In Vitro Models Of Brain For Study Of Molecular Mechanisms In Brain Disorder

by

Christina L. Wilson

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Christina L. Wilson, Ph.D.

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Advisor: Srivatsan Kidambi

Brain disorders are as diverse as they are devastating with few effective treatment options. The current challenge in diagnosing and treating neurological disorders is a lack in understanding regarding root causes leading to observed behavioral and biochemical alterations. Clinical and animal models provide substantial information but these models are complex with systemic and multicellular response to injury making mechanistic study difficult and vague. Fortunately, advances in our knowledge of *in vitro* cell culture and biomaterials have inspired the engineering of novel platforms for the study of physiologically relevant conditions leading to brain disorder. This work outlines the development of three *in vitro* models for the study of molecular mechanism leading to brain disorder due to aberration in static tissue mechanical properties, traumatic mechanical injury and methamphetamine abuse.

These *in vitro* platforms utilize astrocytes which are the most abundant glial cell in the brain. These cells perform a number of important roles for proper brain function including blood brain barrier manipulation, neurotransmitter recycling, and tissue repair. Due to the overall abundance of cells and diversity of cellular function, astrocytes have been identified as a potential target for therapeutic manipulation to improve disease and injury prognosis. Of particular interest is the astrogliotic phenotype which is observed in

numerous disease and injury pathologies to have both detrimental and healing characteristics. This work establishes and utilizes *in vitro* platforms for the understanding of cellular processes induced by static mechanical properties, dynamic mechanical damage and methamphetamine for greater understanding of astrocytes' role in disorder progression and potential for therapeutic manipulation.

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List of Abbreviations

Blood Brain Barrier (BBB)

Controlled Axonal Injury (CAI)

Copper Zinc Superoxide Dismutase (CuZnSOD)

Enhanced Green Fluorescent Protein (eGFP)

Glial Fibrillary Acidic Protein (GFAP)

Glutamate Aspartate Transporter (GLAST)

Glutamate Transporter 1 (GLT1)

Institutional Animal Care and Use Committee (IACUC)

Methamphetamine (METH)

Polyacrylamide (PA)

Polydimethylsiloxane (PDMS)

Poly-L-Lysine (PLL)

Reactive Oxygen Species (ROS)

Real Time Polymerase Chain Reaction (RT-PCR)

Tissue Culture Polystyrene (TCPS)

Traumatic Brain Injury (TBI)

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1 CHAPTER 1 INTRODUCTION

1.1 Significance

Neurologic disorders are a class of central nervous system ailments that affect individuals of all ages and are among the most devastating and present a vast financial drain on society. Dementia (i.e. Alzheimer's disease, Vascular dementia, etc.) is a class of disease which typically manifests later in life, age 65+, as declining intellectual function, loss of memory and altered emotional control. Although age, family history and environmental factors (such as high fat diet, hypertension, and smoking) are considered risk factors, the origin of dementia is often unknown and is seemingly spontaneous. Recent studies show that 35.6 million people worldwide suffer from dementia presenting a heavy emotional and financial burden on individuals and families. The financial burden alone is approximately $604 billion dollars in medical treatment including informal and formal care not including those associated with emotional and mental strain on the care givers [2, 3]. Conversely, attention deficit/hyperactivity disorder (ADHD) is a neurobehavioral disorder resulting from developmental irregularity of unknown origin. Approximately 9.5% of children ages 4-17 years old and 4.4% of adults ages 18-44 have been diagnosed with ADHD costing approximately $143 billion dollars primarily in lost productivity and income in adults; This cost also includes special education and counselling for children as society seeks to assist parents in providing equal opportunity for their children [4-6]. Neuropsychiatric disorders (such as schizophrenia or bipolar disorder) affect individuals of all ages with a dysregulation of thought processes resulting in false beliefs, depression, and hallucinations. Diagnosis is currently based on an interview with a doctor on a voluntary basis. Therefore it is difficult to quantify how many individuals suffer without

diagnosis but current studies estimate approximately 1.1% of the US population over the age of 18 suffer from these disorders [7, 8]. Schizophrenia alone accounts for approximately $63 billion in treatment, loss of income and societal cost (social services and criminal justice) [9]. The common factors which tie these and many other neurologic disorders is the inability to detect and diagnose the illness in early stages, prior to behavioral deviation, when tissue damage may be reversible and the limited effective treatments. Medical professionals and law makers agree that the best chance society has of finding early detection biomarkers, effective prevention plans and successful treatment regimens is to better understand the molecular mechanisms underlying these disorders. In a few brain disorders (Huntington's Disease, Parkinson's Disease, etc.) specific genetic mutations have been identified to trigger the cascade leading to behavioral changes; however, environmental factors such as drug use, pesticides, excess fatty acids, stroke and traumatic injury can alter disease progression in unknown ways and are therefore risk factors in healthy tissue where no genetic cause is known [10-12]. This highlights the critical need for understanding of the molecular basis of toxicity and injury progression prevention and treatment of disorder which can only be discovered through resolute and precise research efforts.

