IMPACT OF CULTURE CONDITIONS ON PRIMARY ASTROCYTE PHENOTYPE

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ABBREVIATIONS

Aβ	Amyloid beta
ABM	Astrocyte Basal Medium
AD	Alzheimer's disease
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AQP4	Aquaporin-4
BBB	Blood brain barrier
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CXN 43	Connexin 43
CXN 30	Connexin 30
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EAAT2	Excitatory amino acid transporter 2
ECAR	Extracellular acidification rate
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF2	Fibroblast growth factor2
GFAP	Glial fibrillary acidic protein

GLT-1	Glial high affinity glutamate transporter), member 2
GLAST	Glial high affinity glutamate transporter), member 3
GLUT1	Glucose transporter member 1
GSH	Glutathione
HEK	Hexokinase
HD	Huntingtins disease
IL	Interleukin
mGLUT	Metabotropic glutamate receptor
MCT	Monocarboxylate transporter
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMDA	N-methyl-D-aspartate receptor
NDs	Neurodegenerative disease
Nrf2	Nuclear factor erythroid 2–related factor 2
OCR	Oxygen consumption rate
PBS	Phosphate-buffered saline;
PD	Parkinson's disease
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TGF	Transforming growth factors
TNF	Tumor necrosis factor

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CHAPTER 2

SPECIFIC AIMS AND SIGNIFICANCE

Primary astrocyte cultures have been an invaluable tool for studying signaling pathways that regulate astrocytes physiology, function and reactivity. Many functions of astrocyte such as its role in providing trophic support for neurons, promoting formation and function of synapses, roles in brain defense system, controlling neuronal metabolism as well as its role in the initiation and progression of NDs were all learnt in part from *in vitro* cultures. Hence our understanding of astrocyte biology today has been derived from studies conducted with primary cultures, although this *in vitro* culture does not fully mimic the complex events occurring *in vivo* [1]. The early protocol of McCarthy and de Vellis [2] for culturing astrocytes, which was latter modified by other laboratories, played a critical role in providing tools for studying astrocyte biology [3, 4]. This protocol has major limitations which include the use of a culture medium containing fetal bovine serum (FBS) which does not mimic physiological conditions as some of the serum proteins cannot cross the blood brain barrier (BBB) [5, 6] also, astrocytes prepared by the MD method are flat and fibroblast like in morphology whereas adult astrocytes *in vivo* are high process bearing [4]. Almost all studies of astrocyte functions use the MD method of culture preparation and cells cultured under hyperglycemic glucose conditions (25 mM glucose) although hyperglycemia *in vivo* has been shown, to induce astrogliosis and increase expression of inflammatory cytokines [7-9]. Due to the physical stress e.g. shaking (done to remove other cell types) associated with the MD method of astrocyte preparation and the presence of FBS in the MD medium, it has been suggested that astrocytes obtained using this culture system are reactive [1, 3, 10]. Recently it has also been shown that MD-astrocytes have gene profile different from adult astrocytes *in vivo* [4]. Thus raising a concern of the degree to which results obtained using this culture protocol can be translated to astrocyte role *in vivo.* Similarly this

suggests that culture conditions impact astrocyte biology. In order to correct the limitations of the MD method, some labs have developed the immunopanning and florescence activated cell sorting method of isolating astrocytes from rodent brains [4, 11, 12]. This methods produce astrocytes with gene profiles that mimic astrocytes *in vivo* which helped to discover some new properties of astrocytes. Like the MD method, immunopanning (IP) and florescent activated cell sorting (FACS) method of isolating astrocyte also has some limitations. These methods are very capital intensive, and the selection and isolation processes reduce the number of viable cells. There is an urgent need to correct the limitations of the MD, IP and FACS method, by producing astrocyte with a less capital intensive method and a culture medium that will support survival and growth of astrocyte and still mimics *in vivo* resting astrocyte phenotype. To develop a culture condition that mimic astrocyte *in vivo* there is also a need for detail understanding of the factors that affect astrocyte biology in culture.

Our **goal in this study** was to understand how culture conditions impact astrocyte biology and develop a serum free astrocyte culture method that mimic astrocyte biology *in vivo* and provide a critical tool for defining the precise function of astrocyte under physiological and pathological conditions. **Our rationale** for this study was that a well-characterized astrocyte culture method that maintains primary astrocytes in quiescent state as *in vivo* will provide a critical tool for better understanding the structure and function of brain circuits under health and disease.

Heparin–binding epidermal growth factor (HBEGF) has been shown to promote astrocyte survival and proliferation and to affect cell differentiation and morphology in serum free culture conditions [13, 14]. Fibroblast growth factor (FGF2), first purified in bovine brain [15], has been found to maintain astrocyte in nonreactive state. Similarly, FGF2 signaling delays astrogliosis

and accelerates astrocytes deactivation after injury [16, 17]. Furthermore different velocity adherence cell culture method (DVA) has been found to produce cell cultures with high purity [18]. Thus we hypothesized that primary astrocyte culture method employing normal glucose, serum free FGF2/EGF containing media, will produce astrocytes that mimics astrocyte biology *in vivo*. Our hypothesis was tested with, the following aims.

Specific Aims 1: To determine the phenotype and metabolic profile of primary astrocytes under serum-containing MD medium culture and serum-free EGF/FGF2-containing ABM culture conditions.

Specific Aims 2: To determine the phenotypic alteration of serum-containing MD medium cultured and serum-free EGF/FGF2-containing ABM cultured astrocytes upon stimulation of astrogliosis factors.

Specific Aims 3: To determine the effects of hyperglycemia on the phenotypic alteration of cultured astrocytes and implication for astrocyte research.

Innovation and significance

Astrocyte culture provides a useful tool for the study of astrocyte biology. Cells need to be cultured in medium that mimics their physiological conditions *in vivo*, allowing results obtained from studies conducted *in vitro* to be translated to events *in vivo*. This study is significant as it will provide a new perspective on the effect of certain components of culture media on astrocyte phenotype, provided for how the important roles FGF2 and EGF are playing *in vivo* can be exploited to help achieve an optimum astrocytic culture condition and also resolve some of the contradictory views on the effects of certain growth factors on astrogliosis. This study also tested the effect of hyperglycemia on astrocytic function. The results of this study will establish a reliable, cost effective astrocyte primary culture condition needed for the understanding of the

pathological and physiological state of astrocytes so that results obtained can be translated to *in vivo* conditions.

CHAPTER 1

1. INTRODUCTION

1.1

STAR OF NEURODEGENERATIVE DISORDERS: ASTROCYTES AS A CELLULAR TARGET FOR TREATMENT OF NEUROLOGICAL DISORDERS

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1.1.1 ABSTRACT

Astrocytes have traditionally been considered support cells in the Central nervous system (CNS). However it is now evident that astrocytes cooperate with neurons on many levels, including trophic support and defense against oxidative stress, energy metabolism, recycling and trafficking of neurotransmitters and ionic and fluid homeostasis. Astrocytes play these key roles in maintaining the homeostasis of the CNS due to wide range of transporters, receptors, ion channels they express. These features make astrocyte sense and respond to minute changes in CNS microenvironment. The critical dependence of neurons on astrocytes confers a protective and supportive role on astrocytes. Conversely all pathogenic stimuli which disturb astrocyte functions compromise neuronal functionality and viability. Thus understanding the structural features and properties regulating specific roles of astrocytes in maintaining brain homeostasis and how astrocytic dysfunctions contribute to certain cellular events that impact neuronal survival and outcomes in disease state can serve as a potential for drug discovery. This article reviews the various features or molecular entities of astrocytes that underpin their physiological roles. Using Alzheimer's disease, Parkinson disease, epilepsy, amyotrophic lateral sclerosis as examples we discuss the multi-dimensional changes astrocytes undergo that impact disease state. We further identify astrocyte specific pathways that are involved in neuroprotection which may be useful targets in prevention, and treatment of neurodegenerative disorders.

Keywords: Astrocytes, astrocytes-neuron interaction, neurodegenerative disorders, astrocytic dysfunction, brain homeostasis, therapeutic target

1.1.2. INTRODUCTION

Until the last few decades, there have been mostly neuron-centric approaches to neuropathological research. It was thought that neurodegenerative disorders (NDs) were mostly due to the loss of neuronal structures and functions largely in the cerebral cortex, brainstem, basal ganglia, cerebellum, and spinal cord, leading to motor and cognitive decline, dementia, and death. Researchers focused on understanding mechanisms associated with neuronal structural changes, dysfunctions, and damage, with therapy designed to target these neuronal malfunctions. This approach has failed to yield major new discoveries in the treatment and prevention of NDs. With recent advancements in technology, cell identification, culturing methods, and animal models for astrocyte research, a number of previously unsuspected roles of astrocytes have been identified, challenging previous views that astrocytes function only as support cells to neurons. These findings coupled with mounting evidence that astrocytic dysfunctions contribute to CNS disease processes [19, 20] have led to the notion that astrocytes could be a novel therapeutic target in clinical disorders and lesions of the CNS.

Astrocytes outnumber neurons and constitute about 40% of the mammalian brain [21]. They extend several processes and express gap junctions that interconnect them with other neural cells. Astrocytes also express various transporters, ion channels, enzymes and a wide range of receptors for neurotrophic factors, neurotransmitters and cytokines [22-24]. These phenotypical features position astrocytes to detect and respond to minute changes in the CNS microenvironment, maintain homeostasis, regulate and modulate learning and memory as well as breathing and sleeping [25, 26]. It is therefore plausible that alteration in astrocytic structures and properties leading to their dysfunction may be involved in the onset and progression of several neurodegenerative disorders. Ultimately, the development of new strategies and novel specific

therapeutic targets for NDs may hinge on our ability to understand the multifaceted role of astrocytes. In this review, we will provide a detailed summary of (1) the various features and properties regulating specific functions of astrocytes in health, (2) recent advances linking astrocytic dysfunctions to neurodegenerative disease, (3) and propose methods for targeting these dysfunctions in order to improve the CNS microenvironment, maintain homeostasis and treat NDs while keeping in mind current problems in the field and future research directions.

1.1.3. PHYSIOLOGICAL FUNCTIONS OF ASTROCYTES

Neuronal function and survival is compromised when small changes in the CNS microenvironment occur without the cell's ability to effectively adapt to these changes. For instance, minute changes in pH, energy supply, or ionic concentration alter brain synaptic activities. Research suggests that astrocytes play critical roles in maintaining the homeostasis of the CNS through cooperativity with other cells in the CNS and synthesis of growth and neurotrophic factors [27]. In this section we will outline the cellular processes as well as essential features and properties of astrocytes involve in maintaining the homeostasis of the CNS and the mechanisms by which they perform these functions.

Neurotrophic and Antioxidant properties

Oxidative stress is a known contributing factor of certain neuropathological diseases. The CNS is vulnerable to damaging effects of reactive oxygen species (ROS), in part because of its high rate of oxidative metabolism which generates ROS and due to auto oxidation of neurotransmitters which can lead to depletion of modest brain antioxidants and damage to neuronal membranes. Astrocytes are more resistant to oxidative stress due to their production of several antioxidant molecules and ROS detoxifying enzymes. Furthermore, astrocytes shuttle these molecules to neurons promoting neuronal viability [28, 29]. These antioxidant molecules

and ROS detoxifying enzymes include glutathione, ascorbate, vitamin E, glutathione peroxidase, glutathione transferase, thioredoxin reductase, heme-oxygenase, superoxide dismutase and catalase [30, 31]. Several studies have demonstrated the importance of astrocytic antioxidant properties in promoting neuronal survival against oxidative stress. Neuronal death induced by toxic doses of hydrogen peroxide [32] and nitric oxide [33, 34], was reduced when neurons were cocultured with astrocytes or in astrocyte conditioned media. This was attributed to the antioxidant potential of astrocytes. Thus, increasing the antioxidant properties of astrocytes or reducing their ability to produce ROS is a potential strategy to reducing neuronal damage in NDs.

Astrocytes have been shown to produce various neurotrophic factors, most notably: glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (FGF2), brain-derived neurotrophic factor (BDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF) and Nerve growth factor (NGF) [35, 36]. These factors possess some pharmacological and therapeutic potential. For example, it has been shown that administering BDNF protects cholinergic neurons from degenerative changes following axotomy in adult rat brains [37]. This finding implies that the human NDs may be due to alterations in disorder-specific neurotrophic factors, their respective receptors, or neurons undergoing programmed cell death as a result of their inability to obtain sufficient amounts of neurotrophic factors [38]. Hence, therapeutic modulation of the trophic potential of astrocytes should be given more scrutiny in order to develop agents which may improve NDs prognosis.

Energy metabolism

The brain consumes more energy than any organ of the body with glucose being the obligatory energy substrate. Astrocytes with their processes that reach blood vessels, neurons and synapses take up glucose from circulation through their insulin independent glucose transport

protein (GLUT) and provide neuronal elements with energy metabolites. It has been established that glucose metabolism is compartmentalized in the brain. Astrocytes rely heavily on glycolytic metabolism, whereas neurons use the end products of glycolysis for oxidative metabolism [30, 39]. Recent studies have examined why astrocytes oxidize glucose to a lesser extent than neurons even though both possess mitochondria. One line of evidence indicates that, whereas neurons possess low levels of 6-phosphofructose-2- Kinase/ fructose 2-6 bisphosphatase-3, (Pfkfb3) an important regulator of glycolytic enzymes, astrocytes highly express this enzyme [40]. The variation in expression of this enzyme was attributed to the regular proteasomal degradation of Pfkfb3 by anaphase-promoting complex/cyclosome (APC/C)–Cdh1 in neurons [40]. Furthermore it has been suggested that astrocytes rely heavily on glycolysis due to the low activity of pyruvate dehydrogenase complex (PDC) an important enzyme regulating the entry of pyruvate into the Krebs cycle [39].

Glycogen granules located in astrocytes are the largest energy reserve of the brain localized in areas where synaptic density is high [41]. Evidence suggests that astrocytic glycogen is critical as fuel during physiological and pathological emergencies as well as periods of high neuronal activity. During hypoglycemia and ischemia, astrocytic glycogen is broken down to lactate which serves as energy substrate for neurons [42, 43]. The role of astrocytes as the source of energetic fuel for neurons was originally proposed by Pellerin and Magistretti (1994). It was suggested that activation of neurons with the release of the neurotransmitter glutamate stimulated glycolysis in nearby astrocytes, and the lactate so produced was then released by astrocytes to fuel neuronal metabolism. This hypothesis was termed the astrocyte-neuron lactate shuttle hypothesis (ANLSH) [44]. Astrocytes are also involved in brain lipid metabolism and lipoprotein

secreting. These cells secrete apo, ApoE which are involved in lipid metabolism and transporting cholesterol to neurons [45, 46].

Neurotransmitter uptake and recycling

Astrocytic processes are enriched with transporters for the uptake of a variety of neurotransmitters including glutamate, GABA and glycine [47]. These transporters are involved in the sequence leading to the termination of the action of neurotransmitters so as to maintain synaptic homeostasis. In particular, astrocyte specific glutamate transporters GLT-1 and GLAST (excitatory amino acid transporters EAAT2 and EAAT1) are important in removing excess glutamate from the synaptic cleft in order to prevent overstimulation of glutamate receptors which may cause neuronal damage. The importance of these astrocytic glutamate transporters in preventing glutamate induced excitotoxity has been demonstrated by many studies [48-50]. Mice deficient in the main astrocytic glutamate transporter, GLT-1 showed increase vulnerability to hippocampal and cortical injury as well as lethal spontaneous seizures attributed to elevated levels of glutamate in their brain [50]. Likewise, it has been reported that chronic inhibition of the expression or actions of this important astrocytic glutamate transporter by different agents *in vitro* have resulted in neurodegeneration cause by elevated glutamate levels [48, 49]. These experiments suggest that astrocytic glutamate transporters could be a useful pharmacological target in NDs associated with glutamate excitotoxity.

It is important to maintain a neurotransmitter pool in neurons so as to prevent the disruption of both excitatory and inhibitory neurotransmissions as well as to meet the energy demands of neurons. Glutamate taken up by astrocytes can either be metabolized to α-ketoglutarate providing a substrate for TCA cycle, generating ammonia [51] or in a reaction with ammonia, converted to glutamine which is shuttled to neurons [52]. This reactions are catalyzed

by glutamate dehydrogenase and glutamine synthase respectively, enzymes primarily expressed by astrocytes. In neurons, glutamine can be subsequently converted back to glutamate via deamination by phosphate-activated glutaminase [52, 53]. However, in inhibitory neurons glutamate is decarboxylated to the inhibitory neurotransmitter GABA representing the glutamine-glutamate-GABA cycle [52, 53]. Astrocytes also solely express the anaplerotic enzyme, pyruvate carboxylase. This enzyme is critical for astrocytes to replenish the amino acid neurotransmitter pool via glucose utilization [54]. This astrocytic-neuronal cooperation has been demonstrated to be disrupted in certain NDs [53] making them a novel pharmacological target in the search for NDs therapy.

Astrocytes are directly involved in synaptic transmission by releasing synaptically active molecules called gliotransmitters [55]. These molecules which include glutamate, GABA, ATP, adenosine, D-serine are released by astrocytes in response to changes in neuronal synaptic activities that cause astrocyte excitation, evident by elevated Ca^{2+} in astrocytes. This gliotransmitters affect synaptic transmission, for instance astrocytic glutamate by acting on extracellular NMDA receptors mediates neuronal synchrony [56]. Similarly, astroglial ATP has been reported to serve as an extracellular messenger for astrocytes and other neural cell communication [57, 58].

Regulation of extracellular pH, ions and osmolarity

Neuronal activity and its resulting cellular depolarization cause changes in the extracellular ion concentration, pH, and osmolality. If these changes are not closely regulated, synaptic transmission and CNS functions will be compromised. Astrocytes whose processes wrap synapses, closely regulate ionic and water content of the interstitial space. Their processes are rich with transporters and enzymes, and aquaporin 4 (AQP4) water channels which are

involved in the exchange of ions between the extracellular space and cytoplasm, and regulating osmolality in the brain, respectively. Neuronal activity causes substantial local increases in K^+ in the extracellular space. Without tight regulation this alters neuronal membrane potential and seriously compromise CNS function. Astrocytes are permeable to K^+ due to the expression of numerous potassium channels. Also, Na^+ K^+ ATPase and $Na^+/K^+/Cl^-$ cotransporters [23, 59] help astrocytes in maintaining a constant potassium concentration at levels compatible with continued neuronal activity [60]. Astrocytes, in a process known as spatial K^+ buffering, transiently accumulate and redistribute K^+ to maintain K^+ homeostasis and prevent neuronal hyperexcitability [61]. For this to occur, a cell has to form a syncytium for K^+ current to transverse a relatively long distance and these cells should be highly and selectively permeable to potassium [61]. Astrocytes form a coupled intercellular network through gap junctions composed of six connexin subunits that enables movement of small molecular weight molecules between adjacent cells [62]. In addition to gap junctions, they also express inwardly rectifying K^+ (Kir4 and Kv 1.5) channels that surround blood vessels and synapses in the brain [63, 64]. The net accumulation of K^+ in astrocytes is accompanied by osmotically driven water leading to astrocytic swelling [65]. This swelling as a result of decreased external osmolarity is mediated through AQP4.

In addition to slight changes in K^+ concentration, relatively minor changes in pH affects neuronal excitability, synaptic transmission and metabolism and impacts CNS enzymatic function [22]. Astrocytes express carbonic anhydrase (CA), Na^+/H^+ exchangers, Cl^-/HCO_3 antiporters and Na^+-HCO_3^- cotransporters (NBC) that enables them to regulate pH [66-68]. Due to the many important astrocytic features, a model has been proposed in which synaptic transmission is coupled to astroglial pH regulation and neuronal energy supply [22, 69].

According to this model, glutamate uptake via EAAT during periods of increased neuronal activity results in a net uptake of sodium and H^+ accompanied by the release of potassium. CO_2 generated by oxidative metabolism during this period diffuses into astrocytes where it is degraded to protons and bicarbonate via astroglial CA. Sodium and bicarbonate is then extruded in a 1:2 ratio via NBC. The extruded bicarbonate removes acid equivalents and the degradation of CO_2 makes astrocytes act as a CO_2 sink. The intracellular protons facilitate lactate secretion from astrocytes through MCT-1 and 4 which is subsequently taken up by neurons through MCT-2 for use as energy. This model is significant because it shows that defects in one astrocytic protein can lead to several cellular defects and care has to be taken when therapeutically targeting certain astrocyte proteins.

Blood Brain Barrier formation and blood flow regulation

The blood brain barrier (BBB) is formed by endothelial cells, microglia, pericytes, astrocytic end feet, and neuronal processes [70, 71]. The BBB functions to provide essential nutrients for CNS cells regulate ionic content of interstitial fluid (ISF), and separate neuroactive agents and transmitters that regulate brain environment [70, 71]. Tight junctions formed by endothelial cells allow for selective permeability of low molecular weight molecules. Aside from the structural support astrocytes provide to the BBB, the influence of astrocytes on the function of the BBB is not well understood. Some studies provide evidence which favors the role of astrocytes as a cell type which releases factors such as, FGF2, Angiopoietin, and GDNF [72-74] to influence the BBB by promoting vascular stabilization and remodeling, BBB junction protein expression, and reducing BBB leakage or permeability [72-74]. Due to the different cellular models and experimental conditions employed in these studies, which may not necessarily mimic *in vivo* conditions, coupled with the fact other cells surround the BBB which may also provide

these factors at different times during development, more studies using models that mimic *in vivo* conditions are needed to understand the molecular mechanisms astrocytes employ to influence BBB characteristics. Astrocytes also play key roles in controlling blood flow and changes in microcirculation of the CNS. This occur in response to stimulation of neuronal and astrocyte processes leading to increase Ca^{2+} and gliotransmitters release. Increase of these molecules lead to the release of other mediators such as nitric oxide, arachidonic acids metabolites and increase flux of potassium ions which leads to vasodilation or vasoconstriction [75, 76].

1.1.4. ASTROCYTE DIVERSITY; A CRITICAL CONSIDERATION IN ASTROCYTES AS A THERAPEUTIC TARGET.

Astrocytes are heterogeneous. Histological and biomolecular studies have characterized astrocytes into two main types based on their morphology and anatomical localization [77]. The first type called protoplasmic astrocyte is located in the gray matter of the CNS and is characterized by highly complex processes, fewer glial intermediate filaments such as vimentin, nestin, and GFAP. The second type termed fibrous astrocytes express higher levels of vimentin, nestin, and GFAP and are located in white matter with distinguishable processes with moderate to little branching. Other morphologically distinct astrocyte-like-cells have recently been recognized, however their description is beyond the scope of this review. Further extensive studies have shown that astrocytes exhibit gene expression, functional, and physiological heterogeneity across and within brain regions.

In vivo and *in vitro* studies comparing translated mRNAs in hippocampal, cerebellar, major cortical and subcortical astrocytes, have reported differences in the gene expression profile of astrocytes from these brain regions [78-81]. Heterogeneity in astrocyte gene expression

implies that there is physiological and functional diversity among astrocytes [78, 82]. The variation in astrocyte glutamate uptake, coupling, ability to buffer potassium, and support neurotransmission and the metabolic needs of neurons may be associated with differences in gap junction proteins (connexin 43 and connexin 30), potassium channels, and glutamate transporter expression across brain regions [78, 80, 82, 83]. Recent electrophysiological studies reported that striatal astrocytes exhibit lower levels of Kir4.1 than hippocampal astrocytes [84]. Another study illustrated a high degree of variability in glycogen content and storage in astrocytes between and within brain regions. Glycogen immunoreactivity was observed to be higher in the cortex, hippocampus, striatum, and cerebellar molecular layer compared to white matter and most of the subcortical structures [85] suggesting that astrocyte neuron metabolic coupling between and within brain regions may differ. It also raises the possibility that glycogen might not be essential for all neuronal circuit function.

Despite vast evidence that astrocytes are heterogeneous, and recent research suggesting that fibrous and protoplasmic astrocytes respond differently to injury, as has been shown by the differences in their response to ischemia [86, 87], astrocytic functions are yet to be categorized for each astrocytic sub-type. This may be due to the fact that the majority of reported studies relied on astrocytes cultures for studying gene and functional diversity in astrocytes. Since it has been proven that astrocyte culture conditions alter gene expression profiles and phenotypes [4], more investigation is needed using *in vivo* methods to understand the exact roles astrocyte sub-types play in health and disease. It is also important to adopt a context specific approach in interpreting data as results generated using astrocytes from one brain region may not be generalizable to other areas of the brain. Therefore, when designing compounds to target

astrocytes in NDs, regional and subtype specificity should be carefully considered in order to develop treatments that target only astrocytes involved in the disease pathology.

1.1.5. ASTROCYTES IN DISEASED BRAIN

One can expect that astrocytic dysfunctions may result in NDs. Several neurological diseases share common pathogenic mechanisms such as excitotoxicity, oxidative stress, metabolic dysfunction, or inflammation, many of which are neutralize by astrocytes in healthy brain. Thus, disease progression is linked to chronic or escalating exposure to harmful stimuli that exhausts astroglial neuroprotective mechanisms or activates deleterious pathways in astrocytes. Understanding the functional and biomolecular characteristics of astrocytes under pathological conditions will lead to developing therapies that target these astrocyte-specific pathways involved in the outcome of NDs.

Astrogliosis is triggered and modulated by glutamate excitotoxicity, oxidative stress, ischemia associated hypoxia and glucose deprivation, chemokines and growth factors as well as ATP, Amyloid beta and α-synuclein accumulation following injury or neurodegeneration [88]. Astrogliosis is recognized both *in vivo* and *in vitro* by cellular hypertrophy and increase expression in intermediate filament GFAP, vimentin, and nestin [89]. Changes in some functional astrocytic proteins such as gap junctions, connexin 43, glutamate transporters and AQP4 have been proposed to be associated with this phenomenon. The debate about whether reactive astrocytes are harmful or beneficial is still ongoing. Although astrocytes role has been described in a number of brain pathologies, in this section, we will provide an overview of astrocytes roles in Alzheimer's disease, Parkinson disease, epilepsy, amyotrophic lateral sclerosis, onset, and progression as well as highlight the beneficial and detrimental roles of reactive astrocytes in the context of these neuropathologies.

Alzheimer's disease

Alzheimer's disease (AD) is a disorder characterized by progressive memory and cognitive decline, as well as disturbances in mood and behavior. Typical pathological hallmarks of AD are the deposition of extracellular amyloid β (Aβ), intraneuronal neurofibrillary tangles composed of tau protein and astrogliosis [90]. Although the exact pathophysiological mechanism behind AD development and progression has not been fully elucidated, several hypothesis have been proposed to address the pathogenesis of this disease. The amyloid metabolic cascade hypothesis which states that Aβ dyshomeostasis is responsible for the cognitive decline and most cellular and molecular alterations observed in AD is generally accepted [90, 91]. Consequently, many therapeutic strategies based on this hypothesis have been largely ineffective in delaying the progression of AD, meaning the abnormalities of this disease involve several factors including diverse cellular and biochemical processes.

Astrocytes play an important neuroprotective role in AD by internalizing and degrading Aβ to avoid the deposit of toxic extracellular Aβ. Immunohistochemistry on brain tissues from AD patients shows that reactive astrocytes accumulate Aβ in a quantity that correlates to the extent of local AD pathology [92]. When adult astrocytes transplanted into the hippocampus of AD transgenic mice were observed at different times, it was observed that these astrocytes phagocytosed and degraded Aβ through the actions of Aβ degrading proteases expressed by astrocytes [93]. Similarly, when astrocytes seeded on top of Aβ bearing sections from AD patients and AD mice were observed, these astrocytes internalized and degraded Aβ reducing the levels of Aβ in the sections [93, 94]. The precise mechanism by which astrocytes degrade Aβ is not known but apolipoprotein E (Apo E) which is almost exclusively expressed by astrocytes has been proposed to be responsible for this function. ApoE facilitates the degradation of Aβ

through internalization of Aβ bound ApoE receptors which is delivered to lysosomes for degradation [95] and by enhancing the activities of neprilysin and insulysin, the enzymes involved in the degradation of Aβ [96]. ApoE polymorphic alleles are the major genetic determinants of AD with individuals expressing the ε4 allele being at a risk of AD as a result of disruption in Aβ and tau clearance or aberrant hyper phosphorylation [97]. Literature on ApoE in AD is extensive and its detailed revision is outside the scope of this review. Interestingly astrocytes degrade Aβ in the early stages of AD even before plaque formation [98] suggesting a protective role for astrocytes in the early stages of AD. This protection is lost following Aβ accumulation in astrocytes in later stages of the disease.

Additional evidence supports the view that accumulated Aβ compromises astrocytes physiological functions. Aberrant calcium oscillation and waves has been observed in astrocytes proximal to senile plaques in AD brains [99, 100]. Also, acute and chronic exposure of astrocyte cultures to Aβ led to elevated calcium levels and calcium mediated mitochondrial damage and oxidative stress [101, 102]. It has been reported that reactive astrocytes release excess GABA leading to memory decline in AD. Studies have shown that reactive astrocytes in AD APP/PS1 mice have increased GABA content and release contributing to learning and memory impairment. Furthermore, inhibition of GABA production and release from reactive astrocytes restored cognitive decline observed in this transgenic mouse [103]. The increase in GABA release was attributed to increased Monoamine oxidase B (MAO-B) mediated GABA synthesis from putrescine. Astrocytic GABA modulates hippocampal synaptic plasticity during memory processing. In healthy brains GABA released from astrocytes acts on $GABA_B$ receptors of interneurons and exerts a disinhibitory action at the prefrontal cortex to the dentate gyrus (DG). In AD transgenic mice however, astrocytic GABA exerts inhibitory effects in glutaminergic

terminals via $GABA_A$. Using a transgenic mouse model of AD (5xFAD), researchers found that excessive GABA released through astrocyte specific GABA transporters enhanced tonic GABA inhibition in the dentate granule cells. They also reported that reducing tonic inhibition in this 5xFAD mouse by blocking astrocytic GABA release or $GABA_A$ receptors rescued the impairment of LTP and memory deficit [29]. In addition, MAO inhibitors were also found to restore impaired spike probability, synaptic plasticity and learning and memory deficit induced by tonic GABA inhibition on DG neurons in AD transgenic mice [103]. This result supports the idea that tonic GABA inhibition of the DG neurons cause memory decline in AD. Thus, reducing this tonic inhibition by selectively inhibiting $GABA_A$ receptors, astrocytic GABA synthesis or release may serve as an effective therapeutic strategy for treating memory impairment in AD.

Impairment in glutaminergic neurotransmission has also been implicated in AD progression. The ability of astrocytes to sense and respond to glutamate levels, following Aβ induced damage may contribute to alteration in neuronal microenvironment and cause overstimulation of glutamate receptors. This abnormal stimulation of glutamate receptors has been proposed as a mechanism of synapses damage in AD. Studies have demonstrated that Aβ decrease the expression or activity of GLT-1 and GLAST in astrocytes thereby disrupting the clearance of glutamate leading to neuronal excitotoxity [104-106]. Some mechanisms by which GLT-1 expression is decreased in AD have been proposed. Astrocytic Adenosine receptor ($A_{2A}R$) has been reported to be involved in modulating the activity and long term reduction in the expression of glutamate transporters [107]. In a study where the effects of $A\beta_{1-42}$ on GLT-1 and GLAST expression was tested in both astrocytes obtained from wild type mice and $A_{2A}R$ global knock out mice, the ability of $A\beta_{1-42}$ to decrease the expression of GLT-1 and GLAST in wild type mice was completely absent in astrocytes from $A_{2A}R$ global knock out mice. Rather GLT-1

expression was increase upon $A\beta_{1-42}$ exposure in astrocytes from $A_{2A}R$ global knock out mice [105] indicating that Aβ induce decrease in astrocytic glutamate transporter expression might occur via A_{2A} receptor making this receptor a novel therapeutic target. GLT-1 repression could also be regulated at both transcriptional and translation levels and this could be a mechanism by which glutamate toxicity is associated with AD. $NF\text{-}_KB$ and N-myc have been shown to be involved in TNF-α mediated transcriptional repression of GLT-1 [108]. In AD brains N-myc was found to be overexpressed in astrocytes [109] indicating that N-myc levels could be the underling mechanism causing decreased GLT-1 expression. Apart from causing glutamate excitotoxicity decrease expression of GLT-1 has also been linked with cognitive and memory deficit associated with AD. In animal models, GLT-1 knockdown and $A\beta_{1-42}$ administration leading to decrease GLT-1 expression has been reported to cause and accelerate cognitive and memory decline [105, 110]. Thus the targeting of GLT-1 should improve memory and reduce cognitive decline as well as neurotoxicity associated with AD.

The involvement of the cholinergic pathway in AD memory loss is indicated by studies showing loss of cell bodies in the nucleus basalis of Meynert (the source of cholinergic projections) in postmortem brains of demented patients with AD [111], reduction in choline acetyltransferase activity following increasing number of plaques in these individual [112] as well as studies showing the decline in cognitive ability associated with increasing use of anticholinergic agents [113]. The alteration in the levels of some important neurotrophic factors may account for disruption in the cholinergic system, the accumulation of Aβ plaque and its associated dementia. NGF is synthesized in the cortex and hippocampus and retrogradely transported to the basal forebrain cholinergic neurons and involved in cholinergic system development, neuronal survival and stimulation of choline acetyltransferase activity for

acetylcholine synthesis [114]. NGF levels have been reported to be reduce following chronic Aβ administration in mice [115]. Also *in vivo* and *in vitro* studies have shown that NGF levels regulates the phosphorylation of APP, β secretase activity with decrease NGF favoring Aβ peptide formation [114, 116]. Furthermore NGF treatment has been shown to improve cognitive ability and reduce amyloidogenesis [114, 117]. BDNF which is important for neuronal survival and function in addition to its role in mediating synaptic plasticity as well as assisting in early and long term potentiation has also been shown to be decrease in AD brain [118, 119]. The loss of this neurotrophic factors and functions in AD could be as a result death of neurons involve in their production or reduce synthesis by astrocytes following Aβ accumulation. It is also possible that this alteration in neurotrophic factors could be a cause of neuron death, Aβ accumulation and cognitive impairment.

In conclusion astrocytes play both a protective and deleterious role in AD. As Aβ accumulates and the disease progress, astrocyte protective roles diminish while deleterious pathways are activated. Therapeutic targeting of the major mechanisms by which astrocytes exert neurotoxic effects in AD may modify their functions.

Epilepsy

Epilepsy comprises a family of neurological disorders characterized by periodic, unpredictable and unprovoked seizures. Aberration in GABAergic and glutaminergic pathways is associated with seizure activity with the widely used antiepileptic drugs targeting GABA metabolism and transport. Astrocytes are important contributors to this aberration. In human epileptic tissues, excessive glutamate levels in the epileptic hippocampus have been reported before and during seizure [120, 121]. Studies in humans probing whether this excess extracellular glutamate is as a result of changes in the expression of glutamate transporters have

produced conflicting reports. Using immunohistochemistry and densitometry some studies reported a reduced expression of EAAT1 and EAAT2 in the hippocampus and temporal lobe of human patients with temporal lobe epilepsy (TLE) [122, 123] while other studies using in situ hybridization and immunoblot reported no change in the expression levels of EAAT1 and EAAT2 [124, 125]. These inconsistencies may be a result of differences in tissue processing, or the experimental procedure/time after seizure and techniques use in measuring the expressed levels of the transporters. Other studies in animal models have shown that knock down or deficiency of GLT-1 in astrocytes leads to seizures and mortality [50, 126]. This suggests that alteration in glutamate transporters plays a critical role in epileptogenesis. One other potential mechanism hypothesized to account for the abnormally high level of glutamate in the hippocampus of the human epileptic brain is the loss of glutamine synthase (GS). When the distribution, quantity and activity of GS were assessed in hippocampus of patients with mesial temporal lobe epilepsy (MTLE), it was observed that GS expression and activity was decreased as compared to non-MTLE sample [124]. Similarly, when rats were chronically infused with a GS inhibitor methionine sulfoximine (MSO) the concentration of glutamate increased in MSO treated compared to saline treated rats. Recurrent seizures also ensued with key features of human TLE with hippocampal sclerosis, confirming the role of GS in glutamate toxicity and seizure generation [127, 128]. In addition to affecting glutamate and GABA levels, downregulation of GS activity may lead to accumulation of ammonia. In primary astrocyte cultures hyperammonemia has been reported to reduce glutamate transport and contribute to neurotoxicity [129]. The above studies make astrocytic GS an important target for anti-epileptic therapy.

