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ELEVATED L-LACTATE DRIVES MAJOR CELLULAR PATHOLOGIES ASSOCIATED WITH NEURODEGENERATION

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ELEVATED L-LACTATE DRIVES MAJOR CELLULAR PATHOLOGIES ASSOCIATED WITH NEURODEGENERATION

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Dedication

“To my family and friends for their life-long love and support”

Table of Contents

List of Figures vi

Preface vii

Acknowledgements viii

List of abbreviations x

Abstract xii

1 Literature Review 1

1.1 Introduction 1

1.2 Neurodegenerative Diseases 3

1.3 Glutamate Excitotoxicity 7

1.4 Lactate 9

1.5 Cerebrospinal Fluid Abnormalities 13

1.6 Hypothesis 15

2 Elevated L-lactate Drives Major Cellular Pathologies Associated with Neurodegeneration 16

2.1 Abstract 16

2.2 Introduction 17

2.3 Methods 21

2.3.1 NGF-Derived PC12 Cell Culture 21

2.3.2 Preparation of Neuronal Cultures 22

2.3.3 Cell Treatment 22

2.3.4 Flow Cytometry 23

2.3.5 Real-Time Imaging 24

2.3.6 Neuronal mRNA Expression Levels 26

2.3.7 Statistical Analysis 27

2.3.8 Chemicals 27

2.4 Results 27

2.4.1 L-lactate Increases Cytosolic Calcium in Primary Neuronal Cultures 27

2.4.2 L-lactate Increases Cytosolic ROS in Primary Neuronal Cultures and Dopaminergic-Like PC12 Cells 29

2.4.3 L-lactate Increases Intracellular Acidification in Primary Neuronal Cultures and Dopaminergic-Like PC12 Cells 32

2.4.4 L-lactate Alters Expression of Synaptic Plasticity Genes c-Fos and EGR1 in Primary Neuronal Cultures 33
2.4.5 L-lactate Induces Unfolded Protein Response in Primary Neuronal Cultures 34
2.4.6 L-lactate Increases VMAT2 mRNA Expression in Primary Neuronal Cultures 35
2.4.7 L-lactate Increases Cell Death in Primary Neuronal Cultures and NGF-Derived PC12 Cells 36
2.5 Discussion 39
3 Summary, Limitations, and Future Directions 46
3.1 Summary 46
3.2 Limitations 47
3.3 Future Directions 48
4 References 49
A Raw Data and Statistics 55

List of Figures

Figure 1-1. Proposed Sequence of Events Occurring During Ischemia/Reperfusion Injuries. 5

Figure 1-2. Calcium-Induced Apoptotic Pathway via Calcium Channel Activation. 8

Figure 1-3. Pertinent Chemical Structures. 9

Figure 1-4. Monocarboxylate Transporter (MCT) Dysregulation. 10

Figure 1-5. The Cori Cycle. 11

Figure 1-6. Lactate Accumulation Contribution to Cytotoxicity. 15

Figure 2-1. Hypothesis Model for Lactate-induced Cell Death. 21

Figure 2-2. L-lactate Increases Cytosolic Calcium in Neurons. 28

Figure 2-3. L-lactate Increases Cytosolic ROS in Neurons. 30

Figure 2-4. L-lactate Increases Cytosolic ROS in PC12 Cells. 31

Figure 2-5. Real Time pH Imaging. 33

Figure 2-6. Effect of L-lactate on mRNA Expression Levels in Primary Neuronal Cultures. 35

Figure 2-7. L-lactate Increases Cell Death in Primary Neuronal Cultures. 37

Figure 2-8. L-lactate Increases Cell Death in PC12 Cells. 39

Preface

Chapter 2 presents the original research titled, “Elevated l-lactate drives major cellular pathologies associated with neurodegeneration.” This article is used with permission as a collaborative work with co-authors, Andrew D. Chapp (A.D.C), Taija Hahka (T.H.), Zhiying Shan (Z.S.), Qing-Hui Chen (Q.H.C.).

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List of Abbreviations

Aβ Amyloid-Beta

aCSF Artificial Cerebrospinal Fluid

AD Alzheimer's Disease

ALS Amyotrophic Lateral Sclerosis

AMPA-R Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Proprionic Acid Receptor

ATP Adenosine Triphosphate

BBB Blood-Brain Barrier

BREMS Beyesian Risk Estimate for MS

c-Fos Cellular Phosphoprotein

CHOP DNA-Damage-Inducible Transcript 3

CNS Central Nervous System

CoA Coenzyme A

DMEM Dulbecco's Modified Eagle Media

EGR1 Early Growth Response Hormone 1

GRP78 Glucose Regulated Protein 78

IEG Immediate Early Gene

I/R Ischemia/Reperfusion

LCIS Live Cell Imaging Solution

LDH Lactate Dehydrogenase

PCR Polymerase Chain Reaction

MCT Monocarboxylate Transporter

MS Multiple Sclerosis

NGF Neural Growth Factor

NMDA-R N-Methyl-D-Aspartate Receptor

NO Nitric Oxide

PNS Peripheral Nervous System

PD Parkinson's Disease

ROS Reactive Oxygen Species

RNS Reactive Nitrogen Species

TCA Tricarboxylate Acid

VMAT2 Vesicular Monoamine Transporter 2

Abstract

Within the past few decades, lactate research has expanded from initial findings deeming lactate as a dead-end metabolic product to recognition of lactate's role as a potential energy substrate in the CNS. Due to the tight relationship between lactate and energy metabolism, interest in the scientific community has been mounting around associations among metabolic dysregulation, elevated lactate and neurodegenerative states such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, ischemia/reperfusion (AD, PD, ALS, I/R injuries), and physiological aging, however underlying cellular mechanisms and/or facilitators for neuronal degeneration pathologies still remain unknown. Here, we tested several hypotheses that implicate L-lactate to various neurodegenerative mechanisms: increases in cytosolic calcium, increases in cytosolic reactive oxygen species (ROS), increases in intracellular acidification, and subsequently, increases in cellular death in primary neuronal cultures and dopaminergic-like NGF-derived PC12 cells. Treatment of cells with a pathologic concentration of L-lactate (4 mM) induced significant increases in cytosolic calcium, cytosolic ROS, and intracellular acidification measured in real time with fluorescent markers and quantified using ImageJ software. Furthermore, treatment with L-lactate (4 mM) induced significant increases in cell death, measured in real time and using flow cytometry in support. We conclude that L-lactate increases cellular events commonly observed in cells undergoing

degeneration, providing support for the implications of lactate in neurodegenerative disorders.

1 Literature Review

1.1 Introduction

Despite hundreds of years of study, the mechanisms behind the complex network of physiological processes that constitute pathological human conditions remain under investigation. At the molecular level, cells have the ability to communicate and adapt to changes in the internal environment of the body, which provide a dynamic means of survival and allows for continual function. However, when cells are exposed to continuous stressors and/or fail to effectively adapt, injury and apoptosis or necrosis ensue. Maintaining cellular homeostasis is crucial to the proper functioning of the various bodily systems in concert with one another. The nervous system provides the necessary machinery for communication within the body, sensory perception of the world around us, motor function to allow us to traverse the external environment, and autonomic function to control organ and endocrine systems within us. The brain and spinal cord of the central nervous system (CNS) communicate via cranial and spinal nerves with the peripheral nervous system (PNS). Proper neuron function is extremely important to human survival, so there are specialized adaptive measures in the body to protect the nervous system. Neuroglial cells, such as oligodendrocytes, microglia, and astrocytes, function to metabolically support and provide a means of protection for neuron cells. The brain is separated from the rest of the body by the blood brain barrier (BBB) and the cerebrospinal fluid (CSF)-brain barrier, which create a filtered environment for neurons specialized

to pH, ion, and energy substrate preferences. The BBB consists of endothelial cells connected with tight junctions, basement membrane, and astrocytes. Oxygen, carbon dioxide, and water can freely pass through the BBB, however larger and chemically charged molecules require regulated transport, allowing for further protection of the CNS.

Energy homeostasis within the brain is closely regulated and a laboring physiological task, as it accounts for nearly 20% consumption of total resting cardiac output and oxygen (14) . The importance of homeostasis within the CNS cannot be understated, as minutes without blood and/or oxygen supply can have permanent damaging effects (63). Under ischemic conditions, the lack of blood flowing to and from the brain can lead to the build-up of toxic by-products of metabolism, increasing the rate of neuronal damage. One of the major energy substrates used by the brain is glucose, however for this the neurons rely on blood glucose or glycogen supplied from neuroglia. Recent studies have shown that the brain can aerobically utilize lactate as an energy substrate via the krebs cycle (26), and have also implicated the importance of lactate as an oxidative energy substrate (8, 14, 56, 73).

Disruption of cellular and energy homeostasis is the hallmark of various neurodegenerative disorders and injury to the brain. Characterized by the deterioration of neuronal circuits, homeostasis dysregulation impairs cell-to-cell communication critical for healthy bodily function (10). Neuronal degeneration also occurs in the normal aging process, leading to cognition and memory impairment. Through extensive cellular and molecular research, common

mechanisms underlying neurodegenerative pathologies have been identified, however precise etiology behind these conditions still remains largely unsolved.

1.2 Neurodegenerative Diseases

Traumatic brain injury, aging, and degenerative changes to neurons can affect the ability to effectively process information and can damage cognitive function. A hallmark of many neurodegenerative disorders is cognitive impairment, which reduces the ability to perceive sensory input, process information, form new memories and recollect old ones. Although neuropathological diseases encompass varying symptoms or injuries, there are similar underlying mechanisms associated with neurodegeneration such as oxidative stress (5, 23, 47, 57, 65), excitotoxicity (10, 15, 19, 20, 44, 62), dysfunction of mitochondria (16, 28, 31, 32, 39, 43, 66, 68), protein aggregation (23, 47), and alterations in gene expression (15, 47). Regardless of the processes, it is likely to be a combination of genetic predispositions and environmental factors that contribute to the extent and severity of these digressions.

Aging is an inevitable process of life that encompasses similar physiological changes that occur in pathologic neurodegeneration. A 2016 population study demonstrated that autopsies from brains of aged people lacking a neurodegenerative disease diagnosis still presented with amyloid-β (Aβ), neurofibrillary tau protein tangles, Lewy bodies, neuronal loss, and reduction in

brain volume (22, 69). It still remains under investigation as to whether these features are antecedents of neurodegenerative disease or artifacts of aging. Advances in modern medicine have resulted in growth among the population of people aged 60 and older, with numbers anticipated to increase to account for 28% of the total population in North America and 35% in Europe by 2050 (United Nations, 2017). With such a strong similarity between aging and neurodegeneration, precise cellular mechanisms need to be further elucidated.

In ischemic brain conditions, there is a halt in glucose delivery within minutes, and under anaerobic conditions this can lead to increased lactate production and exhaustion of ATP. The majority of energy use by nervous tissue is used in maintaining ionic concentration gradients (such as the Na+/K+-ATPase pump), which can account for fluctuations in ions such as sodium, calcium, and potassium that are instrumental to neuronal communication and function. If the ischemic episode is not controlled, total brain death ensues. Even if the ischemic tissue is reperfused, this has been documented to contribute to secondary brain injury via inflammation and noxious by-products, which can compound the injuries received from ischemia (Figure 1-1). Of note are the role reactive oxygen (ROS) and nitrogen species (RNS) have in ischemia reperfusion injuries, regulating apoptosis (59, 63). Under anaerobic conditions, cellular stores of ATP are rapidly depleted, requiring cells to produce energy via the less-effective anaerobic respiration. This anaerobic environment impairs the ability of the citric acid (TCA) cycle to re-oxidize NAD+ to NADH, can lead to accumulation in the build-up of lactate, damage mitochondria and their ability to scavenge free

radicals, increase levels of intracellular ROS and RNS, and exacerbate cell death. The use of antioxidants as treatment to mitigate the free radical insult is being explored (9, 30, 50, 65).

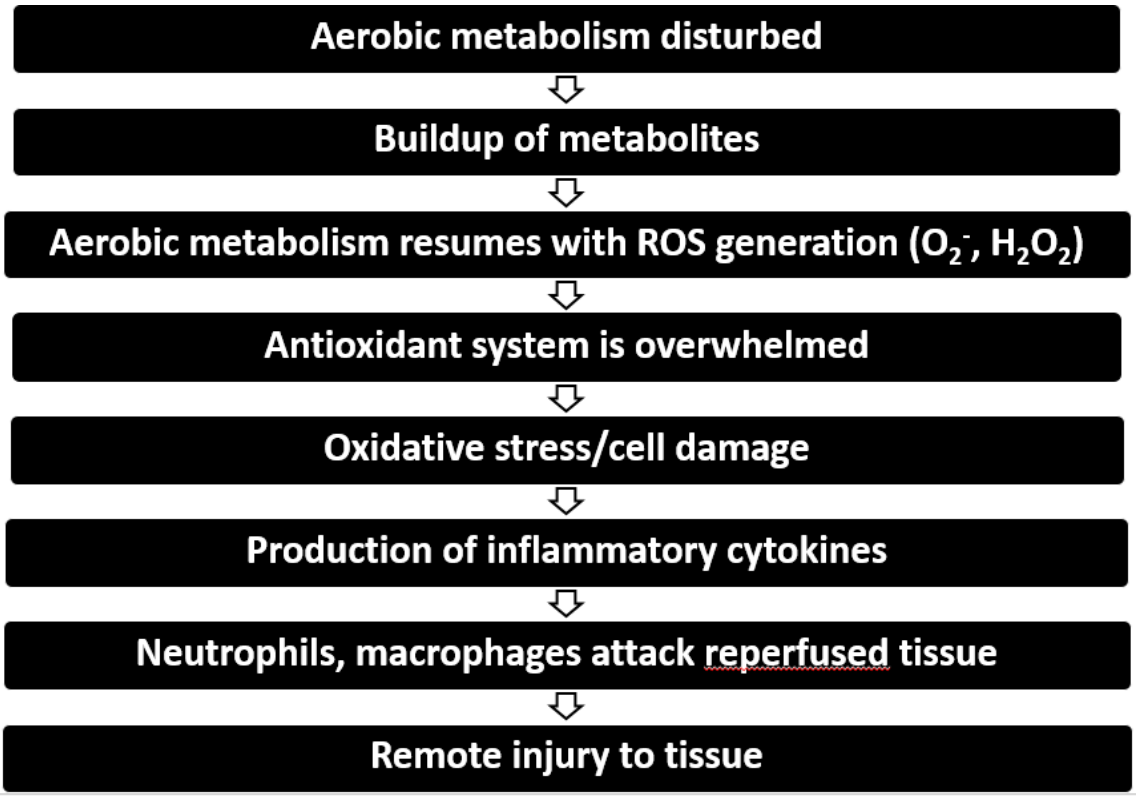

Figure 1-1. Proposed Sequence of Events Occurring During Ischemia/Reperfusion Injuries.