Advancement in analytical methods of molecular biology has vastly improved our ability to study brain diseases and injury. The ability to accurately characterize the gene, protein and metabolites expressed in patients has allowed for the engineering of improved disease models, animal and *in vitro*, which can more accurately represent the disease state in experiments [13-15]. Much of our current understanding correlating symptoms to tissue alterations we can attribute to animal models. However, *in vivo* models have numerous

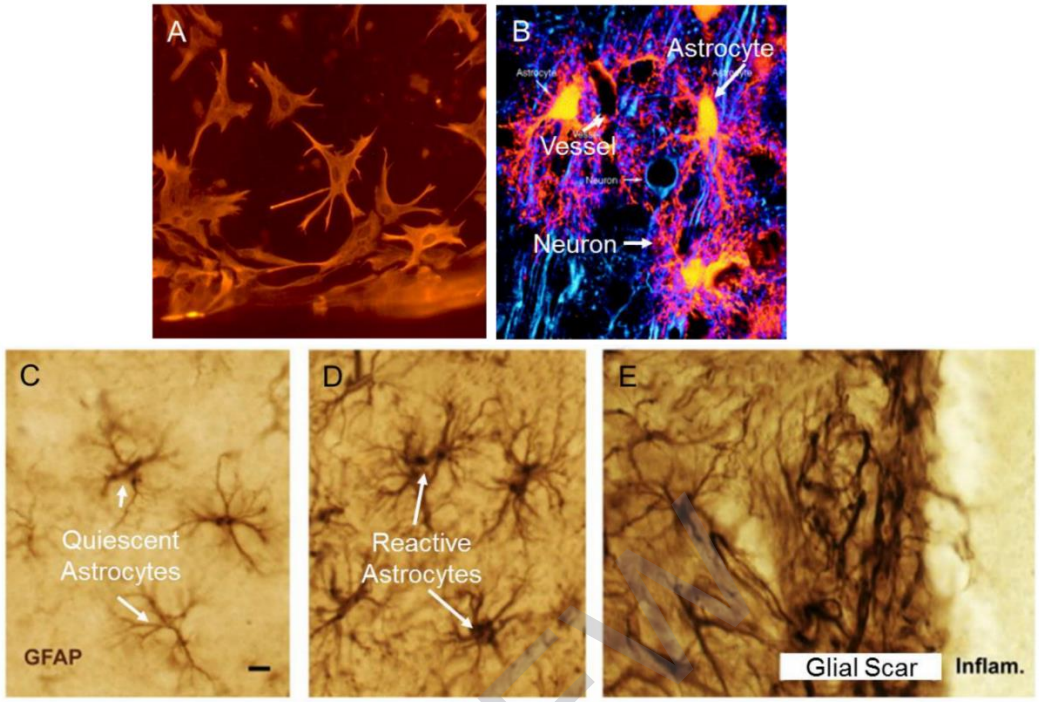
limitations including (1) the challenge associated with identifying molecular mechanisms due to systemic response, (2) difficulties in reproducing identical injury or extent of disease progression in multiple experiments and (3) the inability to identify biochemical contributions of specific cell types [16]. The challenges are particularly undermining as scientists seek to develop novel therapies which manipulate the cellular microenvironment to promote innate healing in the tissue. For instance, several recent reviews suggest manipulation of glial cells in the treatment of drug abuse, traumatic brain injury and stroke as they are observed contribute to tissue health and repair in nature. However, this requires intimate understanding of the contributions of individual cell types to disease progression and healing mechanisms which we do not possess [17-19]. *In vitro* models allow the investigation of isolated phenomenon in a well-defined environment, which is free from complex system interactions and defined by the investigator. The standard and most simplistic *in vitro* model is the cellular monoculture on TCPS but advances in knowledge of biomaterials provides avenues of engineering *in vitro* disease models through controlled chemical release, well-defined topography, mechanical stimulation, and cellular organization through patterning. Furthermore, the study of induced pluripotent patient cells and heterogeneous population in spatially controlled interactions allows a relevance to drug discovery and disease understanding previously unachievable [20-23].