Seizure activity additionally involves aberration in ion and water homeostasis. This has been studied extensively using *in vivo* and *in vitro* models as well as brains of epileptic patients. Alteration in brain osmolarity and volume of the extracellular space (ECS) contribute directly or indirectly to neuronal discharge or epileptic activity [130]. Astrocytes role in water and ion homeostasis is associated with this phenomenon. For instance, astrocytes role in regulating K^+ homeostasis has been shown to be impaired in TLE. Using ion-sensitive microelectrode and patch-clamp studies, down-regulation of Kir currents and impaired Kir channel expression in specimens from patients with TLE has been observed [131, 132]. Also, down regulation and knockout of Kir4 in animals has been reported to lead to neuronal hyperexcitability and the development of seizures [133, 134] suggesting that Kir channels could be a novel therapeutic target. Astrocytic gap junctions are important in spatial redistribution of glutamate and K^+. Thus, alteration in astrocytic gap junction coupling may affect seizure generation. In a study analyzing astrocytic gap junction coupling in hippocampal specimens from patients with mesial temporal lobe epilepsy (MTLE) with or without sclerosis using different experimental techniques, it was found that MTLE specimens with sclerosis completely lacked classical astrocyte gap junction coupling whereas coupled astrocytes were abundant in non-sclerotic specimens [135].

One important gliotransmitter released by astrocytes, ATP, has also been shown to impact seizure generation. ATP released by astrocytes is converted into adenosine monophosphate (AMP) and then adenosine by extracellular nucleosidases. When adenosine acts on A1 receptors located on presynaptic neurons it inhibits excitatory neurotransmission and promotes inhibitory neurotransmission by decreasing the release of various excitatory neurotransmitters [136]. Adenosine level is reduced by enzymatic phosphorylation into Adenosine monophosphate (AMP) by astrocyte specific enzyme adenosine kinase (ADK). An

alteration in ADK is thus relevant to seizure generation. In animal models and the hippocampus of patients with TLE, ADK overexpression has been observed with its associated decrease in Adenosine [137, 138]. Additionally, inhibitors of ADK, adenosine receptor agonist and intracranial injection of Adenosine have been reported to prevent seizures [139-141]. Therefore, adenosine augmentation therapies could be an effective strategy for seizure prevention.

Whether all of these astrocytic dysfunctions contribute to the initiation of seizures or are compensatory mechanisms after the seizure has developed remains to be investigated. Nevertheless, their modulation and regulation therapeutically could be an innovative approach to the prevention and treatment of epilepsy.

Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder, pathologically characterized by loss of dopaminergic neurons in the substantia nigra pars compacta, and a presence of Lewy bodies, and accumulation of α-synuclein [142, 143]. Clinically PD is a movement disorder with patients presenting with symptoms of rigidity, bradykinesia, resting tremor and postural instability [143, 144]. Since there is no cure for this disease, PD treatments focus on managing the disease symptoms with patients often receiving a dopaminergic replacement therapy, levodopa and carbidopa. However continuous administration of this therapy results in motor fluctuations and dyskinesia, confusion and hallucination [143]. Hence there is a need for further research into the etiology of this disorder in order to find a better treatment. Studies have implicated astrocytes in the progression of this disease. A-synuclein accumulation in astrocytes has been reported in PD with neurons shown to transfer this substance to astrocytes in cases of overload [145, 146]. It has been reported that accumulation of α -synuclein in astrocytes induces pro-inflammatory genes, cytokines and chemokines causing

astrogliosis [146] as well as an increase in ROS generation resulting in oxidative damage to neurons [147]. In human brain tissue it has been shown that the disease is associated with mutations in astrocytes specific genes [148, 149]. Defects in the genes for DJ-1 (PARK7), Parkin (PARK2), α-synuclein (SNCA), Group VI Ca^{2+} -independent phospholipase A_2 (PLA2G6) and PTEN- induced putative kinase 1 (PINK1), are known to be associated with the disruption of astrocytic functions, leading to deterioration of the brains microenvironment which affects neuronal survival [150-154].

Like other neurodegenerative disorders, astrocytic regulation of glutamate in the extracellular space is altered in PD, due to decreased expression and function of glutamate transporters. Lipid rafts are dynamic membrane microdomains enriched with cholesterol and glycosphingolipids, flotillin-1 (flat-1) and caveolin-1 (can-1), being the main protein components in astrocytes [151]. They are found in both plasma membranes and cell organelles and act as a site for the communication and assembly of proteins involved in endocytosis, membrane sorting and trafficking, cell polarization and signal transduction [155]. Studies have reported that DJ-1 is important in regulating the assembly and stability of lipid rafts in astrocytes [151, 156]. Lipid raft disruption caused by decreased expression of flotillin-1 (flat-1) and caveolin-1 (can-1) has been shown in Park7 Knockout and mutant astrocytes. This leads to decreased protein expression of the astrocyte-specific glutamate transporter EAAT2 whose assembly, localization and function are known to be due to its association with lipid rafts, leading to decrease glutamate uptake [151, 157] resulting in neuron excitotoxity. Astrocytes exert some neuroprotective effect by producing neurotrophic and antioxidant factors as well as serving as scavengers of ROS. GDNF, MANF, and BDNF are important neurotrophic factors produced by astrocytes and shown to protect against dopaminergic neuron death [158-160]. Intrastriatal injection of MANF, for

instance, has been found to be neurorestorative in the murine 6-hydroxydopamine (6-OHDA) model of PD [159, 161]. In PD it is believed that impaired trophic and antioxidant functions of astrocytes are involved in disease progression. For instance, DJ-1 deficiency has been reported to reduce GDNF and BDNF expression in astrocytes [162]. Similarly, GSH a ROS scavenger whose level is regulated by astrocytic Parkin has been shown to be lower in Parkin-null mice than in wild-type [163]. To Further confirm the protective role of astrocytes in PD, Nuclear factor E2-related factor 2 (Nrf2) an important transcription factor regulating the expression of several antioxidant molecules, and preferentially induced in astrocytes [164] was overexpressed in astrocytes, this restricted overexpression was shown to enhance neuronal survival in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of PD [165].

Whether all these changes occur in all the different types of astrocytes remains to be seen. Some studies have shown that PD related genes and α-synuclein are concentrated more in protoplasmic astrocytes and not fibrous astrocytes [148, 149, 166]. Determining the roles of the various astrocytic sub-types will ensure selective and effective therapeutic targeting of a particular subset of astrocytes and, hopefully, produce a more desirable clinical outcome.

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a progressive fatal neurodegenerative disorder characterized by sequential or simultaneous loss of upper and lower motor neurons in the brain and spinal cord [167]. Abnormalities in motor neuron physiology leads to loss of muscular synapses, motor weakness and skeletal muscle atrophy causing paralysis and death due to respiratory failure. About 90% of cases are sporadic (SALS) with little genetic component identified. The remaining cases are inherited dominantly (FALS) and linked to causative genes such as Cu/Zn superoxide dismutase (SOD1 [168]. ALS develops under several complex

pathophysiological mechanisms which with their combined effects lead to the death of motor neurons. Following the discoveries of mutations in genes encoding for the antioxidant enzyme Cu/Zn superoxide dismutase and other genes in patients with FALS, several studies using transgenic models expressing these mutant genes showed that astrocytes do not only react to damage in degenerating neurons but are also involved in the complex events in neurodegeneration in ALS. When astrocytes isolated from postmortem tissue of patients with FALS and SALS were introduced in motor neuron cultures it was observed that these astrocytes induce motor neuron death [167]. In this same study suppressing SOD1 in astrocytes isolated from SALS and FALS patients confers significant motor neuron protection when they were introduced in motor neuron culture.

Impairment in the homeostatic function of astrocytes to regulate glutamate levels in the synaptic cleft is one of the mechanisms hypothesized to explain the motor neuron loss in ALS. Using different experimental techniques, loss of astroglial glutamate transporter GLT-1 and GLAST has been observed in patients with ALS and animal models of ALS [20, 169, 170]. Although the loss of glutamate transporters in ALS cannot be debated, whether this loss is a primary cause in the cascade leading to neuron degeneration or a secondary event to cell death is still inconclusive. Using transgenic mice overexpressing SOD1, Howland et al observed a focal loss of GLT-1 in the ventral horn of the spinal cord before motor neuron degeneration with loss increasing as the disease progresses [169]. However Bendotti and colleagues using similar transgenic mice expressing mutant SOD1 showed a decrease in GLT-1 immunoreactivity after motor neuron impairment and motor neuron loss but not before the appearance of clinical symptoms [171]. It is also important to note that oxidative reactions triggered by SOD1 mutants in ALS inactivates GLT-1 as shown in animal models of FALS [172], suggesting that GLT-1

inactivation leading to glutamate induced toxicity contributes to disease progression but is not the main cause of motor neuron death. By regulating the calcium entry through AMPA receptors on motor neuron, astrocytes also protect motor neurons against glutamate induced excitotoxic death. AMPA receptors are important in mediating fast excitatory neurotransmission. Excessive stimulation and Ca^{2+} permeability of this receptor mediate neurotoxicity in some degenerative disorders [173]. AMPA receptors are composed of four subunits (GluR1-4). The absence or presence of the GluR2 subunit regulates the degree of Ca^{2+} permeability, with the absence or low expression of this subunit permitting excessive Ca^{2+} permeability [174]. Relative expression of GluR2 in functional AMPA receptors in motor neurons is regulated by astrocytes. Studies have shown that the ability of astrocytes to control the expression of this subunit of AMPA glutamate receptor on motor neurons is lost in SOD1 mutant astrocytes. This leads to a decrease in the GluR2 subunit and increased calcium influx through AMPA receptors leading to AMPA mediated neurotoxicity [175].

One other way in which astrocytes contribute to motor neuron death in ALS is altered secretion of important neurotrophic factors. Astrocytes promote motor neuronal survival by secreting neurotrophic factors such as NGF, GDNF and BDNF. NGF is critical for neuronal survival and differentiation through activation of the tyrosine kinase receptor, TrkA. It can also stimulate neuronal death by stimulating the p75 neurotrophic receptors isoform [176]. Adult motor neurons lack both of these receptors and are unresponsive to NGF. However under disease conditions such as seen in ALS patients and transgenic mice, p75 neurotrophic receptors isoform is re-expressed [177]. In SOD (G93A) mice, reactive astrocytes were found to express NGF in high amounts inducing apoptosis in p75 neurotrophic receptors isoform expressing motor neurons by a mechanism involving nitric oxide and peroxynitrite formation thereby contributing

to the progression of the disease [178]. Astrocyte derived lactate is shuttled to neurons through Monocarboxylate transporters and serves as an energy substrate for neurons. Studies have been conducted to investigate the metabolic dysfunctions in ALS which may be a compensatory effect during disease progression or the cause of neuron death. The shuttling of lactate between astrocytes and neurons for energy is impaired in ALS due to a decreased expression of the astrocyte lactate transporter (Monocarboxylate transporter 4) as shown in mutant SOD1 expressing astrocytes [179, 180]. Reduced energy supply to motor neurons may contribute to their dysfunctions and possibly death. These findings demonstrate the roles of astrocytes in ALS disease progression in both patients and animal models. Further studies are needed to establish, at the microcellular level, molecular features linked to astrocyte dysfunction to allow new pharmacological targets to be identified.

1.1.6. THERAPEUTIC POTENTIALS OF ASTROCYTE

Since astrocyte dysfunctions may play a role in the initiation and progression of NDs, approaches that restore, modulate or improve astrocyte functions, or target their signaling or biochemical pathways may aid in the development of novel treatments for NDs. In this section we will discuss some approaches and compounds that have been shown to restore proper astrocytic functions and improve CNS disease prognosis. In neurodegenerative disorders such as PD, ALS and AD where imbalance between ROS/oxidative stress and antioxidant molecules are culprit in neuronal loss, increasing the availability of molecules to scavenge ROS or inhibit their production is an important neuroprotective strategy. Transcription factor Nrf2 a cytoplasmic protein binds to its antioxidant response element (ARE) and regulate the expression of several antioxidant genes like SOD, as well as the two enzymes involved in the synthesis of GSH , glutamate cysteine ligase and glutathione synthase increasing the antioxidant defense [181].

Hence activation of the Nrf2-ARE pathway could be therapeutically beneficial. Since Nrf2 is preferentially activated in astrocytes, Nrf2 overexpression in astrocytes or activation has been reported to be neuroprotective in neurodegenerative conditions. Overexpression of Nrf2 in astrocytes enhanced neuronal survival and extend disease onset in ALS and PD animal model [165, 182]. Several molecules which activate the Nrf2-ARE pathway have been shown in both *in vivo* and *in vitro* models to decrease accumulation of aberrant proteins and protect against neuronal loss [181, 183, 184]. Examples includes the triterpenoids, 2-cyano-3, 12 dioxooleana-1,9-dien-28-oic acid ethylamine (CDDO-EA) and 2-cyano-3, 12 dioxooleana-1,9-dien-28-oic acid-trifluoroethylamine (CDDO-TFEA), tert-Butylhydroquinone and Sulforaphane [181, 183]. Whether these strategies are beneficial in counteracting the loss of neurons in all NDs needs to be investigated as they have been mostly screened in PD and ALS animal models. Since most of these studies used acute models, there is a need to investigate these compounds in chronic models of NDs to see if inducing this pathway could be a sure long-term neuroprotective strategy.

In addition to antioxidant molecules, various neurotrophic factors (NTF) produced by astrocytes and neurons are associated with different neurodegenerative conditions [115, 119, 178, 185]. Chief among this are BDNF, NGF and GDNF. Delivery of some neurotrophic factors to the brain could increase neuronal viability and survival in neurodegenerative disease. However neurotrophic factors are hydrophilic relatively large molecular weight molecules which are restricted by the BBB after systemic administration. To achieve an effective concentration at the target site, several strategies have been developed recently to deliver these trophic factors to the brain, including intra-cerebroventricular injection (ICV), intranasal administration, and viral vector mediated delivery of neurotrophin genes. Safety issues with the introduction of a virus and the invasiveness of these methods coupled with their side effects made this approach

undesirable. Since astrocytes can serve as source of trophic support to neurons, transplantation of astrocyte restricted precursors genetically engineered to produce growth factors could represent a more efficient delivery method. Most neurotrophic factors secreted by astrocytes binds and act differently at Trk and p75NTR receptors as earlier highlighted. NGF binds to p75 inducing the death of motor neurons instead of producing trophic effects associated with most neurotrophin [186]. Developing small molecular weight compounds that stimulates release of a specific NTF from astrocytes or act as agonist or antagonist at the specific NTF receptors could serve as therapy with improved pharmacological and pharmacokinetic properties. This concept is supported by the a study in which an NGF-like peptide BB14, an agonist at TrkA receptors have been shown to increase neurotrophic activity on cells and reduce reactive gliosis[187].

As earlier highlighted, glutamatergic dysfunction and excitotoxicity has been implicated in the pathogenesis of many NDs, such as epilepsy, AD, HD, PD and ALS. Loss of astroglial glutamate transporters have been linked to excitotoxicity induced neurodegeneration. Hence, drugs targeting astrocytic glutamate transporters to enhance their function and expression present potential target for NDs associated with excitotoxicity. Astrocyte glutamate transporter. GLT-1 expression and activity is regulated at transcriptional and post-transcriptional levels [188]. Therefore, upregulation of GLT-1 could be achieved at these levels with many compounds tested for this purpose. Rothstein and colleagues having screened a diverse library of FDA approved drugs and nutritionals have discovered over 20 compounds that could increase the transcription of GLT1 [189]. Among these compounds the β-lactam antibiotic including penicillin and its derivatives as well as cephalosporin were highly effective in upregulating GLT-1 expression. The study also revealed the antibiotic ceftriaxone as one of the best candidates among this group. Ceftriaxone crosses the BBB and has been demonstrated to increase brain GLT-1 expression and

function. In animal models of PD and HD, ceftriaxone was found to reduce glutamate excitotoxity [190, 191]. Ceftriaxone has been found to ameliorate tau pathology improve memory and learning in AD models via glutamate transporter regulation [192]. Furthermore, in ALS animal models ceftriaxone showed efficacy in delaying motor neuron degeneration thus prolonging survival [193]. Although ceftriaxone was well tolerated by ALS patients in stage I and II clinical trials, it was discontinued in stage III due to its failure to meet predetermined criteria for efficacy [193]. It is possible that ALS is not the best disease condition for these drugs hence the failure of the trials. Therefore, clinical trials of ceftriaxone are needed in different disease conditions. Development of ceftriaxone derivatives or analog with better efficacy and pharmacokinetic properties is also needed. Other compounds like riluzole an FDA approved drug for ALS known to prevent glutamate release from synaptic neurons, estrogen [194], and insulin [195] also showed similar effects in upregulating GLT-1 expression. Cotton et al executed a high throughput screening to search for translational enhancers and found 61 components that increase GLT-1 translation [196]. They selected and developed pyridazine base analogs after extensive studies for further investigation. The pyridazine derivative LDN/OSU-0212320 was found to protect cultured neurons from glutamate induced excitotoxic death, delays motor function decline and extend life span in ALS animal models via GLT-1 translation enhancing [197, 198]. Other test in a range of species and disease models where glutamate toxicity is a concern are required to confirm the therapeutic potential of this compound. The translational regulation of GLT-1 may be a better therapeutic strategy as faster effect and greater selectivity may be achieved. Another compound that allosterically modulate the function of GLT-1, Parawixin 1 have been discovered [199]. This compound promotes direct and selective enhancement of glutamate uptake by GLT-1 by facilitating conformational transitions involved

in glutamate translocation. Therefore compounds that allosterically act on GLT-1 modulate glutamate clearance and be therapeutically beneficial in preventing glutamate induced neuron death.

1.1.7. CONCLUSION

Astrocytes are key homeostatic cells of the CNS. It has become clear that the neuron-centric approach to brain function and disease is unrealistic. Due to their physiological role in maintaining normal brain function, any insult to the brain is likely to impact astrocytes. Mounting evidence indicates that dysfunctional astrocytes play a critical role in the physiopathology of many NDs. Thus understanding astrocyte function and regulation in both physiological and pathological states may lead to better identification of therapeutic targets to treat NDs. Furthermore strategies that modulate and protect astrocytes may be a better therapeutic approach rather than targeting neuronal functions or directly protecting neurons from insult.

1.1.8. TABLES

Table 1.1 Physiological functions of astrocytes

Roles	Physiological functions	Ref.
Metabolic support	The brain depends on glucose for its energy needs; Astrocytes store glycogen and release lactate for neuronal use	[42, 200]
Synaptic transmission	Astrocytes release Gliotransmitters to activate synapses directly, and can modulate neuronal excitability through their own Ca^{2+} concentrations; growth factors can regulate synaptic transmission over longer time periods. Astrocytes modulate synapse formation and pruning. They express transporters and enzymes for neurotransmitter recycling.	[52, 55, 201]
pH and ion regulation	Astrocytes have specialized channels such as AQP4 that regulate fluids and Kir4.1, gap junction proteins and Na^+/K^+ ATpase that regulate K^+ and carbonic anhydrase that regulate pH	[22, 63, 69]
Trophic support	Astrocytes release growth factors such BDNF, NGF and antioxidant molecules such as GSH, SOD needed for neuron growth and survival	[30, 31, 36]
Vascular support	Astrocytes help form the blood-brain-barrier by secreting Chemicals that induce and modulate barrier properties and second messengers such as nitric oxide, arachidonic acid metabolites that regulate blood flow.	[72, 74, 76]

Table 1.2. Influence of astrocytes on the physiopathology of neurodegenerative disorders

Disease	Cellular Presentation	Evidence of astrocytic dysfunction	Ref
AD	Accumulation of Aβ plaque and tau neurofibrillary tangles	Astrocytes accumulate Aβ Astrogliosis Calcium dysregulation Impaired glucose metabolism Reduction in glutamate transporters Glutamate and GABA dyshomeostasis Aberrant trophic factor release	[100, 101, 103, 106, 202]
Epilepsy	Excessive neuronal excitation	Impaired GABAergic inhibition Reduced expression of GLT-1 and glutamine synthase. Calcium, potassium water and neurotransmitter dyshomeostasis	[123, 124, 136, 203]
ALS	Majority of cases are sporadic 10% due to familial mutations	Accumulation of mutant SOD1 Reactive astrocytes Decreased expression of GLT-1 Alteration in glutamate and neurotrophic receptor expression Altered potassium channels and AQP4 expression	[169, 176, 204, 205]
PD	Loss of dopaminergic neurons Presence of Lewy bodies	Accumulation of mutant α-synuclein Increase release of pro-inflammatory cytokines and neurotoxic species Loss of glutamate transporter EAAT2	[146, 147, 151, 157, 206]

1.2

PRIMARY ASTROCYTE CULTURES; THEIR VALUE IN UNDERSTANDING ASTROCYTE BIOLOGY

1.2.1. INTRODUCTION

The advancement in understanding the previously unsuspected roles of astrocyte listed in the first chapter and challenging the "neuron-centrism" in neuroscience has been part derived from studies conducted using primary cultures. *In vitro* astrocyte cultures allow the influence of the complex brain environment to be overcome, providing a critical tool to study astrocyte functions at the cellular and molecular levels. It allows detail studies on the mechanisms and pathways regulating astrocytic dysfunctions as well as real time responses to compounds targeting these dysfunctions. Biochemical, genetic and molecular tools to study astrocyte features which cannot be use in *vivo* can be employ in primary cultures. *In vitro* astrocyte culture has also contributed greatly to our understanding of astrocyte roles in the initiation and progression of neurodegenerative diseases such as Stroke, AD, PD and hepatic encephalopathy (HE) [1]. The use of ammonia treated primary astrocytes provided substantial evidence of the role of ammonia in HE pathophysiology and the principal affected cells in HE [207-209]. Similarly subjecting primary astrocytes to an *in vitro* model of cerebral ischemia; oxygen glucose deprivations (OGD) have demonstrated the significant role and alterations astrocytes undergo during ischemia [210, 211]. The beneficial role astrocytes play during AD has also been learnt using primary cultures. Cultured astrocytes have been shown to phagocytize and degrade Aβ [94, 212]. Although many of the findings in cultures are also observed *in vivo*. Concerns have

been raised whether primary astrocyte cultures are reliable in accurately mirroring all the complex events occurring *in vivo* and whether obtained findings can be reproducible between laboratories as culture protocols may differ. In the following sections we will provide various protocols use to culture astrocytes and discuss a couple of their limitations.

1.2.2. CULTURE METHODS

Cell culture is the general term used to describe the removal of cells, tissues or organs from different species and their placement into artificial environment conducive for their survival and proliferation. Astrocyte culture protocol developed by McCarthy and De Vellis (MD) with minor modifications has been used for most studies of astrocyte biology [2-4]. This method involves the isolation and the digestion of CNS tissue from postnatal days 1 to 3 pups by Trypsin, TrypLE, or a cocktail of enzymes consisting of papain and DNase and trituration of the digested tissues to obtain a single cell suspension. The single cell suspension is then seeded into a coated or uncoated plate and incubated at 37 ^{o}C with CO_2 to confluent. When the cells are confluent they are then shaken to remove cell debris or other neural cells and split into different plates and incubated until they are ready to use.

This culture method herein referred to as the MD method has several limitations to its use. First MD method isolates astrocytes progenitor cells from neonatal brains. This astrocytes divide rapidly and continuously, are not high process bearing and are able to be passaged for months while adult astrocytes *in vivo* are highly process bearing and exhibit limited division [1, 4, 11]. Secondly MD-astrocytes isolation does not involve directly selecting and isolating a specific cell and involve many steps extending for weeks and are only prepared from neonatal brains at the start of their generation. Whereas astrocyte progenitor cell isolation from neonatal brain by MD method generates highly proliferative cells, the modifications made to the MD protocol isolates

astrocytes from postnatal days 1 to 4 (P1 to P4) rodent brains. The cell proliferation and propagation of isolated astrocytes from this pups is limited [213]. Since *in vivo* astrocytes proliferation is mostly complete by P14 [214], we believe astrocytes for experimentation after 14 days *in vitro* have phenotypes similar to mature astrocytes. This has been proven by studies where they found that astrocytes isolated from P1 to P4 mouse pups have mature astrocyte phenotype between days 21 to 28 *in vitro* [213]. Thirdly this culture is obtained in an undefined serum (FBS) containing medium which is highly non-physiological as most serum proteins are unable to cross the blood brain barrier [4]. It has also recently being shown that MD-astrocytes have a different gene expression profile different from astrocyte *in vivo* and acutely isolated postnatal day 16 and 7 astrocytes and this was attributed to variability in FBS, number of days *in vitro*, sub culturing , culture plate coating and contamination by other cell types [1, 3, 79]. To ensure data reproducibility when using this culture method, it is necessary to optimize the concentration of the digestion medium and the time for digestion, the number of times the cells are splitted and the plate coating medium.

The importance of culturing cells is that a single cell type can be studied and any result obtained is attributed to that cell type ,the purity of astrocytes in culture is therefore very important as the presence of other cell types could lead to inaccurate interpretation of the physiological roles of astrocytes. Different methods have been use to get rid of contaminating cells in astrocyte cultures. In the MD method cells are shaken overnight in an incubator whereas other groups employ a 15 to 20 mins differential velocity adherence (DVA) method to get rid of cell debris [18]. Due to the physical stress e.g. shaking (done to remove other cell types) associated with the MD method of astrocyte preparation and the presence of FBS, it has been suggested that astrocytes obtained using this culture system is in the reactive state. In order to

correct the limitations of the MD method, some labs have developed the immunopanning (IP) and florescence activated cell sorting (FACS) method base on either GFAP, ALDH1L1 or GLT-1 promoter driven green florescent protein to isolate astrocytes from rodent brains [4, 11, 12]. These methods directly select and isolate astrocytes from postnatal days 1 to 14 mice and rat brains, reducing the number of days required to culture astrocytes with the MD method. They also produce astrocytes with gene profiles that mimic astrocytes *in vivo* which helped to discover some new properties of astrocytes. Like the MD method, immunopanning (IP) and florescent activated cell sorting (FACS) method of isolating astrocyte also has some limitations. These methods are very capital intensive, and the selection and isolation processes reduce the number of viable cells. Hence there is a need for continuous research into a less capital intensive culture method that will isolate astrocyte that mimics astrocyte phenotype *in vivo*.

1.2.3. CULTURE MEDIUM

Culture medium is known to affect culture purity, gene expression profile and response to compounds and starvation [3-5, 10]. DMEM is the mostly used medium to culture astrocyte. This culture medium is typically composed of glucose, vitamins, amino acids, inorganic salts, trace elements and supplemented with antibiotic and fetal bovine serum (FBS). Components of astrocyte culture medium are to regulate pH of the medium, provide source of energy, binding proteins and help maintain osmotic balance [5]. FBS is a complex medium supplement that contains several factors needed to promote cell survival and proliferation and to also fulfill many of the metabolic requirements for cultured cells [215, 216]. It provides factors necessary for the proper attachment of cells, nutrients to sustain exponential growth, binding proteins and acts as pH buffers. FBS is collected from cattle, thus genetic diversity of herd, seasonal and continental variation, animal diet as well as manufacturing procedures makes the complete composition

difficult to determine, and inter-vendor as well as inter-batch variability hard to control. There is also a likelihood of the presence of contaminants in FBS. Recently it has been found that FBS contains significant levels of lipopolysaccharides which may affect the production of cytokines by certain cells [217]. Additionally FBS has been shown to contain extracellular vesicles (EV) like exosomes and apoptotic vesicles which have recently been reported to alter cell biology and phenotype [215, 218, 219]. Astrocytes *in vivo* do not contact serum as many component of serum do not cross the blood brain barrier (BBB) unless under pathological conditions when BBB is damage. The culturing of astrocytes in serum contained medium affects the gene expression profile and produce astrocytes with morphology different from *in vivo* astrocytes [3-5, 10]. The presence of FBS in a media also affects the purity of the culture by providing growth factors and directly or indirectly stimulating astrocytes to secret factors such as CSF-1 that stimulate microglia growth [10].

In terms of experimentation, the presence of serum in the culturing medium makes it difficult to study the effects of certain hormones and drugs on astrocytes *in vitro* as serum components may antagonize or potentiate the actions of certain agents under investigation in astrocyte culture. Additionally the presence of FBS in the culture medium makes it hard to study the release into the medium of certain secreted products. Due to the concerns regarding the use of FBS to maintain astrocytes, efforts have been made to develop defined medium or serum free media whose complete composition is known. This media contains several factors that perform the known functions of FBS in the culture media. Examples of such factors are Insulin, Transferrin, hydrocortisone, growth hormones and selenium. Cell permeable cAMP analogue dbcAMP has also been included in serum free astrocyte culture medium to increase the

expression of astrocyte specific proteins and to cause morphological changes in astrocytes [220]. However astrocytes produced by this defined media have not been fully characterized.

Most studies of astrocyte functions using the MD method or the immunopanning or FACS culture astrocytes under hyperglycemic glucose conditions (25 mM glucose). This is due to the believe that glucose concentration close to 6 mM is rapidly depleted by astrocytes in culture. However this approach has many draw backs as raising glucose concentration induces experimental diabetes and may alter astrocytes phenotype *in vitro* as hyperglycemia *in vivo* has been shown, to induce astrogliosis and increase expression of inflammatory cytokines [7-9].

1.2.4. CONCLUSION

In summary primary cultures have played immense role in understanding astrocyte biology in health and disease. Despite this remarkable achievement questions have been raised about the validity of this culture methods. Since culture conditions influence astrocytes phenotypes one needs to be cautious in the interpretation of data obtained from this cultures. Efforts still need to be made to ensure that *in vitro* conditions and astrocytes closely mimic *in vivo* environment and astrocytes.

CHAPTER 2

A NOVEL SERUM FREE PRIMARY ASTROCYTE CULTURE METHOD THAT MIMICS QUIESCENT ASTROCYTE PHENOTYPE

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2.1 ABSTRACT

Primary astrocyte cultures have been used for decades to study astrocyte functions in health and disease. The current primary astrocyte cultures are mostly maintained in serum-containing medium which produces astrocytes with a reactive phenotype as compared to *in vivo* quiescent astrocytes. The aim of this study was to establish a serum-free astrocyte culture medium that maintains primary astrocytes in a quiescent state. Serum free astrocyte base medium (ABM) supplemented with basic fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) (ABM-FGF2-EGF) or serum supplemented DMEM (MD-10%FBS) was used to culture primary astrocytes isolated from cerebral cortex of postnatal day 1 C57BL/6 mice. Compared to astrocytes cultured in MD-10%FBS medium, astrocytes in ABM-FGF2-EGF had higher process bearing morphologies similar to *in vivo* astrocytes. Western blot, immunostaining, quantitative polymerase chain reaction and metabolic assays revealed that astrocytes maintained in ABM-FGF2-EGF had enhanced glycolytic metabolism, higher glycogen content, lower GFAP expression, increased glutamine synthase, and glutamate transporter-1 mRNA levels as compared to astrocytes cultured in MD-10% FBS medium. These observations suggest that astrocytes cultured in ABM-FGF2-EGF media compared to the usual FBS media promote quiescent and biosynthetic phenotype similar to *in vivo* astrocytes. This media provides a novel method for studying astrocytes functions in vitro under physiological and pathological conditions.

Keywords: Astrocyte, basic fibroblast growth factor, epidermal growth factors, fetal bovine serum, astrogliosis, primary culture.

2.2. INTRODUCTION

Astrocytes constitute about 40% of mammalian brain cells outnumbering neurons [221, 222]. Astrocytes have been long thought to play primarily passive support roles in the function of the central nervous system (CNS). However, decades of physiological and pathological research using various state of the art techniques and equipment have shown that astrocytes are cells with functional ion channels, receptors, and transporters that play pivotal roles critical for the development and function of the CNS. For instance astrocyte plays a crucial role in maintaining brain homeostasis, neuronal development, and survival by regulating ionic and water content of the interstitial space, uptake and recycling of neurotransmitters, and producing various neurotrophic factors, antioxidant molecules and reactive oxygen species (ROS) detoxifying molecules [28, 55]. Astrocytes are also critical for the formation, maintenance and function of synapses, blood flow regulation, blood brain barrier formation, as well as providing the energy needs for neurons [30, 223, 224]. Additionally, astrocytes are considered important components in the regulation and modulation of sleep, as well as learning and memory [26, 225]. It is therefore not surprising that astrocyte dysfunctions have been implicated in the onset and progression of many neurodegenerative disorders [226].

In spite of the aforementioned advances, our understanding of the mechanisms and pathways regulating astrocytic dysfunctions and their roles in the initiation and progression of neurodegenerative diseases is still rudimentary. This is as a result of the complex interwoven nature of the different cells in the CNS. Understanding brain cells at the cellular and molecular levels is the cornerstone of modern neuroscience. The complexities of brain cells structure and function require unusual methods of *in vitro* culture to determine the function of brain cells and their interaction under physiological and pathological conditions devoid of the complex brain

environment. Over the years, primary astrocytes, mostly prepared and maintained in fetal bovine serum (FBS)-containing medium according to the protocol developed by McCarthy and de Vellis in 1980, named as MD method [2] with minor modifications, have served as a useful tool in understanding many of the complex astrocytic functions in both health and disease. However, concerns have been raised as to whether these primary astrocyte cultures are reliable in accurately mirroring events *in vivo*, and whether such obtained findings can serve as a basis for effective therapeutic targeting of astrocytes in pathological conditions.

FBS is a complex medium supplement that contains unspecified growth factors, proteins and vitamins needed to promote cell survival and proliferation [215]. Since FBS is collected from cattle, genetic diversity of a herd, seasonal and continental variation, and animal diet as well as manufacturing procedures make the complete composition difficult to determine and inter-vendor as well as inter-batch variability become challenging to control. Recently it has been found that FBS contains significant levels of lipopolysaccharides and extracellular vesicles (EV) which have been reported to alter cell biology and phenotype [215, 217, 219]. Astrocytes *in vivo* do not contact serum as many components of serum do not cross the blood brain barrier (BBB) except in certain pathological conditions. It was reported that astrocytes cultured in serum free media had a gene profile more representative of *in vivo* astrocytes as compared with those cultured in FBS containing media [3, 4, 79]. Additionally media composition has been reported to affect astrocyte culture purity and the expression of astrocytes specific proteins [4, 10]. Due to the physical stress (e.g. shaking; done to remove other cell types) associated with the MD method of astrocyte preparation and the presence of FBS, it has been suggested that astrocytes obtained using this culture system are in the reactive state [227]. There is a need to purify and

culture astrocytes under conditions that mimic the *in vivo* environment thus increasing translational value of the result derived from these cultures.

Efforts have been invested in the development of serum-free primary astrocyte culture systems, including a recent method combining negative immunopanning and a serum-free heparin-binding EGF-containing medium which has been demonstrated to resemble astrocytes *in vivo* [4, 12, 228] . However, the application of this method has been dramatically limited due to the labor-intensive, costly 5/6-step negative immunopanning of microglia, oligodendrocyte, endothelium, and neurons. [4]. Additionally this method was not completely devoid of serum, as astrocytes were exposed to fetal calf serum (FCS) in the process of detaching purified astrocytes from the dishes [4, 12, 228].