The cells that require the highest energy usage, neurons and myocytes, have been the focus around the degenerative processes that encompass Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and aging in general (23, 27, 69). A growing body of evidence points towards the dysfunction of mitochondria as an underlying cause of many neurodegenerative diseases such as PD (7, 27, 43), AD (5, 13, 16, 20),

and ALS (27, 43, 44, 60, 72). The mitochondria are key players in energy metabolism and largely function to produce ATP. Through the glycolytic pathway, glucose is converted to pyruvic acid in the cytosol, which then enters the mitochondria. Once inside, pyruvate is quickly converted to acetyl coenzyme A (coA), which enters the tricarboxylic acid (TCA) cycle. Glycolysis and the TCA cycle only account for a net gain of 4 ATP from the original glucose molecule. Most of the ATP used to supply cellular functions arise from the last stage of cellular respiration: the oxygen-dependent electron transport chain, which results in a net gain of 34 ATP. Since most of the energy generated in mitochondria depends on the presence of oxygen to facilitate this process, cells cannot survive efficiently for prolonged periods without fresh oxygen supply. It has been documented that electrons can escape from the electron transport chain, and this can be exacerbated during mitochondrial dysfunction, leading to the production of superoxide anions, generating ROS (39). As a means of regulating ROS formation via scavenging, mitochondria are equipped with antioxidant systems. Only when the formation of ROS exceeds the formation of antioxidants, will the cell undergo oxidative stress. This can occur due to abnormally high production of ROS and/or dysfunction in free radical scavenging capabilities. ROS can stimulate the uptake of glucose into the mitochondria, further generating more ROS (39). Increased levels of cytosolic ROS are implicated in many pathological states, especially AD and PD (59).

1.3 Glutamate Excitotoxicity

In many neurological disorders, neuronal damage can be facilitated through a host of mediators such as excitatory neurotransmitters, free radicals, nitric oxide (NO), inflammatory cells, and apoptotic responses. Excitotoxic injury to the brain occurs as a result of excessive activity of excitatory neurotransmitters and their interactions with respective receptors, and can be acute (stroke, ischemia, trauma) or chronic (as seen in neurodegenerative disorders) in nature. The primary excitatory neurotransmitter in the brain is glutamate, which facilitates numerous high-order functions such as memory, cognition, motor function, and sensory experiences. The action of glutamate occurs at glutamate family receptor ion channels, such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) and the N-methyl-D-aspartate receptor (NMDA-R). Of particular interest in studies of neurodegeneration are ligand-gated calcium channels, which facilitate entrance of calcium into the intracellular space, leading to increased firing and duration of action potentials fired by neurons. Primary literature suggests calcium channel-driven implications in CNS injury, as excessive influx of calcium into the intracellular space can initiate a calcium-cascade of events that include acidification of the cell and the release of enzymes that propagate cellular destruction (19, 48, 59, 71) (Figure 1-2).

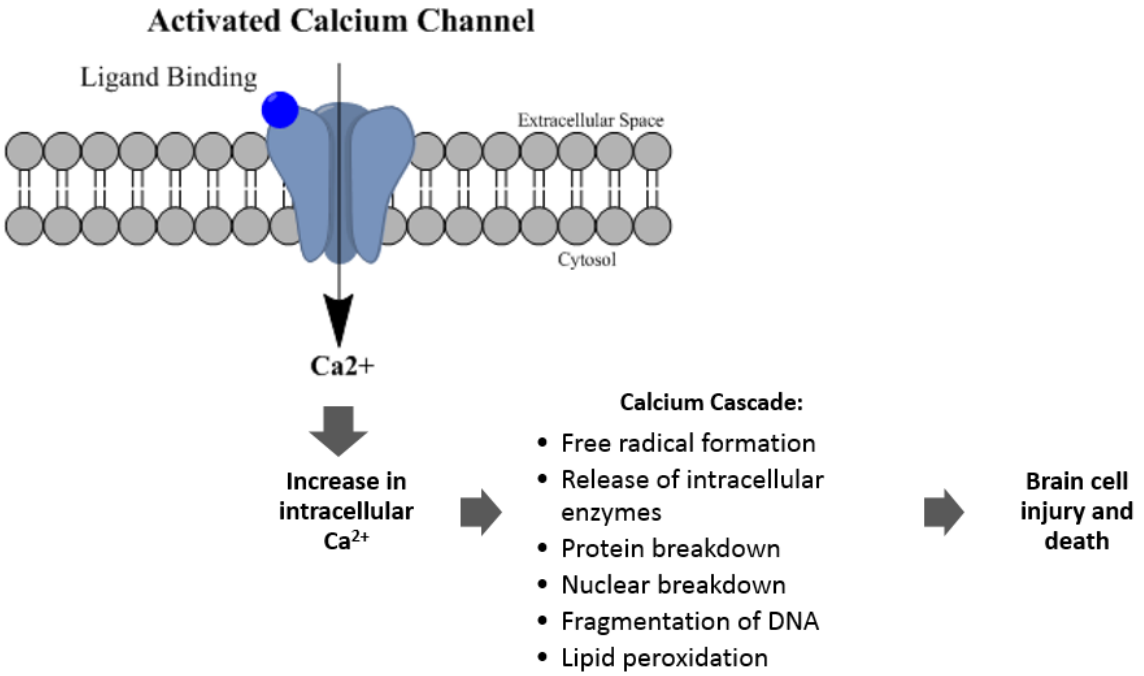

Figure 1-2. Calcium-Induced Apoptotic Pathway via Calcium Channel Activation.

Acute glutamate excitotoxicity can be remedied through the removal of excess glutamate or by blocking its downstream effects. One of the cornerstones of AD diagnosis is the presence of Aβ plaques or oligomers (38, 45, 61). Mounting evidence points to the role of Aβ oligomers in disrupting glutamate neurotransmission (19, 20), and Aβ has been shown to induce NMDA-R calcium currents, oxidative stress, and apoptosis in primary cortex and hippocampal cultures (19, 48, 51). The clinical significance of calcium channels in neurodegeneration is supported through the Food and Drug Administration (FDA), with their approval of Memantine, a non-competitive NMDA-R pore blocker, as the current treatment strategy in reducing neuronal loss associated with AD (19, 20, 25, 61).

1.4 Lactate

Most of the research conducted in the 1900's was evaluating the role of lactic acid/lactate as a by-product of energy metabolism that occurs in exercising muscle (33, 36, 37). While this holds true, further advances in cellular and molecular techniques are opening up avenues of further investigation of lactate as a potentially significant energy substrate for the CNS.

Lactate (and its conjugate acid, lactic acid) has a similar chemical structure to glutamate, which may mimick the carboxyl terminals located on each end of glutamate (Figure 1-3). It is through this feature that lactate can partially agonize glutamate sites at glutamatergic ion channel receptors such as NMDA-R.

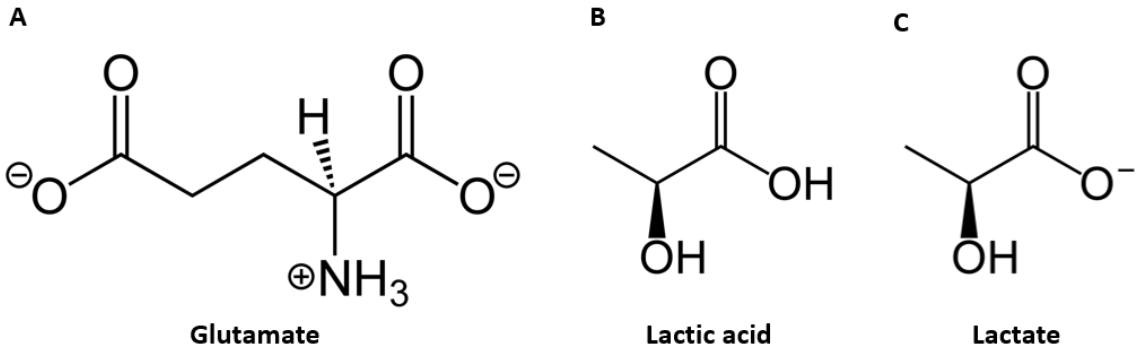

Figure 1-3. Pertinent Chemical Structures.
Chemical structures of A) glutamate, B) lactic acid, and C) lactate.

One of the most significant physiologic roles of lactate is in regards to the Cori cycle. Also known as the lactic acid cycle, it is the process by which lactate provides an energy substrate for muscles during the anaerobic conditions of exercise. Under normoxic conditions, the ATP needed for muscular activity is supplied via pyruvate conversion through the TCA cycle and especially through

subsequent oxidative phosphorylation. Under hypoxic conditions, as occurs in chronically working skeletal muscles, ATP supply is dependent on the less sufficient anaerobic metabolism. Instead of entering the TCA cycle, pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH), which oxidizes NADH to NAD+ in order to reduce pyruvate into lactate (Figure 1-4).

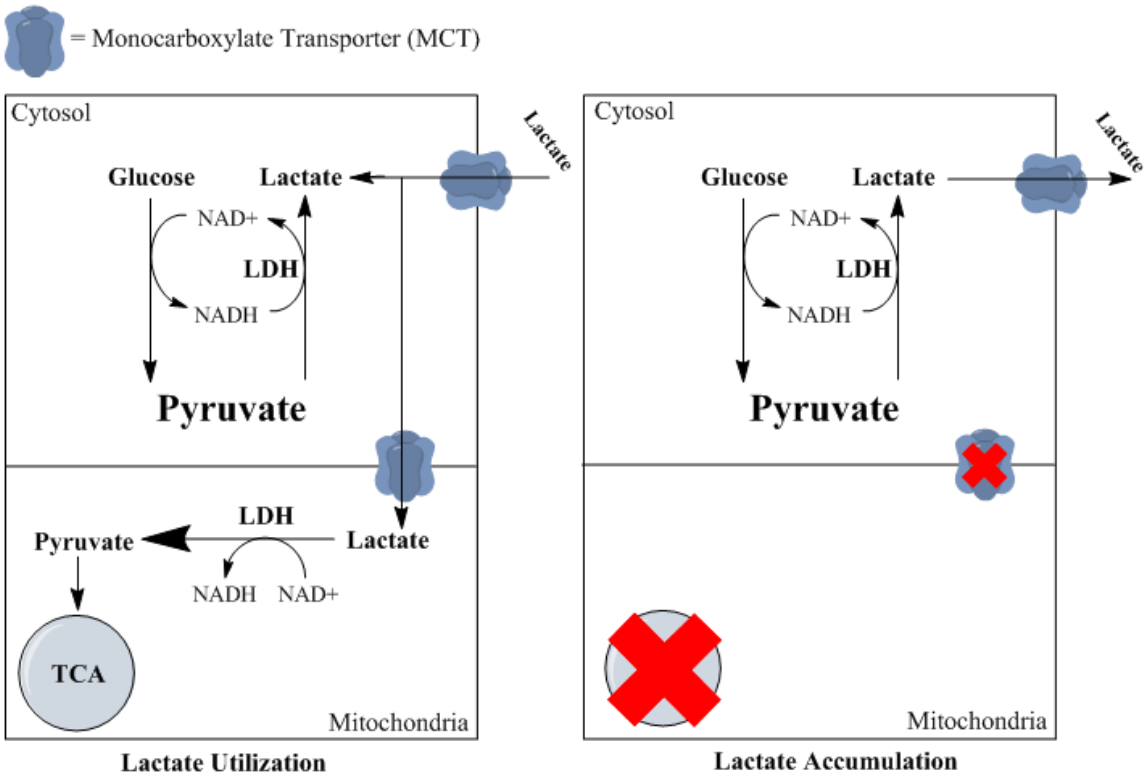

Figure 1-4. Monocarboxylate Transporter (MCT) Dysregulation.
Lactate is utilized in the mitochondria to convert to pyruvate and fuel the tricarboxylate acid cycle (TCA). When there is cellular dysfunction, either producing excess lactate that overwhelms the system, or through dysfunction in MCTs, the TCA cycle does not take place, creating a feed forward mechanism whereby lactate accumulates in the tissue.

As a means of reducing lactic acidosis experienced in muscles in an anaerobic environment, gluconeogenesis occurs in the liver. Through this

process, lactate is converted back into pyruvate via LDH, with pyruvate then converted back to glucose. This glucose travels from the liver to the muscles through the bloodstream, where the cycle repeats itself. Gluconeogenesis is a costly process, in terms of ATP consumed, so the Cori cycle cannot be sustained chronically without overwhelming the cellular systems (Figure 1-5). If lactic acidosis is sustained, this can lead to increased intracellular calcium, protein misfolding, excitotoxicty, and subsequent cell death.

