathi, 2004). This delayed, or secondary, cell death may occur hours, weeks, or even months after the initial insult (McIntosh, et al., 1998), causing prolonged or permanent loss of sensory, motor, and/or cognitive functions (Smith, et al., 1994; Adelson, et al., 2000; Povlishock and Katz, 2005). Specifically, secondary cell death is attributed to many factors including breakdown of the blood-brain barrier, inflammation, transient alterations in membrane permeability, and excitotoxicity, resulting in the initiation of deleterious intracellular signaling cascades and changes in gene expression (Gennarelli, 1993; McIntosh, 1994; McIntosh, et al., 1998; McIntosh, et al., 1999; Gaetz, 2004). Breakdown of the blood-brain barrier occurs through hemorrhages from broken blood vessels causing cells to lose their source of nutrients and oxygen, resulting in ischemia/hypoxia. There are also disruptions to intracellular homeostasis through alterations in cell permeability, activation of transmembrane signaling complexes and/or activation of ion channels (LaPlaca, et al., 1997; LaPlaca and Thibault, 1998; Goforth, et al., 1999; Geddes, et al., 2003; Lusardi, et al., 2004). Furthermore, the extracellular concentration of excitatory amino acids (e.g., glutamate) increases precipitously after cell damage due to cell lysis, transient permeability changes, depolarization-dependent vesicular release, and reversed transporter operation (Rossi, et al., 2000; Sattler and Tymianski, 2000). These increases in extracellular glutamate concentrations then lead to further excitatory stimulation and glutamate release, possibly depleting intracellular energy to the point of excitotoxic cell death (Goforth, et al., 1999; Weber, et al., 1999).
Thus, intracellular signaling cascades may be altered following a mechanical insult; however, many of these altered signaling cascades have been linked to increases in intracellular Ca$^{2+}$ concentrations, potentially leading to prolonged cellular dysfunction and passive (necrosis) or programmed (apoptosis) cell death (Choi, 1994; Goforth, et al., 1999; Weber, et al., 1999; Sattler and Tymianski, 2000). Due to long-term mechanisms of cell dysfunction/death initiated at the time of injury, TBI may be considered both an acute inflammatory disease as well as a longer-term neurodegenerative disease (McIntosh, et al., 1998).

Further physical and biochemical changes are initiated by a mechanical insult to the CNS that can drastically alter the post-injury microenvironment. Physical disruption and compromise of the blood-brain barrier contributes to a widespread reactive response involving many cell types including astrocytes, macrophages, microglia, oligodendrocytes, and meningeal cells (see (Fawcett and Asher, 1999) for review). This results in an inflammatory immune response in the normally immune privileged brain, consisting of macrophage infiltration, microglia activation, and the release of free radicals, chemokines, and other toxic substances which are potentially damaging to cells (Giordana, et al., 1994; Giulian, 1994; Holmin, et al., 1995). A subset of these activated cell types coalesce into the glial scar – a physical and chemical barrier sequestering tissue acutely injured from tissue that is not severely damaged; however, the chronic glial scar consists primarily of astrocytes. The formation of a glial scar appears to have short-term positive effects as secondary damage may be lessened through isolation of the immune response and re-establishment of the blood-brain barrier (Bush, et al., 1999). However, ultimately the formation of the glial scar appears to hinder regeneration as specific
extracellular matrix (ECM) components are increased that have been shown to be inhibitory to neurite outgrowth (McKeon, et al., 1995; McKeon, et al., 1999; Asher, et al., 2000). An increased understanding of the mechanisms underlying secondary cell death and glial scar formation may eventually lead to protective treatments and the augmentation of repair and regeneration. Strategies of these types can potentially lessen the long-term effects of TBI; however, to date, no clinically affective treatments exist to combat altered signaling cascades, secondary death mechanisms, and the anti-regenerative post-injury environment.

Figure 1.1: Simplified conceptual rendition of primary and secondary responses following a mechanical insult to the CNS.
**Treatment for TBI**

Current treatments for TBI are directed at patient stabilization and a reduction in secondary damage induced by brain swelling, increased intracranial pressure, and hemorrhaging (Chesnut, 1997). Clinical treatments have routinely included hyperventilation, cerebrospinal fluid drainage, corticosteroids, barbiturates, and mannitol; however, when the studies included the appropriate controls, none of these treatments were effective in reducing morbidity and mortality (Roberts, et al., 1998). Furthermore, mannitol, used to reduce cerebral edema (Bareyre, Wahl et al., 1997), has been found to increase inflammation and apoptosis (Famularo, 1999), and may have damaging long term side effects when used to treat severe TBI (Polderman, van de Kraats et al., 2003). A recent clinical trial evaluating the effects of methylprednisolone, a common treatment for spinal cord injury, had to be discontinued due to an increased risk of mortality within the first two weeks following injury (Sauerland and Maegele 2004; Edwards, Arango et al. 2005). Such clinical results are a testament to the heterogeneity of injuries collectively designated as TBI, with a multitude of complex causations, variable severity levels, and differential manifestations on a per-patient basis; matters further complicated by the potential of variable therapeutic windows. The long-term goal of pharmacological intervention in the acute phase is to block endogenous destructive pathways and thus attenuate tissue degeneration; however, beyond that phase the goal will be to enhance functional recovery through activation of endogenous repair mechanisms or replacement of lost cells (Faden, 1996; Faden, 1996; Kanelos and McDeavitt, 1998; McIntosh, et al., 1998; Cao, et al., 2002).
Delivery of Neural Stem Cells to the Injured Central Nervous System

Due to the prolonged degeneration and complex environmental alterations following TBI, it is likely that a sustained effort will be required to alleviate or reduce neurological disability. Cellular replacement therapy may be advantageous over pharmacological strategies due to the ability to dynamically target a wide range of biochemical alterations over a prolonged period of time; transplanted cells may produce, release, and process biomolecules in response to both external and internal signals. Transplanted cells may provide bulk trophic support, mediate cell-cell repair, and aid in the construction of neuronal circuitry given the proper choice of cell type and microenvironment. Thus, cells with multipotent characteristics may have the ability to replace the function of missing or damaged cells and also to halt secondary damage in surrounding tissue. To this end, it may be advantageous to transplant multipotent neural stem cells (NSCs) after trauma, due to the ability to form multiple and specific cell phenotypes based on physical and temporal allocation of specific environmental cues. NSCs are capable of differentiating into the major cells of the CNS (neurons, astrocytes, and oligodendrocytes), a potential advantage when more than one cell type may be needed to mediate recovery of injured tissue (Rao, 1999; Whittemore, 1999; Gage, 2000). In addition, NSCs have been shown to produce trophic factors when transplanted into injured CNS tissue (Lu, et al., 2003; Yan, et al., 2004) and can rescue host cells from degeneration (Ourednik, et al., 2002; Llado, et al., 2004). Transplantation of neural cells into the injured brain and spinal cord has been shown to attenuate degeneration and enhance functional recovery to some degree; however, this methodology has had limited success in terms of donor cell survival and graft integration (Vanderwolf, et al., 1990;
Hoovler and Wrathall, 1991; Kanelos and McDeavitt, 1998; Cao, et al., 2002; Riess, et al., 2002; Lu, et al., 2003; Picard-Riera, et al., 2004; Boockvar, et al., 2005). NSC survival and integration, and hence the potential therapeutic benefits, are dictated by the characteristics of the post-injury degenerating and reactive astrogliotic environment; however, the dominant factors influencing NSC behavior upon transplantation have yet to be elucidated due to the complexity of the in vivo environment.

**Modeling Traumatic Brain Injury**

Models of TBI provide researchers with arenas to study cellular and systemic alterations initiated by a mechanical insult as well as serving as platforms to evaluate strategies to augment repair and/or enhance recovery. Depending on the complexity of the model, researchers may evaluate changes on the intracellular, extracellular, tissue, and systemic levels, and these models have been applied to study effects ranging from acute mechanisms of neural dysfunction to developing reactive responses. *In vivo* models of TBI have been developed to study neural tissue damage, account for the pathophysiological response of the whole animal, and assess post-injury alterations in cognitive, motor, and sensory abilities. *In vivo* models of brain injury have been developed which mimic both contact (focal) and inertial (diffuse) injuries, and such models include fluid percussion (Dixon, et al., 1987), cortical impact (Nilsson, et al., 1977), weight drop (Marmarou, et al., 1994) and acceleration/deceleration models (Ommaya, et al., 1966; Gennarelli, et al., 1982). These models are successful in eliciting damage to brain tissue and causing reproducible functional impairment representative of clinical TBI. Furthermore, with the aid of physical and computer simulations, these
models may link pathological manifestations with predicted biomechanical parameters (Margulies and Thibault, 1989; Margulies, et al., 1990; Meaney, et al., 1995; Zhang, et al., 2001; Zhang, et al., 2001; Zhang, et al., 2004). With these models, however, the effects of traumatic loading conditions on individual neural cells are difficult to evaluate as control over spatial and temporal parameters is limited. However, well-characterized animal models are a necessity in pre-clinical testing of pharmacological or other intervention strategies that aim to attenuate secondary cell death or augment endogenous repair capabilities.

Although in vivo studies model the systemic and behavioral deficits of TBI, in vitro approaches provide a powerful framework for investigating isolated mechanisms. Cellular models range from transection (Gross, et al., 1983; Lucas, et al., 1985; Tecoma, et al., 1989; Regan and Choi, 1994; Mukhin, et al., 1996) and compression models (Balentine, et al., 1988; Shepard, et al., 1991; Murphy and Horrocks, 1993; Wallis and Panizzon, 1995) (contact loading) to more complex stretching and acceleration devices (Ellis, et al., 1995; Cargill and Thibault, 1996; LaPlaca and Thibault, 1997; Smith, et al., 1999; Geddes and Cargill, 2001) (inertial loading). The aforementioned in vitro models have primarily been utilized to deform cells in a planar (2-D) orientation, which may fail to simulate complex strain combinations; in fact, most utilize a single mode of deformation. Also, the skewed distribution and types of cell-cell/cell-matrix interactions found in 2-D cultures may affect pathological mechanotransduction mechanisms. Three-dimensional (3-D) models of neural trauma may better recapitulate the biomechanics of traumatic loading to the brain, which results in the generation of complex, heterogeneous strain fields at the tissue and cellular levels. In vitro models have been developed to
evaluate the injury response in brain tissue slices – thus preserving a 3-D configuration (Morrison, et al., 1998; Sieg, et al., 1999; Adamchik, et al., 2000); however, in brain slice culture systems the ability to control specific attributes (e.g., cell types present, matrix characteristics) is limited.

Two-Dimensional and Three-Dimensional Cell Culture

Although in vitro models are invaluable in the systematic elucidation of cell behavior in a highly controlled setting, the interpretation of cellular responses in traditional 2-D models may be confounded by altered cell-cell/cell-matrix interactions and atypical cellular morphology. Cells cultured in a 3-D environment have been shown to better represent in vivo cellular behavior than cells cultured in monolayer (e.g., osteoblastic cells (Granet, et al., 1998); fibroblasts (Grinnell, 2000); breast cells (Wang, et al., 1998); and neural cells (Fawcett, et al., 1989; Fawcett, et al., 1995)), and accordingly have been found to exhibit higher compatibility and survival upon transplantation in vivo (Fawcett, et al., 1995). Fundamental differences exist between cells cultured in monolayer versus 3-D configurations in terms of access to soluble factors and the distribution and types of cell-cell/cell-matrix interactions (Cukierman, et al., 2001; Cukierman, et al., 2002; Schmeichel and Bissell, 2003; Yamada, et al., 2003). These phenomena, in turn, may have ramifications on morphology (Grinnell, 2003), growth and proliferation (Granet, et al., 1998), viability (Fawcett, et al., 1995), gene and protein expression (Masi, et al., 1992; Berthod, et al., 1993), and in the response to biochemical (Hoffman, 1993) and/or mechanical stimuli (LaPlaca, et al., 2005). Overall, due to fundamental deviations of planar cell culture systems from the in vivo situation,
cells cultured in 2-D may be inherently unable to depict traits exhibited by *in vivo* systems.

**Project Motivation**

The overall goal of this work was to develop and characterize a novel three-dimensional (3-D) *in vitro* paradigm of neural trauma integrating a robust 3-D neural co-culture system and a well-defined biomechanical input representative of clinical TBI. Mechanically injured 3-D co-cultures were then interfaced with neural stem cells (NSCs) to serve as an *in vitro* test-bed for the evaluation of factors influencing NSC survival and integration in a controlled setting. This project developed a series of novel technologies prior to the execution of the overall goal. First, a requisite objective was the development of a 3-D neuronal-astrocytic co-culture system and the characterization of elements potentially influencing the fidelity of the model (CHAPTER II). The next step towards the overall objective was the development and validation of a novel system capable of reproducibly imparting variable rate shear deformation to 3-D cell-containing matrices, producing a heterogeneous local cellular stain in these cultures (CHAPTER III). The ramifications of 3-D biomechanics were further explored by evaluating the neuronal response to loading in comparison to a similar 2-D model (CHAPTER IV). The novel 3-D neural co-culture (CHAPTER II) and mechanical trauma (CHAPTER III) models were then used in combination to evaluate the neuronal-astrocytic response to mechanical loading, specifically evaluating alterations in culture viability and markers of astrocyte reactivity (CHAPTER V). NSCs were then delivered into mechanically-injured 3-D co-cultures in order to assess factors in a degenerating and astrogliotic environment that may
influence NSC survival and integration (CHAPTER VI). This work may establish a valuable experimental platform that may be further exploited for the \textit{in vitro} study of the neural response to trauma and serve as a test-bed for potential therapeutic interventions.
CHAPTER II

DEVELOPMENT OF THREE-DIMENSIONAL NEURONAL AND NEURONAL-ASTROCYTIC CULTURES

Abstract

In vitro models utilizing neural cells in three-dimensional (3-D) culture may be a more accurate representation of the in vivo environment while maintaining the benefits of two-dimensional (2-D) models. We have developed and optimized two 3-D neural cell culture systems: 1) primary cortical neurons, and 2) primary cortical neurons and astrocytes in co-culture. Morphology, viability, cell phenotype/differentiation, synaptic protein markers and proliferation were compared between 3-D and analogous 2-D models. Electron microscopy revealed distinct morphological differences between planar cultures, which present a flat morphology, compared to cells distributed throughout a matrix, which present a bulbous morphology with cell-matrix contact in all dimensions. Viability in 3-D neuronal cultures was highly dependent on cell density (with optimal viability at 3750 cells/mm$^3$ for 500 µm thick cultures) and did not differ from viability in 2-D neuronal cultures up to 21 days in vitro (DIV). Co-cultures were evaluated at a 1:1 or 1:5 neuron:astrocyte plating ratio; the viability for both ratios was > 95% in 3-D and 2-D cultures up to 21 DIV. In neuronal-astrocytic co-cultures in 3-D, proliferation was dependent upon the initial neuron:astrocyte ratio, whereas in 2-D, proliferation was independent of the initial ratio, suggesting complex proliferative regulatory mechanisms existing in 3-D. Furthermore, by 7 DIV both culture models in 3-D and 2-D presented
immunolocalization for synapsin in discrete puncta, suggestive of functional synapse formation; however there was a substantial increase in synapsin$^+$ puncta per neuron in neuronal-astrocytic co-cultures versus neuronal cultures. These 3-D models may more accurately represent in vivo neural responses and develop enabling technologies for neurobiological and tissue engineering applications.

**Introduction**

In vitro models are invaluable in systematic elucidation of cell behavior in a highly controlled setting. However, the interpretation of cellular responses in traditional planar (2-D) models may be confounded by altered cell-cell/cell-matrix interactions and atypical cellular morphology. Three-dimensional (3-D) models consisting of multiple neural cell types are capable of maintaining many positive aspects of in vitro modeling while closer approximating the cytoarchitecture of the brain. Cells cultured in a 3-D environment have been shown to better represent in vivo cellular behavior than cells cultured in monolayer (e.g., osteoblastic cells (Granet, et al., 1998); fibroblasts (Grinnell, 2000); breast cells (Wang, et al., 1998); and neural cells (Fawcett, et al., 1989; Fawcett, et al., 1995)), and accordingly have been found to exhibit higher compatibility and survival upon transplantation in vivo (Fawcett, et al., 1995). Fundamental differences exist between cells cultured in monolayer versus 3-D configurations in terms of access to soluble factors and the distribution and types of cell-cell/cell-matrix interactions (Cukierman, et al., 2001; Cukierman, et al., 2002; Schmeichel and Bissell, 2003; Yamada, et al., 2003). These phenomena, in turn, may have ramifications on morphology (Grinnell, 2003), growth and proliferation (Granet, et al., 1998), viability.
(Fawcett, et al., 1995), gene and protein expression (Masi, et al., 1992; Berthod, et al., 1993), and in the response to biochemical (Hoffman, 1993) and/or mechanical stimuli (LaPlaca, et al., 2005). Furthermore, culture models consisting of multiple cell types closer approximate the heterogeneity of in vivo tissue. With respect to the nervous system, cellular heterogeneity is important to represent such interactions as physical support and metabolic coupling between neurons and astrocytes; see (Tsacopoulos and Magistretti, 1996; Aschner, 2000; Tsacopoulos, 2002) for reviews.

Neuronal models in 3-D have been developed from dissociated primary cells as reaggregate cultures by rotation-induced reassociation (Hsiang, et al., 1989; Choi, et al., 1993) or by distribution throughout an extracellular matrix (ECM) material (Woerly, et al., 1996; O'Connor, et al., 2000; O'Connor, et al., 2001). Difficulty exists in controlling ECM components and cellular distribution of reaggregate cultures, although cell interactions, growth, and function appear to approximate in vivo behavior. Other models have been developed in which neurons are plated above a matrix material (Coates and Nathan, 1987; Coates, et al., 1992; O'Shaughnessy, et al., 2003), which recapitulate aspects of 3-D morphology; however, cell-cell and cell-matrix interactions are spatially limited. Models consisting of dissociated dorsal root ganglia have been developed where those cells are distributed throughout a hydrogel matrix, with neurite outgrowth occurring with or without receptor-mediated cell-matrix interaction (Balgude, et al., 2001). However, embryonic cortical neurons or astrocytes plated within 3-D matrices of collagen, hydrogels with immobilized collagen, or hydrogels without a bioactive matrix have exhibited varying degrees of cell viability with enhanced process extension in matrices utilizing collagen, demonstrating the need for specific cell-matrix interactions.
for the support of primary cortical cells (Woerly, et al., 1996; O'Connor, et al., 2000; O'Connor, et al., 2001). Co-cultures consisting of neurons and astrocytes are typically 2-D models, although multi-cellular reaggregate cultures have been developed (Pulliam, et al., 1998; Pardo and Honegger, 2000). In 2-D neuronal-astrocytic co-cultures, the two cell types naturally arrange themselves into a base layer of astrocytes (which become confluent) with neurons on top (Ahmed, et al., 2000). Although this distribution spatially limits both neuron-astrocyte and neuron-neuron interactions, such models have proven useful in establishing the pivotal role of astrocytes in neuronal survival and synapse formation (Steinschneider, et al., 1996; Nakanishi, et al., 1999).

The neural cell culture models described here have been developed using Matrigel matrix, a reconstituted basement membrane that is biologically active for neural cells through matrix-protein (e.g., collagen, laminin)(Kleinman, et al., 1986) and cytokine-related interactions (Vukicevic, et al., 1992) that has been shown to promote neurite outgrowth (Madison, et al., 1985)(see APPENDIX D). Cellular models consisting of neurons and astrocytes distributed throughout a 3-D matrix more closely approximate the heterogeneous composition and architecture of native tissue and thus may exhibit more in vivo-like behavior than previous in vitro models consisting of 2-D monotypic populations. The objective of the current study was to develop and characterize two 3-D neural cell culture models – a model consisting predominantly of neurons and a model consisting of astrocytes and neurons in controlled ratios. These 3-D models potentially provide enhanced physiological relevance and will be valuable in the mechanistic study of cell growth, interactions, and the responses to chemical or mechanical perturbations.
Materials and Methods

Isolation of Primary Cortical Neurons (Embryonic Day Seventeen)

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. Timed-pregnant Sasco Sprague-Dawley rats (Charles River, Wilmington, MA) were anesthetized using halothane (Halocarbon, River Edge, NJ) and decapitated. The uterus was removed by Caesarian section and placed in Hanks Balanced Salt Solution (HBSS, Invitrogen). Each fetus was removed from the amniotic sac, rapidly decapitated, and the brains removed. The cortex was isolated and the hippocampal formation removed. To dissociate the tissue, pre-warmed trypsin (0.25%) + 1 mM EDTA (Invitrogen, Carlsbad, CA) was added for 10 min at 37°C. The trypsin-EDTA was then removed and deoxyribonuclease I (0.15 mg/mL, DNase, Sigma, St. Louis, MO) in HBSS was added. The tissue was then triturated with a flame-narrowed Pasteur pipet and then centrifuged at 1000 rpm for 3 minutes after which the supernatant was aspirated and the cells were resuspended in neuronal media (Neurobasal medium + 2% B-27 + 500 µM L-glutamine (Invitrogen). Cell concentration and viability were determined using a hemocytometer and evaluating the exclusion of trypan blue (0.2%, Sigma).

Isolation of Cortical Astrocytes (Postnatal Day One)

Postnatal (day 0-1) Sasco Sprague-Dawley rats (Charles River) were anesthetized using halothane and rapidly decapitated. The brain was removed and the cortical region isolated as described above. Upon isolation of the cortical regions, the tissue was minced and pre-warmed trypsin (0.25%) + EDTA (1 mM) was added and placed in at 37°C for 5 minutes. DNase was added and the tissue was triturated using a flame-narrowed Pasteur
pipet. Medium was added (DMEM/F12 + 10% FBS) and the cells were centrifuged (1000 rpm, 3 minutes) after which the supernatant was aspirated, the cells were resuspended in DMEM/F12 + 10% FBS and transferred to T-75 tissue culture flasks. To isolate a nearly pure population of type I astrocytes, procedures described elsewhere were followed (McCarthy and de Vellis, 1980). Briefly, at various time-points over the first week post-dissection, the flasks were mechanically agitated to dislodge less adherent cell types (e.g. neurons, oligodendrocytes). As the cells approached ~90% confluency they were resuspended using trypsin-EDTA, centrifuged, and replated at a density of 300 cells/mm$^2$. To permit maturation, astrocytes were used between passages 4 - 12 for the generation of 3-D and 2-D cultures.

3-D and 2-D Primary Cortical Neuron Cultures

Cultures were plated in custom-made cell culture chambers consisting of a glass coverslip (no. 1½ thickness) below a circular silicone-based elastomer mold (Sylgard 184 and 186, Dow Corning; Midland, MI; cross-sectional area = 2 cm$^2$). Prior to plating, the chambers were pre-treated with 0.05 mg/mL poly-L-lysine (PLL, Sigma) followed by Matrigel (0.5 mL/well at 0.6 mg/mL, Becton Dickinson Biosciences; Bedford, MA) in Neurobasal medium (each treatment was > 4 hours). Neuronal cultures in 3-D were plated within Matrigel matrix (final protein concentration of 7.5 mg/mL). Matrigel exhibits fluid-like behavior at 4°C (which permits even dispersion of dissociated cells throughout matrix material) and subsequent gelation at or near physiological temperature (which entraps cells in 3-D matrix) (Kleinman, et al., 1986). 3-D cultures were plated at cell densities ranging from 1250 to 7500 cells/mm$^3$ within a 500 - 750 µm thick matrix. These cultures were placed at 37°C to permit matrix gelation and 3-D cell entrapment,
after which 0.5 mL media was added to each well. For 2-D neuronal cultures, cells were plated at 300-1250 cells/mm$^2$ in 0.5 mL medium. Cultures were maintained at 37°C and 5% CO$_2$-95% humidified air and fed every 3 - 4 days by replacing the media with fresh media pre-warmed to 37°C.

**3-D and 2-D Primary Cortical Neuron / Secondary Cortical Astrocyte Co-Cultures**

Co-cultures were plated in pre-treated custom-made chambers with neurons and astrocytes that were separately isolated and dissociated (as described above) using coculture medium (Neurobasal medium + 2% B-27 + 1% G-5 + 500 µM L-glutamine). Co-cultures in 3-D were plated at 2500 cells/mm$^3$ at a 1:1 or a 1:5 neuron:astrocyte ratio in Matrigel (final matrix concentration 7.5 mg/mL) at a thickness of 500 - 750 µm. Co-cultures in 2-D were plated at a neuron:astrocyte ratio of 1:5 or 1:1 at a density of 200-300 cells/mm$^2$. Control astrocyte-only cultures were plated in 3-D (2500 cells/mm$^3$) and 2-D (200-300 cells/mm$^2$) using matched conditions as described above. The 2-D plating density was chosen to match the theoretical 3-D cell-cell distance in the 3-D cultures (to negate the potential effects of contact-inhibition or other modes of cell-cell influence). Also, to normalize the influence of potentially stimulatory factors present in Matrigel, acellular Matrigel (7.5 mg/mL in medium) was placed above the 2-D cultures to match the amount of matrix used in the 3-D system. After matrix gelation in the 3-D and 2-D cultures, 0.5 mL co-culture medium was added per well. Cultures were maintained at 37°C and 5% CO$_2$-95% humidified air and medium was replaced at 24 hours and every 2 days thereafter.
Morphologic Analysis: Scanning Electron Microscopy

Cell morphology and the spatial distribution of cell-cell and cell-matrix interactions were qualitatively assessed through low voltage, high resolution scanning electron microscopy (LVHR-SEM). Cultures were plated on pre-treated silicon wafers and at 7 days in vitro (DIV) were fixed using 2.5% EM-grade glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 24 hours to provide complete intracellular and extracellular proteinaceous cross-linking. Cultures were washed in di-H₂O and then post-fixation of phospholipid moieties was accomplished by immersion into 1% OsO₄ in 0.1M cacodylate buffer (pH 7.4) for 1 hour and then rinsed several times. A graded series of ethanol (30, 50, 70, 3 x 100%, 15 min each) was used to substitute culture fluids prior to wrapping individual gels in parafilm. Samples were loaded into a Polaron E-3000 critical point dryer (CPD) and exchange with CO₂ was made while monitoring the exhaust gas rate. The CPD was thermally regulated to the critical temperature and pressure and, following phase transition, the CO₂ gas was released at a constant flow rate. Prior to LVHR-SEM, all specimens were sputter coated with 3 nm of chromium.

Cell Viability

Cell viability was assessed using fluorescent probes for distinguishing live and dead cells. Cell cultures were incubated with 4 µM ethidium homodimer-1 (EthD-1) and 2 µM calcein AM (both from Molecular Probes, Eugene, OR) at 37 °C for 30 min and then rinsed with 0.1 M Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen). The percentage of viable cells was calculated by counting the number of live cells (fluorescing green by AM-cleavage) and the number of cells with compromised membranes (nuclei fluorescing red by EthD-1). In 3-D neuronal cultures, the viability
was assessed at cell densities of 1250, 2500, 3750, 5000, 6250, and 7500 cells/mm$^3$ at 7 DIV (n = 6, 10, 10, 10, 6, 6, respectively). In neuronal cultures at a density of 3750 cells/mm$^3$, viability was assessed at 7 - 8, 13 - 14, and 21 DIV (n = 5, 6, 4, respectively) and compared to viability in 2-D cultures (n = 5, 6, 5, respectively). In the neuronal-astrocytic co-cultures, viability was also assessed at 7, 14, and 21 DIV in 3-D (n = 18, 4, 4, respectively) and 2-D (n = 8, 4, 4, respectively).

**Phenotypic Identification**

Cultures were stained for specific phenotypic markers by standard immunocytochemical procedures. Briefly, cells were fixed in 3.7% formaldehyde (Fisher, Fairlawn, NJ) for 30 min, rinsed in PBS and permeabilized using 0.3% Triton X100 (Kodak, Rochester, NY) + 4% goat serum (Invitrogen) for 20 minutes. Primary antibodies were added (in PBS + 4% serum) at 18-24 °C for 4 hours. After rinsing, the appropriate secondary fluorophore-conjugated antibodies (FITC or TRITC-conjugated IgG, Jackson Immuno Research, West Grove, PA or Alexa 488 or 546-conjugated IgG, Molecular Probes) were added (in PBS + 4% serum) at 18-24 °C for 2 hours. Both neuronal cultures and neuronal-astrocytic co-cultures were immunostained using primary antibodies recognizing a combination of the following intracellular proteins: 1) glial fibrillary acidic protein (GFAP) (AB5804, 1:400; MAB360, 1:400, Chemicon, Temecula, CA), an intermediate structural filament found exclusively in astrocytes (Debus, et al., 1983), 2) tau-5 (MS247P, 1:200, NeoMarkers, Fremont, CA), a microtubule-associated protein expressed predominantly in neurons (Binder, et al., 1985; Migheli, et al., 1988; Goedert, et al., 1991), and 3) synapsin I (A6442, 1:200, Molecular Probes), a synaptic vesicle protein localized in presynaptic specializations (Fletcher, et al., 1991).
Counterstaining was performed using Hoechst 33258 (1:1000, Molecular Probes). The purity of the neuronal cultures was assessed by quantifying the number of GFAP$^+$ cells versus the total number of cells in 3-D ($n = 5$) and 2-D ($n = 6$) at 7 DIV. In co-cultures at 7 DIV, the percentage of neurons in culture was assessed by determining the numbers of tau$^+$ cells in 3-D and 2-D with GFAP and Hoechst counter-staining (initial plating ratio 1:1, $n = 5$ in 3-D and $n = 6$ in 2-D; initial plating ratio 1:5, $n = 7$ for each). In 2-D neuronal cultures and 2-D and 3-D neuronal-astrocytic co-cultures, the number of synapsin$^+$ puncta per neuron was quantified at 7, 14 and 21 DIV (initial co-culture neuron:astrocyte ratio of 1:1, $n = 3 - 6$ per culture condition per time-point).

**Cell Proliferation Assay**

Cell proliferation was assessed in 3-D and 2-D neuronal-astrocytic co-cultures (initial neuron:astrocyte ratio of 1:5) at 1, 4, and 7 DIV ($n = 3 - 4$ per culture per time-point) and in 3-D and 2-D astrocyte-only cultures at 12 hours post-plate ($n = 8$ for each) through 5-bromo-2’-deoxy-uridine (BrdU) incorporation and subsequent antibody-mediated detection (Roche Molecular Biochemicals, Mannheim, Germany). The cultures were incubated with BrdU (10$\mu$M in co-culture medium) at 37°C for 60 min, which integrates into nuclear DNA of proliferating cells. After rinsing, the cultures were fixed (70% EtOH + 30% 50 mM glycine buffer, pH 2.0) for 30 min at -20°C. Then, anti-BrdU monoclonal antibody was added (1:10 in incubation buffer) at 37°C for 30 min followed by anti-mouse-Ig-fluorescein (1:10 in DPBS + 1:1000 Hoechst). The percentage of proliferating cells was quantified by counting the number of BrdU-positive nuclei with respect to the total number of nuclei.
Data Collection and Statistical Analysis

After viability and immunocytochemistry assays, cells were viewed using fluorescent microscopy techniques on an epifluorescent microscope (Eclipse TE300, Nikon, Melville, NY) or a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). For epifluorescent microscopy, images were digitally captured (DKC5T5/DMC, Sony, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Confocal images were acquired across the full thickness of the cultures and were analyzed using LSM Image Browser (Zeiss). Three to five randomly selected regions per culture were digitally captured and counted for statistical analysis. Data are presented as mean ± standard deviation. All confocal photomicrographs from 3-D cultures are 100 µm thick reconstructions (from 500-750 µm thick cultures). General linear model ANOVA was performed followed by Tukey’s pairwise comparisons (p-value < 0.05 was considered significant).

Results

3-D Cell Culture Configuration

Culture configuration (3-D versus 2-D, see Figure 2.1) was qualitatively observed throughout experimentation to ensure that the desired architecture was maintained up to 21 DIV, the longest time-point evaluated. Reconstructions of confocal z-stacks comparing 3-D and 2-D configurations for neuronal cultures and neuronal-astrocytic cocultures are at http://www.neuro.gatech.edu/groups/ laplaca/3Dreconstruction.html (live cells are labeled green and the nuclei of dead cells are labeled red). Cells in 3-D extended numerous processes at all orientations while cells in 2-D remained planar.
There was not a significant difference in viability based on the z-position in a culture, demonstrating consistent cell behavior throughout the culture thickness. In 3-D neuronal-astrocytic co-cultures, there was a significant amount of matrix contraction that was not observed in neuronal cultures, demonstrating an enhanced matrix-remodeling capability of astrocytes (Gottschall and Deb, 1996; Wells, et al., 1996). Furthermore, this observation may be indicative of a net mechanical response of matrix fibrils in resisting tension associated with process outgrowth, a phenomenon observed in similar 3-D collagen (e.g., fibroblasts (Grinnell, 2000)) and collagen-glycosaminoglycan matrices (e.g., peripheral nerve explants (Spilker, et al., 2001)).

Figure 2.1: Schematic representation of the 3-D and 2-D cell culture models used in this study (not to scale). All cultures were plated above a layer of acellular matrix (up to 100 µm thick). (A) Cells in 3-D (neuronal cultures and neuronal-astrocytic co-cultures) were homogeneously dispersed throughout a 500 - 750 µm matrix. (B) Neuronal-astrocytic co-cultures in 2-D were plated and, after allowing for cell adhesion, acellular matrix was added to a height of 500 - 750 um.
Cell Viability in Neuronal Cultures

In order to determine the optimal cell density for neuronal viability in 3-D culture, neurons were plated at various densities (Figure 2.2.1). In this model, cell viability demonstrated a parabolic relationship with cell density, with an optimum viability of ~90% at 3750 cells/mm$^3$. All other cell densities, with the exception of 5000 cells/mm$^3$, presented a statistically significant decrease in viability ($p < 0.05$) (Figure 2.2.2). Throughout various 3-D platings, it was observed that cell culture chambers of smaller cross-sectional area (~0.3 cm$^2$) did not support viable cells at the culture thicknesses utilized in this study (data not shown). This observation suggests an interaction between culture thickness and the cross-sectional area for diffusion which exists independent of cell density.
Figure 2.2.1: Neuronal survival was assessed as a function of 3-D cell density. (A-F) Fluorescent confocal reconstructions of representative neuronal cultures plated at various cell densities. At 7 DIV, cells were stained to discriminate live cells (green) and the nuclei of dead cells (red). Scale bar = 50 µm.
Figure 2.2.2: Neuronal survival was assessed as a function of 3-D cell density. The percentage of viable cells in 3-D neuronal cultures at 7 DIV as a function of cell density (cells per mm$^3$). In this culture model, cell density was found to be a significant factor in neuronal survival. *Groups varied significantly from the peak cell viability attained at a cell density of 3750 cells/mm$^3$ (p < 0.05).