A reliable serum free astrocyte culture system that greatly promote the survival of astrocytes to the same degree as FBS containing medium as well as produce astrocytes with a phenotype closely resembling *in vivo* quiescent astrocytes will increase the translational value and reliability of the results obtained from primary cultures. Aside from transferrin, selenium, hormones, and other molecular components known to perform certain functions attributed to FBS in media [229, 230], various growth factors have been found to play critical role for astrocyte survival and proliferation *in vitro*. Heparin–binding epidermal growth factor (HBEGF) has been shown to promote astrocyte survival and proliferation and to affect cell differentiation and morphology in serum free culture conditions [13, 14]. FGF2, first purified in bovine brain [15], has been found to maintain astrocyte in nonreactive state. Similarly, FGF signaling delays astrogliosis and accelerates astrocytes deactivation after injury [16, 17, 231]. In the current study, we developed a serum free FGF2-EGF containing medium for primary astrocyte culture. We employed metabolic assays, morphological and gene expression analysis to compare the

phenotype of primary astrocytes under FBS containing medium and serum free FGF2-EGF containing medium culture conditions. Our study demonstrated that serum free FGF2-EGF containing medium supports astrocyte growth while mimicking an *in vivo* quiescent astrocyte phenotype.

2.3. MATERIALS AND EXPERIMENTAL METHODS

Materials and reagents

Cell culture dishes, plates, cell strainers were purchased from Greiner bio-one (USA) and Genesee scientific (USA). Micro cover glasses were purchased from VWR. Trypsin-EDTA, trypan blue, poly-L-Lysine solution, bovine serum albumin, transferrin, putrescine dihydrochloride, progesterone, sodium selenite, and N-Acetyl Cysteine were purchased from Sigma-Aldrich (USA). TrypLE, Dulbecco's Modified Eagle's medium (DMEM), Neurobasal medium, Penicillin-streptomycin, sodium pyruvate, Glutmax was purchased from Gibco/Life Technologies (USA). FBS was purchased from Atlanta Biologicals (USA). Normal goat serum was purchased from Jackson Immunoresearch (USA). Gold antifade mountant with DAPI and BD cytofix/cytoperm^{TM} fixation and permeabilization solution were purchased from Invitrogen (USA) and BD Biosciences (USA) respectively. Human FGF-basic and Human EGF were purchased from PeproTech (USA).

Primary antibodies against GFAP (monoclonal cell signaling, USA), Vimentin (monoclonal Cell signaling, USA), IBA-1, ALDH1L1 (polyclonal Abcam, USA), AMPKα and AMPKβ (monoclonal Cell signaling, USA), GS and ACC (Monoclonal Cell signaling, USA), mTOR (monoclonal Cell signaling, USA), Actin and GAPDH (monoclonal Santa Cruz, USA) and Alexa-fluor conjugated secondary antibodies from Invitrogen (USA) as well as non-conjugated secondary antibodies from Jackson laboratory (USA) were used for immunostaining

or Western blot. PE anti-mouse/human CD11b Antibody, purchased from Biolegend and GFAP Monoclonal Antibody (GA5), Alexa Fluor 488, eBioscience™ all in USA were used to label astrocytes and microglial for flow cytometry. Information regarding primers used for quantitative Polymerase Chain Reaction (qPCR) to quantify mRNA expression of some astrocyte specific genes is listed in Table 1.

Culture media

Astrocytes cultures were prepared in differing media conditions, defined as follows. The serum containing media known as MD medium is composed of Dulbecco's Modified Eagle's medium (DMEM with 5.5 mM glucose, 4 mM L-glutamine, 1mM sodium pyruvate) containing 10% FBS and streptomycin (100 units/ml) - penicillin (100 μg/ml). Serum free media defined as astrocyte based media (ABM) is composed of Neurobasal medium and DMEM (1:1 v/v) supplemented with 5.5 mM glucose, Penicillin (100 units/ml)-streptomycin (100 μg/ml), 1 mM sodium pyruvate, Glutmax, bovine serum albumin (100 μg/ml), transferrin (100 μg/ml), putrescine dihydrochloride (16 μg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), and N-Acetyl Cysteine (5 mg/ml). ABM was supplemented with 2, 5 or 10 ng/ml of Human FGF-basic, Human EGF, or a combination of both.

Primary astrocyte culture

All procedures for primary astrocyte cultures were approved by the Institutional Animal Care and Use Committee of the university of North Texas Health Science Center (UNTHSC). Primary astrocytes were prepared according to previous published methods with minor modifications [2, 210]. Briefly, one day old pups were anesthetized by hypothermia followed by decapitation with sharp surgical scissors. Cerebral cortices were dissected and meninges were removed under aseptic conditions. Cortical tissue was digested in TrypLE, at 37 ^{o}C for 15

minutes. Cell suspension was prepared by repeated pipetting of digested cortical tissue through different bore sized Pasteur pipettes. The cell suspension was strained through 40 µM size cell strainers and cells were counted with a hemocytometer using trypan blue staining. Cells were then seeded into plates coated with poly-l-lysine in MD (10% FBS) or ABM supplemented with different growth factors referred to as ABM-FGF2, ABM-EGF, ABM-FGF2-EGF for 15 minutes. Media was replaced to get rid of debris in a process referred to as variable cell attachment rate. The cells were then cultured in a humidified incubator at 37 ^{o}C with 5% CO_2. Half of the media was changed every 3 days and when the cells became 90% confluent, the plates were constantly shaken for 24 hours in a CO_2 incubator at 37 ^{o}C to eliminate microglia. The cells were split into new plates, incubated for 2-3 days before use, and generally used within 2 weeks at passage 1.

Cell viability assay and apoptosis analysis

Cell viability was determined by Calcien AM and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay at day 2 after primary astrocytes were prepared in different media conditions. For the Calcien AM assay, 40,000 cells were seeded in poly-l-lysine coated 12-well plates in different media conditions on the day of primary astrocyte preparation and incubated at 37 ℃ with 5% CO_2. On day 2 of culturing, media was removed and replaced with a 1 µM solution of Calcien AM in PBS. Cells were incubated for 5 minutes at 37 ℃ and fluorescence was measured using a Tecan Infinite F200 plate reader (excitation 485 emission 530). For the MTT assay, plates were removed from the incubator and 20 µl MTT (5 mg/ml in PBS) was added per well. The plates were agitated gently to mix the MTT into the media and then returned to the incubator for 2 hours. After 2 hours the media was removed and 100 µl of DMSO was added to each well. The plate was mixed by gentle agitation and the absorbance was measured (560 nm with a reference of 670 nm) with a Tecan Infinite F200 plate

reader. For flow cytometry analysis, primary astrocytes were cultured in various media and grow to confluence. The cells were then collected and seeded in 6 well plates. On the 7th day the cells were stained with annexin-V and propidium iodide and flow cytometry analysis was performed (BD LSR II, San Jose, CA, USA).

Immunostaining of astrocytes

Cells were seeded at 3 x 10^4 cells/well in 24-well plates on poly-L-lysine coated coverslips in serum free ABM-FGF2-EGF or MD medium containing 10% FBS. When confluent, cells were fixed with cytofix overnight at 4 ^oC. Cells were incubated with goat serum blocking solution and 0.2 % triton –X 100 for 1 hour. After 3 times washing with PBS, cells were incubated overnight with primary antibodies at 4 ^oC. Cells were then washed and incubated with FITC-labelled secondary antibodies for 1 hour at room temperature and stained with DAPI. Images were obtained with a confocal microscope (Olympus fluoview FV1200). The purity of astrocytes was calculated by the percentage of cells both stained with ALDH1L1 and DAPI to cells stained with DAPI only or Iba-1, using 5 random views per slides. The cell's morphology and dimensional structure were analyzed using Imaris (bitplane) and FIJI-ImageJ as previously described [232] .

For Flow cytometry to determine culture purity, astrocytes cultured in the different media conditions were trypsinized and collected. Cells were incubated in 2% PFA for 15 minutes and permeabilized using 0.1% triton X 100 for 10 minutes. Supernatant was discarded after the cells were centrifuged. The cells were incubated by resuspension in Magnetic-Activated Cell Sorting (MACS) buffer with fluorochrome conjugated anti-CD11b and anti-GFAP antibodies for 1 hour. Cells were centrifuged again and supernatant removed. MACS buffer was used to re-suspend cells for analysis via flow cytometry (BD LSR II, San Jose, CA, USA).

Growth curve and cell cycle assay

Astrocytes were seeded at a density of 40,000 cells/well in 12-well plates or 25,000 cells per well in 24-well plates with different culture medium. At the indicated days after seeding, cells were harvested using trypsin-EDTA and counted using a hemocytometer. Four wells were assigned to each group and cell counting was conducted by a researcher who was blinded to the group assignment using an inverted phase contrast Zeiss Invertoskop microscope.

For cell cycle analysis astrocytes were seeded at a density of 50,000 cells/well in 12-well plates in various culture media. On day 7 after culture, cells were harvested using trypsin-EDTA and washed with buffer (PBS) twice to remove trypsin. Cells were fixed in ice-cold 70% ethanol for 24 hours at 4 °C. Then, cells were incubated with propidium iodide (PI) (40 μg/ml) and RNase (10 μg/ml) for 30 minutes at 37 ^{o}C. The stained cells were analyzed using a Beckman Coulter FC500 Flow Cytometry Analyzer for quantification of cell cycle distribution (G1, S or G2/M).

ATP assay

Total cellular ATP levels were determined using an ATP kit (Invitrogen) as previously described in [210]. Astrocytes were seeded in 12-well plates at a density of 10 X 10^4 cells/well under different culture conditions and grew for 14 days. On the day of the experiment cells were treated with oligomycin for 2 hours. The cells were then detached with trypsin (Sigma-Aldrich, St Louis, MO), washed with PBS twice and lysed with ATP assay buffer (500 mM Tricine buffer, pH 7.8, 100 mM 100 mM MgSO4, 2 mM EDTA, and 2 mM sodium azide) containing 1% Triton X-100. The ATP reaction buffer (30 μg/ml D-luciferin, 20 μM DTT, and 25 μg/ml luciferase) was added to each 10-μl cell lysate in white 96-well plates (in triplicate) along with an ATP standard. Luminescence was measured using a Tecan Infinite F200 plate reader. Protein

concentration was measured simultaneously using the Pierce 660 nm Protein Assay (660 nm absorbance). ATP values were determined from a standard curve and normalized to the protein content of each sample.

Glycogen and lactate assays

Total glycogen level and amount of lactate produced by astrocytes cultured under various conditions was determined using a glycogen and lactate assay kit following the manufacturer's instructions (Sigma-Aldrich, St Louis, MO). Glycogen levels and lactate concentrations were normalized to the protein concentration of the samples determined by colorimetric analysis using the Pierce 660 nm Protein assay reagent (Thermo Scientific) and a Tecan Infinite M200 plate reader.

Hexokinase activity

Flex station (Molecular Devices) was used to measure hexokinase (HEK) activity as previously described [210]. Astrocytes were seeded in 12-well plates at a density of 10 X 10^4 cells/well under different culture conditions and grew for 14 days. On the day of the experiment, cells were lysed in RIPA buffer (50 mM of Tris. HCL, pH 7.4,150 mM NaCl, 1mM EDTA, 1%Triton X). In a 96 well plate, 10 µl of cell lysate was added to 120 µl of Tris $MgCl_2$ buffer (0.05 M Tris*HCl buffer, pH 8.0 with 13.3 mM $MgCl_2$), 50 µl of glucose (0.67 M) and 10 µl each of ATP (16.5 mM), NAD (6.8 mM) and glucose-6-phosphate dehydrogenase (300 IU/ml). The change in absorbance was determined at 340 nm. Enzyme activities were expressed as units per milligram of protein after the protein concentration of each sample was measured.

Extracellular flux analysis

Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and cell metabolic potential was monitored using Agilent Seahorse XFe96 analyzer. Astrocytes were

seeded at a density of 15,000 cells/well in 96-well Seahorse XF cell culture microplates under different culture conditions for 7 days. One day prior to the experiments a sensor cartridge was hydrated in Seahorse calibrant in a non-CO_2 incubator at 37 ^oC overnight. One hour before the experiment, assay medium was prepared by supplementing Seahorse XF base medium with 1 mM pyruvate, 2 mM glutamine and 5.5 mM glucose. Assay medium was warmed and adjusted to pH 7.4 with 0.1N NaOH. For the Mito stress test, oligomycin, carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone/antimycin were diluted in XF base medium and loaded into the accompanying cartridge port to achieve a final concentration of 1 μM, 1 μM and 0.5 μM, respectively. For cell energy phenotype determination, oligomycin and FCCP were diluted in XF medium together to achieve a final concentration of 1 μM for both oligomycin and FCCP, which was loaded into one port. Various metabolic parameters were monitored with each cycle set as mix for 3 minutes, delay for 2 minutes and then measure for 3 minutes. Values were normalized to the cell number of each well determined by Calcien AM assay. Using wave software, metabolic potential was calculated by dividing stressed OCR and ECAR by baseline OCR and ECAR respectively.

Glucose uptake

Using glucose analog 2-NBDG, glucose uptake in astrocytes was determined as previously described [233]. Astrocytes were cultured in 96-well culture plates (10,000 cells/well) under different culture conditions. Cells were washed twice and incubated in glucose-free Krebs Ringer HEPES (KRH) buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $KH2PO_4$, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES; pH 7.4) for 30 minutes on the day of the experiment. The astrocytes were then incubated in glucose free KRH buffer containing 100 μM of 2-NBDG

for 5 minutes. Glucose uptake was determined using a Tecan Infinite M200 plate reader (Excitation/Emission for 2-NBDG ~465/540 nm).

Western blot and real time qPCR analysis

Astrocytes were seeded at a density of 400,000 cells/well in 6-well plates in different media conditions and grew to confluency for 7 days. For induction of astrocyte activation, 10 ng/ml TGF-β1 was added to cells for 3 days or 100 ng/ ml LPS for 15 hours. The cells were collected and lysed in RIPA buffer (50 mM of Tris. HCL, pH 7.4,150 mM NaCl,1mM EDTA, 1%Triton X) with protease and phosphatase inhibitors (1:100). Protein assay reagent Pierce 660 nm (Thermo Scientific) was used to determine the protein content of the samples. The samples were resolved on SDS gel and transferred to a nitrocellulose membrane. The membranes were incubated overnight in primary antibodies against GFAP, Aldh1L1, vimentin, AMPKα and AMPKβ, glycogen synthase; GS and ACC, mTOR, Actin and GAPDH followed by secondary antibody. Using Biospectrum 500 UVP imaging system, chemiluminescence signal was detected and normalized to actin.

For RNA extraction and real time qPCR, total RNAs were extracted using PicoPure® RNA Isolation Kit following the manufacturer's manual (Invitrogen). Complement DNA synthesis was performed using SuperScript® III First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Quantitative PCR was performed using Fast SYBR® Green Master Mix (Invitrogen) on a 7300 Real-Time PCR System (Invitrogen). Data were analyzed with 7300 system software and $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression.

Glutamate clearance/uptake assay

Primary astrocytes were plated in 24-well tissue culture plates at a density of 10×10^4 cells/well and allowed to grow confluent for one week in different media condition. On the day

of the experiment, glutamate (400 μM) was dissolved in phenol-free astrocyte medium and added into each well. Clearance was assayed at 4 and 8 hr. The assay was performed and analyzed according to manufacturer's guidelines (Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit, Life Technologies).

Scratch/migration assay

Astrocytes were seeded at a density of 100,000 cells/well in 12-well plate in different media conditions. Using a sterile 200 μl pipette tip, straight scratches were made on the cell layer, simulating a wound. The cells were then washed with PBS to remove cell debris and replaced with fresh media. At different time points after scratch, cells were stained with Calcien AM (10 μM), and fluorescent images taken randomly with a Zeiss fluorescent Microscope.

Astrocyte calcium signaling

Astrocytes were seeded at a density of 20,000 cells/well in poly-l-lysine coated black-walled 96-well plate and grew confluent for a few days. Cells were washed and incubated with 10 μM Fluo-4 AM (Invitrogen F14201) for 45 minutes. Fluo-4 AM fluorescence was measured before and, after stimulating the cells with 0.1 mM glutamate, 50 μM Adenosine and 100 μM ATP. Calcium concentration were measured with Flex station 3 (Molecular Devices, Sunnyvale, CA) for 600 seconds (Excitation 490/Emission 510). The baseline calcium signal was measured for 30 seconds followed by injection of stimulant.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7. Results are expressed as mean ± standard error mean (SEM). When comparing two groups a t-test was used to identify any significant differences. Significant difference among groups with one independent variable was determined by one-way ANOVA with a Turkey's multiple comparisons test for planned

comparisons between groups when significance was detected. For comparison of groups with two independent variables, two-way ANOVA was used and post-hoc Bonferroni analysis was conducted for planned comparisons between groups when significance was detected. $P < 0.05$ was considered statistically significant.

2.4. TABLE

Table 2.1

Markers quantified by qPCR.

	Gene name	Physiological function
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1	Astrocyte specific marker
GFAP	Glial fibrillary acid protein	Astrocyte intermediate filament
VIM	Vimentin	Astrocyte intermediate filament
GS	Glutamine synthase	Converts glutamate to glutamine
HEK1	Hexokinase type 1	Phosphorylates glucose
HEK2	Hexokinase type 2	Phosphorylates glucose
HEK3	Hexokinase type 3	Phosphorylates glucose
MCT1	Monocarboxylate transporter 1	Lactate transport
Kir4.1	Inward rectifier potassium channel 10	Potassium uptake or buffering
AQP4	Aquaporin-4	Mercurial-insensitive water channel
CNX-43	Gap junction alpha-1 protein	Gap-junction component (coupling)
CNX-30	Gap junction beta-6 protein	Gap junction component (coupling)
GLUT1	Glucose transporter member 1	Glucose uptake
GLT-1	Glial high affinity glutamate transporter), member 2	Glutamate transporter
GLAST	Glial high affinity glutamate transporter), member 3	Glutamate transporter
IL-6	Interleukin -6	Cytokine
IL-1β	Interleukin - 1β	Cytokine
LCN-2	Lipocalin-2	Inflammatory mediator
TNFα	Tumor necrosis factor-α	Cytokine

2.5. RESULTS

FGF2 and EGF collectively support survival and growth of primary astrocytes in serum free media.

The effect of the serum free media and the various concentrations of growth factors to promote survival and growth of astrocytes prepared and seeded using the variable cell attachment rate method was assessed (Fig. 2.1A). At 48 hours after seeding the cells, in the absence of growth factors, the ABM only promoted 12% cell survival. We also found that 2, 5, and 10 ng/ml FGF2 increased the survival rate to 19.14% ± 2.03, 29.714% ± 2.52, and 38.57 ± 2.467, respectively, in serum free ABM as compared to 72.00% ± 3.901 survival rate of astrocytes cultured in MD (10% FBS) medium. Similarly, 2, 5, and 10 ng/ml EGF increased the survival rate to 25.34% ± 2.95, 40.6% ± 3.22, and 47 ± 3.40, respectively, in serum free ABM (Fig. 2.1B). Furthermore, a combined 2, 5 and 10 ng/ml FGF2 and EGF was more effective in promoting astrocyte survival as compared to each growth factor by increasing the survival rate to 38.12% ± 3.5, 57.12% ± 3.52, and 56.57% ± 3.45, respectively, in the serum free ABM. No statistical significant difference in cell survival was observed between 5 and 10 ng/ml FGF2-EGF and MD (10% FBS) culture conditions (Fig. 2.1B). When the effect of 5 ng/ml FGF2, 5 ng/ml EGF, or 5 ng/ml ABM-FGF2-EGF together was assessed for growth rate of astrocytes cultured in different medium for 2 weeks, we observed that cells grown in ABM-FGF2-EGF proliferated faster than those grown in ABM-EGF, or ABM-FGF2 (Fig. 2.1C). When the growth rate of astrocytes in ABM culture conditions was compared to MD (10% FBS) for 7 days, we found that the growth rate in serum containing MD medium was higher than those in ABM-FGF2 and ABM-EGF conditions but not ABM-FGF2-EGF. (*$p<0.05$, ***<0.001 day 7 comparison) (Fig. 2.1C). Astrocytes were seeded at various densities in MD (10% FBS) and

ABM-FGF2-EGF to assess if the seeding density has any effect on our growth assay. We observed no significant difference in the growth rate of astrocyte seeded in the two media conditions after 4 days in culture (Fig 2.1D).

We speculated that the decreased viability in different conditions could be as a result of reduced cell proliferation or increase cell death. Flow cytometry analysis of cells stained with Annexin V and PI after 7 days in the various culture conditions demonstrated that 14.2%, 18.7%, and 8.466% of ABM-FGF2, ABM-EGF, ABM-FGF2-EGF cultured astrocytes, respectively, were in their early apoptotic stages whiles 3.50%, 1.12%, and 1.53% of ABM-FGF2, ABM-EGF, ABM-FGF2-EGF cultured astrocytes, respectively, were in their late apoptotic stages (Fig. 2.1E). Additionally, 25%, 14%, and 12% of astrocytes seeded in ABM-FGF2, ABM-EGF, and ABM-FGF2-EGF, respectively, were in their necrotic stages of cell death. Comparatively, 14.4%, 0.65%, and 5.03% astrocytes cultured in MD (10% FBS) condition were in their early, late and necrotic apoptotic stages, respectively (Fig 2.1E).

Cell cycle distribution of astrocytes grown in serum free ABM-FGF2, ABM-EGF, ABM-FGF2-EGF, and MD (10% FBS) demonstrated a significant increase in the percentage of cells in the G2/M phase of cells grown in ABM-FGF2, ABM-EGF with proportional decrease of cell number in G0/G1 phase at day 7 after culture (Fig 2.1F). Among the 4 culture conditions, the proportion of cells in the S phase was higher in cells cultured in ABM-FGF2 (Fig 2.1F). A notable observation was that after day 6 in culture, the growth rates of astrocytes in serum free medium supplemented with growth factors began to decline. We speculated that this could be as a result of decreased growth factors concentration to provide trophic support to the cells. Hence it is necessary to replace half of the media every 3 days (Fig. 2.1C).

Astrocyte cultures in ABM-FGF2-EGF mimic quiescent astrocyte morphology with high purity.

Using FIJI-ImageJ with simple neurite tracer (SNT) plugin, an open source software that aids in reconstructing the structure of astrocytes as previously described [232], morphological analysis indicated that primary astrocytes cultured in serum free ABM-FGF2-EGF medium have more branching processes as compared to astrocytes cultured in MD medium (Fig. 2.2A). Immunostaining of astrocytes cultured for 2 weeks showed decreased GFAP expression and smaller cell size of primary astrocytes cultured in ABM-FGF2-EGF conditions as compared to MD (10% FBS) cultured astrocytes (Fig. 2.2B). When the purity of both cultures were compared after 2 weeks by flow cytometry of CD11b and GFAP staining, we observed that 97% and 92% of cells were GFAP positive in the primary astrocytes cultured in the serum free ABM-FGF2-EGF medium and MD (10% FBS) medium, respectively whereas 2% and 7% of the cells cultured in serum free ABM-FGF2-EGF and MD (10% FBS) medium respectively stained positively for CD11b (Fig. 2.2C). Immunostaining of astrocytes using ALDH1L1, neuronal marker Tuj1, and Iba1 revealed a similar pattern of purity, with most cells staining positive for the astroglial marker ALDH1L1 (Supplementary Fig. 2.1).

TGF-β1 activates primary astrocytes cultured in serum free ABM FGF2/EGF medium.

Astrogliosis defined as changes in astrocytes in response to all forms of CNS injury and disease is characterized by cellular hypertrophy and upregulation of intermediated filament proteins such as GFAP [17, 234]. We examined reactive astrogliosis markers in primary astrocytes cultured in different conditions using Western blot, PCR, and immunostaining. There was a significant decrease in GFAP expression in primary astrocytes cultured in ABM supplemented with either FGF2, EGF or a combination of FGF2 and EGF, as compared to

astrocytes cultured in MD media with 10% FBS (Fig. 2.3A). Similarly, ABM-FGF2 and ABM-FGF2-EGF cultured astrocytes, but not ABM-EGF cultured astrocytes, had decreased vimentin expression as compared to astrocytes cultured in MD media with 10% FBS (Fig. 2.3A). When growth factors were withdrawn for 2 days before the experiment, astrocytes cultured in ABM without growth factors have GFAP and vimentin expression levels similar to that of MD cultured astrocytes, indicating the key role of the EGF-FGF2 in maintaining primary astrocytes in their quiescent state (Fig. 2.3A). Consistently, Real time PCR of GFAP and vimentin in astrocytes cultured under different conditions showed a significant decrease in GFAP and vimentin expression only in astrocytes maintained in ABM-FGF2 and ABM-FGF2-EGF, but not ABM-EGF as compared to MD (10% FBS) (Fig. 3.3B).

TGF-β1 has been known to induce astrocyte activation with an increase of GFAP expression [235]. Our quantitative PCR analysis indicated that TGF-β1 treatment (10 ng/ml for 3 days) significantly increased the expression of GFAP in primary astrocytes cultured in ABM-FGF2-EGF and MD (10% FBS) medium as compared to the non-treatment groups (Fig. 2.3C). Consistently, GFAP and vimentin protein levels were increased in both culture conditions after insult (Fig. 2.3D). Similarly LPS treatment significantly increased the expression of IL-6, IL-1β, TNFα and LCN-2; genes that are involve in inflammatory or immune responses (Supplementary Fig 2.2) . Astrogliosis is involved in wound healing after injury. Wound healing assay demonstrated that scratch induced primary astrocytes activation in primary astrocytes cultured in serum free ABM-FGF2-EGF medium, although the wound healing was slower than those cultured in MD (10% FBS) condition (Fig. 2.4A).

Increased calcium concentrations are associated with reactive astrogliosis [236, 237]. Using Fluo-4 AM we investigated the calcium waves in primary astrocytes cultured in different

conditions. We observed that primary astrocytes responded to the entire stimulus in both media. Higher basal level of calcium was observed in serum containing MD medium cultured astrocytes. Nonetheless, stimulation by adenosine, ATP, and glutamate increased calcium concentration in primary astrocytes cultured in serum containing MD medium and ABM-FGF2-EGF medium (Figure. 2.4B).

Astrocytes cultured in serum free ABM-FGF2-EGF medium have a biosynthetic phenotype.

The effects of culture conditions on astrocyte metabolism were examined using Seahorse XFe 96 analyzer. We determined OCR and ECAR before and after injecting oligomycin, FCCP, and rotenone/antimycin A. We observed a significant increase in basal (~19%) and maximal respiration (~35%) as well as ATP production (~13%) but no significant difference in proton leak and non-mitochondrial oxygen consumption (non-MOC) in ABM-FGF2-EGF and MD (10% FBS) cultured astrocytes (Fig. 2.5A). Similarly, a higher baseline and stressed ECAR was observed in ABM-FGF2-EGF cultured astrocytes (Fig. 2.5B). Assessment of cell energy phenotype indicated a more glycolytic phenotype of the ABM-FGF2-EGF cultured astrocytes as compared with FBS-containing MD medium cultured astrocytes (Fig. 2.5C). We measured lactate production in astrocytes cultured under different conditions. Consistent to the glycolytic phenotype, there was a 20% increase in lactate production in ABM-FGF2-EGF cultured primary astrocytes as compared to MD medium cultured astrocytes (Fig. 2.5D). Our result also showed no significant difference in hexokinase activity in astrocytes cultured in both conditions (Fig. 2.5E). However, using PCR, we observed that the mRNA levels of HEK-1 and MCT-1 were significantly increased in serum free ABM-FGF2-EGF cultured primary astrocytes as compared with MD medium cultured astrocytes (Fig. 2.5F).

ATP is generated by both glycolysis and mitochondrial phosphorylation in astrocytes. We observed a high ATP level in ABM-FGF2-EGF cultured primary astrocytes as compared to MD cultured astrocytes (Fig. 2.6A). We speculated that the increase the higher ATP levels observed in ABM-FGF2-EGF cultured astrocytes was likely attributed to glycolysis. To investigate this, oligomycin (ATP synthase inhibitor) was added 2 hours prior to ATP assay to inhibit oxidative phosphorylation. As expected, even with the inhibition of oxidative phosphorylation we observed 24% and 54% ATP content in MD and serum free ABM-FGF2-EGF cultured primary astrocytes, respectively, indicating that glycolysis contributed to the increase ATP production in serum free ABM-FGF2-EGF cultured astrocytes. Furthermore, glycogen content, high molecular weight glucose polymer which serve as energy reserve in astrocytes, was higher in serum free ABM-FGF2-EGFcultured primary astrocytes than in MD cultured astrocytes (Fig. 2.6B)

AMP-Activated protein kinase pathway (AMPK) is an important regulator of cellular energy homeostasis with predominate expression in neuron in the brain. We observed a decrease in expression and phosphorylation of AMPKα in ABM-FGF2-EGF cultured primary astrocytes as compared to MD cultured astrocytes whiles increase mTOR phosphorylation was observed in ABM-FGF2-EGF cultured astrocytes (Fig. 2.7A). Consistently, a significant decrease of ACC and GS phosphorylation, downstream of AMPK activation, was observed in ABM-FGF2-EGF cultured primary astrocytes as compared to MD cultured astrocytes (Fig. 2.7B). These data indicated that primary astrocyte cultured in serum free ABM-FGF2-EGF medium had a biosynthetic phenotype as compared with serum-containing MD medium cultured astrocytes.

The mRNA expression levels of astrocytes cultured in serum free ABM-FGF2-EGF condition depicts resting astrocyte phenotype.

The mRNA expression of genes commonly associated with astrocyte functions was assessed by quantitative PCR. No significant difference in S100β (Fig. 2.8A) and GLAST (Fig 2.8B) mRNA levels were observed in primary astrocytes cultured in ABM-FGF2-EGF and MD culture media. On the other hand, higher mRNA levels of GLT-1 (Fig. 2.8C), GS (Fig. 2.8D), GLUT-1 (Fig. 2.8E) were observed in primary astrocytes cultured in ABM-FGF2-EGF for 14 days as compared to MD cultured astrocytes. A decreased mRNA level of CXN-43 in primary astrocytes cultured in ABM-FGF2-EGF medium was observed as compared to serum containing MD cultured astrocytes (Fig. 2.8F). Comparing the mRNA expression of other important astrocytic factors, we observed no significant difference in the levels of AQP4 (Fig 2.8G) and Kir4.1 (Fig. 2.8H) between primary astrocytes cultured in serum free ABM-FGF2-EGF medium and astrocytes cultured in serum containing MD media.

Astrocytes are involved in the uptake and clearance of neurotransmitters at the synaptic cleft. Using an assay kit to measure the amount of glutamate present in the media after the addition of 400 μM glutamate for 4 and 8 hours. We observed a significantly higher rate of glutamate clearance by ABM-FGF2-EGF cultured (70.17±3.962) astrocytes at 8, but not 4, hours as compared to MD cultured astrocytes (10% FBS) (53±4.926) (Fig. 2.8I). Higher glucose uptake was also observed in serum free ABM-FGF2-EGF cultured primary astrocytes as compared to MD (10% FBS) astrocytes (Fig. 2.8J).

2.6. FIGURES AND LEGENDS

Figure 2.1.

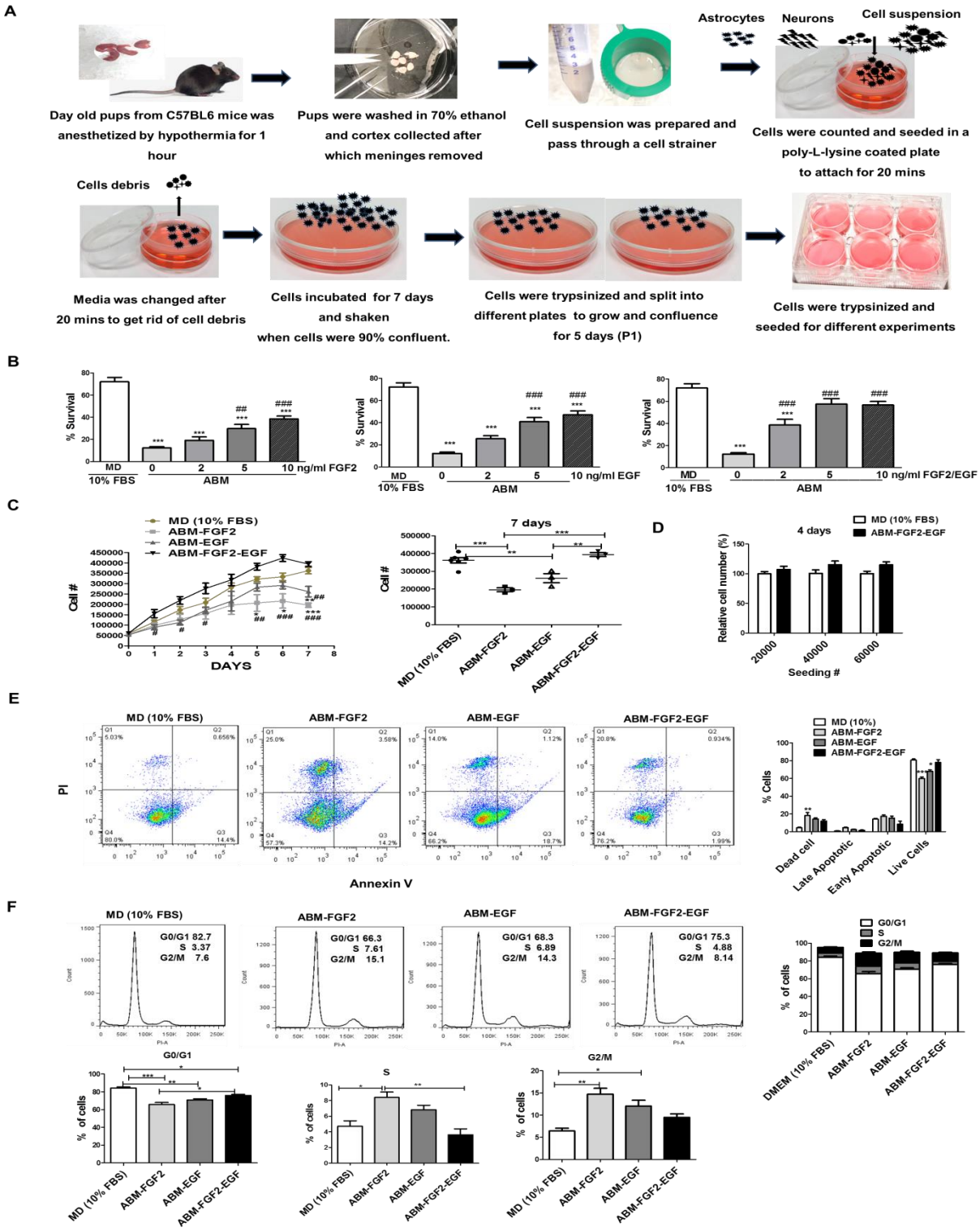


Figure 2.1. FGF2 and EGF synergistically support the survival and growth of primary astrocytes in serum free media. (A) Schematic diagram depicts the variable cell attachment experimental procedure of astrocyte culture (B) Viability assay done after seeding 40,000 cells in ABM (supplemented with different amount of growth factors) and MD (10% FBS) for 48 hours. The Y axis represent % of viable cells determined from the seeded number (*p< 0.05, **p<0.01, ***p<0.001 vs MD (10% FBS)), (# p< 0.05, ###p< 0.001 vs ABM (no growth factor) n= 4). (C) Growth curve assay of primary astrocytes cultured in different medium for 2 weeks. 40,000 cells were seeded in 12-well plates and cells counted daily for 7 days (*p< 0.05, ***p<0.001 vs MD (10% FBS) n= 6) (D) Different numbers of cells were seeded in 12-well plate and growth was analyzed by Calcien AM at day 4 after seeding (n= 6). (E) Flow cytometry and quantitatively analysis of Annexin V and PI stained primary astrocytes at 7th day after culture in MD and ABM media (*p< 0.05, **p<0.01, ***p<0.001 n= 3). (F) Cell cycle analysis of primary astrocytes cultured in ABM (supplemented with different growth factors) and MD (10% FBS). Represented comparison among 4 groups *p< 0.05, **p<0.01, ***p<0.001 n= 3.