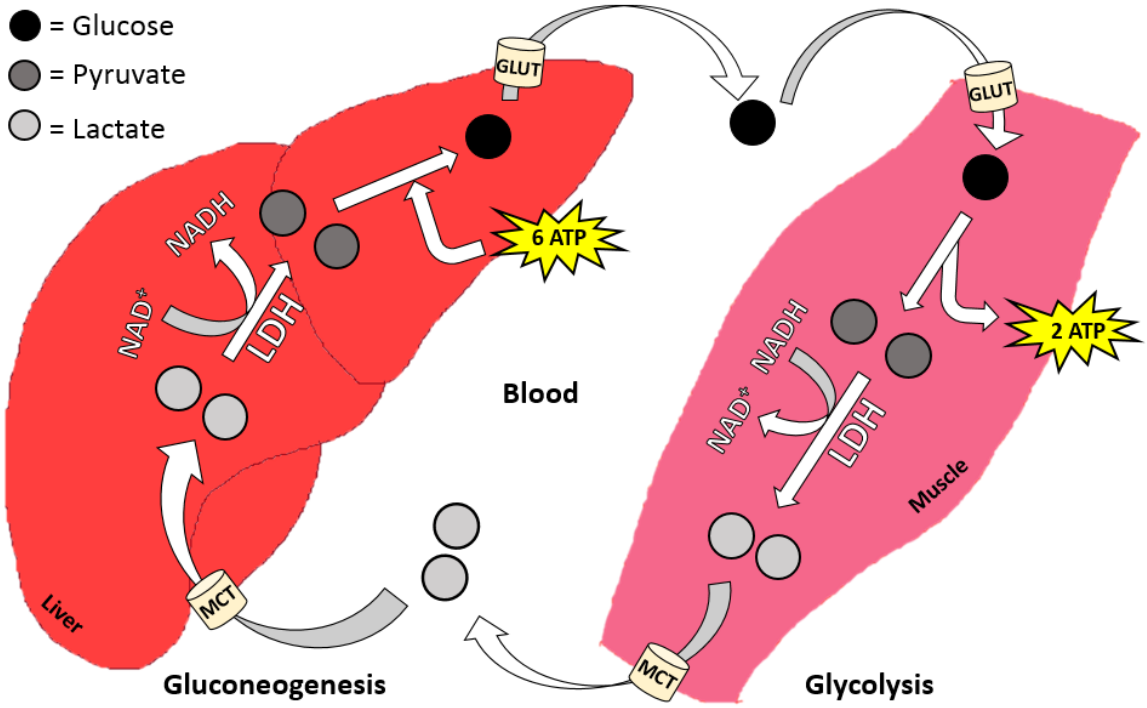

Figure 1-5. The Cori Cycle.
In exercising muscle, glucose is transported into muscle tissue by glucose transporter (GLUT), where glycolysis then takes place, converting one glucose molecule into two pyruvate molecules and generating a net gain of 2 ATP. Lactate dehydrogenase (LDH) converts the two molecules of pyruvate into two molecules of lactate, oxidizing NADH to utilize the electrons. Lactate is transported out of muscle, into the bloodstream, and into liver cells through the monocarboxylate transporter (MCT). In the liver, gluconeogenesis takes place, converting lactate back into glucose, at the expense of 6 ATP.

Lactate transfer across cell membranes is facilitated by transmembrane proteins called monocarboxylate transporters (MCTs). As their name suggests, these co-transporters allow for bi-directional movement of monocarboxylates (such as lactate and pyruvate) and protons across cellular membranes in a concentration and pH-dependent manner (34). These transporters allow for the flow of lactate from astrocytes to neurons to be used as an energy substrate in anaerobic conditions (8, 49, 53, 64, 70), and also intracellular expression for movement of lactate in and out of mitochondria (29, 34, 60). Dysregulation of MCT's are implicated in rises in lactate concentrations and subsequent acidosis and if prolonged, cell death (53).

Recent literature is pointing towards the significance of lactate as an energy substrate. The biochemical make-up of blood, extracellular fluid, and CSF are varying, with specific regulatory measures for maintaining proper homeostasis. These different fluids have varying content of energy substrates: it has been reported via microdialysis data collected from humans and rats that baseline glucose and lactate concentration in the extracellular fluid of the cortex and hippocampus is about 1-2 mM and 2-5 mM (respectively), with blood glucose and lactate concentrations reaching about 5-7 mM and 1-2 mM (respectively) (1). Another study with a rat model showed that *in vivo*, a shift in energy substrate concentrations occurred in extracellular fluid upon electrical stimulation. Increased neuronal activity induced a decrease in glucose concentration and a

significant increase in lactate concentrations (36). Taken together, these findings point towards the potential of lactate as a preferred energy source for neurons.

Interestingly, in majority of persons diagnosed with ALS, a neurodegenerative disease characterized by gradual loss of motor neurons and subsequent muscular atrophy, the muscular function of the eyes remain working. It has been shown that ocular muscles and nerves use lactate as an energy substrate, thus these tissues do not fatigue when exposed to lactic acid as is seen in skeletal musculature (4). Furthermore, it has been reported that LDH activity is detected in eye muscles (37) and oculomotor neurons (33). Taken together, these findings suggest that the eye muscles and associated neurons possess the cellular machinery to withstand abnormally high amounts of lactate without subsequent cellular degeneration. The existence of an ATP-dependent muscular-neuronal lactate shuttle (MNLS) has been proposed as a means of sustaining lactate homeostasis among motor neurons and muscles (44). Dysfunction among LDH, mitochondria and/or the MNLS are all proposed mechanisms by which lactate can accumulate at the neuromuscular junction (NMJ) and contribute to the toxicity factor in the pathogenesis of ALS.

1.5 Cerebrospinal Fluid Abnormalities

Cerebrospinal fluid (CSF) lactate concentrations are used as an analytical tool in diagnosing nervous system infections, such as bacterial and aseptic meningitis (54). Alteration in lactate CSF levels has been gaining momentum in

the research world as an indicator of many neurodegenerative diseases. A 2016 study reported elevated concentrations of CSF lactate in relapsing-remitting multiple sclerosis (MS) patients (n=118) that increased with increased disease progression, indicated by factors such as progression index (PI), MS severity scale (MSSS), and Beyesian risk estimate for MS (BREMS) (2). Also indicated in this study was a positive and significant correlation between CSF lactate and CSF concentrations of tau and neurofilament proteins, well-accepted markers for neurodegenerative disorders. A recent study of patients who were diagnosed with a variety of neurodegenerative diseases such as ALS, PD, and Huntington's disease, demonstrated an increase in CSF lactate levels of neurodegenerative patients aged 65 years and older compared to their younger counterparts, and a positive correlation between CSF lactate and glucose levels.

An elevation of lactate in the CSF (typically considered > 2.1 mM) can be a crucial indicator of mitochondrial dysfunction, which is implicated in neurodegenerative disease progressions as mentioned. It has been shown however, that patients with mitochondrial disease may have normal CSF lactate concentrations except when enduring cellular stress, as experienced in metabolically demanding tasks such as strenuous exercise (32). If a disease presents with mitochondrial dysfunction, this would facilitate an environment of increased metabolic stress, thus increasing the levels of lactate that accumulate in the CSF.

1.6 Hypothesis

Major findings in cellular mechanistic neurodegenerative studies include: increased ROS, glutamate excitotoxicity, increased cytosolic calcium, and cell and body acid/base issues (Figure 1-6).

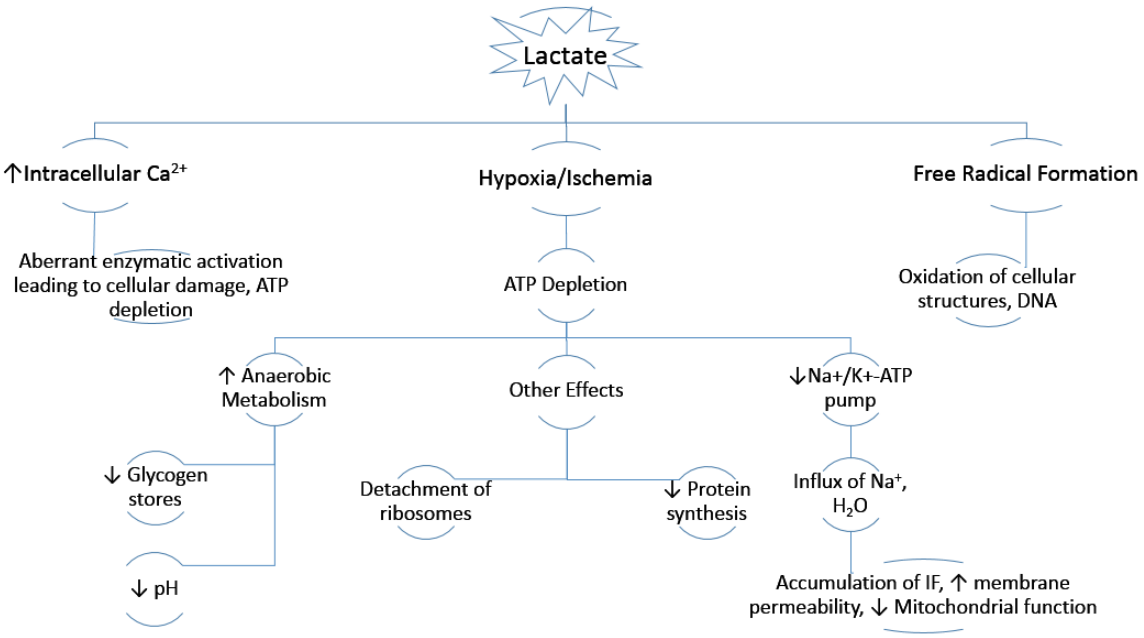

Figure 1-6. Lactate Accumulation Contribution to Cytotoxicity.

We hypothesized that since a majority of neurodegenerative diseases present with elevated lactate concentrations, the lactate may serve as an exictatory feed forward mechanism propagating not only cell death, but also contributing to β-amyloid and cognitive decline. Therefore, in this study we explored the effects of lactate on apoptosis, cytsolic reactive oxygen species, intracellular calcium concentrations, and cytosolic pH in dopaminergic-like PC12 cells.

2 Elevated L-lactate Drives Major Cellular Pathologies Associated with Neurodegeneration

2.1 Abstract

Within the past few decades, lactate research has expanded from initial findings deeming lactate as a dead-end metabolic product to recognition of lactate's role as a potential energy substrate in the CNS. Due to the tight relationship between lactate and energy metabolism, interest in the scientific community has been mounting around associations among metabolic dysregulation, elevated lactate and neurodegenerative states such as AD, PD, ALS, I/R injuries, and physiological aging, however underlying cellular mechanisms and/or facilitators for neuronal degeneration pathologies still remain unknown. Here, we tested several hypotheses that implicate L-lactate to various neurodegenerative mechanisms: increases in cytosolic calcium, increases in cytosolic reactive oxygen species (ROS), increases in intracellular acidification, and subsequently, increases in cellular death in primary neuronal cultures and dopaminergic-like NGF-derived PC12 cells. Treatment of cells with a pathologic concentration of L-lactate (4 mM) induced significant increases in cytosolic calcium, cytosolic ROS, and intracellular acidification measured in real time with fluorescent markers and quantified using ImageJ software. Furthermore, treatment with L-lactate (4 mM) induced significant increases in cell death, measured in real time and using flow cytometry in support. We conclude that L-lactate increases cellular events commonly observed in cells undergoing degeneration, providing support for the implications of lactate in neurodegenerative disorders.

2.2 Introduction

Cell homeostasis is critical to healthy bodily functions, and fluctuations in the cell environment lead to the activation of various compensatory mechanisms to ensure appropriate function. Proper homeostatic function is especially critical in the central nervous system (CNS), as pathological changes in intracellular and extracellular environment of neurons are observed in patient's experiencing neurodegeneration, both in disease models and as a result of normal aging (11, 15, 49, 69).

. A key player in energy homeostasis within cell bodies, including neurons, are the mitochondria. Active mitochondria generate ATP and NAD+ for cells, and in neurons this provides energy to keep pre- and post-synaptic sites functioning properly (70, 73). The early symptoms of dementia such as confusion and memory loss have been linked to synaptic dysfunction due to build up of β-amyloid oligomers characteristic of AD (20) and increased oxidative stress in mitochondria (47, 65, 66). Age has also been identified as one of the greatest risk factors for development of dementia (69), however additional factors such as glutamate excitotoxicity (8, 44, 45, 62), intracellular calcium-induced cascades (30, 63), oxidative stress (23, 57), and inflammatory factors (35, 47) can also increase the risk of developing neuronal loss characterized in degenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and normal human aging. Mitochondrial dysfunction can lead to reduced energy/ATP generation

(11), impaired free radical scavenging capabilities (52) and dysfunctional monocarboxylate transporters (29, 34, 70). It is through these impaired cellular processes that ROS accumulates in cell bodies such as neurons, eventually triggering an apoptotic-cascade ending in neuronal death. The Alzheimer's Association estimates that 5.5 million Americans live with AD (2017), with majority of AD patients at least 65 years of age or older. Due to advances in medicine and subsequent prolonged life expectancy, the prevalence of neurodegenerative disorders is predicted to dramatically increase, pressing a huge demand on the scientific and medical community for better understanding of neurodegeneration to employ more effective treatments.

For most of the 19th and 20th centuries, lactate was only regarded as a dead-end product in exercising muscles after metabolism, but current research is reconsidering the role and significance of lactate as: (1) a fuel source to meet the energetic requirements of neurons (4, 8, 14, 26, 29, 44, 53, 56, 64, 70, 73) and (2) a necessary signaling molecule for neuronal plasticity (71). Furthermore, research has been conducted in primary culture mouse neurons and *in vivo* showing that L-lactate can function as a signaling molecule that stimulates plasiticity-related gene expression in neurons (71), in addition to functioning as an energy substrate for neurons. On a molecular basis, L-lactate functions as a mediator for long-term plasticity and memory through the induction of expression of immediate early genes (IEGs), such as early growth response 1 (Egr1), c-Fos, and Arc. This is achieved via action at N-methyl-D-aspartate receptors (NMDA-

R), as MK801, an NMDA-R antagonist, completely abolished L-lactate-induced Arc and Egr1 expression (71).

There is a growing basis of research indicating that accumulation of lactate in the central nervous system, through impairment of MCTs (29, 34, 70), altered activity of lactate dehydrogenase (LDH), and/or hypoxic/ischemic conditions (63), can lead to metabolic acidosis, increased ROS, and increased levels of intracellular calcium. In anaerobic conditions, as occurs in hypoxia or ischemia, mitochondrial oxidative phosphorylation is impaired, leading to an increased production of lactate and if prolonged, deleterious cellular responses such as cytosolic acidosis (42). Protein structure and function is largely regulated by chaperone molecules, such as GRP78 and CHOP, and lactic acidosis can contribute to conformational changes to these molecules, reducing their activity and further exacerbating the acidosis. Blood lactate concentrations are usually 1-2 mM at rest, however with excess accumulation as experienced during intense exercise, these levels can exceed 20 mM (12). Blood or CSF lactate increases (>2.1 mM) (32) can be an indicator of mitochondrial disease, however this dysfunction can go undetected in patients due to normal pyruvate and lactate levels except when the individual is exercising or has been subjected to adverse metabolic conditions (such as seen in ischemic injuries and neurodegenerative states). Rises in CSF lactate have been well established in neurodegenerative disease models (2, 3, 44, 49, 62, 71, 72).