Using the optimal 3-D cell density of 3750 cells/mm$^3$ and comparing to 2-D cultures (Figure 2.3.1), viability was assessed up to 21 DIV. There were no statistical differences in cell viability between the 3-D and 2-D cultures at 7 DIV, 14 DIV or 21 DIV. Furthermore, there was not a significant difference between either the 7 DIV to 14 DIV or the 14 DIV to 21 DIV for either configuration; however, there was a significant decrease in culture viability at 21 DIV compared to 7 DIV in both the 3-D and 2-D models (p < 0.05) (Figure 2.3.2).
Figure 2.3.1: Viability in 3-D and 2-D neuronal cultures was determined up to 21 DIV. Fluorescent confocal reconstructions of representative neuronal cultures at 8 DIV plated (A) in 3-D throughout a matrix or (B) in 2-D above a matrix. Live cells are stained green while the nuclei of dead cells are stained red. Scale bars = 50 µm.
Figure 2.3.2: Viability in 3-D and 2-D neuronal cultures was determined up to 21 DIV. The percentage of viable cells in 3-D and 2-D neuronal cultures was quantified at 7 - 8, 13 - 14, and 21 DIV. Neurons in 3-D were plated at the optimal cell density for this system (3750 cells/mm$^3$). There was not a significant difference between 3-D and 2-D viability at any time point; however, both 3-D and 2-D neuronal cultures had a significant decrease in cell viability at 21 DIV compared to 7 - 8 DIV (p < 0.05)*.

Cell Viability in Neuronal-Astrocytic Co-Cultures

Based on an adjustment to the optimal cell density established in neuronal cultures, neuronal-astrocytic co-cultures were developed where each cell type was derived from a separate source and mixed in a controlled ratio (1:1 or 1:5). At either plating ratio, the viability from 7 DIV to 21 DIV remained > 95% for 3-D co-cultures and > 98% for 2-D co-cultures; however, there was not a statistically significant difference between these groups at any time-point evaluated (Figure 2.4).
Figure 2.4: Viability in 3-D and 2-D neuronal-astrocytic co-cultures was assessed up to 21 DIV. Fluorescent confocal reconstructions of representative neuronal-astrocytic co-cultures at 7 DIV in (A) 3-D and (B) 2-D and at 21 DIV in (C) 3-D and (D) 2-D. Live cells are stained green while the nuclei of dead cells are stained red. The viability for both 3-D and 2-D co-cultures was > 95% at all time-points evaluated; there was not a statistically significant difference in the viability between these culture configurations at any time-point. Scale bar = 50 µm.
Theoretical Analysis of Diffusional Parameters

A mathematical analysis of diffusional parameters was performed to theoretically describe mass transport phenomena that may potentially affect the health and viability of neural cells cultured in 3-D. Mass transport in these 3-D cultures relies on passive diffusion, which alters the cellular microenvironment by driving nutrients in and waste products out. Fick’s 2\textsuperscript{nd} Law of Diffusion was applied in one-dimension, approximating the cell-containing matrix as a disc (with cross-sectional area, $A$, and thickness, $w$) and assuming a constant diffusion coefficient, $D$.

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial z^2} \quad (2.1)
\]

This relationship is adequate to account for the passive diffusion into the matrix; however, the rate at which a particular nutrient is consumed must be included. If the process by which a diffusing nutrient is immobilized on or within a cell (and is metabolized or trapped) proceeds rapidly compared with the diffusion process, local equilibrium may be assumed to exist between the extracellular (free) and immobilized components of the diffusing nutrients. Accordingly, the concentration $S$ of metabolized substance was assumed to be directly proportional to the concentration $C$ of nutrient free to diffuse by an immobilization rate constant, $k$, described by the relationship $S = kC$.

The immobilization (i.e. consumption) rate constant will be specific to a particular compound, as some nutrients freely cross the cell membrane while others exhibit specific mechanisms of entry. Also, consumption will vary as a function of cell number, cell type and metabolic activity. Accordingly, Fick’s 2\textsuperscript{nd} Law may be modified to become

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial z^2} - \frac{\partial S}{\partial t} = D_c \frac{\partial^2 C}{\partial z^2}, \text{ where } D_c = \frac{D}{k + 1} \quad (2.2)
\]

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where a new diffusion coefficient, $D_c$, is the diffusion coefficient given nutrient consumption, and is a synthesis of the free diffusion coefficient to the consumption rate constant. Assuming a large nutrient source (i.e. bulk medium volume above the matrix), the resulting concentration profile within the cell culture is given by

$$C(z, t) = \frac{4C_a}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \cdot \exp \left( \frac{-D_c (2n+1)^2 \pi^2 t}{4w^2} \right) \cdot \cos \left( \frac{(2n+1)\pi z}{2w} \right)$$

(2.3)

This equation may be used to predict the diffusion of molecules into the cell-containing matrix. The mass entering the matrix may be described by the integration of the concentration profile over the effective area for diffusion ($A_{eff}$) through the matrix:

$$M = \int_0^w C(z, t) A_{eff} \, dz$$

(2.4)

where the effective area for diffusion is the total cross-sectional area of the matrix minus the average area (per plane) occupied by cells and matrix.

$$A_{eff} = A_{total} - A_{cells}$$

(2.5)

Thus, given the initial assumptions, the mass transport of this system is 1) governed by the diffusion/consumption characteristics of a particular compound, 2) proportional to the effective cross-sectional area for diffusion, and 3) effectively delayed by the culture thickness. The effective area for diffusion is the total cross-sectional area of the culture minus the area occupied by cells (the open porosity of the matrix will not change based on cell density and is assumed to be constant) and thus is inversely proportional to cell density. Changes in diffusional area are demonstrated in confocal reconstructions of 3-D neuronal cultures plated at various cell densities (Figure 2.2.1). Increases in cell density will increase the tortuosity of the system as an enhanced cell-neurite network necessitates increasingly convoluted pathways for diffusion. Although
this analysis has focused on nutrient consumption, it is trivial to modify the initial rate relationship so as to describe the rate of production and subsequent diffusion of metabolic waste products out of the cellular microenvironment. That process will analogously be adversely affected by increases in cell density as the rate of waste product production will increase, possibly creating an unhealthy microenvironment for the cells. Mass transport must surpass specific metabolic thresholds for 3-D culture systems to support viable cells, and given the inter-relationship between cell density and culture thickness, changes in one parameter may compensate for another provided mass transport thresholds remain surpassed. Overall, this analysis serves as a theoretical tool demonstrating that increasing the cell density in 3-D effectively hinders mass transport by decreasing the available area for diffusion, increasing tortuosity, and increasing the rates of nutrient consumption and waste product production.

**Cell Morphology in Neuronal Cultures and Neuronal-Astrocytic Co-Cultures**

SEM revealed morphological differences between cells plated within a 3-D matrix and cells on a 2-D substrate. Cells plated in the neuronal cultures and in the neuronal-astrocytic co-cultures in 3-D presented a bulbous morphology with intercellular contacts in all spatial directions (Figure 2.5.1-2.5.2). Neurons in 2-D culture had a flatter base and interactions occurred on the substrate plane or up the height of the cell body (Figure 2.5.1). Co-cultures in 2-D arranged themselves into a base layer of astrocytes (which approached confluency) with neurons segregated to a second plane above (Figure 2.5.2).
Figure 2.5.1: Electronmicrographs of neuronal cultures in 3-D and 2-D. (A-B) Neurons in 3-D present a rounded morphology with matrix interactions possible in all spatial dimensions, while (C-D) neurons in 2-D have a flattened morphology.
Figure 2.5.2: Electronmicrographs of neuronal-astrocytic co-cultures in 3-D and 2-D. (A-B) Neuronal-astrocytic co-cultures in 3-D are rounded with an apparently even dispersion of neurons and astrocytes, compared to 2-D where the cell types segregate themselves into (C) a top plane of neurons with (D) a base layer of flattened astrocytes below.
Cell Composition in Neuronal Cultures and Neuronal-Astrocytic Co-Cultures

Cellular phenotypic identification was assessed using specific immunocytochemical markers. The level of astrocyte contamination in the neuronal cultures was assessed through GFAP immunostaining in conjunction with Hoechst staining. The percentage of GFAP+ cells was 2.9 ± 1.5% in 3-D and 3.2 ± 1.6% in 2-D, without a significant difference between these groups. The percentage of neurons in co-culture with astrocytes was assessed via immunocytochemical staining. The neuronal population was determined by staining for tau and GFAP in conjunction with Hoechst labeling (Figure 2.6.1).

At 7 DIV, the neuronal component in 3-D co-cultures was 22.0 ± 5.4% (initially 50%) and 14.4 ± 5.5% (initially 16.7%). In 2-D co-cultures the neuronal percentage was 19.8 ± 7.2% (initially 50%) but only 1.0 ± 0.3% (initially 16.7%) (Figure 2.6.2). Thus, for an initial plating ratio of 1:1, there was a similar alteration in the neuron:astrocyte

Figure 2.6.1: The neuronal composition in neuronal-astrocytic co-cultures in 3-D and 2-D was determined at 7 DIV. Immunofluorescent photomicrographs of representative neuronal-astrocytic co-cultures (plated at a ratio of 1:5) in (A) 3-D and (B) 2-D triple labeled to denote the astrocyte marker GFAP (red), the neuronal marker tau (green), and the nuclear stain Hoechst (blue). Scale bar (in both) = 50 µm.
ratio in 3-D compared to 2-D. However, for an initial plating ratio of 1:5, the relative neuronal population in 2-D co-culture presented a substantial decrease from the initial neuronal population and is a statistically significant deviation from the neuronal presence in 3-D co-cultures \( (p < 0.001) \). Similar neuronal composition percentages were determined by staining for MAP-2, another microtubule-associated protein expressed predominantly in neurons (data not shown).

**Figure 2.6.2: The neuronal composition in neuronal-astrocytic co-cultures in 3-D and 2-D was determined at 7 DIV.** Graphical representation of the percentage of neurons at 7 DIV for two different initial plating ratios. For neuron:astrocyte co-cultures plated at 1:5 (initially 16.7% neurons), the percentage of neurons decreased significantly in 2-D but not in 3-D \( (p < 0.001) \). At a plating ratio of 1:1 (initially 50% neurons), the neuronal composition significantly decreased in 3-D and 2-D, but these groups did not differ significantly from each other.
Synapse Formation in Neuronal and Neuronal-Astrocytic Co-Cultures

The formation of synapses was assessed via immunocytochemical staining for neuronal cultures and neuronal-astrocytic co-cultures. Immunolocalization of synapsin$^+$ puncta along neuronal processes suggests the presence of active synapses (Fletcher, et al., 1991; Hartley, et al., 1999; O'Shaughnessy, et al., 2003). The continuum transition from an immature to a mature neuronal phenotype is marked by established cellular polarity and electrochemical activity (i.e. synaptic communication). The vast majority of cells in neuronal culture expressed the neuronal marker tau, and by 7 DIV these cells presented synapsin distributed in discrete puncta along the processes or somas that increased in number out to 21 DIV (Figure 2.7.1). Synapsin$^+$ puncta were also observed along tau$^+$ somas and processes in co-cultures in 3-D and 2-D (Figure 2.7.1), and there was a similar trend of increased synapsin staining as a function of DIV. There was a significant increase in the number of synapsin$^+$ puncta per neuron in neuronal-astrocytic co-cultures versus the neuronal cultures (Figure 2.7.2). However, there was not a change in the formation of synapsin$^+$ puncta based on culture dimensionality.
Figure 2.7.1: Synapse formation in neuron and neuron-astrocyte co-cultures.
Neuronal cultures and neuronal-astrocytic co-cultures were immunolabeled at 7, 14 and 21 DIV to identify neurons and synaptic puncta. Representative confocal reconstructions of (A) 2-D neuronal culture, (B) 2-D neuronal-astrocytic co-culture, and (C) 3-D neuronal-astrocytic co-culture (all at 21 DIV). Synapsin$^+$ puncta (green) can be seen along tau$^+$ processes (red) for all culture types (with blue nuclear counterstain). Scale bar $= 20 \mu$m in (A) and (B); scale bar $= 10 \mu$m in (C).
Neuronal cultures and neuronal-astrocytic co-cultures were immunolabeled at 7, 14 and 21 DIV to identify neurons and synaptic puncta. There was a significant increase in the number of synapsin$^+$ puncta per neuron as a function of DIV for all culture types. There was an increase in synapsin$^+$ puncta per neuron in neuron-astrocyte co-cultures versus the neuron cultures, signifying an enhanced rate of synapse formation or retention in the presence of astrocytes. However, culture dimensionality did not affect the formation of synapsin$^+$ puncta per neuron.

Cell Proliferation in Neuronal-Astrocytic Co-Cultures and Astrocytic Cultures

Cell proliferation was assessed in 3-D and 2-D neuronal-astrocytic co-cultures and astrocyte-only cultures using a standard proliferation assay based on BrdU incorporation at various time-points. Proliferation was assessed in astrocyte-only cultures in 3-D and 2-D at 12 hours post-plate to assess the immediate effects of culture configuration on astrocyte proliferation. There was a significant decrease in astrocyte proliferation in 3-D compared to 2-D ($p < 0.001$), signifying that culture configuration affects proliferative mechanisms for monotypic populations (Figure 2.8). Next, proliferation was assessed in neuronal-astrocytic co-cultures (initial ratio of 1:5) at 1, 4,
and 7 DIV in order to evaluate neural cell proliferation throughout a time-course of culture development. This initial plating ratio was chosen for these experiments to test the hypothesis that enhanced astrocyte proliferation in 2-D compared to 3-D was the mechanism responsible for the decrease in the relative neuronal population in these cultures at 7 DIV. At all time-points evaluated, there was a significant decrease in cell proliferation in 3-D versus 2-D co-cultures (p < 0.05), suggesting that neuron-astrocyte interactions as well as culture configuration affect the proliferative behavior of neural cells in co-culture (Figure 2.9). Similar results were obtained comparing higher density 2-D co-cultures to 3-D co-cultures (data not shown).
Figure 2.8.1: Cell proliferation was determined in astrocyte-only cultures in 3-D and 2-D at 12 hours post plating to assess the immediate effect of culture configuration on proliferation. Cultures are stained with the nuclear marker Hoechst (blue) and immunolabeled using antibodies recognizing BrdU, a nucleotide incorporated into the nuclei of dividing cells (green). (A) Phase contrast and (B) immunofluorescent photomicrographs of astrocytes in 3-D and (C) phase contrast and (D) immunofluorescent photomicrographs of astrocytes in 2-D. Scale bar = 50 µm.
Figure 2.8.2: The percentage of proliferating cells was determined in astrocyte-only cultures in 3-D and 2-D at 12 hours post plating. There was a significant increase in the percentage of proliferating astrocytes in 2-D versus 3-D (p < 0.001), signifying that culture configuration influences the proliferative behavior of astrocytes.
Figure 2.9: The percentage of proliferating cells in 3-D and 2-D neuronal-astrocytic co-cultures was assessed at 1, 4, and 7 DIV. Immunofluorescent photomicrographs from representative neuronal-astrocytic (initially 1:5) co-cultures in (A) 3-D and (B) 2-D. The nuclei of all cells are stained blue with proliferating cells labeled green. (C) Graphical representation of the percentage of proliferating cells. There was a significant decrease in the percentage of proliferating cells in 3-D versus 2-D at each time-point assessed (p < 0.05). Scale bar = 50 µm.
Discussion

We have developed and characterized two 3-D culture systems consisting of either predominantly primary cortical neurons or separately isolated primary cortical neurons and cortical astrocytes. Extensive neuronal networks were evident in both models, yet SEM revealed morphological differences between cells in planar culture, which present a flat morphology, versus cells distributed throughout a matrix, which present a bulbous morphology with cell-matrix contact in all spatial dimensions. Plating density was a critical parameter for neurons in 3-D, with an optimal cell viability obtained at 3750 cells/mm$^3$. Time in culture was also a significant factor in neuronal viability, as neurons in 3-D and 2-D demonstrated a decreasing trend in viability up to 21 DIV (the longest time-point assessed); however, cell viability did not decrease below 94% up to 21 DIV in the neuronal-astrocytic co-cultures in either 3-D or 2-D. These data suggest that co-culture conditions (i.e. glial support) may be required for the long-term culture of primary cortical neurons, yet even under those circumstances the culture viability has been shown to steadily decrease (Lesuisse and Martin, 2002).

Characterization of the ratio of cells is an important factor when establishing culture models. The neuronal culture model demonstrated a nearly pure population of neurons. In 3-D neuronal-astrocytic co-cultures, there was preservation of the initial neuron:astrocyte ratio for cultures plated at 1:5 but not 1:1, with the latter approaching a 1:5 ratio at 7 DIV. However, in the 2-D co-cultures there was a decrease in the relative percentage of neurons at both plating ratios. Investigation of cell proliferation revealed an increase in mitotically-active cells in 2-D versus 3-D co-culture. However, in 3-D the primary regulator for proliferation may be the ratio of neurons to astrocytes rather than
three-dimensionality, and past work has demonstrated the prominence of contact-mediated neuron-astrocyte interaction on the regulation of astrocyte proliferation (Hatten, 1987). Given that the relative percentage of astrocytes increases in co-culture, it is likely that the majority of proliferation occurs in astrocytes; however, it has been shown that differentiating neocortical neurons in 2-D culture can be mitotically active (Jacobs and Miller, 2000), maintaining the possibility that neurons in our model proliferate as well. Furthermore, there was a decrease in proliferation in astrocyte-only cultures in 3-D versus 2-D. These data indicate complex mechanisms regulating proliferation in 3-D that may not be responsive in 2-D cultures for these neural cell types. Such regulatory mechanisms involve astrocyte-matrix interactions in 3-D as well as neuron-astrocyte interactions. Studies of proliferation in non-neural cell types in 3-D have produced mixed results, where some cell types demonstrate an increase in proliferation for monotypic cell populations in 3-D versus 2-D (e.g., osteoblastic cells (Granet, et al., 1998)).

The neuronal cell culture model was characterized based on viability at various cell densities and time-points. There was a parabolic relationship between cell plating density and cell viability in the 3-D neuronal cultures, suggesting an important balance between diffusional requirements (affecting the higher density cultures) and a threshold for cell-cell interactions (both physical and chemical, adversely affecting the low density cultures). Because these cultures rely on passive diffusion for mass transport, Fick’s 2\textsuperscript{nd} Law of Diffusion was applied to analyze the cellular microenvironment by describing the diffusion of waste products out of the matrix into the medium, and conversely, the diffusion of nutrients into the matrix from the medium. This analysis yielded a
mathematical description of the mass transport through the matrix and revealed a synergistic interaction of the effective area for diffusion, which is a function of cell density, and the culture thickness. It was determined that increasing the cell density in 3-D effectively decreases the available area for diffusion, increases tortuosity factors, and increases the overall rates of nutrient consumption and waste product production. It is likely that mass transport limitations are the predominant reason that 3-D neuronal cultures exhibited poor viability at high cell densities ($\geq 6250\text{cells/mm}^3$), given that all other parameters were consistent between these cultures. These experimentally-derived parameters for 3-D cell culture density still remain far from the densities of $10^5$-$10^6$ cells/mm$^3$ reported in various cortical regions (Gabbott and Stewart, 1987; Braitenberg, 2001); however, given a heterogeneous neural cell population and a perfusion system, it may be possible to approach this cell density \textit{in vitro}. Thus, there are important design considerations and limitations that must be acknowledged for 3-D \textit{in vitro} systems. Cells cultured in 3-D may experience diffusional transport limitations, causing essential nutrients to not reach all of the cells and possibly leading to accumulation of toxic waste products in close proximity to the cells. Such phenomena may result in altered gene expression and a detrimental effect on protein production and fidelity, proliferation and viability, leading to an appreciable deviation from \textit{in vivo} behavior (Alves, et al., 1996). However, properly designed and optimized 3-D models, where diffusional limits are not approached, may more faithfully recapitulate elements of native tissue than 2-D models.

There are fundamental differences between cells cultured in 3-D versus 2-D environments. There are differences in the types, quantity, and distribution of cell-cell and cell-matrix interactions (Cukierman, et al., 2001; Cukierman, et al., 2002;
Schmeichel and Bissell, 2003; Yamada, et al., 2003), with cells in a 3-D bioactive matrix contacting ECM proteins and experiencing cell-cell interactions (e.g., receptor-mediated, synaptic) in all spatial directions. Cells in 2-D configurations may only experience such interactions in a single plane since a majority of the cell surface is exposed. From a physical perspective, cells grown in 3-D versus 2-D environments have a starkly different morphology and cytostructure as demonstrated by this study and others (Grinnell, 2003). The soma and growth cones of neurons in 2-D are flatter compared to cells in 3-D which present a rounder, more bulbous shape (Balgude, et al., 2001). Thus, inherent differences in cell-cell/cell-matrix interactions coupled with corresponding alterations in cell morphology and alterations in the cellular microenvironment (e.g., access to trophic factors) may have an impact on intracellular signaling and gene expression. Furthermore, differences in gene expression, growth characteristics, and viability are found when comparing cells cultured in 3-D and 2-D to cells in vivo. Previous neural transplantation studies have shown a significantly higher attrition rate for cells cultured in 2-D prior to implantation versus those cultured in 3-D (Fawcett, et al., 1995). Also, cells cultured in 2-D have shown an increase in sensitivity to chemical treatments independent of changes in surface area (Miller, et al., 1985). Overall, due to fundamental deviations in 2-D cell culture from the in vivo environment, cells cultured in 2-D may be inherently unable to recapitulate traits exhibited in vivo, whereas properly designed and regulated 3-D systems may represent a step closer to in vivo.

In neuronal and neuronal:astrocytic co-cultures in 3-D and 2-D, cells exhibited the neuronal cytoskeletal marker tau and discrete puncta containing the synaptic vesicle protein synapsin. This immunolocalization was suggestive of the presence of maturing
neuronal phenotype and active synapses from 7 to 21 DIV. The methodology used in this study to identify synapses via immunolocalization has previously been correlated with the formation of active synapses via whole-cell patch clamp recordings in similar neuronal culture models (Hartley, et al., 1999; O'Shaughnessy, et al., 2003). Our results, along with those of others, demonstrate that astrocytic presence is not a requirement for synapse formation, although evidence suggests that neuronal-glial interactions may be necessary to elicit proper neuronal differentiation and fully functional synapses (Pfrieger and Barres, 1997). In vivo, astrocytes and neurons have an intricate coupling as astrocytes provide metabolic support by regulating the blood-brain barrier, providing trophic factors, and maintaining proper glutamate and glutathione metabolism, thus creating interdependence between these cell types in assuring proper energetics, neuromodulation, and termination of pathologic states such as excitotoxicity (Aschner, 2000). Astrocytes also exhibit a physical role as they serve as substrates for neuronal migration, guides for neuronal process outgrowth, and form barriers to segregate axon types. Astrocytes play an important role in neuronal function through regulation of the synaptic microenvironment by controlling the number and stability of synapses and by ensheathing synapses to maintain an appropriate ionic environment (Ullian, et al., 2001). Neuronal-astrocytic interdependence was demonstrated in this study as there was an increase in the number of synapses per neuron in neuron-astrocyte co-cultures versus cultures that were predominantly neuronal. Overall, it may be necessary for neurons to be directly associated with astrocytes in a 3-D environment in order for proper function of these cell types and accurate responses to chemical or physical disturbances.
Conclusions

*In vitro* models of neural cells have proven to be effective in the systematic identification of specific mechanisms of cellular signaling pathways and responses. The primary attributes of *in vitro* models include control of cellular composition, control of local environment, accessibility for imaging, experimental manipulability, and elimination of systemic effects. The 3-D models presented here maintain all these advantages while more closely approximating the *in vivo* environment than many previous models. Specifically, the *in vitro* models of neural cells here presented are capable of recapitulating many aspects of the *in vivo* environment including cell cytoarchitecture, distribution of cell-cell/cell-matrix interactions, and the multicellular composition. Properly designed cellular models may serve as a more accurate guide to cellular growth, interaction, and responses to biochemical and/or mechanical stimuli.
CHAPTER III

HIGH RATE SHEAR STRAIN OF THREE-DIMENSIONAL NEURAL CELL CULTURES:
A NOVEL IN VITRO TRAUMATIC BRAIN INJURY MODEL

Abstract

The fidelity of cell culture simulations of traumatic brain injury (TBI) that yield tolerance and mechanistic information relies on both the cellular models and mechanical insult parameters. We have designed and characterized an electro-mechanical cell shearing device (CSD) in order to produce a controlled high strain rate injury (up to 0.50 strain, 30 s⁻¹ strain rate) that deforms three-dimensional (3-D) neural cultures in an extracellular matrix scaffold. Theoretical analysis revealed that these parameters generate a heterogeneous 3-D strain field throughout the cultures that is dependent on initial cell orientation within the matrix, resulting in various combinations of normal and shear strain. The ability to create a linear shear strain field over a range of input parameters was verified by tracking fluorescent microbeads in an acellular matrix during maximal displacement for a range of strains and strain rates. In addition, cell death was demonstrated in rat cortical neurons in response to high rate, high magnitude shear strain. Furthermore, cell response within the 3-D neuronal cultures depended on orientation, with higher predicted shear strain correlating with an increased loss of neurites, indicating that culture configuration may be an important factor in the mechanical, and hence cellular, response to traumatic insults. Collectively, these results suggest that
differential responses exist within a 3-D culture subjected to mechanical insult, perhaps mimicking the *in vivo* environment, and that this new model can be used to investigate the complex cellular mechanisms associated with TBI.

**Introduction**

Traumatic brain injury (TBI) is a severe health and socioeconomic problem, for which there are few effective clinical treatments (Roberts, et al., 1998). TBI results from mechanical loading to the head and therefore models that seek to reveal injury mechanisms should accurately simulate the related biomechanics. Traumatic loading to the head can involve several components, including contact and/or inertial loading (Gennarelli, 1993). The severity of neurological disability depends on the initial insult and the ensuing cellular cascades, which may be complex and persistent.

Although *in vivo* studies model the systemic and behavioral deficits of TBI, *in vitro* approaches provide a powerful framework for investigating isolated mechanisms. Cellular models range from transection (Gross, et al., 1983; Lucas, et al., 1985; Tecoma, et al., 1989; Regan and Choi, 1994; Mukhin, et al., 1996) and compression models (Balentine, et al., 1988; Shepard, et al., 1991; Murphy and Horrocks, 1993; Wallis and Panizzon, 1995) (contact loading) to more complex stretching and acceleration devices (Ellis, et al., 1995; Cargill and Thibault, 1996; LaPlaca and Thibault, 1997; Geddes and Cargill, 2001) (inertial loading). Inertial loading has been correlated with severe, diffuse brain injury that is attributed to stretching neural components (Margulies and Thibault, 1992; Gennarelli, 1993; Ommaya, et al., 1994). The spatial and temporal patterns of strain associated with inertial loading are the basis for several *in vivo* and *in vitro* injury
models that apply large strains (see (Gennarelli, 1994; Morrison, et al., 1998) for reviews). In addition to the strain magnitude, the tensorial nature of strain is a determining factor in tissue outcome. In fact, the primary mode of failure in neural tissue is shear (Holburn, 1943; Shuck and Advani, 1972). Models that employ rapid mechanical deformation often exhibit a strain- and/or strain rate-dependent injury, thus substantiating the clinical observation that angular acceleration and injury severity are causally related.

Planar (2-D) cultures differ markedly from the three-dimensional (3-D) cytoarchitecture of the brain. Cell behaviors of 2-D versus 3-D cell configurations have shown that 3-D cultures more closely resemble those of native tissue (e.g., osteoblastic cells (Granet, et al., 1998), hepatocytes (Takeshita, et al., 1998), breast cells (Wang, et al., 1998), and neural cells (Fawcett, et al., 1989; Fawcett, et al., 1995)), suggesting that 3-D models may yield more accurate secondary responses. Cells cultured in 2-D have different cell-cell and cell-matrix interactions than 3-D cultures (Gumbiner and Yamada, 1995; Cukierman, et al., 2001; Cukierman, et al., 2002; Schmeichel and Bissell, 2003; Yamada, et al., 2003), potentially impacting mechanotransduction associated with traumatic insults. In this context, brain slices (ranging in thickness from 200 to 500µm) have been utilized to determine molecular responses to injury (Wallis and Panizzon, 1995; Morrison, et al., 1998; Sieg, et al., 1999). Although slice models are invaluable to in vitro investigations, the ability to control cell types, ratio of cell types, and extracellular components—factors that may be important to the injury response—is limited.

A device that incorporates the features of 3-D cell cultures under conditions of simple shear strain would be a significant addition to the repertoire of cell injury devices
in the study of TBI. To this end, we have developed a 3-D cell shearing device (CSD) that delivers a prescribed shear strain to 3-D cell cultures. The strain is controlled by a closed loop system, ensuring precise and repeatable deformation. The objectives of the current study are (1) to analytically describe the 3-D strain field for a range of shear displacement angles, (2) to measure the strain field throughout a 3-D gel configuration, and (3) to test the ability of the system to induce cell injury in 3-D primary neural cultures.

**Materials and Methods**

**Three-Dimensional Cell Shearing Device (3-D CSD)**

The primary design goal for the 3-D CSD was to have control over the magnitude and rate of displacement. The 3-D CSD has two major components: the **cell chamber** and the **actuator/control system** (Figure 3.1.1). The cell chamber is designed to contain enough cells for molecular analyses in a 3-D configuration. The actuator/control system develops shear strains up to 0.50 (shear angle up to 45°) at rates from 1 s⁻¹ to 30 s⁻¹ in order to simulate the spatial and temporal strain patterns associated with inertial TBI (Margulies and Thibault, 1992).
Figure 3.1.1: 3-D Cell Shearing Device (3-D CSD) components. A schematic representation of the 3-D CSD. The device can be mounted on a confocal microscope to obtain 3-D images before, during, and after mechanical deformation. A closed-loop control system (PID controller with feedback from a DVR T) governs a linear actuator, inducing motion of the cell chamber top-plate (not to scale).

Cell Chamber

The cell chamber is comprised of the cell reservoir and the top plate (Figure 3.1.2). The cell reservoir is a rectangular polycarbonate well with a glass floor (no. 1½ coverslip). Parallel shoulders were machined into the upper surface of the two long sides of the polycarbonate walls to provide proper alignment and constrain motion of the top plate to a single axis. The 3-D cultures (two per reservoir) are confined within an elastomer mold composed of Sylgard 184 and 186 (Dow Corning; Midland, MI), which were mixed 1:1 (w/w) and cast to the desired thickness. For culture containment, two cutouts (inner dimensions of each, 10mm x 10mm or 15mm x 15 mm) were made with a razor punch. Throughout experimentation, the mold adhered firmly to the bottom glass coverslip without any movement. The top plate is composed of a polycarbonate body.
with two C-shaped protrusions on the bottom surface. The C-shaped protrusions act as struts to transfer the transverse motion of the top plate to the cell culture via a porous, hydrophilic polyethylene filter (15-45 µm pore size; Porex Corp; Atlanta, GA) which is attached to the C-shaped protrusions on the topside and directly to the cell culture on the bottomside. The porous filter permits transfer of nutrients and waste. The top plate is attached to the linear actuator by a polycarbonate extension plate that is mounted to a linear bearing system (Thomson Bearings; Port Washington, NY).

![Figure 3.1.2: 3-D Cell Shearing Device (3-D CSD) cell culture chamber.](image)

The cell chamber consists of a top-plate with polyethylene filters to interface with the 3-D cell cultures. The top plate is mounted above the cell reservoirs and connected to the linear actuator to impart high rate deformation.

**Actuator and Closed Loop Motion Control System**

The 3-D CSD generates a dynamic shear strain by the linear motion of the top plate with respect to the cell reservoir (Figure 3.1.3) via a linear voice coil actuator (BEI Kimco; San Marcos, CA) and a closed loop control system consisting of a proportional-
integral-derivative (PID) controller (Feedback Inc.; Hillsborough, NC), an amplifier (Advanced Motion Controls; Camarillo, CA), and a differential variable reluctance transducer (DVRT) (Microstrain; Burlington, VT). The linear actuator produces a rigid body translation of the top plate assembly using a function generator (Hewlett Packard; Palo Alto, CA). The input signal is the reference input to the PID controller and is compared internally to the actual displacement of the top plate provided by the DVRT. The closed loop controller produces a self-correcting signal to minimize the error between the actual and desired position. PID control reduces steady-state error between the input signal and the motion of the top plate, eliminates possible oscillations in the response, and increases sensitivity of the system.

![Diagram of 3-D Cell Shearing Device (3-D CSD) mechanical action.](image)

**Figure 3.1.3: 3-D Cell Shearing Device (3-D CSD) mechanical action.** The horizontal motion of the linear actuator drives the displacement of the cell chamber top-plate, inducing shear deformation in the Sylgard mold and matrix (with either cells or microbeads) (not to scale).
Analysis

The 3-D strain field for a range of displacements was analyzed using a continuum mechanics approach. A neural cell was modeled as a line element and placed in various orientations. This kinematic analysis of the shear strain field in the 3-D CSD may aid in validating our experimental findings and lend insight into the mechanisms of shear-induced injury. The amount of neurite loss in injured neurons was then analyzed as a function of theoretical strains in the 3-D constructs to test the hypothesis that high shear strain fields will result in increased cell dysfunction.

Strain Field Characterization

The shear displacement field was validated using a 3-D acellular construct. Microbeads were embedded in Matrigel basement membrane matrix (Becton Dickinson; Bedford, MA) and tracked on an epifluorescent laser scanning confocal microscope (Zeiss LSM 510 UV). The testing sample was cast by mixing Matrigel and Dulbecco’s Modified Eagle Media (DMEM) (1:1) with FluoSpheres carboxylate-modified microspheres (0.5µm diameter, 9 x 10⁶ beads/ml; Molecular Probes; Eugene, OR). The mixture was triturated and a sufficient amount to create 500µm thick constructs was distributed into each mold and allowed to set for 4 hrs at 37°C. 3-D constructs were subsequently mounted on the 3-D CSD. A symmetric trapezoidal reference function was generated for each of the following deformation profiles: strain 0.25, strain rate 1 s⁻¹, n = 4; strain 0.25, strain rate 10 s⁻¹, n = 5; strain 0.25, strain rate 20 s⁻¹, n = 4; strain 0.50, strain rate 1 s⁻¹, n = 4; strain 0.50, strain rate 10 s⁻¹, n = 3; strain 0.50, strain rate 20 s⁻¹, n = 4. A long dwell time (strain-and-hold) was used to allow the microscope to scan through multiple planes. Images from the confocal microscope were obtained for 20
planes (2µm thickness) throughout the cultures. The relative positions of one to four beads for each plane were measured pre-strain and during maximum strain in order to calculate the displacement of each bead.