Figure 2.2.

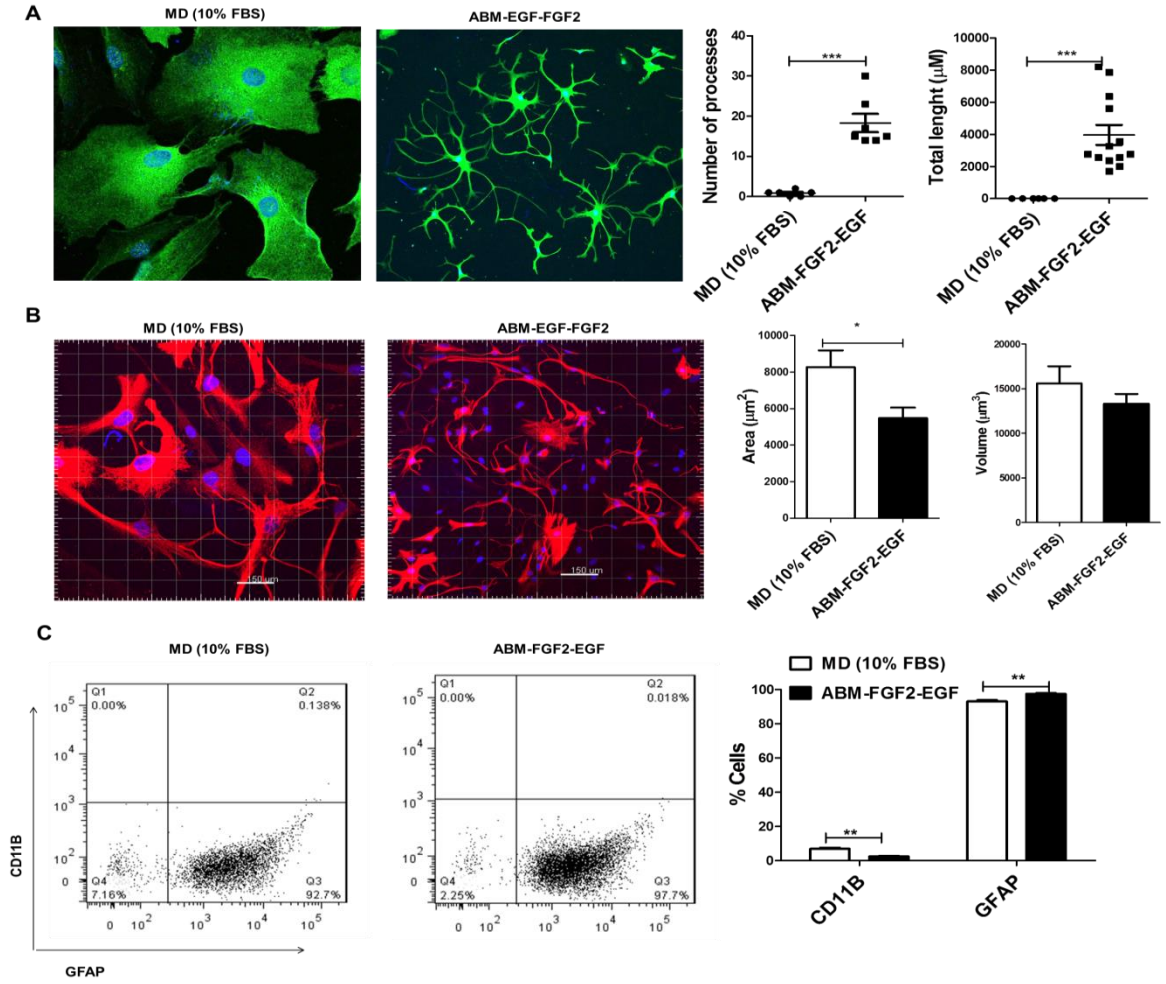


Figure 2.2. Astrocyte cultured in ABM-FGF2-EGF mimic resting astrocyte morphology with high purity. (A). Representative florescent microscopic images of ALDH1L1 immunostaining in primary astrocytes cultured for 2 weeks and quantitative analysis of number and length of astrocyte process in MD and ABM culture condition. (B) Representative image of GFAP staining of astrocytes cultured for 2 weeks in MD (10% FBS) and ABM-FGF2-EGF; bar graph showing quantitative Imaris software analysis of astrocyte size (*p< 0.05 vs. MD (10% FBS) n= 8). (C) Flow cytometry analysis of GFAP and CD11B staining of primary astrocytes cultured in MD and ABM culture condition. (**p<0.01 vs MD (10% FBS) n=3)

Figure 2.3.

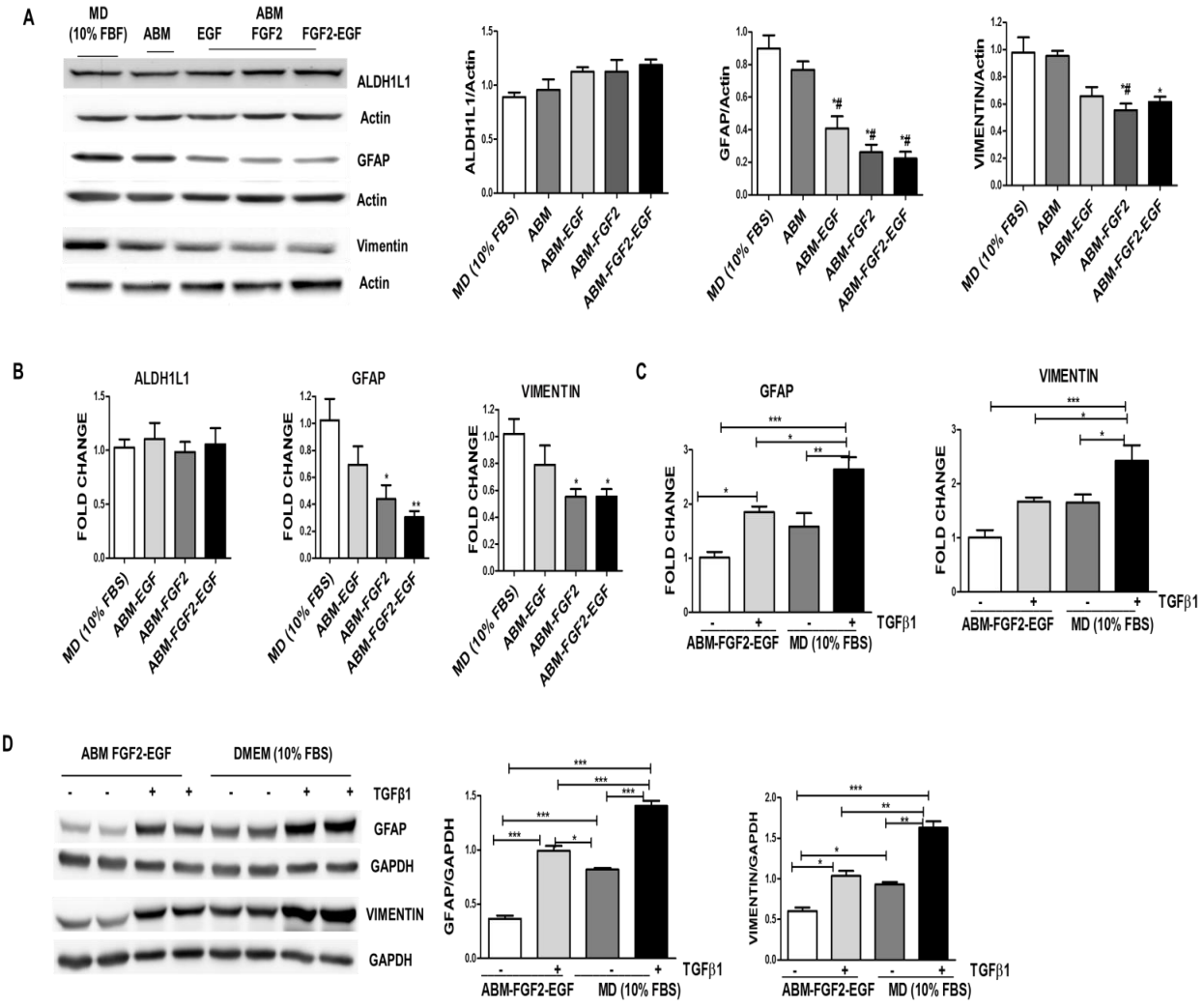


Figure 2.3. Serum free ABM FGF2/EGF medium maintains primary astrocyte in their resting state which could be activated by TGF-β1. (A). Representative and quantitative Western blot analysis of ALDH1L1, GFAP, vimentin expression after 7 days of culture in serum free ABM Containing 5 ng/ml EGF/FGF2 or MD (10% FBS) . (*p< 0.05 vs. MD 10% FBS n= 6-8). # p< 0.05 vs ABM (no growth factor), n=6. (B) Real-time PCR analysis of ADL1L1, GFAP and vimentin expression in astrocytes culture in MD media condition and ABM condition. (*p< 0.05, **p<0.01 vs. MD 10% FBS, n= 6-8). (C) Real-time PCR analysis of GFAP and vimentin

expression in astrocytes culture in MD media condition and ABM media condition and activated with TGFβ (10ng/ml), Represented comparison among 4 groups *p< 0.05, **p<0.01, ***p<0.001 n= 6-8. (D) Representative and quantitative Western blot analysis of GFAP and vimentin expression after astrocytes were culture in MD media condition and ABM media condition and activated with TGFβ (10 ng/ml). Represented comparison among 4 groups *p< 0.05, **p<0.01, ***p<0.001 n= 6-8.

Figure 2.4.

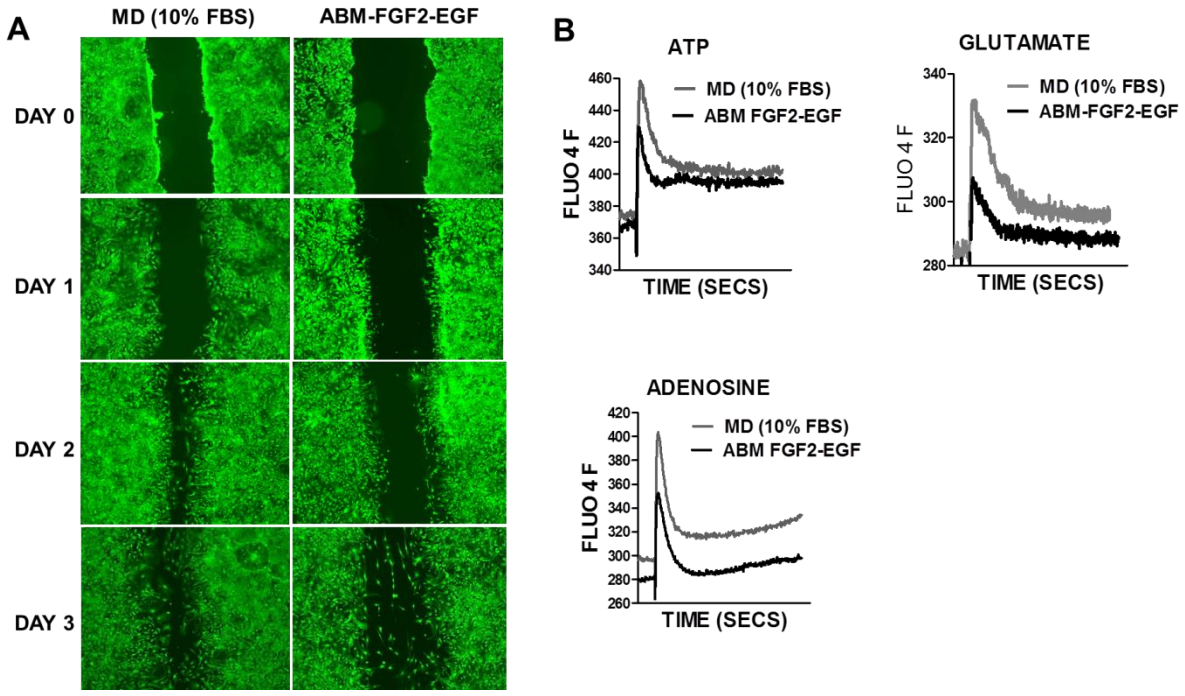


Figure 2.4. Activation of primary astrocytes cultured in serum free ABM-FGF2-EGF medium. (A) Astrocytes maintained in serum free ABM-FGF2-EGF and MD (10% FBS) for 14 days and then seeded for scratch assay, with cell migration monitored from day 0 to day 3 after scratch (B) Astrocytes calcium response to different stimuli measured by Fluo 4AM Florescence (Fluo 4 F).

Figure 2.5.

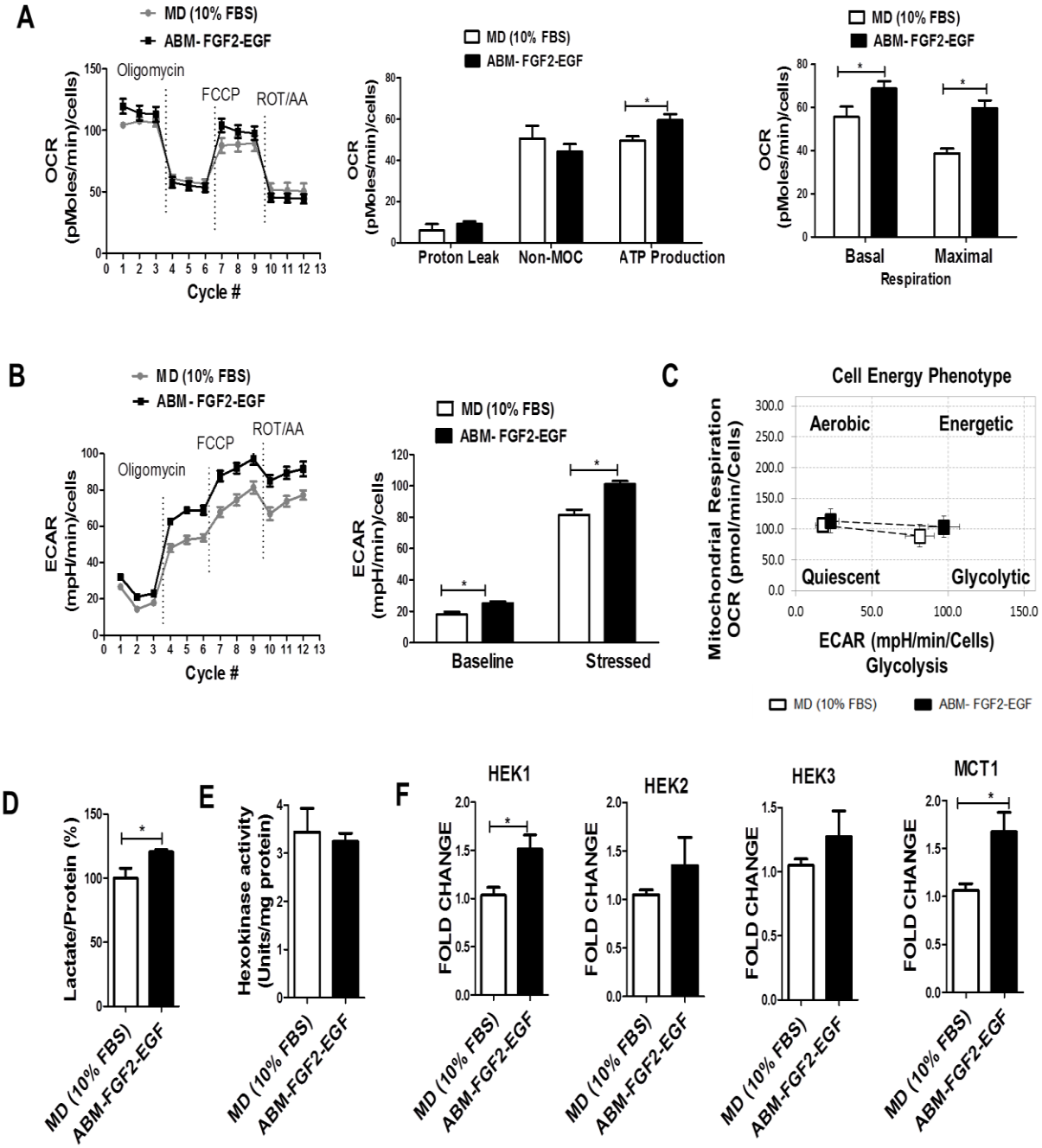

A
MD (10% FBS)
ABM- FGF2-EGF
Oligomycin
FCCP
ROT/AA
OCR (pMoles/min)/cells
Cycle #
Proton Leak
Non-MOC
ATP Production
Basal
Maximal
Respiration
B
ECAR (mpH/min)/cells
Baseline
Stressed
C
Cell Energy Phenotype
Aerobic
Energetic
Quiescent
Glycolytic
Mitochondrial Respiration OCR (pmol/min/Cells)
ECAR (mpH/min/Cells) Glycolysis
D
Lactate/Protein (%)
E
Hexokinase activity (Units/mg protein)
F
HEK1
HEK2
HEK3
MCT1
FOLD CHANGE

Figure 2.5. Primary astrocytes cultured in serum free ABM-FGF2-EGF has enhanced glycolysis and increased extracellular acidification and oxygen consumption rate. Seahorse extracellular flux analysis of Oxygen consumption rate (OCR); bar graph indicate basal and maximal respiration, proton leak, non-mitochondrial oxygen consumption (non-MOC) and ATP production linked to mitochondrial respiration. (B) Extracellular acidification rate (ECAR); bar graph indicate baseline and stressed ECAR. (C) Cell metabolic potential of astrocytes cultured in MD (10%) and ABM-FGF2-EGF. (D) Lactate production of astrocytes cultured in different media condition (E) quantitative analysis of hexokinase activity in astrocytes (F) Real-time rtPCR analysis of hexokinase and MCT1 expression of astrocytes after cells were cultured in different media condition. (* $p < 0.05$ vs MD (10% FBS), n = 6-10).

Figure 2.6.

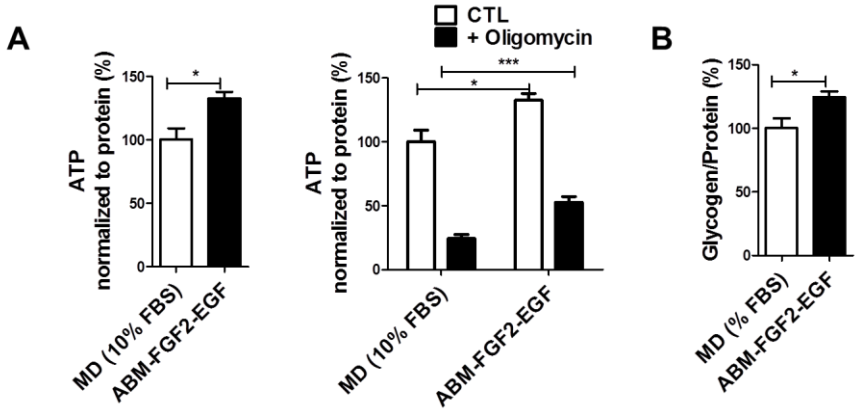


Figure 2.6. Primary astrocyte cultured in serum free ABM-FGF2-EGF have increased total ATP content, glycogen storage and increased glucose uptake. (A) Total ATP level of primary astrocytes cultured in MD (10% FBS) and ABM-FGF2-EGF (B) Glycogen assay of primary astrocytes cultured in MD (10% FBS) and ABM-FGF2-EGF

Figure 2.7.

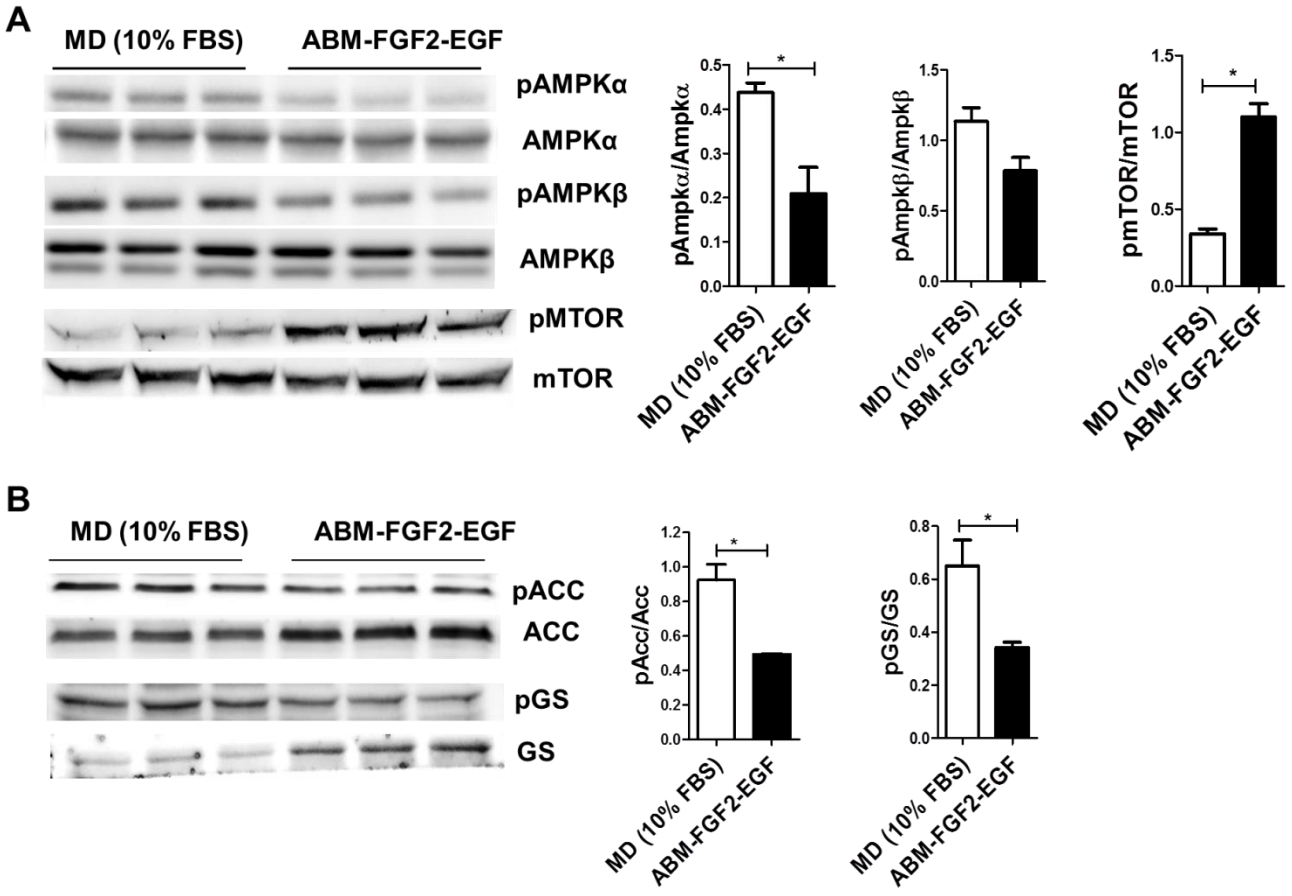


Figure 2.7. ABM cultured primary astrocytes have lower AMPK activation as compared with MD cultured astrocyte. (A) Representative western blots and quantitative analysis of total and phosphorylated AMPKα, pAMPKα, AMPKβ, pAMPKβ, and mTOR, pmTOR after 2 weeks of astrocyte culture in ABM-FGF2-EGF and MD (10% FBS) (n=6 *p< 0.05). (B) Representative Western blots and quantitative analysis of total and phosphorylated ACC, pACC, GS, pGS after 2 weeks of astrocyte culture in ABM-FGF2-EGF and MD (10% FBS) (*p< 0.05 n=6).

Figure 2.8

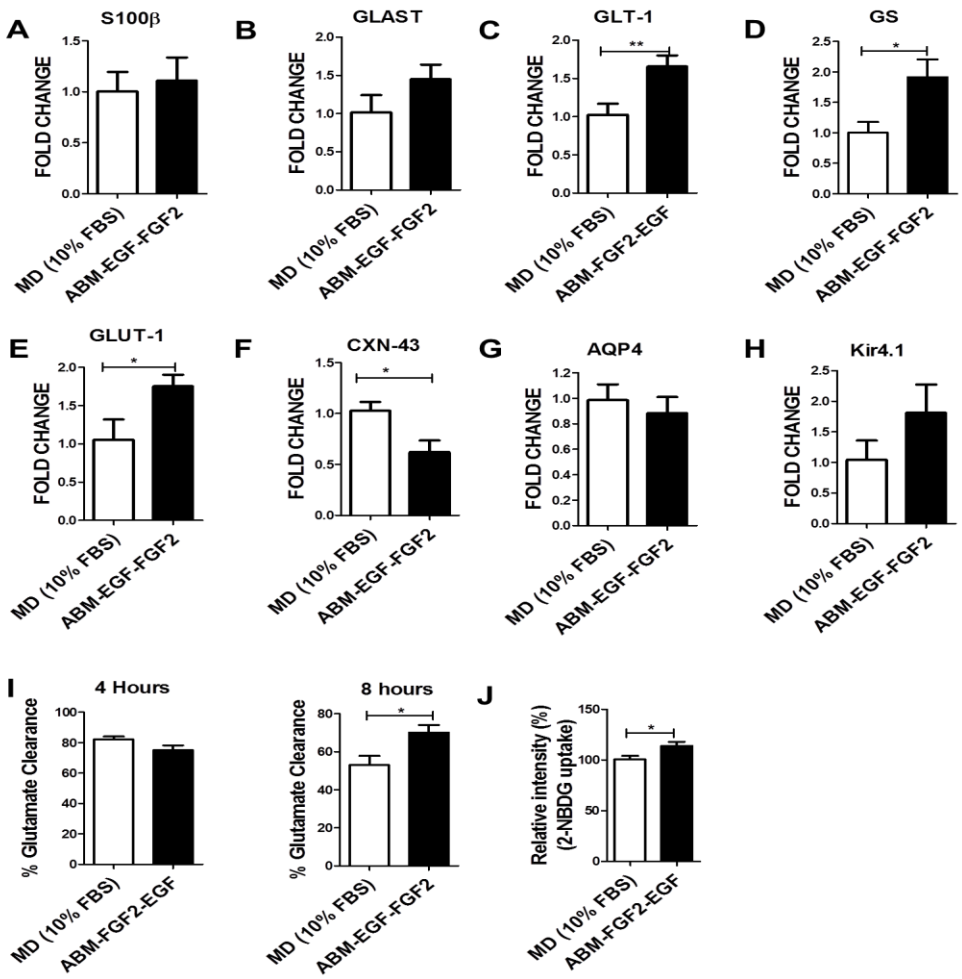
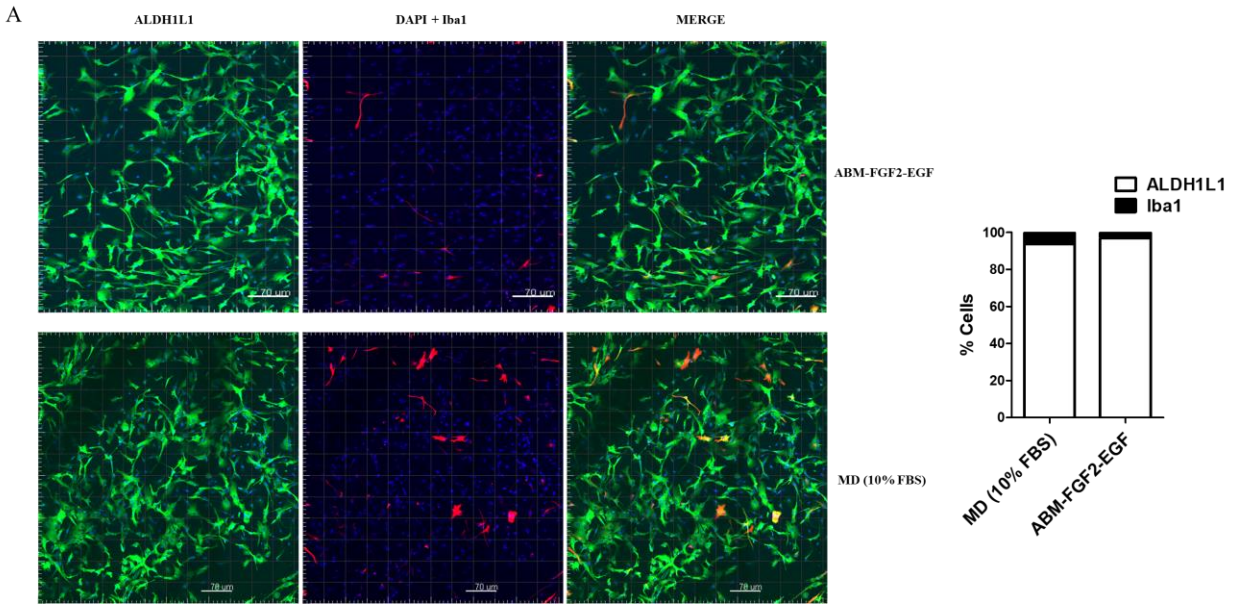
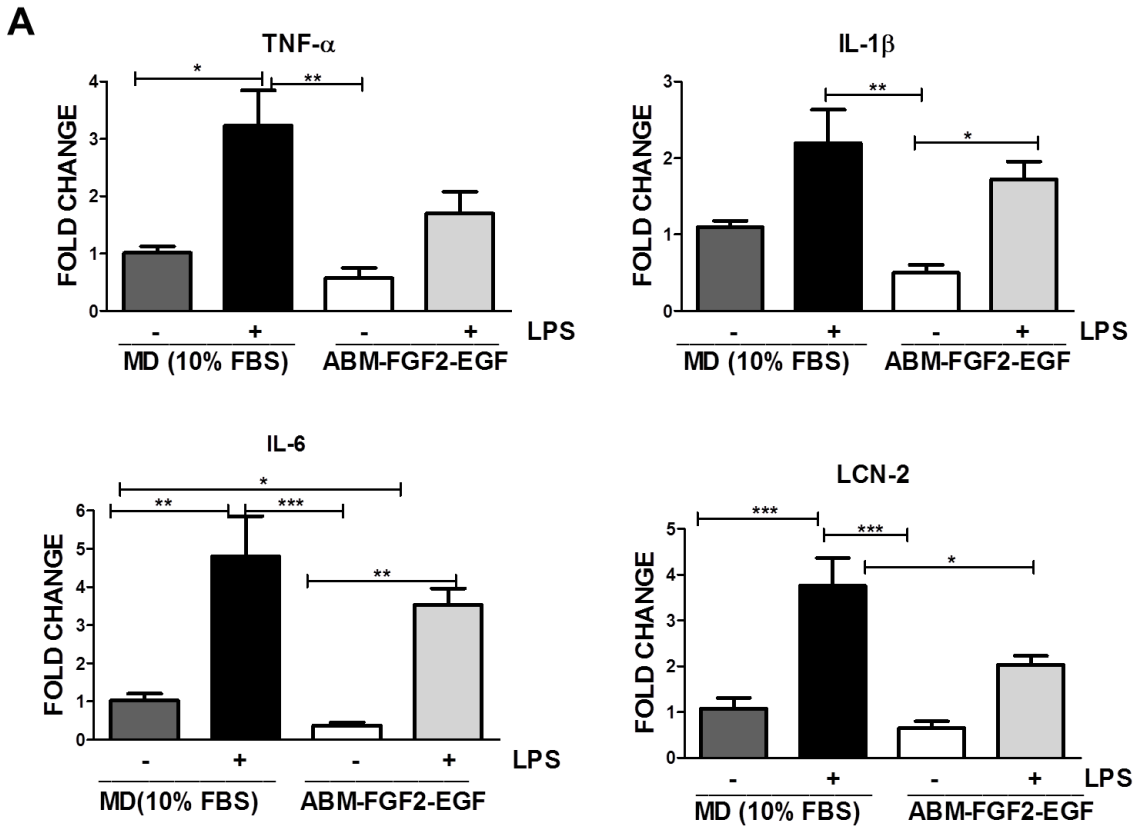


Figure 2.8. Comparison of mRNA expression profile of astrocyte specific factors of Serum free ABM-FGF2-EGF astrocytes with MD (10% FBS) astrocytes. Real time PCR analysis of astrocytes associated factors (A) S100β (B) GLAST (C) GLT-1 (D) GS (E) GLUT-1 (F) CXN-43 (G) AQP4 (H) Kir4.1 (*p< 0.05, **p<0.01 vs. MD 10% FBS n= 6-8). (I) Glutamate clearance measured at different time points in primary astrocyte cultured in MD and ABM conditions. (J) Quantitative analysis of 2-NBDG uptake shows increased 2-NBDG uptake in primary astrocytes cultured in ABM-FGF2-EGF. (* p< 0.05 ***p<0.001 vs MD (10 FBS), n = 6-8).

Sup Fig 2.1.



Sup Fig 2.1. (A). Representative image of ALDH1L1 and Iba1 immunostaining of astrocytes cultured in MD (10% FBS) and ABM-FGF2-EGF; bar graph showing calculated percentage of cells both stained with ALDH1L1 and DAPI to cells stained with DAPI only or Iba-1, using 10 views per slide. (n= 4).



Supplementary Fig 2.2. LPS activates and induce the expression of inflammatory mediators in astrocytes. (A) Real-time PCR analysis of IL-6, IL-1β, TNFα and LCN-2 expression in astrocytes culture in MD media condition and ABM media condition and activated with LPS (100ng/ml) for 12 hours, Represented comparison among 4 groups *p< 0.05, **p<0.01, ***p<0.001 n= 6-8

2.7. DISCUSSION

In vitro systems allowing maintenance and experimentation on primary astrocyte cultures have been in use for decades, which are essential for understanding astrocyte functions in health and to mimic various pathological conditions. The use of a serum containing media makes astrocytes obtained via this method exhibit a flat and fibroblast like morphology [4, 14, 228]. Additionally the use of FBS in the medium makes it difficult to study the effects of certain compounds and drugs on astrocytes *in vitro,* as serum components may antagonize or potentiate the actions of certain agents under investigation [5]. Astrocytes obtained by this method also have gene profiles significantly different from astrocytes *in vivo* [3, 79]. Correcting the limitations of this astrocyte culture method and producing astrocytes which closely resemble *in vivo* astrocytes will ensure results obtained using primary culture can be attributed to events occurring *in vivo*.

Culture media are to ensure cell growth and proliferation. We report that our serum free media promotes proliferation of astrocytes similar to astrocytes cultured in FBS containing medium. Consistent with recently established serum free primary culture methods [4, 14, 228], we observed that the morphology of astrocytes cultured in the serum free ABM culture condition was similar to *in vivo* resting astrocytes with a greater degree of branching.

Studies have indicated that different sera leads to different degrees of microglia contaminations as serum components may affect proliferation of microglia and hence astrocytes growth [10]. Our current study used a serum free-ABM-FGF2-EGF culture condition with a variable cell attachment rate method to produce a culture of high purity. Similar to earlier studies conducted by [4] using immunopanning to directly isolate and culture astrocytes in a serum free

medium, astrocytes cultured in our serum free ABM-FGF2-EGF medium have similar degree of purity.