In this study we attempt to eluicidate more precise cellular mechanisms associated with the cognitive and memory decline surrounding

neurodegeneration. As the CNS functions primarily though the use of neurotransmitters, such as the excitatory transmitter glutamate, disregulation or aberrant expression of neurotransmitters has been well established in CNS pathologies. The excitotoxic result of excessive glutamate binding occurs through increased calcium influx into the cell, triggering a cascade of neurotoxic cellular events, such as neuronal depolarization and binding with calmodulin to lead to increased nitric oxide (NO) formation. This can further exacerbate neurodegeneration. Based on mounting evidence, we posit that lactate is a key player in many of the pathological states that occur during the development and progression of neurodegeneration. Elevated levels of cerebral spinal fluid (CSF) lactate, chronically, can lead to cytotoxic insult and ensuing cell death (as observed in strokes, ischemia reperfusion injuries, and neurodegeneration). Therefore, we hypothesized that administration of lactate to primary neuronal cultures and dopaminergic-like NGF-derived PC12 cells would elicit increases in cell death, intracellular calcium, intracellular reactive oxygen species (ROS), and cytosolic acidification; all hallmark aspects of neurodegeration processes (Figure 2-1).

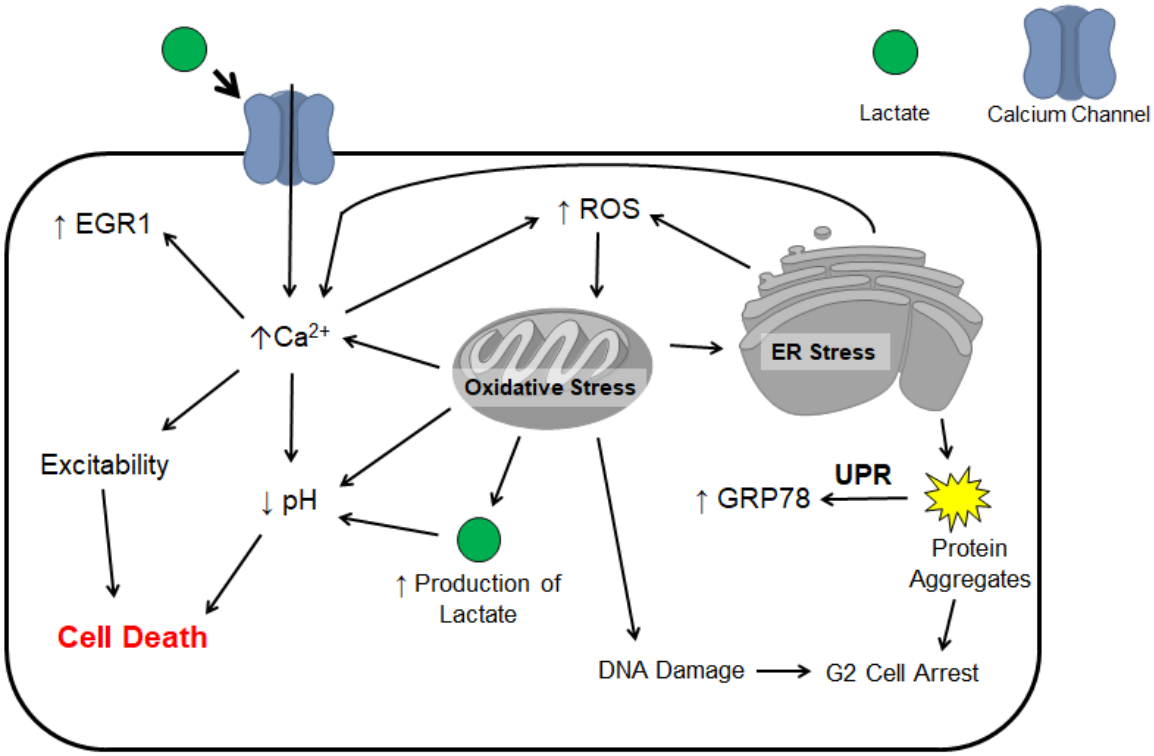

Figure 2-1. Hypothesis Model for Lactate-induced Cell Death.
Lactate binds with calcium channel receptors that induce an influx of calcium into the cell body. Intracellular calcium elicits increases in neuronal excitability and decreases in cytosolic pH that can directly contribute to cell death. Cytosolic calcium also increases reactive oxygen species (ROS) formation, creating a positive-feedback loop, inducing oxidative stress in the mitochondria, leading to increased production of lactate, decreases in pH, and further increases intracellular calcium. Oxidative stress is often followed by ER stress in dysfunctional cellular environments, further increasing cytosolic ROS. If ER stress is prolonged, protein aggregates begin accumulating, eliciting the unfolded protein response (UPR). Activation of the UPR leads to increased mRNA expression of GRP78, and the accumulation of protein aggregates can contribute to G2 cell cycle arrest. DNA damage due to oxidative stress is another contributor to G2 cell arrest. Taken together, these mechanisms can further exacerbate cell death.

2.3 Methods

2.3.1 NGF-Derived PC12 Cell Culture

PC12 cells (American Type Culture Collection, USA) were prepared by placing cells in a 37.9 °C media cocktail containing: 50 mL of Dulbecco's Modified Eagle Medium (DMEM), 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% penicillin, and nerve growth factor (NGF) at 20 ng/mL. Cells were plated according to the experiment to be run (24 well plates; ThermoFisher) and placed in a 37.5 °C humidified CO_2 (5%) incubator. Cells were grown to 75-80% confluency and not passaged more than 10 times before new cells were used.

2.3.2 Preparation of Neuronal Cultures

Whole brain neuronal cultures were made from 1 day old SD rats according to our previous publication (58). Briefly, rats were anesthetized with pentobarbital (50 mg/kg) and the brains extracted, dissected, combined, brain cells dissociated and cells plated on poly-L-lysine pre-coated culture dishes. Two days following plating, cultures were treated for two days with cytosine arabanoside (ARC, 10μM) to kill off rapidly dividing cells (astroglial and astrocytes) followed by fresh ARC free media for the remainder of cultures. Neuronal cultures contained > 90% neurons with the remainder primarily astroglial. Neuronal cell cultures were grown for 10-14 days before use in cell culture experiments.

2.3.3 Cell Treatment

Before application of treatments, cells were observed for healthy neurite outgrowth. Once cells were deemed viable, the previous cell media was aspirated and cells were rinsed with 12 mL artificial cerebral spinal fluid (aCSF) to wash away any residual media (0.5 mL/well). The lactate treatment stock solution was was diluted with DMEM to the correct concentration. The lactate treatment was heated to 37.9 °C, added to the 24 well plate (0.5 mL/well), and then placed back into the 37.5 °C humidified CO_2 (5%) incubator for 4 h. This treatment protocol was followed for all treatments in this research, with treatment times specified (1 h or 4 h).

2.3.4 Flow Cytometry

Prior to flow cytometry preparations, PC12 cells were observed for healthy neurite outgrowth/cell viability. The previous treatment media was aspirated from the wells and cells were rinsed with 12 mL artificial cerebral spinal fluid (aCSF) to wash away residual media (0.5 mL/well). Cells were then treated with 12 mL (0.5 mL/well) trypsin EDTA warmed to 37.9 °C, and gently shaken for several minutes until cells were loosened from the bottom of the wells. To neutralize the trypsin, 12 mL (0.5 mL/well) of DMEM (with 10% HS and 1% antibiotics) was added to the wells and gently swirled to ensure proper mixing of the trypsin and DMEM. This mixture from each well was transferred to individual 1.5 mL centrifuge tubes (ThermoFisher) and centrifuged for 2 m at 1000 rotations per minute (RPM's). The supernatant was aspirated from each tube and cells were re-suspended in

12 mL (0.5 mL/well) of live cell imaging solution (LCIS, ThermoFisher). One drop of propidium iodide (PI) ReadyProbes (ThermoFisher) was added to each tube of cells/LCIS, and the tubes were then vortexed to break up the cell pellet at the bottom of the tube. The tubes were covered with aluminum foil to protect against light damage and were incubated at room temperature for 20 m before starting data collection on the flow cytometer (BD Accuri C6; BD Bioscience). Flow cytometry was conducted in triplicates for each experiment with 10,000 cells per run. The PI staining was measured in percentages based off control groups and analysis parameters were kept consistent among treatment and control groups.

To measure cytosolic reactive oxygen species (ROS), CellROX Orange (ThermoFisher) probe was used instead of PI. The same cell preparation procedures were used as above, with the CellROX Orange prepped with LCIS in accordance with the protocol provided by ThermoFisher. The LCIS/CellROX cocktail was added into each 1.5 mL centrifuge tube (0.5 mL/tube), tubes were covered with aluminum foil to protect against light damage, and cells were allowed to incubate at room temperature for 30 m. After 30 m, the tubes were centrifuged at 1000 RPM's for 2m. Supernatant was aspirated, 12 mL fresh LCIS (37.9 °C) was added to the centrifuge tubes (0.5 mL/tube), and the tubes were vortexed before data collection was carried out on the flow cytometer.

2.3.5 Real-Time Imaging

Following 4 h treatment with DMEM (control) or L-lactate (4 mM) in DMEM, NGF-derived PC12 cells were prepared for real-time imaging with probes to measure for: calcium (Fluo-4AM, ThermoFisher), apoptosis (propidium iodide, ReadyProbe, ThermoFisher), ROS (CellROX Orange, ThermoFisher), and pH (pHrodo red AM, ThermoFisher).

For the calcium imaging, previous treatment media was aspirated from each well, rinsed with aCSF (0.5 mL/well), and aspirated again. Fluo-4AM dye was added to aCSF to a final concentration of 3 μM and was applied to the cells (0.5 mL/well). Cells were incubated at 37.9 °C for 30 m and then the media was aspirated from the wells. LCIS was added (1 mL/well) and viewed under an inverted microscope (Leica) with the appropriate fluorescent filters, a digital camera, mercury burner, and connection to a computer equipped with image capture software (Leica). After images were captured for each well, the backgrounds of the images were normalized and fluorescence was quantified using ImageJ software prior to background normalization. The analysis was completed using 6 randomly selected cells per treatment group and differences in intensity were compared among groups.

For the apoptosis imaging, treatment media was aspirated and each well was rinsed with aCSF (0.5 mL/well) to remove residual media. The aCSF was aspirated, cells were re-suspended in LCIS (0.5 mL/well), and 1 drop of PI ReadyProbe dye and 1 drop of NucBlue ReadyProbe (ThermoFisher) dye was added to each well according to the manufacture's instructions . The cells were covered in foil and incubated at room temperature for 30 m before imaging.

The oxidative stress detection probe, CellROX Orange, was prepared according to ThermoFisher's protocol to final concentration of 5 μM. Previous treatment media was aspirated from each well, cells were re-suspended in the CellROX reagent, and incubated at 37.9 °C for 30 m. This media was removed and 0.5 mL of fresh LCIS with 1 drop of NucBlue was added to each well, incubated at room temperature (protected from light) for 20 m before imaging.

To measure intracellular pH, pHrodo red-AM was prepared in LCIS according to manufacturer's protocol. Previous treatment media was aspirated and each well was rinsed once with LCIS (0.5 mL/well). The LCIS was aspirated, cells were re-suspended in the prepared pHrodo red-AM staining solution (0.5 mL/well), and incubated at room temperature (protected from light) for 30 m. The staining solution was removed and cells were re-suspended in fresh LCIS (0.5 mL/well) before imaging.

2.3.6 Neuronal mRNA Expression Levels

Whole brain primary neuronal cell cultures were treated with DMEM (control) or L-lactate (4mM) in DMEM for 1 h or 4 h prior to extraction. An RNA extraction kit (Qiagen) was used in accordance with the manufacturer's instructions to extract the mRNA. The mRNA was converted to cDNA by the use of SuperScript VILO (ThermoFisher), with concentrations normalized to 250 ng/μL. Real time PCR (StepOnePLus real-time PCR system, Applied Biosystems) was conducted to measure mRNA levels of GAPDH (control), c-Fos, EGR1, GRP78, CHOP, and

VMAT2 using the respective primers (Applied Biosystems). Experiments were performed in quadruplicates and data was analyzed via Data Assist V. 3.01 (Applied Biosystems) for changes in mRNA expression levels using GAPDH as the selected control.

2.3.7 Statistical Analysis

Data values were reported as mean ± SE. Group means were compared using either unpaired Student's *t*-test, or a one-way ANOVA (depending upon the experiment). Significant difference between means was considered at $p < 0.05$. Bonferroni post hoc tests were used for multiple pair-wise comparisons among differences. Statistical analyses were performed using GraphPad Prism, version 5.0.

2.3.8 Chemicals

All chemicals/cell culture reagents and supplies were obtained from ThermoFisher, USA, with the exception of nerve growth factor (NGF, Sigma Aldrich) and L-lactate (Sigma Aldrich).

2.4 Results

2.4.1 L-lactate Increases Cytosolic Calcium in Primary Neuronal Cultures

Primary neuronal cultures were incubated with DMEM (control) (Figure 2-2A) or L-lactate (4 mM) in DMEM (Figure 2-2B) for 4 h, co-stained with Fluo-4AM

(ThermoFisher), a Ca^{2+} binding fluorescent dye, and NucBlue, and imaged in real-time under an inverted microscope.

Fluorescence intensity was quantified using ImageJ software. Treatment of cells with L-lactate (4 mM) significantly ($p < 0.05$) increased the amount of cytosolic calcium in both neurons and PC12 cells (Figure 2-2C), compared to control.