**Cell Culture**

Neurons were isolated from embryonic day 17 rat fetuses. Each fetus was removed by Caesarian section, rapidly decapitated, and the brains were removed. The cortices were isolated and digested in HBSS with trypsin (0.25% + 1mM EDTA) at 37°C for 10 min. The trypsin-EDTA was then removed and the tissue was trituated in HBSS containing DNase I (0.15mg/mL). The cells were centrifuged at 1000 rpm for 3 min and the cells were resuspended in neuronal plating medium (Neurobasal medium + 2% B27 + 500µM L-glutamine) immediately prior to 3-D plating.

Cell chambers were autoclaved and the cell reservoirs were prepared by coating all sides in poly-L-lysine (0.0015%), facilitating Matrigel adsorption to the edge of the Sylgard mold and the bottom glass. 3-D cultures were made by mixing suspended neurons (in neuronal plating medium) with liquid Matrigel (1:1) and adding a sufficient volume to create 500 µm thick cultures (final cell density of 5000 neurons/mm³). Following Matrigel gelation at 37°C, medium was added to the cell reservoirs above the matrices.

All procedures using animals conformed to guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Georgia Tech Institutional Animal Care and Use Committee. All cell reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO) unless otherwise noted.
Cell Deformation

Cells were mechanically loaded following mounting the cell reservoir within the 3-D CSD and attaching the top plate to the extension plate. Neuron cultures (7 days post 3-D plating) were either subjected to deformation in the 3-D CSD (strain 0.50, strain rate 20 s\(^{-1}\), n = 6; strain 0.50, strain rate 30 s\(^{-1}\), n = 8), placed into the device with the top plate (static control, n = 6) or left undisturbed (naïve control, n = 4). A symmetric trapezoidal pulse was employed with the following rise times (in msec): 12.5 (0.25, 20 s\(^{-1}\)), 25 (0.50, 20 s\(^{-1}\)), and 16.6 (0.50, 30 s\(^{-1}\)). After the deformation was applied, warm medium was added and the cultures were returned to the incubator until cell viability was assessed.

Cell Viability and Neurite Orientation

Neuron survival was assessed using fluorescent probes (LIVE/DEAD Viability/Cytotoxicity Kit; Molecular Probes, Eugene, OR) and a confocal microscope (Zeiss). Twenty-four hrs following deformation or static control conditions, cells were removed from the incubator, rinsed in phosphate buffered saline (PBS, pH 7.4) and incubated with 2 \(\mu\)M calcein AM and 4 \(\mu\)M ethidium homodimer (EthD-1) at 37°C for 30 min. After rinsing with PBS, confocal z-stacks were acquired across the thickness of the cultures. After deformation or static control conditions, the percent of viable cells and the distribution of neurites in viable neurons were assessed using the DSM Confocal Image Viewer (Zeiss). Live and dead cells were manually quantified through the thickness of the cultures. The number of neurites was determined at discrete orientation angles by rotating the viewing plane about the \(x_1\) axis (corresponding to \(\phi \in [-70^\circ, 70^\circ]\), see Results for definition) and manually quantifying the number of in-plane neurites that were greater than 10\(\mu\)m in length. Three to five planes per orientation
were counted; the field of view for each plane was \((460.7 \mu m)^2\). As an additional control, 3-D neuronal cultures \((n = 3)\) were subjected to an excitotoxic insult \((100 \mu M\) for 6 hours) at 7 days post-plating and the orientation of viable neurites was assessed 24 hours later (see APPENDIX E). The mean range of the number of neurites counted per plane was 25.1 - 64.2 for static control cultures, 21.4 - 36.8 for excitotoxic cultures and 5.0 - 20.9 for injured cultures. The number of neurites was then converted to percentage of neurites for each orientation in order to compare the injury to the static control conditions.

**Statistical Analysis**

Regression analysis was performed on the measured bead displacements for each loading condition. The slopes of the lines were then compared to theoretical values for simple shear strain using a t-test with \(p < 0.05\) considered statistically significant. Vertical bead displacement was measured (in the \(x_3\) direction) and compared to total displacement in the \(x_2\) direction. The percent of viable cells for each condition was analyzed using a two-way ANOVA (with strain and strain rate as independent variables and viability as the dependent variable) followed by a Tukey’s multiple comparison test. The percentage distribution of neurites at various orientation angles was analyzed using two-way repeated measures ANOVA with a Tukey’s multiple comparison test. A p-value \(< 0.05\) was considered significant.
Results

Kinematic Analysis of 3-D Strain Field

It was hypothesized that the orientation of the cell within the 3-D matrix contributes to the strain transferred to the individual cells from bulk deformation. It was assumed that the overall shear strain field was homogeneous, isotropic, and the culture was incompressible. To describe the motion of points within the deformable matrix, a fixed reference frame in 3-D space was established where the Cartesian coordinates of a point were denoted by \( \mathbf{x} = (x_1, x_2, x_3) \). The positions of any two adjacent reference points in an undeformed state were defined as \( P : (x_1, x_2, x_3) \) and \( Q : (x_1 + dx_1, x_2 + dx_2, x_3 + dx_3) \). The same two reference points were described in a deformed state as \( P : (\xi_1, \xi_2, \xi_3) \) and \( Q : (\xi_1 + d\xi_1, \xi_2 + d\xi_2, \xi_3 + d\xi_3) \). Thus, deformation was a one-to-one mapping from a reference configuration to a deformed configuration and can be represented by a continuous function \( \xi_i = \xi_i(x_1, x_2, x_3, t) \) for \( i = 1, 2, 3 \), where \( t \) denotes the time over which the deformation occurs. The initial angles between points projected into the \( x_3, x_2 \) plane and in the \( x_1, x_2 \) plane were denoted as \( \phi \) and \( \alpha \), respectively (Figure 3.2).
Figure 3.2. Definitions of coordinate system used in theoretical strain analysis. The angles $\phi$ and $\alpha$ are defined as the angles between arbitrary points of reference projected to the $x_3,x_2$ plane and the $x_1,x_2$ plane, respectively. The distance along a fixed axis between these two arbitrary points is defined as $dx_1$ along the $x_1$ axis, $dx_2$ along the $x_2$ axis and $dx_3$ along the $x_3$ axis. The shear deformation of the cell-containing matrices (proportional to the angle $\gamma$) occurs in the $x_3,x_2$ plane.

$$\tan(\alpha) = \frac{dx_1}{dx_2}$$

$$\tan(\phi) = \frac{dx_3}{dx_2}$$
The distance between two points was described as $dS$ in an undeformed state and $\overline{dS}$ in a deformed state. Accordingly, in reference to the initial configuration, the change in the squared distance between the two points becomes:

$$(d\overline{S})^2 - (dS)^2 = 2E_{ij}dx_idx_j$$

(3.1)

where $E_{ij}$ is the Green’s strain tensor. In the specific case of simple shear deformation, the location of a point in the deformed configuration may be described in terms of its initial location and the applied deformation:

$$d\xi_1 = dx_1 \quad , \quad d\xi_2 = dx_2 + dx_3 \tan(\gamma) \quad , \quad d\xi_3 = dx_3$$

(3.2.1-3.2.3)

where $\gamma$ is the angle of shear deformation (Figure 3.3) and vertical displacement is assumed to be zero. Accordingly, the components of the Green’s strain tensor for this system are:

$$E_{ij} = \frac{1}{2} \begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & \tan(\gamma) \\ 0 & \tan(\gamma) & \tan^2(\gamma) \end{pmatrix}$$

(3.3)

The Green’s strain tensor is sufficient to describe the bulk deformation behavior of the cultures. However, we are interested in the state of strain relative to a local coordinate system fixed to a cell. These strains can be determined though coordinate transformations from the initial coordinate system to a coordinate system aligned with a particular unit vector of interest (representing, for example, a cellular process). A series of rotational transformations relate the initial strain tensor, $[E]$, and a transformed strain tensor, $[E']$, based on the transformation matrix $[R]$.

$$[E'] = [R][E][R]^T$$

(3.4)
The transformation matrix was derived such that the new $x_2$ axis direction was aligned with the unit vector of interest through an initial rotation about the $x_1$ axis, followed by a space-fixed rotation about the original $x_3$ axis (Figure 3.3).

Figure 3.3: Axis rotation descriptions. Axis rotations were used to align the $x_2$ axis with a direction of interest. The first rotation is about the $x_1$ axis by $\phi$ degrees. The second rotation is space-fixed about the $x_3$ axis by $\alpha$ degrees. This methodology permits the derivation of the Green's strain tensor specific for a cell process or soma at any orientation with respect to the strain field induced by the 3-D CSD.

The angle of rotation about the $x_1$ axis, $\phi \in [-90^\circ, 90^\circ]$, and the angle of rotation about the $x_3$ axis, $\alpha \in [-90^\circ, 90^\circ]$, were derived based on the ratio of the distances between points of interest.

$$\tan(\phi) = \frac{dx_1}{dx_2} \quad \tan(\alpha) = \frac{dx_1}{dx_2} \quad (3.5.1-3.5.2)$$
Accordingly, the strain tensor as a function of initial orientation is:

\[
\left[ E' \right] = \frac{1}{2} \tan(\gamma) \begin{pmatrix}
0 & -\sin(\phi)\sin(\alpha) & -\cos(\phi)\sin(\alpha) \\
-\sin(\phi)\sin(\alpha) & \sin(2\phi)\cos(\alpha) + \sin^2(\phi)\tan(\gamma) & \cos(2\phi)\cos(\alpha) + \frac{1}{2}\sin(2\phi)\tan(\gamma) \\
-\cos(\phi)\sin(\alpha) & \cos(2\phi)\cos(\alpha) + \frac{1}{2}\sin(2\phi)\tan(\gamma) & -\sin(2\phi)\cos(\alpha) + \cos^2(\phi)\tan(\gamma)
\end{pmatrix}
\] (3.6)

The transformed Green’s strain tensor represents the strain along vectors oriented at arbitrary angles with respect to the shear deformation field. Designation of the angles of orientation in the \(x_3, x_2\) plane and in the \(x_1, x_2\) plane (\(\phi\) and \(\alpha\), respectively) is necessary to specify the strain along a vector of interest upon shear deformation. The normal strains along vectors at various orientations are \(E'_{11}\) (lateral strain), \(E'_{22}\) (axial strain), and \(E'_{33}\) (vertical strain), of which \(E'_{22}\) and \(E'_{33}\) are plotted for the maximum shear angle (\(\gamma = 45^\circ\)) used in this study (Figure 3.4.1). The lateral strain was equal to zero regardless of the strain tensor orientation, thus motion between points within the \(x_1, x_2\) plane maintains a fixed distance. Depending on the initial orientation, cellular components may undergo a wide range of axial and vertical strains causing both extension and compression.
Figure 3.4.1. Normal strain distribution for maximum shear ($\gamma = 45^\circ$).
Normal elements of the Green’s strain tensor as a function of the angles of orientation, $\phi$ and $\alpha$, between a vector of interest and the $x_3$, $x_2$ and $x_1$, $x_2$ plane, respectively. (A) $E'_{22}$ is the axial-normal strain and (B) $E'_{33}$ is the vertical-normal strain. Based on the convention defined in this analysis, the lateral-normal strain, $E'_{11}$, is zero independent of orientation.

The shear strains along vectors at various orientations, $E'_{12}$ (lateral-shear strain), $E'_{13}$ (vertical-shear strain), and $E'_{23}$ (axial-shear strain) are plotted at the maximum shear angle evaluated (Figure 3.4.2). Lateral and vertical-shear strains are non-zero only when the vector of interest is not parallel to the $x_3$, $x_2$ plane ($\alpha \neq 0$), and increase in magnitude as $\alpha$ increases. The deformation continuum that the cell-containing matrices undergo as the matrix is driven from an undeformed state ($\gamma = 0^\circ$) to a state of maximum deformation can result in: 1) continuous extension, 2) continuous compression, or 3) initial compression followed by extension. Furthermore, this cycle is reversed upon return to the initial, undeformed state.
Figure 3.4.2. Shear strain distribution for maximum shear ($\gamma = 45^\circ$). Shear elements of the Green’s strain tensor as a function of the angles of orientation, $\phi$ and $\alpha$, between a vector of interest and the $x_3,x_2$ and $x_1,x_2$ plane, respectively. (A) $E'_{12}$ is the lateral-shear strain, (B) $E'_{13}$ is the vertical-shear strain and (C) $E'_{23}$ is the axial-shear strain. Depending on orientation, vectors of interest may experience a range of combinations of normal and shear strain with varying magnitudes and signs (signifying compression or extension) during loading with the 3-D CSD. (D) A plot of $E'_{22}$, $E'_{33}$, and $E'_{23}$ when $\alpha = 0^\circ$ (all other strains are zero). The highest magnitude normal and shear strains occur at angles of orientation out of the horizontal plane.

This analysis demonstrates that the orientation of the principal axes changes with the magnitude of the shear deformation. The highest magnitude normal strains occur when

$$\phi = \frac{1}{2} \tan^{-1}\left[\frac{-2}{\tan(\gamma)}\right] \quad \alpha = 0$$

(3.7.1-3.7.2)
The solutions to Equation 7.1 yield the directions of principal normal strain in the initial coordinate system with the direction of maximum extension being located between 45° and 90°, and the direction of maximum compression being located between 0° and -45°. Within these ranges, as the shear angle increases, the directions of maximum extension and compression both increase (orthogonal principal normal axes rotate counterclockwise about the \( x_1 \) axis). Furthermore, the principal shear strains are located at axes 45° to the direction of the principal normal strains. These trends are depicted based on the shear strains utilized in this study (Table 3.1). This derivation was based on large deformation continuum mechanics and yielded results consistent with those previously attained for the orientation of the principal axis upon simple shear deformation (Ogden, 1984). Accordingly, with reference to the initial coordinate system, both the directions of principal shear strains and principal normal strains vary as functions of the shear angle.

**Table 3.1: Orientation of the principal axes depends on shear angle.** The bulk shear strain refers to the overall shear strain for the cell-containing matrices based on a given shear angle. The axes of principal normal and shear strain were derived based on position in the initial, undeformed coordinate system and the angles describe values of \( \phi \) (projected angle in the \( x_3,x_2 \) plane) when \( \alpha \) (projected angle in the \( x_1,x_2 \) plane) is zero. As the shear angle increases, the direction of maximum extension increases towards 90° while the angle of maximum compression moves towards 0°. The directions of principal shear strain are located at axes 45° to the directions of principal normal strain.

<table>
<thead>
<tr>
<th>Bulk shear strain</th>
<th>Shear angle ((\gamma))</th>
<th>Directions of principal normal strain</th>
<th>Directions of principal shear strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>26.6°</td>
<td>-38.0°, 52.0°</td>
<td>-83.0°, 7.0°</td>
</tr>
<tr>
<td>0.50</td>
<td>45.0°</td>
<td>-31.7°, 58.3°</td>
<td>-76.7°, 13.3°</td>
</tr>
</tbody>
</table>
This analysis suggests that the orientation for 3-D neural cells within a defined strain field may contribute to the heterogeneity of the strain at the cellular level. This heterogeneity (i.e. normal and shear strain combinations) cannot be readily reproduced in 2-D systems, and thus the 3-D CSD may provide an intermediate level of complexity between 2-D and in or ex vivo models.

**Acellular Characterization of Displacement**

Characterization of the microbead displacement throughout the acellular gel construct indicated that the beads followed a linear path regardless of the strain magnitude or strain rate (Figure 3.5). In order to obtain a complete set of images throughout the 3-D structure it was necessary to use a strain-and-hold method, rather than the short dwell time used for the cell experiments. The slopes of the lines were statistically the same for a given strain magnitude and therefore these conditions were combined. Displacements in the horizontal plane ($x_1,x_2$) were measured based on linear deformation, where vertical displacement ($x_3$ direction) was minimal (< 5 µm, which is within 2% of the maximum $x_2$ displacement) and therefore was assumed to be zero, validating the theoretical assumption. The bead displacement in the $x_2$ direction was dependent on the shear angle and the vertical position within the gel. Further regression analysis revealed that there was a difference between the measured paths and theoretical simple shear strain ($p < 0.01$), indicating that the shear strain field is complex and likely contains elements of both simple and pure shear. However, if there is an assumption of no-slip at the bottom plate (i.e., forcing the experimental $x_3$ intercept to zero) the slopes of the measured and theoretical shear strain profiles are statistically the same for the 0.25
strain cases ($p > 0.2$), indicating a better approximation for simple shear for the lower of the strain magnitudes tested.

![Microbead tracking in acellular matrix](image)

**Figure 3.5: Microbead tracking in acellular matrix.** Representative photomicrographs of a single plane (A) before deformation and (B) during deformation with the 3-D CSD (scale bar = 50 µm). Bead displacements across the thickness of the gel from the bottom to the top (vertical displacements of 0 µm and 500 µm, respectively) were measured at maximum deformation. Graphical representation of measured displacements for (C) 0.25 strain, all rate cases and (D) 0.50 strain, all rate cases. Measured bead displacements are presented as a mean value and SEM (data points with error bars and a best-fit solid line). The theoretical displacement is presented as a dashed line.
Neuron Viability and Neurite Orientation Following 3-D Shear Strain

Neurons subjected to high strains at two different rates (20 s\(^{-1}\) and 30 s\(^{-1}\)) were both injured more than static or naïve controls (p < 0.001) at 24 hrs post-deformation, demonstrating that this system is capable of injuring neural cells in a 3-D matrix (Figure 3.6.1). However, there was not a significant difference in the injury response between these two strain rates tested (Figure 3.6.2).

![Figure 3.6.1: Neuron viability and neurite distribution following deformation.](image)

Confocal reconstructions of 3-D neuronal cultures at 24 hrs post-loading or static control conditions. Live cells are stained green and the nuclei of dead cells are stained red for (A) a static control culture and (B) a culture loaded at 0.50 strain, 30 s\(^{-1}\) strain rate (scale bar = 50 µm).
Figure 3.6.2: Neuron viability following deformation. The percentage of viable neurons was quantified following loading or static control conditions. There was a significant decrease in neuronal viability versus static control for both loading regimes evaluated with neuronal cultures (*, p < 0.001). Error bars represent standard deviation.

The distribution of neurites within 3-D neuronal cultures was examined after high strain, high strain rate injury (0.50, 30 s$^{-1}$) in order to assess whether neurite orientation within the culture, and hence varying strain combinations, contributed to severity of cell injury. The distribution of viable neurites was compared to that of static control cultures as well as cultures subjected to an excitotoxic insult. The neurite density across all orientation angles was lower in mechanically injured cultures (72.6 ± 42.4) and excitotoxic cultures (131.0 ± 37.7) versus static control conditions (212.5 ± 92.3) (mean # neurites / mm$^2$ ± standard deviation) (Figure 3.7.1).
Figure 3.7.1: Neurite density following excitotoxic treatment or mechanical deformation. The density of viable neuronal processes was quantified and averaged over all orientation angles following static control, excitotoxic treatment or high rate deformation. There was a significant reduction in the neurite density following excitotoxicity treatment and high rate deformation compared to static controls (*, p < 0.05). This reduction in neurite density parallels the reduction in viability induced by these factors (see Figure 3.6.2 and APPENDIX E). Error bars represent standard deviation.

The percentage of neurites per orientation angle (corresponding to $\phi$, the projected angle in the $x_3,x_2$ plane) after mechanical loading or excitotoxic treatment was compared to the neurite distribution found in neurons subjected to static control conditions at corresponding orientation angles. After high strain, high strain rate injury there was a significant loss of neurites ($p < 0.05$) at orientation angles of –10, 0, 10, and 20 degrees (representing $\phi$), which is indicative of degeneration and subsequent cell death (Schwab and Bartholdi, 1996; Sievers, et al., 2003) (Figure 3.7.2). These angles bracket the range at which axial-shear strain ($E'_{23}$) is the highest (see Figure 3.4.2), suggesting that strain...
combinations are important to cellular response in a dynamic loading regime. However, no such preferential pattern of neurite loss was found following excitotoxic treatment. The percentage distribution of neurites over all orientation angles following excitotoxicity matched the distribution of neurites in static control cultures, indicating that neurite degeneration and cell death occur independent of cell orientation following a chemical insult. This pattern of preferential neurite loss following high rate deformation signifies the need to consider cell orientation as a factor for response to mechanical stimuli in 3-D configurations.

![Graph showing neurite distribution](image)

**Figure 3.7.2: Neurite distribution following excitotoxic treatment or mechanical deformation.** The distribution of viable neurites for static control cultures and cultures subjected to an excitotoxic insult (100µM glutamate treatment) were compared to the distribution of neurites remaining after loading at 0.50 strain, 30s⁻¹ strain rate. After excitotoxic treatment the distribution of remaining neurites was similar to that of control cultures indicating that there was not a preferential loss of neurites based on orientation for a chemical insult. However, after mechanical loading, there was a significant reduction in the percentage of total neurites at angles of orientation (φ, the projected neurite orientation in the x₃,x₂ plane) close to the horizontal plane (†, p < 0.05), correlating with orientations predicted to experience maximum axial-shear strain (E’₂₃). Error bars represent standard deviation.
Discussion

We have designed a device that is capable of mechanically deforming 3-D neural cell cultures and demonstrated its use as a quantifiable, reproducible model of \textit{in vitro} traumatic injury. A shear deformation was achieved through the parallel motion of the top plate of a cell chamber with respect to the bottom surface, thus uniformly deforming the 3-D cell culture. A linear shear strain field for several combinations of strain and strain rate was determined using microbead tracking, illustrating the ability of this system to simulate shear strain—the deformation pattern associated with diffuse brain injury (Holburn, 1943; Chu, et al., 1994; Donnelly and Medige, 1997). The extent of matrix deformation is controlled and therefore the cell response can be correlated to loading parameters. We showed that cell death in neurons was dependent on a combination of high strain and strain rate providing evidence that the mode of loading is important to the validation of cell injury systems.

Measuring the displacement of fluorescent microbeads revealed deviations from the simple shear assumption, with mean regression slopes of the measured paths consistently approximately 20% lower than the theoretical slopes. While an explanation of these differences may be explained by slight rotation of the culture (i.e. an underestimation of $\gamma$), vertical displacement of the beads was less than 2% of the $x_2$ displacement, indicating that the confinement of the system limits rotation of the culture. Strain patterns within the culture are complex and these results suggest that elements of both pure and simple shear may be present in this system. The strain profile at 0.25 strain was shown to be a better approximation of simple shear than the 0.50 case, suggesting that larger displacement angles may lead to deviation from the theoretical simple shear
strain. In addition, based on finite deformation theory, the application of traction forces at the end faces may be required for a true simple shear deformation. Because the Matrigel was contained in an elastic Sylgard mold, the motion was constrained and dictated by the overall displacement of the mold. The forces at the end faces, however, are governed by the attachment of the Sylgard mold to the Matrigel and, while the matrix is contained, during dynamic loading we cannot rule out the possibility that nonuniform forces result from the bulk deformation that may result in a variation from ideal simple shear. This characterization over a range of input parameters, however, served to demonstrate that the CSD consistently induces the prescribed deformation in a linear manner and that we can use a theoretical analysis to approximate the complex strain patterns within the 3-D culture.

We demonstrated that the 3-D CSD is capable of injuring primary cortical neurons in a 3-D configuration. This study correlates with previous studies on neural or neural-like cultures, in that high rate mechanical deformation results in cellular dysfunction and death (Ellis, et al., 1995; Cargill and Thibault, 1996; LaPlaca and Thibault, 1997; Morrison, et al., 1998). Although high rate loading of neurons resulted in significant cell death, there was no difference between the two rates tested, suggesting a rate threshold in the cellular response. Moreover, the 3-D CSD is capable of eliciting cellular responses under a range of loading conditions, thus allowing for the investigation of cellular thresholds.

In contrast to 2-D injury models, the current 3-D configuration may lead to increased heterogeneity of strains at the cellular level. A uniform distribution of cells within a largely isotropic 3-D matrix results in a corresponding distribution of neurite
orientations with respect to the horizontal plane, and therefore a wider range of local cellular strain for the same bulk shear deformation. The results of the theoretical strain analysis of this system show that cells with a 3-D orientation within the shear field experience alternate combinations of normal and shear deformation, and overall higher magnitudes of deformation than cells in a planar geometry. We demonstrated that these combinations of strain, and in particular high shear strain, may preferentially contribute to cell death based on a preferential loss of neurites at orientation angles receiving a maximal amount of axial-shear strain (compared to a non-preferential loss of neurites following an excitotoxic insult). The assumption was made that neurite orientation at the time of loading or static control conditions was represented by neurite orientation assessed 24 hrs later. While it is possible that some degree of network re-organization may occur following mechanical trauma or excitotoxicity, we do not observe any soma or neurite movement in this system at this time point, as may be the case in different cell types. Overall, the heterogeneity of the strain field as a function of cellular orientation may be further exploited to investigate the effect of various combinations of normal and shear strains on cellular response.

While 3-D cultures exposed to shear strain may have a non-uniform strain field at the cellular level, other features of the system may contribute to the response. For example, 3-D cultures may be more susceptible to secondary injury cascades than 2-D models due to the altered mass transport properties of the 3-D configuration. The high ratio of extracellular to intracellular volume in 2-D cultures and the sink-like property of the bathing medium may serve to dilute secreted or released molecules at the time of injury. Thus, a 3-D configuration with more physiologically relevant cell-cell
interactions (Gumbiner and Yamada, 1995; Schmeichel and Bissell, 2003) may provide a setting in which to investigate of the role of autocrine and paracrine signaling in response to mechanical trauma. In addition, different cell types may respond differently to the microenvironment and properties of the surrounding matrix (Flanagan, et al., 2002).

To our knowledge, this is the first report of a biomechanically-controlled traumatic injury model using 3-D cultures. This 3-D model produces cellular deformation in a more in vivo-like configuration than traditional monolayers, but still provides the benefit of the absence of systemic factors. The deformation rates and magnitudes that were used in this study are in the range of those that occur in inertial human head injuries (Gennarelli, et al., 1982; Margulies, et al., 1990; Margulies and Thibault, 1992). This model will aid in elucidating the complex biochemical and molecular cascades that occur after a traumatic insult and may be used to investigate a wide range of shear loading profiles in a variety of cell types. Biomechanically characterized in vitro models such as the 3-D CSD, used in combination with animal models and computer simulations, will lead to a better understanding of TBI and positively impact the development of treatment strategies.
CHAPTER IV

DIFFERENTIAL RESPONSE OF NEURONS CULTURED IN TWO-VERSUS THREE-DIMENSIONS TO HIGH RATE SHEAR DEFORMATION

Abstract

Many cellular models of traumatic brain injury (TBI) deform cells in a planar (2-D) configuration, a contrast from three-dimensional (3-D) brain cytoarchitecture, resulting in strain fields that may fail to represent the complex deformation patterns associated with neural injury in vivo. Cell culture models in 3-D may more accurately represent in vivo cellular behavior than planar models due to differences in cytostructure, cell-cell/cell-matrix interactions, intracellular signaling, and access to trophic factors; however, the effects of culture configuration on the response to high rate deformation have not been evaluated. We examined cell viability following a defined mechanical insult to primary cortical neurons plated in 3-D (distributed throughout a bioactive matrix) or 2-D (below a bioactive matrix). After high rate loading, (20 s\(^{-1}\) or 30 s\(^{-1}\), 0.50 strain), there was a significant decrease in neuron viability in both 3-D and 2-D; however, neurons in 3-D presented greater cell death based on matched bulk loading parameters. Computer simulations predicted local cellular strains experienced by neurons in 3-D or 2-D, revealing that neurons in 3-D were subjected to a heterogeneous strain field simultaneously consisting of tensile, compressive and shear strains; conversely, neurons in 2-D experienced a less complex, shear-dominated deformation regime. These results
show differential susceptibility to mechanical loading between neurons in 2-D and 3-D that may be due to differences in strain manifestation at the cellular level. Models of TBI that accurately represent the related cellular biomechanics and pathophysiology are important to develop mechanistically-driven intervention strategies and can therefore serve as valid pre-animal test beds.

**Introduction**

Traumatic brain injury (TBI) is caused by a mechanical insult to the head and may result in temporary or permanent brain dysfunction. After a mechanical insult to brain tissue, cells may undergo immediate death if structural thresholds are surpassed; however, secondary mechanisms initiate cellular alterations that can progress for weeks to months after the insult (Gennarelli, 1997; McIntosh, et al., 1998). As a result, functional impairment may be prolonged after the incident due to altered signaling cascades and the limited regenerative ability of the brain. Rapid acceleration/deceleration of the head (inertial loading) has been shown to produce tissue strain throughout the brain, and has been linked to such clinical manifestations as diffuse brain injuries (Margulies, et al., 1990; Margulies and Thibault, 1992; Gennarelli, 1993). Although previously reported *in vitro* models of TBI are able to recreate various aspects of primary and secondary damage in a controlled setting, such models have ranging physiological and biomechanical relevance to clinical head injury (reviewed in (Morrison, et al., 1998)). Various models utilize deformable membranes that are stretched biaxially (Ellis, et al., 1995; Cargill and Thibault, 1996; Geddes and Cargill, 2001) or uniaxially (Pfister, et al., 2003; Lusardi, et al., 2004) to transfer deformation to attached cells, some with the
capability of deforming neurites aligned longitudinally to the strain field (Galbraith, et al., 1993; Smith, et al., 1999). In such models, difficulty may exist in correlating membrane strain with individual cell strain due to variable cell adhesion to the deformable substrate. Furthermore, a commonality between these models of neural trauma is that they deform cells via tension; however, the physical properties of brain tissue make shear the dominant mode of deformation upon mechanical loading since brain tissue has a high compressive modulus and a low shear modulus (Holbourn, 1943; Sahay, et al., 1992). Devices utilizing shear deformation have been developed; for instance, hydrodynamic models utilizing fluid shear to deform cells (LaPlaca and Thibault, 1997; Nakayama, et al., 2001). The aforementioned models have primarily been utilized to deform cells in a planar (2-D) orientation, which may fail to simulate complex strain combinations; in fact, most utilize a single mode of deformation. Also, the skewed distribution and types of cell-cell/cell-matrix interactions found in 2-D cultures may affect pathological mechanotransduction mechanisms. Three-dimensional (3-D) models of neural trauma may better recapitulate the biomechanics of traumatic loading to the brain, which results in the generation of complex, heterogeneous strain fields at the tissue and cellular levels. In vitro models have been developed to evaluate the injury response in brain tissue slices – thus preserving a 3-D configuration (Morrison, et al., 1998; Sieg, et al., 1999; Adamchik, et al., 2000). However, in these models the ability to control cellular and extracellular-matrix constituents are limited, thus making the mechanistic elucidation of the roles of specific factors in the injury response difficult.

Correlating the extent of cell death and functional impairment with the mechanical parameters inducing brain injury is crucial to accurately elucidate cellular
tolerances and secondary responses. Models of neural trauma in 3-D may more faithfully recapitulate such secondary responses due to improved physiological relevance to the *in vivo* situation. Interpretation of cellular responses in 2-D models may be confounded by fundamental differences in terms of the cellular microenvironment (e.g., access to trophic factors), atypical cellular morphology (Balgude, et al., 2001; Grinnell, 2003), and altered cell-cell/cell-matrix interactions (Cukierman, et al., 2001; Cukierman, et al., 2002; Schmeichel and Bissell, 2003; Yamada, et al., 2003). These phenomena may have ramifications on growth and proliferation (Granet, et al., 1998), viability (Fawcett, et al., 1995), gene and protein expression (Masi, et al., 1992; Berthod, et al., 1993), and in the response to biochemical stimuli (Miller, et al., 1985); possibly limiting the expression of certain traits in 2-D cultures, thus deviating from *in vivo* systems. Overall, *in vitro* models of TBI utilizing neural cells in 3-D culture may be a more accurate representation of the *in vivo* situation based on enhanced physiological and biomechanical relevance while maintaining the experimental control of previous *in vitro* systems.

The goal of the current study was to evaluate the response of 2-D and 3-D neuronal cultures to a defined, high rate mechanical insult. Also, comparisons were made between neurons cultured in 2-D and 3-D to establish baseline parameters having potential ramifications on the injury response, such as culture viability, morphology, neuronal marker expression, neurite outgrowth and astroglial composition. Neuronal cultures were subjected to high rate deformation and alterations in cell viability were assessed as a function of loading parameters. Furthermore, simulations of representative cellular strain fields were generated to gain insight into the cellular biomechanics of high strain rate induced cell death for neurons cultured in 2-D versus 3-D.
Materials and Methods

Cortical Neuron Harvest and Dissociation

Procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. Neurons were obtained from timed-pregnant (embryonic day 17) Sasco Sprague-Dawley rats (Charles River, Wilmington, MA). Anesthetized dames were rapidly decapitated and the uterus was removed by Caesarian section and placed in Hanks Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA). Each fetus was removed from the amniotic sac, rapidly decapitated, and the brains removed. The cortices were isolated and placed in pre-warmed trypsin (0.25%) + 1 mM EDTA (Invitrogen) for 10 min at 37°C. The trypsin-EDTA was removed and the tissue was triturated HBSS + DNase I (0.15 mg/mL, Sigma, St. Louis, MO) using a flame-narrowed Pastuer pipet. The tissue was then centrifuged at 1000 rpm for 3 minutes and the cells were resuspended in a defined medium (Neurobasal medium + 2% B-27 + 500 μM L-glutamine (Invitrogen)).