Glucose metabolism in the brain occurs in a compartmentalized way. Astrocytes are the primary contributors to glycolysis, the cytosolic and anaerobic arm of glucose metabolism, whiles neurons are more responsible for oxidative metabolism [30]. Energy metabolism of astrocytes is coupled to neurons as the end product of glucose metabolism in astrocytes, lactate, is released into the extracellular space through (Monocarboxylate transporters) MCT1 and taken up by neurons through MCT2 to be used for oxidative metabolism to generate ATP [238]. Our study indicates that astrocytes cultured in serum free media have increased mitochondrial respiration, enhanced glycolysis, increased lactate levels, and ATP content compared to astrocytes in serum containing media. We speculated that the increase of ATP and lactate production as well as the enhanced glycolysis could be due to increased expression of HEK1, MCT1, GLUT-1, and increased glucose uptake observed in serum free ABM-FGF2-EGF astrocytes.

AMPK is an important regulator of multiple metabolic pathways. AMPK activation stimulates catabolism and concomitantly inhibits anabolism [239]. Given the increase ATP and glycogen content of primary astrocytes cultured in the serum free ABM-FGF2-EGF condition, it might not be a surprise that our serum free culture condition will impact AMPK signaling. In the brain, AMPK are predominately localized and activated in neurons. We observed that primary astrocytes cultured in serum free ABM-FGF2-EGF condition have a decreased AMPK activation as compared to astrocytes cultured in MD media evidenced by lower AMPK phosphorylation. Consistent with the lower AMPK activation in primary astrocytes cultured in serum free medium, we observed a concomitant increase anabolism evidenced by decrease phosphorylation and

activation of acetyl CoA carboxylase (ACC) and glycogen synthase. Consistently, serum free media could produce astrocytes with a biosynthetic or anabolic phenotype similar to resting *in vivo* astrocytes.

It has been previously reported that astrocytes cultured in serum containing media have higher GFAP levels [227]. Studies have shown that astrocytes express several FGF2 receptors and FGF2 signaling maintains astrocytes in their resting state [17, 235]. Consistently, we observed a decrease of GFAP and vimentin levels in primary astrocytes cultured in serum free FGF2-EGF medium as compared with astrocytes cultured in FBS containing medium, indicating that primary astrocytes cultured in the serum free FGF2-EGF media are in their resting state. Nevertheless, reactive astrogliosis could be induced in primary astrocytes cultured in serum free FGF2-EGF containing medium by pro-inflammatory cytokine and scratch injury [240].

Calcium waves can be stimulated in astrocytes by several stimuli including ATP, adenosine, glutamate, and KCl [4, 241]. The increased calcium levels observed in the absence of stimuli in serum containing MD media was similar to what was reported in previous study [4]. Increased calcium waves have been found to be associated with reactive astrocytes or astrocytes in pathological conditions [236, 237]. Consistently, primary astrocyte cultured in serum free ABM-FGF2-EGF medium has lower calcium as compared with astrocytes cultured in serum containing MD medium. However, stimulation of adenosine, ATP, and glutamate increased calcium concentration in primary astrocytes cultured in serum containing MD medium and ABM-FGF2-EGF medium.

Astrocytes play a critical role in the uptake and metabolism of glutamate thereby regulating glutamate level at the synaptic cleft via glutamate transporters expressed at presynaptic astrocytic processes [52]. Glutamate taken up by astrocytes can serve as a transmitter precursor for the

synthesis of the inhibitory amino acid GABA via its conversion to glutamine by glutamine synthase [52]. In various neurodegenerative disorders characterized by astrogliosis, this important astrocytic function is lost leading to glutamate induced neurotoxic death [226]. Additionally activated astrocytes both *in viv*o and *in vitro*, have also been shown to have decreased GLT-1 expression making them less efficient in clearing glutamate, while quiescent astrocytes express higher GLT-1 [242, 243]. In our study we observed that primary astrocytes cultured in serum free ABM-FGF2-EGF medium have higher GLT-1 mRNA expression levels as compared to astrocytes cultured in FBS containing MD medium. Consistently, an increased clearance of glutamate as well an increase GS mRNA expression were observed in primary astrocytes cultured in the serum free medium ABM-FGF2-EGF medium as compared with FBS containing MD medium. One other astrocytic protein, whose expression is increased in reactive astrocytes and various diseases as well as in injuries, is CNX-43 [244-246]. We found a decrease level of CNX-43 mRNA levels in astrocytes cultured in serum free medium. Interestingly there have been conflicting reports on the regulation of astrocytes glutamate transporters by CNX 43 levels [247, 248]. Our findings are consistent with recent data that showed decreased levels of connexin 43 regulates the increase in the expression of astrocyte glutamate transporters [248]. Taken together, the increased GLT-1 and CXN-43 expression and glutamate clearance provided additional evidence that our serum free ABM-FGF2-EGF medium maintains astrocytes in their quiescent state. Indeed, it has been indicated that both FGF2 and EGF reduced CNX-43 expression in astrocytes [249, 250] and that EGF induce GLT-1 expression [251].

Conclusion

We demonstrated that the novel serum free ABM-FGF2-EGF medium supports astrocytes growth and enhanced glycolytic metabolism with higher glycogen content, lower GFAP and vimentin expression, and increased glutamate transporter mRNA levels as compared to astrocytes cultured in the MD-10% FBS medium. Our study suggests that our serum free culture method produces quiescent astrocytes with a biosynthetic phenotype and morphology similar to *in vivo* resting astrocytes. Additionally ABM- FGF2-EGF cultured primary astrocytes could be activated by various pathological conditions. Thus our developed serum-free and EGF/FGF2-containing astrocyte basal medium will provide a critical tool for defining the precise function of astrocytes under physiological and pathological conditions.

CHAPTER 3

HYPERGLYCEMIA IMPACTS PHENOTYPE AND FUNCTIONAL PROPERTIES OF CULTURED ASTROCYTES

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3.1. ABSTRACT

Astrocytes are glial cells that maintain brain homeostasis by regulating energy and neurotransmitter metabolism, synaptic function and antioxidant defense. Astroglial dysfunctions are involved in the pathogenesis of many neurodegenerative disorders. In these disorders activated astrocytes play critical roles in the pathological processes. Primary astrocyte culture has become a valuable tool for studies of astrocyte functions and signaling pathways that regulate astrocyte physiology and reactivity. Most studies using primary astrocyte cultures maintain astrocytes in high glucose (25 mM) conditions. This condition can induce experimental diabetes. Astrocytes as components of the blood brain barrier are involve in glucose uptake from the blood and provide for neuronal energy demands via astrocytes-neuron coupling hence changes in glucose levels might impact astrocytes metabolism and biology. In these present study we determined the effects of high glucose culture condition on astrocyte metabolism, phenotype and function. Astrocytes prepared from C57BL6 pups were cultured in high glucose (25 mM) and normal glucose (5 mM) serum free medium. Hyperglycemic culture condition enhanced glycolysis, increased ATP and glycogen content of the cell. High glucose also induced cell cycle arrest and inhibited astrocyte proliferation. Furthermore we observed that high glucose altered astrocyte morphology and produced astrocytes with a reactive phenotype. In addition maintaining astrocytes in high glucose condition decreased the expression of functional astrocytes proteins and impacted astrocytes functions. In summary our study indicated that hyperglycemia triggers cellular alterations and affect astroglial functions and provide a reason for careful consideration of astrocyte culture conditions.

3.2. INTRODUCTION

Astrocytes are the main glial cells responsible for the maintenance of brain homeostasis. They regulate extracellular ion concentration, and pH of the microenvironment [28]. Astrocytes are also involved in neurotransmitter metabolism and homeostasis, metabolic support for neurons as well as trophic support and antioxidant defense for neurons [28, 30, 55]. Astrocytes swiftly respond to subtle changes in the microenvironment including that from blood supply because of their end feets that are tightly attached to blood vessels [28, 223]. The brain is the most expensive organ in terms of glucose expenditure with cerebral activities consuming about 20% available glucose [252]. To meet this strong energy demand of the brain, glucose is provided by the blood to brain tissues through cell uptake and processed by different metabolic pathways [30, 252, 253]. Astrocytes due to their anatomical localization and abundant expression of glucose metabolizing enzymes and glucose transporters (GLUT-1) at their end feet are mainly responsible for glucose uptake and metabolism in the brain [252, 254]. Neurons obtain part of their energy from extracellular lactate, a glucose metabolite produced by astrocytes [44, 255]. Therefore any change is the amount of glucose available to astrocyte could impact neuronal function. For example in hyperglycemic situations neuronal glucose availability increases leading to neuronal dysfunction and damage, a phenomenon referred to as glucose neurotoxicity [256].

Primary astrocyte cultures over the years have been a useful tool in understanding astrocytes biology in health and disease. They provide an avenue for studying a single cell type so that any effect observed is astrocyte specific. Primary astrocyte cultures also enables functional studies of astrocytic proteins and receptors and their real time response to compounds, mechanisms involve in astrocytic dysfunctions, as well as permit knockdown or overexpression

of genes to investigate changes in disease conditions [1]. Most studies using primary astrocyte cultures rely on the early protocol developed by McCarthy and de Vellis, with minor modifications [2, 10]. Due to the limitations of this culture method, others use immunopanning, and florescent activated cell sorting to isolate astrocytes [4, 11]. One common feature of these culture protocols is the use of high glucose media (20 and 25 mM) to culture astrocytes. This may be due to the assumption that normal glucose concentration close to 6 mM is rapidly depleted by astrocytes in culture. Once glucose is rapidly depleted in the culture medium, astrocytes start depending on lactate as a substrate for oxidative metabolism [257]. However high glucose concentration in primary cell cultures induces experimental diabetes and does not mimic *in vivo* physiological condition.

Diabetes mellitus is a metabolic disorder that also affects the central nervous system (CNS) by raising brain glucose levels. It is associated with high risk of many neurological disorders. It has also been shown that people with diabetes are at greater risk of stroke and hyperglycemia increases brain damage after ischemic stroke [258-260]. High glucose concentration within the CNS has been demonstrated in animal models to impair brain cognitive functions and expression of neurotrophic factors such as BDNF [261]. Hyperglycemia induces the expression of pro-inflammatory cytokines and reactive oxygen species production in astrocytes [9, 262]. In an earlier study conducted by our group using serum contained medium, we found that high glucose condition alters astrocyte metabolism and inhibits astrocyte proliferation [263]. Since astrocytes play critical role in glucose metabolism and brain homeostasis due to their phenotypic features, and culture conditions have been shown to alter astrocyte phenotype [3, 10], we speculated that glucose concentration in a serum free culture

medium might alter astrocyte's phenotypic features and functions, hence outcomes of studies design to obtain information on astrocytic mechanisms and functions.

The purpose of this study is to determine the consequences of glucose concentration in culture media on primary astrocytic phenotype and functions. Using a serum free medium supplemented with fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) which we demonstrated to maintain astrocytes in the resting state, we investigated if glucose conditions in the media impact astrocyte morphology, function, metabolism and protein expression.

3.3. MATERIALS AND EXPERIMENTAL METHODS

Materials and Reagents

Propidium iodide (PI) and annexin V apoptotic staining kit was purchased from BD Bioscience (USA), Rotenone, Carbonyl cyanide-4- (trifluoromethoxy) phenylhydrazone (FCCP) mito-stress and phenotype kit was purchased from Agilent technologies (USA). Micro cover glasses were purchased from VWR. Trypsin-EDTA, trypan blue, poly-L-Lysine solution, bovine serum albumin, transferrin, putrescine dihydrochloride, progesterone, sodium selenite, and N-Acetyl Cysteine were purchased from Sigma-Aldrich (USA). TrypLE, Dulbecco's Modified Eagle's medium low and high glucose (DMEM), Neurobasal medium, Penicillin-streptomycin, sodium pyruvate, Glutmax was purchased from Gibco/Life Technologies (USA). Normal goat serum was purchased from Jackson Immunoresearch (USA). Gold antifade mountant with DAPI and BD cytofix/cytoperm^{TM} fixation and permeabilization solution were purchased from Invitrogen and BD Biosciences (USA) respectively. Human FGF-basic and Human EGF were purchased from Pepro Tech (USA). Primary antibodies against GFAP (monoclonal cell signaling), Vimentin (monoclonal Cell signaling), AMPKα and AMPKβ (monoclonal Cell signaling), GS and ACC (Monoclonal Cell signaling), mTOR (monoclonal Cell signaling), Actin

and GAPDH (monoclonal Santa Cruz) and Alexa-fluor conjugated secondary antibodies from Invitrogen as well as non-conjugated secondary antibodies from Jackson laboratory all in USA were used for immunostaining or Western blot. Information regarding primers used for quantitative Polymerase Chain Reaction (qPCR) to quantify mRNA expression of some astrocyte specific genes using SYBR Green PCR Master mix (Promega, USA) and real time PCR system from applied Biosystems (USA) are listed in Table 3.1.

Culture media

Serum free media defined as astrocyte based media (ABM) is composed of Neurobasal medium and DMEM (1:1 v/v) supplemented with 5.5 mM, 11 mM or 25 mM glucose, Penicillin (100 units/ml)-streptomycin (100 μg/ml), 1 mM sodium pyruvate, Glutmax, bovine serum albumin (100 μg/ml), transferrin (100 μg/ml), putrescine dihydrochloride (16 μg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), and N-Acetyl Cysteine (5 mg/ml). ABM was supplemented with combination of 5 ng/ml of Human FGF-basic and Human EGF.

Primary Astrocyte culture

The institutional animal care and use committee of UNTHSC has approved all procedures performed during preparation or isolation of primary astrocyte from C57bL6 mice (Jackson's lab). Primary astrocyte was prepared according to previous published methods with minor modifications [2, 210]. Briefly day old C57BL6 pups were anesthetized by hypothermia followed by decapitation with sharp surgical scissor. The cerebral cortices were dissected and meninges removed under aseptic conditions. 0.25% trypsin (sigma) was used to digest the cortices at 37 ^{o}C for 20 minutes shaking it after every 5 mins. Single cell suspension were prepared by pipetting (pipette, up and down 3-5 times) through 3 different sized pipettes and cell suspension strained through 40 μM size cell strainer. The cells were plated in 10 cm culture plate

in ABM-5.5 mM, ABM-11 mM, and ABM-25 mM glucose culture condition and incubated in a humidified incubator at 37 ^{o}C with 5% CO_2 for 15 mins after which media replaced to get rid of debris. When the cells become 90% confluent; the plates were constantly shaken for 24 hours in CO_2 incubator at 37 ^{o}C to eliminate microglia and other types of cell debris. The media was replaced every two days and the cells were used at Passage 2.

Cell viability assay and apoptosis analysis

Primary astrocytes were seeded into the different media conditions at different densities to confluent for 3 days in a 12 or 6 well poly-L-lysine coated plates. On the day of experiment, media was removed and replaced with a 1 µM solution of Calcien AM in PBS. Cells were incubated for 5 minutes at 37 ℃ and fluorescence was measured using a Tecan Infinite F200 plate reader (excitation 485 emission 530). Cell apoptosis was analyzed using flow cytometry (BD LSR II, San Jose, CA, USA) with annexin-V and propidium iodide staining according to the manufactures protocol 4 days after they were seeded in a 6 well plate

Growth curve and cell cycle assay

Astrocytes were seeded at a density of 40,000 cells/well in 12-well plate in different culture medium. At the indicated days after seeding, cells were harvested using trypsin-EDTA and counted using a hemocytometer. Four wells were assigned to each group and cell counting was conducted by a researcher who was blinded to the group assignment using an inverted phase contrast Zeiss Invertoskop microscope.

For cell cycle analysis astrocytes were seeded at a density of 50,000 cells/well in 12-well plates in various culture media. On day 3 after culture, cells were harvested using trypsin-EDTA and washed with PBS twice to remove trypsin. Cells were fixed in ice-cold 70% ethanol for 24 hours at 4 ℃. Ethanol was removed by washing twice with PBS, and the cells incubated with

propidium iodide (PI) (40 μg/ml) and RNase (10 μg/ml) for 30 min at 37 ^{o}C. The stained cells were analyzed using a Beckman Coulter FC500 Flow Cytometry Analyzer for quantification of cell cycle distribution.

Cellular bioenergetics and metabolism

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was monitored using Agilent Seahorse XFe96 analyzer. Astrocyte were seeded at a density of 15,000 cells per well in 96-well Seahorse XF cell culture microplates in different media conditions and allowed to attach and confluent for 7 days. On the day of the experiment the culture media was replace with Seahorse XF base medium supplemented with 1 mM pyruvate, 2 mM glutamine and 5.5 mM or 25 mM glucose media and incubated for 1 hour in a non CO_2 incubator at 37 ^{o}C. Rotenone/antimycin, FCCP, and oligomycin were diluted into XF media and loaded into the accompanying cartridge to achieve final concentrations of 0.5 μM, 1 μM, and 1 μM respectively. Injections of the drugs into the medium occurred at the time points specified. Each cycle was set as: mix for 3 minutes, delay for 2 minutes and then measure for 3 minutes. For cell energy phenotype determination, oligomycin and FCCP were diluted in XF medium together to achieve a final concentration of 1 μM for both oligomycin and FCCP, which was loaded into one port. Values were normalized to the cell number of each well determined by Calcien AM assay.

ATP, NAD/NADH and lactate assay was performed using a commercial kit from Invitrogen Eugene, OR, USA and Sigma-Aldrich MO, USA respectively. ATP, NAD/NADH and lactate concentrations were normalized to the protein concentration of each sample measured with a protein assay kit (Thermos Scientific, Rockford, IL, USA). All this assays were performed using the protocols designed by the different manufactures.

Glucose uptake and glycogen analysis were determined as describe in [210] , briefly for glucose uptake glucose analog 2-NBDG was used. Cells were washed and incubated in glucose free Krebs Ringer HEPES (KRH) buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES; pH 7.4) for 30 min and then incubated in glucose free KRH buffer containing 100 mM of 2-NBDG for 10 min after which the cells were washed and glucose uptake determined using a Tecan infinite M200 plate reader (excitation/emission for 2-NBDG 465/540 nm).

Western blot, Immunostaining and real time qPCR analysis

Astrocytes were seeded at a density of 400,000 cells/well in 6-well plates in different media conditions and grew to confluency for 14 days. The cells were collected and lysed in RIPA buffer (50 mM of Tris. HCL, pH 7.4,150 mM NaCl,1mM EDTA, 1%Triton X) with protease and phosphatase inhibitors (1:100). Protein assay reagent Pierce 660 nm (Thermo Scientific) was used to determine the protein content of the samples. The samples were resolved on SDS gel and transferred to a nitrocellulose membrane. The membranes were incubated overnight in primary antibodies against GFAP, Aldh1L1, vimentin, AMPKα and AMPKβ, glycogen synthase; GS and ACC, mTOR, Actin followed by secondary antibody. Using Biospectrum 500 UVP imaging system, chemiluminescence signal was detected and normalized to actin.

For immunostaining cells were fixed with cytofix overnight at 4 ^{o}C. Cells were incubated with goat serum blocking solution and 0.2 % triton –X 100 for 1 hour. After washing 3 times with PBS, cells were incubated overnight with primary antibodies at 4 ^{o}C. Cells were then washed and incubated with FITC-labelled secondary antibodies for 1 hour and stained with

DAPI. Images were obtained with a confocal microscope (Olympus fluoview FV1200). The cell's morphology and dimensional structure were analyzed using Imaris (bitplane)

For RNA extraction and real time qPCR, total RNAs were extracted using PicoPure® RNA Isolation Kit following the manufacturer's manual (Invitrogen). Complement DNA synthesis was performed using SuperScript® III First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Quantitative PCR was performed using Fast SYBR® Green Master Mix on a 7300 Real-Time PCR System (Invitrogen). Data were analyzed with 7300 system software and $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression.

Wound healing/migration assays

Astrocyte were seeded at a density of 100000 cells/well in a 12 well plate in different media conditions and allowed to attach and confluent for 7 days. Using a pipette tip, a straight scratch simulating a wound was made on the cell layer. The plates were then washed with PBS to remove cell debris and media replaced. At different time points after scratch, cells were stained with Calcien AM (10 μM), and fluorescent images taken randomly with a Zeiss fluorescent Microscope (USA).

Glutamate clearance/uptake assay

Primary astrocytes were plated in 24-well tissue culture plates at a density of 10×10^4 cells/well and allowed to grow confluent for one week in different media condition. On the day of the experiment, glutamate (400 μM) was dissolved in phenol-free astrocyte medium and added into each well. Clearance was assayed at 8 hr. The assay was performed and analyzed according to manufacturer's guidelines (Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit, Life Technologies).

Astrocyte calcium signaling

Astrocytes were seeded at a density of 20,000 cells/well in poly-l-lysine coated black-walled 96-well plate and grew confluent for a few days. Cells were washed and incubated with 10 μM Fluo-4 AM (Invitrogen F14201) for 45 minutes. Fluo-4 AM fluorescence was measured before and, after stimulating the cells with 0.1 mM glutamate, 50 μM Adenosine and 100 μM ATP. Calcium concentration were measured with Flex station 3 (Molecular Devices, Sunnyvale, CA) for 600 seconds. The baseline calcium signal was measured for 30 seconds followed by injection of stimulant.

Reactive oxygen analysis (ROS)

ROS fluorescent indicator H_2DCFDA (Anaspec) was used to examine changes in reactive oxygen species using a fluorescent microplate reader and flow cytometry. For flow cytometry, cells were seeded at a density of 100,000 cell/ well in a 6 well plate in different media to attach. The media was then removed and cells washed with PBS and then incubated in PBS containing 10 μM H_2DCFDA for 15 mins 37^{o}C. The PBS containing H_2DCFDA was replaced with fresh PBS and the DCF fluorescence was determined with a Beckman Coulter FC-500. For the fluorescent microplate reader, cells were seeded at a density of 3000 cells/well and incubated overnight in 96 well plate. The media was removed and cells were incubated in PBS containing 10 μM H_2DCFDA for 45 minutes at 37^{o}C. The PBS was removed and cells were incubated for additional 15 minutes at 37^{o}C in KRH. Cells were then washed with PBS and DCF florescence measured using a Tecan infinite F200 plate reader (excitation 485, emission 530).

Mitochondrial membrane potential analysis

Astrocytes were seeded in a 12 well plate at a density of 50,000 cells per well in different media for 7 days. Media was then replaced with PBS containing 1 μM Tetra methyl rhodamine,

Ethyl Ester (TMRE) mitochondrial (Invitrogen, USA) for 20 min at 37 ^{o}C. Florescence intensity was measured using a Tecan infinite F200 plate reader (Excitation 594, emission 575).

Super oxide dismutase and Glutathione assay.

Astrocytes were seeded at a density of 70,000 cells/well in a 12 well plate and allowed to confluent. Super oxide dismutase (SOD) activity and GSH was measured using assay kits (Sigma Aldrich) and according to the manufacturers protocol. Values were normalized to the cell number of each well determined by Calcien AM assay.

Statistical Analysis

Statistical differences between control and treated samples were evaluated with student's unpaired *t*-test (2-tailed) or one-way ANOVA with post hoc Bonferroni test for multiple comparisons. Mean ±SE are shown. p values ≤ 0.05 were significant.

3.4. TABLE

Table 3.1

Markers quantified by qPCR.

	Gene name	Physiological function
GFAP	Glial fibrillary acid protein	Astrocyte intermediate filament
VIM	Vimentin	Astrocyte intermediate filament
GS	Glutamine synthase	Converts glutamate to glutamine
HEK1	Hexokinase type 1	Phosphorylates glucose
HEK2	Hexokinase type 2	Phosphorylates glucose
HEK3	Hexokinase type 3	Phosphorylates glucose
MCT1	Monocarboxylate transporter 1	Lactate transport
Kir4.1	Inward rectifier potassium channel 10	Potassium uptake or buffering
AQP4	Aquaporin-4	Mercurial-insensitive water channel
CNX-43	Gap junction alpha-1 protein	Gap-junction component (coupling)
CNX-30	Gap junction beta-6 protein	Gap junction component (coupling)
GLUT1	Glucose transporter member 1	Glucose uptake
GLT-1	Glial high affinity glutamate transporter), member 2	Glutamate transporter
GLAST	Glial high affinity glutamate transporter), member 3	Glutamate transporter
IL-6	Interleukin -6	Cytokine
IL-10	Interleukin -10	Cytokine
IL-4	Interleukin -4	Cytokine
IL-1β	Interleukin - 1β	Cytokine
LCN-2	Lipocalin-2	Inflammatory mediator
TNFα	Tumor necrosis factor-α	Cytokine

3.5. RESULTS

High glucose slows astrocyte proliferation

Primary astrocytes were cultured in ABM-FGF2-EGF culture condition supplemented with 5.5, 11 or 25 mM glucose, for one week. The cells were then split and cultured for another week before 40,000 cells were seeded per well for growth curve analysis. Astrocytes cultured in high glucose (25 mM) proliferated significantly slower than those cultured in 5.5, and 11 mM glucose. There was no difference in the growth rate of astrocytes cultured in 5.5 mM and 11 mM glucose medium (Fig 3.1A). When the media was switched from 25 mM to 5.5 mM, 2 days after seeding 40,000 cells in a 12 wells plate to determine if the slow proliferation rate of astrocytes cultured in high glucose media is reversible, no change in the proliferation rate was observed after switching glucose from 25 to 5.5 mM (Fig 3.1B). Additionally, we observed that astrocytes proliferation was inhibited when glucose concentration in the media was switched from 5.5 mM to 25 Mm (Fig 3.1B), suggesting that high glucose effects on astrocyte proliferation may not be reversible after switching to normal glucose concentration. To assess if seeding density has any effect on growth assay and if hyperglycemia affects astrocytes viability, astrocytes were seeded in a 12 well plate at densities of 20000, 40000, and 60000 cells/well in normal glucose (5.5 mM) and high glucose (25 mM) and the cell viability was assessed using calcien AM on day 3 of culture. There was a significant decrease in the viability of astrocytes cultured in high glucose medium (25 mM) as compared to those seeded in normal glucose media (5.5 mM) (Fig 3.1C). We speculated that the reduced number of astrocytes seeded in high glucose media could be attributed to the reduction of cell proliferation or increase cell death. Flow cytometry analysis of cells stained with Annexin V and PI after 4 days in the various culture conditions demonstrated

that high glucose (25 mM) did not impact cell death (Fig 3.1D). Cell cycle analysis of astrocytes grown in normal (5.5 mM) and high (25 mM) glucose media showed that high glucose (25 mM) increased cells in the G2/M phase suggesting cell cycle arrest (Fig 3.1E). The mRNA expression of cell cycle cyclins showed that cyclin D1, D2 and D3 expression were significantly decreased in astrocytes cultured in high glucose as compared to those cultured in normal glucose media for 3 days while there was no change in the mRNA levels of cyclin A1, A2, E1, and E2 (Fig 3.1F). This data suggests that high glucose (25 mM) inhibited astrocytes proliferation by inducing cell cycle arrest at G2/M phase.

High glucose activates astrocytes and alters astrocyte morphology

Shin and colleagues have shown that hyperglycemia changes the morphology of retinal astrocytes [264]. We observed that astrocytes cultured in normal glucose have much branching processes than astrocytes maintained in high glucose medium (Fig 3.2A). Astrogliosis is characterized by cellular hypertrophy and upregulation of intermediate filament proteins such as GFAP [17, 234]. Activated astrocytes are involved in wound healing processes after injury. Scratch (Wound healing) assay demonstrated that astrocytes cultured in high glucose migrated faster towards the scratch than astrocytes maintained in normal glucose although the wound healing was slow in both culture conditions (Fig 3.2B). We also examined activated astrocyte markers in primary astrocytes cultured in normal and high glucose using Western blot, and rtPCR. We found that astrocytes cultured in high glucose media expressed GFAP highly as compared to astrocytes in normal glucose media (Fig 3.2C and D). Increased calcium concentrations are associated with activated astrocytes. Using Fluo-4 AM we measured calcium waves in astrocytes cultured for 2 weeks in normal and high glucose media. We observed that primary astrocytes cultured in high glucose media have higher basal level of calcium and

stimulation by Adenosine, ATP and glutamate increased their calcium concentration as compared to astrocytes cultured in normal glucose media (Fig 3.2E).

High glucose enhanced astrocytes glycolysis and metabolic potential

The effects of media glucose concentration on astrocyte metabolism were evaluated using seahorse XFe96 analyzer. OCR and ECAR were monitored in astrocytes following the sequential injection of oligomycin, FCCP, and rotenone/antimycin A. No significant change was observed in baseline OCR. However we observed a significant increase in stressed OCR in astrocytes cultured in high glucose media for 14 days (Fig 3.3A). Baseline and stressed ECAR was higher in astrocytes cultured in high glucose media for 2 week as compared to those cultured in normal glucose media (Fig 3.3B). Similarly astrocytes cultured in high glucose condition have a higher metabolic potential (Fig 3.3C). Consistent with an increased ECAR is a significant increase in lactate production (Fig 3.3D) and hexokinase activity (Fig 3.3E) in astrocytes cultured in high glucose media suggesting increase glycolysis. Using PCR we determined the effects of high glucose on the expression levels of hexokinases and astrocytes lactate transporter Monocarboxylate transporter 1 (MCT1). We observed that astrocytes cultured in high glucose media have higher HEK1 and MCT1 mRNA level as compared to those cultured in normal glucose media (Fig 3.3F).

The ATP content of astrocytes cultured in both culture conditions was determined. We observed a significant increase in ATP content in astrocyte cultured in high glucose (25 mM) media. We speculated that the higher ATP levels observed in astrocytes cultured in high glucose media is likely attributed to glycolysis. To investigate this, we treated the cells with oligomycin (ATP synthase inhibitor) 2 hours prior to ATP assay to inhibit oxidative phosphorylation. With the inhibition of oxidative phosphorylation we observed 18% and 58% ATP content in astrocytes

cultured in normal glucose and high glucose media, respectively, indicating that glycolysis contributed to the increase ATP production in high glucose media cultured astrocytes (Fig 3.4A). Consistent with an increase ATP content of astrocyte cultured in high glucose media is a reduced NAD/NADH+ ratio (Fig 3.4B) confirming that the increase ATP produced is likely due to glycolysis. Furthermore as expected with the abundance of glucose in the media, we observed an increased glycogen content (Fig 3.4C) in astrocytes cultured in high glucose culture conditions.

AMPK is a conserved sensor and regulator of cell energy. It is activated by increased AMP/ATP or ADP/ATP levels. Activated AMPK stimulates glycolysis and fatty acid oxidation and inhibits glycogenesis and lipogenesis by phosphorylating and inhibiting glycogen synthase (GS) and Acetyl-CoA carboxylase (ACC) respectively [265]. After culturing astrocytes in high glucose media for 2 weeks, we observed an increased AMPK phosphorylation and a significant decrease in phosphorylation of mTOR in astrocytes cultured in high glucose media (Fig 3.5A). Culture of astrocytes in high glucose media also decreased the phosphorylation of GS and ACC (Fig 3.5B). These data indicated that primary astrocyte cultured in high glucose medium had an enhanced glycolysis and a biosynthetic phenotype.

High glucose induced ROS production and the expression of pro and anti-inflammatory cytokines.

In vivo studies have established that hyperglycemia induced ROS and inflammatory cytokines production participates in normal and abnormal processes [266, 267]. However it is not certain whether high glucose (25 mM) induces oxidative stress or ROS and cytokine productions in primary astrocytes. Oxidative stress occurs as a result of an imbalance between the production of ROS and antioxidant molecules by a biological system. When levels of ROS was measured in astrocytes cultured for 7 days in high glucose (25 mM) using flow cytometry

and microplate reader, we observed that high glucose increases ROS production (Fig 3.6A and B). Glutathione and super oxide dismutase (SOD) are produced by astrocytes and shuttle to neurons to maintain cellular redox status and protect against oxidative stress [28, 30, 31]. We investigated using assay kits GSH levels and SOD activity in astrocytes cultured in different medium condition. We found a decrease in GSH levels in astrocytes cultured in high glucose media for 7 days (Fig 3.6C) but no significant change in SOD activity in astrocytes cultured in high glucose medium as compared to those seeded in normal glucose media (Fig 3.6D). We also investigated if high glucose also impact mitochondrial membrane potential. We observed no difference in the mitochondrial membrane potential of astrocytes cultured in normal and high glucose medium (Fig 3.6E).

Real time PCR was used to evaluate the expression of inflammatory cytokines in astrocytes cultured in normal and high glucose medium. We observed that high glucose stimulation increased the mRNA expression levels of pro inflammatory cytokines; IL-1β (Fig 3.6 F), TNF-α (Fig 3.6G), and anti-inflammatory cytokines IL-4 (Fig 3.6H) and IL-10(Fig 3.6I). However there was no significant change in the mRNA expression levels of IL-6 (Fig 3.6J) and LCN-2 (Fig 3.6K) in astrocytes stimulated by high glucose as compared by astrocytes in normal glucose media.

High glucose alters mRNA expression of astrocyte specific proteins.

The mRNA expression of genes commonly associated with astrocyte functions was assessed using quantitative PCR. We observed no significant difference in CXN-43 (Fig 3.7A) and GS (Fig 3.7B) mRNA levels between astrocytes cultured in normal glucose and high glucose media. Lower mRNA levels of GLT-1 (Fig 3.7C), GLAST (Fig 3.7D), GLUT-1 (Fig 3.7E), CXN-30 (Fig 3.7F), Kir4.1 (Fig 3.7G) and AQP4 (Fig 3.7H) were observed in astrocytes

cultured in high glucose media for 14 days as compared to those maintained in normal glucose media. Consistent with a decrease mRNA expression of glutamate transporters is a significant 25% decrease in glutamate clearance of astrocytes cultured in high glucose media measured using glutamate assay kit (Fig 3.7I). Higher glucose uptake was also observed in primary astrocytes cultured in high glucose media as compared to normal glucose astrocytes (Fig 3.7J).