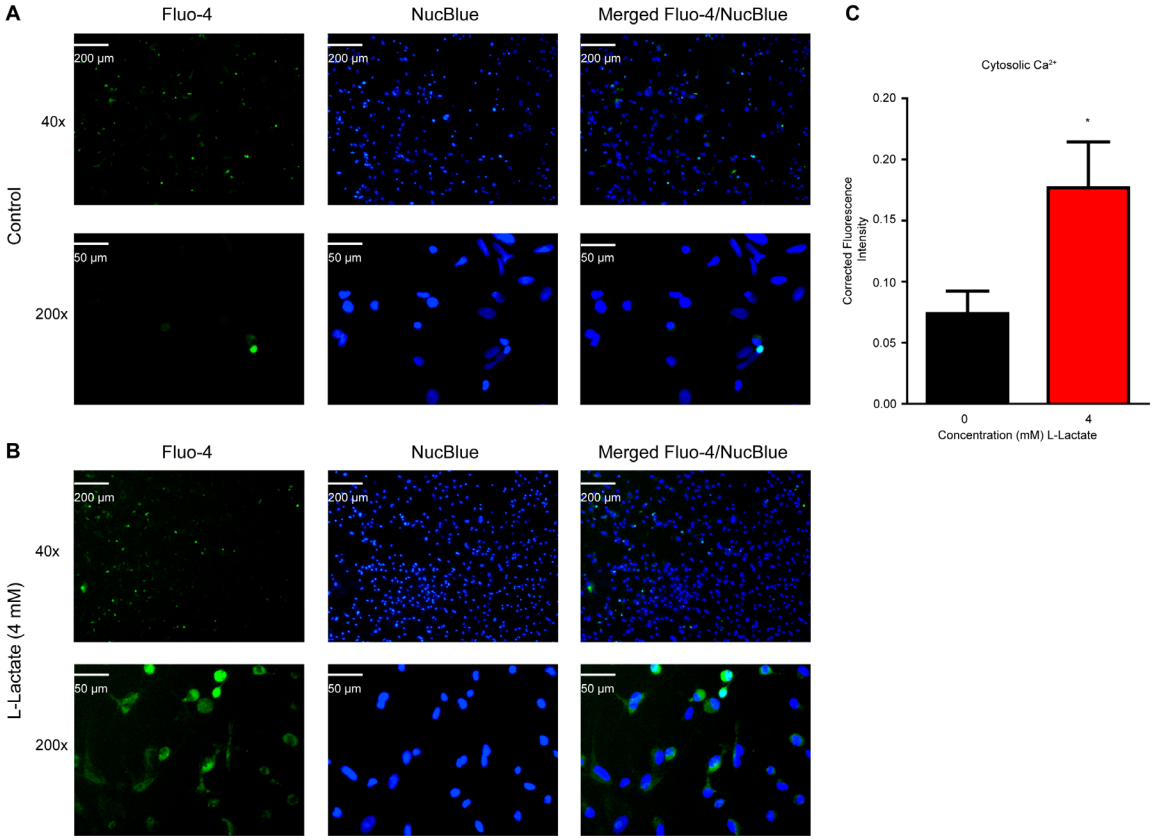

Figure 2-2. L-lactate Increases Cytosolic Calcium in Neurons.
(A) Control cells were treated with DMEM for 4 h before being co-stained with Fluo-4AM and NucBlue. Real-time images were taken at 40x and 200x magnification. (B) Cells were treated for 4 h with L-lactate (4 mM) before being prepped for imaging. Real-time images were taken at 40x and 200x magnification. (C) The fluorescence intensity of the neurons treated with L-lactate (4 mM) or DMEM (control) and stained with Fluo-4AM, a cytosolic calcium stain, was quantified. The lactate treatment significantly ($p < 0.05$) increased the amount of cytosolic calcium compared to control.

2.4.2 L-lactate Increases Cytosolic ROS in Primary Neuronal Cultures and Dopaminergic-Like PC12 Cells

Primary neuronal cell cultures were treated with either control DMEM (Figure 2-3A) or 4 mM L-lactate (Figure 2-3B), co-stained with cellROX orange and NucBlue (ThermoFisher), and analyzed in real time under an inverted microscope with appropriate fluorescent burner. Fluorescence intensity was quantified using ImageJ software. Treatment of cells with 4 mM L-lactate (n = 6) significantly increased the amount of cytosolic reactive oxygen species (ROS) compared to control (n = 6) (Figure 2-3C).

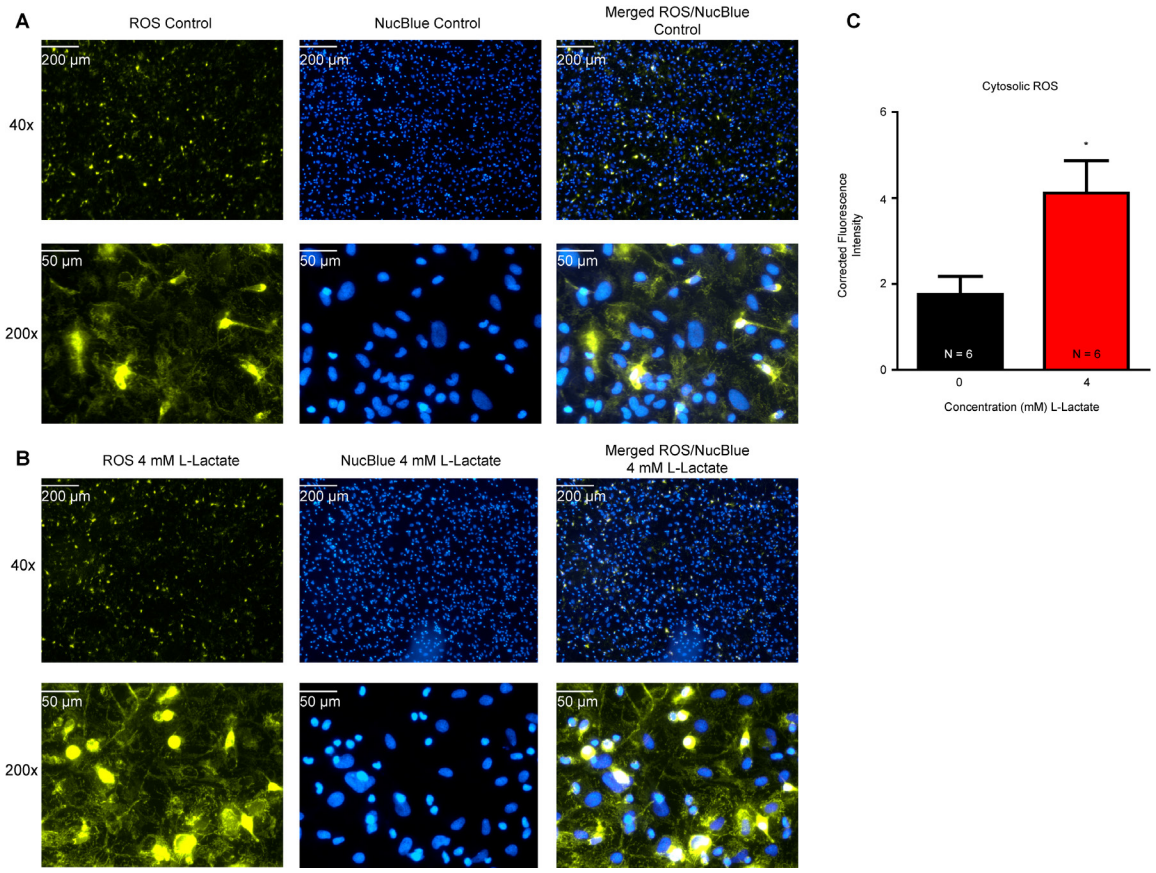

Figure 2-3. L-lactate Increases Cytosolic ROS in Neurons.
(A) Control cells were treated with DMEM for 4 h before being co-stained with CellROX orange and NucBlue. Real-time images were taken at 40x and 200x magnification. (B) Cells were treated for 4 h with L-lactate (4 mM) before being prepped for imaging. Real-time images were taken at 40x and 200x magnification. (C) The fluorescence intensity of the neurons treated with L-lactate (4 mM) or DMEM (control) and stained with CellROX orange, a cytosolic ROS stain, was quantified. The lactate treatment significantly ($p < 0.05$) increased the amount of cytosolic ROS compared to control.

NGF-derived PC12 cell cultures were treated with either control DMEM (Figure 2-4A) or 4 mM L-lactate (Figure 2-4B), co-stained with cellROX orange and NucBlue (ThermoFisher), and analyzed in real time under an inverted microscope with appropriate fluorescent burner. Fluorescence intensity was quantified using ImageJ software. Treatment of cells with 4 mM L-lactate (n = 6)

significantly increased the amount of cytosolic reactive oxygen species (ROS) compared to control (n = 6) (Figure 2-4C).

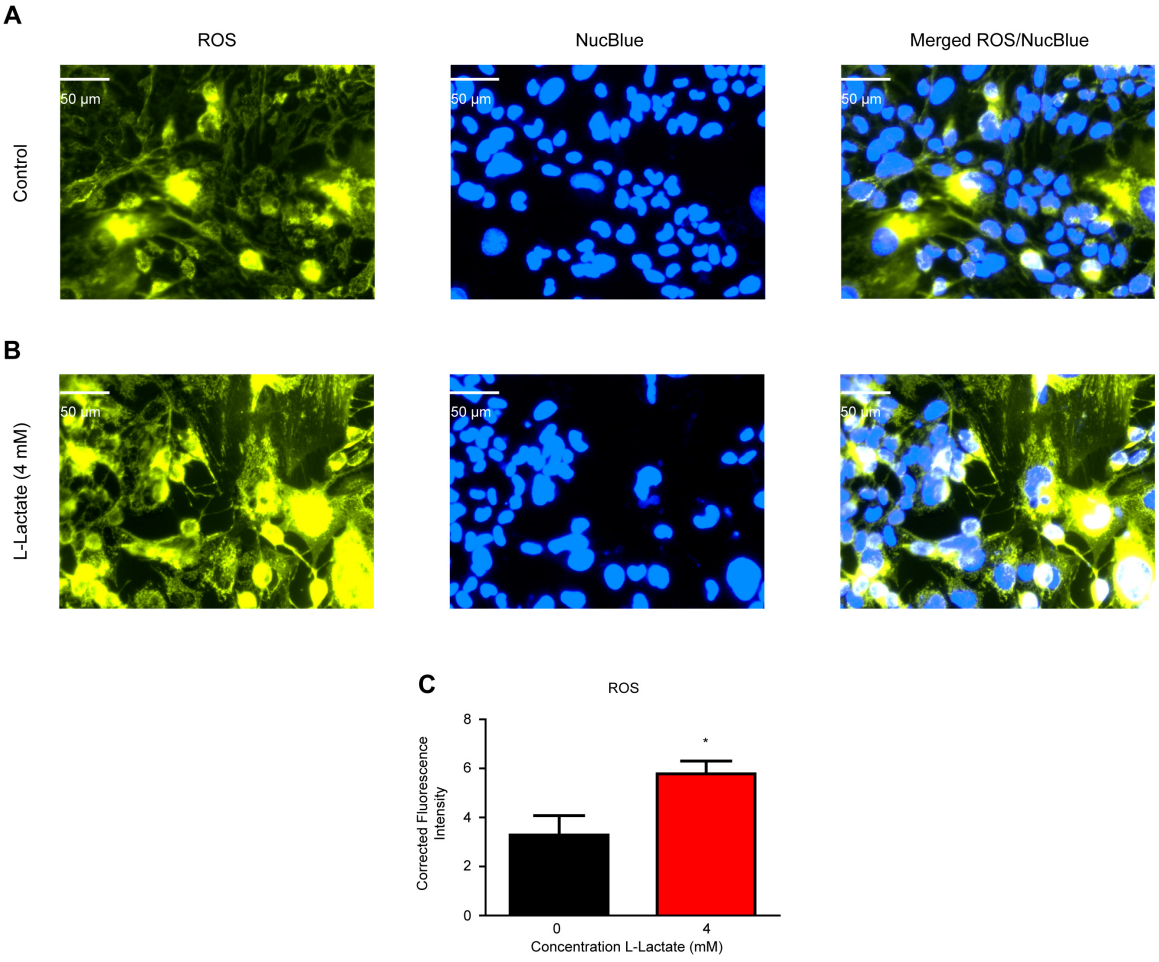

Figure 2-4. L-lactate Increases Cytosolic ROS in PC12 Cells.
(A) Control cells were treated with DMEM for 4 h before being co-stained with CellROX orange and NucBlue. Real-time images were taken at 200x magnification. (B) Cells were treated for 4 h with L-lactate (4 mM) before being prepped for imaging. Real-time images were taken at 200x magnification. (C) The fluorescence intensity of the PC12 cells treated with L-lactate (4 mM) or DMEM (control) and stained with CellROX orange, a cytosolic ROS stain, was quantified. The lactate treatment significantly ($p < 0.05$) increased the amount of cytosolic ROS compared to control.

2.4.3 L-lactate Increases Intracellular Acidification in Primary Neuronal Cultures and Dopaminergic-Like PC12 Cells

Primary neuronal cultures (Figure 2-5A) and NGF derived PC12 cells (Figure 2-5C) were treated with either control DMEM or L-lactate (4 mM) for 4 hrs and then stained with pHrodo-red (ThermoFisher), a pH indicator that increases in fluorescence as pH becomes more acidic, and analyzed in real time. Images were captured for both treatment groups at 200x magnification and for PC12 cells at 40x magnification.

Fluorescence intensity was quantified using ImageJ software. Treatment of cells with 4 mM L-lactate significantly ($p < 0.01$) increased the amount of intracellular acidification compared to control, in both neuronal cultures (Figure 2-5B) and PC12 cells (Figure 2-5D).