2-D and 3-D Primary Cortical Neuronal Cultures

Cultures of 2-D and 3-D primary cortical neurons were plated in cell chambers designed to interface with our custom-built injury device. Prior to plating, chambers were pre-treated with 0.05 mg/mL poly-L-lysine (PLL, Sigma) followed by Matrigel (0.5 mL/well at 0.6 mg/mL, Becton Dickinson Biosciences; Bedford, MA) in Neurobasal medium (each treatment was > 4 hours). Neuronal cultures in 3-D were plated within Matrigel (final protein concentration of 7.5 mg/mL) at a cell density of 3750-5000 cells/mm³ within a 500-750 μm thick matrix. Cultures were placed at 37°C to permit
matrix gelation and 3-D cell entrapment, after which 0.5 mL of warm medium was added. For 2-D neuronal cultures, cells were plated at 1250-2500 cells/mm² in 0.5 mL medium. After allowing for neuronal adhesion, Matrigel (7.5 mg/mL) was placed above the 2-D cultures to match the amount of matrix used in the 3-D system (Figure 4.1). After matrix gelation, 0.5 mL warm medium was added per culture. Cultures were maintained at 37°C and 5% CO₂-95% humidified air and fed at 24 hours post-plating and every 2-3 days thereafter. All experiments were performed at 7-8 days in vitro (DIV).

**NEURONAL CELL CULTURES**

**MECHANICAL DEFORMATION**

![Schematic representation of neuronal culture and mechanical deformation models](image)

**Figure 4.1:** Schematic representation of the neuronal culture (left) and mechanical deformation (right) models (not to scale). Neuronal cultures in 2-D and 3-D were plated above a layer of acellular matrix and were laterally constrained by an elastomer mold. Neuronal cultures in 3-D were homogeneously dispersed throughout a bioactive matrix, whereas neuronal cultures in 2-D were plated and, after allowing for cell adhesion, were covered with additional bioactive matrix. Mechanical deformation is imparted to cell-containing matrices through the action of the 3-D Cell Shearing Device (3-D CSD), a custom-built electromechanical device. A closed-loop control system (PID controller with positional feedback from a DVRT) governs a linear actuator, causing horizontal displacement of the cell chamber top-plate inducing shear deformation in the elastomer mold and cell-imbedded matrices.
Morphologic Analysis: Scanning Electron Microscopy

Cell morphology and the spatial distribution of cell-cell and cell-matrix interactions were qualitatively assessed through low voltage, high resolution scanning electron microscopy (LVHR-SEM). Neuronal cultures in 2-D and 3-D were plated on pre-treated silicon wafers and were fixed using 2.5% EM-grade glutaraldehyde in 0.1M cacodylate buffer at 4°C for 24 hours to provide proteinaceous cross-linking. Post-fixation of phospholipid moieties was accomplished using 1% OsO₄ in 0.1M cacodylate buffer for 1 hour followed by dehydration using a graded series of ethanol. Critical point drying was done with CO₂ followed by sputter coating with chromium (~3 nm).

Cell Viability Using Fluorescent Staining

Neuron viability was assessed using fluorescent probes for distinguishing live and dead cells. Cell cultures were incubated with 4 µM ethidium homodimer-1 (EthD-1) and 2 µM calcein AM (both from Molecular Probes, Eugene, OR) at 37°C for 30 min and then rinsed with 0.1M Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen). The percentage of viable cells was calculated by counting the number of live cells (fluorescing green by AM-cleavage) and the number of cells with compromised membranes (nuclei fluorescing red by EthD-1).

Phenotypic Identification Using Immunocytochemistry

Neuronal cultures were immunostained using primary antibodies recognizing the following intracellular proteins: 1) tau-5 (MS247P, 1:200, NeoMarkers, Fremont, CA), a microtubule-associated protein expressed predominantly in maturing neurons (Binder, et al., 1985; Migheli, et al., 1988; Nunez, 1988; Goedert, et al., 1991) or 2) glial fibrillary acidic protein (GFAP) (AB5804, 1:400; MAB360, 1:400, Chemicon, Temecula, CA), an
intermediate filament found in astrocytes (Debus, et al., 1983). Briefly, cells were fixed
in 3.7% formaldehyde (Fisher, Fairlawn, NJ) for 30 minutes, rinsed in PBS and
permeabilized using 0.3% Triton X100 (Kodak, Rochester, NY) + 4% goat serum
(Invitrogen) for 20 minutes. Primary antibodies were added (in PBS + 4% serum) at 18-
24°C for 4 hours. After rinsing, the appropriate secondary fluorophore-conjugated
antibodies (FITC or TRITC-conjugated IgG, Jackson Immuno Research, West Grove, PA
or Alexa 488 or 546-conjugated IgG, Molecular Probes) were added (in PBS + 4%
serum) at 18-24°C for 2 hours. Hoechst 33258 (1:1000, Molecular Probes) was used as a
counterstain. The percentage of neurons in culture was assessed by determining the
numbers of tau⁺ cells versus the total number of cells (n = 4 in 3-D and n = 8 in 2-D).
Similarly, the percentage of astrocytes was assessed by quantifying the number of GFAP⁺
cells (n = 6 in 2-D and 5 in 3-D).

**Protein Expression by Western Blotting**

The amount of neurite outgrowth between neurons cultured in 2-D and 3-D was
evaluated based on the expression of neuron-specific microtubule-associated proteins
(MAPs), tau-5 (n = 3 each) and MAP-2 (n = 3 each). Neuronal cultures in 2-D and 3-D
were treated with lysis buffer to extract cellular proteins which were then separated using
SDS-PAGE. Primary antibodies specific for tau-5 (MS247P, 1:200, NeoMarkers) and
MAP-2 (AB5622; 1:200; Chemicon) with appropriate secondary antibodies were used to
assess the relative expression of those proteins.

**Mechanical Loading Using the 3-D Cell Shearing Device**

Neuronal cultures in 2-D and 3-D were mechanically loaded using the 3-D Cell
Shearing Device (CSD), a custom-built electromechanical device capable of quantifiably
imparting high strain rate shear deformation to 3-D cell-containing matrices (LaPlaca, et al., 2005). At the time of injury, cultures were removed from the incubator and mounted within the 3-D CSD. The mechanical action of the device was driven by a linear-actuator (BEI Kimco; San Marcos, CA) governed by a proportional-integral-derivative (PID) (Feedback Inc.; Hillsborough, NC) controller with closed-loop motion control feedback from a differential variable reluctance transducer (DVRT, Microstrain; Burlington, VT) (Figure 4.1). A trapezoidal input was provided by custom code written in LabVIEW® (National Instruments; Austin, TX) software. 2-D neuronal cultures were deformed (strain 0.50, strain rate 20 s\(^{-1}\), \(n = 16\); strain 0.50, strain rate 30 s\(^{-1}\), \(n = 11\)) or placed into the device with the top plate (static control, \(n = 14\)). 3-D neuronal cultures were deformed (strain 0.50, strain rate 20 s\(^{-1}\), \(n = 12\); strain 0.50, strain rate 30 s\(^{-1}\), \(n = 17\)) or placed into the device with the top plate (static control, \(n = 17\)). After mechanical deformation, warm medium was added and the cultures were returned to the incubator. Viability was also assessed in naïve control cultures in 2-D (\(n = 11\)) and 3-D (\(n = 11\)).

**Simulations of Local Cellular Strain Using Matlab®**

A kinematic analysis of the strain field generated by the 3-D CSD was previously performed to relate the bulk deformation of the matrix to the local cellular strain dependent on soma/neurite orientation within the matrix (see CHAPTER III and (LaPlaca, et al., 2005)) This work is extended here to theoretically compare the local cellular strain fields experienced by neurons in 2-D versus 3-D. The orientation of a soma/neurite within the matrix was defined based on a fixed Cartesian reference frame as the initial angles projected into the \(x_3,x_2\) plane and in the \(x_1,x_2\) plane (denoted as \(\phi \in [-90^\circ, 90^\circ]\) and \(\alpha \in [-90^\circ, 90^\circ]\), respectively) (Figure 4.2).
Figure 4.2. Description of cell orientation used in theoretical strain analysis. The orientation of a neuron was described based on the departure angle from horizontal (defined as $\phi$, a projection into the $x_3, x_2$ plane) and the angle from vertical (defined as $\alpha$, a projection into the $x_1, x_2$ plane). The shear deformation of the cell-containing matrices (proportional to the angle $\gamma$) occurs in the $x_3, x_2$ plane. The Green’s strain tensor describing the bulk deformation of the matrix was translated to a local cellular strain using a series of rotational transformations. This methodology permits the determination of the components of the Green’s strain tensor specific for a soma/neurite at any orientation with respect to the strain field induced by the 3-D CSD.

Using high resolution confocal microscopy, the angles of orientation describing the departure trajectory of neurites from the soma were measured from neurons in 2-D and 3-D ($n = 12$ neurons each from 4 cultures, 1-4 neurites per neuron). The local normal and shear strains for neurites at discrete orientation angles within the strain field generated by the 3-D CSD were then calculated.

Data Collection and Statistical Analysis

After viability and immunocytochemistry assays, cells were viewed using fluorescent microscopy techniques on an epifluorescent microscope (Eclipse TE300, Nikon, Melville, NY) or a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). For epifluorescent microscopy, images were digitally captured.
(DKC5T5/DMC, Sony, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Confocal images were acquired across the full thickness of the cultures and were analyzed using LSM Image Browser (Zeiss). Three to five randomly selected regions per culture were counted for statistical analysis. The results from Western blots were digitally imaged (FLA-3000, FujiFilm, Tokyo, Japan) and analyzed using Image Reader (FujiFilm). Data are presented as mean ± standard deviation. General linear model ANOVA was performed followed by Tukey’s pairwise comparisons (p-value < 0.05 was considered significant).

Results

Culture Characterization

Neuronal cultures in 2-D and 3-D were evaluated based on viability, morphology, neurite outgrowth, neuronal marker expression and glial presence since baseline culture parameters have potential ramifications on the neuronal response to high rate deformation. Cells in 3-D extended numerous processes at all orientations while cells in 2-D remained nearly planar, as expected. The viability in 2-D and 3-D neuronal cultures was ~90%, with no statistical difference between the culture configurations (Figure 4.3). In 3-D, there was not a significant difference in viability based on the z-position in a culture, demonstrating consistent cell behavior throughout the culture thickness.
Figure 4.3: Baseline viability in 2-D and 3-D neuronal cultures. Fluorescent photomicrographs of representative neuronal cultures plated (A) in 2-D between layers of matrix or (B) in 3-D dispersed throughout matrix. Cells were stained to discriminate live cells (green) and the nuclei of dead cells (red). There was not a significant difference between neuronal viability in 2-D and 3-D. Scale bar = 20 µm.
Confocal and scanning electron microscopy revealed morphological and cytostructural differences between neurons distributed throughout a matrix compared to neurons in planar culture, although the latter technique required the assessment of traditional 2-D cultures (i.e., not buried beneath a bioactive matrix). Neurons in 3-D present a bulbous morphology with neurites departing the soma in all spatial dimensions, whereas neurons in 2-D culture present a restricted cytostructure with neurites departing the soma mainly in-plane (Figure 4.4).

Figure 4.4: Electronmicrographs of neuronal cultures in 2-D and 3-D. Scanning electron microscopy of traditional 2-D neuronal cultures (i.e., not buried beneath a bioactive matrix) and 3-D neuronal cultures. (A) Neurons in 2-D present a flattened morphology while (B) neurons in 3-D present a rounded morphology.
Immunocytochemistry revealed a consistent percentage of cells staining positive for a maturing isoform of the neuronal cytoskeletal tau-5 in 2-D and 3-D (~90% each), and the astrocytic presence was virtually identical (~3% each) in these two culture configurations (Figure 4.5.1-4.5.2).

Figure 4.5.1: Neuronal cultures in 2-D and 3-D were immunolabeled to identify neurons and astrocytes. Fluorescent confocal reconstructions of representative neuronal cultures in (A) 2-D or (B) 3-D immunolabeled for tau-5 (green) to denote neurons with Hoechst counterstaining (blue) to denote nuclei. The astrocytic presence was similarly determined by immunolabeling for GFAP (images not shown). Scale bars = 10µm.
Figure 4.5.2: Neuronal cultures in 2-D and 3-D were immunolabeled to identify neurons and astrocytes. Fluorescent confocal reconstructions of neuronal cultures in 2-D or 3-D were immunolabeled for tau-5 to denote neurons or GFAP to denote astrocytes with nuclear counterstaining. The neuronal and astrocytic presence in 2-D and 3-D was quantified. There was not a significant difference between the percentage of neurons or astrocytes in 2-D and 3-D.

The relative expression of the microtubule-associated proteins tau-5 and MAP-2 was used to approximate the amount of neurite outgrowth in the two culture configurations. Although there was no statistical difference in MAP-2 expression between neurons in the two configurations, there was a 1.9-fold increase (p < 0.05) in the amount of tau-5 expressed by neurons cultured in 3-D compared to 2-D (Figure 4.6). This suggests that a 3-D configuration may result in an increase in neurite (axonal) outgrowth or an enhanced state of neuronal maturation compared to a 2-D configuration.
Figure 4.6: The expression of neuron-specific cytoskeletal proteins was determined in 2-D and 3-D. The expression of the cytoskeletal protein tau-5 was assessed to evaluate relative amounts of neurite outgrowth based on culture configuration. The relative blot intensity was quantified for tau-5 in 2-D and 3-D. There was a 1.9-fold increase in the expression of tau-5 in 3-D compared to 2-D (p < 0.05).

Altogether, these results demonstrate alterations in neuronal cytoarchitecture and neurite outgrowth in 2-D versus 3-D cultures; however, both configurations produce viable cultures with similar neuronal and astroglial compositions. A more detailed evaluation of neurons cultured in 2-D versus 3-D over various stages of culture development has been performed (see CHAPTER II).
Response to High Rate Deformation

A standard live/dead assay was performed in conjunction with confocal microscopy in order to assess post-injury cell viability as a function of culture configuration (Figure 4.7.1).

Figure 4.7.1: Post-injury cell viability in networks of neurons cultured in 2-D and 3-D. Cells were subjected to high strain rate and magnitude shear deformation using the 3-D CSD. Viability was assessed 24 hours after the application of control or deformed conditions. Neuronal cultures in 2-D after (A) static control or (B) 0.50 strain, 30 s$^{-1}$ strain rate. Neuronal cultures in 3-D after (C) static control or (D) 0.50 strain, 30 s$^{-1}$ strain rate. Scale bar = 50 μm.
Cell viability was found to significantly depend on culture configuration (2-D versus 3-D, p < 0.05), injury level (p < 0.001), and interactions between the two variables (p < 0.05), indicating a synergistic dependence between culture configuration and injury level (Figure 4.7.2).

![Graphical representation of cell viability assessed 24 hours after the application of control or deformed conditions. There was not a significant difference in viability in naïve controls compared to static controls for either 2-D or 3-D; however, there was a significant decrease in both 2-D and 3-D viability after high rate deformation. *Indicates a significant decrease in viability in 2-D versus 3-D under matched loading conditions (p < 0.05). †Indicates a significant decrease in viability versus uninjured controls (p < 0.05).]

**Figure 4.7.2: Post-injury cell viability in networks of neurons cultured in 2-D and 3-D.** Cells were subjected to high strain rate and magnitude shear deformation using the 3-D CSD. Graphical representation of cell viability assessed 24 hours after the application of control or deformed conditions. There was not a significant difference in viability in naïve controls compared to static controls for either 2-D or 3-D; however, there was a significant decrease in both 2-D and 3-D viability after high rate deformation. *Indicates a significant decrease in viability in 2-D versus 3-D under matched loading conditions (p < 0.05). †Indicates a significant decrease in viability versus uninjured controls (p < 0.05).

There was not a statistically significant difference between the static and naïve controls in either 2-D or 3-D cultures. For the 2-D cultures, there was a significant decrease in cell viability versus the static control cultures for the 20 s⁻¹, 0.50 strain injury...
and 30 s\(^{-1}\), 0.50 strain injury (p < 0.05). Similarly, there was a significant difference in percentage of viable cells under both loading conditions for the injured 3-D cultures versus the static control 3-D cultures (p < 0.001) (Figure 4.7.2). There was not a strain rate dependence on viability for the strain rates evaluated in either the 2-D or the 3-D cultures. However, there was a statistically significant decrease in cell viability based on matched loading conditions between the 2-D compared to 3-D cultures, signifying that culture configuration affects the response to high rate deformation.

**Simulations of Local Cellular Strain**

A kinematic analysis of the strain field generated by the 3-D CSD was performed to theoretically compare the local cellular strains experienced by neurons in a 2-D versus 3-D configuration based on cellular orientation with respect to the bulk deformation of the matrix. The Green’s strain tensor for the case of simple shear deformation describing the bulk culture deformation is given by

\[
E_{ij} = \frac{1}{2} \begin{pmatrix}
0 & 0 & 0 \\
0 & 0 & \tan(\gamma) \\
0 & \tan(\gamma) & \tan^2(\gamma)
\end{pmatrix}
\] (4.1)

where \(\gamma\) is the angle of shear deformation (Figure 4.2). The bulk strain tensor was transformed to a local strain tensor aligned with a vector of interest representing the orientation of a neurite (described by \(\phi\) and \(\alpha\)) within the matrix based on the transformation matrix \([R]\).

\[
[E'] = [R][E][R]^T
\] (4.2)
The transformation matrix was derived such that the strain tensor was aligned longitudinally with a neurite. The strain tensor as a function of initial neurite orientation within the matrix is described by

$$
\begin{bmatrix}
0 & \sin(\phi)\sin(\alpha) & -\cos(\phi)\sin(\alpha) \\
-\sin(\phi)\sin(\alpha) & \sin(2\phi)\cos(\alpha) + \sin^2(\phi)\tan(\gamma) & \cos(2\phi)\cos(\alpha) + \frac{1}{2}\sin(2\phi)\tan(\gamma) \\
-\cos(\phi)\sin(\alpha) & \cos(2\phi)\cos(\alpha) + \frac{1}{2}\sin(2\phi)\tan(\gamma) & -\sin(2\phi)\cos(\alpha) + \cos^2(\phi)\tan(\gamma)
\end{bmatrix}
$$  \hspace{1cm} (4.3)

The local normal strains are the longitudinally-aligned axial strain, $E'_{22}$, and the radially-aligned vertical strain, $E'_{33}$; the local shear strains are $E'_{12}$ (lateral-shear strain), $E'_{13}$ (vertical-shear strain), and $E'_{23}$ (axial-shear strain). Based on initial orientation, neurites may experience a range of axial and vertical strains resulting in both extension and compression (Figure 4.8).
Figure 4.8: Demonstration of potential strain fields experienced by cells in 2-D and 3-D. (A) Conceptual rendition of the local cellular strain aligned with a neuron. (B) Range of theoretical local cellular strains as a function of neurite departure angle from horizontal ($\phi$ only, $\alpha$ equal to 0° for conceptual simplification). Neurons in 3-D, with departure angles from the horizontal ranging from -90° to 90°, experience a range of potential strain fields consisting of normal (tensile or compressive) and shear strains. However, neurons in 2-D were found to have departure angles from the horizontal contained from -10° to 10°, causing these neurons to experience a more homogeneous, shear-dominated strain field.

In a 3-D configuration, neurites were arranged at all possible orientation angles ($\phi \in [-90^\circ, 90^\circ]$ and $\alpha \in [-90^\circ, 90^\circ]$), whereas in 2-D neurite departure angles were found to be more restricted ($\phi \in [-10^\circ, 10^\circ]$). As expected, there were no differences in the neurite departure trajectories in the $x_1, x_2$ plane (i.e., $\alpha$) leading to a broad range of
lateral-shear and vertical-shear stains with no significant differences in 2-D versus 3-D. Axial-shear strains were found to be consistently large for neurites in the 2-D configuration (0.33 ± 0.10), but were more variable for neurites in 3-D (0.10 ± 0.29). Normal strains followed similar trends, with higher variability in the longitudinally-aligned axial strain and the radially-aligned vertical strain for neurites in 3-D; in fact, axial strains were close to zero for the majority of the neurites in 2-D. Thus, for neurites in 3-D, arbitrary combinational strain fields may be experienced, consisting of normal-dominated strain fields (i.e. extension and compression) or shear-dominated strain fields, at times within the same neuron (Figure 4.9.1).
3-D Neurons

<table>
<thead>
<tr>
<th></th>
<th>φ</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>-28.4°</td>
<td>19.5°</td>
</tr>
<tr>
<td>b</td>
<td>45.0°</td>
<td>-44.1°</td>
</tr>
<tr>
<td>c</td>
<td>9.5°</td>
<td>53.8°</td>
</tr>
</tbody>
</table>

![top view](image)

side view (rotated 30° from horizontal)

**Figure 4.9.1: Results of local cellular strain simulations for neurons in 3-D.**
Representative local cellular strain fields for neurons in 3-D. Neurites projecting from a neuron in 3-D culture were theoretically subjected to variable strains, consisting of (a) compression-dominated, (b) extension-dominated, or (c) shear-dominated strain fields. Neurons in 3-D experience more heterogeneous local cellular strains than neurons in 2-D, possibly causing a more detrimental outcome after high rate deformation for neurons in a 3-D configuration.

Conversely, strain fields experienced by cells in 2-D were more homogeneous and consisted primarily of shear strains (Figure 4.9.2). Furthermore, considering dynamic loading conditions as the matrix is driven from an undeformed state (γ = 0°) to a state of maximum deformation (γ = 45°), the strain continuum of a neurite may result in: 1) continuous extension, 2) continuous compression, or 3) initial compression followed by extension, but such strain fields were only possible for neurites in a 3-D configuration. It may be the simultaneous application of complex strain fields, consisting of significant
normal and shear elements, which results in maximal neuronal damage, and such combinational strain fields were found primarily in a 3-D configuration.

2-D Neurons

<table>
<thead>
<tr>
<th></th>
<th>ϕ</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>d</td>
<td>1.0°</td>
<td>59.7°</td>
</tr>
<tr>
<td>e</td>
<td>0.6°</td>
<td>-29.4°</td>
</tr>
<tr>
<td>f</td>
<td>-2.8°</td>
<td>21.0°</td>
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</tbody>
</table>

Figure 4.9.2: Results of local cellular strain simulations for neurons in 2-D. Local cellular strain fields for representative neurons in 2-D. Neurites in 2-D were theoretically subjected to a more homogeneous strain regime, (d, e, f) found to be shear-dominated with relatively low axial strains.
Discussion

A novel model of TBI has been developed to subject 2-D or 3-D cell cultures to high strain rate deformation to allow for direct comparison based on culture configuration. Prior to evaluating the cellular response to mechanical loading, neuronal cultures in 2-D and 3-D were characterized to establish a baseline in culture parameters as fundamental differences existing between these culture models — independent of mechanical stimulation — may contribute to the injury response. There were no significant differences in culture viability or in the percentage of neurons and astrocytes based on culture configuration; however, there were cytostructural differences and an increase in neurite outgrowth per neuron in the 3-D versus 2-D configuration. After high magnitude, high rate loading, there was a significant decrease in viability for neurons cultured in both 2-D and 3-D; however, there was a significant decrease in neuronal viability in 3-D compared to 2-D under matched loading parameters. Simulations to predict the local cellular strains for representative cells in 2-D and 3-D determined that neurons in 3-D experience a heterogeneous strain regime simultaneously consisting of normal and shear strains; conversely, neurons in 2-D experience a more homogenous strain regime dominated by a single mode of deformation, namely, shear strain. This in vitro model of TBI is capable of incorporating a heterogeneous strain regime, representative of the in vivo situation, while controlling the culture configuration, matrix constituents, and cellular composition – thus presenting a degree of experimental control that may be further exploited for the mechanistic elucidation of the contribution of the aforementioned parameters in the injury response.
Factors affecting the translation of forces from bulk (macro) deformation to cellular (micro) deformation may differ markedly in 3-D versus 2-D configurations. As demonstrated by this study, the neuronal orientation within the bulk strain field, and hence the local cellular strain, influences the post-injury viability of neuronal cultures. However, other factors influencing the related biomechanics should be noted, such as cell morphology, matrix mechanical properties, and cell-matrix/cell-cell interactions which may play differential roles in the transfer of strain to the cells, and/or differential susceptibility to localized stress formations. This study and others have noted variations in cellular morphology and cytostructure between cells grown in 3-D versus 2-D environments (Balgude, et al., 2001; Grinnell, 2003). Such variations in cytoarchitecture may contribute to a differential response to mechanical loading as the neuronal network in 2-D was contained within a region parallel to the direction of the bulk deformation. Also, the bioactivity and physical properties of a matrix material may affect the transfer of bulk deformation to cellular deformation. Properties such as viscoelasticity (i.e., viscous and elastic moduli) and porosity may affect the fidelity of the deformation transfer to cells contained within a 3-D matrix, and such properties may be tailored to approximate that of brain tissue (Shuck and Advani, 1972; Donnelly and Medige, 1997; Darvish and Crandall, 2001). The quantity and spatial distribution of cell-matrix and cell-cell interactions may also affect the magnitude and locations of stresses developed on and within cells. Differences in the types, quantity, and distribution of cell-cell and cell-matrix interactions between cells in 2-D versus 3-D have previously been characterized for various cell types (Cukierman, et al., 2001; Schmeichel and Bissell, 2003), supporting the assertion that neurons in a 3-D bioactive matrix will contact matrix factors and
experience cell-cell interactions (e.g., receptor-mediated, synaptic) in all spatial directions. Cells in traditional 2-D configurations (i.e., not exposed to a bioactive matrix) may experience such interactions in a more limited or one-sided fashion. Receptor-ligand adhesions may serve as areas of stress concentrations where cellular deformation is constrained (intra-membrane binding proteins may not shift to accommodate deformation), creating regions that are more likely to fail during loading. Such cell-matrix and cell-cell interactions will be a function of the receptor density, the relative clustering of those receptors, the strength of binding, and the surface area of the cells. For neurons in a 3-D configuration, the ~2-fold increase in neurite outgrowth may result in an enhanced contribution of these adhesions on the response to high rate deformation. The effects of cellular orientation, cytoarchitecture, matrix properties, and cell-matrix/cell-cell interactions likely work in concert to create the strains and stresses realized at the cellular level, and these factors may contribute in different proportions in 2-D versus 3-D cell deformation.

This injury model adds to the repertoire of TBI models as the first capable of generating a controlled, homogeneous deformation to cell-containing matrices where local strain heterogeneity is predictably realized based on the neurite/soma orientation with respect to the overall strain field (LaPlaca, et al., 2005). Although stretch models employing brain slices will achieve such heterogeneity in cellular strain (Morrison, et al., 1998), the overall deformation is inherently inhomogeneous due to unconstrained boundary conditions at the top surface, confounding predictions of local cellular strain. In vitro models employing planar cultures are valuable in studying the effects of 1-D and 2-D strain fields on neural cultures; however, the ramifications of such simplified strain
fields on the acute injury response must be taken into account. For example, non-specific plasma membrane permeability alterations, a generally accepted mechanism of cell injury (Pettus, et al., 1994; Pettus and Povlishock, 1996; Singleton and Povlishock, 2004), has been demonstrated in vitro when subjecting neurons to biaxial strain regimes (Ellis, et al., 1995; LaPlaca, et al., 1997; Geddes, et al., 2003; Schiffman, et al., 2004) but only after axotomy in a uniaxial strain field (Smith, et al., 1999; Wolf, et al., 2001). However, uniaxial stretch models may exploit the unique ability to separate specific modes of altered intracellular ionic homeostasis (e.g., ion channels) from nonspecific modes (e.g., mechanoporation). Also, in 2-D stretch models cell strain may be inherently lower than membrane strain, and variable dependent upon cell adhesion and morphology (Winston, et al., 1989; Barbee, et al., 1994). Thus, relative levels of cell death or measures of dysfunction between neural cell types may be due to differences in the transfer of substrate strain to cell strain, rather than accurately representing differential loading thresholds (e.g., cerebral cortical neurons versus hippocampal neurons (Geddes, et al., 2003) and neurons versus astrocytes (Ahmed, et al., 2000)).

The 3-D CSD is able to deliver a bulk shear strain to 3-D cell-containing matrices, the deformation pattern associated with diffuse brain injury, and elicit significant cell death at levels that are relevant to the in vivo injury situation (Gennarelli, et al., 1982; Margulies, et al., 1990; Margulies and Thibault, 1992). This model may produce a more accurate representation for cell death/dysfunction thresholds than previous 2-D in vitro models of TBI due to an in vivo-like cell configuration, the ability to precisely control the rate and magnitude of the bulk matrix deformation, and cell deformation being constrained by the overall matrix deformation. Bulk tissue strains resulting in diffuse
Axonal injury (DAI) was approximated to be shear strains of 0.10-0.50 at onset-times corresponding to approximate strain rates of 10-50s$^{-1}$ (Margulies and Thibault, 1989; Margulies, et al., 1990; Meaney, et al., 1995). In some cases, neural cellular tolerances to mechanical loading in 2-D trauma models must be interpreted with caution as model-specific phenomena may result in variable development of stresses and strains, necessitating mechanical inputs far greater than those correlating with in vivo injury. For instance, some uniaxial stretch models have required strains $>0.50$ or rates up to 90s$^{-1}$ to elicit axonal pathology (Thibault and Gennarelli, 1989; Smith, et al., 1999; Pfister, et al., 2003). The rates and magnitudes used in this study were chosen to correspond to those of severe inertial injury, and the degree of cell death in our model at these injury levels indicates a correlation with diffuse brain injury thresholds. Given the results of this study suggesting that local cellular strain may influence the response to bulk mechanical inputs, coupled with the well-documented dependence on strain rate and strain magnitude (Cargill and Thibault, 1996; LaPlaca, et al., 1997; Geddes and Cargill, 2001; Geddes, et al., 2003), it is reasonable to propose that heterogeneity in the neuronal response to mechanical inputs may be based on variations in local stress formations. Therefore, future derivations of neuronal tolerances to mechanical loading should focus on predictions of local cellular stress, which will have contributions based on cell orientation with respect to the overall strain field, cell and matrix viscoelastic properties, morphology/cytostructure, and cell-cell/cell-matrix adhesions, among potentially other factors.

In vitro models of neural trauma in 3-D may more accurately recapitulate secondary responses than 2-D cultures due to fundamental differences in the cellular
Injuries to the brain are among the most likely traumatic insults to result in death or permanent disability and few, if any, clinically effective treatments exist (Roberts, et al., 1998) – facts that substantiate the need for improved laboratory models of TBI. Models employing 3-D neural cell cultures subjected to high rate, heterogeneous strain fields may more accurately represent the response of in vivo cells to a mechanical insult including not only acute cell death, but also secondary mechanisms of cell dysfunction/death. Such models may reveal cellular thresholds to loading with accuracy not possible from 2-D models of neural trauma. Also, as the baseline physiological states for cells in 3-D more closely approximates the in vivo situation, 3-D models of neural trauma may more faithfully represent the effects of pharmacological intervention and thus serve as valid pre-animal test beds for potential treatments for TBI. This model may be further exploited as more sophisticated 3-D neural cell cultures are developed (e.g.,

microenvironment (e.g., access to trophic factors) and receptor-mediated signaling (Cukierman, et al., 2002; Schmeichel and Bissell, 2003; Yamada, et al., 2003), potentially impacting growth and proliferation, survival, and gene and protein expression (Masi, et al., 1992; Berthod, et al., 1993; Fawcett, et al., 1995; Granet, et al., 1998). In traditional 2-D cultures, the sink-like property of the bathing medium may serve to dilute secreted or released molecules at the time of injury; conversely, 2-D cultures have demonstrated an increase in sensitivity to chemical treatments independent of changes in surface area (Miller, et al., 1985), possibly making 2-D models less suitable for studies evaluating pharmacological intervention after neural trauma. Thus, a 3-D configuration with a more physiologically relevant microenvironment may provide a more useful setting in which to investigate secondary cellular responses to a traumatic insult.
multi-cellular composition, matrix constituents more representative of those in the central nervous system) which may serve as a more accurate guide to cellular damage/death thresholds while better recapitulating post-injury alterations in cell biochemistry and secondary death mechanisms.
CHAPTER V

MECHANICAL INDUCTION OF CELL DEATH AND REACTIVE ASTROGLIOSIS IN THREE-DIMENSIONAL NEURONAL-ASTROCYTIC CO-CULTURES

Abstract

A mechanical insult to the CNS can drastically alter the post-injury microenvironment. Specific cell types become reactive after a perturbation to neural homeostasis and coalesce into the glial scar—a physical and chemical barrier sequestering acutely injured tissue. Although elements of this reactive environment have been characterized, much of the in vitro work elucidating mechanisms of astrogliotic induction have been performed using two-dimensional (2-D) models which may fail to recapitulate certain induction mechanisms, such as extracellular-matrix-bound factors and elements of autocrine/paracrine signaling. The goal of this study was to evaluate reactive astrogliosis using a three-dimensional (3-D) in vitro model for a range of mechanical loading regimes to assess the effects of a direct mechanical insult and local cell death on astrogliotic induction. Neuron-astrocyte co-cultures were plated in 3-D within a bioactive matrix (1:1 initial neuron:astrocyte ratio). At 21 days post-plating, these cultures were subjected to mechanical loading (0.50 strain at 1, 10, or 30 s\(^{-1}\) strain rate), TGF-\(\beta\) treatment (an astrogliosis-inducing factor) or control conditions. At 48 hrs and 5 days post-insult, cultures were characterized by evaluating cell viability and specific
markers of reactive astrogliosis. At 48 hrs post-insult, there was a significant increase in cell death after high rate (10 and 30 s\(^{-1}\)) loading, but not after quasi-static (1 s\(^{-1}\)) loading or TGF-β treatment. Following 2 days of treatment with TGF-β, there was a robust astrogliotic response consisting of increased GFAP expression, astrocyte hypertrophy, and increases in expression of chondroitin-sulfate proteoglycans (CSPGs). After mechanical loading, the astrogliotic response varied based on strain-rate. Quasi-static loading (1 s\(^{-1}\)) resulted in increased in 3-D cell density (suggesting a hyperplasic response) and increased glycosaminoglycan content by 2 days post-insult. Moderate rate loading (10 s\(^{-1}\)) also produced significant increases in cell density, as well as significant increases in GFAP reactivity and astrocyte hypertrophy by 2 days post-insult. However, such changes took longer to manifest following high rate deformation (30 s\(^{-1}\)), and were not statistically significant until 5 days post-insult, potentially related to the degree of cell death induced by this injury level. This work demonstrates an augmentation in reactive astrogliosis at sub-lethal injury levels as well as levels inducing significant cell death, presenting the capability to control astrogliosis and cell death based on injury parameters.