3.6. FIGURES AND LEGENDS

Figure 3.1

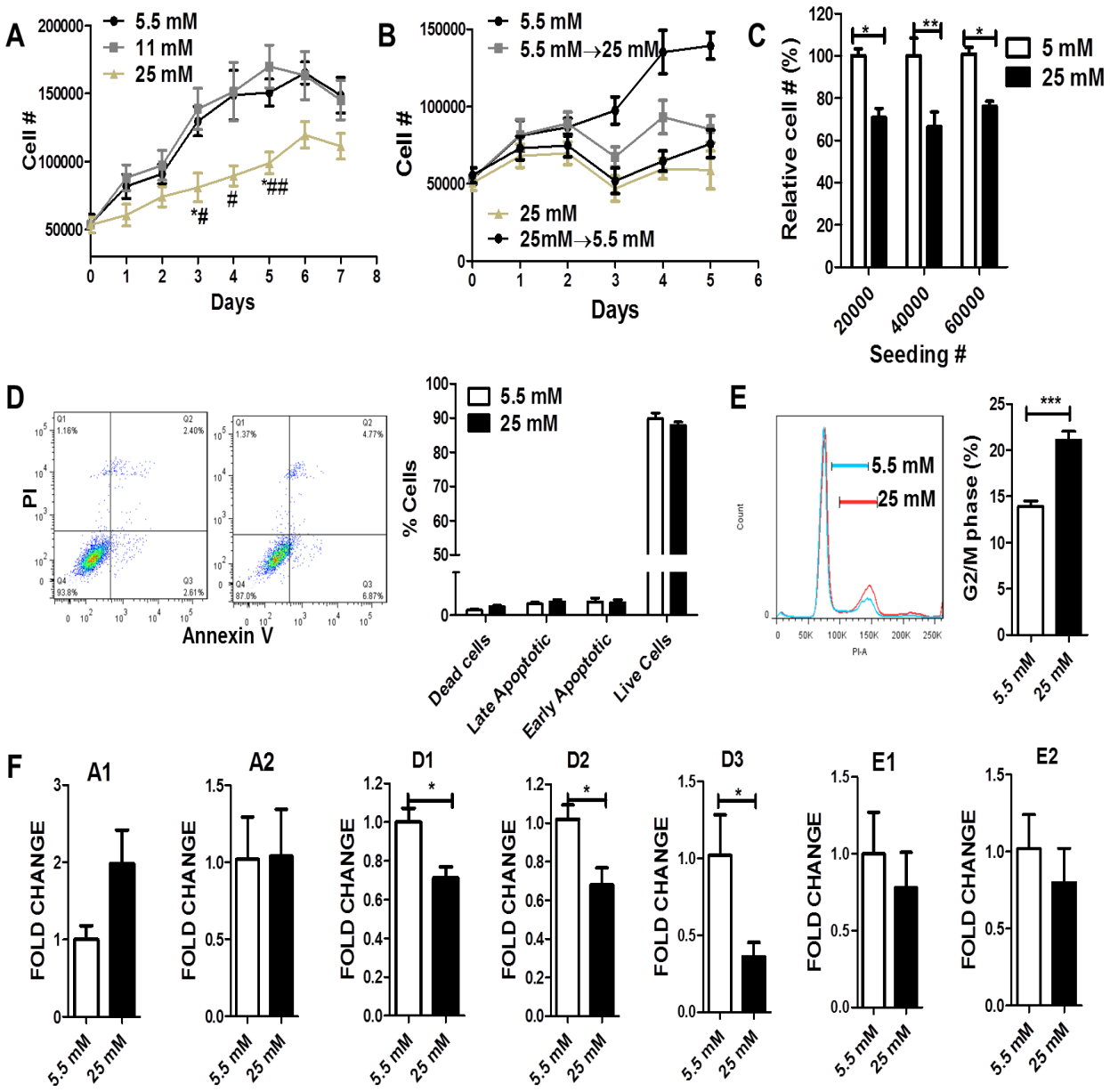
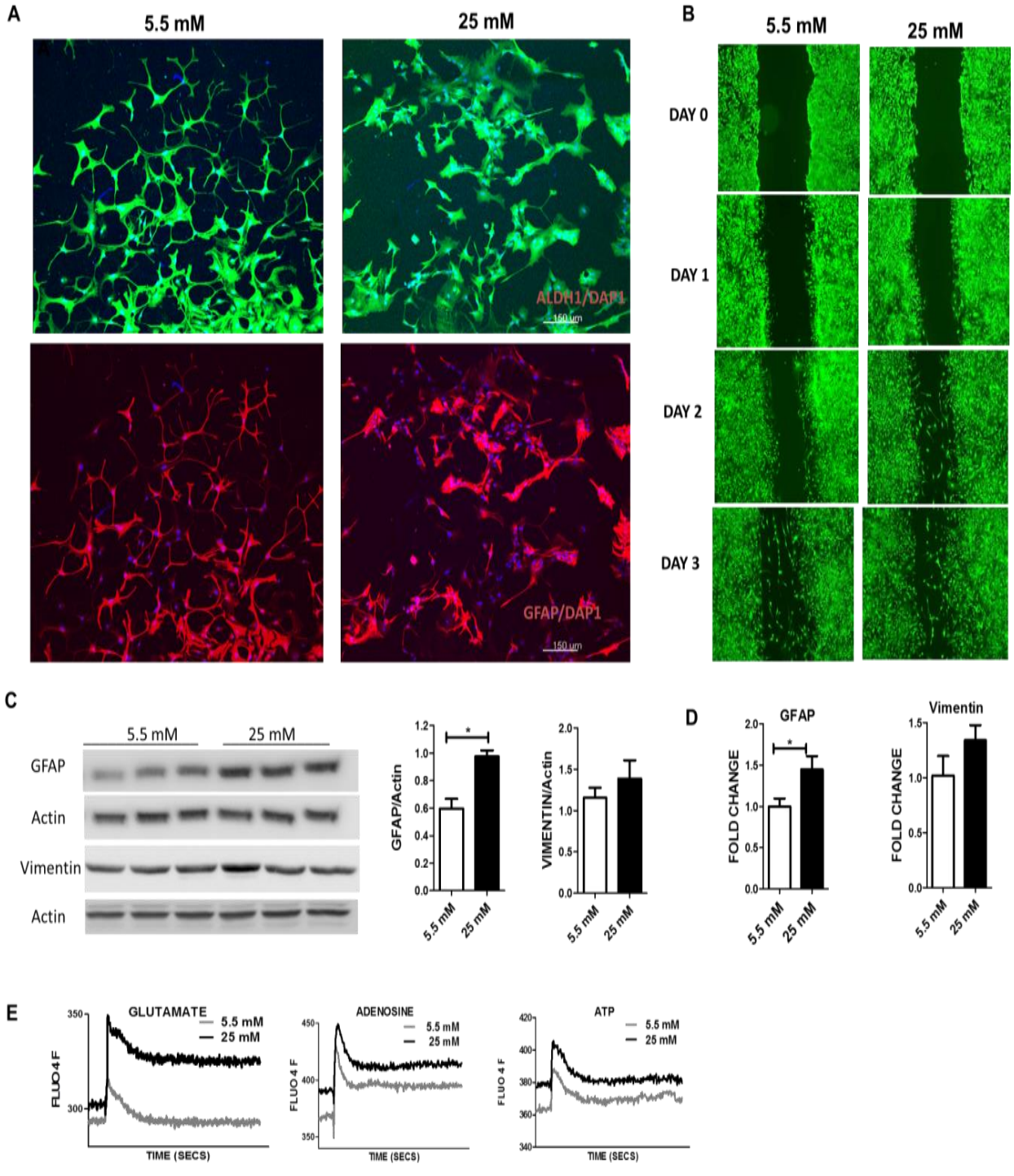


Figure 3.1. High glucose slows astrocytes growth. (A) Growth curve assay of primary astrocytes cultured in normal glucose (5.5 mM) and high glucose (11 or 25 mM) for 2 weeks. 40000 cells were seeded in 12 well plate and cells counted daily for 7 days (*p< 0.05, 5.5 mM vs 25 mM) # p< 0.05, ## p< 0.01, 11 mM vs 25 mM n=6). (B) Growth curve of astrocytes with glucose condition switched on day 2 (n=6) (C) Calcien AM viability assay of different number of astrocytes seeded in 12 well plate and cultured in normal and high glucose for 3 days (*p< 0.05, **p<0.01, ***p<0.001 n= 6). (D) Flow cytometry and quantitatively analysis of Annexin V and PI stained primary astrocytes at 4 days after culture in normal glucose (5.5 Mm) and high glucose (25 mM) media (E) Cell cycle analysis of primary astrocytes cultured in normal glucose (5.5 Mm) and high glucose (25 mM) for 3 days. (F) Real time rtPCR analysis of cyclin expression in astrocytes cultured in normal glucose (5.5 Mm) and high glucose (25 mM) for 3 days (*p< 0.05, **p<0.01, ***p<0.001 n= 6

Figure 3.2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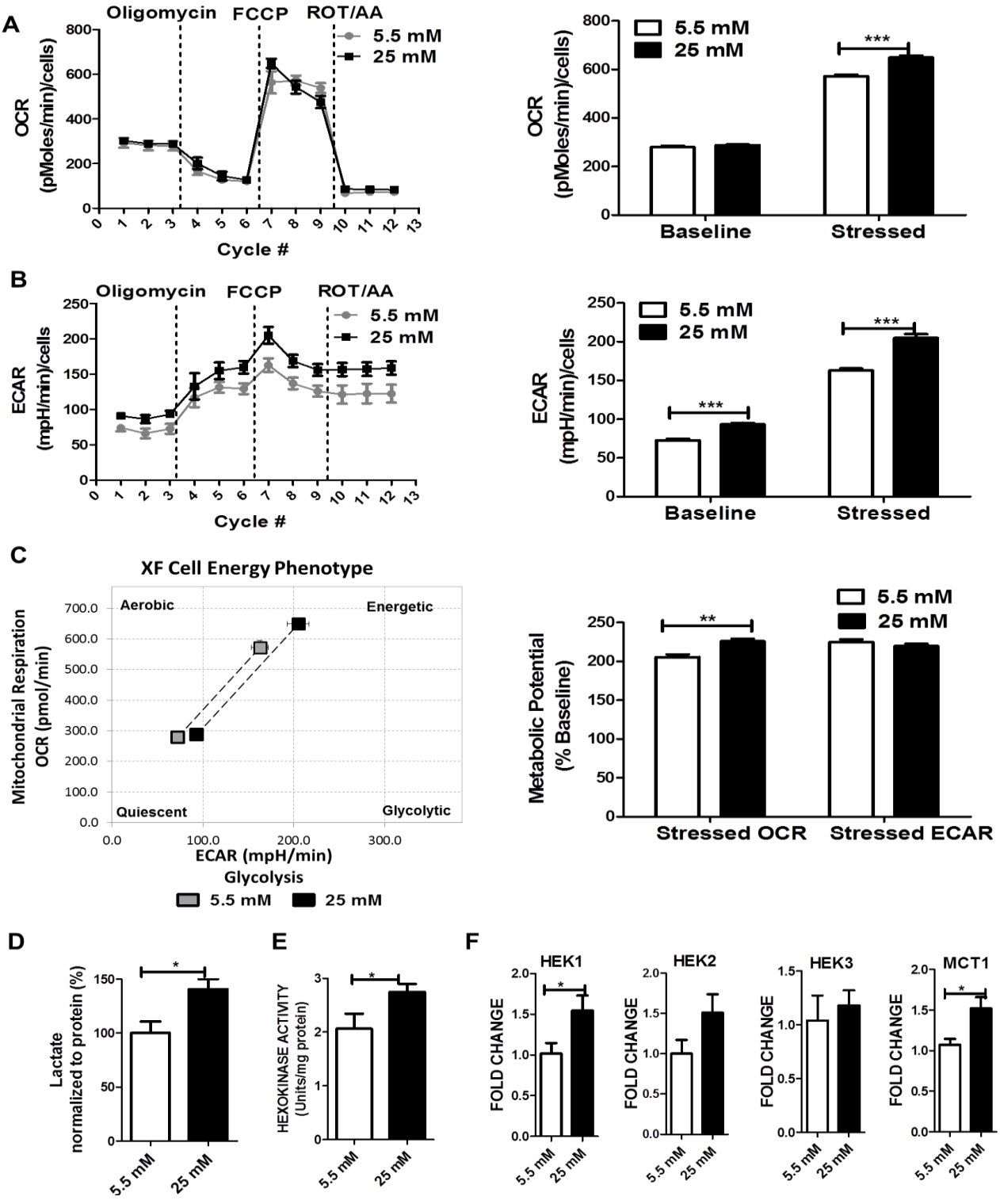
25 mM
ALDH1/DAPI
GFAP/DAPI
150 um
B
5.5 mM
25 mM
DAY 0
DAY 1
DAY 2
DAY 3
C
5.5 mM
25 mM
GFAP
Actin
Vimentin
Actin
GFAP/Actin
VIMENTIN/Actin
D
GFAP
Vimentin
FOLD CHANGE
E
GLUTAMATE
ADENOSINE
ATP
5.5 mM
25 mM
FLUO 4 F
TIME (SECS)

Figure 3.2. High glucose activates and alters astrocyte morphology (A). Representative florescent microscopic images of ALDH1L1 and GFAP immunostaining in primary astrocytes cultured for 2 weeks in normal glucose (5.5 mM) and high glucose (25 mM) ABM-FGF2-EGF culture condition. (B) Astrocytes migration monitored from day 0 to 3 after cells cultured in normal (5.5 mM) and high glucose (25 mM) ABM-FGF2-EGF culture condition for 2 weeks were seeded for scratch assay. (C) Representative and quantitative Western blot analysis of GFAP, vimentin expression after 14 days of culture in normal glucose (5.5 mM) and high glucose (25 mM) serum free medium (D) Real-time PCR analysis of GFAP and vimentin expression in astrocytes cultured in normal glucose (5.5 mM) and high glucose (25 mM) serum free medium for 2 weeks (*$p < 0.05$ vs. MD 10% FBS n= 6-8). (E) Astrocytes calcium response to different stimuli monitored by flex station after astrocytes was stained with calcium florescent dye Fluo 4 AM.

Figure 3.3


A
Oligomycin
FCCP
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5.5 mM
25 mM
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B
ECAR (mpH/min)/cells
C
XF Cell Energy Phenotype
Aerobic
Energetic
Quiescent
Glycolytic
Mitochondrial Respiration OCR (pmol/min)
ECAR (mpH/min)
Glycolysis
Baseline
Stressed
Stressed OCR
Stressed ECAR
Metabolic Potential (% Baseline)
D
Lactate normalized to protein (%)
E
HEXOKINASE ACTIVITY (Units/mg protein)
F
HEK1
HEK2
HEK3
MCT1
FOLD CHANGE

Figure 3.3. High glucose increases glycolysis and astrocytes metabolic potential (A) Seahorse extracellular flux analysis of Oxygen consumption rate (OCR) of astrocytes cultured in normal and high glucose for 14 days. (B) ECAR recordings and quantitative analysis of baseline and after addition of oligomycin, FCCP and Rotenone/Antimycin. (C) Cell metabolic potential and phenotype of astrocytes cultured in normal and high glucose media. (D) Lactate production of astrocytes cultured in normal and high glucose media (E) quantitative analysis of hexokinase activity in astrocytes (F) Real-time rtPCR analysis of hexokinase and MCT1 expression of astrocytes cultured in normal and high glucose media for 2 week. (* $p< 0.05$, ** $p< 0.01$ vs high glucose, $n = 6\text{-}10$).

Figure 3.4

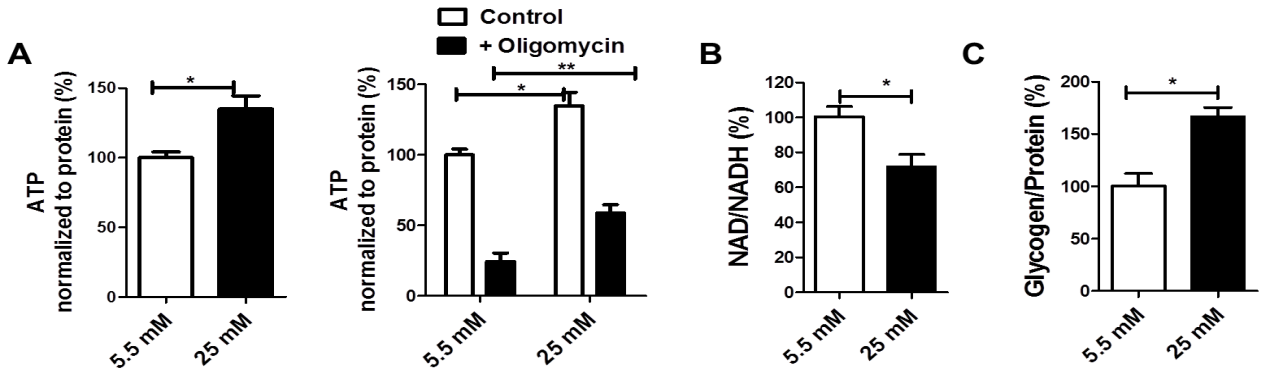

Figure 3.4. High glucose increased glucose uptake, ATP and glycogen content. (A) Total ATP content of primary astrocytes cultured in normal (5.5 mM) and high (25 mM) glucose (B) NAD/NADH ratio of primary astrocytes cultured in normal (5.5 mM) and high (25 mM) glucose (C) Glycogen assay of primary astrocytes cultured in normal (5.5 mM) and high (25 mM) glucose (*p< 0.05, **p<0.01, vs. high glucose (25 mM) n= 6-8).

Figure 3.5

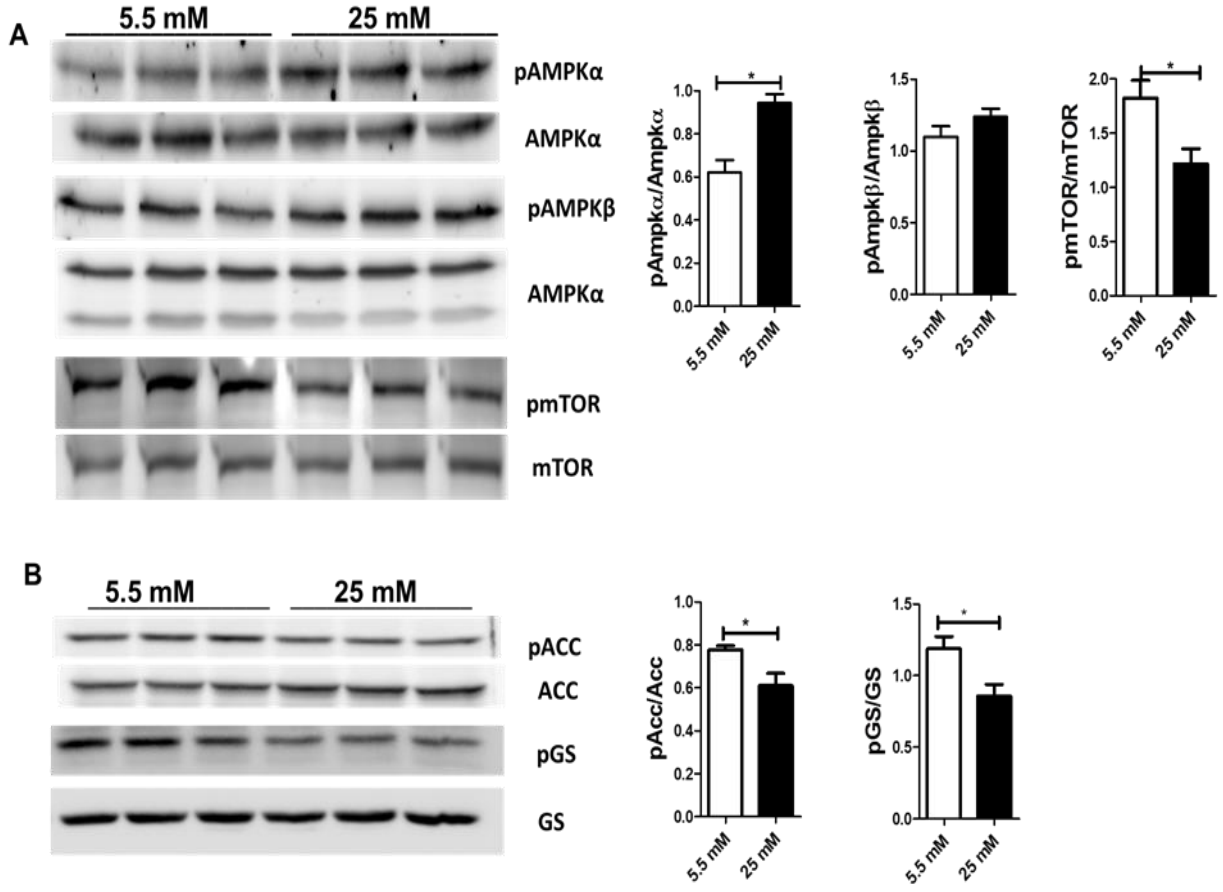

Figure 3.5. High glucose activate AMPK pathway (A) Representative western blots and quantitative analysis of total and phosphorylated AMPKα, pAMPKα, AMPKβ, pAMPKβ, and mTOR, pmTOR after 2 weeks of astrocyte culture in normal glucose (5.5 mM) or high glucose (25 mM) (n=6 *p< 0.05). (B) Representative Western blots and quantitative analysis of total and phosphorylated ACC, pACC, GS, pGS after 2 weeks of astrocyte culture in normal glucose (5.5 mM) or high glucose (25 mM). (*p< 0.05 n=6).

Figure 3.6

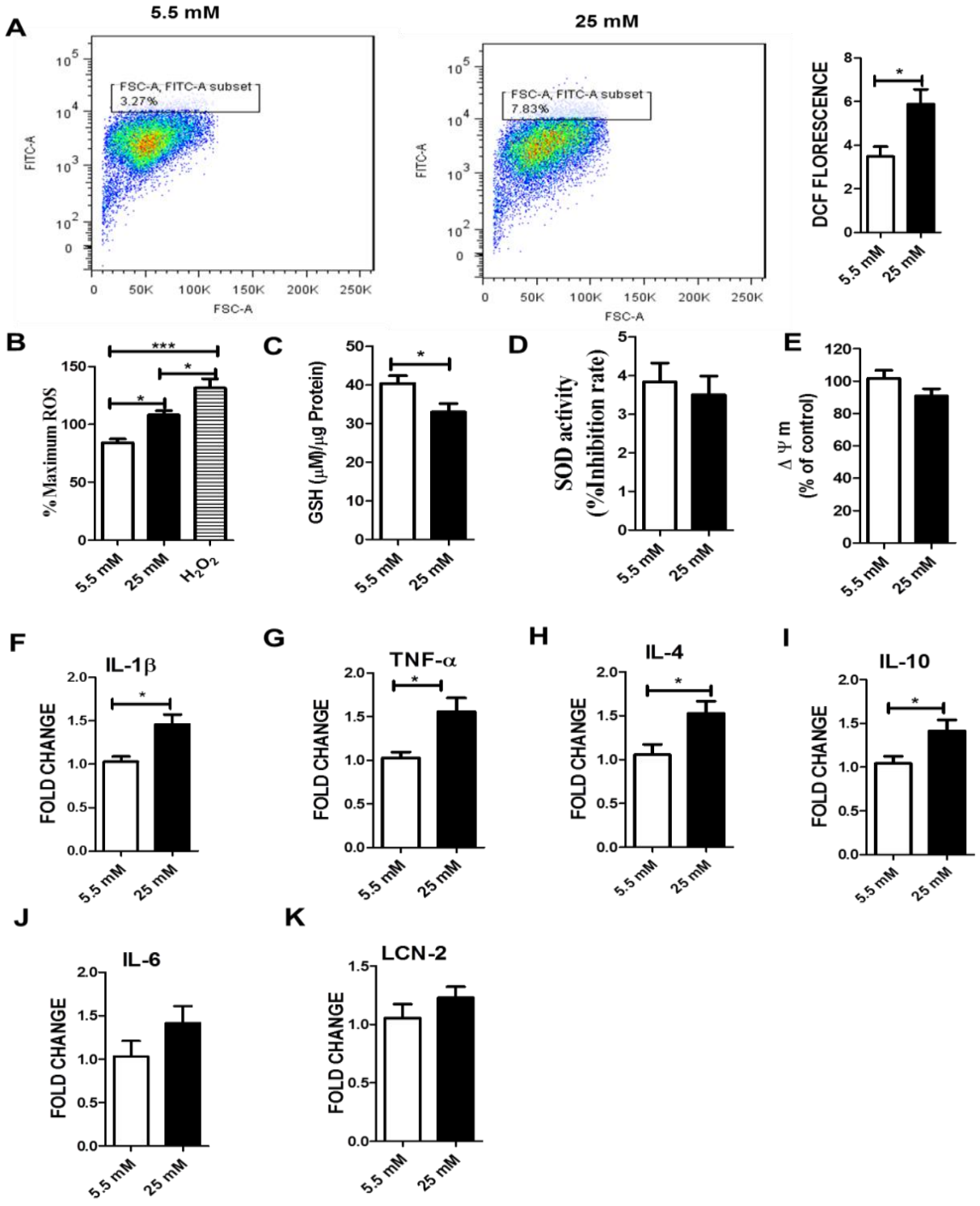
A
5.5 mM
25 mM
FSC-A, FITC-A subset
3.27%
FSC-A, FITC-A subset
7.83%
FITC-A
FSC-A
DCF FLORESCENCE
B
% Maximum ROS
C
GSH (μM)/μg Protein)
D
SOD activity (%Inhibition rate)
E
Δ Ψ m (% of control)
F
IL-1β
G
TNF-α
H
IL-4
I
IL-10
J
IL-6
K
LCN-2
FOLD CHANGE
5.5 mM
25 mM
H2O2

Figure 3.6. High glucose induces ROS production and expression of inflammatory cytokines. (A) DCF flow cytometry assay depicts an increase in ROS production in astrocytes cultured in high glucose (25 mM) media for 7 days, *p< 0.05 n= 4. (B) DCF plate reader assay depicts an increase ROS production in astrocytes cultured in high glucose (25 mM) media for 7 days. Assay kits were used to evaluate the (C) glutathione (E) SOD activity of astrocytes cultured in high glucose (25 mM) media for 7 days. Tetra methyl rhodamine, Ethyl Ester (TMRE) plate reader assay of astrocytes cultured in normal and high glucose media for 7 days. Real-time PCR analysis of (F) IL-1β (G) TNF-α (H) IL-4 (I) IL-10 (J) IL-6 (K) LCN-2 expression in astrocytes cultured in normal glucose (5.5 Mm) and high glucose (25 mM). *p< 0.05, **p<0.01, ***p<0.001 n= 6-8

Figure 3.7

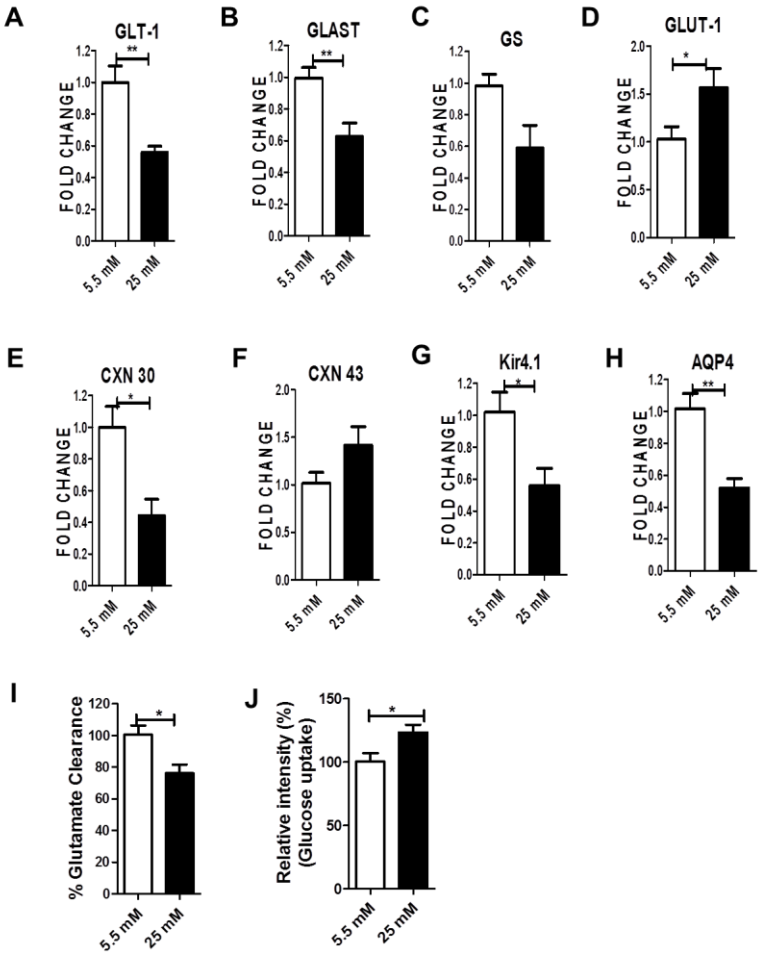

Figure 3.7. Comparison of mRNA expression profile of astrocyte specific factors in astrocytes cultured in normal and high glucose. Real time PCR analysis of astrocytes associated factors (A) CXN-43 (B) GS (C) GLT-1 (D) GLAST (E) GLUT-1 (F) CXN 30 (G) Kir4.1 (H) AQP4 (*p< 0.05, **p<0.01 vs. high glucose (25 mM) n= 6-8) (I) High glucose effect on glutamate uptake (J) Effects of hyperglycemia on glucose uptake

3.7. DISCUSSION AND CONCLUSION

Glucose is the primary energy source in the CNS. It is transported into the brain mainly through insulin-independent glucose transporter proteins GLUT-1 and GLUT-3 [268]. Astrocytes have unique morphology and phenotypes which enables them to sense and integrate signals such as brain glucose levels and trigger adaptive responses [269]. There is increasing evidence that changes in brain microenvironment alter astrocyte functions and phenotype. For instance the expression and activity of glutamate transporters is altered by changes in brain microenvironment [270, 271]. Similarly activation of somatosensory cortex alters astrocytic glucose uptake [272]. Furthermore in certain neurodegenerative disorders characterized by protein accumulations which changes brain microenvironment, astrocytic functions and phenotype is altered [28, 270]. Glucose is required in the culture of astrocytes and other neural cells. Growth of cells in glucose conditions above 6.6 mM is a pathophysiological condition not *mimicking in vivo* astrocyte microenvironment. In this current study we found that glucose condition in culture media alters astrocyte phenotype. We report that hyperglycemia decreased astrocyte proliferation irreversibly likely through cell cycle arrest.

The activities of the major glucose transporters in the CNS which are expressed by astrocytes are independent of insulin action but rather, glucose uptake depends on the extracellular glucose concentration [256, 273, 274]. We observed that increasing glucose concentration in the culture medium led to an increase glucose uptake. High glucose in the media also enhanced glycolysis and increased lactate production, ATP and glycogen content of astrocytes. This increase in glycolysis could be as a result of the increase glucose uptake stimulating increase expression and activity of hexokinase, the enzyme catalyzing the rate limiting step of glycolysis. Astrocytes shuttle lactate to neurons through the astrocytic lactate

transporter MCT-1. While lactate can be use as energy source for neurons, excessive lactate has been reported in diabetic patients and cause neuronal damage in certain neurological pathology such as ischemic stroke [275]. We expect that high glucose induced metabolic phenotype might affect glial to glial communication as well as astrocyte neuronal coupling leading to neuronal dysfunction. Hence studying astrocyte-neuronal coupling in high glucose media will affect experimental outcome. AMPK is a conserve sensor and regulator of cell energy. We observed following astrocytes exposure to high glucose an enhanced AMPKα activation evident by the increasing AMPKα phosphorylation. Consistently inhibitory action of high glucose on downstream mTOR was observed as seen by the decrease phosphorylation of mTOR. Since activated AMPK contribute to inhibition of cell proliferation [276], we speculated that AMPK activation by high glucose may contribute to its inhibitive action on astrocyte proliferation . The increasing AMPK activation was not correlated with decrease phosphorylation of ACC and GS. We speculated that increase glycogen content and decrease phosphorylation or inhibition of GS and ACC despite activated AMPK in astrocytes might be due to the high glucose condition in the culture providing excessive glucose for biosynthesis and increasing the metabolic potential of the cells.

Oxidative stress can be induced by hyperglycemia. High glucose can stimulate ROS generation through the formation of glycated end products, impairments of mitochondrial functions and cellular antioxidant capacity [256, 277, 278]. We observed that high glucose increased ROS production in cultured astrocytes. This increase ROS production could be due to decrease antioxidant levels such as the decrease GSH levels observed in astrocytes maintained in high glucose media. The decrease in GSH observed may be linked to the consumption of NADPH by pathways activated in hyperglycemic conditions thus affecting the regeneration of

oxidized glutathione (GSSG) to GSH [279, 280]. Downstream consequences of ROS overproduction include oxidative damage to lipid molecules and cell cycle arrest [281]. Thus the decrease cell proliferation may also be due to ROS mediated cell cycle arrest.

It is known that depleted GSH and oxidative stress induces neuroinflammation and are associated with the pathophysiology of many neurodegenerative disorders such as Parkinson's disease and diabetic neuropathy [282, 283]. Most of these neurodegenerative disorders are characterized by astrogliosis, evident by increase GFAP expression, change in cell morphology and astrocytic protein expression. In uncontrolled diabetes astrogliosis may exacerbate diabetes associated neurodegeneration [284] .We observed an increase GFAP expression and cells with little or no branching processes in astrocytes cultured in high glucose medium. Astrocytes activation can be triggered by oxidative stress and activated astrocytes release a variety of cytokines that modulate the activities of other glia cells. It has been suggested that astrocytes regulate microglial inflammatory responses by releasing cytokines that activate or suppress microglial activation [285, 286]. In our study we observed that hyperglycemia increases the expression of both pro inflammatory and anti-inflammatory cytokine. This suggests that activated astrocytes in diabetes can have very diverse or even opposing roles which may be implicated in diabetic complications of the CNS. Thus high glucose culture condition mimics *in vivo* situations in disease brain.

In order to prevent excitotoxicity astrocytes uptake and regulate glutamate concentration at the synaptic cleft. Activated astrocytes have been shown to have decrease GLT-1 expression as seen in CNS pathology characterized by neurodegeneration [52, 242, 243]. In our study We observed a decrease mRNA expression of astrocyte glutamate transporters GLT-1 and GLAST in astrocytes cultured in high glucose condition. Consistently we also observed decrease glutamate

uptake in astrocytes maintained in high glucose media. It is possible that the decrease glutamate transport observed could be due to the increase release of cytokines observed in astrocytes maintained in high glucose media. Since it's known that TNF-alpha can potentiate glutaminergic excitotoxicity by decreasing glutamate transport in astrocytes [287]. Glutamate taken up by astrocytes can also be used as a precursor to synthesis other neurotransmitter or GSH the major antioxidant defense of the CNS [288]. We speculate a link between astrocyte glutamate uptake and antioxidant capacity in diabetes and other neurodegenerative disorders. Consistent with other studies we also observed a decrease mRNA levels of CNX30 and Kir4.1 channels in astrocytes maintained in high glucose media [289, 290]. The decrease expression of this important astrocytic channel could imply a decrease potassium uptake and neural cell communication leading to loss of brain homeostasis.

Our data shows that culturing astrocytes in high glucose media triggers cellular alteration such as astrogliosis, morphological change, and production of ROS and cytokines. High glucose culture condition also affects important astroglial functions such as astrocyte metabolism, expression and activity of astrocytic functional proteins. Altered astroglial functions might be involved in diabetes complications of the CNS. High glucose also activates astrocytes and alters their morphology. Taken together, these data stress the importance of considering glucose concentration in astrocyte culture medium as part of the experimental design so that results obtained could mimic in vivo astrocytes under physiological condition.

CHAPTER 4

Cholesterol Sulfate Alters Astrocyte Metabolism and Prevents Glutamate-Induced cell death.

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4.1 Background

This chapter illustrates an important role of primary cultures in studying the effects of compounds on neural cells. Studying the effects of cholesterol sulfate (CS), an important known sterol sulfate in tissues on astrocyte metabolism and its neuroprotective properties, we employed the use of charcoal stripped FBS in our culture medium since FBS contains steroids which may potentiate or antagonize the effects of CS. It was evident also from our study that the presence of FBS and culture condition affected our experimental outcome as we observed the effects of CS in ABM-FGF2-EGF cultured astrocytes at lower doses than in either 10% FBS or charcoal stripped medium.

This chapter is an illustration of the impact of culture condition on astrocyte biology and experimentation and the critical considerations that have to be made when testing compounds *in vitro.*

4.2. ABSTRACT

Cholesterol sulfate (CS) is one of the most important known sterol sulfates in human plasma and is present as a normal constituent in a variety of human tissues. In both the brain and periphery, CS serves as a substrate for the synthesis of sulfonated adrenal steroids such as pregnenolone sulfate and Dehydroepiandrosterone (DHEA) sulfate and as a constituent of many biological membranes including red blood cells where it functions as a stabilizing agent. It also acts as an endogenous regulator of cholesterol synthesis. However, the role of CS in neurological insult and brain metabolism is unknown. Our goal in this study is to investigate the neuroprotective action of CS as well as its effect on brain energy metabolism. Primary astrocytes were prepared from the cortex of postnatal day 0-2 C57BL/6 pups and seeded in Dulbecco's modified eagle medium (DMEM) with 10% charcoal-stripped FBS under normal glucose (5.5 mM). HT-22 cells were maintained in high glucose (25 mM) DMEM supplemented with charcoal-stripped FBS. The neuroprotective effect of CS and its role on cell metabolism were determined in primary astrocyte and HT-22 cells using Calcein AM and MTT cell viability assay, flow cytometry, Seahorse extracellular flux analysis, and metabolism assay kits.