1.2 Background

1.2.1. Astrocytes

The brain is a highly organized, heterogeneous organ that relies on a complex interplay of cells, extracellular matrix and biomolecules to sustain life and thought [24]. A unique characteristic of the brain is the requirement of neuronal ultrafast signaling which

relies heavily on a tightly controlled microenvironment which is constantly monitored and maintained by glial cells. The term “glial cells” refers to the non-neuronal cells of the brain (including oligodendrocytes, microglia, and astrocytes) which each make crutial contributions for healthy neuron development and function. Astrocytes are the most abundant glial cell type, which act in numerous processes including ion homeostasis, neurotransmitter recycling, ECM remodeling, blood-brain barrier integrity and tissue repair [25]. They are named such for the star-like appearance (Figure 1A) when stained for standard marker Glial Fibrillary Acidic Protein (GFAP) which highlights processes that reach out to maintain communication and monitor all cell types, especially neurons and blood vessels (Figure 1B), through a variety of receptors and transporters. Regulation of extracellular ions relies on a series of astrocyte ion receptors which trigger uptake and/or blood brain barrier (BBB) manipulation assisting in ion or water transfer for the purpose of restoring ideal conditions [26, 27]. Astrocytes also encourage transport of nutrients, especially oxygen and glucose, across the BBB to provide for brain energy production and consumption [28]. Astrocytes also monitor and assist in clearance of neurotransmitters utilized by neurons to transfer signals across the synaptic cleft. This process is especially important to prevent excitotoxicity which is a common mechanism of neurodegeneration that results from prolonged activation of neuronal neurotransmitter receptors. This is especially true in the case of glutamate, the most abundant excitatory neurotransmitter, as neurons do not make sufficient glutamine synthetase, a required enzyme for efficient glutamate conversion [29]. Furthermore, astrocytes manipulate extracellular matrix (ECM) content through the expression of a large range of proteoglycans and numerous other small molecules and proteases during development and after injury to create the ideal



<u>Figure 1:</u> Images of astrocytes in vitro and in vivo. (A) Astrocytes in monoculture isolated from rat hippocampal tissue labeled with antibody to Glial Fibrillary Acidic Protein (GFAP; red). Image adapted from Wilson, RSC Advances 2015[1]. (B) Image displaying the complex spatial organization of astrocytes, neurons, and blood vessels in rat cortex. Neurons are labeled with antibody to microtubule-associated protein 2 (MAP-2; blue), whereas astrocytes are expressing enhanced green-fluorescent protein (eGFP; yellow). A small vessel is outlined by eGFP-positive astrocytic endfeet (X. Wang and M. Nedergaard, unpublished). Astrocytes in cortical tissue at various degrees of astrogliosis stained for GFAP wtih immunohistochemical stain. (C) Astrocytes in healthy tissue, (D) reactive astrocytes in response to bacterial antigen, lipopolysaccharide and (E) glial scarring next to a region of severe traumatic injury and inflammation (inflam). Scale bar = 8 um Image adapted from Sofroniew. Trends in Neuroscience 2009[30].

environment for tissue healing and function [30-32]. Astrocytes also "react" to injury through induction of a unique phenotype known as "astrogliosis" which results in change in morphology, population size and molecular (i.e. small molecule, gene and protein) expression [30]. Astrocytes typically reside in neighboring domains in a quiescent morphology (Figure 1C) but become hypertrophic and proliferative with increased expression of intermediate filaments (e.g. glial fibrillary acidic proteins (GFAP) and vimentin), pro-inflammatory mediators and proteoglycans invading adjacent domains

when reactive. Advanced astrogliosis ultimately leads to formation of a glial scar (Figure 1E) which acts as physical barrier between the injury and healthy tissue. This can inhibit axonal regeneration into the injured region and has led many to question whether the change in phenotype is useful for healing or leads to further progression of injury [24]. Furthermore, animal studies imply that astrocytes play an important role in the progression of several diseases including Alzheimer's disease, Parkinson's disease, epilepsy, and others [33-35]. Much is still uncertain about the deviant molecular mechanisms expressed by reactive astrocytes and whether astrocytes could be manipulated toward greater healing potential. This has incited much support and exploration into astrocyte based *in vitro* models which could aid in advancing the therapeutic power of astrocytes [19, 30].

1.2.2. Modeling Brain Disorder Related To Change In Static Tissue Mechanical Properties

Most *in vitro* studies of tissues in health and disease focus on biochemical aspects as this is an easily manipulated variable in cell culture. However, many diseases and injuries are also associated with change of the tissue mechanical properties (i.e. stiffness) inducing cellular changes. The stiffness of a tissue is defined as the rigidity or resistance to change shape and is usually described as a ratio of applied stress to material strain in terms of Young's or shear modulus (Pa) depending on the direction of applied stress. Variation in stiffness is a prominent feature in cases of liver fibrosis or breast tumor development with a drastic increase in the tissue modulus [36]. The brain is one of the softest tissues in the body which has been observed to be nonlinear, viscoelastic and varies slightly depending on brain region and age [37]. Recent studies show that changes in brain structure and composition during development into an adult structure results in a stiffness

range of 100 to 400 Pa [38-40]. Further changes in tissue stiffness are observed in a number of disease states including brain tumor, stroke and traumatic brain injury [41-45]. In the case of brain tumors, this change in stiffness between the tumor and surrounding tissue is attributed to the increased efficacy of ultrasound detection. Furthermore, glioma cells have been observed to be sensitive to the change in stiffness leading to cancer progression [46]. This and other studies bring to light the mechanical sensitivity of brain cells and how this might affect the progression or healing of various disorders.

