Understanding the Impact of Metabolic Alterations on Brain Development

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Discipline of Anatomy

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A dissertation submitted to Trinity College Dublin, the University of Dublin in candidature for the degree of Masters in Anatomy

Year Degree Awarded: 2025

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Thesis word count: 18,155

Candidate's signature: 

Date: 30/07/2024

Acknowledgments

I'd primarily like to thank Dr. Denis Barry for the opportunity to undertake this MSc and to be part of the Anatomy Department at Trinity. His patience and oversight have been invaluable over the past two years. I hope the working relationship we have made will continue into the future.

I'd like to acknowledge the help of those who have been involved in the research showcased in this thesis, particularly Lauren, Mary-Kate, Karl, and Sarah.

The technical staff in the Department of Anatomy, all have been so kind and willing to help. I'd particularly like to thank Claire Murphy and Mary Lynch, both of whom remembered me from my undergraduate anatomy days and helped me through every step of my thesis. You will never be forgotten, and I thank you immensely.

To all the amazing friends I've made during this journey.

From the first day, when David made me feel very much at home, and taught me all he knew from his own journey in research. Abdallah, who's kind words and humour never failed to make me laugh. Ciara, my lab mate, who I would bore endlessly with random facts, and who allowed me to tote I know someone from Harvard (Brigham). I wish them all the best in the future.

To the second-year gang, Ashley, Dearbhla, Jay, and Claire: my phylum. I cannot thank you enough for the amazing year you have given me. Your support and friendship will never be forgotten, and I hope that we will continue to work together far into our future careers.

To my parents and family. Thank you for your continuous support, interest, and enthusiasm in my studies and ambitions.

Thank you to everyone who has made the past two years some of the best and most memorable so far.

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List of Abbreviations

Abbreviation	Term	Abbreviation	Term
AcAc	Acetoacetate	MCTs	Monocarboxylic transporters
ACAT1	Acetyl-CoA transferase		
AD	Alzheimer's disease	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
ASD	Autism Spectrum Disorder	NB	Neuroblastoma
ATP	Adenosine triphosphate	NGS	Normal goat serum
BBB	Blood-brain-barrier	NSCs	Neural stem cells
CNS	Central nervous system	OXCT1	3-Oxoacid-CoA Transferase
Cpt1a	Carnitine palmitoyl transferase 1a	OXPHOS	Oxidative phosphorylation
DMEM	Dulbecco's modified eagle medium		
DMSO	Dimethyl sulfoxide	PBS	Phosphate buffered saline
DNA	Deoxyribonucleic acid	PD	Parkinson's disease
ENO1	Enolase 1	PDH	Pyruvate dehydrogenase
FAO	Fatty acid oxidation	PFA	Paraformaldehyde
Fasn	Fatty acid synthase	RG	Radial glia
FBS	Foetal bovine serum		
GALE	Urine diphosphate galactose 4-epimerase	RG	Regular glucose
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase		
GF	Glucose free	RM	Regular medium
GFAP	Glial fibrillary acidic protein	ROS	Reactive oxygen species
h	Hours	SCFAs	Short-chained fatty acids
HBSS	Hank's balanced salt solution	SCOT	Co-A transferase
HDACs	Class I histone deacetylases		
hESCs	Human embryonic stem cells	SEM	Standard error of the mean
HK2	Hexokinase 2		
ICC	Immunocytochemistry	SVZ	Subventricular zone
KD	Ketogenic diet	TBI	Traumatic brain injury
KM	Kaplan Meier	TLE	Temporal lobe epilepsy
LDH	Lactate dehydrogenase	TOMM20	Translocase of outer mitochondrial membrane 20
		VZ	Ventricular zone
LG	Low glucose	BDH	β-OHB dehydrogenase
MCAO	Middle cerebral artery occlusion	βIII	β-III tubulin
MCFAs	Medium chain fatty acids	β-OHB	β-hydroxybutyrate

Thesis Overview

Abstract

Evaluating the metabolic requirements of neural stem cells, including the balance between glucose and ketone body utilisation, is important in understanding their role in brain development and disease. This study examined how changes in metabolism affect brain development by testing the hypothesis that glucose is crucial for neural stem cell growth and survival, while ketone bodies act as an important energy source during glucose deprivation. Additionally, it was hypothesised that genomic analysis of glycolytic and ketolytic genes in neuroblastoma would reveal increased glycolytic expression linked to MYCN amplification and worse prognosis, while higher ketolytic expression would correlate with better outcomes. To address these hypotheses, the aims were to perform a literature review to clarify the roles of glucose and ketone bodies in brain development, disease, and therapeutic potential, to analyse glycolytic and ketolytic gene expression in neuroblastoma to understand their role in tumour progression and prognosis, with relevance to neural stem cell metabolism, and, to conduct in vitro experiments to evaluate how glucose deprivation and ketone body supplementation impacts neural stem cell density, metabolic viability, and cellular metabolism. Findings demonstrated that glucose, the primary energy source for neural stem cells, is critical for homeostasis, with deprivation significantly reducing cell density and viability, whereas ketone body supplementation elicited only minor effects. Genomic analysis of neuroblastoma revealed increased glycolytic gene expression in MYCN-amplified metastatic tumours, correlating with poor prognosis, and lower ketolytic gene expression associated with better survival outcomes. These results demonstrate the essential role of metabolic pathways in neural stem cell behaviour and brain development, highlighting potential metabolic targets for therapeutic interventions and prognosis prediction in neurological and oncological conditions. These studies therefore contribute to a better understanding of neural stem cell metabolism and its implications for development, disease, and treatment.

1. Chapter One: Introduction

1.1 Overview of Central Nervous System Development

The central nervous system (CNS) is one of the earliest organ systems to develop in the body, while also being the last to finish developing postnatally with development continuing into the mid-20s (Silbereis et al., 2016). This extended period of neural development as well as a long period of dependency following birth is found nearly exclusively in humans and therefore allows environmental factors to weigh in to mould the development of the cognitive, emotional, and social capabilities attributed to our species (Silbereis et al., 2016). The CNS is made up of numerous cell types. Major cell types of the CNS include neurons and glial cells such as astrocytes, oligodendrocytes, and ependymal cells. Microglia are the immune cells of the CNS. Choroid plexus cells secrete cerebrospinal fluid, and a vast pool of distinct cells are seen within the spinal cord (Kovacs, 2017; Lu et al., 2015). The development of the CNS is a complex, well-orchestrated, and temporal process involving the formation of the brain, spinal cord, and other neuroectodermal-derived cell types.

1.1.1. Brain

The brain is arguably the most complex and vital organ within the human body. Without it, basic homeostatic functions as well as higher cognitive functions including speech and higher reasoning would not be possible. Given its importance and complexity, it's not surprising that the development of the brain is a lengthy and complex process, spanning from early gestation and beyond childhood (Arain et al., 2013; Silbereis et al., 2016). Neural development begins during the third and fourth weeks. Proliferation of the ectoderm during the third-week results in the formation of the neural plate, a single layer of pseudostratified epithelial cells distinct from non-neural ectoderm. Day 20 of development signals the start of neurulation. During this process, neural plate cells elongate, invaginate, and curl towards the midline thereby creating a groove: the neural groove, and folds on either side known as neural folds. Continued curling of the neural folds eventually leads to contact on day 22. By day 23, the neural fold has closed, creating the neural tube (Fig. 1.1).