We found that CS attenuates against glutamate and rotenone induced cell death in HT-22 cells, decrease glutamate induced mitochondria membrane potential collapse, and ROS production. Additionally CS activates the Akt/Bcl_2 pathway. We also observed that CS impacts astrocyte metabolism by increasing mitochondrial phosphorylation, ATP, and glycogen contents. Our study demonstrated that CS modulates brain energy metabolism and its neuroprotective effects might be due to the activation of Akt signaling or its ability to decrease ROS production.

4.3. INTRODUCTION

The central nervous system (CNS) is particularly vulnerable to the damaging effects of reactive oxygen species (ROS) due to its high rate of oxidative metabolism, auto-oxidation of neurotransmitters and other substrates, and the modest antioxidant potential of CNS cells [291, 292]. Oxidative stress occurs as a result of an imbalance between the production of ROS and antioxidant defenses by a biological system and has been implicated in the initiation and progression of various neuropathological conditions such as Parkinson's disease, Alzheimer's disease, and aging [292-294]. It is therefore not surprising that therapeutic strategies involving antioxidant and ROS detoxifying enzymes have become attractive in the treatment of various neuropathologies. Although *in vivo* and *in vitro* studies have demonstrated the neuroprotective effects of many antioxidant compounds, most of these compounds have failed to yield major new discoveries in the treatment and prevention of neurodegenerative disorders in clinical settings [295, 296]. It is, therefore, necessary to explore other compounds as neuroprotective agents or alternative strategies for detoxifying reactive intermediates. Nature has been a source of many important drugs or structurally diverse compounds in use today. Therefore, it is reasonable to screen natural nutritional products for their therapeutic or neuroprotective potential.

Cholesterol sulfate (CS), a naturally occurring endogenous substance, is an enzymatic product of sulfoconjugation of cholesterol by sulfotransferase (SULT2B1b). CS is a major known sterol sulfate in human plasma with its concentration ranging from 135 to 260 μg/ml [297-299]. Although cholesterol concentration in mammalian membranes varies over a wide range, it is always found in exceedingly greater quantity than CS [300]. CS is distributed in seminal fluid, urine, adrenal glands, liver, hair, nails and uterine endometrium [298, 301-303]. In the brain, CS has been reported to be significantly high in the cerebellum compared to other

regions, although the specific cerebellum associated role remains undefined [304]. CS is well documented in erythrocytes as well as stratum corneum of the epidermis where abnormally high levels lead to ichthyosis [298, 305, 306]. In membranes rich with CS the ratio of cholesterol to CS is thought to be a critical regulator of membrane function [300].

CS plays an important role in cell membrane stabilization, cell adhesion, and protection against osmotic induced cell lysis [298]. Furthermore, it was reported that CS plays a role in the immune response serving as a regulator of T-cell signaling as well as an inhibitor of hepatitis C virus NS3 helicase [307, 308]. Additionally, it has been demonstrated that CS is an inhibitor of gluconeogenesis in hepatocytes and is an agonist on the retinoic acid orphan receptor α (RORα) [297, 309]. In both, the brain and periphery, CS serves as a substrate for the synthesis of sulfonated adrenal steroids such as pregnenolone sulfate and Dehydroepiandrosterone (DHEA) sulfate [310, 311]. CS regulates the synthesis of cholesterol which is vital in synaptogenesis [312, 313]. However, the role of CS in neurological insult and brain metabolism is largely unknown.

Astrocytes, the most abundant glial cells in the CNS, are critical in maintaining CNS homeostasis. These cells are involved in clearance and metabolism of neurotransmitters, regulation of extracellular ions, blood-brain barrier formation, synaptic transmission regulation, and controlling local blood flow through secretion of vasodilators and constrictors[28, 314]. Due to their unique structural features, astrocytes play an active role in brain energy production, storage, and utilization to meet the metabolic demands of neuron via astrocyte-neuron metabolic coupling [30]. It has been widely accepted that energy metabolism plays a fundamental role in the pathogenesis of many neurodegenerative diseases and energy metabolism could be a potential target in the prevention and treatment of neuropathologies [315, 316]. Similarly, impairments in astrocyte metabolism have been linked to neurodegenerative processes [317].

The goal of this study was to test the hypothesis that CS alters brain bioenergetics and offers some neuroprotection. This is based on several studies which demonstrated the role of CS as a substrate for the synthesis of neurosteroids and that some neurosteroids display neuroprotective properties [318].

4.4. MATERIALS AND EXPERIMENTAL PROCEDURE

Materials and Reagents

5'AMP-activated protein kinase (AMPK) (monoclonal cell signaling), Glycogen synthase (GS) and Acetyl CoA Carboxylase (ACC) (Monoclonal Cell signaling), mammalian target of rapamycin (mTOR) (monoclonal cell signaling), Actin and GAPDH (monoclonal Santa Cruz) as well as secondary antibodies from Jackson laboratory were used for western blot. Cell culture dishes, 24 well plates, 12 well plates, and 40 μM cell strainers were all purchased from Greiner bio-one (USA). In addition, 6 and 96 well plates were purchased from Genesee scientific (USA). L-Glutamic acids, Trypsin-EDTA, trypan blue and poly-L-Lysine solution were purchased from Sigma Aldrich (USA). Penicillin-streptomycin, sodium pyruvate, TrypLE, and glutmax were purchased from Gibco/life technologies (USA). Cholesterol sulfate was purchased from Cayman chemical. Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, USA) high and low glucose were used to culture the cells for the experiments.

Primary astrocyte culture

The institutional animal care and use committee of the University of North Texas Health Science Center UNTHSC approved all procedures performed during the isolation or preparation of primary astrocytes from C57BL6 mouse (Jackson's lab). As previously described [210] primary astrocytes were prepared from 1-day old C57BL6 pups. Briefly, hypothermia was used to anesthetize day old C57BL6 mouse pups followed by decapitation with a sharp surgical

scissor. The cerebral cortices were dissected and meninges removed under aseptic conditions. TrypLE was used to digest the cortices at 37 ^{o}C for 15 minutes with shaking after every 5mins. A single cell suspension was prepared by pipetting through 3 different sized pipettes after which the cell suspension was strained through 40 μM size cell strainer. The cells were counted and plated in a culture dish in Dulbecco's Modified Eagle's medium (DMEM with 5.5 mM glucose, 4 mM L-glutamine, 1mM sodium pyruvate, thermos scientific) containing charcoal-stripped 10 % fetal bovine serum and streptomycin (100 units/ml) - penicillin (100 μg/ml) or ABM-FGF2-EGF and allowed to attach for 15 minutes in a humidified incubator at 37 ^{o}C with 5% CO_2 after which media was changed. When the cells became 80% confluent; the plates were constantly shaken for 24 hours in a CO_2 incubator at 37^{o}C to eliminate microglia and other cell debris.

HT-22 Cell Culture

A murine hippocampal cell line HT-22 cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with charcoal-stripped 10% fetal bovine serum, 1 mM pyruvate and 4 mM glutamine in addition to streptomycin (100 units/ml) - penicillin (100 μg/ml) in tissue culture dishes. The cells were incubated at 37 ^{o}C under 5% CO_2. The medium was changed two times weekly, and cells split at confluence. The cells were used between passages 10 to 25.

Neuroprotective analysis

Cell viability assay

The ability of cholesterol and CS to protect cells against glutamate, rotenone and glucose oxidase induced cell death was assessed using different methods as previously described [319]. Briefly, HT-22 cells were washed with PBS and trypsinized to collect and count the cells. Three thousand cells/well were seeded in 96 well plates in 100 μl of DMEM (25 mM glucose, 1 mM

pyruvate and 10% charcoal strip FBS) and allowed to attach for 6 hours at 37 ^{o}C with 5% CO_2. The cells were either pretreated with varying concentrations of CS for 12, 24 and 36 hours or the cells were co-treated with cholesterol sulfate, and neurotoxic substances. Cell death was induced with the following treatment: 20 mM glutamate for 12 hours, 5 μM rotenone treatments for 24 hours, and 2 U glucose oxidase treatments for 2 hours. Hours after treating the cells and incubating them at 37^{o}C with 5% CO_2, MTT assay or Calcein AM viability assay was done using a microplate reader. For the MTT assay, plates were removed from the incubator, and 20 μl of 5 mg/ml MTT in PBS was added per well and returned into the incubator for 2 hours after gentle agitation. After 2 hours, the media was removed and 100 μl of DMSO was added to each well. Absorbance was measured at 560 nm with a reference of 670 nm on a Tecan Infinite F200 plate reader. For the Calcein AM assay the media was replaced with 1 μM solution of Calcein AM in PBS. The cells were then incubated at 37 ^{o}C for 5 minutes, and fluorescence was measured using a Tecan plate reader (excitation 485 emission 530). For fluorescent microscopy, following cell co-treatment with compounds and 20 mM glutamate for 12 hours, cells were incubated at 37^{o}C in PBS containing 1 μg/ml Calcein AM and 5 μg/ml PI (BD Bioscience) for 15 minutes after which images were taken using Zeiss Observer Z1 microscope.

Apoptosis Analysis.

70,000 HT-22 cells/well were seeded in a 6 well plate and incubated overnight in 1 ml of DMEM (25 mM glucose, 1 mM pyruvate and 10% FBS) at 37^{o}C with 5% CO_2. Varying concentrations of cholesterol sulfate and 20 mM glutamate were added to each well and incubated for 12 hours at 37^{o}C with 5% CO_2. After 12 hours, both floating and attached cells were collected and cell apoptosis was analyzed using flow cytometry (BD LSR II, San Jose, CA,

USA) following annexin–V and propidium iodide staining (BD Bioscience) according to the manufacturer's protocol.

Reactive oxygen species (ROS) analysis

ROS fluorescent indicator H2DCFDA (Anaspec) was used to examine changes in reactive oxygen species using a fluorescent microplate reader and flow cytometry as previously described [319]. HT-22 cells were seeded at a density of 70,000 cells/well in a 6 well plate to attach overnight. Cells were treated with different concentrations of cholesterol sulfate, and then 20 mM glutamate and incubated for 12 hours at 37^{o}C with 5% $CO_{2.}$ 10 μM H2DCFDA in PBS was added to cells for 15 minutes, after which PBS containing H2DCFDA was replaced with fresh PBS, and the DCF fluorescence was determined with a Beckman Coulter FC-500. For the fluorescent microplate reader, HT-22 cells were seeded at a density of 3000 cells/well and incubated overnight in a 96 well plate containing 100 μl of DMEM (25 mM glucose, 1 mM pyruvate and 10% FBS) at 37 ^{o}C with 5% CO_2. Varying concentrations of CS and 20 mM glutamate were added to each well and incubated for 12 hours at 37^{o}C with 5% $CO_{2.}$ The cells were then incubated in PBS containing 10 μM H2DCFDA for 45 minutes at 37 ^{o}C and KRH for an additional 15 minutes. DCF florescence was measured using a Tecan Infinite F200 plate reader (excitation 485, emission 530) after washing the cells with PBS.

Mitochondrial membrane potential analysis

HT-22 cells were seeded in a 12 well plate at a density of 20,000 cells/well in DMEM (high glucose, 1 mM pyruvate and 10% FBS) overnight to attach. Cells were treated with different concentrations of CS and 20 mM glutamate after which cells were incubated for 8 hours at 37^{o}C with 5% $CO_{2.}$ Media was then replaced with PBS containing 1 μM Tetramethylrhodamine, Ethyl Ester (TMRE) working solution (Abcam, USA) for 20 min at 37^{o}C.

Florescence intensity was measured using a Tecan Infinite F200 plate reader (Excitation 594, emission 575).

Super oxide dismutase and Glutathione Assay

HT-22 cells were seeded at a density of 70,000 cells/well in a 6 well plate and allowed to attach overnight. Cells were treated with different concentrations of CS for 24 hours and 20 mM glutamate for 12 hours. Super oxide dismutase (SOD) activity and GSH was measured using assay kits (Sigma Aldrich) according to the manufacturer's protocol.

Cell metabolism assays

Extracellular flux analysis

These experiments were conducted using both HT-22 cells and primary astrocytes. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was monitored using a Seahorse Bioscience XFe96 Extracellular Flux Analyzer. Astrocytes were seeded in 96 well seahorse microplates with DMEM (low glucose for astrocytes and high glucose for HT-22, 1 mM pyruvate and 10% FBS) and were treated with different concentrations of CS for 72 hours. On the day of the experiment, the culture media was replaced with XF base media (supplemented with 1 mM pyruvate, 2 mM glutamine and 5.5 mM glucose adjusted to pH 7.4) and incubated for 1 hour in a non-CO_2 incubator at 37^{o}C. Rotenone/Antimycin A, FCCP, and oligomycin were diluted into the XF base media and loaded into the accompanying cartridge to achieve final concentrations of 0.5 μM, 1 μM, and 1 μl, respectively. Initial baseline readings were established followed by injections of the drugs into the medium at the time points specified. For cell energy phenotype determination, oligomycin and FCCP was diluted in XF medium together to achieve a final concentration of 1 μM oligomycin and 1 μM FCCP and loaded into one port. Various metabolic parameters were monitored with each cycle set as mix for 3 minutes, delay for 2

minutes, and then measure for 3 minutes. Values were normalized to the cell number of each well determined by Calcein AM assays.

ATP and NAD/NADH assay

Astrocytes were plated and allowed to attach for 2 days at 37^{o}C and CO_2. The cells were then treated with different concentrations of CS for 72 hours. ATP concentrations in cell lysates were determined using an ATP determination Kit (Invitrogen USA) according to the manufacturer's protocol. Briefly, cells were washed with PBS and lysed with ATP releasing buffer (500 mM Tricine buffer, pH 7.8, 100 mM MgSO4, 2 mM EDTA, and 2 mM sodium azide, 1% Triton X-100). ATP standards and 10 µl of cell lysate were added to a 96 well plate after which 100 µl of ATP reaction buffer was added to each well. Luminescence was immediately determined using a Tecan Infinite F200 plate reader. Total ATP levels were normalized to protein content of the samples determined using Pierce 600 nm Protein assay (600 nm absorbance). NAD/NADH was performed using a commercial kit from Sigma-Aldrich MO, USA. NAD/NADH levels were normalized to the protein concentration of each sample measured with a protein assay kit.

Glycogen assay

Total glycogen in astrocytes following treatment of the cells with different concentrations of CS for 72 hours was determined. Briefly, astrocytes were collected using 0.25% trypsin (Sigma) to detach the cells, washed twice with PBS, and resuspended in distilled water. Glycogen assay kits (Sigma-Aldrich, St Louis, MO) were used to measure glycogen content according to the manufacturer's manual. Glycogen levels were normalized to the protein concentration of each sample.

Lipid analysis

Lipid content was analyzed using Nile red staining. Astrocytes were treated with different concentrations of CS and 60 μM propranolol and cyclosporine for 72 hours. The cells were fixed with 10% formalin for 15 minutes at room temperature and stained with PBS containing 1 μM Nile red solution and 5 μg/ml Hoechst 33342 for 30 minutes. Florescence was measured using a Tecan Infinite F200 plate reader at various wavelengths: Neutral lipid droplets 488/560, phospholipids 532/>620, Hoechst 33342; 361/486. Values of fluorescence were normalized to those of Hoechst fluorescence to control for cell number. Flow cytometry analysis was also done after staining the cells with Nile red.

Western Blot

Cells were collected and lysed in RIPA buffer (50 mM of Tris. HCl, pH 7.4,150 mM NaCl,1mM EDTA, 1%Triton X) with protease and phosphatase inhibitors (1;100). Protein assay reagent Pierce 660 nm (Thermo Scientific) was used to determine the protein content of the samples. Protein lysate was run in polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking for 1 hour, nitrocellulose membranes were incubated with primary antibodies: GS (polyclonal Abcam), AMPKα and AMPKβ (monoclonal cell signaling), glycogen synthase; GS and ACC (Monoclonal Cell signaling), mTOR (monoclonal cell signaling), Blc2, Akt, (cell signaling), Actin (monoclonal Santa Cruz) overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. Using biospectrum 500 UVP imaging system, chemiluminescence signals were detected and protein densities analyzed.

Statistical analysis

All data are presented as the mean ± S.E.M. The significance of differences among groups with one independent variable was determined by one-way ANOVA with Bonferroni's

correction for pairwise comparisons between groups when significance was detected. The significance of differences among groups where two independent variables were present was determined by two-way ANOVA with Bonferroni post-hoc test for planned comparisons between groups when significance was detected. For all tests, $p < 0.05$ (*) was considered significant.

4.5. RESULTS

CS protects HT-22 cells against glutamate and rotenone induced cell death

The protective effects of CS were characterized by HT-22 cells using glutamate, glucose oxidase and rotenone neurotoxicity models. The cells were either pre-treated with CS for 24 or 36 hours before being exposed to glutamate for 12 hours or treated with CS in the presence of glutamate or rotenone. In both Calcein AM and MTT assay, CS dose-dependently protected HT-22 cells against glutamate-induced cell death (Fig 4.1A and B). Florescent images of HT-22 cells stained with Calcein AM and PI also showed that CS increased cell viability when the cells were exposed to a toxic dose of glutamate (Fig 4.1C). In the Calcein AM assay, CS protected HT-22 cells with an EC50 of 17.6 µM when the cells where co-treated with CS and glutamate for 12 hours (Fig 4.1D). When HT-22 cells were pre-treated with CS for 24 and 36 hours before glutamate induced cell death, CS increased cell viability with an EC50 of 22.6 µM and 11.4 µM respectively (Fig. 4.1D). Flow cytometry of annexin V and PI staining of HT22 cells treated with CS for 24 hours and exposed to glutamate for 12 hours showed that a total of 1.54%, 2.38% and 22.8% cells treated with glutamate and CS were in the early, late and necrotic stages of cell death whereas 9.64%, 14.1%, and 48.2% of cells treated with glutamate-only were in the early, late and necrotic stages of cell death respectively (Fig 4.1E). Although 30 and 50 µM CS prevented

rotenone-induced cell death, CS failed to increase HT-22 viability in the presence of toxic doses of glucose oxidase (Fig 4.2A and B).

CS inhibited glutamate-induced ROS production, loss of mitochondria membrane potential and activated AKT pathway.

The effect of CS on glutamate-induced ROS generation was measured using fluorescent indicator H_2DCFDA which is converted into DCF by ROS. Flow cytometry assay revealed that glutamate caused a significant increase in ROS which was attenuated by CS (Fig 4.3A). The inhibitory action of CS on ROS generation in HT-22 cells was also verified using a microplate reader. CS decreased ROS production measured as total cellular ROS (Fig 4.3B). High levels of ROS affected mitochondrial membrane potential causing loss of a proton gradient and membrane depolarization. Treatment of HT-22 cells with glutamate caused a loss of mitochondrial membrane potential evidenced by TMRE microplate reader analysis which was attenuated by treatment of cells with CS (Fig 4.3C). We also investigated the effects of CS on glutamate-induced GSH depletion and observed that CS dose-dependently inhibited glutamate-induced loss of GSH (Fig 4.4A). The effects of CS on the activity of superoxide dismutase, an enzyme that converts superoxide radicals to hydrogen peroxide and molecular oxygen [320] was also measured. We observed that CS does not affect SOD activity (Fig 4.4B). We determined the effect of CS on the Akt anti-apoptotic pathway. HT-22 cells were treated with CS for 36 hours. Western blot analysis showed that CS dose-dependently increased Akt phosphorylation and increased Bcl-2/Bax ratio (Fig 4.5A).

CS increased ATP content, glycogen storage and mitochondrial respiration in astrocytes

The effects of CS on astrocyte metabolism were determined using a Seahorse XFe 96 analyzer. We measured parameters of oxygen consumption rate (OCR) such as basal and

maximal respiration, ATP-linked respiration, non-mitochondrial respiration, proton leak and extracellular acidification rate (ECAR) before and after sequential injection oligomycin, FCCP and Rotenone/Antimycin A. After 72 hour treatments of astrocytes with different concentrations of CS we observed that 50 μM CS increased basal and maximal respiration, spare respiratory capacity, and ATP production linked to OCR (Fig 4.6A). We also observed an increase in non-mitochondrial respiration, a parameter of OCR (Fig 4.6A). No significant increase was observed in the extracellular acidification rate following astrocyte treatments with CS for 72 hours and the cell energy metabolic phenotype shifted toward mitochondrial oxidative phosphorylation (Fig 6B). A similar effect was also observed in CS-treated HT-22 cells (Sup Fig 4.1). We determined the effects of CS on ATP levels in astrocytes. CS significantly increased total ATP content (Fig 7A and Sup Fig 4.2) and corresponding lactate levels in the culture media (Fig 4.7B) after astrocytes and HT-22 were treated with CS for 72 hours. Furthermore, increased glycogen (Fig 4.7C), and lipid content (Fig 4.7D) was observed following astrocyte treatment with CS.

CS increased AMPK activation

We determined the effects of CS on the AMPK pathway following 72 hours of astrocyte treatment. Western blot analysis indicated that CS significantly decreased phosphorylation of AMPK (Fig 4.8A) while increased mTOR phosphorylation or activation was also observed (Fig 4.8A). Seventy-two hour treatments of astrocytes with CS also decreased phosphorylation of Acetyl-CoA (ACC) and glycogen synthase (GS) (Fig 4.8A). Taken together these data indicated that CS alters astrocyte metabolism by enhancing mitochondria respiration, glycogenesis, and lipogenesis and increasing AMPK signaling.

4.6. FIGURES AND LEGENDS

Figure 4.1

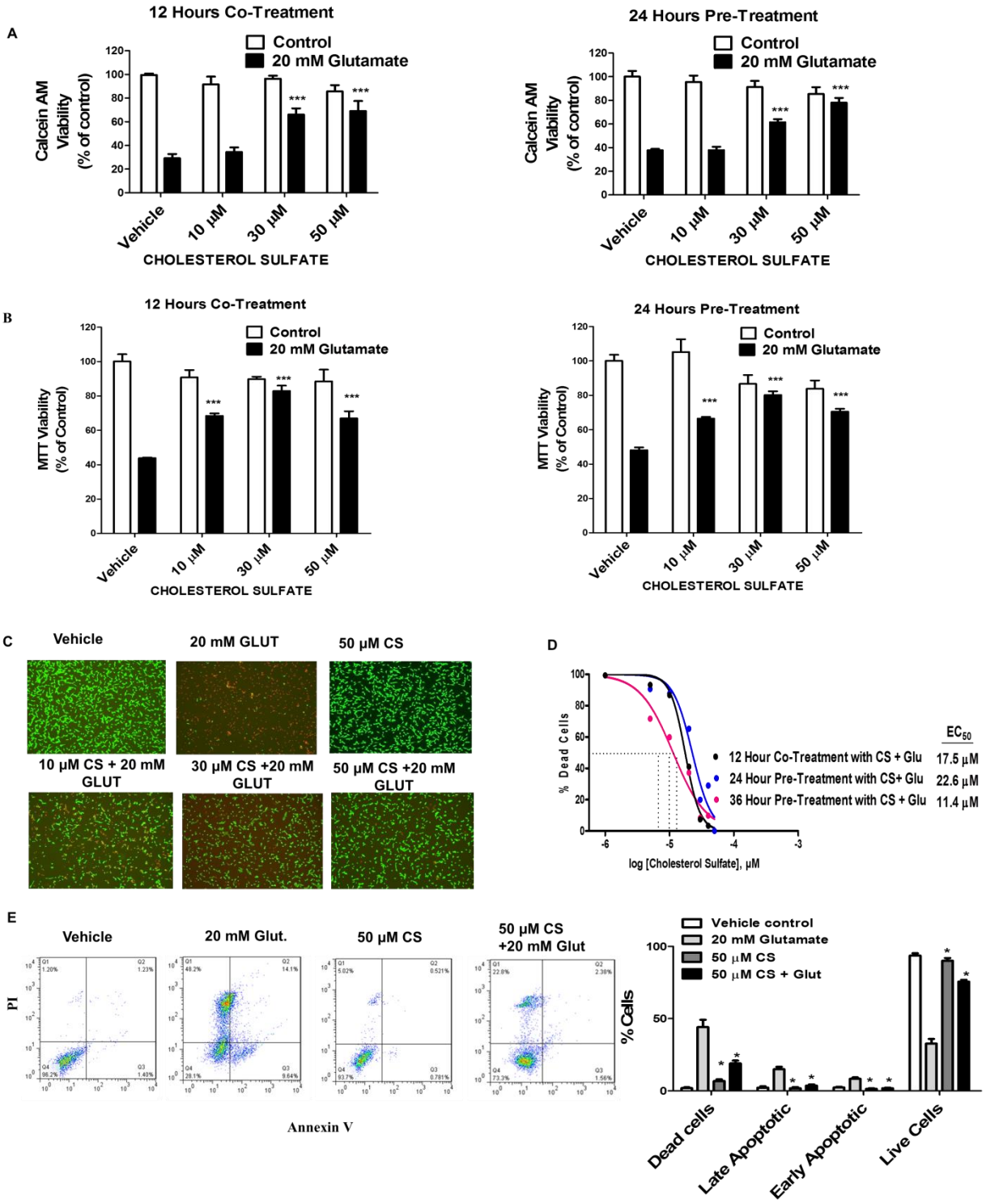


Figure 4.1. CS protects HT22 cells against glutamate-induced cell death. Quantitative analysis of HT-22 viability by (A) Calcein AM assay (B) MTT assay after 12 hours 20 mM glutamate insult demonstrates that cholesterol sulfate CS has a neuroprotective effect. *p< 0.05, **p<0.01 ***p< 0.001 compared to 20 mM glutamate treatment (N= 8-10). (C) Representative Calcein AM fluorescent images of PI (red) and Calcien AM (green) depict a dose-dependent neuroprotective effect of CS in HT-22 cells after 20 mM glutamate induce insult. (D) Dose-response curve of CS treatment of HT-22 Cells at various time points against glutamate-induced toxicity. (E) Representative flow cytometry analysis of Annexin-V and PI staining after 24 hours pre-treatment with CS. *p< 0.05, **p<0.01 ***p< 0.001 compared to 20 mM glutamate treatment (N= 4).

Figure 4.2

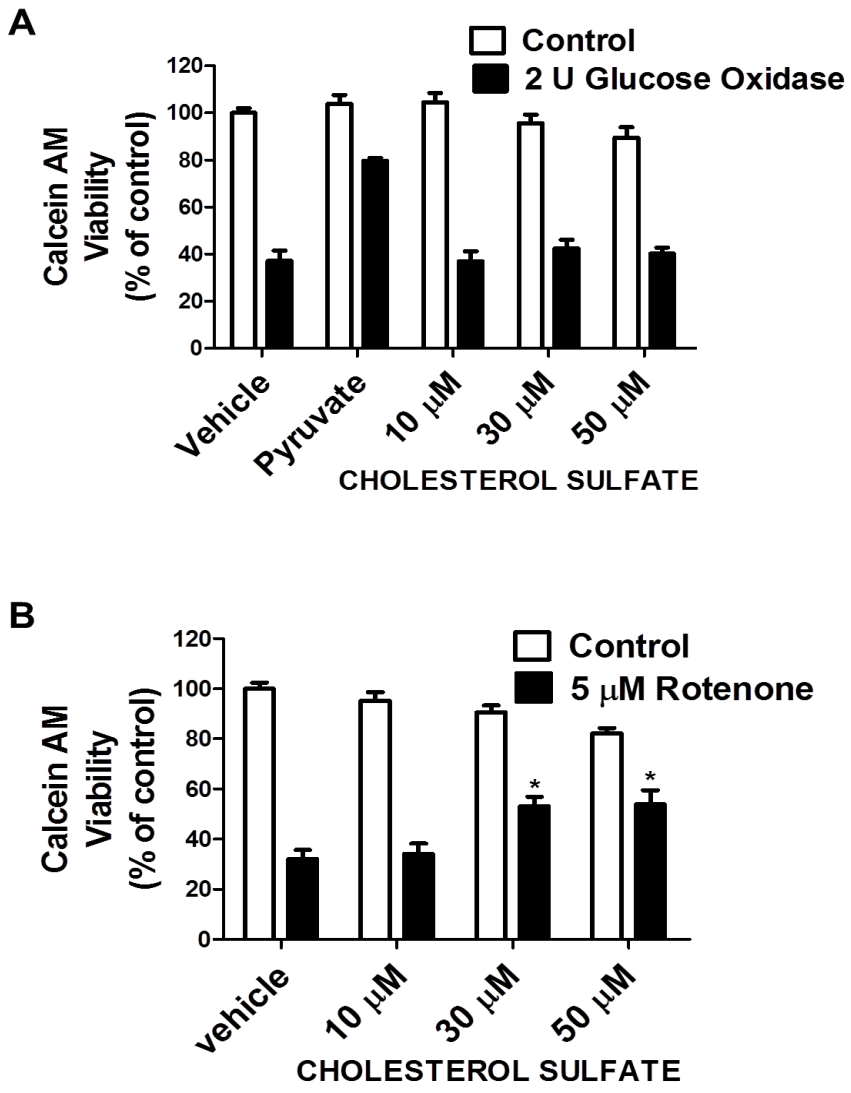


Figure 4.2. CS inhibited rotenone-induced neurotoxicity in HT-22. (A) 24 hours pre-treatment of HT-22 cells with CS failed to protect cells against glucose oxidase induced cell death (B) Effects of CS on rotenone-induced cell death. Cells were treated with 5 μM rotenone for 24 hours in the presence of CS. *$p < 0.05$ compared to 5 μM rotenone treatment (N= 8-10).

Figure 4.3

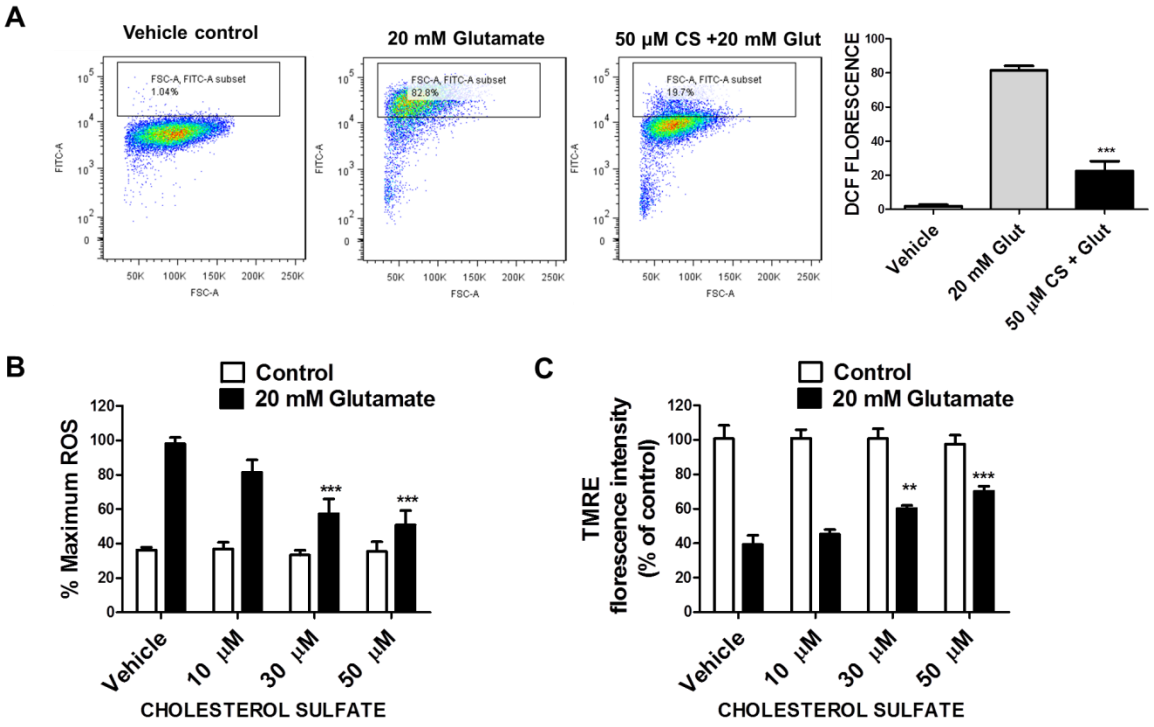


Figure 4.3. CS attenuates glutamate-induced ROS production and mitochondrial membrane depolarization. (A) Representative DCF flow cytometry assay depicts a significant increase in ROS after 12 hours exposure to 20 mM glutamate which was attenuated by CS. (B) DCF microplate reader assay depicts a significant increase in ROS after 12 hours exposure to 20 mM glutamate which was dose-dependently attenuated by CS. (C) TMRE plate reader assay shows CS attenuates glutamate-induced mitochondrial membrane potential loss. *p< 0.05, **p<0.01 ***p< 0.001 compared to 20 mM glutamate treatment (N= 8-10).

Figure 4.4.

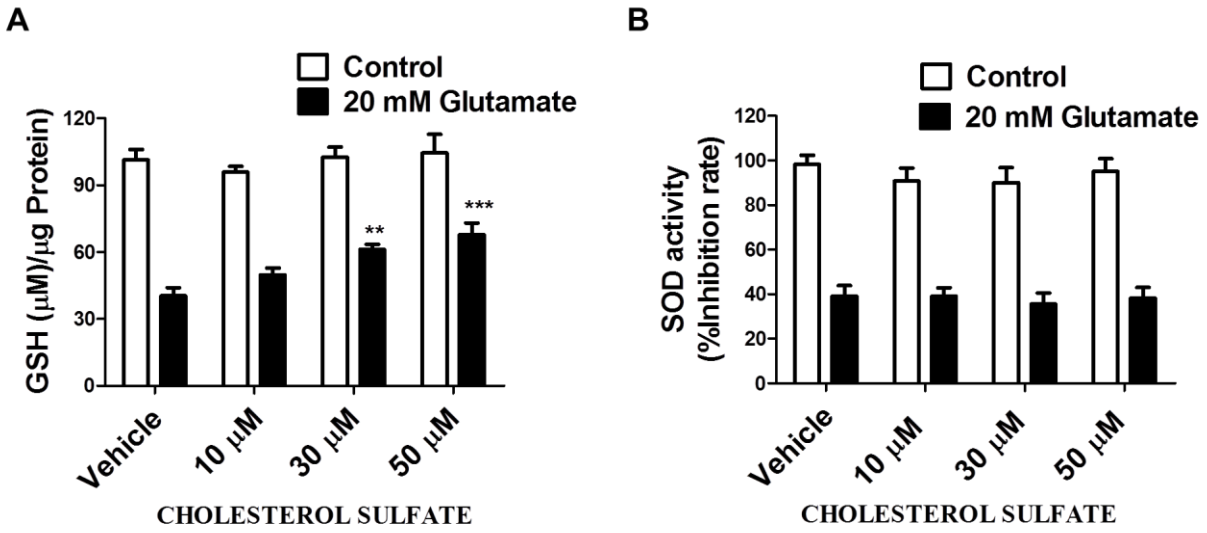


Figure 4.4. Pretreatment with CS prevented glutamate induced GSH depletion. (A) Total GSH assay in HT-22 cells treated with CS and 20 mM glutamate (B) CS has no effect on SOD activity following HT-22 exposure to glutamate *p< 0.05, **p<0.01 ***p< 0.001 compared to 20 mM glutamate treatment (N= 8-10).