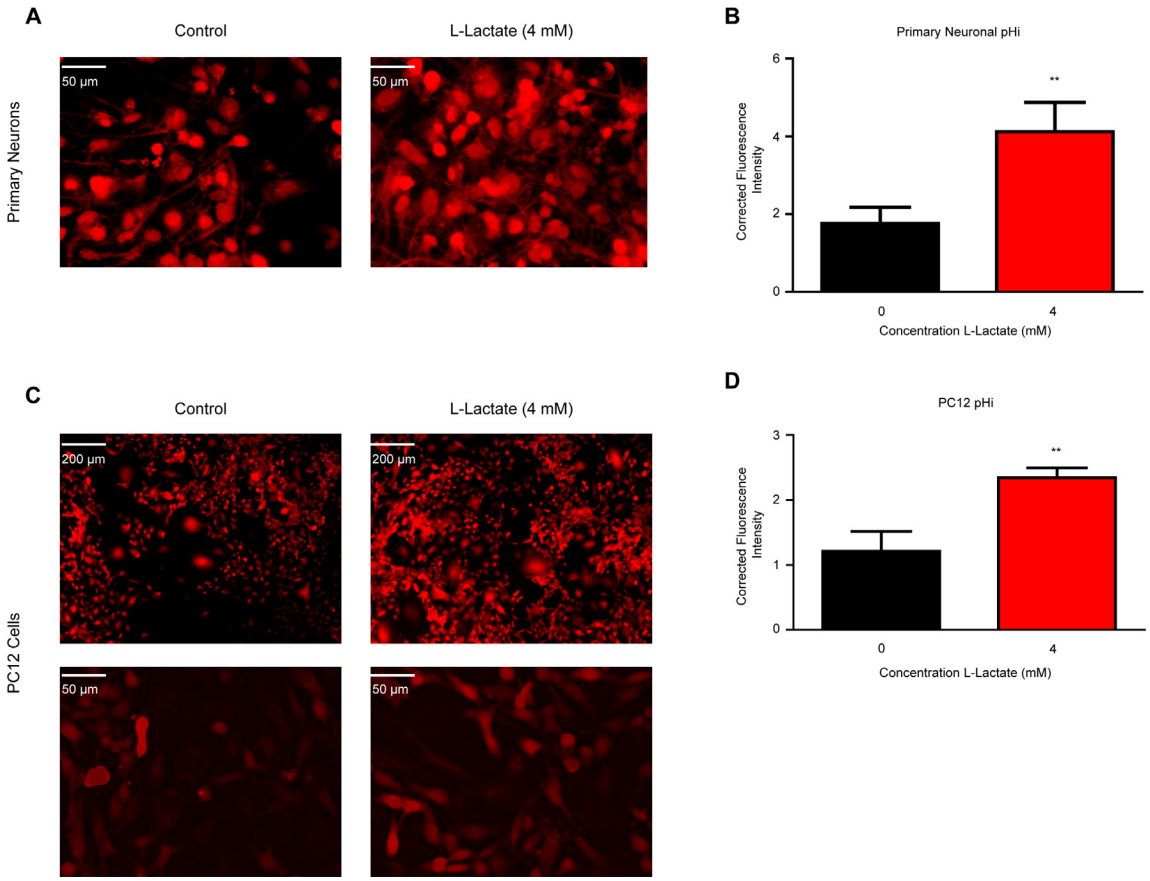

Figure 2-5. Real Time pH Imaging.
(A) Neurons were treated with DMEM as a control or L-lactate (4 mM) and stained with pHrodo red. Images were taken at 40x. (B) L-lactate significantly ($p < 0.01$) increased the amount of cytosolic acidification in neuronal culture treated for 4 h. (C) PC12 cells were treated with DMEM or L-lactate (4 mM) for 4 h before being stained with pHrodo red. Images were taken at 40x and 200x magnification. (D) L-lactate significantly ($p < 0.01$) increased the amount of cytosolic acidification in PC12 cells treated for 4 h.

2.4.4 L-lactate Alters Expression of Synaptic Plasticity Genes c-Fos and EGR1 in Primary Neuronal Cultures

Primary neuronal cultures incubated with L-lactate (4 mM) had significantly ($p < 0.0001$) increased mRNA expression levels of the immediate early gene (IEG), c-Fos (Figure 2-6A), at 1 h versus control (5.77 ± 0.38 vs 1.01 ± 0.06 fold). The mRNA expression level of c-Fos at 4 h decreased significantly

($p < 0.0001$) versus 1 h (0.10 ± 0.03 vs 5.77 ± 0.38) and control (0.10 ± 0.03 vs 1.01 ± 0.06 fold).

Neurons treated with L-lactate (4 mM) induced a significant ($p < 0.05$) increase in mRNA expression of the immediate early gene (IEG) and transcription factor, early growth response 1 (EGR1) at 1 h compared to control (2.16 ± 0.17 vs 1.01 ± 0.06 fold). No difference was found at 4 h, as levels returned towards baseline. (Figure 2-6B).

2.4.5 L-lactate Induces Unfolded Protein Response in Primary Neuronal Cultures

Primary neuronal cultures treated with L-lactate (4 mM) elicited a significant ($p < 0.0001$) decrease in the mRNA expression levels of CHOP (also known as DDIT3), a protein activated during ER stress, at 4 h versus control (0.06 ± 0.00 vs 1.01 ± 0.06), with no change in mRNA levels found at 1 h (Figure 2-6C).

A significant ($p < 0.05$) increase in mRNA expression levels of GRP78 (also called HSPA5 or BiP) an ER chaperone, was induced in neurons at 4 h following L-lactate (4 mM) treatment versus control (1.40 ± 0.09 vs 1.01 ± 0.06, although no significance was found at 1 h (Figure 2-6D).

2.4.6 L-lactate Increases VMAT2 mRNA Expression in Primary Neuronal Cultures

Primary neuronal cultures treated with L-lactate (4 mM) elicited a significant ($p < 0.0001$) increase in the mRNA expression levels of VMAT2, a protein involved in maintenance of neuronal vesicles, at 1 h versus control (0.06 ± 0.00 vs 1.01 ± 0.06), with no change in mRNA levels found at 4 h (Figure 2-6E).

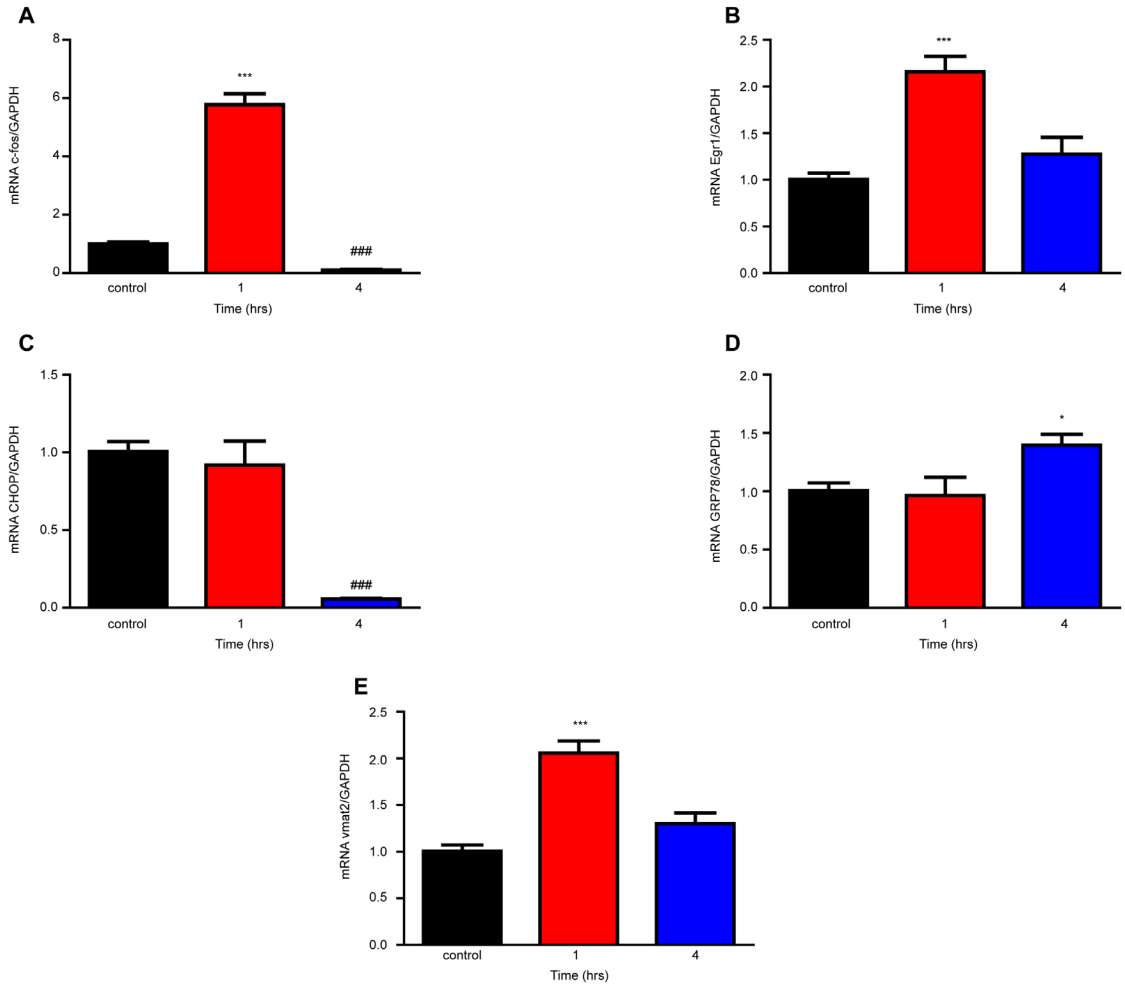

Figure 2-6. Effect of L-lactate on mRNA Expression Levels in Primary Neuronal Cultures.

(A) L-lactate (4 mM) significantly ($p < 0.0001$) increased the mRNA expression level of c-Fos at 1 h compared to control, and significantly decreased the mRNA levels of c-Fos at 4 h compared to 1 h (*** vs control, ### vs 1 h). (B) L-lactate (4 mM) significantly ($p < 0.0001$) increased the mRNA expression level of EGR1 at 1 h compared to control, although no difference was observed at 4 h (*** vs control). (C) L-lactate (4 mM) significantly ($p < 0.0001$) decreased the mRNA expression level of CHOP at 4 h compared to control (### vs control). No difference was observed at 1 h. (D) L-lactate significantly ($p < 0.01$) increased the mRNA expression level of GRP78 at 4 h compared to control (* vs control). (E) L-Lactate significantly ($p < 0.0001$) increased mRNA expression level of VMAT2 at 1 h compared to control (*** vs control).

2.4.7 L-lactate Increases Cell Death in Primary Neuronal Cultures and NGF-Derived PC12 Cells

Neuronal cultures were treated with either control DMEM or 4 mM L-lactate in DMEM, loaded with PI and NucBlue ready probes (ThermoFisher), and analyzed in real time. The 4 mM L-lactate treatment stimulated an increase in cells staining positive for PI compared to control (Figure 1E). There was no significant ($p < 0.05$) difference between the amount of neurons staining positive for NucBlu between control and the treatment group. Images were captured for controls at 40x magnification and 200x magnification (Figure 1A) and 4 mM L-lactate at 40 and 200x magnification (Figure 1B).

Neuronal cultures treated with either control DMEM (n=5) or 4 mM L-lactate (n=6) were stained with PI and analyzed via flow cytometry. The amount of cells staining positive for PI increased in the 4 mM L-lactate treatment group (31.6%) compared to the control group (21.4%) (Figure 1D).

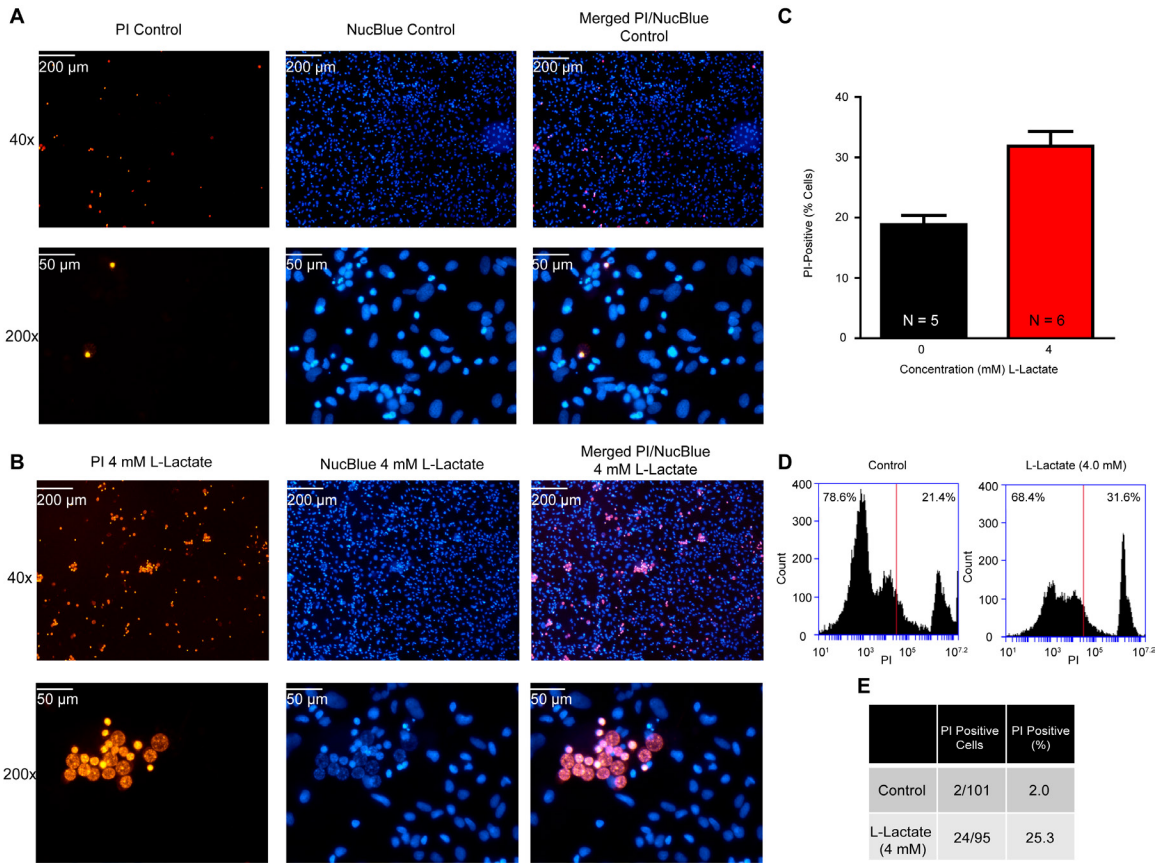

Figure 2-7. L-lactate Increases Cell Death in Primary Neuronal Cultures.
(A) Cells were treated with DMEM as a control, **co**-stained with PI and NucBlue, and imaged using an inverted microscope (40x and 200x magnification). (B) Cells were treated with 4 mM L-lactate for 4 h, **co**-stained with PI and NucBlue, and imaged using an inverted microscope (40x and 200x magnification). (C) L-lactate (4 mM) had a trend to increase PI stained cells compared to control, although no statistical difference was found. (D) L-Lactate PI Flow Cytometry. Cells treated with control DMEM for 4 h experienced 21.4% of the population staining positive for PI. Cells treated with 4 mM L-lactate for 4 h experienced 31.6% of the population staining positive for PI. (E) PI Fluorescence Quantification. The ratio and percentage of neurons that fluoresced positively for PI staining when treated for 4 h with either control or 4 mM L-lactate.