**Introduction**

Complex physical and biochemical alterations are initiated by a mechanical insult to the central nervous system (CNS) that drastically alter the post-injury microenvironment. A perturbation to neural homeostasis may induce a potent reactive response resulting in the formation of the glial scar, isolating tissue acutely injured from tissue that is not severely damaged. The post-insult reactive response involves the recruitment/activation of many cell types including astrocytes, macrophages, microglia,
oligodendrocytes, and meningeal cells; although the chronic glial scar consists primarily of reactive astrocytes (Fawcett and Asher, 1999). The formation of a glial scar appears to have short-term positive effects after CNS trauma as secondary damage may be lessened through isolation of the immune response and re-establishment of the blood-brain barrier (Bush, et al., 1999). However, ultimately the formation of the glial scar appears to hinder regeneration as specific extracellular matrix (ECM) components are increased that have been shown to be inhibitory to neurite outgrowth (McKeon, et al., 1995; McKeon, et al., 1999; Asher, et al., 2000). Reactive astrocytosis is an element of this overall response, hallmarks of which are alterations in astrocyte function including increased expression of intermediate filaments (e.g., GFAP), hypertrophy, increased matrix production/secretion (e.g., chondroitin sulfate proteoglycans (CSPGs), laminin) and hyperplasia. An increased understanding of the mechanisms underlying induction of reactive astrogliosis and chronic glial scarring may eventually lead to protective treatments and the augmentation of repair and regeneration.

The reactive astrogliotic environment and chronic glial scar have been extensively characterized using a range of in vivo, ex vivo, and in vitro systems, which have proven valuable in studying mechanisms of astrogliotic induction. Characterization of the time-course and degree of the inflammation and reactive astrogliotic response has been performed in vivo following brain and spinal cord injuries (Janeczko, 1989; Cervos-Navarro and Lafuente, 1991; Fitch, et al., 1999; Goussev, et al., 2003; Moon, et al., 2004). This work had noted a robust inflammatory and reactive astrogliotic response proportional to injury severity. The implantation of nitrocellulose filters into the cerebral cortex have been used as an in vivo model of the chronic glial scar, providing insight into
the physiology of reactive astrocytes and associated matrix alterations (McKeon, et al., 1999). Such \textit{in vivo} models are well suited to characterize the totality of the reactive astrogliotic response; however, isolating specific elements or differentially regulating proposed induction mechanisms remains complex. Furthermore, the differentiation of causative factors versus epiphenomena may be challenging. Accordingly, \textit{in vitro} models permit a high degree of experimental control and minimization of potentially confounding systemic variables; however, much of the \textit{in vitro} work elucidating mechanisms of astrogliotic induction have been performed using planar (2-D) models. Such 2-D models may be affected by altered cell-cell/cell-matrix interactions and atypical cellular morphology, thus potentially failing to capture certain induction mechanisms such as extracellular-matrix-bound factors and elements of autocrine/paracrine signaling. Three-dimensional (3-D) models consisting of multiple neural cell types may offer a degree of intermediate complexity by maintaining many positive aspects of \textit{in vitro} modeling while closer approximating the cytoarchitecture of the brain.

The goal of this study was to evaluate alterations in neural cell viability and reactive astrogliotic augmentation following a defined mechanical insult to 3-D neuronal-astrocytic co-cultures. These cell cultures were subjected to a range of mechanical loading regimes using a custom-built electromechanical device capable of subjecting 3-D cell-containing matrices to controlled shear deformation of variable rate and magnitude (see CHAPTER III and (LaPlaca, et al., 2005)). Neural cells have been found to display a strain rate-dependent response to mechanical loading (Ellis, et al., 1995; Cargill and Thibault, 1996; LaPlaca and Thibault, 1997; Geddes and Cargill, 2001); however, such an analysis has only recently been performed on neural cells cultured in 3-D (see
CHAPTER III). The strain regimes chosen for this study varied the rate of deformation and contain levels shown to be sub-lethal as well as levels which produced significant cell death, thus potentially assessing the effects of a direct mechanical insult and local cell death on astrogliotic induction in a 3-D environment. Specifically, astrocytotic parameters (e.g., hypertrophy, hyperplasia, GFAP expression) and ECM alterations (e.g., increases in CSPG expression) were evaluated. It was hypothesized that subjecting 3-D neuronal-astrocytic co-cultures to high rate, high magnitude deformation will induce cell death and alterations consistent with reactive astrogliosis. Upon characterizing the degree of cell death and reactive astrogliosis induced by various loading regimes, this model may be further exploited as an in vitro test-bed to determine factors in the injured/reactive environment that may influence neural stem cell behavior in a controlled environment (CHAPTER VI).

Materials and Methods

Isolation of Primary Cortical Neurons

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. Timed-pregnant Sasco Sprague-Dawley rats (embryonic day 17, Charles River, Wilmington, MA) were anesthetized using halothane (Halocarbon, River Edge, NJ) and decapitated. The uterus was removed by Caesarian section and placed in Hanks Balanced Salt Solution (HBSS, Invitrogen). Each fetus was removed from the amniotic sac, rapidly decapitated, and the brains removed. The cerebral cortices were isolated and the hippocampal formations were removed. To dissociate the tissue, pre-warmed trypsin (0.25%) + 1 mM EDTA
(Invitrogen, Carlsbad, CA) was added for 10 minutes at 37°C. The trypsin-EDTA was then removed and deoxyribonuclease I (0.15 mg/mL, Sigma, St. Louis, MO) in HBSS was added. The tissue was tritutated with a flame-narrowed Pastuer pipet and then centrifuged at 1000 rpm for 3 minutes after which the supernatant was aspirated and the cells were resuspended in co-culture medium (Neurobasal medium + 2% B-27 + 1% G-5 + 500 µM L-glutamine (Invitrogen)).

**Isolation of Primary Cortical Astrocytes**

Postnatal (day 0-1) Sasco Sprague-Dawley rats (Charles River) were anesthetized using halothane and rapidly decapitated. The brains were removed and the cerebral cortices isolated as described above. Upon isolation of the cortical regions, the tissue was minced and pre-warmed trypsin (0.25%) + EDTA (1 mM) was added and placed in at 37°C for 5 minutes. DNase was added and the tissue was triturated using a flame-narrowed Pasteur pipet. Medium was added (DMEM/F12 + 10% FBS) and the cells were centrifuged (1000 rpm, 3 minutes) after which the supernatant was aspirated, the cells were resuspended in DMEM/F12 + 10% FBS and transferred to T-75 tissue culture flasks. To isolate a nearly pure population of type I astrocytes, procedures described elsewhere were followed (McCarthy and de Vellis, 1980). Briefly, at various time-points over the first week post-dissection, the flasks were mechanically agitated to dislodge less adherent cell types (e.g., neurons, oligodendrocytes). As the cells approached ~90% confluency they were resuspended using trypsin-EDTA, centrifuged, and replated at a density of 300 cells/mm². Astrocytes were used between passages 4-12 for the generation of 3-D cultures to permit phenotypic maturation.
3-D Primary Cortical Neuronal / Secondary Cortical Astrocytic Co-Cultures

Co-cultures were plated using neurons and astrocytes that were separately isolated and dissociated (as described above) in custom-made cell culture chambers consisting of a glass coverslip (no. 1½ thickness) below a circular silicone-based elastomer mold (Sylgard 184 and 186, Dow Corning; Midland, MI; cross-sectional area = 2 cm$^2$). Prior to plating, the chambers were pre-treated with 0.05 mg/mL poly-L-lysine (PLL, Sigma) followed by Matrigel (0.5 mL/well at 0.6 mg/mL, Becton Dickinson Biosciences; Bedford, MA) in Neurobasal medium (each treatment was > 4 hours). Co-cultures in 3-D were plated within Matrigel matrix, which exhibits fluid-like behavior at 4°C (which permits even dispersion of dissociated cells throughout matrix material) and subsequent gelation at or near physiological temperature (which entraps cells in the 3-D matrix) (Kleinman, et al., 1986). 3-D co-cultures were plated at 2500 cells/mm$^3$ at a 1:1 neuron:astrocyte ratio (final Matrigel concentration 7.5 mg/mL) at a thickness of 500-750 µm. These cultures were placed at 37°C to permit matrix gelation and 3-D cell entrapment, after which 0.5 mL of co-culture medium was added to each well. Cultures were maintained at 37°C and 5% CO$_2$-95% humidified air and medium was exchanged at 24 hours and every 2 days thereafter.

Mechanical Loading Using the 3-D Cell Shearing Device

Neuronal-astrocytic co-cultures in 3-D were mechanically loaded using the 3-D Cell Shearing Device (CSD), a custom-built electromechanical device capable of quantifiably imparting high strain rate shear deformation to 3-D cell-containing matrices (CHAPTER III and (LaPlaca, et al., 2005)). At the time of injury, cultures were removed from the incubator and mounted within the 3-D CSD. The mechanical action of the
device was driven by a linear-actuator (BEI Kimco; San Marcos, CA) coupled to a custom-fabricated digital proportional-integral-derivative (PID) controller (25kHz sampling rate, 16 bit sampling resolution) with closed-loop motion control feedback from an optical position sensor (RGH-34, 400 nm resolution; Renishaw). A trapezoidal input was provided by code written in LabVIEW® software v6.1 (National Instruments; Austin, TX). Rapid lateral motion of the cell chamber top plate with respect to the fixed base of the cell chamber imparts simple shear deformation to the elastically-contained 3-D cell-containing matrices (Figure 5.1). At 21 days in vitro (DIV), 3-D co-cultures were deformed (strain 0.50, strain rate 1 s⁻¹, n = 10; strain 0.50, strain rate 10 s⁻¹, n = 10; strain 0.50, strain rate 30 s⁻¹, n = 18) or left as control cultures (uninjured control, n = 16). After mechanical deformation, warm medium was added and the cultures were returned to the incubator.
Figure 5.1: Schematic representation of the 3-D neuronal-astrocytic co-culture (left) and mechanical deformation (right) models (not to scale). Neuronal-astrocytic co-cultures in 3-D were plated throughout a matrix and were laterally constrained by an elastomer mold. Mechanical deformation is imparted to cell-containing matrices through the action of the 3-D Cell Shearing Device (3-D CSD), a custom-built electromechanical device. A closed-loop control system (PID controller with positional feedback from an optical position sensor (OPS)) governs a linear actuator, causing horizontal displacement of the cell chamber top-plate inducing shear deformation in the elastomer mold and cell-imbedded matrices.

Cytokine Treatment

Beginning at 21 DIV, a set of co-cultures were treated with transforming growth factor beta 1 (TGF-β1, 10 ng/mL) diluted in co-culture medium (n = 14). These cultures were designed to serve as a positive reactivity control as this cytokine has previously been found to induce astrogliosis (Logan, et al., 1994).
Cell Viability

Cell viability was assessed using fluorescent probes for distinguishing live and dead cells. Cell cultures were incubated with 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM (both from Molecular Probes, Eugene, OR) at 37° C for 30 minutes and then rinsed with 0.1M Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen). The percentage of viable cells was calculated by counting the number of live cells (fluorescing green by AM-cleavage) and the number of cells with compromised membranes (nuclei fluorescing red by EthD-1). At 23 DIV (2 days following experimental treatment), co-culture viability was assessed following mechanical loading at 1, 10 and 30 s\(^{-1}\) (n = 5, 5, 7, respectively), TGF-β treatment (n = 5), and control conditions (n = 7).

Markers of Astrogliosis

Baseline parameters were determined from co-cultures at 1 and 21 – 23 DIV (n = 3 each). Astrogliotic induction was evaluated at 2 days following control, TGF-β treatment (n = 5 each), or mechanical loading at 1s\(^{-1}\), 10s\(^{-1}\), or 30s\(^{-1}\) (n = 5, 5, 7, respectively). A subset of the co-culture conditions were taken out for analysis at 5 days post-insult (the co-culture parameters used to interface with neural stem cells; see CHAPTER VI). Accordingly, astrogliotic induction was also analyzed 5 days following control, TGF-β treatment or mechanical loading at 30 s\(^{-1}\) (n = 4 each).

Immunocytochemistry

Astrogliosis was assessed using immunocytochemistry for antibodies recognizing specific markers associated with astrocyte hypertrophy/reactivity, proliferation, and matrix alterations. Briefly, 3-D neuronal-astrocytic co-cultures were fixed with 3.7%
formaldehyde (Fisher, Fairlawn, NJ) for 60 minutes and then placed in 30% sucrose (Sigma) overnight at 4°C. Co-cultures were then placed in OCT Embedding Compound (Sakura, Tokyo, Japan), flash frozen in liquid nitrogen, sectioned on a cryostat (20 µm thick), and mounted on glass slides. Sections were rinsed in PBS and permeabilized using 0.3% Triton X100 (Kodak, Rochester, NY) + 4% goat serum (Invitrogen) for 60 minutes. Primary antibodies were added (in PBS + 0.3% Triton X100 + 4% serum) overnight at 4 °C covered in a humidified chamber. After rinsing, the appropriate secondary fluorophore-conjugated antibodies (FITC or TRITC-conjugated IgG, Jackson Immuno Research or Alexa 488 or 546-conjugated IgG or IgM, Molecular Probes) were added (in PBS + 0.3% Triton X100 + 4% serum) for 2 hours at 18-24 °C covered in a humidified chamber. Sections were immunostained using primary antibodies recognizing: 1) glial fibrillary acidic protein (GFAP) (AB5804, 1:400; MAB360, 1:400, Chemicon), an intermediate filament found exclusively in astrocytes (Debus, et al., 1983), 2) Neurocan (MAB5212, 1:1000, Chemicon), a CSPG, 3) CS-56 (C8035, 1:100, Sigma), a general CSPG marker, 4) laminin-1 (AB2034, 1:200; Chemicon), an ECM protein, and 5) phosphorylated histone H3 (1:100, Upstate), a marker of mitosis (n = 3 – 6 per group per marker; each sample stained in triplicate). Counterstaining was performed using Hoechst 33258 (1:1000, Molecular Probes).

CSPG Secretion

Two days following experimental treatment, medium was sampled for analysis from co-cultures treated with TGF-β (n = 5), subjected to mechanical loading at 1, 10 and 30 s⁻¹ (n = 3, 4, 9, respectively), or controls (n = 7). CSPG production and extracellular expression in 3-D cultures was quantified using a colorimetric assay (Blyscan®; Biocolor
Assays, Newtownabbey, Northern Ireland). Briefly, medium was sampled from the 3-D cultures and treated with 1,9-dimethylmethelyne blue (DMMB), a specific label for the sulfated polysaccharide component of proteoglycans and/or the protein-free sulfated glycosaminoglycan chains. Colorimetric alterations were assessed using a plate reader and results quantified using a standard curve for CSPG.

Development of Outcome Measures

Astrocyte reactivity and increases in CSPG expression have been well-documented in vivo following injury; however, these responses required validation in our in vitro co-culture system. Co-cultures were subjected to longer-term TGF-β treatment and immunostained for markers of reactive astrogliosis in order to determine acceptable outcome measures for this system. Accordingly, 3-D neuronal co-cultures were treated with TGF-β from 21 – 36 DIV (10 ng/mL, added fresh every other day; n = 4) or left as untreated controls (n = 4). At 36 DIV, cultures were fixed, processed and immunolabeled for GFAP and CS-56 with Hoechst counterstaining (as described above). Also, tissue sections from injured brains 7 days following controlled cortical impart were processed in parallel for comparison (see APPENDIX F). In 3-D co-cultures, TGF-β treatment for 14 days was found to induce dramatic alterations in astrocytic process density and hypertrophy compared to untreated controls. Furthermore, the extracellular expression of CSPGs increased substantially in TGF-β treated cultures versus controls (Figure 5.2). This work established specific outcome measures (astrocytic hypertrophy and CSPG expression) for subsequent experiments and validated the use of TGF-β as a positive control for induction of reactive astrogliosis (previously described in (Logan, et al., 1994)).
**Figure 5.2: Derivation of markers of astrogliosis.** In order to develop the reactive astrogliotic outcome measures utilized in this study, (A,C) control co-cultures were compared to (B,D) co-cultures treated with TGF-β for 14 days. (A,B) TGF-β treatment induced alterations in astrocyte morphology (GFAP, green) as many hypertrophic processes were observed (arrows). (C,D) Also, there were robust increases in CSPG expression in the matrix following longer-term TGF-β treatment. Scale bar = 20 µm.
Data Collection and Statistical Analysis

After viability and immunocytochemistry assays, cells were viewed on an epifluorescent microscope (Eclipse TE300, Nikon, Melville, NY) or a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). For epifluorescent microscopy, images were digitally captured (DKC5T5/DMC, Sony, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Confocal images were analyzed using LSM Image Browser (Zeiss). Three to five randomly selected regions per section were counted for statistical analysis. **Viability:** The percentage of viable cells was calculated and a one-way ANOVA was used with treatment as the independent variable and viability as the dependent variable. **Immunocytochemistry:** The number of hypertrophic processes per unit volume was calculated. Matrix CSPG and laminin expression were semi-quantitatively assessed using Image-Pro Plus. The number of proliferating cells per unit volume was calculated. General linear model ANOVA was performed on quantified results from the immunocytochemistry studies. **CSPG secretion:** a one-way ANOVA was performed with CSPG concentration as the dependent variable. In all cases, when significant differences exist between groups, post hoc Tukey’s pair-wise comparisons were performed with p < 0.05 required for significance. Data are presented as mean ± standard deviation.

Results

Baseline Remodeling in 3-D Co-Cultures

Specific baseline parameters of the 3-D neuronal-astrocytic co-cultures have previously been presented (see CHAPTER II). Table 5.1 presents a subset of parameters
that were observed in these co-cultures shortly after plating (1 DIV) compared to a later
time-point following the development of mature neural networks (21 DIV). Although
culture viability remains high, there are stark increases in cell density, potentially a by-
product of the modest levels of astrocyte proliferation (see Figure 2.9) and overall matrix
contraction. The matrix contraction, potentially indicative of the matrix-remodeling
capability of astrocytes (Gottschall and Deb, 1996; Wells, et al., 1996), may be due to a
net mechanical response of matrix fibrils in resisting tension associated with process
outgrowth, a phenomenon observed in similar 3-D collagen (e.g., fibroblasts (Grinnell,
2000)) and collagen-glycosaminoglycan matrices (e.g., peripheral nerve explants
(Spillker, et al., 2001)). Furthermore, by 21 DIV the percentage of neurons relative to the
total cell population in co-culture had reduced to ~10%, possibly a function of astrocyte
proliferation or an indication of some neuronal death during culture development.

Table 5.1: Properties of 3-D co-cultures at the time of plating compared to
developed cultures, after a dense network of processes has formed. Although cell
viability remains high, there was a sharp increase in cell density concurrent with a
reduction in the neuronal presence. Moreover, dramatic alterations were observed in the
culture diameter.

<table>
<thead>
<tr>
<th></th>
<th>0 DIV</th>
<th>21 DIV</th>
</tr>
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<tbody>
<tr>
<td><strong>Viability</strong></td>
<td>-</td>
<td>95.4 ± 1.87%</td>
</tr>
<tr>
<td><strong>Cell Density (cells/mm³)</strong></td>
<td>2500</td>
<td>8903.8 ± 1173.4</td>
</tr>
<tr>
<td><strong>Diameter (mm)</strong></td>
<td>15.8</td>
<td>7.93 ± 0.52</td>
</tr>
<tr>
<td><strong>Neuronal Presence (%)</strong></td>
<td>50</td>
<td>9.75 ± 3.65</td>
</tr>
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</table>
To further assess the matrix remodeling capabilities of the cells in co-culture, immunocytochemistry was performed to assess the astrocyte presence and the expression of laminin and CSPGs, two ECM molecules present in Matrigel. Astrocyte development can be observed from recently plated cultures to the formation of mature networks, as at 1 DIV there are only GFAP$^+$ somas, but later the cells assume a more compact, process-bearing morphology (Figure 5.3.1). Also, the expression of laminin and CSPGs in immature versus developed 3-D co-cultures was examined. There was not a significant difference in the expression of either laminin or CSPGs in immature versus developed co-cultures, signifying that there was not a net change in the density of these matrix constituents over time in culture (Figure 5.3.2).
Figure 5.3.1: Expression of laminin and CSPGs in recently plated versus developed 3-D co-cultures. The expression of CSPGs (red) and GFAP (green) with nuclear counter-staining (blue) at (A) 1 DIV compared to (B) 23 DIV. The expression of laminin (green) with nuclei (blue) at (C) 1 DIV and (D) 23 DIV. Also, an increase in cell density was apparent from 1 DIV compared to 23 DIV. Scale bar = 20 µm.
Figure 5.3.2: Laminin and CSPG expression in recently plated versus developed 3-D co-cultures. The relative expression of laminin and CSPGs were quantified. There was not a significant difference in the expression of either laminin or CSPGs in immature versus developed co-cultures.

**Cell Viability Following Mechanical Loading or TGF-β Treatment**

3-D neuronal-astrocytic co-cultures were subjected to mechanical deformation, TGF-β treatment, or control conditions at 21 DIV and culture viability was assessed 2 days later. Fluorescent confocal microscopy revealed that the highest rate deformation (30s⁻¹) resulted in a significant reduction in culture viability (Figure 5.4.1). However, there was not a statistically significant change in the percentage of viable cells from either the quasi-static deformation (1 s⁻¹) or the moderate rate deformation regime (10 s⁻¹) compared to controls. Furthermore, TGF-β treatment had no effect on culture viability compared to controls (Figure 5.4.2).
Figure 5.4.1: Cell viability following mechanical loading or TGF-β treatment.
3-D neuronal-astrocytic co-cultures were subjected to mechanical deformation, TGF-β treatment, or control conditions at 21 DIV and culture viability was assessed 2 days later. Fluorescent confocal reconstructions of representative co-cultures after (A) control conditions, (B) TGF-β treatment, or mechanical loading at (C) 0.50 strain, 1s⁻¹ rate, (D)
0.50 strain, 10 s$^{-1}$, or (E) 0.50 strain, 30 s$^{-1}$. High rate deformation resulted in a significant reduction in culture viability while cytokine treatment had no effect on culture viability. Scale bar = 50 µm.

![Figure 5.4.2: Culture viability following mechanical loading or TGF-β treatment.](image)

**Figure 5.4.2: Culture viability following mechanical loading or TGF-β treatment.** Cytokine treatment or quasi-static deformation (1 s$^{-1}$) did not affect culture viability, whereas high rate deformation resulted in a significant reduction in viability* (p < 0.05).

It was also observed that there were increases in total cell density (live and dead cells) following some of the treatments. Although there was an increasing trend in overall cell density in all the treatment groups versus controls, these increases were only statistically significant for the quasi-static and moderate rate deformation regime (Figure 5.4.3). These data suggest a proliferative response initiated by mechanical deformation that was induced over a range of loading rates. Also, due to an overall increase in cell density, it was possible that detrimental effects on culture viability were masked when calculating the percentage of viable cells. Accordingly, the density of dead cells was analyzed, revealing that there was a significant increase in the dead cell density following both moderate and high rate deformation (Figure 5.4.4).
Figure 5.4.3: Cell density following mechanical loading or TGF-β treatment. There was a significant increase in cell density following mechanical loading† (p < 0.05).

Figure 5.4.4: Density of dead cells following mechanical loading or TGF-β treatment. There was a significant increase in the density of dead cells following moderate and high rate deformation‡ (p < 0.05).

Cell Proliferation Following Mechanical Loading or TGF-β Treatment

Given the stark alterations in cell density following mechanical loading and that hyperplasia is a common astrocytic response to trauma, cell proliferation was assessed in
3-D co-cultures at 2 days following mechanical loading or TGF-β treatment (Figure 5.5.1). At this time-point, there were no statistically significant differences in the density of proliferating cells in any of the treatment groups versus controls (Figure 5.5.2). Each of the co-culture groups had less than 1% of the cells mitotically active. This may indicate that the initial proliferative response begins shortly following an insult and such proliferative mechanisms have been down-regulated by 2 days post-insult.

**Figure 5.5.1: Cell proliferation following high rate deformation or TGF-β treatment.** A series of fluorescent micrographs of a co-culture treated with TBF-β for 2 days and immunolabeled for (A) GFAP (red), an astrocyte marker, with nuclear counterstaining (blue) and (B) phosphorolated histone-3 (pH3, green), a marker of mitosis. (C) An overlay of (A) and (B) to illustrate the nuclear co-localization of pH3 and GFAP (scale bar = 20 µm).
Figure 5.5.2: Cell proliferation following high rate deformation or TGF-β treatment. The number of proliferating cells per unit volume was quantified for control, TGF-β treated and mechanically loaded cultures. At his time-point, there were no significant differences in the number of proliferating cells per mm³ for any of the conditions tested; no group had more than 1% of the cells mitotically active.

Astrocyte Hypertrophy Following Mechanical Loading or TGF-β Treatment

Co-cultures were immunolabeled for GFAP with nuclear counterstain at 2 and 5 days post-insult to assess the overall density of astrocytic processes and the density of hypertrophic processes (Figure 5.6.1). There was increased GFAP reactivity (intensity and amount) and process density following TGB-β treatment and mechanical deformation compared to controls. At 2 days post-insult, TGF-β treatment and moderate rate deformation (10 s⁻¹) induced significant alterations in the density of hypertrophic processes (Figure 5.6.2). The density of cell nuclei also increased following loading versus controls, corroborating the earlier results attained using 3-D confocal microscopy.
Figure 5.6.1: Astrocyte process growth and hypertrophy at 2 days following TGF-β treatment or mechanical deformation. Co-cultures were immunolabeled for GFAP (red) with nuclear counterstain (blue) at 2 days following (A) control, (B) TGF-β treatment, (C) quasi-static deformation, or (D) moderate rate deformation. There was increased GFAP reactivity and process density following TGF-β treatment and moderate rate deformation. Also, the density of astrocytes increased versus controls. Scale bar = 20 µm.
Figure 5.6.2: Astrocyte hypertrophy at 2 days following insult. The number of hypertrophic GFAP$^+$ processes was quantified 2 days following control, TGF-β treatment, or mechanical loading of 0.50 strain at strain rates of 1 s$^{-1}$, 10 s$^{-1}$, or 30 s$^{-1}$. TGF-β treatment and moderate rate deformation (10 s$^{-1}$ only) induced significant alterations in the density of hypertrophic processes$^*$ (p < 0.05).

A similar analysis was performed at 5 days post-insult for a subset of the treatment conditions (Figure 5.7.1). This analysis revealed that TGF-β treatment and high rate deformation (30 s$^{-1}$) both induced significant alterations in the density of hypertrophic processes (Figure 5.7.2). The relatively high levels of cell death produced by the high strain rate injury may influence the apparent lag in the increase in hypertrophic process density, thus necessitating recovery time for the process density to increase beyond pre-injury levels.
Figure 5.7.1: Astrocyte process growth and hypertrophy at 5 days following insult. Co-cultures were immunolabeled for GFAP 5 days following (A) control, (B) TGF-β treatment, or (C) high rate mechanical loading (0.50 strain, 30s⁻¹ strain rate). There were increases in the density of hypertrophic processes (arrows) following loading and TGF-β treatment. Scale bar = 20 µm.
Figure 5.7.2: Astrocyte hypertrophy at 5 days following the insult. The number of hypertrophic GFAP^+ processes was quantified 5 days following control, TGF-β treatment, or mechanical loading of 0.50 strain at a strain rate of 30 s^-1. TGF-β treatment and high rate deformation induced significant alterations in the density of hypertrophic processes^* (p < 0.05).

Expression of CSPGs Following Mechanical Loading or TGF-β Treatment

Matrix alterations characteristic of reactive astrogliosis were evaluated following mechanical loading or TGF-β treatment. This was evaluated using a general marker for all CSPGs and a marker for a specific CSPG, neurocan. At 2 days post-insult, the matrix content of CSPGs increased following TGF-β treatment compared to controls (Figure 5.8.1). Following high rate deformation, increases in CSPG were not observed at this time-point. Similarly, the matrix content of neurocan increased following treatment with TGF-β, but such alterations were not apparent following high rate deformation at this time-point (Figure 5.8.2).
Figure 5.8.1: Expression of CSPGs following high rate deformation or TBF-β treatment. Fluorescent micrographs of representative neuronal-astrocytic co-cultures at 23 DIV, 2 days following (A) control conditions, (B) TGF-β treatment or (C) high rate deformation (scale bar = 20µm). Cultures are immunolabeled for CSPGs (red) and GFAP (green) with nuclear counterstain (blue). There was a significant increase in the expression of CSPGs following TGF-β treatment (p < 0.05).
Figure 5.8.2: Neurocan expression following high rate deformation or TBF-β treatment. Representative fluorescent micrographs of neuronal-astrocytic co-cultures at 23 DIV, 2 days following (A) control conditions, (B) TGF-β treatment or (C) high rate deformation (scale bar = 10µm). Cultures are immunolabeled for neurocan (red), a specific CSPG, and GFAP (green) with nuclear counterstain (blue). There was a significant increase in neurocan expression following TGF-β treatment (p < 0.05).
**Soluble GAG Content Following Mechanical Loading or TGF-β Treatment**

Using a standard colorimetric assay, the content of soluble glycosaminoglycan (GAG) expression in the medium around the co-cultures was quantified at 2 days following the insult. The total GAG content in the medium was maximal for the cultures subjected to quasi-static deformation (1 s\(^{-1}\) strain rate) in comparison to control cultures (Figure 5.9). Interestingly, the soluble GAG profile mirrored that of the viable cell density profile, possibly indicating that only at very high cell densities were these cultures able to increase to detectable levels the [GAG] released into the medium.

![Figure 5.9: Soluble GAG content following TGF-β treatment of mechanical loading.](image)

The content of soluble GAGs in the medium around the co-cultures was quantified using a standard assay at 2 days following the insult. The total amount of GAGs in the media was greatest for the cultures subjected to a low rate mechanical insult (0.50 strain, 1s\(^{-1}\) strain rate)\(^*\) (p < 0.05).
Discussion

The goal of this study was to characterize elements of neural cell death and reactive astrogliosis in response to mechanical trauma using a 3-D in vitro co-culture model. Mature 3-D neuron-astrocyte co-cultures were subjected to a range of mechanical loading regimes (0.50 strain at 1, 10, or 30 s\(^{-1}\) strain rate), TGF-β treatment (a factor inducing astrogliosis (Logan, et al., 1994)) or control conditions and then characterized by evaluating cell viability and markers of reactive astrogliosis. This analysis revealed that moderate and high rate deformation caused significant cell death in the co-cultures, but quasi-static deformation and TGF-β treatment did not impact culture viability. Also, following quasi-static and moderate rate deformation, there were significant increases in cell density, potentially indicating an astrocytic hyperplastic response to trauma. Furthermore, increases in astrocyte reactivity and hypertrophy were observed following mechanical trauma and TGF-β treatment. However, matrix alterations in CSPG content following high rate deformation were not manifested to the degree of that induced by TGF-β treatment. This may indicate an insufficient number of viable cells following high rate deformation to significantly alter the matrix or different reactive responses in affected astrocytes based on the mode of induction. Also, following quasi-static deformation, there was an increase in the soluble GAG content in the medium, perhaps indicating enhanced GAG synthesis following a deformation that did not detrimentally affect culture viability.

The relative alterations in cell viability following the loading regimes used in this study are consistent with those following both in vivo and in vitro models of neural trauma (Gennarelli, et al., 1982; Ellis, et al., 1995; Meaney, et al., 1995; Ahmed, et al.,
2000). Also, the strain rate-dependent response to mechanical loading has previously been reported for neural cells (Ellis, et al., 1995; Cargill and Thibault, 1996; LaPlaca and Thibault, 1997; Geddes and Cargill, 2001); however, this is the first report of such a response in 3-D mixed cultures. This model is well-suited for the investigation of cellular tolerances to mechanical injury on a per cell-type basis (e.g., neuron versus astrocyte survival). This can be explored based on the overall injury parameters (e.g., strain magnitude and rate) as well as the specific mode of deformation (e.g., shear versus normal), an analysis currently not possible with other models of neural trauma. Establishing such tolerances may be beneficial in permitting targeted treatment for a specific neural cell-type based on parameters of the injury.

This model may be appropriate for the further exploration of neural responses to trauma, including cellular degeneration/death and the reactive astrogliotic response. Neuronal degeneration/death attained in this model may be compared to that attained in neuron-only cultures (CHAPTERS III and IV) in order to investigate potential rescuing capabilities of astrocytes. For instance, the production and secretion of neurotrophic factors such as basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF), which may be released by astrocytes and may enhance neuronal survival following trauma (McIntosh, et al., 1999). Specifically, astrocytes have been found to play a role in the neuronal repair process by inhibiting neuronal Ca\textsuperscript{2+} currents following trauma. Furthermore, this response may require cross-talk between these two cell types, as inflammatory cytokines secreted by injured neurons may indirectly enhance neuronal survival by inducing astrocytes to proliferate and enhance synthesis of the aforementioned pro-survival neurotrophic factors.
(McIntosh, et al., 1999). Also, the proliferative response may be further explored in injured astrocyte only-cultures versus these mixed cultures to assess the influences of neuron-astrocyte interactions in regulating hyperplasia.

The strain regimes chosen for this study varied the rate of deformation and contain levels shown to be sub-lethal as well as levels which produced significant cell death. This distinction permitted the assessment of the effects of a direct mechanical insult with and without local cell death on astrogliotic induction in a 3-D environment. Previous models for the *in vitro* evaluation of specific astrogliotic induction mechanisms following mechanical trauma have utilized 2-D culture systems, which may fail to fully capture certain induction mechanisms, such as ECM-bound factors and elements of autocrine/paracrine signaling. However, an ablation injury to astrocytes cultured in 2-D was found to significantly increase bFGF by 2 hours following injury, levels that peaked at 12 hours and declined by 2 days (Li, et al., 1997). This study also observed up-regulation of proliferating cell nuclear antigen (PCNA) and GFAP by 2 hours and 6 hours post-injury, respectively, and enhanced GFAP expression with corresponding astrocytic hypertrophy (peaking 2 days post-insult). The authors noted that the prominent event of reactive astrogliosis was the hypertrophy of astrocytes, which was attributed to autocrine bFGF as the promoter. Using a 2-D stretch model of traumatic neural injury, enhanced GFAP expression, astrocyte hypertrophy and proliferation were observed at 24 and 48 hours following injury (Muir, et al., 2002). In a follow-up study, it was demonstrated that 2-D stretch injury induced a strain magnitude and strain-rate dependent activation of extracellular signal-regulated protein kinase (ERK), a key regulator of cellular proliferation and differentiation, as early as 1 minute post-injury, which was maximal
from 10 - 30 minutes and remained elevated for 3 hours (Neary, et al., 2003). This study implicated extracellular ATP release and increases in intracellular Ca\(^{2+}\) as mechanisms of astrogliotic induction. Thus, isolated induction mechanisms are complex and multifaceted and may be mediated by a combination of initial calcium influx leading to autocrine and paracrine signaling involving release of further astrocytotic-inducing factors such as bFGF and ATP.