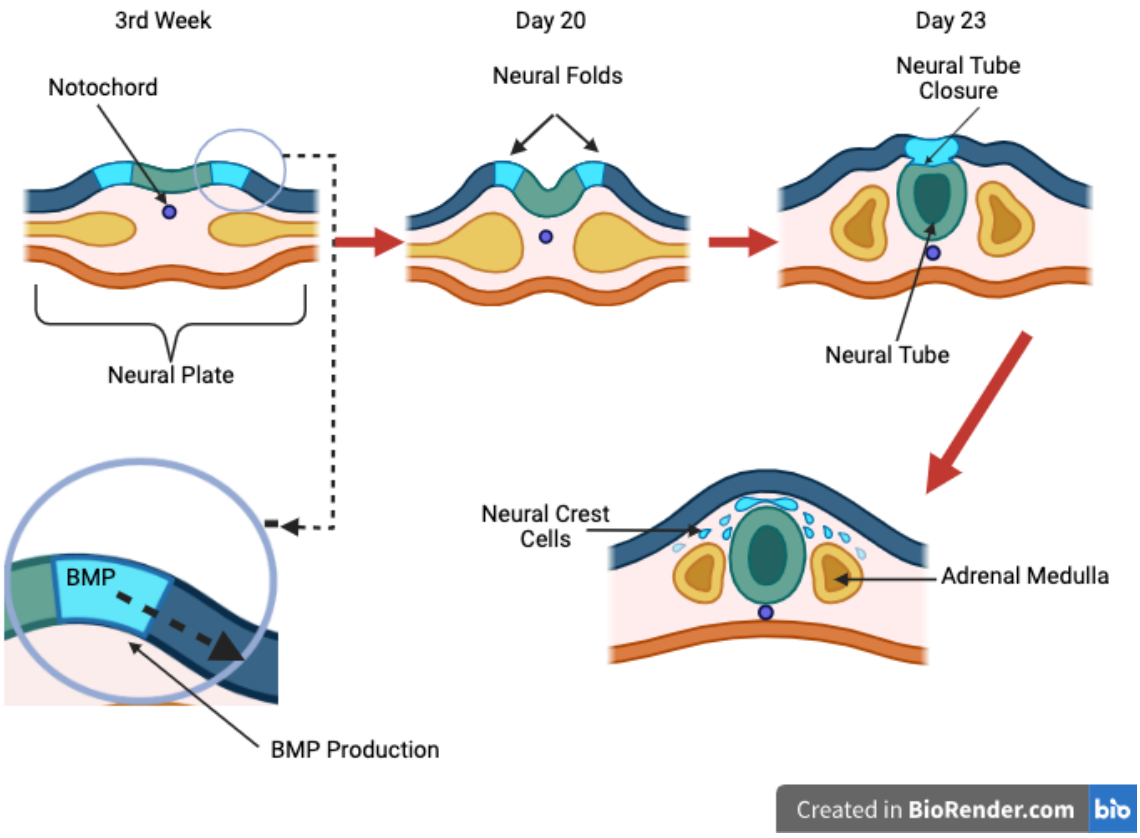


Figure 1.1. Schematic of the process of neurulation. Generated using BioRender.com.

Neurulation is followed by the process of vesiculation, the formation of the primary vesicles of the brain. Vesiculation takes place just before the closure of the neural tube giving rise to the prosencephalon, mesencephalon, and rhombencephalon. In the fifth week, the primary brain vesicles differentiate further. The prosencephalon gives rise to the telencephalon and diencephalon. Further development of these structures results in the cerebral hemispheres, thalamus, and hypothalamus, respectively. The mesencephalon will become the midbrain, while the rhombencephalon forms three structures: the metencephalon, myelencephalon, and spinal cord. The metencephalon derives the pons and cerebellum, and the myelencephalon develops into the medulla oblongata in the fully developed brain (Borsani et al., 2019; Sadler, 2005). The ventricular system of the brain begins to form, containing the choroid plexus which produces cerebrospinal fluid, filling the ventricles and bathing the brain and spinal cord. The formation of the olfactory bulbs begins during the sixth week. During the seventh week, the optic stalk, hypophysis, thalamus, hypothalamus, and epiphysis begin to form. The

last two months of gestation sees the formation of the gyri, fissures, and sulci of the cerebral hemispheres (Sadler, 2005).

Neural crest cells, a group of cells arising at the crest of the neural folds, are also formed during brain development. These cells originate in the neuroectoderm, migrate, and thereafter differentiate into a variety of cell types. Neural crest cells may differentiate into the parasympathetic ganglia of the gastrointestinal tract, the dorsal ganglion or chain and collateral ganglia of the spinal cord, and also the meninges surrounding the brain (Sadler, 2005).

1.1.2. Cellular Expansion of the Brain

All the cells of the brain are derived from the ectoderm, with the exception of microglia, the resident macrophages of the brain, which arise from the mesoderm germ layer (Ginhoux & Prinz, 2015; Liu & Niswander, 2005). Neurogenesis, the production of neurons, followed by their migration begins during the 8th gestational week (Steiner, 2019). The ventricular zone (VZ), a layer of pseudostratified neuroepithelial cells, lines the neural tube. From these cells, all neurons and macroglia originate as they serve as stem or progenitor cells. A substantial number of progenitor cells are generated from the division of the cells in the neuroepithelial layer as each division gives rise to two progenitor daughter cells in a process of symmetrical division (Silbereis et al., 2016). Following this, the VZ cells generate the earliest neurons from the emerging cortical plate (CP). During this process neurons are generated at a rate of approximately 3.86 million per hour (Silbereis et al., 2016). Subsequently, the subventricular zone (SVZ) arises above the VZ and drastically increases in size from early to mid-foetal development. These zones together give rise to pyramidal neurons within the telencephalon and thereafter glial cells, with glial cell proliferation peaking around the time of birth (Silbereis et al., 2016; Steiner, 2019). Furthermore, in early embryonic neurogenesis, the neuroepithelial progenitor cells within the VZ transition into another stem cell/ progenitor cell type called radial glia (RG) (Silbereis et al., 2016). These RG are characterised by their long radial processes, and they play a vital role in neural development as they guide newborn neurons from the VZ to the mantle regions, but also act as key progenitor cells within the brain as they comprise the majority of mitotically active cells in the VZ (Barry et al., 2014; Campbell & Götz, 2002).

Glial cells such as astrocytes and oligodendrocytes also originate from RG cells, and they continue to proliferate, migrate, and differentiate up until three years postnatally (Barry et al., 2014; Silbereis et al., 2016; Steiner, 2019). This activity is in line with a peak in synapse formation and neural network reorganisation often seen at this time in childhood (Steiner, 2019) (Fig. 1.2).

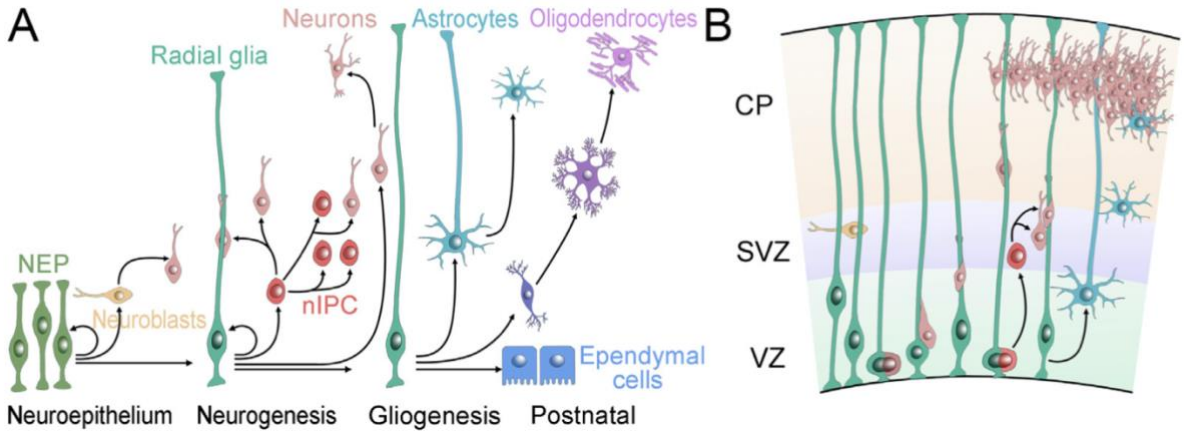


Figure 1.2. Progression of cellular development in the brain. Adapted from *Barry et al. 2014.* CP; cortical plate. SVZ; subventricular zone. VZ; ventricular zone.

1.2. Cellular Metabolism in Brain Development

The main source of energy for neurons in the adult brain is glucose supplied via the cerebral circulation. However, during periods of starvation, alternative fuels such as ketone bodies and lactate may also be used (Vergara et al., 2019). A varying supply of energy sources is important in maintaining brain homeostasis because the brain is an energy-intensive organ, consuming approximately 20% of total body oxygen to carry out aerobic respiration to meet its energy requirements (Rink & Khanna, 2011; Vergara et al., 2019). Under normal conditions, the supplied glucose is used to generate adenosine triphosphate (ATP) either by glycolysis or oxidative phosphorylation (OXPHOS), with the latter providing the majority (Hall et al., 2012; Steiner, 2019). Glycolysis is a cytosolic process where the supplied glucose is broken down to pyruvate, releasing energy as ATP. Pyruvate can then be converted to acetyl-CoA by the enzyme pyruvate dehydrogenase (PDH). Acetyl-CoA can subsequently enter the Krebs cycle, allowing for the further production of more ATP (Alabduladhem & Bordoni, 2022; Chaudhry & Varacallo, 2022;

Kumari, 2018). OXPHOS however, is the major energy-producing pathway within cells. The ATP derived from this process is used by neural cells for various homeostatic functions (Du et al., 2008). A more detailed description of the events involved in the cellular metabolism in brain development can be found in Chapter Two of this thesis.

1.2.1. (Aerobic) Glycolysis

At birth, the newborn brain accounts for approximately 60% of the body's daily energy requirements despite only comprising 13% of lean body weight (Steiner, 2019). The metabolic requirements of the brain correspond with its development and maturation, with the peak in demand occurring during postnatal years two and three, a time of increased synapse formation and myelination (Goyal et al., 2018). The newborn brain is incredibly energy intensive, so much so that measurements of the cerebral metabolic rate for glucose demonstrated that the glucose consumption rate far exceeded OXPHOS by 34% (Steiner, 2019). Due to this high requirement for glucose, in early pregnancy, there is a surge in the production of placental lactogen, progesterone, and prolactin, stimulating appetite and glycolysis (Horton et al., 1985). Increased glucose metabolism was observed in areas of the brain responsible for the emergence of various important behaviours in a child's early years, such as the primary motor and sensory cortices (Chugani, 1998). The importance of glucose in the developing brain is not limited to the generation of energy, but it also used in biosynthetic pathways (Steiner, 2019) (Fig 1.3.) A more detailed description of the glycolytic events involved in brain development can be found in Chapter Two of this thesis.