Numerous cell types are mechano-responsive but not to the same degree of sensitivity. The underlying cellular characteristic which appears to dictate sensitivity is the degree of interaction that they achieve with the extracellular matrix [47, 48]. An example of this is the observation of traction forces created by neurons and fibroblasts on polyacrylamide gels. It was observed that fibroblasts could strongly deform surfaces of 10 kPa while neurons could only deform gels between 50-200 Pa. This was attributed to the ability of fibroblasts to transfer more mechanical energy through greater degree of cell-surface interaction [49]. Through cell matrix and cell-cell interaction via cytoskeletal proteins, integrins and cadherins, cells are able to biochemically respond to mechanical stimuli in their environment regulating a number of processes including proliferation, migration and stress pathways [50]. *In vitro* studies, by varying culture surface stiffness, has provided much of what we know regarding cell-matrix interactions and response to extracellular mechanical stimuli [51].

In engineering a construct for study of matrix stiffness driven cellular behavior there are several design constraint to consider which dictates appropriate biomaterials. These include 1) the achievable range of stiffness, 2) the chemical uniformity of the matrix

and 3) topographical uniformity of the matrix. First, consideration of a modulus range regards the physiological relevance of the study and must correlate with the material ability to support cellular growth within the stiffness range. This is most often achieved by varying crosslinking or concentration of the material matrix which can be limiting as the material must be solid to ensure cells interact with the matrix. Chemical uniformity of a material is also important as has been seen in recent tissue culture models which successfully pattern cellular growth by various surface chemistry [52]. Cells are influenced by the chemistry of culture platform therefore the uniformity of surface chemistry present is extremely important to ensure that study observations arise solely from surface compliance and not difference in chemistry [53]. Matrix uniformity is also crucial as heterogeneity in matrix composition can influence cellular behavior. Although cell size can range from 15-100 μm it has been observed that many cell types are influenced by topographical change on the nanoscale [54, 55]. Therefore, to ensure compliance is the sole influential variable, matrix homogeneity is a key surface property for cellular study.

Considering the previous design constraints there are several materials which have been employed for stiffness induced cellular behavior studies. Protein based hydrogels (i.e. collagen, fibrin or the proprietary blend Matrigel) and natural hydrogels (alginate and agarose) are most common. Polymerization in these matrices can be induced by the addition of specific enzymes or altered solution temperature. These materials have many advantages as they are biocompatible and employ materials found in the cellular microenvironment [56, 57]. However, the natural derivation of the foundational material can make these gels expensive and the modulus is altered by modifying polymer mass therefore chemical and topographical heterogeneity can occur [58]. Polyacrylamide (PA)

gels have emerged as an alternative with the ability to separate chemical from mechanical signals with a nearly chemically inert gel (adhesive molecules must be covalently bonded to PA to promote interaction). Also, the modulus is varied by the concentration of crosslinker, dimeric bisacrylamide, keeping the construct concentration uniform across the stiffness range. This avoids changes in surface topography and chemistry seen in previously discussed hydrogels. The disadvantages of PA gels are associated with the technical difficulty in covalently bonding adhesive molecules which is required for cell-matrix interaction and concerns associated with unpolymerized acrylamide [58]. Finally, silicon polymers (i.e. polydimethylsiloxane or PDMS) provide an economical, chemically inert, and wide range of achievable modulus [59, 60]. The surface can be easily modified to introduce adhesive proteins via standard cell culture methods and the elasticity is based on the concentration of crosslinker assuring a uniform topography and chemistry. A possible disadvantage is the toxicity associated with unpolymerized material however, as with the PA gels, washing or surface treatment should minimize this risk [61].

1.2.3. Modeling Brain Disorder Related To Dynamic Change Of Tissue Mechanical Properties

Traumatic Brain Injury (TBI) is defined as any brain damage resulting from an external mechanical force (rapid acceleration/deceleration, blast waves, impact, or penetration) which results in clinical signs of brain alteration such as loss of consciousness, amnesia, muscle weakness, loss of balance or any alteration in mental state [62, 63]. TBI is the leading cause of disability in people under 40 years of age and severely disables 150-200 per million people annually resulting in heavy emotional and financial burden on society [64-66]. Furthermore, individuals that experience TBI, especially multiple