Figure 4.5

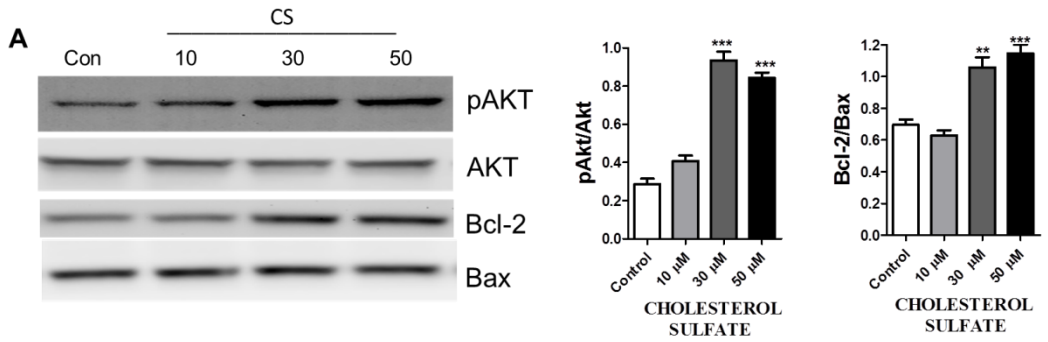


Figure 5. CS activates AKT pathway in HT-22 cells. (A) Representative Western blots and statistical analysis of AKT and Bcl-2 after HT-22 were treated with CS for 36 hours**.**

Figure 4.6.

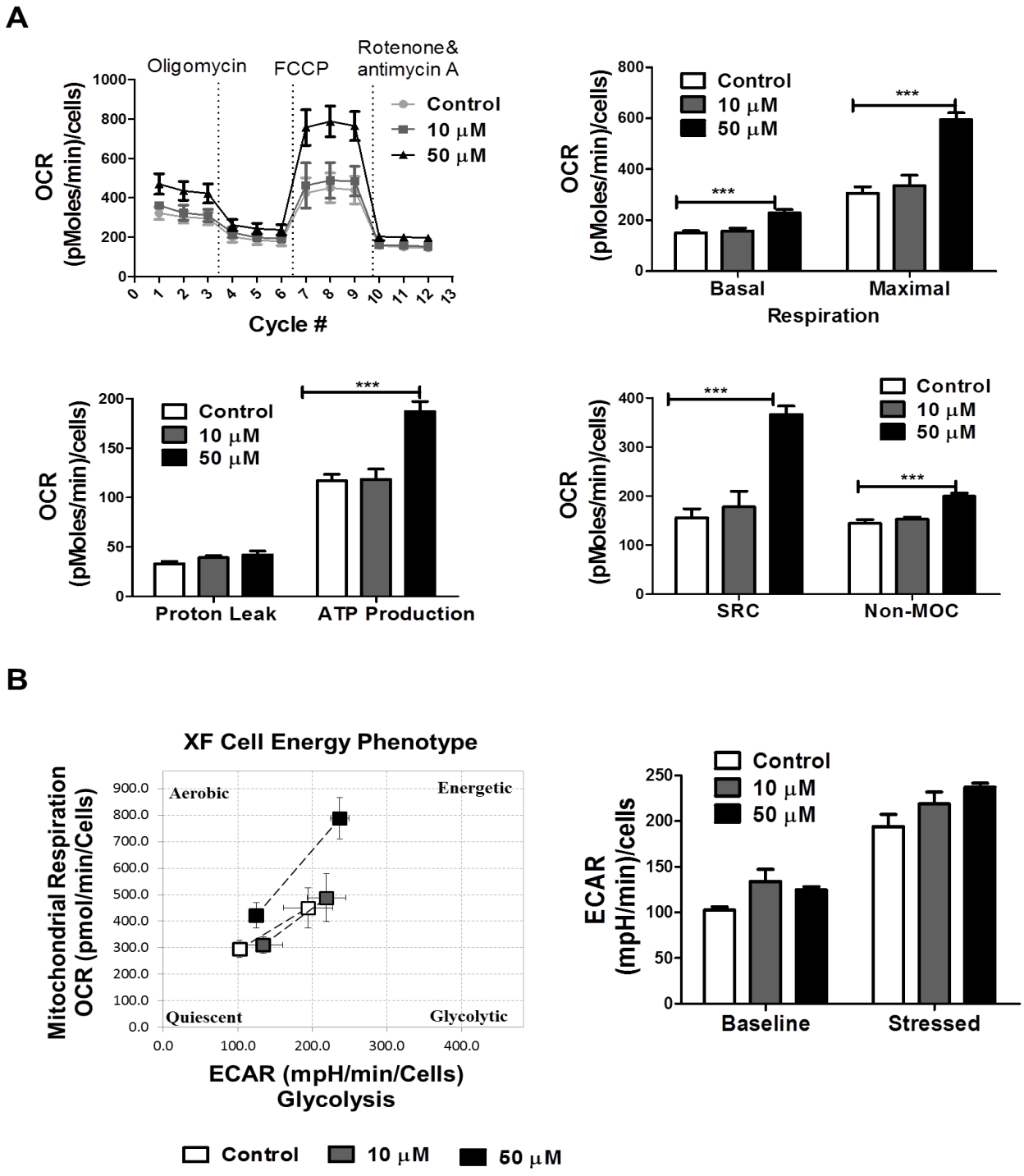

A
Oligomycin
FCCP
Rotenone& antimycin A
OCR (pMoles/min)/cells)
Cycle #
Control
10 μM
50 μM
Basal
Maximal
Respiration
Proton Leak
ATP Production
SRC
Non-MOC
***
B
XF Cell Energy Phenotype
Mitochondrial Respiration OCR (pmol/min/Cells)
Aerobic
Energetic
Quiescent
Glycolytic
ECAR (mpH/min/Cells)
Glycolysis
ECAR (mpH/min)/cells
Baseline
Stressed

Figure 4.6. CS increases oxygen consumption rate (OCR) in primary astrocytes. (A) OCR recordings at baseline and after treatment with oligomycin, FCCP and rotenone/Antimycin A. Bar graphs indicate basal and maximal respiration, proton leak, non-mitochondrial oxygen consumption (non-MOC), ATP production linked to mitochondrial respiration and spare respiratory capacity (SRC). (B) Cell energy phenotype after treatment of astrocyte with CS. Bar graph indicates the baseline and stressed extracellular acidification rate (ECAR).

Figure 4.7.

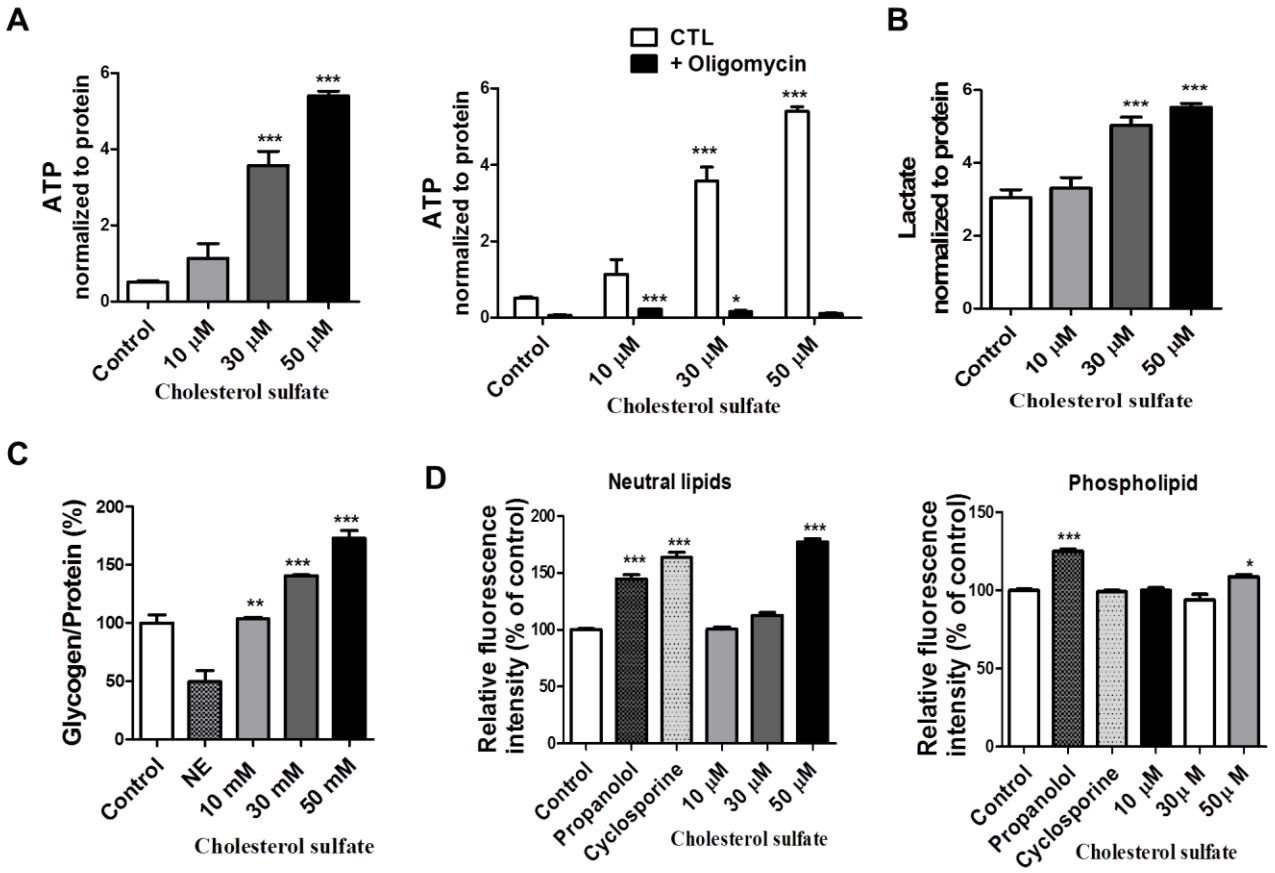


Figure 4.7. CS enhanced ATP production and increased glycogen content in astrocyte. (A) Total ATP content in primary astrocytes was analyzed using an ATP kit. (B) Lactate levels of primary astrocytes after treatment with CS (C) Glycogen content of primary astrocytes treated with CS (D) Quantitative analysis of lipid content in astrocytes treated with CS. * p< 0.05, * p< 0.01 ***p<0.001 vs control (10 FBS), n = 6-8.

Figure 4.8.

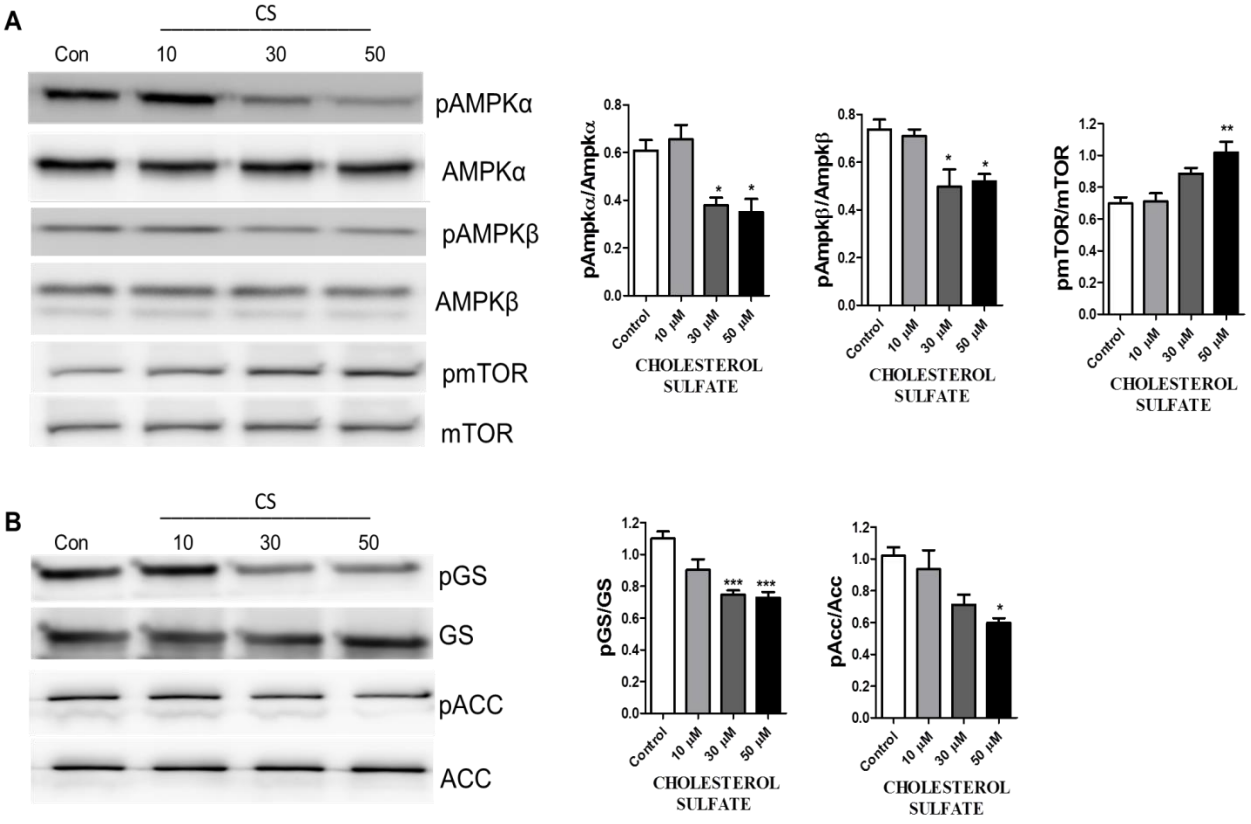
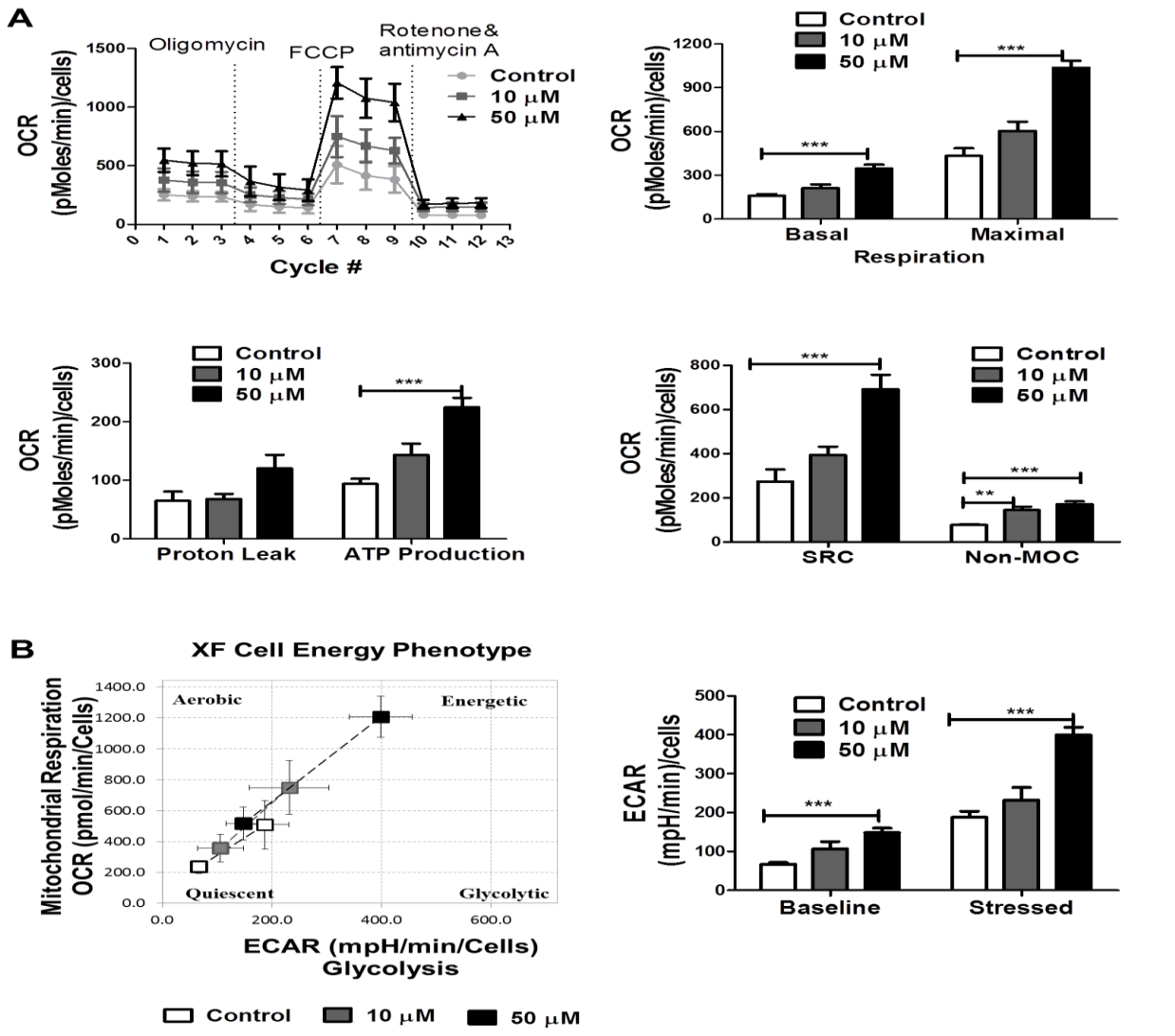


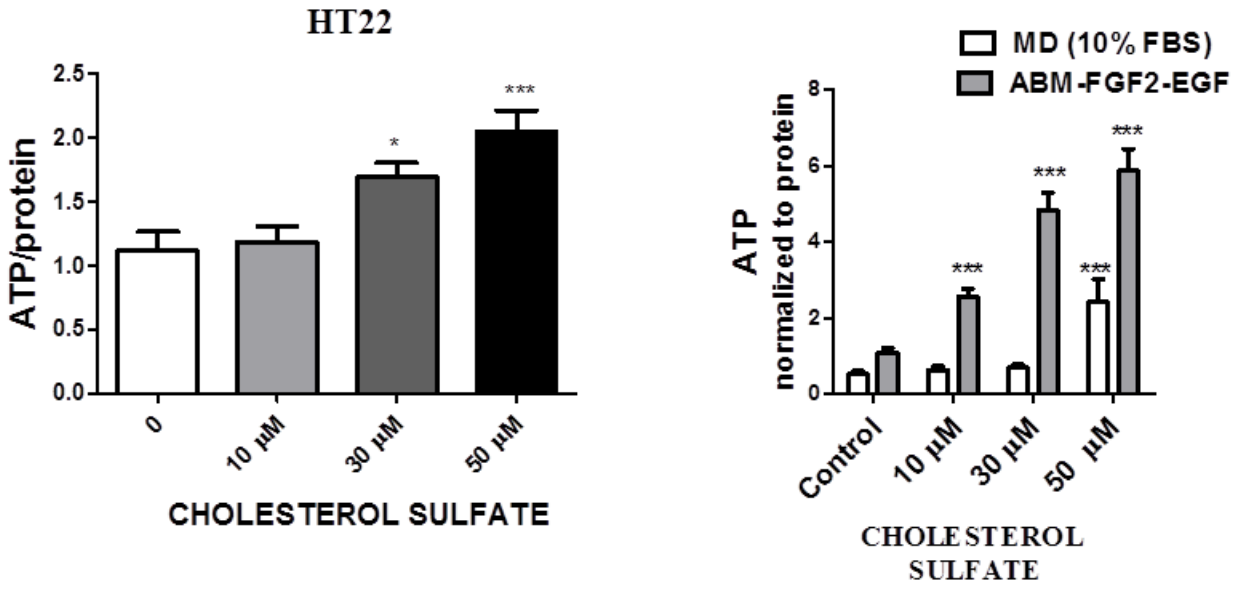
Figure 4.8. CS inhibits AMPK pathway in astrocytes. (A) Representative Western blots and statistical analysis of AMPK and mTOR pathway after astrocytes were treated with CS for 72 hours (B) Western blots and statistical analysis of GS and ACC after astrocytes were treated with CS for 72 hours indicated a reduction in p-GS and P-ACC.

Supplementary Figure 4.1.



SUPP Figure 4.1. CS increases oxygen consumption rate (OCR) in primary HT-22. (A) OCR recordings at baseline and after treatment with oligomycin, FCCP and rotenone/Antimycin A. Bar graphs indicate basal and maximal respiration, proton leak, non-mitochondrial oxygen consumption (non-MOC), ATP production linked to mitochondrial respiration and spare respiratory capacity (SRC). (B) Cell energy phenotype after treatment of astrocytes with CS. Bar graph indicates the baseline and stressed extracellular acidification rate (ECAR).

SUPP Figure 4.2



SUPP Figure 4.2. CS enhanced ATP production in HT-22 and Astrocytes maintained in MD (10% FBS) and ABM-FGF2-EGF medium. (A) Total ATP content in HT-22 was analyzed using an ATP kit. (B) Total ATP content of astrocytes cultured under different conditions and treated with CS

4.7. DISCUSSION

Neuroprotective strategies are aimed at protecting and preserving the CNS cell's structure and function from insults arising from toxins and neurodegenerative diseases. Several cellular mechanisms are involved in the initiation and progression of events leading to CNS cell structure and functional loss. This includes mitochondrial dysfunction, excitotoxicity, defects in energy metabolism and oxidative stress [28, 321, 322]. ROS production facilitates a vicious cycle of accelerated excitotoxity, lipid peroxidation, accelerated mitochondrial damage, and inflammation leading to neuronal loss [210]. Hippocampal oxidative stress also plays an important role in cognitive impairment caused by normal aging and neurodegenerative diseases [323]. In this current study, we investigated the neuroprotective and antioxidant role of CS in HT-22 cells using various *in vitro* neurotoxic models.

Our study demonstrates that CS protects HT-22 cells against glutamate and rotenone-induced cell death. We also found that treatment with CS decreased glutamate-induced increase in total ROS levels, glutamate-induced mitochondrial membrane potential collapse, and rescued cells from apoptotic cell death. In HT-22 cells glutamate blocks the glutamate/cysteine antiporter with saturating concentrations of extracellular glutamate resulting in glutathione (GSH) depletion leading to cell death [324, 325]. Our data indicate that CS prevents glutamate-induced GSH depleting and this could be the mechanism for attenuating total ROS. Cell survival requires the active inhibition of apoptosis, which is accomplished by inhibiting the expression of pro-apoptotic factors as well as promoting the expression of anti-apoptotic factors. AKT and its target B cell lymphoma-2 (Bcl-2) have been recognized as an anti-apoptotic factor [326, 327]. Blc2 is a large family of proteins that regulate apoptosis. Bcl-2 is an anti-apoptotic protein whereas Bcl-2 associated X protein (Bax) is a pro-apoptotic protein [328]. In our study, we

observed that CS significantly increased AKT phosphorylation and activation as well as increased the expression of Bcl2 with no significant change in Bax expression. Our data suggest that the anti-apoptotic effect of CS in the presence of glutamate could be attributed to its ability to increase anti-apoptotic factors. Interestingly *in vivo* and *in vitro* studies have shown that many neurosteroids have neuroprotective effects. For instance DHEA, DHEAS, and progesterone have been shown to protect hippocampal neurons against glutamate-induced neurotoxicity [329]. DHEA and DHEAS neuroprotective effects have been attributed to its anti-apoptotic effect via AKT/Bcl-2 pathway [330-332]. Since cholesterol sulfate serves as a substrate for the synthesis of DHEAS, it is possible that the neuroprotective effects of CS observed could be because CS is converted to DHEAS by the cells *in vitro*.

The brain's metabolism is tightly regulated to ensure adequate energy substrate delivery in accordance with neuronal activity. Astrocytes play a critical role in brain glucose metabolism. They provide for the energy needs of neurons via glucose uptake and metabolism [30]. AMPK regulates energy metabolism under physiological and pathological conditions in the brain. It is activated by conditions that compromise cellular ATP or increase ATP consumption whereas high ATP concentration antagonizes AMPK activation [333]. In our study, we observed that CS increases astrocyte ATP production in astrocytes cultured under different conditions, thus inhibiting AMPK pathway. Interestingly in astrocytes cultured in serum free ABM-FGF2-EGF culture conditions, the effects of CS on ATP production was observed at lower concentrations as compared to our observations in FBS medium.

Glucose metabolism and ATP generation occur in a compartmentalized way in the brain. Whereas astrocytes rely heavily on glycolytic metabolism, neurons use the end products of glycolysis for oxidative metabolism [30, 39]. Interestingly our cellular bioenergetics analysis

indicates that CS significantly increases the mitochondrial oxygen consumption rate but has no effect on extracellular acidification rate although we observed an increase in lactate production by the cells. The increase in ATP production might be due to increasing OCR as a result of CS-induced increase mitochondrial efficiency. Loss of AMPK signaling increases ATP levels and promotes anabolism. Consistent with the inhibition of AMPK we observed an increase in lipogenesis and glycogenesis evident by decrease inactivation of ACC and GS.

Glycogen a polymer of glucose is exclusively localized in astrocytes in the adult brain. It has been reported that almost half of the brain's glucose is taken up by astrocytes under physiological condition. It has been demonstrated that increasing astrocytic glycogen stores preserves the neuronal function and viability under limited conditions of restricted ATP availability, such as in hypoglycemia [30, 42, 334]. We found a significant increase in glycogenesis in astrocytes following CS treatment indicated by the decrease in phosphorylated glycogen synthase and increased glycogen content. The enhancement of glycogen stores and glycogenesis might also contribute to the increase in ATP production, and OCR observed following CS treatment. We speculate that CS activation of glycogen synthesis and increase ATP production might provide some neuroprotective effect in pathological conditions.

In summary, our current study demonstrates the CS protects HT-22 cells against glutamate-induced cell death and increases the expression of anti-apoptotic factor Bcl2. In addition CS also enhances astrocytes metabolism, mitochondrial oxidative phosphorylation and increases astrocyte energy storage. We also observed that

Further, *in vivo* studies are needed to investigate the effects of CS on aging and other neurodegenerative disorders associated with oxidative stress and metabolic syndrome.

4. OVERALL DISCUSSION AND FUTURE DIRECTION

Molecular, cellular, physiological and developmental mechanisms by which the CNS maintains homeostasis and response to infections, disease, damage, repair and regeneration has been the main focus of neuroscience over the years. To this end understanding the roles the diverse cell types in the CNS play in maintaining homeostasis and response to injury is of great importance in understanding the signaling pathways and mechanisms involve in CNS dyshomeostasis. However for years, studies aimed at understanding CNS mechanisms in health and disease has focus on neurons, sometimes more and sometimes less specifically aimed at a particular neuronal population. This reflected the general neuron-centrism in neuroscience, whereby other components of the brain such as astrocytes were seen irrelevant. In the last two decades, with the development of new research tools and techniques, research efforts aiming at providing a better understanding of astroglial cell function and mechanisms have revealed previously unsuspected roles of these glial cells which were long thought to be passive supportive cells. It has now become very clear that astrocytes and neurons cooperate to maintain CNS homeostasis. As a result of the growing appreciation of the role of astrocytes in health and disease, the traditional neuron-centric conception of the CNS has been challenged. An important advancement that aided the understanding of astrocyte development and function was the development of an astrocyte culture preparation method from rodent brains [2]. This *in vitro* astrocyte culture method provided a model of studying astrocyte biology devoid of the complex CNS microenvironments so that findings obtained are attributed to astrocyte. The primary astrocyte protocol developed by McCarthy and de Vellis has been used by most studies of astrocyte role in health and disease [2]. Concerns have been raised as to whether astrocytes produced from this method mimic *in vivo* astrocytes in terms of their morphological, metabolic

and functional phenotype. It has recently been found that the widely used MD-protocol produces astrocyte with gene, morphological and functional profiles different from *in vivo* astrocytes [3, 4, 10]. Attempts to develop other methods that produce astrocytes with phenotype quite similar to *in vivo* astrocytes is ongoing. There is a need to delineate factors that impact astrocyte phenotype *in vitro* in order to design a cost effective method of astrocyte culture that produces astrocyte with *in vivo* phenotype, and allow experimentation under different conditions. This will enable results obtained in a given context to be translated to events *in vivo*. In that line of thought, the chapters presented in this dissertation emphasize the importance of primary astrocyte in understanding astrocyte biology in health and disease and the impacts of culture condition on their phenotype. Studies also presented here highlight how the important roles growth factors such as FGF2 and EGF are playing *in vivo* can be exploited to help achieve an optimum astrocytic culture condition that mimics physiological conditions *in vivo.* In our *in vitro* astrocyte experimental conditions we observed two important factors that affect primary astrocyte phenotype; FBS, culture purity and glucose concentration in culture medium. In this dissertation I present the findings from 3 different studies which deal with how the presence of these factors in a culture medium impacts astrocytes phenotype and experimentation.

FBS provides for the growth and metabolic needs of astrocytes *in vitro*, Nevertheless its complete composition is difficult to determine [335]. There is also inter vendor and inter batch variability hence validation experiments have always been done by our lab and other labs before the use to ensure that a new batch provides the same growth support as previous ones [336]. The development of a defined serum free media has ensured the reproducibility and decreased variability in results obtained from primary cultures [337]. However the effects of media supplemented with FBS and serum free media on astrocytes phenotype has not been fully

defined although it's known that serum components do not cross the BBB unless there is a pathological damage [338]. Astrocytes *in vivo* are in constant communication with other neural cells which release molecules inducing the expression of certain astrocytic factors [251]. Therefore the presence of certain factors release by CNS cells in a culture media could mimic *in vivo* situation. In the first study presented we isolated astrocytes from postnasal days 1 to 2 C57BL6 pups and employed the differential variable cell attachment rate method to seed single cell suspension obtained from the digestion and titration of cortical tissues in FBS medium and serum free ABM-FGF2-EGF condition in a poly-lysine coated plate. We found that both our serum (FBS) contained and serum free medium (ABM-FGF2-EGF) supported astrocytes growth to the same extent. However the presence of FBS in the culture medium affected culture purity even after repetitive shaking to get rid of cell contaminant. It is possible that FBS directly or indirectly supports the growth of other cell types other than astrocytes by affecting the secretion of CSF-1 or other growth factors that are known to impact the proliferation of microglia and other cells in primary culture [10]. The purity of a culture ensures that the observations are attributed to a single cell type. In this study cultures prepared in serum free FGF2 and EGF medium showed lowest amount of microglia contamination. Hence the absence of serum and the presence of this growth factors (which may have inhibited microglia proliferation) appear to improve the purity of the astrocyte culture similar to results obtained in primary cultures of neuron. The presence of microglial as seen in FBS cultured astrocytes might affect experimental results such as experiments done to obtain the cytokine release pattern of *in vitro* astrocytes as microglia are known to release cytokines.

We also observed that culture conditions alter astrocytes metabolic profile. We observed using seahorse extracellular flux analyzer, ATP, glycogen and lactate assay that astrocytes

cultured in FBS medium have a decrease biosynthetic phenotype different from astrocytes cultured in a serum free FGF2/EGF medium. This may be due to FBS inhibiting the expression of some astrocytic proteins and enzymes involve in astrocytes metabolism. Similar to the metabolic phenotypes of astrocyte *in vivo*, astrocytes cultured in the serum free medium have biosynthetic phenotype. Astrocyte *in vivo* are high process bearing, consistent with other studies we found that astrocytes cultured FBS medium are flat and fibroblast like in morphology whereas those in serum free medium are high process bearing [4, 18]. This could be due to the non-physiological nature of FBS which impacts astrocytes genes expression and cell cytoskeletons hence morphology [3, 4].

Astrogliosis is a spectrum of changes in astrocytes that occurs in response to all forms and severities of CNS injury and disease, including subtle perturbations which may alter astrocyte activities through gain or loss of functions [234]. It is recognized *in vivo* by alterations in molecular or gene expression, cellular hypertrophy, proliferation and scar formation and rapid intermediate filament synthesis (GFAP, vimentin, nestin) [17, 234]. The specific functions astrocytes in their reactive state play are not well defined and the degree to which astrocytes in their reactive state adopt new functions that they do not normally exert and if such new functions are beneficial or detrimental to surrounding cells is also not clear [339]. Therefore there is a need to prepare astrocytes in their resting state so as to mimic these cells *in vivo* in the absence of injury. We found that culturing astrocytes in FBS medium as compared to serum free medium produce astrocytes with a reactive phenotype. This is evident by the increase GFAP, vimentin expression and the decrease expression of many astrocytic proteins found in activated astrocytes. Out data is consistent with other studies that shows that astrocytes cultured by the MD method in an FBS medium have reactive phenotype [227]. In this same study we observed that our

developed serum free FGF2-EGF medium produced astrocytes with quiescent phenotype at the same time allowing reactive astrogliosis to be induced in this primary astrocytes by pro-inflammatory cytokines and events that mimic *in vivo* injury. It has been reported that FGF2 signaling maintains astrocytes in their resting state and delays astrocytes activation after injury {Kang, 2014 #20}. We suggest that the quiescent phenotype of astrocytes in the ABM-FGF2 culture condition could be attributed to the presence of FGF2 in the culture medium. Employing metabolic assay, morphological and gene expression analysis we found that culture conditions impact astrocytes phenotype. We also report a serum free FGF2-EGF medium that supports astrocytes growth and mimic *in vivo* quiescent astrocyte phenotype. This medium will provide a critical tool to define the precise mechanism regulating astrocyte functions and astrogliosis. Nevertheless, further studies are needed to see the impact of culture conditions on astrocytes from different brain region, and different species.

In our second study we investigated the impact of glucose concentration in a culture medium of primary astrocyte phenotype. In other to study astrocyte physiological mechanism and pathways, there is a need to maintain astrocyte in physiological condition. However most astrocyte studies are conducted under hyperglycemic condition. Since glucose is primarily transported by astrocytes, we speculated that hyperglycemia may have a direct impact on astrocyte metabolism and phenotype. We found that hyperglycemia irreversibly inhibit astrocyte proliferation likely mediated by cell cycle arrest without impacting apoptosis. As expected we observed high glucose (25 mM) increases astrocyte metabolic potential and also enhances glycolysis. Additionally astrocytes cultured in high glucose media have a biosynthetic phenotype.

Evident by increase ROS production and cytokine release, high glucose condition mimic pathological environment hence could affect results obtained when studying astrocytes in healthy

brain. One interesting finding from this studies is that, unlike previous data published by our lab, where hyperglycemia did not activate astrocytes culture in MD conditions [263], we observed that hyperglycemia activated astrocytes cultured in ABM-FGF-EGF medium, this may be to the fact that astrocytes in the MD condition are already activated and effects of hyperglycemia may not evident. However in ABM-FGF2-EGF condition, astrocytes are in a resting state hence permitting astrogliosis to be induced or studied. Taken together this data highlight the effect of culture condition on astrocyte phenotype and experimentation and provides a potential role of growth factors in vivo on astrocyte biology. This study also provides a potential mechanism which may underline hyperglycemia induced neuropathy and warrant further *in vivo* study to determine the effect of hyperglycemia on astrocyte phenotype.

Our third study illustrates an important role of primary cultures in studying the effects of compounds on neural cells. Studying the effects of cholesterol sulfate (CS), an important known sterol sulfate in tissues on astrocyte metabolism and its neuroprotective properties, we employed the use of charcoal stripped FBS in our culture medium since FBS contains steroids which may potentiate or antagonize the effects of CS. It was evident also from our study that the presence of FBS and culture condition affected our experimental outcome as we observed the effects of CS on ATP production in ABM-FGF2-EGF cultured astrocytes at lower doses than in either 10% FBS or charcoal stripped medium.

This chapter is an illustration of the impact of culture condition on astrocyte biology and experimentation and the critical considerations that have to be made when testing compounds *in vitro.*

FUTURE DIRECTION

The impact of this culture medium on neuron also needs to be investigated using astrocyte -neuron mixed culture. This will enable us investigate the effect astrocyte phenotype on neuron biology. Additionally the mechanisms by which the components of the medium, such as FGF2, EGF affect astrocyte phenotype also needs more elucidation. Studies are also needed to compare astrocytes isolated by FACS and IP to astrocytes produce by our method in terms of their gene expression and metabolic and reactive phenotype.

Furthermore *in vivo* studies are needed to investigate how hyperglycemia alters astrocyte function and phenotype and how this contributes to neurological defects associated with diabetics.

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