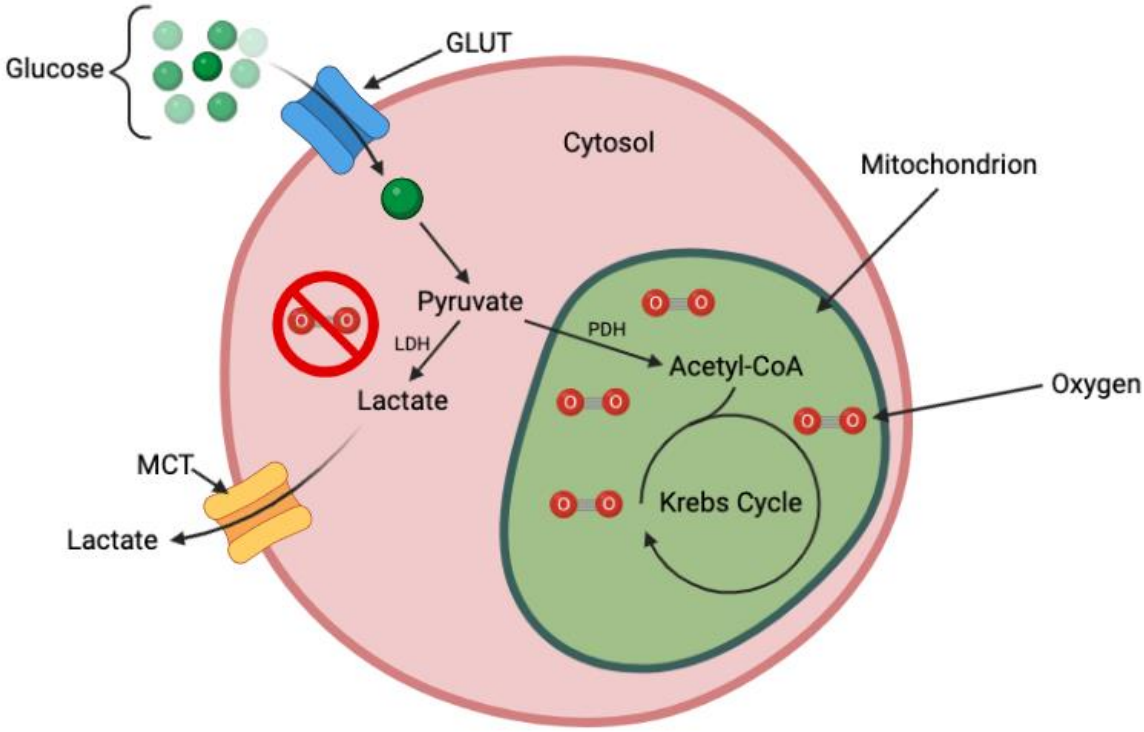


Figure 1.3. Pyruvate utilisation during hypoxia and normoxia in mammalian cells. Generated using BioRender.com.

1.2.2. Ketones Bodies

Despite glucose being the main energy supply in the adult brain, during neurodevelopment and maturation, this supply is often over-exhausted (Cunnane & Crawford, 2014; Vergara et al., 2019). In this scenario, ketone bodies become an essential supply of energy, particularly β-hydroxybutyrate (β-OHB), making up 70% of available circulating ketones, as well as acetoacetate and acetone (Achanta & Rae, 2017; Barry et al., 2018; Cotter et al., 2013; Cunnane, Courchesne-Loyer, Vandenberghe, et al., 2016). Ketone bodies are short-chained fatty acids (SCFAs) generated through β-oxidation of fatty acids in hepatocytes, process known as ketogenesis (Barry et al., 2018; Cotter et al., 2013; Cunnane & Crawford, 2014). The rate of hepatic ketogenesis is regulated by factors including hormones, gene transcription, and post-translational events (Puchalska & Crawford, 2017). Following β-oxidation, ketone bodies are transported to extrahepatic tissues, such as the brain, where they then cross the blood-brain barrier (BBB) and into cells via monocarboxylic transporters (MCTs). Cells within the brain then oxidise β-OHB to generate acetoacetate. Further metabolism of acetoacetate generates acetyl-CoA which

can then enter the Krebs cycle and the downstream electron transport chain (Barry et al., 2018). When circulating glucose is present, ketone bodies may be used to mediate other physiological processes (Morris, 2005).

Ketone bodies start being utilised in neurodevelopment while in utero, where they can cross the placenta efficiently (Vilaró et al., 1987). The importance of ketone bodies remains postnatally as the infant switches from a constant supply of energy via the placenta, to intermittent feeding (Adam et al., 1975; Nehlig & Pereira de Vasconcelos, 1993). Newborn possess the ability needs to metabolise the medium-chained fatty acids (MCFAs) from breast milk into SCFAs, which can then be used as an energy source (Erecinska et al., 2004; Nehlig & Pereira de Vasconcelos, 1993). This demonstrates that not only is glucose an important factor in neurodevelopment, but ketone bodies could be considered equally as vital (Fig. 1.4).

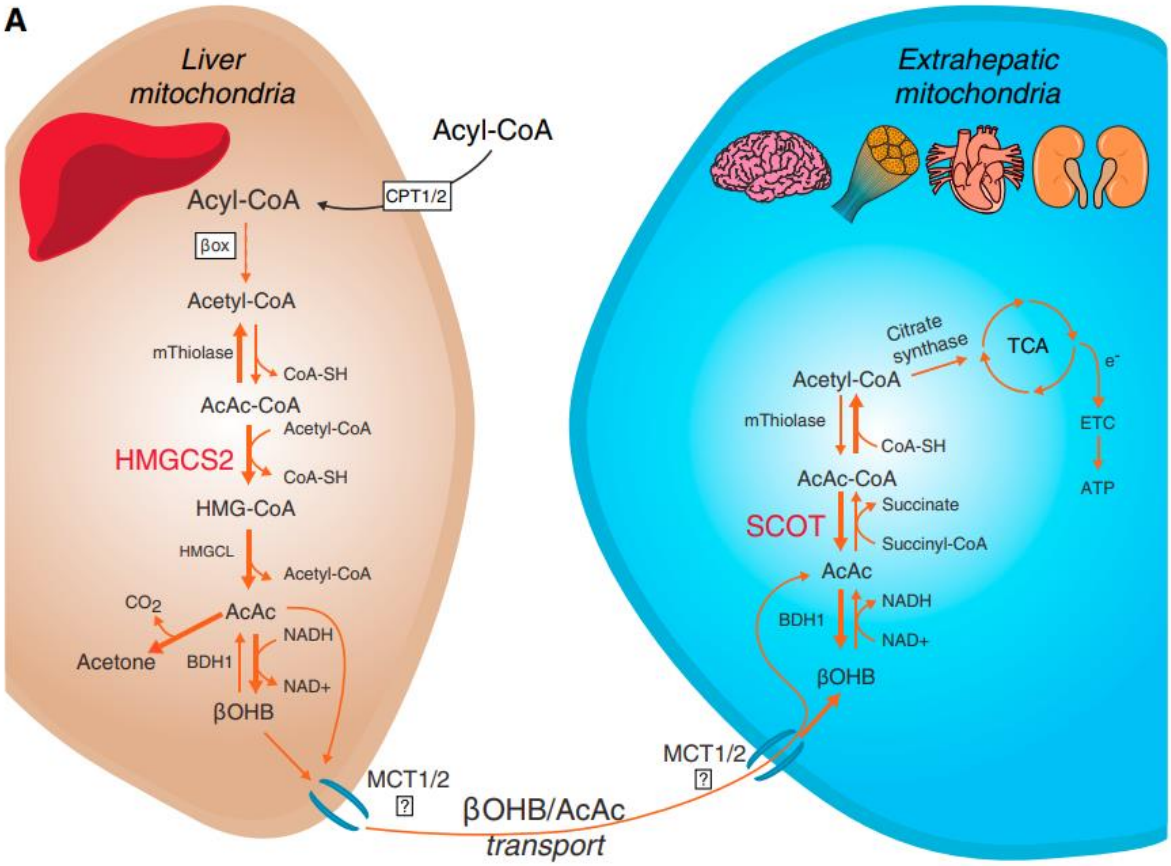


Figure 1.4. Ketone body metabolism. Adapted from *Puchalska and Crawford, 2017.*

For further discussion on glucose and ketone body metabolism in NSCs, in addition to their effects on health and disease, a comprehensive review of the topic can be found in Chapter Two of this thesis: “The Interplay Between Glucose and Ketone Bodies in Neural Stem Cell Metabolism”.