NGF-derived PC12 cells treated with either DMEM as a control (Figure 2-8B) or increasing concentrations of L-lactate [1.5 mM (Figure 2-8C), 2 mM (Figure 2-8D), 3 mM (Figure 2-8F), 4 mM (Figure 2-8G), 8 mM (Figure 2-8H)] increased in percentage of cells staining positive for propidium iodide (PI) in a

dose dependent manner (14.2, 18.5, 20.8, 27.2, 36.8, 53.4, respectively), analyzed via flow cytometry.

A dose curve was plotted for the increasing concentrations of L-lactate, and the EC_{50} was calculated to be 3.28 mM (Figure 2-8A). The experiment was repeated at the L-lactate concentration of 4 mM and there was a significant ($p > 0.05$) increase in percentage of cells staining positive for PI compared to control (Figure 2-8E).

PC12 cells were treated with either control DMEM (Figure 2-8I) or 4 mM L-lactate in DMEM (Figure 2-8J, 2-8K), pre-loaded with PI and NucBlue ready probes (ThermoFisher), and their fluorescence was quantified. Cells treated with 4 mM L-lactate significantly ($p < 0.05$) increased in percentage stained positive for PI compared to control, however there was no significant difference in percentage stained positive for NucBlue between the two groups.

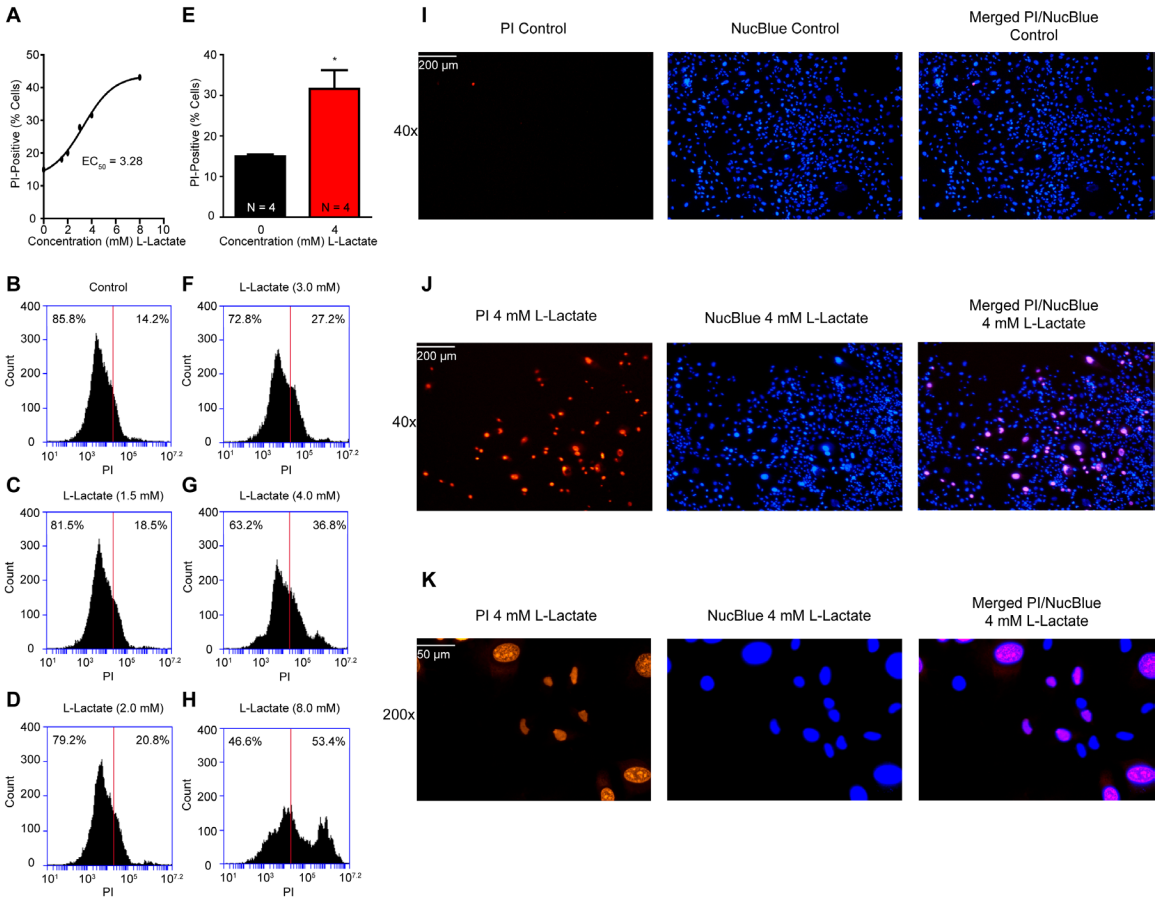

Figure 2-8. L-lactate Increases Cell Death in PC12 Cells.
(A) L-lactate induced a dose-dependent increase in cells staining positive for PI, with an EC50 value of about 3.3 mM. Cells were treated with (B) control DMEM, (C) 1.5 mM L-lactate, (D) 2 mM L-lactate, (F) 3 mM L-lactate, (G) 4 mM L-lactate, or (H) 8 mM L-lactate for 4 hours before being stained with PI and analyzed via flow cytometry. The percentage of cells stained positive for PI is the value to the right of the red line. (E) 4 mM L-lactate significantly ($p < 0.05$) increased the percentage of cells stained positive for PI compared to control. (I) Cells were treated with DMEM as a control, co-stained with PI and NucBlue, and imaged using an inverted microscope (40x magnification). (J) Cells were treated with 4 mM L-lactate for 4 h, co-stained with PI and NucBlue, and imaged using an inverted microscope (40x magnification) and (K) 200x magnification.

2.5 Discussion

The present study evaluated the role L-lactate contributes towards toxicity and neuronal degeneration in both primary neuronal cultures and dopaminergic-like NGF-derived PC12 cells. While a majority of literature on lactate/lactic acid

from the 20th century concluded this substrate as being a dead-end metabolic product present in exercising muscles, current research is growing towards more important implications in CNS function and dysfunction. As such, we report molecular mechanisms by which L-lactate can induce degenerative cascades in primary neuronal cultures and dopaminergic-like PC12 cells.

The cytotoxic role of calcium ions to neurons has been well established in the literature. Calcium build-up occurs intracellularly as a result of impaired transporters, mitochondrial dysfunction, ER dysfunction, or a combination of these factors. Increased levels of intracellular calcium can lead to excessive neuronal depolarization, leading to “excitotoxicity” and subsequent cell death. Due to the similarity in chemical structure between glutamate, the primary excitatory neurotransmitter, and lactate, we hypothesized that elevated lactate concentrations would elicit increases in intracellular calcium. To evaluate the effects of L-lactate on intracellular calcium levels, we treated primary neuronal cultures with 4 mM L-lactate for 4 h and stained the cells with Fluo-4AM, a fluorescent cytosolic calcium dye. Real-time imaging quantification revealed that L-lactate significantly increased the percentage of cells staining positive for Fluo-4AM. It is likely that a combination of events contribute to the sustained increase of calcium, such as activation of Ca^{2+}-dependent receptors such as NMDA-R, or Ca2+ leak from organelles such as the mitochondria during oxidative stress, or the endoplasmic reticulum during ER-stress.

Oxidative stress is a cellular condition that occurs when the generation of reactive oxygen species (ROS) surpasses the ability of the body to neutralize and effectively eliminate the ROS. It was not surprising for us to see increases in cytosolic ROS following L-lactate (4 mM) treatment, as this has been established in the literature from mitochondrial generated ATP. Mitochondrial dysfunction can lead to reduced energy/ATP generation (11), impaired free radical scavenging capabilities (52) and dysfunctional monocarboxylate transporters (29, 34, 70). It is through these impaired cellular processes that ROS accumulates in cell bodies such as neurons, eventually triggering an apoptotic-cascade ending in neuronal death.

A crucial component of cellular homeostasis is intracellular pH levels, and this is often disturbed in neurodegenerative models (AD, PD) as well as ischemia (42). Modest decreases in pH have been shown to provide protective defenses for cells, however severe acidosis (intracellular pH less than 6.4) increases cellular damage observed in the pathologies listed above. It has been demonstrated that oxidative stress can disrupt oxidative phosphorylation, leading to increased production of lactate, with ensuing acidosis (42). As such, in this study we were able to demonstrate that L-lactate, at elevated concentrations commonly found in neurodegenerative disease models (4 mM), induces cytosolic acidification, perhaps as a response to increased intracellular calcium, increased ROS, or a combination.

Research has been conducted in primary culture mouse neurons and *in vivo* showing that L-lactate can function as a signaling molecule that stimulates

plasiticity-related gene expression in neurons (71). L-lactate functions as a mediator for long-term plasticity and memory through the induction of expression of immediate early genes (IEGs), such as early growth response 1 (EGR1) and c-Fos. The expression of EGR1 is widely regulated by multiple environmental factors and is implicated heavily in synaptic plasticity and information encoding processes, suggesting a role for EGR1 in perceving the environment and forming an appropriate response to stimuli. Furthermore, EGR1 has been associated in the literature with neuropsychiatric illnesses where synaptic plasticity and activity is dysregulated, such as stress-related mood disorders, schizophrenia, and addiction models (21). Also, increased levels of EGR1 have been found in AD brains, with implications in regulating tau phosphorylation leading to acummulated proteins as is found in AD pathology (41). Interestingly, it has been reported that while acute stressors elicit an increase in mRNA expression levels of EGR1 and c-Fos in brain areas such as the neocortex, hippocampus, nucleus accumbens, and the paraventricular nucleus (PVN) located in the hypothalamus, chronic exposure to the same stressor produces a blunted IEG expression response (18, 46, 55, 67). Consistent with current literature, in this study we demonstrated that L-lactate (4 mM) induces increased mRNA expression of the IEGs c-Fos and EGR1 at 1 h, with expression levels returning back towards baseline at 4 h. This finding suggests a key role in L-lactate's ability to impact synaptic learning processes, which are often dysregulated in neurodegenerative models.

Protein structure and function is largely regulated by chaperone molecules and lactic acidosis can contribute to conformational changes to tertiary protein structure, leading to the misfolding of protein, thus reducing their activity and further exacerbating the acidosis. This protein misfolding is largely implicated in the pathological development of AD (6). A 2005 study reported increased protein levels of GRP78, a molecular chaperone during the unfolded protein response (UPR), in AD temporal cortex and hippocampus via Western blot (35). In concurrence, in this study we demonstrated that L-lactate (4 mM) signficantly increases mRNA expression levels of GRP78 at 4 h, suggesting a role of L-lactate in eliciting the UPR. It has been shown in the literature that chronic UPR can result in increased expression of CHOP (DDIT3), a pro-apoptotic transcription factor implicated in the ER stress response. In the current study we showed that L-lactate (4 mM) induced a signficant decrease in mRNA expression levels of CHOP at 4h but not 1h, suggesting that the neurons were experiencing uncontrollable protein misfolding and subsequent death.

A hallmark of neuronal homeostasis and regulation lies through vesicular transport. Vesicular monoamine transporter 2 (VMAT2) is expressed primarily in the CNS, with roles in organizing and releasing neurotransmitters. Dysregulation of VMAT2 through exogenous stimulant exposure has been implicated in neurotoxicity and addiction (24). Dyfunction of VMAT2 has also been implicated in PD pathologies (40). Furthermore, a 2005 study showed VMAT2 knock-out mice were unable to store/release dopamine in their synapses, indicating the importance of VMAT2 in proper neurotransmission (17). Consistent with current

literature, in this study we demonstrated that VMAT2 mRNA expression is increased at 1 h, with levels returning back towards baseline at 4 h. This suggests that acute exposure to a stimulant such as L-lactate can induce increased vesicular transport in neuronal synapses through acidification and/or calcium levels, however chronic exposure can diminish the ability of VMAT2 to successfully release and take-up neurotransmitters in the synapse, leading to further cellular dysregulation.

Using flow cytometry, we confirmed that NGF-derived PC12 cells incubated with L-lactate for 4 h increased positive PI staining in a dose dependent manner (Figure 1). A dose-response curve revealed an EC_{50} value for L-lactate of 3.28 mM (Figure 1A). Blood lactate concentrations are usually 1-2 mM at rest, however with excess accumulation as experienced during intense exercise, these levels can exceed 20 mM (12). Rises in CSF lactate have been well established in neurodegenerative disease models (2, 3, 44, 49, 62, 71, 72). It is also reported that blood or CSF lactate increases (>2.1 mM) (32) can be an indicator of mitochondrial disease. Our EC_{50} value for L-lactate-induced increases in cell death (represented by increased percentage of cells staining positive for PI) corresponds with the apparent increases in brain L-lactate concentrations in neurodegenerative models reported thoroughly in the literature. In support of the quantitative cell death observed via flow cytometry, we also observed significant increases in fluoresced PC12 cells following L-lactate (4 mM) treatment via real-time imaging versus control. The flow cytometry and real-time imaging experiments were re-conducted using primary neuronal cultures

with similar trends observed, further supporting the role of lactate in modulating CNS activity. There is a growing basis of research indicating that accumulation of lactate in the central nervous system, through impairment of MCTs (29, 34, 70), altered activity of lactate dehydrogenase (LDH), and/or hypoxic/ischemic conditions (63), can lead to metabolic acidosis, increased ROS, and increased levels of intracellular calcium; all precursors to cell death individually and in combination. This current study provides further evidence for the role of lactate in neurodegeneration in the CNS, and allows for continued studies into precise cellular mechanistic pathways.