Future studies elucidating specific mechanisms of astrogliotic induction using this 3-D co-culture model may incorporate a number of additional measures to enhance the sensitivity of the system. For instance, TGF-\(\beta\) treatment (with variable concentration) may be used in conjunction with mechanical loading (beginning before or at the same time) to better regulate the degree of astrogliosis and cell death. This would create groups consisting of the combinational effect of cell death/degeneration and a robust cytokine-induced response that may not be possible from mechanical loading alone. Also, more severe mechanical injury levels may be explored; however, there may be a non-linear relationship between level of mechanical injury and degree of astrogliosis as more severe loading could produce cultures too unhealthy for these changes to occur. Astrogliosis may be more pronounced at longer/shorter time-points from those chosen, especially in regard to matrix remodeling. Furthermore, additional factors may be added to this model to assess a broader range of potential astrogliotic induction mechanisms. For instance, the addition of microglia may enhance the astrogliotic response as factors secreted by these cells have been linked to astrogliosis (e.g., TGF-\(\beta\)). However, through the removal of some potentially dominant mechanisms of astrogliotic induction such as blood-brain compromise (systemic influences) and microglia stimulation, this model
permits evaluation and manipulation of specific facets of the reactive response in a controlled setting.

**Conclusions**

This study utilized a well-controlled, 3-D *in vitro* model to investigate alterations in cell viability and the induction of specific astrogliotic parameters following mechanical loading or TGF-β treatment. Specifically, TGF-β treatment augmented astrogliosis while not affecting culture viability whereas mechanical loading induced significant cell death and some markers of developing astrogliosis. This model, exhibiting localized cell death and astrogliotic parameters, may be further exploited as an *in vitro* test-bed to determine factors in this surrogate injured/reactive environment that may influence neural stem cell behavior upon transplantation into this environment (CHAPTER VI).
CHAPTER VI

ASSESSMENT OF FACTORS INFLUENCING NEURAL STEM CELL SURVIVAL USING AN IN VITRO MODEL OF TRAUMATICALLY INDUCED CELL DEATH AND REACTIVE ASTROGLIOSIS

Abstract

A series of deleterious cascades ensue following a traumatic insult to the brain, including excitotoxicity, inflammation, and reactive astrogliosis, creating a hostile environment that induces cell death and inhibits regeneration. Neural stem cell (NSC) transplantation after injury has had limited success and has been shown to mediate some neurological recovery; however, this technique has been limited by poor NSC survival and minimal control of function. NSC survival and integration are influenced by properties of the post-injury environment, but the determination of specific factors exerting influence in vivo has remained elusive due to the inherent complexity of the system. Accordingly, the goal of this study was to utilize a controlled, three-dimensional (3-D) in vitro model exhibiting traumatically-induced cell death and markers of reactive astrogliosis to elucidate factors influencing NSC survival and differentiation upon introduction to an injury environment. This test-bed consisted of mature 3-D neuronal-astrocytic co-cultures that were subjected to TGF-β treatment (inducing astrogliosis while not affecting culture viability), high rate mechanical loading (reducing culture viability and augmenting astrogliosis) or left undisturbed (controls having high culture viability
and minimal astrogliosis). At 48 hours post-insult, NSCs (E14.5, FGF-responsive) expressing green fluorescent protein were delivered into the center of these co-cultures to evaluate the effects of local cell death and reactive astrogliosis on NSC survival and differentiation. At 24 hours following delivery, NSC viability was approximately 95% across all groups. However, by 72 hours following delivery, NSC survival in the mechanically injured co-cultures was reduced to less than 75% while NSC survival in the TGF-β treated and control co-cultures remained approximately 95%. Furthermore, assessment of the differentiation profiles of NSC 72 hours following delivery revealed that the majority had progressed towards an astrocytic lineage (GFAP⁺), while modest percentages expressed nestin, NG2, and Tuj-1. However, these phenotypic expression patterns were not affected by treatment, signifying that a subset of NSCs may differentiate independent of cues affecting survival. This study indicates factors in the mechanically injured, but not astrogliotic, environment that are detrimental to NSC survival without affecting the differentiation of the remaining NSCs. This work demonstrates the utility of an in vitro model as a test-bed for evaluating factors potentially influencing the efficacy of treatment strategies for traumatic brain injury.

Introduction

Traumatic brain injury (TBI) is caused by physical deformation of the brain and may result in prolonged or permanent loss of sensory, motor, and/or cognitive functions (Smith, et al., 1994; Adelson, et al., 2000; Povlishock and Katz, 2005). TBI represents a major health and socioeconomic problem as each year 1.4 million Americans are affected, causing over 50,000 deaths (Langlois, et al., 2004). At least 5.3 million
Americans, close to two percent of the U.S. population, are chronically affected by TBI-related disorders (Thurman, et al., 1999). Primary cell death occurs during and immediately following a mechanical insult when local cellular stresses surpass thresholds. This initial insult sets into motion a series of secondary complications including excitotoxicity, inflammation, and blood-brain barrier breakdown, creating a hostile environment that may induce secondary, or delayed, cell death for hours, weeks, or even months after the initial insult (Smith, et al., 1997; McIntosh, et al., 1998). The post-injury microenvironment is further altered by an initial reactive response involving the recruitment and activation of various cell types including astrocytes, macrophages, microglia, oligodendrocytes, and meningeal cells. This initial response, collectively termed reactive gliosis, results in the formation of the glial scar – a physical and chemical barrier sequestering tissue acutely injured from surrounding tissue (see (Fawcett and Asher, 1999) for review). The formation of a glial scar appears to have short-term positive effects as secondary damage may be lessened through isolation of the immune response and re-establishment of the blood-brain barrier (Bush, et al., 1999). However, ultimately the chronic glial scar, consisting mainly of a tightly interwoven meshwork of hypertrophic astrocytes and their processes (bound together by tight and gap junctions), appears to hinder regeneration as chondroitin-sulfate proteoglycans (CSPGs) that have been shown to be inhibitory to neurite outgrowth are increased in the matrix (McKeon, et al., 1995; McKeon, et al., 1999; Asher, et al., 2000). Due to prolonged cellular dysfunction/death initiated at the time of injury, TBI may be considered both an acute inflammatory response as well as a longer-term neurodegenerative disease (McIntosh, et al., 1998). An increased understanding of the mechanisms underlying secondary cell
death and chronic glial scarring may eventually lead to protective treatments and the augmentation of repair and regeneration. Strategies of these types can potentially lessen the long-term effects of TBI; however, to date, no clinically affective treatments exist to combat altered signaling cascades, secondary death mechanisms, and the anti-regenerative post-injury environment.

Due to the prolonged degeneration and complex environmental alterations following TBI, it is likely that a sustained effort will be required to alleviate or reduce neurological disability. Cellular replacement therapy may be advantageous over pharmacological strategies due to the ability to dynamically target a wide range of biochemical alterations over a prolonged period of time; transplanted cells may produce, release, and process biomolecules in response to both external and internal signals. Transplanted cells may provide bulk trophic support, mediate cell-cell repair, and aid in the construction of neuronal circuitry given the proper choice of cell type and permissive microenvironment. Thus, cells with multipotent characteristics may have the ability to replace the function of missing or damaged cells and also to halt secondary damage in surrounding tissue. To this end, it may be advantageous to transplant multipotent neural stem cells (NSCs) after trauma, due to the ability to form multiple and specific cell phenotypes based on physical and temporal allocation of specific environmental cues. NSCs are capable of differentiating into the major cells of the CNS (neurons, astrocytes, and oligodendrocytes), a potential advantage when more than one cell type may be needed to mediate recovery of injured tissue (Rao, 1999; Whittemore, 1999; Gage, 2000). In addition, NSCs have been shown to produce trophic factors when transplanted into injured CNS tissue (Lu, et al., 2003; Yan, et al., 2004) and can rescue host cells from
degeneration (Ourednik, et al., 2002; Llado, et al., 2004). Transplantation of neural cells into the injured brain and spinal cord has been shown to attenuate degeneration and enhance functional recovery to some degree; however, this methodology has had limited success in terms of donor cell survival and graft integration (Vanderwolf, et al., 1990; Hoovler and Wrathall, 1991; Kanelos and McDeavitt, 1998; Cao, et al., 2002; Riess, et al., 2002; Lu, et al., 2003; Picard-Riera, et al., 2004; Boockvar, et al., 2005). NSC survival and integration, and hence the potential therapeutic benefits, are dictated by the characteristics of the post-injury degenerating and reactive astrogliotic environment; however, the dominant factors influencing NSC behavior upon transplantation have yet to be elucidated due to the complexity of the in vivo environment.

The goals of the current study were to deliver NSCs into a simulated injury environment, expressing mechanically-induced cell death and reactive astrogliosis, in order to elucidate factors influencing NSC survival and differentiation in a controlled setting. Accordingly, NSCs were delivered within mature 3-D neuronal-astrocytic co-cultures that had been subjected to the following experimental conditions: 1.) cytokine treated (TGF-β, 10 ng/mL); 2.) mechanically injured (0.50 shear strain, 30 s⁻¹ strain rate); or 3.) untreated control conditions. The effects of these treatments on co-culture viability and specific astrogliotic parameters have previously been characterized (see CHAPTER V). Briefly, the baseline conditions in untreated control co-cultures were determined to consist of viable (~90%) neuronal-astrocytic co-cultures, where the astrocytes mostly present a non-hypertrophic morphology, and the matrix surrounding the cells contains a modest CSPG content. Treating the co-cultures with TGF-β produced a robust astrogliotic response after two days of exposure, consisting of hypertrophic alterations in
astrocyte morphology and an increase in matrix CSPG content; however, there was no effect on culture viability. Subjecting the 3-D neuronal-astrocytic co-cultures to high rate mechanical deformation induced significant neural cell degeneration by two days following the insult (viability decreased to < 70%) and signs of developing astrogliosis (increased hypertrophy) that was significant by five days following the insult. This model permits evaluation of NSC survival and differentiation in a well-controlled injured/reactive environment, presenting a subset of potentially influential factors found following injury in vivo. In vitro models that are well-characterized, highly-controlled, and easy to manipulate and visualize are well-suited for mechanistic evaluation of factors influencing NSC survival and integration, and can therefore serve as valid pre-animal test-beds.

Materials and Methods

3-D Neuron-Astrocyte Co-Culture

Neurons were isolated from embryonic day 17 rat fetuses. Each fetus was removed by Caesarian section, rapidly decapitated, and the brains were removed. The cerebral cortices were isolated and digested in HBSS with trypsin (0.25% + 1mM EDTA) at 37°C for 10 minutes. The trypsin-EDTA was then removed and the tissue was triturated in HBSS containing DNase I (0.15mg/mL). The cells were centrifuged at 1000 rpm for 3 minutes and the cells were resuspended in plating medium (Neurobasal medium + 2% B27 + 1% G-5 + 500µM L-glutamine) immediately prior to 3-D plating.

Astrocytes were harvested from postnatal day 1 rat pups. The pups were rapidly decapitated, and the cerebral hemispheres were placed in sterile Hank’s Balanced Salt
Solution (HBSS). The isolated cortices were minced and the tissue fragments were then digested in trypsin (0.25% + 1 mM EDTA) for 3-5 minutes at 37°C, followed by DNase I (0.15 mg/ml) treatment and gentle mechanical trituration. Cells were then centrifuged at 1000 rpm for 3 minutes, dispersed in DMEM/F12 with 10% fetal bovine serum (FBS), and plated in a flask. Mechanically agitation was used to detach less adherent cell types at 24 and 72 hours and the primary astrocyte culture (> 95% type I astrocytes) was passaged upon reaching ~90% confluency for four weeks. Astrocytes were used between passages 4-12 for plating in 3-D culture (see below).

Cultures were plated in custom-made cell culture chambers consisting of a glass coverslip below a silicone-based elastomer mold (Sylgard 184 and 186, Dow Corning; Midland, MI; cross-sectional area = 2 cm²). Prior to plating, the chambers were pre-treated with 0.05 mg/mL poly-L-lysine (PLL, Sigma) followed by Matrigel (0.5 mL/well at 0.6 mg/mL, Becton Dickinson Biosciences; Bedford, MA) in Neurobasal medium (each treatment was > 4 hours). 3-D neuronal-astrocytic co-cultures were plated at a 1:1 initial neuron:astrocyte ratio at a density of 2500 cells/mm³ in Matrigel matrix (500-750 μm thick; final concentration 7.5 mg/mL). Following Matrigel gelation, plating medium was added to the co-cultures (Neurobasal medium + 2% B-27 + 1% G-5 + 500μM L-glutamine). Co-cultures were maintained at 37°C and 5% CO₂-95% humidified air and medium was replaced at 24 hours and every 2 days thereafter. Experiments were initiated at 21 days in vitro (DIV).

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. All cell reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO) unless
otherwise noted.

**Induction of Cell Death and Reactive Astrogliosis**

Neuronal-astrocytic co-cultures were subjected to mechanical deformation, cytokine treatment, or control conditions. Co-cultures were mechanically injured using the 3-D Cell Shearing Device (CSD), a custom-built electromechanical device capable of quantifiably imparting high strain rate shear deformation to 3-D cell-containing matrices (see CHAPTER III and (LaPlaca, et al., 2005)). At the time of injury, cultures were removed from the incubator and mounted within the device. The mechanical action of the device was driven by a linear-actuator (BEI Kimco; San Marcos, CA) governed by a custom-fabricated digital proportional-integral-derivative (PID) controller (25kHz sampling rate, 16 bit sampling resolution) with closed-loop motion control feedback from an optical position sensor (RGH-34, 400 nm resolution; Renishaw, New Mills, United Kingdom). A trapezoidal input was provided by code written in LabVIEW® (version 6.1, National Instruments; Austin, TX). Rapid horizontal motion of the cell chamber top plate with respect to the fixed base of the cell chamber imparts simple shear deformation to the elastically-contained 3-D cell-containing matrices (Figure 6.1). At 21 days *in vitro* (DIV), 3-D co-cultures were subjected to high rate deformation (strain 0.50, strain rate 30 s\(^{-1}\), n = 12) or left as control cultures (uninjured control, n = 12). After mechanical deformation, warm medium was added and the cultures were returned to the incubator. Also beginning at 21 DIV, a set of co-cultures were treated with transforming growth factor beta 1 (TGF-β1, 10 ng/mL diluted in co-culture medium) to serve as a positive reactivity control (n = 12).
Figure 6.1: Schematic representation of the 3-D neuronal-astrocytic co-culture (left) and mechanical deformation (right) models (not to scale). Neuronal-astrocytic co-cultures in 3-D were plated throughout a matrix and were laterally constrained by an elastomer mold. Mechanical deformation was imparted to cell-containing matrices through the action of the 3-D Cell Shearing Device (3-D CSD), a custom-built electromechanical device. A closed-loop control system (PID controller with positional feedback from an optical position sensor (OPS)) governs a linear actuator, causing horizontal displacement of the cell chamber top-plate inducing shear deformation in the elastomer mold and cell-imbedded matrices.

Neurosphere Isolation and Culture

Neural progenitor cells were harvested from timed-pregnant C57/BL6J mice containing a transgene expressing green fluorescent protein (GFP) to permit post-transplant identification. Dames were sacrificed on gestational day 14.5 and the embryos were removed by Cesarean section. The germinal zone was isolated and mechanically dissociated into a single cell solution in HBSS. Cells were maintained in suspension culture in serum-free DMEM:F12 media containing insulin (25 µg/mL), transferrin (100 µg/mL), putrescine (60 µM), sodium selenite (30 nM), progesterone (20 nM), and
glucose (6 µg/mL). Human recombinant FGF2 (20 ng/mL) was added fresh every other day to maintain the cells as proliferating neurospheres, which were passaged every 7-10 days. Neurospheres were used between passages 3 - 6 for transplantation.

**Delivery of NSCs into 3-D Neuronal-Astrocytic Co-Cultures**

NSCs were delivered via controlled injection designed to mimic in vivo delivery protocols implemented by our group (Tate, et al., 2002; Shear, et al., 2004). Within a laminar flow hood, a micromanipulator was fixed to a stereoscope base to visualize the injection. At 48 hours (23 DIV) following mechanical loading, cytokine, or control conditions, most of the medium was removed (leaving 100 µL) from the cultures and NSCs were delivered within the 3-D neuron-astrocyte co-cultures using a micro-syringe (Hamilton, 10 µL) gradually penetrating to a depth of 200 µm below the culture surface. The delivery consisted of 2.5 µL containing 1.5 x 10⁴ cells (dispersed in medium) delivered over 1.5 - 2.0 minutes. The cultures were returned to a tissue culture incubator for 60 minutes, after which 500 µL medium (without G-5) was added per culture.

**NSC Outcome Measures (see Figure 6.2 for experimental time-line)**

At various time-points following NSC delivery (60 minutes, 24 hours and 72 hours) the 3-D co-cultures were visualized using low-magnification fluorescent microscopy to assess the presence and distribution of the NSCs. At 24 and 72 hours following delivery, 3-D neuronal-astrocytic co-cultures (+NSCs) were fixed with 3.7% formaldehyde (Fisher, Fairlawn, NJ) for 60 minutes and then placed in 30% sucrose (Sigma) overnight at 4°C. Co-cultures were then placed in OCT Embedding Compound (Sakura, Tokyo, Japan) flash frozen in liquid nitrogen, sectioned on a cryostat (20 µm thick), and mounted on glass slides.
Assessment of NSC survival

Assessment of NSC dispersion and differentiation

21 DIV  23 DIV  24 DIV  26 DIV

3-D co-cultures subjected to:
1. TGF-β treatment
2. mechanical injury
3. control conditions

NSCs delivered

Assessment of NSC dispersion and differentiation

Figure 6.2: Timeline of experiments.

Quantity and 3-D Distribution

The total number of NSCs per sample was quantified at 72 hours following delivery into 3-D co-cultures by counting GFP+ cells in sequential horizontal sections throughout the entire thickness of a sample. The 3-D distribution of GFP+ NSCs was then determined based on the presence of GFP+ cells as a function of position throughout the 3-D co-cultures (n = 3 - 4 per group) (see Data Collection and Statistical Analysis).

Differentiation

The expression of specific neural phenotypic markers was assessed at 72 hours following transplantation using immunocytochemistry. Sections containing NSCs were chosen prior to staining (n = 3 – 4 per group; each sample was stained in triplicate). Briefly, sections were rinsed in PBS and permeabilized using 0.3% Triton X100 (Kodak, Rochester, NY) + 8% goat serum (Invitrogen) for 60 minutes. Primary antibodies were added (in PBS + 0.1% Triton X100 + 2% serum) overnight at 4°C covered in a
humidified chamber. After rinsing, the appropriate secondary fluorophore-conjugated antibodies (TRITC-conjugated IgG, Jackson Immuno Research or Alexa 546-conjugated IgG, Molecular Probes) were added (in PBS + 0.1% Triton X100 + 2% serum) for 2 hours at 18-24°C covered in a humidified chamber. Sections were immunostained using primary antibodies recognizing: 1) glial fibrillary acidic protein (GFAP) (MAB360, 1:400, Chemicon, Temecula, CA), an intermediate filament found in astrocytes (Debus, et al., 1983), 2) Tuj-1 (β-tubulin, MMS-435P, 1:2000; Covance, Denver, PA), an intermediate filament found in immature neurons, 3) neuronal-glial antigen-2 (NG2) (AB5320, 1:200; Chemicon), a CSPG expressed by oligodendrocyte precursor cells (and other cell types), and 4) nestin (MAB353, 1:200; Chemicon), an intermediate filament expressed by NSC remaining undifferentiated and by reactive astrocytes. Counterstaining was performed using Hoechst 33258 (1:1000, Molecular Probes).

**Cell Death**

The number of NSCs with DNA strand breaks and DNA fragmentation (indicative of cell death) was determined at 24 and 72 hours post-delivery. Sections containing NSCs were chosen prior to staining (n = 3 – 5 per group per time-point; each sample was stained in triplicate). Sections were stained using a commercially available terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) labeling kit (NeuroTACS II; Trevigen, Gaithersburg, MD). Briefly, cells were permeabilized using NeuroPore for 30 minutes at 18-24 °C. After rinsing, the slides were immersed in TdT labeling buffer for 5 minutes, followed by the addition of labeling reaction mix (containing TdT d NTP mix, Mn²⁺, TdT enzyme and labeling buffer) for 60 minutes humidified at 37 °C. Next, the slides were immersed in a TdT stop buffer
followed by rinsing. Then, strep-HRP was added for 10 minutes humidified at 18-24 °C. After rinsing, the slides were immersed in a DAB solution followed by a counterstain solution. Microscopic analysis was performed to assess the number TUNEL+ nuclei colocalized with GFP.

**Data Collection and Statistical Analysis**

Cultures were viewed using fluorescent microscopy techniques on an epifluorescent microscope (Eclipse TE300, Nikon, Melville, NY) or a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). For epifluorescent microscopy, images were digitally captured (DKC5T5/DMC, Sony, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Confocal images were analyzed using LSM Image Browser (Zeiss). At 72 hours following delivery, the total number of GFP+ cells was quantified and the group mean was calculated. The 3-D distribution of GFP+ cells was calculated as the distance along the z-axis containing 95% (3 standard deviations) of the GFP+ cells. NSC survival was assessed using epifluorescent and brightfield microscopy (Eclipse 80i, Nikon, Melville, NY) through Stereoinvestigator (Microbrightfield) based on co-localization of TUNEL+ nuclei with GFP, and the mean percentage of TUNEL+ NSCs was calculated per group per time-point. Similarly, NSC phenotypic expression was assessed via colocalization of GFP with the primary/TRITC or Alexa 546-conjugated secondary antibody specific for a particular marker. Data are presented as mean ± standard deviation. General linear model ANOVA was used with 3-D co-culture treatment (control, cytokine-treated, or mechanically injured) and time after delivery (24 or 72 hours) as independent variables and NSC outcomes as dependent variables (e.g., survival, phenotype). Post hoc Tukey’s
pairwise comparisons were then performed (for either test $p < 0.05$ required for significance).

**Results**

The goal of this study was to utilize an *in vitro* model presenting elements of the injured and reactive astrogliotic environment to assess the influences of local cell death and reactive astrocytes on neural stem cell (NSC) survival and integration. Accordingly, neural stem cells (NSCs) were delivered into 3-D neuronal-astrocytic co-cultures subjected to TGF-β treatment (inducing astrogliosis while not affecting viability), mechanically injured (inducing cell death and some elements of astrogliosis), or control conditions (minimal astrogliosis with high viability).

**Neural Stem Cell Delivery and Distribution**

At various time-points following delivery into 3-D co-cultures, fluorescent microscopy was used to visualize the presence and distribution of NSCs in culture. A primary cluster of NSCs was clearly visible at 60 minutes and 24 hours following delivery (Figure 6.3). At 24 hours following delivery, the localized fluorescence decreased indicating fewer NSCs clustered at the delivery site. This may be due to NSC death or dispersion/migration away from the delivery site. After fixation and processing of the sections, a total count of the GFP⁺ NSCs was acquired for the different groups. At 72 hours following delivery, there was not a significant difference in the total count of NSCs between the different co-culture treatments. These data were used to calculate the distribution of the NSCs throughout the cultures, which was defined as the thickness containing 95% of the cells. There was no statistical difference in the 3-D distribution of the NSCs between these groups (Figure 6.4).
Figure 6.3: Delivery of NSCs to mechanically injured, cytokine treated, or control 3-D co-cultures. NSCs were delivered into 3-D neuronal-astrocytic co-cultures subjected to (A-B) control conditions, (C-D) TGF-β treatment, or (E-F) mechanical loading (0.50 strain, 30s^{-1} strain rate). Fluorescent photomicrographs showing the primary cluster of NSCs at (A,C,E) 60 minutes and (B,D,F) 24 hours following delivery. At 24 hours following delivery, the localized fluorescent has decreased indicating fewer NSCs clustered at the delivery site. Scale bar = 50 μm.
Figure 6.4: 3-D Distribution of NSCs. The thickness across the 3-D co-cultures containing 95% of the NSCs was calculated as the NSC dispersion. There was not a significant difference between the 3-D distributions of NSC between the different groups.

Neural Stem Cell Differentiation

The patterns of phenotypic marker expression of NSCs were assessed at 72 hours post-delivery via immunocytochemistry for several neural phenotypic markers. In each of the co-culture treatment groups, GFP$^+$ NSCs co-labeled with nestin (intermediate filament found in undifferentiated NSCs as well as in reactive astrocytes), GFAP (intermediate filament expressed by reactive astrocytes), NG2 (CSPG expressed by various neural sub-types), and Tuj-1 (intermediate filament expressed by immature neurons) (Figure 6.5.1). There was some degree of NSC differentiation independent of environmental parameters used in this study. In fact, ~50% of the NSCs labeled for one of these four markers across all groups. Also, there were two distinct morphologies of nestin$^+$ NSCs, one that was process bearing (usually interwoven with host nestin$^+$ processes) and another that was more spherical. This may indicate the difference
between NSCs that were following an astrocytic lineage versus those remaining undifferentiated; however, further analysis would have to be performed to test this hypothesis. The majority of NSCs differentiated to express GFAP (~30% across all groups), followed by nestin, NG2, and Tuj-1. However, there was not a significant difference in the differentiation profiles between NSCs derived into the different cocultures treatments, potentially indicating that differentiation was governed by the properties of the matrix (e.g., laminin-rich) rather than factors associated with degeneration or reactive astrogliosis (Figure 6.5.2).
Figure 6.5.1: Photomicrographs of NSCs expressing phenotypic markers. NSCs were immunolabeled for specific phenotypic markers at 72 hour following delivery into control, TBF-β treated, or mechanically injured 3-D co-cultures. GFP⁺ NSCs co-labeled with immunostaining recognizing (A) nestin, an intermediate filament found in undifferentiated NSCs as well as in reactive astrocytes; (B) GFAP, an intermediate filament expressed by reactive astrocytes; (C) NG2, a CSPG expressed by various neural sub-types; and (D) Tuj-1, an intermediate filament expressed by immature neurons. Scale bar = 10 µm.
Figure 6.5.2: Plot of NSC phenotypic expression patterns. NSCs expressed various phenotypic markers at 72 hour following delivery into control, TGF-β treated, or mechanically injured 3-D co-cultures. The majority of NSCs expressed GFAP, followed by nestin, NG2, and Tuj-1. However, there was not a significant difference in the expression profiles between NSCs derived into the different co-cultures groups.

Neural Stem Cell Survival

NSC survival was assessed at 24 and 72 hours following delivery using a standard TUNEL assay, which indicates dying cells by labeling fragmented DNA. Few TUNEL$^+$ NSCs were found in either the control, TGF-β treated, or mechanically injured 3-D co-cultures at 24 hours following delivery. In each of these groups, only ~5% of the NSCs were TUNEL$^+$, with no statistical difference in the percentage of TUNEL$^+$ NSCs at this time-point. At 72 hours following delivery, the pattern of TUNEL$^+$ NSCs remained unchanged in the control and TGF-β treated co-cultures; however, there was a sharp increase in the percentage of TUNEL$^+$ NSCs delivered into mechanically injured 3-D co-cultures, which rose to ~30% (Figure 6.6.1). There were often clusters of NSCs in
mechanically injured co-cultures that presented a relatively large number of dying NSCs (Figure 6.6.1). This increase in TUNEL$^+$ NSCs was statistically significant compared to the NSC survival in the control and TGF-β treated co-cultures (Figure 6.6.2).

Figure 6.6.1: Photomicrographs of NSCs labeled using a TUNEL assay. Images of NSCs 72 hours following delivery into 3-D co-cultures subjected to (A) control conditions, (B) TGF-β treatment, and (C) mechanical loading (TUNEL$^-$ nuclei appear brown). Scale bar = 10 µm.
Figure 6.6.2: Plot of TUNEL$^+$ NSCs. NSCs were labeled using TUNEL at 24 and 72 hours following delivery into control, TBF-β treated, or mechanically injured 3-D co-cultures. At 24 hours following delivery, there was not a significant difference in the percentage of TUNEL$^+$ NSCs between the different co-culture groups. However, by 72 hours following delivery, there were significantly more TUNEL$^+$ NSCs in the mechanically injured 3-D co-cultures than in the control and TGF-β treated co-cultures\(^*\) (p < 0.05).

**Discussion**

This study has demonstrated the utility of a well-controlled, 3-D *in vitro* model to serve as a test-bed for the elucidation of factors in a reactive/degenerating environment that may influence NSC survival and integration. This test-bed consisted of 3-D neuronal-astrocytic co-cultures, the basis of which was independent control of localized cell death and astrogliotic parameters. Specifically, NSCs were delivered within control
3-D co-cultures (exhibiting minimal astrogliosis with > 90% cell viability) or into 3-D co-cultures 48 hours following administration of TGF-β treatment (producing significant markers of astrogliosis with no effect on cell viability) or high rate mechanical loading (exhibiting significant cell death and some markers of developing astrogliosis). At 72 hours following delivery of NSCs into the various 3-D co-cultures, there were no differences in the total numbers of NSCs, their 3-D distributions, or their differentiation profiles; however, NSC survival was negatively affected by delivery into mechanically injured co-cultures, causing a near 6-fold increase in the percentage of dying NSCs compared to that of control or TGF-β treated co-cultures.

Across all co-culture conditions, there was on average only 1/6 of the target delivery number of $1.5 \times 10^4$ NSCs counted in the processed sections 72 hours following delivery. This may indicate variability in delivery or processing, variable initial adhesion within the matrix, or NSC death across all groups. NSCs may have been pushed through the porosity of the matrix as injection occurred or may have been rinsed away as medium was added to the cultures, only permitting a subset of the NSCs to adhere within the matrix. Widespread NSC cell death is also possible, although the mean percentage of TUNEL+ NSCs for the control and TGF-β treated cultures was only 6.0% and 3.5%, respectively, making this possibility less likely; however, NSC death may have occurred within the first 24 hours following delivery. Furthermore, analogous problems arise following NSC delivery in vivo, leading to difficulty in locating and quantifying NSCs upon histological processing, and resulting in only a small subset of the delivered NSCs to be available for subsequent analysis. Although most in vivo transplant studies fail to examine NSC presence at early time-points, making it difficult to rule out the possibility
that specific parameters of the delivery process are detrimental to NSC survival. For instance, a high density of NSCs being delivered may lead to hypoxic shock upon transplant or shear stresses upon needle injection may physically damage all of some of the NSCs. Furthermore, NSC passage and culture has traditionally been performed at ambient oxygen concentrations of ~20%, whereas the oxygen tension in the brain is less than 5%, a dramatic reduction that may shock the NSCs upon transplant. Due to a combination of these possibilities, and likely others, it is believed that most NSCs delivered in vivo do not survive, but the questions of when and how these cells fail to survive has not thoroughly been addressed, underscoring the need to better understand delivery parameters and host environmental factors that may be inducing NSC death.

The model presented in this study may provide a suitable testing ground to evaluate some of the issues surrounding the delivery process, permitting improvement without the sacrifice and labor involved with additional animal studies.

Assessment of the 3-D distribution of the NSCs after delivery was chosen as a potential metric of NSC migration, albeit an indirect measure. There are factors in the reactive astrogliotic environment that may influence NSC migration. For instance, there are changes in cell-cell surface receptors on astrocytes that may make them more permissive for migration. Also, the secretion of soluble factors and ECM alterations may affect NSC migration. The ECM component laminin, produced by reactive astrocytes and a major component of the matrix used in this system, has been found to promote NSC adhesion and migration in 2-D in vivo (Tate, et al., 2004). NSCs delivered in vivo do not appear to be hindered by the physical boundary and ECM composition of the glial scar, and have been reported to migrate through it, and can continue relatively large
distances through the injured brain (Tate, et al., 2002). NSCs have been reported to secrete specific matrix-metalloproteases (e.g., MMP-2) which may permit enzymatic access through matrix elements of the glial scar (Heine, et al., 2004). At the relatively early time-points investigated in this study, there was not a difference in the dispersion of NSCs based on the different co-culture conditions. NSC migration could be more thoroughly addressed by repeated confocal imaging of the same cultures at earlier as well as later time-points. Furthermore, the addition of specific function-blocking antibodies could elucidate cellular and matrix characteristics that are permissive or inhibitory for 3-D NSC migration.

The differentiation profile of the NSCs was assessed following delivery using a range of neural cell markers. The panel of markers chosen was based on the in vitro differentiation of NSCs as well as that following delivery into the traumatically-injured brain (Tate, et al., 2002; Shear, et al., 2004; Tate, et al., 2004). Our analysis showed that the majority of NSCs were GFAP⁺, likely following an astrocytic lineage, which may be the default cell type for in vitro differentiation (Tate, et al., 2004). In a recent long-term in vivo transplant study, the majority of the NSCs were found express NG2⁺, a marker expressed by multiple neural cell types (Shear, et al., 2004). Following delivery into 3-D co-cultures, a small percentage of NSCs began to express this marker; however, questions remain as to the lineage progression of these cells. A number of NSCs also expressed nestin, an indication of remaining in an undifferentiated state, or conversely, progressing towards a astrocytotic state. Finally, a relatively small percentage of NSCs were progressing towards a neuronal phenotype (as assessed by Tuj-1 staining). However, this neuronal yield is high (and may be due to the high laminin content in the matrix).
compared to other work characterizing differentiation *in vitro*, often revealing GFAP\(^+\) (astrocytes) and nestin\(^+\) (undifferentiated) with a paucity of neuronal marker expression without specific manipulation (Santa-Olalla and Covarrubias, 1995) (Tate, et al., 2004). In future studies, other lineage markers may be utilized to more fully explore the differentiation profiles of NSCs under these environmental conditions, and it may also be desirable to stain for markers of mature phenotypes (e.g., heavy chain neurofilament in neurons) and assess metrics of cell functionality (e.g., propagation of action potentials in neurons). Furthermore, potential differences in the differentiation patterns of NSC delivered into injured/reactive co-cultures versus controls may take longer than 72 hours to manifest, thus necessitating longer time-points.