1.3. Cell Lines in Neurobiology

Cell lines are highly useful tools in neurobiology. Cell lines are cultures of animal, human, or plant cells that can be propagated repeatedly or even indefinitely (Bols et al., 2011). Their clonality and being of the one-cell type enables experimentation on a target cell type (Lendahl & McKay, 1990). Their ability to replicate indefinitely makes them highly beneficial regarding their ease of use, cost-effectiveness, and reproducibility of experiments (Mirabelli et al., 2019). The first cell line arose from work done by Carrel in 1912. His cell line was derived from fragments of explanted chicken embryo heart (Rodríguez-Hernández et al., 2014). This discovery has led to great advancement in biomedical research. Modern cell lines may be used in numerous roles including vaccine production, to assess gene function, and tissue generation, among others (Kaur & Dufour, 2012). The next breakthrough in the development of cell lines was the development of the first immortal cell line, the HeLa cell line in 1951 by Dr. George Gey (Callaway, 2013; Nambisan, 2017). Cell lines used in neurobiological research are commonly derived from two sources: a non-CNS cancer or from the CNS itself.

1.4. Cell Lines Used in This Research

1.4.1. SH-SY5Y Cell Line

The first neuronal cell line was acquired from the liver, lymph nodes, and infiltrated bone marrow of children suffering from neuroblastoma (NB), which is a cancer of sympathoadrenal progenitor cells derived from the neural crest (Brodeur, 2003; Goldstein et al., 1964). This cell line was further improved upon over the coming years, first giving rise to the SK-N-SH NB cell line and then the now commonly used SH-SY5Y NB line (Biedler et al., 1973; Biedler et al., 1978). The development of this cell line can be attributed to June Beidler in 1973 (Biedler et al., 1973). SH-SY5Y cells can grow continuously as

undifferentiated cells exhibiting a neuroblast-like morphology as well as immature markers. Upon differentiation, SH-SY5Y cells appear like primary neurons morphologically (Gordon et al., 2013). The SH-SY5Y cell line is now widely used in neurophysiological research (Gardiner & Freeman, 2016). Their most common application is in Parkinson's disease (PD) research and neuronal function and disease studies (Barth et al., 2022; Xicoy et al., 2017). SH-SY5Y cells are chosen for this purpose because of their human origin, catecholaminergic neuronal properties as dopaminergic neuronal loss is a common feature of PD pathogenesis, as well as their general ease of use (Xicoy et al., 2017). The disadvantage of SH-SY5Y cells is the presence of genetic aberrations which is a direct result of their cancerous origin. Nevertheless, the dysregulated pathway being investigated in PD is still dysregulated in these cells (Krishna et al., 2014).

1.4.2. NE4C Cell Line

The NE4C cell line derives from the cloning of anterior brain vesicles of 9-day-old $p53^{-/-}$ mouse embryos. NE4C cells may differentiate into astrocytes and neurons when treated with all-trans retinoic acid or if in the presence of primary astrocytes (Demeter et al., 2005). These cells were found to be capable of incorporating into the normal neural framework of the brain if implanted into the embryological mouse, however, in adult mice, survival of these cells didn't occur and was restricted to areas of inherent neurogenesis (Demeter et al., 2004). Therefore, the NE4C cell line can be of use in examining neurogenesis. NE4C cells lack the P53 genes. P53 is a tumour suppressor that transcriptionally modulates various cellular processes such as DNA damage repair, cell cycle arrest, and senescence. There are conflicting studies on its function in NSCs. P53 has been found to inhibit neuronal differentiation of NSCs, but alternative studies indicate that p53 may be needed for neuronal differentiation (Xiong et al., 2020).

1.5. Aims and Hypotheses of Thesis:

1.5.1. Aims

1. To perform a literature review to understand the impact of glucose availability and ketone body supplementation on brain development and disease.
2. To conduct genomic analysis of glycolytic and ketolytic genes in NB. This study will investigate the effect of glycolytic and ketolytic genes in rapidly dividing glycolytic

cells. Therefore, the results from this analysis reveal insights into the roles of ketolytic and glycolytic genes on energy metabolism in neural stem cells.

3. To perform in vitro experiments using NSCs to investigate the impact of glucose availability and ketone body supplementation on neuroepithelial and neuronal stem cell precursor cells.

1.5.2. Hypotheses

1. The literature will demonstrate the importance of glucose metabolism in brain development and health and shed light on the functioning of ketone bodies in NSCs.
2. Genomic analysis of glycolytic and ketolytic gene expression in NB will show increased glycolytic expression in MYCN-amplified, metastatic tumours compared to non-amplified, non-metastatic tumours.
3. In vitro experiments will demonstrate that glucose is needed for NSC growth, metabolic viability, and to perform glycolysis. Supplementation with ketone bodies may improve the density and metabolic viability of NSCs.

2. Chapter Two.

The Interplay Between Glucose and Ketone Bodies in Neural Stem Cell Metabolism

Written by Joseph W. Molloy and Dr. Denis Barry

Manuscript DOI: https://doi.org/10.1002/jnr.25342

2.1. Abstract

Glucose is the primary energy source for neural stem cells (NSCs), supporting their proliferation, differentiation, and quiescence. However, the high demand for glucose during brain development often exceeds its supply, leading to the utilization of alternative energy sources including ketone bodies. Ketone bodies, including β-hydroxybutyrate, are short-chain fatty acids produced through hepatic ketogenesis and play a crucial role in providing energy and the biosynthetic components for NSCs when required. The interplay between glucose and ketone metabolism influences NSC behavior and fate decisions, and disruptions in these metabolic pathways have been linked to neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. Additionally, ketone bodies exert neuroprotective effects on NSCs and modulate cellular responses to oxidative stress, energy maintenance, deacetylation, and inflammation. As such, understanding the interdependence of glucose and ketone metabolism in NSCs is crucial to understanding their roles in NSC function and their implications for neurological conditions. This article reviews the mechanisms of glucose and ketone utilization in NSCs, their impact on NSC function, and the therapeutic potential of targeting these metabolic pathways in neurological disorders.

2.2. Significance

This review highlights the significance of glucose and ketone metabolism in NSCs, showcasing their roles in determining brain cell behavior and fate. Disruptions in glycolytic and oxidative phosphorylation metabolic pathways can result in neurodevelopmental, neuropsychiatric, and neurodegenerative conditions. Examining the interplay between glucose and ketone metabolism in NSCs allows insights into their roles in maintaining developing brain homeostasis to be better understood, which may open therapeutic avenues for targeting metabolic pathways to treat neurological disorders.

2.3. Introduction

Neural stem cells (NSCs) are multipotent progenitor cells that differentiate to give rise to glial and neuronal cells. These cells were first described by Camillo Golgi in the 19th century, and are found in the brains of developing embryos, neonates, and adults

(Martínez-Cerdeño & Noctor, 2018). Glucose is the main source of energy for NSCs (Alherz et al., 2021; Jády et al., 2016) and the developing brain requires a vast amount of glucose to meet its metabolic needs. Rapid brain development from the fifth gestational month leads to an increase in the brain's weight from 27% of its final weight to 80% by two years of age (Steiner, 2019). However, the quantities of glucose required by NSCs are not strictly used for energy production. Glucose can be converted to lactate, during aerobic glycolysis, which is a biosynthetic constituent vital for synapse formation, pruning, and myelination during central nervous system (CNS) development (Steiner, 2019).

Glucose may be depleted during neurodevelopment and maturation in times of starvation, fasting, and prolonged exercise amongst other physiological conditions (Cunnane & Crawford, 2014; Vergara et al., 2019). In these circumstances, ketone bodies become an essential energy supply, particularly β-hydroxybutyrate (β-OHB), which comprises 70% of available circulating ketone bodies, in addition to acetoacetate and acetone (Achanta & Rae, 2017; Cotter et al., 2013). Ketone bodies are short-chain fatty acids (SCFAs) which are generated through the β-oxidation of fatty acids in the hepatic mitochondrial matrix, a process known as ketogenesis (Cotter et al., 2013; Cunnane & Crawford, 2014). When circulating glucose is abundant, ketone bodies can be used to mediate other physiological processes such as the synthesis of lipids used in neural development, including myelin and cholesterol (Morris, 2005).

As such, cell metabolism plays a critical role in NSC behavior during proliferation, differentiation, and maintaining quiescence. NSC proliferation is associated with higher levels of glycolysis, while the differentiation of NSCs is driven by oxidative phosphorylation (OXPHOS) (Agostini et al., 2016; Maffezzini et al., 2020; Zheng et al., 2016). This metabolic reprogramming from glycolysis to OXPHOS is essential during mid-embryogenesis and is partly responsible for directing NSC fate decisions (Angelopoulos et al., 2022). Lipid metabolism is also important in regulating adult NSC fate and can control their proliferative activity, with fatty acid oxidation (FAO) maintaining their phenotype, and lipogenesis driving them toward differentiation (Maffezzini et al., 2020). NSC behavior may also be affected by the accumulation of lipids, as well as the expression of proteins such as spot14, fatty acid synthase (Fasn) (Knobloch et al., 2013), and carnitine palmitoyl transferase 1a (Cpt1a) (Knobloch et al., 2017). A breakdown in these metabolic pathways

has been shown to lead to intellectual disabilities (Bowers et al., 2020), neuropsychiatric disorders, and autism spectrum disorder (ASD) (Xie et al., 2016).