3 Summary, Limitations, and Future Directions

3.1 Summary

Dysregulation of homeostatic mechanisms in the CNS remain a cornerstone of neurodegenerative diseases, ischemia reperfusion injuries, and physiological aging. While neurodegenerative disorder studies focus largely on the clinical presentation of these diseases such as protein aggregation, little research has been done to explore the effects of potential biochemical markers that facilitate the homeostatic dysregulation. As such, we explored the effects of lactate on neuronal function.

In the current study we explored whether lactate could induce cytotoxic mechanisms that are heavily implicated in neuronal degeneration. We demonstrated that L-lactate activates a cellular cascade that results in increased cytosolic calcium, increased cytosolic ROS and increased intracellular pH. Furthermore, lactate induced increases in mRNA expression levels of EGR1 and c-Fos, proteins that are upregulated during synaptic plasticity processes. Lactate also induced increases in mRNA expression levels of GRP78 and CHOP, proteins upregulated during ER stress and UPR. In support of the cytotoxic mechanisms explored above, L-lactate also induced significant increases in cell death experienced in both primary neuronal cultures and dopaminergic-like PC12 cells. Taken together, this suggests that lactate-induced cell death is a result or contribution of several cellular pathways explored in this study, and can provide

further insight to the development and maintenance of neurodegenerative diseases.

3.2 Limitations

It is important to note several limitations of the present study. First, all experiments were conducted on primary neuronal cultures, not isolated from specific areas of the brain. This could have implications for the data sets, as various brain areas are correlated with various levels of expression of substrates and receptors. In addition, the flow cytometry machine that was used to carry out the PI flow cytometry data collection does not contain a UV laser line, a necessary component for the use of the NucBlue stain. NucBlue permeates all cell membranes (intact and compromised), which can provide a clearer understanding of the effects of L-lactate on the whole neuronal population. To improvise this equipment shortcoming, we used PI stain alone, which stains compromised membranes but cannot permeate and stain cells with an intact membrane, still allowing us to quantify the cell death experienced upon L-lactate treatment.

Our current study with lactate only utilized cell culture. As is common in research practice, studies progress from cell culture, to animal models and eventually make it to human trials. A limitation for this study is that we lack animal model support. As such, future studies will include animal models and the

contributions of lactic acid/lactate to the development of neurodegenerative disease.

3.3 Future Directions

Since we have identified lactate to contribute to increases in intracellular calcium concentrations, we would like to continue our research into more precise signaling pathways. Specifically, we plan on exploring the effects of lactate on NMDA-R pathways, as it has been demonstrated in the literature that oligomeric forms of β-amyloid, as well as lactate, have been shown to interact with NMDA-R, leading to increases in levels of intracellular calcium, mitochondrial calcium excess, oxidative stress, and apoptosis (19).

We would also like to expand our research to routes further exploring the role of lactate in the UPR and subsequent protein aggregation that is common among neurodegenerative disorders such as AD, PD, and dementia with lewy bodies.

It would be beneficial for the study to include acute versus chronic exposure to lactate, as it is pertinent to further understanding lactate's role in neurodegenerative diseases, which progress chronically over time. This could be achieved through acute administration of lactate through tail vein infusion, and the chronic intracerebrovascular (ICV) infusion of lactate over four weeks to look for neurodegenerative markers for degeneration.

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A Raw Data and Statistics

Table A-1. Two-tailed T-test for mRNA Expression of GRP78 (HSPA5)

Table Analyzed	HSPA5 Neurons 3_13_18
Column A	control
vs	vs
Column C	4 mm Lactate 4 hr
Unpaired t test	
P value	0.0145
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.398 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column C	1.395 ± 0.09447 N=4
Difference between means	-0.3893 ± 0.1146
95% confidence interval	-0.6696 to -0.1090
R square	0.6581
F test to compare variances	
F,DFn, Dfd	2.127, 3, 3
P value	0.5513

P value summary	ns
Are variances significantly different?	No

Table A-2. Two-tailed T-test for mRNA Expression of EGR1

Table Analyzed	EGR1 Neurons 3_13_18
Column A	control
vs	vs
Column B	4 mM Lactate 1 hr
Unpaired t test	
P value	0.0007
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.410 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column B	2.157 ± 0.1675 N=4
Difference between means	-1.151 ± 0.1796
95% confidence interval	-1.591 to -0.7118
R square	0.8726
F test to compare variances	
F,DFn, Dfd	6.684, 3, 3

P value	0.1530
P value summary	ns
Are variances significantly different?	No

Table A-3. Two-tailed T-test for mRNA Expression of c-FOS (Control vs 1 h)

Table Analyzed	c-fos Neurons 3_13_18
Column A	control
vs	vs
Column B	4 mM Lactate 1 hr
.	
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=12.52 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column B	5.773 ± 0.3751 N=4
Difference between means	-4.767 ± 0.3807
95% confidence interval	-5.699 to -3.836
R square	0.9632
F test to compare variances	

F,DFn, Dfd	33.53, 3, 3
P value	0.0166
P value summary	*
Are variances significantly different?	Yes

Table A-4. Two-tailed T-test for mRNA Expression of c-FOS (Control vs 4 h)

Table Analyzed	c-fos Neurons 3_13_18
Column A	control
vs	vs
Column C	4 mm Lactate 4 hr
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=12.52 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column C	0.1003 ± 0.03217 N=4
Difference between means	0.9058 ± 0.07233
95% confidence interval	0.7288 to 1.083
R square	0.9631

F test to compare variances	
F,DFn, Dfd	4.054, 3, 3
P value	0.2804
P value summary	ns
Are variances significantly different?	No

Table A-5. Two-tailed T-test for mRNA Expression of c-FOS (1 h vs 4 h)

Table Analyzed	c-fos Neurons 3_13_18
Column B	4 mM Lactate 1 hr
vs	vs
Column C	4 mm Lactate 4 hr
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=15.07 df=6
How big is the difference?	
Mean ± SEM of column B	5.773 ± 0.3751 N=4
Mean ± SEM of column C	0.1003 ± 0.03217 N=4
Difference between means	5.673 ± 0.3765
95% confidence interval	4.752 to 6.594

R square	0.9743
F test to compare variances	
F,DFn, Dfd	135.9, 3, 3
P value	0.0021
P value summary	**
Are variances significantly different?	Yes

Table A-6. Two-tailed T-test for mRNA Expression of CHOP (DDIT3) (Control vs 1 h)

Table Analyzed	Ddit3 Neurons 3_13_18
Column A	control
vs	vs
Column B	4 mM Lactate 1 hr
Unpaired t test	
P value	0.6199
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5228 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column B	0.9180 ± 0.1555 N=4

Difference between means	0.08806 ± 0.1684
95% confidence interval	-0.3241 to 0.5002
R square	0.04357
F test to compare variances	
F,DFn, Dfd	5.760, 3, 3
P value	0.1844
P value summary	ns
Are variances significantly different?	No

Table A-7. Two-tailed T-test for mRNA Expression of CHOP (DDIT3) (Control vs 4 h)

Table Analyzed	Ddit3 Neurons 3_13_18
Column A	control
vs	vs
Column C	4 mm Lactate 4 hr
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=14.63 df=6
How big is the difference?	

Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column C	0.05552 ± 0.004758 N=4
Difference between means	0.9506 ± 0.06496
95% confidence interval	0.7916 to 1.110
R square	0.9727
F test to compare variances	
F,DFn, Dfd	185.4, 3, 3
P value	0.0013
P value summary	**
Are variances significantly different?	Yes

Table A-8. Two-tailed T-test for mRNA Expression of VMAT2 (Control vs 1 h)

Table Analyzed	vmat2 Neurons 3_13_18
Column A	control
vs	vs
Column B	4 mM Lactate 1 hr
Unpaired t test	
P value	0.0003
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.251 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column B	2.057 ± 0.1297 N=4
Difference between means	-1.051 ± 0.1450
95% confidence interval	-1.406 to -0.6963
R square	0.8976
F test to compare variances	
F,DFn, Dfd	4.007, 3, 3
P value	0.2842
P value summary	ns
Are variances significantly different?	No

Table A-9. Two-tailed T-test for mRNA Expression of VMAT2 (Control vs 4 h)

Table Analyzed	vmat2 Neurons 3_13_18
Column A	control
vs	vs
Column C	4 mm Lactate 4 hr
Unpaired t test	
P value	0.0661
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.243 df=6
How big is the difference?	
Mean ± SEM of column A	1.006 ± 0.06478 N=4
Mean ± SEM of column C	1.301 ± 0.1142 N=4
Difference between means	-0.2945 ± 0.1313
95% confidence interval	-0.6158 to 0.02684
R square	0.4560
F test to compare variances	
F,DFn, Dfd	3.109, 3, 3
P value	0.3765
P value summary	ns

Are variances significantly different?	No

Table A-10. Two-tailed T-test for PI Quantification in Neurons

Table Analyzed	PI Lactate 4 hr neurons 2_15_18
Column A	0
vs	vs
Column B	4
Unpaired t test	
P value	0.0021
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.255 df=9
How big is the difference?	
Mean ± SEM of column A	18.86 ± 1.535 N=5
Mean ± SEM of column B	31.83 ± 2.456 N=6
Difference between means	-12.97 ± 3.049
95% confidence interval	-19.87 to -6.077
R square	0.6680
F test to compare variances	
F,DFn, Dfd	3.074, 5, 4
P value	0.2992

P value summary	ns
Are variances significantly different?	No

Table A-11. Two-tailed T-test for RT-ROS Quantification in Neurons

Table Analyzed	ROS lactate
Column A	0
vs	vs
Column B	4
Unpaired t test	
P value	0.0214
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.725 df=10
How big is the difference?	
Mean ± SEM of column A	1.755 ± 0.4230 N=6
Mean ± SEM of column B	4.115 ± 0.7557 N=6
Difference between means	-2.360 ± 0.8660
95% confidence interval	-4.289 to -0.4302
R square	0.4261
F test to compare variances	
F,DFn, Dfd	3.192, 5, 5

P value	0.2285
P value summary	ns
Are variances significantly different?	No

Table A-12. Two-tailed T-test for RT-pH Quantification in Neurons

Table Analyzed	pHi lactate
Column A	0
vs	vs
Column B	4
Unpaired t test	
P value	0.0088
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.246 df=10
How big is the difference?	
Mean ± SEM of column A	0.3227 ± 0.04263 N=6
Mean ± SEM of column B	0.5975 ± 0.07315 N=6
Difference between means	-0.2748 ± 0.08466
95% confidence interval	-0.4634 to -0.08614
R square	0.5130
F test to compare variances	

F,DFn, Dfd	2.944, 5, 5
P value	0.2610
P value summary	ns
Are variances significantly different?	No

Table A-13. Two-tailed T-test for RT-Calcium Quantification in Neurons

Table Analyzed	Calcium Lactate
Column A	0
vs	vs
Column B	4
Unpaired t test	
P value	0.0116
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.156 df=9
How big is the difference?	
Mean ± SEM of column A	0.05953 ± 0.004102 N=5
Mean ± SEM of column B	0.2159 ± 0.04470 N=6
Difference between means	-0.1564 ± 0.04956
95% confidence interval	-0.2685 to -0.04428
R square	0.5252

F test to compare variances	
F,DFn, Dfd	142.5, 5, 4
P value	0.0003
P value summary	***
Are variances significantly different?	Yes

Table A-14. Two-tailed T-test for RT-PI Quantification in Neurons

Table Analyzed	PI Lactate Column
Column A	0
vs	vs
Column B	4
Unpaired t test	
P value	0.0116
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.585 df=6
How big is the difference?	
Mean ± SEM of column A	14.95 ± 0.5188 N=4
Mean ± SEM of column B	31.58 ± 4.608 N=4
Difference between means	-16.63 ± 4.637
95% confidence interval	-27.97 to -5.277
R square	0.6817

F test to compare variances	
F,DFn, Dfd	78.89, 3, 3
P value	0.0047
P value summary	**
Are variances significantly different?	Yes

Table A-15. Two-tailed T-test for RT-pH Quantification in PC12 Cells

Table Analyzed	pHi
Column A	Control
vs	vs
Column B	4 mM L-Lac
Unpaired t test	
P value	0.0080
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.303 df=10
How big is the difference?	
Mean ± SEM of column A	1.210 ± 0.3064 N=6
Mean ± SEM of column B	2.341 ± 0.1530 N=6
Difference between means	-1.131 ± 0.3424
95% confidence interval	-1.894 to -0.3680

R square	0.5217
F test to compare variances	
F,DFn, Dfd	4.012, 5, 5
P value	0.1536
P value summary	ns
Are variances significantly different?	No

Table A-16. Two-tailed T-test for RT-ROS Quantification in PC12 Cells

Table Analyzed	Cyto ROS
Column A	Control
vs	vs
Column B	4 mM L-Lac
Unpaired t test	
P value	0.0246
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.644 df=10
How big is the difference?	
Mean ± SEM of column A	3.285 ± 0.7930 N=6
Mean ± SEM of column B	5.786 ± 0.5157 N=6
Difference between means	-2.501 ± 0.9459

95% confidence interval	-4.609 to -0.3935
R square	0.4114
F test to compare variances	
F,DFn, Dfd	2.364, 5, 5
P value	0.3668
P value summary	ns
Are variances significantly different?	No

Table A-17. Non-linear fit for dose-dependent response of PC12 cells to L-lactate

Boltzmann sigmoidal	
Best-fit values	
Bottom	12.33
Top	43.83
V50	3.282
Slope	1.285
Std. Error	
Bottom	7.845
Top	4.669
V50	0.7646
Slope	0.9474
95% Confidence Intervals	
Bottom	-4.088 to 28.75
Top	34.06 to 53.61
V50	1.682 to 4.883

Slope	-0.6979 to 3.268
Goodness of Fit	
Degrees of Freedom	19
R square	0.6958
Absolute Sum of Squares	923.9
Sy.x	6.973
Number of points	
Analyzed	23