NSC differentiation has been found to be dependent upon a number of intrinsic and extrinsic factors. Intrinsic factors include location in the CNS, time of harvest, and culture passage number, factors that were maintained as consistently as possible for these experiments. Conversely, extrinsic factors found in the NSC microenvironment such as cytokines, growth factors, matrix constituents, and host cell parameters (reactivity, size, density) may have profound effects on differentiation (Irvin, et al., 2003; Mondal, et al., 2004). A subset of these extrinsic factors may have been different between co-cultures subjected to TGF-\(\beta\) treatment, mechanical injury, or control conditions. Growth factors and cytokines potentially secreted by reactive astrocytes may influence NSC differentiation and proliferation. For instance, epidermal growth factor (EGF), transforming growth factor-\(\alpha\) (TGF-\(\alpha\)), and basic fibroblast growth factor (bFGF) have been found to influence proliferation, whereas nerve growth factor (NGF) and TGF-\(\beta\) have not been found to induce proliferation (Santa-Olalla and Covarrubias, 1995; Irvin, et
al., 2003). Further studies would have to be performed in this system to assess the effects on NSC proliferation, but given the number of factors potentially produced by reactive astrocytes that may influence mitotic behavior, this issue merits further exploration. Furthermore, in a number of precursor cell types, TGF-β has been found to regulate differentiation (Moses and Serra, 1996). However, regulation of NSC differentiation may be more complex, and may rely on other factors such as cell-cell/cell-matrix interactions or exposure to specific growth factors in combination. For instance, neuronal differentiation and neurite outgrowth were enhanced by administering bFGF in combination with EGF compared to each factor alone (Santa-Olalla and Covarrubias, 1995). The complex differentiation-regulating pathways apparent in vivo are only made more confounding by the post-injury environment, thus the 3-D system developed in this study may provide an intermediate level of complexity for the elucidation of specific regulatory mechanisms prior to evaluation in vivo.

NSC survival was found to be negatively affected by the post-injury environment, but not the astrogliotic environment alone, suggesting that there are factors associated with the neural response to injury that are detrimental for NSC survival. In the post-injury environment, detrimental effects may be non-specific or they may specifically activate degenerative signaling pathways. Non-specific effects may be mediated by an unregulated extracellular microenvironment initiated by local cell death in the 3-D neural co-cultures, potentially resulting in loss of ionic/osmotic homeostasis and unregulated enzymatic activity. Also, changes in Ca^{2+} homeostasis, associated with delayed neuronal death and degeneration, have been implicated with the activation of potent degrading proteases and the generation of oxygen free radicals, which may damage transmembrane
proteins and membrane phospholipids, respectively (McIntosh, et al., 1999). The post- 
injury environment may more specifically affect NSCs due to the action of some of the 
neurochemical mediators of secondary neural injury, of which this in vitro model 
potentially contains a subset. Injured neurons, glia and other cell types within the brain 
may synthesize and secrete inflammatory cytokines such as tumor necrosis factor-α 
(TNF-α) and interleukins (IL-1, IL-1β, IL-6). These factors have been implicated in 
excitotoxic neurodegeneration, inflammation, and may mediate diffuse neuronal damage 
(McIntosh, et al., 1999). In addition to proinflammatory effects, both IL-1β and TNF-α 
may mediate the synthesis and release of potentially neurotoxic molecules such as 
arachidonic acid and its metabolites. Furthermore, increases in excitatory amino acids 
(e.g., glutamate, aspartate) or monoamine neurotransmitters (e.g., norepinephrine, 
epinephrine) are found following brain injury, but it is unclear if NSCs will respond to 
these compounds specifically. Conversely, NSC survival may be supported by the 
presence of neurotrophic substances (growth factors) which increase following injury. 
Such neurotrophic factors are NGF, bFGF, brain-derived neurotrophic factor (BDNF), 
glial-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3). Some of these 
growth factors may have been released by reactive astrocytes in this model; therefore, 
NSCs delivered into the astrogliotic co-cultures may have been exposed to pro-survival 
growth factors without potentially damaging inflammatory cytokines.

This work may effectively be followed up with studies to systematically test 
specific elements of the injured environment that may be exerting influence on NSC 
survival. This may be accomplished by deactivating or removing factors hypothesized to 
exert a negative influence on survival, promoting access to or concentrations of pro-
survival factors, or modifying the NSCs prior to delivery to better survive in this injured environment. Information derived from such studies may immediately feed into in vivo delivery methodologies in order to promote maximum NSC survival and integration after delivery.

Much of the work evaluating interactions between NSCs and other neural cell types has been done using 2-D in vitro models or a 2-D interface with a 3-D ex vivo preparation (e.g., cells placed above a slice of brain tissue). Although this work has produced much valuable information as to factors that may influence NSC survival, growth, and differentiation, fundamental differences between these test environments and 3-D interfaces should be noted. When cells are placed above a culture system, cell-cell and cell-matrix interactions differ markedly as growth and migration may occur over an unobstructed surface, whereas growth and migration through a 3-D environment often requires physical or enzymatic burrowing. These differences in matrix interactions and cell morphology have been found to result in different profiles and distributions of cell-cell and cell-matrix receptors (Cukierman, et al., 2001; Cukierman, et al., 2002; Schmeichel and Bissell, 2003; Yamada, et al., 2003). Also, the local microenvironment may differ as secreted and/or soluble factors may be diluted into the culture medium, whereas in 3-D they may be maintained locally or become matrix-bound, possibly leading to a more in vivo-like presentation and access to these factors. Also, cells cultured in a 3-D environment have been shown to better represent in vivo cellular behavior than cells cultured in monolayer (Fawcett, et al., 1989; Fawcett, et al., 1995; Granet, et al., 1998; Wang, et al., 1998; Grinnell, 2000), and accordingly have been found to exhibit higher compatibility and survival upon transplantation in vivo (Fawcett, et al.,
1995). Overall, results gained from 2-D interface systems must be balanced against the potentially altered presentation of matrix interactions, cell-cell couplings, and access to secreted factors.

Further complexity may be added to this model to systematically evaluate the effects of a given variable on NSC survival and integration. For instance, a potentially dominant influence on NSC survival in vivo may be mediated by microglia, activated scavenger cells recruited and expanded at a site of CNS injury. The absence of microglia may equate to the removal of a negative factor on NSC survival as reactive microglia may represent a significant portion of the initial host interface with transplanted cells, and may directly and potently induce death of transplanted NSCs (e.g., via free radical release). The goal of this initial work, however, was to elucidate the effects of mechanically-induced cell death and specific elements of the reactive astrogliotic response (e.g., ECM-CSPG and hypertrophic astrocytes) on NSC survival and integration. However, it would be valuable for subsequent iterations of this model to assess the influence of such additional factors on NSC behavior.

This work has developed a highly simplified, yet versatile, in vitro model to systematically elucidate factors in an injured/reactive environment that may exert influence over NSCs. Advantages of this model are the 3-D interface, high degree of experimental control and ability to expand in complexity, approaching that of an in vivo interface, in a systematic manner. This system was developed to utilize an in vivo-like, 3-D interface, which differs markedly from other 2-D interface systems. Also, the cell types present, ratios of those cell types, cell density and degree of culture development may be experimentally controlled. The matrix constituents, concentrations, and physical
and mechanical properties may also be controlled, ranging from what is considered bioactive to bioinert, with variable levels of permissively or inhibition. Furthermore, this 3-D co-culture interface system may be subjected to a range of molecular treatments (e.g., cytokines or growth factors), biochemical insults (e.g., excitotoxicity, hypoxia) or mechanical injuries (e.g., variable levels of focal or diffuse insults). Collectively, this degree of control provides a broad experimental framework, which may be incrementally expanded upon to add other variables present in the \textit{in vivo} situation, allowing precise experimental manipulation of the interface environment prior to introduction of NSCs. Also, a more broad application of this model is possible to assess interfaces with other neural cell types, differentiated or non-differentiated, such as neurons (to assess integration and axonal in-growth) or myelinating cells (e.g., oligodendrocytes, Schwann cells; to assess function). Further exploitation of this system may allow a comprehensive characterization of factors in an injured environment that may direct NSC survival, migration, and differentiation and lead to a more controlled manipulation of that environment \textit{in vivo} to exert maximum benefit from NSC related treatment for CNS disorders.

\textbf{Conclusions}

This study has demonstrated the utility of a well-controlled, three-dimensional \textit{in vitro} model, exhibiting elements of the reactive/degenerating environment found following injury \textit{in vivo}, to serve as a test-bed for the elucidation of dominant modes through which this environment may influence NSC survival and integration. Specifically, the ability to delivery NSCs into this 3-D environment was demonstrated
and the baseline reaction of NSCs was described based on survival and integration parameters. This work revealed that elements present in a mechanically injured/degenerating environment, but not those in a purely reactive environment, negatively influence NSC survival at very relatively early time-points following delivery. However, the differentiation pattern of these NSCs was less affected by these parameters and progress largely independent of environmental differences. Further modeling of the interface between injured/reactive tissue and NSCs will allow mechanistic studies of survival and integration for use in optimization of tissue engineered scaffolds, which may mediate maximal neural stem cell survival and controlled differentiation and integration. This research may impact cell transplantation strategies and provide a mechanistic basis for the development of clinically effective treatments for TBI as well as other CNS disorders.
CHAPTER VII

CLOSING

Summary

Traumatic brain injury (TBI) remains a major clinical problem with few effective treatments and often results in persistent dysfunction due to complex secondary cascades and the poor regenerative capabilities of the brain. The severity of neurological disability depends on the initial insult and the ensuing deleterious cascades, which may be complex and persistent. TBI results from mechanical loading to the head and therefore models that seek to reveal injury mechanisms should accurately simulate the related biomechanics. In vitro models are invaluable in systematic elucidation of cell behavior in a highly controlled setting; however, the interpretation of cellular responses in traditional planar (2-D) models may be confounded by altered cell-cell/cell-matrix interactions and atypical cellular morphology. Three-dimensional models consisting of multiple neural cell types are capable of maintaining many positive aspects of in vitro modeling while closer approximating the cytoarchitecture of the brain.

This work has succeeded in the development of a robust experimental platform consisting of a versatile, 3-D neural co-culture system used in conjunction with a biomechanically well-characterized device capable of subjecting 3-D cell-containing matrices to variable loading regimes. These systems were characterized independently, exploring fundamental neurobiological phenomena and addressing the role of 3-D biomechanics in the response of neural cells to high rate deformation. The novel culture
system and injury paradigm were then used together as 3-D neural co-cultures were subjected to various loading regimes to assess the roles of local cell death and direct mechanical deformation on reactive astrogliotic induction. Finally, this controlled model of a degenerating / reactive astrogliotic environment was interfaced with neural stem cells (NSCs) to serve used as an in vitro test-bed for the elucidation of microenvironmental parameters influencing NSC survival and integration. The successful application of technologies developed through this work may be further developed for the realization of practical devices and robust experimental platforms.

Conclusions

Development of 3-D Neural Cultures

This work developed and optimized two 3-D neural cell culture systems: 1) primary cortical neurons, and 2) primary cortical neurons and astrocytes in co-culture. The roles of culture dimensionality and neuron-astrocyte interactions on basic neurobiological phenomena were elucidated. Cell morphology and cytoarchitecture differed markedly between cells in 2-D and 3-D. Viability was found to be dependent on cell density in 3-D, suggesting important mass transport thresholds given passive diffusion. Also, the presence of astrocytes improved culture viability and also enhanced synapse formation, implicating astrocytes in maintaining neuronal health and function. Similarly, neuronal presence in 3-D co-culture with astrocytes influenced the regulation of astrocytic proliferation. This work characterized baseline parameters in 3-D neuronal-astrocytic co-cultures for further exploration in the neural response to trauma.
Characterization of the 3-D Cell Shearing Device

This work developed and characterized the 3-D Cell Shearing Device (CSD), a novel electromechanical system capable of delivering a reproducible, high magnitude, high rate strain field to 3-D cell cultures in an extracellular matrix scaffold. Theoretical analysis revealed that these parameters generate a heterogeneous 3-D strain field throughout the cultures that is dependent on initial cell orientation within the matrix, resulting in various combinations of normal and shear strain. In addition, cell death was induced following high rate, high magnitude shear strain to 3-D neuronal cultures and neurite degeneration depended on orientation, with higher predicted shear strain correlating with an increased loss of neurites. These results indicate a heterogeneous neuronal response to loading dependent on characteristics of the strain field at the cellular level.

Biomechanics in 2-D versus 3-D

This work examined the effects of neuronal culture dimensionality on the response to a defined mechanical insult. After high rate loading, there was a significant decrease in neuron viability in both 3-D and 2-D; however, neurons in 3-D presented greater cell death based on matched bulk loading parameters. Computer simulations predicted local cellular strains experienced by neurons in 3-D or 2-D, revealing that neurons in 3-D were subjected to a heterogeneous strain field simultaneously consisting of tensile, compressive and shear strains; conversely, neurons in 2-D experienced a less complex, shear-dominated deformation regime. These results show differential susceptibility to mechanical loading between neurons in 2-D and 3-D that may be due to differences in strain manifestation at the cellular level.
Development of an *In Vitro* Degenerating / Reactive Astrogliotic Environment

The study evaluated the augmentation of reactive astrogliosis using a 3-D *in vitro* model for a range of mechanical loading regimes to assess the effects of a direct mechanical insult and local cell death on astrogliotic induction. In 3-D neuronal-astrocytic co-cultures, there was a significant increase in cell death after moderate and high rate (10 and 30 s\(^{-1}\)) loading, but not after quasi-static (1 s\(^{-1}\)) loading or TGF-β treatment. There were also increases in 3-D cell density following moderate and quasi-static deformation, suggesting a hyperplasic response. TGF-β treatment induced robust astrogliotic alterations consisting of astrocyte hypertrophy and increased chondroitin-sulfate proteoglycan (CSPG) expression. Alterations in astrocyte hypertrophy were apparent after moderate rate loading, and at a later time-point following high rate deformation. However, CSPG expression following injury did not increase to the extent of TGF-β treated cultures, although soluble glycosaminoglycan content was maximal following quasi-static loading. These results demonstrate independent control of culture viability and specific reactive astrogliotic parameters based on mechanical injury levels.

Interfacing NSCs with an *In Vitro* Degenerating / Reactive Astrogliotic Environment

The study interfaced NSCs with a controlled, three-dimensional (3-D) *in vitro* model exhibiting traumatically-induced cell death and reactive astrogliosis to elucidate factors influencing NSC survival and differentiation. NSC survival was detrimentally affected following delivery into mechanically injured co-cultures, but not TGF-β treated co-cultures, signifying factors associated with local degeneration but not astrogliosis that negatively influence NSC survival. However, the 3-D distributions and differentiation patterns of the remaining NSCs were similar for the different environmental conditions.
Collectively, this study demonstrated the utility of an *in vitro* model as a test-bed for evaluating factors potentially influencing the efficacy of treatment strategies for neural injury.

**Directions for Future Work**

**Next Generation 3-D Neural Cultures**

Future iterations of *in vitro* models should maintain the experimental control of previous systems, but improve physiological relevance by systematically approaching *in vivo* complexity. This may be accomplished by the addition of other neural cell types, increases in 3-D cell density, and by use of ECM substrates found in the brain. The addition of other neural cell types may profoundly influence the baseline culture physiology and pathophysiology in response to an insult. The addition of myelinating cells may enhance neuronal function, and the addition of microglia may maintain culture health over time by removing debris. Furthermore, increases in 3-D cell density will enhance the degree of cell-cell interactions to more closely mimic brain. This enhancement is challenging and will involve the development of novel technologies to deliver nutrients and remove waste. Also, the use of ECM components found in the brain will permit engineering of more *in vivo*-like cell-matrix adhesions, and a necessary condition is that the cells be able to actively interface with the ECM and remodel it to their specifications. This is also challenging because ECM components in the mature brain (e.g., hyaluronan, proteoglycans) have generally been found to foster static conditions, and may not be suitable for the growth and development of dissociated cultures. Therefore, the solution may be to create a more developmental-like ECM which
may be replaced with more mature ECM complexes and/or deactivated to mimic mature conditions. Models incorporating these improvements may be exploited to study basic neurobiological phenomena, approaching the complexity of the brain and better recapitulating \textit{in vivo} function.

\textbf{Modeling Traumatic Brain Injury in 3-D}

The \textit{in vitro} model of neural trauma developed for these studies incorporates some elements of \textit{in vivo} injury (e.g., 3-D cell-cell/cell-matrix interactions, morphology, and cytoarchitecture) and may more accurately simulate the complex, heterogeneous cellular biomechanics associated with TBI. Having multiple cell types in this model, where the bulk shear strain field is constant throughout a 3-D matrix, would be valuable in determining the mechanical thresholds of different neural cell types based on local cellular strain. Cellular tolerances to mechanical insults should be derived from the cellular level to the tissue/brain region level; however, to date, most tolerance information has been derived from macroscopic models. Tolerance information from the model presented here, which reproduces complex, 3-D biomechanics, may be more accurate inputs into computer simulations used to predict failure in a particular brain region/layer. The next step in these studies will be a comprehensive separation of biophysical (e.g., cellular strain/stress analysis; physical failure criteria) from biochemical (e.g., cell death pathways; enzymatically-mediated failure) responses to injury. This may be accomplished in our 3-D model using permeability uptake as a function of orientation and will provide information directly linking localized failure with specific strain regimes.
A heterogeneous cell population in 3-D may more accurately represent secondary biochemical responses to injury and may model post-injury cell-cell interactions. Upon further establishment that 3-D multi-cellular cultures mechanically deformed with our model successfully recapitulate aspects of *in vivo* injury, the natural progression will be to use this model to assess treatment strategies (either pharmacological or cellular) in attenuating degeneration or enhancing recovery. These agents may target secondary mechanisms of cell death/dysfunction in an attempt to rescue cells or expedite the repair process. This model may provide a useful pre-screening ground for potential treatment prior to administration in more expensive and time-consuming animal studies. Overall, the neural trauma paradigm here presented, consisting of 3-D biomechanics and innovative neural cell culture models, has the potential to accurately approximate the *in vivo* injury situation with all of the control and visualization capabilities of traditional *in vitro* systems.

**Development of Reactive Astrogliotic Model**

The 3-D co-culture model presented in these studies may be further exploited in elucidating the complex mechanisms of astrogliotic induction. This model has an intermediate level of complexity between *in vivo* models and traditional 2-D cultures. The model presented here has the capability to separate certain induction factors such as direct cellular strain and cues from local injured/degenerating cells, and may better recapitulate signals involving 3-D cell-matrix interactions. Also, since this system has the capability to control the matrix constituents and cell types present, and that injury levels are controllable (providing control over degree of astrogliosis and cell death), this model may provide a powerful experimental framework for the study of a number of
neural responses to injury. For instance, a future iteration of this model may be used in conjunction with neurite outgrowth assays where the primary functional goal is the enticement of neurite outgrowth and/or cell migration through the injured matrices. Interface models in 2-D may not present inhibitory factors in an in vivo-like manner (matrix molecules or chemotaxic cues), and brain slice models are less amenable to experimental control. Future iterations of this model may serve as a test-bed for methodologies to modify an injured environment to make it more permissive for endogenous repair.

**Injured/Reactive Test-Bed for Neural Stem Cells**

The interactions between neural stem cells (NSCs) and the degenerating and astrogliotic brain remain poorly understood, and are difficult to elucidate due to the complexity of the system. Thus, initial attempts in delivering NSC into the injured brain have met with mixed results. The 3-D in vitro interface system presented in these studies may provide a powerful experimental framework for the mechanistic elucidation of specific factors present in the post-injury environment that may be beneficial as well as those that may be detrimental to NSC survival. The results obtained from this model may be fed into higher level models (in vivo) as well as lower level models (in vitro NSC culture). For instance, a two-pronged in vitro approach may be undertaken to refine factors in the injured environment that may be detrimental to NSCs. First, media from injured 3-D co-cultures can be added to NSCs in culture to test whether damaging factors are soluble, and if so, the time-course of action and the percentage of NSCs responsive to such factors. As candidate factors are considered from the literature, their presence could be tested (e.g., via mass spectrometry) and parallel tests could be done doping media with
one factor at a time to evaluate the effects of specific detrimental factors (based on concentration). A second in vitro approach will be to further utilize the injured 3-D co-culture test-bed with the addition of either 1) pro-survival/integration factors surmised from the TGF-β treated groups or 2) factors to inhibit the deleterious function of anti-survival/integration factors from the mechanically injured groups. This will further refine hypotheses as to what factors may be delivered with the NSCs to harness the pro-survival factors potentially secreted by reactive astrocytes and inhibit the negative factors released by dead/dying neural cells. However, prior to in vivo application, these results will have to be validated using a more complex iteration of the injured 3-D co-culture test-bed, specifically employing the used of microglia. If NSCs fare worse after the addition of microglia, then it will be known that additional factors need to be co-transplanted to inhibit the potentially detrimental influence of activated microglia. After this systematic characterization of factors in the injured/reactive environment that may influence NSC survival/integration in vitro, these techniques should then be translated to in vivo transplant studies to test their efficacy, where further refinement of co-transplanted factors may be necessary. Also, this framework may be utilized for the in vitro improvement of neural tissue-engineering strategies, which may enhance the survival and control of NSCs upon delivery. This model could serve as an in vitro test interface for transplantation of neurons, astrocytes (reactive or unreactive), myelinating cells (oligodendrocytes, olfactory ensheathing cells, and/or Schwann cells), or undifferentiated progenitor (stem) cells. The iterative, multi-level approach presented here will permit elucidation of the dominant factors affecting NSC survival upon delivery to an injured/reactive environment. Overall, this research may impact cell transplantation
strategies and provide a mechanistic basis for the development of clinically effective treatments for TBI as well as other CNS disorders.
APPENDIX A

3-D CELL SHEARING DEVICE

Overview

Neural cultures were mechanically loaded using the 3-D Cell Shearing Device (CSD), a custom-built electromechanical device capable of quantifiably imparting high strain rate shear deformation to 3-D cell-containing matrices (see also CHAPTERS III and IV and (LaPlaca, et al., 2005)). At the time of injury, cultures were removed from the incubator and mounted within the 3-D CSD. The mechanical action of the device was driven by a linear-actuator (BEI Kimco; San Marcos, CA) coupled to a custom-fabricated digital proportional-integral-derivative (PID) controller (25kHz sampling rate, 16 bit sampling resolution) with closed-loop motion control feedback from an optical position sensor (RGH-34, 400 nm resolution; Renishaw) (Figure A.1). A trapezoidal input was provided by code written in LabVIEW® software v6.1 (National Instruments; Austin, TX). Lateral motion of the cell chamber top plate with respect to the fixed base of the cell chamber imparts simple shear deformation to the elastically-contained 3-D cell-containing matrices.

3-D CSD Operation and Calibration

Open up the custom stimulation control program in LabVIEW®. To turn on the device, first turn the actuator power to “on”, then hold down the “calibrate” button while turning the main power to “on”. This will automatically calibrate the function and...
position of the actuator. In the LabVIEW® software, initiated the program sequence. Set the desired parameters in LabVIEW® including the movement of the top-plate (in millimeters; corresponding to shear strain magnitude) and the desired rise-time (in milliseconds; corresponding to strain rate), hold-time, and ramp-down time. The device will fire when the “stimulate” button in the software is pressed. The device may be mounted above a confocal microscope to image the deformation (Figure A.1).

![Diagram of 3-D Cell Shearing Device (3-D CSD) components.](image)

**Figure A.1: 3-D Cell Shearing Device (3-D CSD) components.** The device can be mounted on a confocal microscope to obtain 3-D images before, during, and after mechanical deformation. A closed-loop control system (PID controller with feedback from an optical position sensor (OPS)) governs a linear actuator, inducing motion of the cell chamber top-plate (not to scale).

**Application of Mechanical Deformation**

First, remove the cell culture chambers from the incubator and transfer to a laminar flow hood. Gently remove the feeding media from the cultures, being careful not to disturb the matrices. Slowly place sterile top-plates (with affixed elastomer mold) on
the injury chambers so that the cell-containing matrices are completely enclosed by
elastomer on five sides and the glass coverslip at the base (Figure A.2).

Figure A.2: 3-D CSD cell culture chamber components. The cell chamber consists of a top-plate with elastomer mold to interface with the 3-D cell cultures. The top plate is mounted above the cell reservoirs and connected to the linear actuator to impart variable rate deformation.

Transfer the enclosed chamber to the 3-D CSD and affix the top-plate to a horizontal extension plate attached to the linear actuator by two machine screws. The base of the chamber will be contained by a depression in a stainless-steel base plate so the chamber base is not free to move. Clamp down the top of the top-plate using the two free-spinning roller-bars. The roller bar adds minimal friction to the system while ensuring that the top-plate will not deflect vertically upon deformation. Stimulate the linear actuator through the LabVIEW® control program, providing a trapezoidal input to the system. This firing of the linear actuator will deflect the top-plate horizontally while the chamber base is held stationary (see Figure A.3). Static control cultures experience
all steps with the exception of device stimulation, thus controlling for the potential effects of media removal, top-plate application, and any temperature fluctuations. After the application of mechanical deformation or static control conditions, detach the chambers from the device and return to a laminar flow hood. Slowly remove the top-plate from the chamber base, add 500µL of pre-warmed media to each well, and return the chamber to a tissue culture incubator (37°C, 5% CO₂, 95% RH).

Figure A.3: Application of mechanical deformation. The horizontal motion of the linear actuator drives the displacement of the cell chamber top-plate, inducing shear deformation in the elastomer mold and matrix (not to scale).
Primary Cortical Neuron Cell Culture Methodology

Outline:

I. Harvest
II. Dissociation
III. Plating
IV. Assessment

Detailed Procedures:

I. Harvest

A. Notes

1. Time-pregnant (embryonic day 17 (E17)) Sasco Sprague-Dawley rats (Charles River)
2. This procedure may also be used for harvest from E18 rat pups
3. All surgical tools should be sterilized by autoclave prior to dissection
4. Dissection is performed within a laminar flow hood
5. Prior to dissection, fill 4-6 100 mm Petri-dishes with 15 mL each of sterile Hanks Balanced Salt Solution (HBSS, Gibco) and place on ice
6. If cortices will be stored rather than dissociated immediately, then prior to dissection, fill a 50 mL centrifuge tube with 25 mL of sterile...
HBSS, and place on ice (for rinse steps). If cortices will be
dissociated immediately following harvest, see “Section II.
Dissociation A. Notes” below.

B. Procedure

1. In fume hood, anesthetize using 2 mL halothane (2-bromo-2-chloro-
1, 1, 1-trifluoroethane, Halocarbon Laboratories) in glass bell jar for
minimum amount of time until animal is unconscious and
unresponsive to tail pinch

2. Rapidly decapitate using guillotine

3. Transfer carcass to sterile dissection hood

4. Lay carcass ventral side up and rinse abdomen thoroughly with 70%
ethanol

5. Cut beginning at the lower abdomen extending rostally to expose the
uterus

6. Remove uterus and place in a petri-dish with ice-cold HBSS

7. Remove each fetus from the amniotic sacs and transfer to a new
petri-dish containing ice-cold HBSS

8. Decapitate each fetus and remove brains by cutting and peeling back
the top portion of the skull

9. Transfer brains to a new petri-dish containing ice-cold HBSS

10. Cortical isolation

   a. Remove hindbrain and perform a midsaggital cut
b. Remove mid-brains and place hemispheres with lateral surfaces facing down

c. Remove olfactory bulbs and turn over hemispheres such that the medial surface faces down

d. Detach meninges and again turn hemispheres over such that lateral surface faces down

e. Remove cortical region from the remaining midbrain and cut the hippocampal region from the cortex (hippocampal regions may also be saved)

f. Transfer cortical regions to a 15 mL centrifuge tube (4-6 cortices per tube) and place on ice

11. Immediately proceed with dissociation procedure or follow storage procedure outlined in next step

12. Storage procedure

   a. Rinse 2X with cold HBSS (removing small debris)

   b. Add 2 mL L-15 supplemented with 2% B-27

   c. Wrap tube in Aluminum foil and place tube on side at 4 °C ensuring that all cortices are submerged

   d. For optimal viability, dissociate within 2 days (> 90% cells viable)

II. **Dissociation**

   A. Notes
1. This procedure is typically for 2-3 brains (4-6 cortical hemispheres); expected yield is 3-5 million cells/cortical hemisphere

2. Prior to dissociation, transfer 5 mL trypsin (0.25% + 1mM EDTA) (Invitrogen) and 400 µL deoxyribonuclease I (DNase, 1.5 mg/mL, Sigma) to 37°C water bath

3. Keep tissue/cells on ice throughout procedure (except trypsin step) and perform rinses with ice-cold Ca\(^{2+}\) and Mg\(^{2+}\)-free Hanks Balanced Salt Solution (CMF-HBSS)

4. Prior to dissociation, fill a 50 mL centrifuge tube with 25 mL of sterile CMF-HBSS and place on ice (for rinse steps, may be same tube prepared prior to dissection)

5. Rinses may be performed using a plugged Pasteur pipette or serological pipette

6. Prepare DMEM/F12 (Invitrogen) + 10% Fetal Bovine Serum (FBS, Invitrogen) and place on ice (to deactivate trypsin)

7. Prepare Neuronal Medium: 100 mL Neurobasal Medium + 2 mL B-27 + 250 µL L-glutamine (0.5 mM final L-glutamine concentration) and place on ice

B. Procedure

1. Acquire cortical (or hippocampal) region from E17 rat fetuses (cortices will be in HBSS or L-15 + B-27, see “Section I. Harvest”)

2. Rinse 2X with CMF-HBSS (remove small debris)
3. Add 5 mL trypsin (0.25% + 1mM EDTA) pre-warmed to 37°C, manually agitate tube once to mix and place tube in water bath
   a. At 5-7 min, manually agitate tube to mix and check clumping
   b. Check periodically – stop trypsinization if tissue is one large clump
   c. Stop at ~10 min (time can be up to 15 min depending on trypsin strength)
4. Remove trypsin being careful not to disturb tissue
5. Rinse with cold DMEM/F12 + 10% FBS (optional) and gently wash 2X with CMF-HBSS
6. Add 1.8 mL CMF-HBSS + 200-400 µL DNase (final concentration 0.15-0.30 mg/mL)
7. Vortex for 30 seconds and/or triturate 5-10 times with a flame-narrowed Pasteur pipette to break up tissue clumps (any clumps remaining after 10 triturations may be removed and discarded)
8. Centrifuge cells at 1000 rpm for 3 min
9. Aspirate supernatant and resuspend pellet in 2 mL of Neuronal Medium (to re-dissociate cells: use a serological pipette, a flame-narrowed Pasteur pipette or re-vortex).
10. Dilute cells to desired density
    a. Ensure the cell solution is well-mixed (gently triturate 3-5 times) and remove a small sample to dilute and count.
       Typically use a 1:40 dilution consisting of 10 µL cell solution
+ 190 μL HBSS and 200 μL trypan blue (let diluted cell solution sit for 30 seconds prior to trypan blue addition)

b. Ensure the diluted cell solution is well-mixed (gently triturate) and transfer 10 μL to each chamber of a Hemocytometer

c. Count cells on hemocytometer to determine total cell number and percent viability via trypan blue exclusion

d. Validation
   i. Total cell yield should be 3-5 million cells/cortical hemisphere. Do not proceed if yield is substantially less than this amount
   ii. If neuronal viability is less than 90% then do not proceed

e. Dilute with Neuronal Medium to desired density
   i. Sample 2-D cell density: $4 \times 10^5$ cells/mL
   ii. Sample 3-D cell density: $7.5 \times 10^6$ cells/mL

III. Plating

A. Solutions
   1. Neuronal Medium: 100 mL Neurobasal Medium + 2 mL B-27 + 250 μL L-glutamine (0.5 mM final L-glutamine concentration)
   2. Poly-L-Lysine (PLL, stock solution 0.10 mg/mL, dilute to working concentration of 0.05 mg/mL in di-H₂O prior to use (1:1 dilution))

B. Surface prep (specific to plating surface)
   1. Sterilize (e.g., autoclave, 70% EtOH, UV, flame (glass only))
2. PLL treatment (0.05 mg/mL)
   a. Add 250 µL/cm² and place in incubator (37°C, 5%CO₂, 95%RH) for 4-12hrs
   b. Aspirate PLL and let excess evaporate by leaving lid open under laminar flow hood for ~2 minutes

3. Optional protein pre-coat (e.g., Ln, Fn, Cn, Matrigel): 0.5-0.6 mg/mL, 250 µL/cm² and place in incubator (37°C, 5%CO₂, 95%RH) for 4-12hrs

C. Procedure

1. Add cells
   a. 2-D final density: 50,000-125,000 cells/cm², volume = 250 µL/cm²
   b. 3-D final density: 3.75-5.00x10⁶ cells/cm³, add appropriate volume for desired thickness, final Matrigel concentration = 7.5 mg/mL

2. Place cultures in incubator (37°C, 5%CO₂, 95%RH)

3. Feed/Change Media
   a. 2-D: change media at 24 hours post-plating and feed every 3-4 days thereafter by replacing 50% media
   b. 3-D: add feeding media at 4-8 hours post-plate, feed at 24 hours and every 3-4 days thereafter by replacing 50% media

4. Remove DNA fragments (2-D only, optional)
a. At 24 hours post-plate, aspirate medium and treat with DNase (7.5 µg/mL) in HBSS for 5 min
b. Aspirate DNase and rinse 2X with HBSS
c. Add Neuronal Medium and return cultures to incubator

IV. **Assessment** (at 14 days *in vitro* (DIV))

A. Cell viability: > 90% (assessed by viability/cytotoxicity assay, separate procedure)
B. Cell type: > 95% neuronal (assessed by immunocytochemistry, separate procedure)
D. Cell density: perform cell counts in a microscopic field of view and divide by area of field of view. Verify correspondence with initial plating density.
C. Cultures should exhibit minimal clustering, meaning there should be few neurons demonstrating soma-soma contact (assessed visually, and will be proportional to adequate surface preparation and minimal glial contamination; see panel below)
D. Unless experiment-specific, cultures should be used at 14 DIV or later to permit neuronal maturation
E. Examples of neuronal cultures:

Mature Neuronal Networks:

Culture Growth and Network Development:

Levels of Neuronal Clustering:

Figure B.1: Examples of neuronal cultures.
Primary/Secondary Cortical Astrocyte Cell Culture Methodology

Dissection/Dissociation of P0-P1 Astrocytes

Notes:

1. This procedure is for one pup brain or two cortical regions

2. The cells are passaged for > 4 weeks prior to use to allow
   a. A more pure population of type 1 astrocytes to be acquired
   b. “maturation” of the astrocyte phenotype