2.4. Mechanisms of Glucose Utilisation

Under normoxic conditions, glucose generates adenosine triphosphate (ATP) either by glycolysis or OXPHOS, with the latter providing approximately 87% of available ATP (Hall et al., 2012; Steiner, 2019). Glycolysis is a cytosolic process where supplied glucose, a six-carbon molecule, is broken down into two three-molecules of pyruvate, and in the process produces energy in the form of ATP. First, the enzyme hexokinase (HK) phosphorylates glucose to become glucose-6-phosphate (G6P). Further enzymatic activity then derives pyruvate (Tatapudy et al., 2017). During glycolysis, one molecule of glucose yields two molecules of ATP. Pyruvate can be further oxidized to acetyl-CoA by pyruvate dehydrogenase (PDH). Acetyl-CoA may subsequently enter the Krebs cycle within the mitochondrial matrix allowing for the further production of ATP. The Krebs cycle then generates 12 molecules of ATP per acetyl-CoA input (Alabduladhem & Bordoni, 2022; Chaudhry & Varacallo, 2022; Kumari, 2018). However, OXPHOS is the main energy producing pathway in mature brain cells. OXPHOS occurs at the inner mitochondrial membrane where electrons supplied by NADH and $FADH_2$, by-products of the Krebs cycle, allow shuttling of protons into the intermembrane space. This process generates 32–34 molecules of ATP (GM, 2000). The ATP derived from this process are used by neural cells primarily to pump sodium and potassium across the cellular membrane to maintain transmembrane ion gradients allowing downstream events to occur such as neurotransmitter cycling, electrophysiological activity, and cell signalling (Du et al., 2008).

The brain's metabolic requirements correspond with development and maturation, with peak demand occurring during postnatal years two and three, a time of increased synapse formation and myelination (Goyal et al., 2018). The requirement for glucose in early pregnancy results in a surge in the production of placental lactogen, progesterone, and prolactin, which stimulates appetite and glycolysis (Horton et al., 1985). The metabolic needs of the new-born brain are immense and measurements of the cerebral glucose metabolic rate showed that glycolysis exceeded OXPHOS by 34% at this time (Steiner,

2019). The importance of glucose in the developing brain is not limited to the generation of energy. Glucose uptake far exceeds oxygen consumption and oxygen is primarily used for OXPHOS. The excess glucose may be used in the biosynthesis of essential macromolecules (Steiner, 2019). Furthermore, a preferential shuttling of pyruvate into the production of lactate, via lactate dehydrogenase (LDH), occurs despite being in the presence of oxygen, in a process known as aerobic glycolysis or the Warburg effect (Warburg et al., 1927). The initiation of macromolecule biosynthesis is thought to be brought about by eptin, slc2a3, proinsulin, g6pd, and hif1α expression, as well as receptor tyrosine kinase signalling, and the production of reactive oxygen species (ROS), thereby promoting glycolysis and the pentose phosphate pathway (PPP) (Love et al., 2014). Developing brain regions displaying increased levels of aerobic glycolysis also showed increased expression levels of genes responsible for synapse formation and growth (Goyal et al., 2014). Brain regions displaying elevated OXPHOS showed high expression of genes related to mitochondrial function and synaptic transmission (Goyal et al., 2014). Lactate, the main product of aerobic glycolysis is involved in cell growth, differentiation, and transformation of proliferating cells. This effect is brought about by the generation of NAD+ during the conversion of pyruvate to lactate via LDH. The reduced form of NAD+, NADH, regulates transcriptional regulator C-terminal binding protein, which is involved in synaptogenesis and cell maturation. This accounts for the high level of lactate production observed in rapidly dividing mammalian cells (Lunt & Vander Heiden, 2011) (Table 2.1).

Table 2.1. Summary table of NSC metabolism, function, and role in brain health

NSC Metabolism	• **NSC proliferation is associated with higher glycolysis levels while NSC differentiation is associated with OXPHOS.** • **Metabolic reprogramming from glycolysis to OXPHOS is essential during mid-embryogenesis.** • **Lipid metabolism influences NSC fate decisions (FAO for stem phenotype, lipogenesis for differentiation) and is vital for adult brain health.**	***Angelopoulos et at, 2023, Agostini et al, 2016. Xinde Zheng et al, 2016. Maffezzini et al, 2020. Knobloch et al, 2013. Knobloch et al, 2017. Bowers et al, 2020. Xie et al, 2016***
Mechanisms of Glucose Utilization	• **The developing brain consumes glucose for ATP production via glycolysis and OXPHOS.** • **Glucose breakdowns to pyruvate, then acetyl-CoA, enters the Krebs cycle for ATP generation.** • **OXPHOS is the main energy-producing pathway in mature neural cells.** • **Glucose is important for biosynthesis of macromolecules involved in axon growth, synapse maturation, and myelination.** • **Aerobic glycolysis produces lactate despite the presence of oxygen.**	***Hall et al, 2012. Steiner et al, 2019. Tatapudy et al, 2017. Alabduladhem & Bordoni, 2022. Chaudhry & Varacallo, 2022. Kumari et al, 2018. GM et al, 2000. Du et al., 2008. Goyal et al., 2018. Warburg et al., 1927. Love et al., 2014. Goyal et al., 2014. Lunt & Vander Heiden, 2011.***
Mechanisms of Ketone Body Utilization	• **Ketone bodies, especially β-OHB, are an essential energy supply during neurodevelopment and maturation.** • **Ketones modulate neuronal firing and neurotransmitter balance.** • **Ketones are involved in gene expression regulation and influence cell signalling.** • **Ketone bodies produced in the liver are transported to the brain, crosses BBB, oxidized to generate acetyl-CoA for Krebs cycle.**	***Cunnane & Crawford, 2014. Vergara et al., 2019. Achanta & Rae, 2017. Cotter et al., 2013. Barry et al., 2018. Puchalska & Crawford, 2017. Newman & Verdin, 2014. García-Rodríguez & Giménez-Cassina, 2021. Katsu-Jiménez & Giménez-Cassina, 2019.***
Role of Ketone Bodies in NSC Function	• **Ketone bodies are a key source of energy and the synthesis of lipids (myelin and cholesterol).** • **Ketones, including musk ketone, promote NSC proliferation and differentiation through the PI3K/Akt pathway.** • **FAO occurs in NSCs, modulating asymmetric division and maintenance.** • **Ketone bodies produce neuroprotective effects, attenuating oxidative stress, maintaining cellular energy supply, and modulating inflammatory responses.** • **P53 acetylation by ketone bodies maintains stem cell quiescence and prevents stem cell exhaustion.**	***Morris, 2005. Novak et al., 2021. Clémot et al., 2020. Zhou et al., 2020. Yang et al., 2019. Meletis et al., 2006. Andersen et al., 2023.***
Relationship Between NSC Metabolism and Brain Health	• **Defects in glucose and ketone metabolism may lead to neurodevelopmental disorders.** • **Genetic mutations in metabolic pathways are associated with inborn errors of metabolism.** • **Disorders such as GLUT1DS, epilepsy, intellectual disorders, and ASD are linked to glucose metabolism defects.** • **Defective PAST-A leads to epilepsy, intellectual disorders, and ASD.** • **HBP disruption contributes to glucose metabolism and neurological defects. Partial loss of hypothalamic NSCs may result in glucose intolerance and obesity and may be improved through inhibition of NF-κb.** • **Ketone metabolism disorders include MCT1 deficiency, FAO defects, and HMG-CoA lyase deficiency. Stem cells influence mitochondrial function and glucose metabolism-related proteins in PD.**	***A et al., 2021. Saudubray et al., 2019. Hwang et al., 2022. Srour et al., 2017. Kim et al., 2023. Li et al., 2014. Grünert & Sass, 2020.***

Therapeutic Potential	• **KD shows neuroprotective effects in ischaemic stroke, TBIs, and neurodegenerative diseases (PD, AD).** • **Ketone bodies may improve effects of TBI and ischaemic brain injury through neuroprotective and anti-inflammatory effects.** • **Transplantation of rat/human NSCs may reduce stroke volume and improve glucose metabolism in ischaemic areas.** • **KD improves cognitive symptoms in AD and may be beneficial in treating schizophrenia, particularly stereotypical behaviour, mood, and auditory hallucinations.** • **KD potential complementary therapy in anorexia nervosa, ASD, bipolar disorder, and narcolepsy, including treatment-refractory illnesses.** • **SCFA drugs potential treatments in AD, PD and Huntington's disease through maintenance of mitochondrial function and regulating inflammation and oxidative stress.** • **Ketone bodies may decrease beta-amyloid deposition in AD and may protect against cognitive decline in prodromal stage of disease.** • **KD may protect against neurodegeneration in PD by improving OXPHOS and enhancing neuronal resistance.** • **KD may increase availability of tyrosine and the bioavailability of L-DOPA, with combination therapy showing some benefit.** • **KD may protect dopaminergic neurons in PD. TOMM20 and Urine diphosphate (UDP)-galactose 4-epimerase have been associated with galactose metabolism, glucose substrate production, and PD.** • **hESCs may improve glucose metabolism in TLE.**	***Makievskaya et al., 2023. Baranovicova et al., 2022. Sakowski & Chen, 2022. Har-Even et al., 2021. Youm et al., 2015. Veech et al., 2012. Yang et al., 2019. Daines, 2021. Ma et al., 2018. Lipton & Sahin, 2014. Kraeuter et al., 2019. Kraeuter et al., 2015. Tillery et al., 2021. Danan et al., 2022. Kim et al., 2022. Yadav et al., 2022. Rusek et al., 2019. Broom et al., 2019. Lilamand et al., 2022. López-Ojeda & Hurley, 2023. Wurtman et al., 2003. Tieu et al., 2003. VanItallie et al., 2005. Boelens Keun et al., 2021. Pietrzak et al., 2022. Cheng et al., 2009. Choi et al., 2021. Grochowska & Przeliorz, 2022. Du et al., 2019.***

2.5. Mechanisms of Ketone Body Utilisation

While glucose is the primary energy source in the adult brain, it's rapid consumption during neurodevelopment may overwhelm supply (Cunnane & Crawford, 2014; Vergara et al., 2019). Ketone bodies then become the brain's main energy source. β-OHB is the most abundant ketone body (Achanta & Rae, 2017; Cotter et al., 2013). The rate of hepatic ketogenesis is regulated by factors including hormones, gene transcription, and post-translational events (Puchalska & Crawford, 2017). Following β-oxidation, ketone bodies are transported to extrahepatic tissues, including the brain where they cross the blood–brain barrier (BBB) into cells via monocarboxylic transporters (MCTs). Brain cells then oxidize β-OHB to generate acetoacetate using β-hydroxybutyrate dehydrogenase. Acetoacetate subsequently takes up CoA from succinyl-CoA to produce acetoacetyl-CoA. Thereafter, Co-A transferase (SCOT) oxidizes acetoacetyl-CoA generating acetyl-CoA which enters the Krebs cycle and the downstream electron transport chain (Newman & Verdin, 2014; Puchalska & Crawford, 2017). Lipogenesis also plays a crucial role in the nervous system, supporting cellular proliferation, differentiation, and maintenance and dysregulation of lipogenesis may lead to neurodegenerative diseases and other neurological conditions (Jeon et al., 2023).

Ketone bodies play crucial roles in regulating neuronal firing and modulating neurotransmitter balance (García-Rodríguez & Giménez-Cassina, 2021). This involves the reduction of oxaloacetate levels, replenished through aspartate transamination by aspartate transaminase. In this process, α-ketoglutarate is used and transformed into glutamate. GABAergic neurons subsequently catalyse glutamate into GABA, which is involved in regulating excessive neuronal firing (García-Rodríguez & Giménez-Cassina, 2021). Increased GABA levels have been observed in the cerebrospinal fluid of children treated with a KD, highlighting the role of ketone bodies in neurotransmitter balance modulation (Dahlin et al., 2005).

Additionally, ketone bodies play a role in gene expression regulation, specifically glutamate-cysteine ligase, class I histone deacetylases (HDACs), and adenosine kinase (García-Rodríguez & Giménez-Cassina, 2021). Glutamate-cysteine ligase is responsible for the initial rate-limiting step in glutathione synthesis, a vital process in cellular functions

such as DNA and protein synthesis, DNA repair, cellular proliferation, and redox signalling (Franklin et al., 2009). HDACs are crucial for embryonic stem cell proliferation through repression of cell cycle inhibitors (Lagger et al., 2002). Furthermore, adenosine kinase regulates cellular adenosine levels (de Oliveira et al., 2018).

Ketone bodies also influence cell signalling pathways in mouse cortical neurons (Katsu-Jiménez & Giménez-Cassina, 2019) through interactions with the G protein-coupled receptor extracellular receptor GPR109a (Fu et al., 2015; Taggart et al., 2005). García-Rodriguez and Giménez-Cassina (2021) discuss the potential impacts of alterations in the ketone body—GPR109a signalling pathway, eliciting pro-inflammatory or anti-inflammatory effects in a variety of neurodegenerative diseases (Table 2.1).

In utero, ketone bodies cross the placenta efficiently, reaching the same levels as those in maternal blood circulation (Vilaró et al., 1987). During the second half of pregnancy, catabolic maternal metabolism predominates, resulting in increased free fatty acid levels in the blood which can subsequently be used by the foetus through ketogenesis (Horton et al., 1985). The importance of ketone bodies continues postnatally as the infant switches from a constant supply of energy via the placenta, to being intermittently fed (Adam et al., 1975; Nehlig & Pereira de Vasconcelos, 1993). Therefore, newborns must quickly begin β-oxidation of fat stores and metabolise medium-chain fatty acids from breast milk into SCFAs (Erecinska et al., 2004; Nehlig & Pereira de Vasconcelos, 1993). It is therefore not entirely surprising that newborns are found to be in a mild state of ketosis, with ketone levels peaking 48 hours after birth up until six months postpartum (Cunnane, Courchesne-Loyer, St-Pierre, et al., 2016; PERSSON & GENTZ, 1966). Thus, the high degree of adiposity in newborns is then followed by a major increase in fat stores available for β-oxidation over the coming months (Kuzawa, 1998).

2.6. Role of Ketone Bodies in Neural Stem Cell Function

When glucose is readily present in the blood, ketone bodies may mediate physiological processes such as the synthesis of lipids used in neurodevelopment, mainly myelin and cholesterol (Morris, 2005). Quiescent NSCs have also been shown to increase FAO; however, during proliferation, an increase in fatty acid synthesis is observed (Novak et al., 2021). The higher rate of FAO may be due to the increased expression of CPT1A. When

the presence of malonyl-CoA is sufficient, NSCs transform from their quiescence state to a proliferative one. FAO is also required in NSCs, where CPT1A and the breakdown of fatty acids from lipid droplets have been shown to modulate asymmetric division and maintenance (Clémot et al., 2020). Furthermore, ketone bodies have a neuroprotective effect through the promotion of NSC proliferation and differentiation in response to cerebral ischemic injury, signalled though the PICK/Akt pathway (Zhou et al., 2020). Ketone bodies are also thought to elicit a neuroprotective response through the attenuation of oxidative stress, maintaining cellular energy supply, modulating deacetylation, and regulating inflammatory responses (Yang et al., 2019). The p53 protein is acetylated by ketones, thereby maintaining stem cell quiescence, and prevents a gradual decline in their regenerative ability and functionality (Meletis et al., 2006); however, the effects of ketone bodies on p53 activation are still unknown in most adult stem cell types (Andersen et al., 2023) (Table 2.1).

2.7. Relationship Between NSC Metabolism and Brain Health

Defects in glucose and ketone body metabolism may result in a spectrum of neurodevelopmental conditions (A et al., 2021) and distinct categories of inborn metabolic disorders have been proposed involving membrane transporters of energetic molecules, cytoplasmic energy defects, and mitochondrial defects (Saudubray et al., 2019).

2.7.1. Glucose Metabolism Disorders

GLUT1 is a cell membrane transporter that shuttles glucose across the BBB. A deficiency in this transporter, known as GLUT1 deficiency syndrome (GLUT1DS), may result in epilepsy and developmental delay (Table 2.1). A treatment type for GLUT1DS is a ketogenic diet (KD)(A et al., 2021). Bypassing defective glucose metabolism pathways provides an alternate energy pathway through ketone bodies (Hwang et al., 2022). Similarly, PAST-A is a BBB glucose transporter in neurons (A et al., 2021; Srour et al., 2017). Defective PAST-A manifests in epilepsy, intellectual disorders, and ASD. Defects in glycolysis and the PPP, such as in transketolase deficiency, can cause developmental delay and growth defects in children. Abnormalities in glycogen synthesis and catabolism lead

to recurrent hypoglycaemia which may result in acquired microcephaly, epilepsy, and developmental delay (A et al., 2021). Likewise, disruption in the hexosamine biosynthetic pathway (HBP) and downstream *O*-linked *N*-acetylglucosamine cycling also result in neurological defects (Kim et al., 2023).

Moreover, a partial loss of hypothalamic NSCs may result in glucose intolerance and weight gain (Li et al., 2014). Inhibition of NF-κB in hypothalamic NSCs was found to promote their survival and neurogenesis upon implantation. Implanting NF-κB-inhibited hypothalamic NSCs may also ameliorate obesity and glucose-related disorders (Li et al., 2014).