3. Yield: A confluent T-75 is ~3 million cells

Astrocyte Media: 90 mL DMEM/F12
                  10 mL FBS
                  (2% Pen-Strep is optional)

Dissociation: Trypsin (0.25%) + EDTA (1 mM)

Procedure:

1. Acquire cortical region from P0 or P1 rat pups (similar to E17 neuronal harvest; tissue clumps will be in HBSS)

2. Minse tissue with micro-scalpel blades

3. Add 900 μL of trypsin (0.25%), “knock” tube to get tissue to float, and place tube in warm water bath for 5-7 min

4. Add 100 μL of DNase and triturate with a fire-polished pipette

5. Add 1 mL plating media (DMEM/F12 with 10% FBS)
   a. Mix to deactivate trypsin
b. Triturate with fire-polished pipette

6. Centrifuge at 1000 rpm for 3 min

7. Resuspend in plating media by trituration with a fire-polished pipette

8. Put suspension in a T-75 flask and bring total volume to 10 mL

9. Day 1: (24 hrs after plating in T-75)
   a. Mechanically agitate flask to suspend non-astrocytic cell types
   b. Remove media
   c. Add new plating media

10. Day 3:
    a. Mechanically agitate flask to suspend non-astrocytic cell types
    b. Remove media
    c. Add new plating media

11. Every 2-3 days: feed cells
    a. Aspirate media in T-75 flasks
    b. Add 10 mL warm media (DMEM/F12 + 10% FBS) to each flask
    c. Return flask to incubator

12. As cells become confluent (~90% confluency, ~1x per week)
    a. Aspirate media in T-75 flasks
    b. Add 4 mL of warm trypsin (37°C) to each flask
    c. Put flasks in incubator for 4-7 minutes
    d. Add 1 mL of media (DMEM/F12 with 10% FBS) to each flask
    e. Transfer media to a centrifuge tube
    f. Spin for 3 minutes at 1000 rpm
g. Aspirate supernatant
h. Re-suspend cells in media (DMEM/F12 with 10% FBS)
i. Transfer desired ratio to a new flask (30,000 cells/cm²; ~1:3-6)
j. Return flask to incubator

**Plating of Neuronal-Astrocytic Co-Cultures**

**Media:**

**Neuron/Astrocyte Plating Media:** 100 mL Neurobasal Media (NBM)
(Co-culture media) 2 mL B-27
1 mL G-5
250 µL L-Glutamine

**Astrocyte Media:** 90 mL DMEM/F12K
10 mL FBS

**Poly-L-Lysine (PLL)** 0.05 mg/mL poly-L-lysine in di-water

**Procedure:**

1. PLL pre-coat
   a. Add 250 µL/cm²
   b. Transfer to incubator for 4-12hrs
   c. Aspirate PLL and let excess evaporate by leaving lid open under laminar flow hood for ~2min

2. Matrigel pre-coat
   a. 2-D and 3-D: 0.5-0.6 mg/mL in NBM, 250 µL/cm²,
b. Transfer to incubator for ~12hrs

c. Immediately prior to cell addition, aspirate but do not let dry

3. Add cells (at desired neuron:astrocyte ratio, typically 1:1 or 1:5)
   a. 2-D: 18,500-50,000 cells/cm², 250 µL/cm²
   b. 3-D: 2.5 x 10⁶ cells/cm³, 7.5 mg/mL Matrigel

4. Feed/Change Media
   a. 2-D: feed at 24hrs and every 2 days thereafter
   b. 3-D: add plating media at 4-8hrs post-plating, feed at 24hrs and every 2 days thereafter

**Viability/Cytotoxicity - Fluorescent Microscopy (Confocal or Traditional)**

Materials:

1. Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes)
2. 1X Dulbecco’s Phosphate Buffered Saline (D-PBS, Gibco)

Principle:

Calcein AM crosses the membrane of all cells and the AM portion of the molecule is cleaved inside cells with esterase activity – indicating the cell is metabolically active – which then fluoresces bright green (ex/em ~495nm/~515nm). Ethidium homodimer is only able to enter cells with a compromised cellular membrane. Upon entry into a cell, this molecule irreversibly binds DNA causing the nuclei of the cell to fluoresce red (ex/em ~495nm/~635nm). Thus, by counting the number of live cells (green-stained cell somas and processes) versus the number of dead cells (red stained nuclei), the percentage of viable cells in the culture may be assessed.
Live/Dead Solution: 5 μL Calcein AM (4 mM in anhydrous DMSO)

20 μL Ethidium homodimer-1 (2 mM in DMSO/H₂O 1:4 (v/v))

10 mL D-PBS

*solution is light sensitive (wrap in AL foil, store at 4°C)

Procedure:

1. Aspirate media, rinse 1X with D-PBS
2. Add live/dead solution (250 μL/cm²)
3. Cover plate (due to light sensitivity)
4. Place in incubator (37°C, 5% CO₂, 95 % relative humidity) for 30 min
5. Aspirate media, rinse 2X with D-PBS, leave in D-PBS
6. Using fluorescent microscopy*, view cells

*Fluorescent microscopy performed using a 1) Nikon Eclipse TE300 interfaced with a Sony digital photo camera DKC5T5/DMC through Image-Pro Plus software or a 2) Confocal Laser Scanning Microscope (Zeiss LSM 510 UV).

**Immunocytochemistry – Fluorescent Microscopy (Confocal or Traditional)**

Principle:

Immunocytochemistry uses the antigen-antibody reaction to bind visible/fluorescent stains to specific cells. The live cells are first fixed with formalin. Next, a detergent solution is added to permeabilize the cells. All subsequent steps are performed using goat serum to prevent non-specific binding. The neurons are then stained through a series of antibody reactions. The primary antibody is bound with the
protein-of-interest present in the cells. A fluorescent-conjugated secondary antibody is added to fluorescently label the cells. Next, the cells are viewed using a fluorescent microscope.

Materials:

3.7% Formaldehyde in 1X Phosphate Buffered Solution (PBS, Gibco)

(a.) 1.0 mL 37% Formaldehyde

(b.) 9.0 mL 1X PBS

0.3% Triton X100 + 4% goat serum in PBS

4% goat serum in PBS

Procedure:

1. Remove medium and fix cells by adding 3.7% Formaldehyde (0.5mL/well) for 30 minutes at 18-24°C

2. Remove the 3.7% Formaldehyde Solution and rinse 2X with PBS (quick), rinse again with PBS (let soak for 10 minutes).

3. Remove Sylgard containment ring and outline culture with hydrophobic pen.

4. Permeabilize cells with (0.3% Triton X100 + 4% goat serum) for 20 minutes at 18-24°C

5. Rinse 5X with PBS (2X quick, 3X let sit 5 minutes)

6. Dilute polyclonal antibody and/or monoclonal antibody in 4% goat serum in PBS. Prior to dilution, spin (centrifuge) primary antibody for 5-10 seconds and then triturate to mix.
7. Incubate cells in above solution (made in step 6, 200µL/well) for 4 hours at 37°C. Secondary only wells (negative control) receive 200µL of 4% Goat Serum + PBS.

8. Rinse 5X with PBS (2X quick, 3X let sit 5 minutes)

9. Dilute goat anti-rabbit (Alexa Fluor 488 (green); 1:500; Molecular Probes) and/or goat anti-mouse (Alexa Fluor 546 (red); 1:500; Molecular Probes) with Hoechst 33258 (blue; 1:1000; Molecular Probes) in 4% goat serum in PBS. Triturate secondary antibodies prior to dilution. Cover mixed solution with foil until usage.

10. Incubate cells in above solution (made in step 9, 200µL/well) for 2 hours at 18-24°C, in the dark (covered with foil)

11. Rinse 5X with PBS for 5-10 minutes each, after which leave cells in PBS

12. Mount cultures by rinsing slides first in di-H2O, then ultrafiltrate water. Add one drop Fluoromount per culture and then place on glass slide.

13. Refrigerate at 4°C for storage.

14. Using phase and fluorescent microscopy*, view cells and capture photomicrographs

*Fluorescent microscopy performed using a Nikon Eclipse TE300 or a Zeiss Confocal 510 UV. Nikon microscope is interfaced with a Sony digital photo camera DKC5T5/DMC through Image-Pro Plus software. Confocal images are viewed using Zeiss Image Browser.
Immunocytochemistry (3-D) – Fluorescent Microscopy (Confocal or Traditional)

*Adapted immunohistochemistry procedure – for 3-D cell-imbedded hydrogels*

Materials:

Coplin jars (hold 10 glass slides each, for bulk rinses, volume = 25-40mL)

3.7% Formaldehyde Solution in 1X Phosphate Buffered Saline (PBS, Gibco)

(a.) 1.0 mL 37% Formaldehyde (Fisher)

(b.) 9.0 mL 1X PBS

30% Sucrose Solution in 1X PBS

0.1-0.8% Triton X100 + 4-8% goat serum in PBS

0.1-0.3% Triton X100 + 2-4% goat serum in PBS

2-4% goat serum in PBS

Sample Preparation Procedure:

1. Remove medium and fix cells by adding 3.7% Formaldehyde (0.5mL/well) for 30-40 minutes at 18-24°C.

2. Remove the 3.7% Formaldehyde Solution and add 30% Sucrose Solution. Let sit overnight at 18-24°C.

3. Rinse 1X in PBS, transfer to plastic Disposable Embedding Molds (Polysciences), remove PBS, add Optimal Cutting Temperature (OCT, Electron Microscopy Sciences) compound.

4. Plunge (partially) into liquid nitrogen until frozen – do not let liquid nitrogen flow over top of the Embedding Mold. Store at -80°C until sectioning.

5. Section on cryostat (15-20 μm), mount on glass slides (3-4 section per slide). Store at 4°C for short-term or -80°C for long-term.
Immunocytochemistry Procedure:

1. Bulk rinse 3X with PBS (5 min each) at 18-24°C in Coplin jars.

2. Permeabilize cells with (0.1-0.8% Triton X100 + 4-8% goat serum in PBS) for 1 hour at 18-24°C in Coplin jars on a rocking platform (gentle agitation).

3. Bulk rinse 1X with PBS in Coplin jars.

4. Dry slides by shaking and then draw around sections with a hydrophobic pen.

5. Dilute polyclonal antibody and/or monoclonal antibody in (0.1-0.8% Triton X100 + 2-4% goat serum in PBS). Prior to dilution, spin (centrifuge) primary antibody for 5-10 seconds and then triturate to mix.

6. Incubate cells in above solution (made in step 5, 20µL/section) overnight at 4°C in a covered, humidified chamber. Secondary-only sections (negative control) receive 20µL of (0.1-0.3% Triton X100 + 2-4% goat serum in PBS).

7. Bulk rinse 4X with PBS (1X quick, 3X let sit 5 minutes) in Coplin jars.

8. Dilute goat anti-rabbit (Alexa Fluor 488 (green); 1:500; Molecular Probes) and/or goat anti-mouse (Alexa Fluor 546 (red); 1:500; Molecular Probes) with Hoechst 33258 (blue; 1:1000; Molecular Probes) in 2% goat serum in PBS. Triturate secondary antibodies prior to dilution. Cover mixed solution with foil until usage.

9. Incubate cells in above solution (made in step 8, 20µL/section) for 2 hours at 18-24°C, in the dark (humidified; covered with foil).

10. Rinse 4X with PBS (1X quick, 3X let sit 5 minutes) in Coplin jars.
11. Dry sections by shaking and mount by adding one drop of Fluoromount-G (SouthernBiotech) per section and then adding a coverslip on top. Work air bubbles away from sample and wipe away excess Fluoromount-G.

12. Cover and refrigerate at 4°C for drying/storage. After drying, excess Fluoromount-G may be removed with 70% ethanol.

13. Using phase and fluorescent microscopy*, view cells and capture photomicrographs

*Fluorescent microscopy performed using a Nikon Eclipse TE300 or a Zeiss Confocal 510 UV. Microscope is interfaced with a Sony digital photo camera DKC5T5/DMC through Image-Pro Plus software. Confocal images are viewed using Zeiss Image Browser.
APPENDIX C

MEDIA COMPONENTS

Neuronal Medium Components

The neuronal cultures used in these studies were plated with Neurobasal Medium (Life Technologies, Prod. #21103) supplemented with 2% B-27 (50X, Life Technologies, Prod. #17504), and 500 µM L-glutamine (Life Technologies, Prod. #25030).

Neurobasal Medium

Neurobasal Medium is basal medium formulated specifically for long-term maintenance of the normal phenotype and growth of neuronal cells.

B-27 Supplement (50X)

B-27 is a serum substitute developed for in vitro viability and growth of primary CNS neurons. B-27 used as a supplement to Neurobasal supports the growth of nearly pure populations of neural cells without the need of an astrocyte feeder layer.

Components: d-Biotin, BSA, Catalase, L-Carnitine HCl, Corticosterone, Ethanolamine HCl, D-Galactose (Anhyd.), Glutathione (Reduced), Insulin (Bovine, Zinc), Linoleic Acid, Linolenic Acid, Progesterone, Putrescine-2HCl, Sodium Selenite (1000X), Superoxide Dismutase, T-3/Albumin Complex, DL Alpha-
Tocopherol, DL Alpha Tocopherol Acetate, Transferrin (Human, Iron-Poor), Vitamin A Acetate (concentrations not published).

**Neuron-Astrocyte Co-Culture Medium Components**

The neuron-astrocyte co-cultures used in these studies were plated with Neurobasal Medium (Life Technologies, Prod. #21103) supplemented with 2% B-27 (50X, Life Technologies, Prod. #17504), 1% G-5 (100X, Life Technologies, Prod. #17503) and 500 $\mu$M L-glutamine (Life Technologies, Prod. #25030).

**G-5 Supplement (100X)**

G-5 is a serum substitute developed for *in vitro* growth and expression of primary/secondary glial cells of astrocytic phenotype.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>500.00 $\mu$g/mL</td>
</tr>
<tr>
<td>Human Transferrin</td>
<td>5000.00 $\mu$g/mL</td>
</tr>
<tr>
<td>Selenite</td>
<td>0.52 $\mu$g/mL</td>
</tr>
<tr>
<td>Biotin</td>
<td>1.00 $\mu$g/mL</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.36 $\mu$g/mL</td>
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<tr>
<td>FGF</td>
<td>0.50 $\mu$g/mL</td>
</tr>
<tr>
<td>EGF</td>
<td>1.00 $\mu$g/mL</td>
</tr>
</tbody>
</table>
APPENDIX D

MATRIGEL MATRIX

The neural cell culture models described here have been developed using Matrigel matrix (Becton Dickinson Biosciences; Bedford, MA), a reconstituted basement membrane extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins (Kleinman, et al., 1986). Matrigel is biologically active for neural cells through matrix-protein (e.g., collagen, laminin)(Kleinman, et al., 1986) and cytokine-related interactions (Vukicevic, et al., 1992) that has been shown to promote neurite outgrowth (Madison, et al., 1985). Matrigel gels at physiological temperature and has fluid-like behavior at low temperatures (~4°C) allowing cells to be evenly dispersed throughout the 3-D matrix prior to gelation. The physical and chemical properties of Matrigel are presented in Table D.1.
Table D.1: Physical and biochemical properties of Matrigel matrix.

<table>
<thead>
<tr>
<th>Basement Membrane Component</th>
<th>Percent</th>
<th>Growth Factor Composition</th>
<th>Concentration</th>
<th>Physical Characteristic (SEM)*</th>
<th>Mean (nm)</th>
<th>Range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td>61%</td>
<td>EGF</td>
<td>&lt; 0.5 ng/mL</td>
<td>Elevations</td>
<td>162 ± 52</td>
<td>76 – 267</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>30%</td>
<td>BFGF</td>
<td>Unknown</td>
<td>Pores</td>
<td>105 ± 70</td>
<td>26 – 359</td>
</tr>
<tr>
<td>Entactin</td>
<td>7%</td>
<td>NGF</td>
<td>&lt; 0.2 ng/mL</td>
<td>Fibers</td>
<td>69 ± 35</td>
<td>8 – 143</td>
</tr>
<tr>
<td>Heparan Sulfate Proteoglycan</td>
<td>2%</td>
<td>PDGF</td>
<td>&lt; 5 pg/mL</td>
<td>Interpore Distance</td>
<td>117 ± 41</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGF-1</td>
<td>5 ng/mL</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>TGF-β</td>
<td>1.7 ng/mL</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>% Protein that gels</td>
<td></td>
<td>83</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*from (Abrams, et al., 2000): physical characterization was performed at a final Matrigel concentration similar to that used in these studies (diluted 1:1 with media).

Matrigel is composed primarily of laminin, collagen IV, entactin, and heparan sulfate proteoglycan (Kleinman, et al., 1986). It also contains growth factors, matrix metalloproteinases (collagenases), plasminogen activators and other undefined components (Vukicevic, et al., 1992). The average pore size of Matrigel is 105 ± 70 nm with a range of 26 – 359 nm (Abrams, et al., 2000). Figure D.1 displays electron micrographs of Matrigel matrix alone and matrix with visible neuronal process growth. Matrigel has been found to closely resemble the structure, composition, physical properties, and functional characteristics of in vivo extracellular matrix, and may actively be remodeled by neural cells contained within.
Figure D.1: Scanning electron micrographs of Matrigel matrix. (A) The nanoscale topography is composed of a meshwork of fibers and pores similar to that previously examined (Abrams, et al., 2000). (B) Neuronal and neuritic growth through Matrigel matrix. Neurites appear (~1 µm diameter) plunging into the matrix as well as growing along the surface.

In the cell deformation paradigm used in these studies, the physical and chemical properties of the matrix are principal factors in the translation of shear deformation to cells contained within the matrix. The mechanical properties of Matrigel at the concentration used for cell culture and subsequent injury experiments (7.5 mg/mL; concentration determined to readily support neurite outgrowth and viable neural networks) were determined using a Bohlin CVO rheometer (East Brunswick, NJ). Briefly, Matrigel matrix (diluted in medium) was subjected to 0.005 oscillatory shear strain under a parallel plate configuration at 37°C in a humidified environment. The stress response was measured, and the elastic, viscous and complex moduli were determined through a frequency sweep of 0.01 to 10 Hz (n = 4). The complex, viscous and elastic moduli are presented in Table D.2. The mechanical properties were comparable to those previously reported for human brain tissue tested using similar methodology (Fallenstein, et al., 1969; Fallenstein, et al., 1969); however others have
reported significantly higher moduli for human brain tissue (Shuck and Advani, 1972). Thus, the viscoelastic profile of Matrigel was found to be similar, albeit on the low end of reported values, to properties of brain tissue under the conditions tested. This supports the assumption that at the cellular level, cells cultured within this matrix may experience a similar mechanical environment as neural cells in vivo upon bulk loading of the matrix.

**Table D.2: Rheological properties of Matrigel matrix.** The oscillatory shear stress-strain relationship was determined over a range of frequencies in order to determine the viscoelastic properties (elastic, viscous and complex moduli) of Matrigel matrix. This scaffold demonstrates similar material properties to that of human brain tissue under oscillatory shear strain.

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Complex Modulus (Pa)</th>
<th>Elastic Modulus (Pa)</th>
<th>Viscous Modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>49.5 ± 4.5</td>
<td>49.0 ± 4.7</td>
<td>7.1 ± 1.6</td>
</tr>
<tr>
<td>0.06</td>
<td>58.8 ± 5.4</td>
<td>58.7 ± 5.4</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>0.32</td>
<td>69.2 ± 7.1</td>
<td>68.9 ± 7.2</td>
<td>6.7 ± 1.6</td>
</tr>
<tr>
<td>1.78</td>
<td>114.5 ± 6.7</td>
<td>114.0 ± 6.7</td>
<td>10.6 ± 1.3</td>
</tr>
<tr>
<td>10.00</td>
<td>660.0 ± 10.9</td>
<td>522.8 ± 18.0</td>
<td>402.7 ± 6.0</td>
</tr>
</tbody>
</table>
APPENDIX E

GLUTAMATE-INDUCED EXCITOTOXICITY IN TWO- VERSUS THREE-DIMENSIONAL NEURONAL CULTURES

Introduction

Traumatic brain injury (TBI) is caused by a mechanical insult to the head and can result in temporary or permanent brain dysfunction. In addition to causing immediate neural cell death, a traumatic insult to the brain may result in disruptions to intracellular Ca$^{2+}$ homeostasis, potentially leading to prolonged cellular dysfunction and possibly death (Goforth, et al., 1999; Weber, et al., 1999; Sattler and Tymianski, 2000). After TBI in animals and humans, the extracellular concentration of glutamate increases precipitously due to cell lysis, transient permeability changes, depolarization-dependent vesicular release, and reversed transporter operation (Li, et al., 1999; Rossi, et al., 2000; Sattler and Tymianski, 2000), coupling a primary mechanical insult with a secondary chemical insult (McIntosh, et al., 1996). Glutamate is the most widely distributed neurotransmitter in the mammalian brain; however, excess glutamate becomes neurotoxic by over-exciting neurons to the point of depleting intracellular energy stores and may induce neuronal death (Goforth, et al., 1999; Weber, et al., 1999; Sattler and Tymianski, 2000). This pathological state, termed excitotoxicity, has been implicated in a host of neurological disorders in addition to trauma, including cerebral ischemia, hypoglycemia, and epileptic seizures as well as neurodegenerative diseases such as Huntington’s and Alzheimer’s disease (Choi, 1994; Jang, et al., 2002). Excitotoxicity often causes
considerable nuclear, mitochondrial, and cytoplasmic changes in neurons (Esrefoglu, et al., 2003). Due to long-term mechanisms of cell dysfunction/death initiated at the time of injury, TBI may be considered both an acute inflammatory disease as well as a longer-term neurodegenerative disease (McIntosh, et al., 1998).

In vitro models of TBI have ranging biomechanical and pathophysiological relevance to clinical head injury; although many models are able to recreate various aspects of primary and secondary damage in a controlled setting. Several models have coupled a primary mechanical injury with a secondary biochemical insult (Cargill and Thibault, 1996; Glass, et al., 2004). Neuronal cultures in 2-D and 3-D may exhibit differing responses to a biochemical or mechanical injury due to differences in culture cytoarchitecture and/or physiological states. The goal of this research was to evaluate the response of 2-D and 3-D neuronal cultures to a defined excitotoxic insult by evaluating post-injury viability. This work will establish a baseline response to an excitotoxic insult between neurons cultures in 2-D and 3-D and may provide insight into the response to high rate deformation (see CHAPTER IV).

Materials and Methods

Cortical Neuron Harvest and Dissociation

Procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. Neurons were obtained from timed-pregnant (embryonic day 17) Sasco Sprague-Dawley rats (Charles River, Wilmington, MA). Anesthetized dames were rapidly decapitated and the uterus was removed by Caesarian section and placed in Hanks Balanced Salt Solution (HBSS,
Invitrogen, Carlsbad, CA). Each fetus was removed from the amniotic sac, rapidly decapitated, and the brains removed. The cerebral cortices were isolated and placed in pre-warmed trypsin (0.25%) + 1 mM EDTA (Invitrogen) for 10 min at 37°C. The trypsin-EDTA was removed and the tissue was triturated HBSS + DNase I (0.15 mg/mL, Sigma, St. Louis, MO) using a flame-narrowed Pasteur pipet. The tissue was then centrifuged at 1000 rpm for 3 minutes and the cells were resuspended in a defined medium (Neurobasal medium + 2% B-27 + 500 µM L-glutamine (Invitrogen)).

2-D and 3-D Primary Cortical Neuronal Cultures

Cultures of 2-D and 3-D primary cortical neurons were plated in custom-made cell culture chambers consisting of a glass coverslip (no. 1½ thickness) below a circular silicone-based elastomer mold (Sylgard 184 and 186, Dow Corning; Midland, MI; cross-sectional area = 2 cm²). Prior to plating, chambers were pre-treated with 0.05 mg/mL poly-L-lysine (PLL, Sigma) followed by Matrigel (0.5 mL/well at 0.6 mg/mL, Becton Dickinson Biosciences; Bedford, MA) in Neurobasal medium (each treatment was > 4 hours). Neuronal cultures in 3-D were plated within Matrigel (final protein concentration of 7.5 mg/mL) at a cell density of 3750-5000 cells/mm³ within a 500-750 µm thick matrix (Figure E.1). Cultures were placed at 37°C to permit matrix gelation and 3-D cell entrapment, after which 0.5 mL of warm medium was added per well. For 2-D neuronal cultures, cells were plated at 1250-2500 cells/mm² in 0.5 mL medium. At 48 hours post-plating, the medium above the 2-D cultures was removed and Matrigel (7.5 mg/mL, 500-750 µm thick) was placed above the 2-D cultures to match the amount of matrix used in the 3-D system (Figure E.1). After matrix gelation, 0.5 mL warm medium was added per culture. Cultures were maintained at 37°C and 5% CO₂-95% humidified air and fed at 24
hours post-plating and every 2-4 days thereafter by replacing half of the media. All experiments were performed at 7-8 days \textit{in vitro} (DIV).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure_E1.png}
\caption{Schematic representation of the 3-D and 2-D neuronal cell cultures used in this study (not to scale). All cultures were plated above a layer of acellular matrix. (A) Neuronal cultures in 3-D were homogeneously dispersed throughout a 500-750 \textmu m matrix. (B) Neuronal cultures in 2-D were plated and, at 2 DIV, acellular matrix was added to a height of 500-750 um.}
\end{figure}

\textbf{Excitotoxic Injury}

Neuronal cultures in 2-D and 3-D were subjected to an excitotoxic insult or control conditions. The cultures were removed from the incubator and various concentrations of glutamate (in Neurobasal media + 500 \textmu M L-glutamine (no B-27)) were added. Glutamate solutions were made based on the following groups: 2-D: control (n = 4), 100 \textmu M (n = 6), 1000 \textmu M (n = 5); 3-D: control (n = 4), 100 \textmu M (n = 3), 1000 \textmu M (n = 3). Cultures were returned to the incubator for 6 hours, after which the treatment solutions were removed, cultures were rinsed twice with HBSS, and then standard neuronal media was added (NBM + 2\% B27 + 500 \textmu M L-glutamine).
Cell Viability Using Fluorescent Staining

At 24 hrs post-insult, neuron viability was assessed using fluorescent probes for distinguishing live and dead cells. Cell cultures were incubated with 4 µM ethidium homodimer-1 (EthD-1) and 2 µM calcein AM (both from Molecular Probes, Eugene, OR) at 37° C for 30 min and then rinsed with 0.1 M Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen). The percentage of viable cells was calculated by counting the number of live cells (fluorescing green by AM-cleavage) and the number of cells with compromised membranes (nuclei fluorescing red by EthD-1).

Data Collection and Statistical Analysis

After viability assays, cells were viewed using fluorescent microscopy techniques on an epifluorescent microscope (Eclipse TE300, Nikon, Melville, NY) or a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). For epifluorescent microscopy, images were digitally captured (DKC5T5/DMC, Sony, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Confocal z-stacks were acquired for all 3-D cultures and were analyzed using LSM Image Browser (Zeiss). Two to four randomly selected regions per culture were counted for statistical analysis. The mean percentage of viable cells for each group was calculated, and data are presented as mean ± standard deviation. A two-way ANOVA was performed (with culture dimensionality and glutamate level as independent variables and culture viability as the dependent variable) followed by Tukey’s pairwise comparisons (p-value < 0.05 was considered significant).
Results

Cell Viability Following Excitotoxic Injury

Viability was assessed in neuronal cultures 24 hours after excitotoxic glutamate treatment or control conditions. Control cultures, which were incubated for 6 hours in medium without B-27, presented similar viability to undisturbed controls at this time point (data not shown), signifying limited or no adverse affects of B-27 deprivation. Following excitotoxic glutamate treatment in both 2-D and 3-D neuronal cultures, there was a significant decrease in cell viability compared to controls (p < 0.05) (Figure E.2.1).

Figure E.2.1: Photomicrographs of 2-D and 3-D neuronal cultures after excitotoxic treatment. Fluorescent confocal photomicrographs of representative 3-D neuronal cultures following (A) control treatment or [glutamate] of (B) 100 µM or (C) 1000 µM. Representative 2-D neuronal cultures following (D) control or [glutamate] of (E) 100 µM or (F) 1000 µM.
However, in 3-D neuronal cultures, the 1000 µM treatment level produced a significant decrease in viability beyond that of both 3-D neurons at 100 µM (p < 0.001) and 2-D neurons at 1000 µM (p < 0.001) (Figure E.2.2). Furthermore, there was not a significant difference between the 100 µM and the 1000 µM for the 2-D cultures. Many neurons in 2-D were positive for both calcein and ethidium homodimer, signifying cell membrane compromise with maintained metabolic activity, potentially indicating an active degenerating state at this time point. These experiments demonstrate a differential susceptibility to excitotoxicity between neurons in 2-D and 3-D.

![Graphical presentation of 2-D and 3-D neuronal viability following excitotoxicity.](image)

**Figure E.2.2:** Graphical presentation of 2-D and 3-D neuronal viability following excitotoxicity. Neurons in both 2-D and 3-D treated with 100 µM or 1000 µM glutamate were significantly injured versus their respective controls (*). However, only neurons in 3-D demonstrated a difference in the response at the two concentrations tested (†). Error bars represent standard deviation.
Discussion and Conclusions

After a defined excitotoxic insult to neuronal cultures in 2-D or 3-D, a decrease in cell viability was found proportional to glutamate concentration. Furthermore, culture dimensionality was determined to be a factor in an excitotoxic response as these experiments revealed an increased sensitivity to glutamate-induced excitotoxicity for neurons cultured in 3-D compared to 2-D. The concentrations of glutamate used in this study would induce an excitotoxic effect in neurons in vivo. Excitotoxicity can be defined as excess extracellular concentration of glutamate resulting in an uptake of $\text{Ca}^{2+}$ via glutamate receptors (e.g., NMDA receptors) and may result in cell death (Sattler and Tymianski, 2000). Over activation of glutamate receptors is responsible for excitotoxic neuronal death after a number of neurological disorders, including TBI (Choi, 1994; Jang, et al., 2002), linking a primary mechanical insult to a secondary chemical insult. Also, glutamate-receptor antagonists have been found to be neuroprotective in animal and cellular models of TBI (Regan and Choi, 1994; McIntosh, et al., 1996), further implicating excitotoxicity in post-TBI cell death.

Several potential differences in neurons cultured in 2-D versus 3-D may be responsible for the differential response to an excitotoxic insult revealed in this study. Such potential differences including baseline viability, astrocyte presence, cell morphology, glutamate receptor expression and distribution, and microenvironment regulation may have been responsible for the different viability outcomes. We have previously demonstrated similar viability profiles in 2-D and 3-D neuronal cultures up to 21 DIV (see CHAPTER II), indicating that baseline culture viability was likely not responsible for the differential excitotoxic response. Astrocytes physically and
metabolically support neuronal function by forming a network on which neurons can grow, providing trophic factors, maintaining proper energetics, and regulating the synaptic microenvironment (Tsacopoulos and Magistretti, 1996). Astrocytes also play an important role in response to perturbation have been specifically implicated in the termination of pathological states such as excitotoxicity (Aschner, 2000). However, we have previously characterized the astrocytic presence in neuronal cultures in 2-D versus 3-D, where each configuration had a modest ~3% astrocytes at 7 DIV (see CHAPTER II), limiting this factor as a cause of the differential neuronal response (although astrocyte behavior may also be affected by dimensionality). There may also be differences in the rate of neuronal maturation based on culture dimensionality, and because neuronal maturation corresponds with an increased concentration of glutamate receptors, more mature neurons may be more susceptible to excitotoxic glutamate concentrations. Also, the extracellular microenvironment may become more damaging in 3-D as neurons begin to die, initiating a local cascade of cell death. There may also be other yet undefined differences between neurons cultured in 2-D versus 3-D that are responsible for the differential response to an excitotoxic insult. Further experiments may be performed to validate the specificity of this excitotoxic insult, for example, by adding additional groups using glutamate receptor antagonists. Also, other time-points post-insult may be evaluated to test whether the time-course of cell death cascades or the mechanism of cell death vary for neurons in 2-D versus 3-D. Future work on this project may also involve coupling this excitotoxic insult with a primary mechanical insult to assess the combined effects of these injuries on neuronal survival. Overall, this work demonstrates an increased susceptibility to an excitotoxic insult for neurons cultured in 3-D compared to
2-D that may have implications in the interpretation of the response to a mechanical insult between neurons in these culture configurations.
APPENDIX F

ASTROCYTE REACTIVITY AND CHONDROITIN-SULFATE PROTEOGLYCAN EXPRESSION FOLLOWING CONTROLLED CORTICAL IMPACT

Overview

This study sought to characterize elements of the reactive astrogliotic response following mechanical trauma in vivo and to validate methodology employed in assessment of reactive astrogliosis augmentation following a defined mechanical trauma to 3-D neuronal-astrocytic co-cultures (CHAPTER V). Seven days following controlled cortical impact (as described in (Shear, et al., 2004)), animals were perfused with paraformaldehyde and the brains were removed. Standard immunohistochemistry was performed (see APPENDIX B for procedure) using antibodies recognizing glial-fibrillary acidic protein (GFAP), an intermediate filament upregulated in reactive astrocytes, and CS-56, a general marker of chondroitin-sulfate proteoglycans. Sections were chosen for staining that exhibited a pronounced injury cavity and comparisons were made between the ipsilateral and contralateral cortices within the same brain. This analysis revealed a large number of reactive astrocytes (GFAP+) in the ipsilateral cortex, but a paucity of GFAP staining in the contralateral cortex (Figure F.1). Furthermore, there was an increased presence of CSPGs in the ipsilateral versus contralateral cortex (Figure F.2). These results were similar to those reported elsewhere evaluating reactive astrogliosis.
following mechanical trauma (see (Fawcett and Asher, 1999; Morgenstern, et al., 2002) for reviews).
Figure F.1: Astrocyte activation 7 days following controlled cortical impact. Few reactive astrocytes (GFAP\(^+\), green; Hoechst nuclear stain, blue) were present in cerebral cortices (A) contralateral to injury compared to (B) ipsilateral, where many reactive astrocytes and processes were apparent (scale bar = 20 µm).
Figure F.2: Alterations in CSPG expression 7 days following controlled cortical impact. CSPG expression (green) in cortex (A) contralateral compared to expression (B) ipsilateral to injury (scale bar = 50µm). There was an increase presence of CSPG in the ipsilateral cortex following controlled cortical impact.
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