2.7.2. Ketone Metabolism Disorders

Ketone body transport occurs through the MCT1 transporter located in the mitochondrial membrane. A deficiency in MCT1 leads to severe developmental delay (A et al., 2021) and may be treated with dextrose infusions. HMG-CoA lyase deficiency may also alter ketogenesis in addition to exhibiting a leucine catabolic defect (Grünert & Sass, 2020). These abnormalities may lead to myelination issues, cerebral atrophy, and basal ganglia deficiencies (A et al., 2021) (Table 2.1).

2.8. Therapeutic Potential of Manipulating Glucose and Ketone Metabolism in NSCs

2.8.1. Ischemic and Traumatic Brain Injuries

The manipulation of NSC metabolism may impact on outcomes following ischemic stroke. Indeed, the KD appears to exert a neuroprotective effect on damaged brain tissue. The administration of β-OHB before or after an ischemic injury was found to improve both permanent and transient middle cerebral artery occlusion (MCAO), as well as recovery from spinal cord injury. β-OHB administration was also found to protect against neuronal cell damage in oxygen and glucose deprivation, in addition to mitochondrial fragmentation (Makievskaya et al., 2023). Metabolomic profiling of blood plasma following ischemic injury, displayed decreased glycolytic intermediates and an increase in plasma ketone bodies, thereby mimicking a state of ketosis induced by the ischemic event. Elevated β-OHB levels in blood plasma may also indicate the neuroprotective effects of ketone bodies post-ischemic injury (Baranovicova et al., 2022). These benefits may be

attributed to their anti-inflammatory effects, a reduction in oxidative stress, epigenetic regulation, restoration of energy supply, or the prevention of fragmentation and damage to mitochondria (Makievskaya et al., 2023). In rat models of ischemic stroke induced by MCAO, the transplantation of rat NSCs into the ventricular space increased glucose uptake within the ischemic region. Similar effects were observed following the administration of human NSCs, demonstrating the capacity of NSCs to facilitate the recovery of glucose metabolism and reducing stroke volume in moderately ischemic areas (Sakowski & Chen, 2022).

In a model of traumatic brain injury (TBI), administration of rat hippocampal NSCs into the parenchyma restored FDG-PET signal at the injury site (Sakowski & Chen, 2022). Similarly, in ischemic brain injury, the KD has also shown a neuroprotective effect in TBIs. A study examining the potential neuroprotective effect of the KD in a TBI mouse model indicated that a KD may be an effective approach to increase the brain's resistance to damage via the amelioration of cognitive deficits, preventing neuronal cell loss, and reducing reactive astrocytes resulting in reduced neuroinflammation(Har-Even et al., 2021). Ketone bodies may also reduce neurodegeneration by inhibiting the NLRP3 inflammasome, subsequently decreasing neuroinflammation (Youm et al., 2015). Moreover, ketone bodies may ameliorate oxidative stress, a process linked to protein oxidation, DNA damage, and inhibition of the electron transport chain, by inhibiting the production of ROS, preventing lipid peroxidation, protein oxidation, and increasing the levels of antioxidants (Yang et al., 2019). Additionally, ketone bodies may close mitochondrial permeability transition pores, protein complexes associated with apoptosis (Veech et al., 2012). Ketone bodies also reduce the expression of mTOR (Ma et al., 2018), a protein involved in neuroinflammation, neuronal cell death, and neurodegeneration following TBI (Lipton & Sahin, 2014). Inhibition of mTOR expression may be an effective treatment option, reducing inflammation and neurodegeneration in TBI patients (Daines, 2021).

Further experiments using ketone esters in rat TBI models have demonstrated a protective effect against TBI-induced morphological and functional deficits. Results revealed that early administration of ketone esters equated to improved outcomes (Almeida-Suhett et al., 2022). Exogenous ketone supplementation, including synthetic

compounds such as ketone salts and esters, is effective in inducing physiological ketosis regardless of dietary macronutrient intake (Kesl et al., 2016; Poff et al., 2021). In particular, ketone esters, including the monoester 3-Hydroxybutyl-3-hydroxybutyrate, are a potent form of synthetic exogenous ketone and will effectively elevate blood ketone levels in a dose-dependent mechanism, offering regulatory advantages in controlling the duration and activity of blood ketones in brain disease conditions. Experiments testing the impact of ketone esters in rat TBI models have demonstrated a protective effect against TBI-induced morphological and functional deficits (Almeida-Suhett et al., 2022), while promising animal research has indicated the potentials of ketone monoesters in ameliorating Alzheimer's-related disease states and in enhancing cognition (Kashiwaya et al., 2013; Pawlosky et al., 2020).

However, ketone ester supplementation increases the risk of ketoacidosis unless their consumption is closely monitored, and the health implications of their long-term use is not fully known. Nonetheless, the potential of ketone esters in maintaining brain health are being explored and trials are underway to understand their pro-cognition and anti-Alzheimer's potentials in humans (Avgerinos et al., 2022; Poff et al., 2021).

2.8.2. Neuropsychiatric Disorders

Studies involving in vivo imaging, post-mortem brain analysis and animal models have highlighted that schizophrenia may be linked to abnormal glucose metabolism within the cerebrum. Schizophrenic animal models subjected to a KD displayed improvements in hyperactivity, stereotypical behaviour, and increased sociability (Kraeuter et al., 2015). The KD also demonstrated an improvement in auditory hallucinations, mood, energy, the ability to concentrate, and sociability in patients with schizoaffective disorder (Kraeuter et al., 2019). A literature review concerning the use of the KD in treating other psychiatric disorders demonstrated that the KD displays merit as a potential complementary treatment option for anorexia nervosa, ASD, bipolar disorder, and narcolepsy, in addition to schizophrenia (Tillery et al., 2021). The KD has also shown to be effective in treating treatment refractory mental illness, resulting in significant improvements in depression and psychosis symptoms (Danan et al., 2022).

2.8.3. Epilepsy

Temporal lobe epilepsy (TLE) is the most common focal epilepsy and is associated with impaired glucose metabolism (McDonald et al., 2018; Vinti et al., 2021). In mouse models of TLE, human embryonic stem cells (hESCs) implantation resulted in the restoration of glucose metabolism, possibly due to their differentiation into astroglial cells highlighting the potential metabolic utility of stem cells in treating neurological conditions (Du et al., 2019; Sakowski & Chen, 2022).

2.8.4. Neurodegenerative Diseases

SCFAs in the past have been synthesized into drugs effective in treating epilepsy, such as sodium butyrate and valproic acid (Kim et al., 2022). These drugs have also shown promise as potential treatments for other neurological pathologies including Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (Kim et al., 2022). SCFAs maintain healthy mitochondrial function and stimulate the maturation of microglia, consequently suppressing the progression of neurodegenerative diseases by regulating inflammation and oxidative stress (Yadav et al., 2022). However, SCFAs such as butyric acid and propionic acid may play a part in the acquisition of ASD (Lei et al., 2016). However, research is not definitive in this area. SFCA levels in children were not associated with ASD, but using the KD to treat children suffering from ASD may ameliorate behavioural symptoms; however, these effects were patient specific (Li et al., 2021; Wang et al., 2019).

Certain pathophysiological events that occur in AD may be attributed to mitochondrial dysfunction and decreased respiratory chain function. These events result in the defective metabolism of amyloid precursor protein, thereby causing increased production and deposition of beta-amyloid fragments, characteristic of an AD brain (Devi et al., 2006). Furthermore, high-sugar diets increase the deposition of these fragments (Taylor et al., 2017). The production of endogenous ketone bodies may drive positive neurological effects such as reducing inflammation and ameliorating mitochondrial function (Rusek et al., 2019). Further studies have demonstrated that the KD decreases beta-amyloid fragment production and deposition (Broom et al., 2019). The KD may also protect against the cognitive symptoms of AD, especially in the prodromal stage of the disease and

enhance cognitive performance and potentially postpone AD-associated cognitive decline (Lilamand et al., 2022).

Abnormal glucose metabolism, mitochondrial dysfunction, and metabolic disturbances have been linked to PD, and the KD has shown a range of benefits in PD patients (López-Ojeda & Hurley, 2023). The KD increases the availability of tyrosine, a precursor for dopamine which relieves the deficits in this neurotransmitter (Wurtman et al., 2003). The KD may also improve OXPHOS in the brain and thereby improve overall brain energy metabolism (Tieu et al., 2003). The KD may improve the bioavailability of L-DOPA, a staple therapeutic used in PD treatment (VanItallie et al., 2005). Furthermore, combination therapy of L-DOPA and KD has been shown to slow the progression of PD symptoms (Boelens Keun et al., 2021; Pietrzak et al., 2022) and the KD may protect dopaminergic neurons from degeneration and improve motor function (Cheng et al., 2009). The use of KD in PD improved patient learning and memory. Preclinical research indicating that factors, such as brain-derived neurotrophic factor and glutamate, often associated with hippocampal synaptic integrity and stabilisation of neural networks, were influenced by ketone bodies (Choi et al., 2021). The KD also enhanced neuronal resistance in the substantia nigra to destruction, preventing the progression of PD symptoms (Grochowska & Przeliorz, 2022). Moreover, key metabolic mitochondrial proteins including translocase of outer mitochondrial membrane 20 (TOMM20) and urine diphosphate galactose 4-epimerase (GALE), which shuttle proteins destined for the mitochondrial matrix, and play important roles in galactose metabolism and the production of glucose substrates respectively, have also been associated with PD pathogenesis (Sakowski & Chen, 2022) (Table 2.1).

2.9. Future Directions

The interplay between NSC metabolism and brain homeostasis emphasizes the importance of examining the molecular and metabolic mechanisms that guide these processes to best improve therapeutic and diagnostic options in various neurological disorders. Further research investigating the regulation of glycolysis, OXPHOS, and lipid metabolism in NSCs may shed light on the temporal and molecular mechanisms controlling these processes during neural development. The therapeutic manipulation of NSC metabolism may give rise to targeted interventions that harness the neuroprotective

and regenerative capabilities of NSCs in ischemic injuries and TBIs, neuropsychiatric disorders, and neurodegenerative conditions. In the following chapters, we will further investigate the importance of ketolytic and glycolytic pathways in NSC metabolism. This will be achieved through genomic analysis of NB datasets and in vitro analysis of two NSC cell line models.

2.10. Conclusion

Glucose serves as the primary energy source for NSCs, integral in proliferation, differentiation, quiescent maintenance, and biosynthetic processes. However, the high energy demands of the developing brain often exceed glucose availability, when ketone bodies are utilised. Ketone bodies, generated through β-oxidation in the liver, may be used by NSCs for energy, in addition to the synthesis of lipids involved in neurodevelopment, including myelin and cholesterol. The regulation of β-oxidation and lipogenesis plays a crucial role in NSC behaviour and fate determination. Disruptions in these metabolic pathways have been associated with neurodevelopmental, neuropsychiatric, and neurodegenerative conditions. Manipulating neural metabolism, including placing patients on a KD, has been highly advantageous in epilepsy management and has shown promise in many other neurological conditions. Such therapies elicit a neuroprotective effect thereby promoting NSC proliferation and differentiation, further highlighting the promise of these approaches as therapeutic interventions. Further investigations into NSC metabolic pathways, as well as elucidating the impact of dysregulated NSC metabolism, will contribute to our understanding of neurodevelopment and perhaps shed new light on novel treatments for the management of neurological conditions.

The review of the literature has addressed the hypothesis that glucose metabolism is vital in brain development and health and that ketone bodies play and important role in NSC metabolism (Table 2.2).

Table 2.2. Proof of hypothesis one. Glucose metabolism is vital in brain development and health and that ketone bodies play and important role in NSC metabolism.

Importance of Glycolysis	• NSC proliferation is associated with higher levels of glycolysis • The developing brain consumes glucose for ATP production via glycolysis and OXPHOS. • Glucose is metabolised to pyruvate, then acetyl-CoA, and enters the Krebs cycle for ATP generation. • Glucose is vital for the biosynthesis of macromolecules involved in axon growth, synapse maturation, and myelination. • Aerobic glycolysis produces lactate despite the presence of abundant oxygen.
Importance of Ketone Bodies	• Important source of energy in the absence of glucose • Promote NSC proliferation and differentiation • Neuroprotective • Regulate inflammation • Mitigate oxidative stress • maintains stem cell quiescence and prevent stem cell exhaustion
Clinical Implications	• Defects in energy metabolism leads to neurodevelopmental disorders. • ASD, epilepsy, and intellectual disorders are associated with glucose metabolism defects. • Ketone bodies elicit a therapeutic effect in neurodevelopmental and neurodegenerative diseases, TLE, stroke and TBIs.
Therapeutic potential	• KD shows neuroprotective and anti-inflammatory effects in ischaemic stroke, TBI, and neurodegenerative diseases (AD, PD). • KD improves cognitive symptoms in AD, schizophrenia, anorexia nervosa, ASD, bipolar disorder, and narcolepsy. • Ketone bodies reduce β-amyloid deposition in AD, protect

	dopaminergic neurons in PD, and enhance OXPHOS. • Rat/human NSCs and hESCs improve glucose metabolism and reduce stroke volume in ischaemic areas and TLE. • SCFA drugs support mitochondrial function and regulate inflammation in AD, PD, and Huntington's disease.

3. Chapter Three.

Fuelling Neuroblastoma: Genomic Analysis of Ketolytic and Glycolytic Gene Expression in Relation to MYCN Oncogene Amplification, Stage, and Prognosis

Written by Joseph W. Molloy, Mary-Kate McLoughlin, Lauren Devitt, Dr. David Lee, Dr. Eric Downer and Dr. Denis Barry

3.1. Abstract

Neuroblastoma (NB) is a childhood cancer of the sympathetic nervous system, and its prognosis is poor. NB cells undergo transcriptional changes to utilise aerobic glycolysis as the primary metabolic pathway, which provides an immediate source of ATP to meet high biosynthetic demands. Alternative metabolic fuel inputs, including ketone bodies which require oxidative phosphorylation, may impact the proliferative capacity of NB.

In this study, the expression of glycolytic and ketolytic genes in the context of *MYCN* oncogene amplification, tumour staging 1 - 4, and Kaplan-Meier survivability were investigated using the R2: Genomics analysis and visualisation platform (http://r2.amc.nl). The R2 database is a platform for gene expression and prognostic data for primary tumour samples. Three NB genomics datasets were accessed and further analysed in GraphPad Prism to investigate the relationships between glycolytic and ketolytic gene expression and prognosis.

Glycolytic gene expression was increased in MYCN-amplified, metastatic tumours, and was associated with worse event-free survival. Ketolytic gene expression was lower in metastatic tumours and was associated with better event-free survivability. The glycolytic gene expression profile of NB suggests that elevated levels correlate with a low probability of survival. Ketolytic gene expression patterns suggest a decreased reliance on ketolytic energy, which may be exploited to slow tumorigenic growth.

This study validates the glycolytic gene expression profiles in metastatic and MYCN-amplified NB tumours and suggests the potential use of these genes in prognosis prediction. Furthermore, this study highlights the reliability and utility of the R2 database as an oncogenomic tool for NB research.

3.2. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumour in childhood and the most common cancer diagnosed during the first year of life. Arising from the primordial crest cells that form the sympathetic nervous system, NB presents as a mass lesion predominantly in the adrenal cortex and paraspinal ganglia yet can arise anywhere from the neck to the pelvis. NB is responsible for a disproportionally high percentage of cancer-related paediatric deaths (Young et al., 1986) and incidence rates can vary from 4.1 to 15.8 per 1,000,000 population (Okawa & Saika, 2022). Current therapeutic approaches show low efficacy due to the biological and immunological features of high-risk NB patients in approximately 60% of cases, and staging is important in determining severity and prognosis (DuBois et al., 2022) (Table 3.1). The current frontline treatment of high-risk NB consists of surgical resection of the tumour, the administration of multiagent chemotherapy, and harvest of peripheral blood stem cells. Recent statistics puts the 5-year event-free survival rate of this regimen to be 51% (DuBois et al., 2022).

Table 3.1. The INSS is the current staging system for the Children's Oncology Group for the risk-classification of patients (Brodeur et al., 1993).

Stage	**Definition**
1	Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive).
2A	Localised tumour with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumour microscopically.
2B	Localised tumour with or without complete gross excision; with ipsilateral non-adherent lymph nodes negative for tumour microscopically.
3	Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; or localised unilateral tumour with

	contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration or by lymph node involvement.
4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4s)
4s	Localized primary tumour (as defined for stage 1, 2A or 2B) with dissemination limited to skin, liver and/or bone marrow (limited to infants < 1 year of age and bone marrow with < 10% tumour cell involvement)

Given the heterogeneous nature of NB cancer population, risk stratification becomes essential. Initial response rates to treatment are suboptimal, highlighting the need for improved induction chemotherapy schedules. Even after seemingly curative initial treatment, the risk of recurrence poses an ongoing challenge. Similar to other malignancies, chemoresistance is an ongoing obstacle, and treatment-related morbidities are core considerations for patient outcomes (Gains et al., 2012)

Complete regression or spontaneous differentiation may occur, resulting in complete recovery without treatment. Conversely, other patients may develop widespread metastatic tumours, associated with increasingly poor prognosis. Tumour staging, along with other prognostic factors, offers a predictive value when choosing patient treatment, treatment response(s) and potential outcomes. MYCN amplification is correlated with advanced stages of disease and is highly predictive of rapid and aggressive tumour progression with poor clinical outcome and drug resistance (Cheng et al., 1993; DeBerardinis et al., 2008). MCYN gene amplification is a single oncogenic driver capable of inducing neoplastic transformation in neural crest-derived cells. It is present at diagnosis and is not acquired during later stages of tumorigenesis, suggesting that increased *MYCN* expression is an early tumorigenic event. As a consequence, dysregulation of cells results in increased self-renewal, apoptotic resistance, and metabolic flexibility (Otte et al., 2021). The MYCN transcription factor promotes a pro-tumour environment in which NB can proliferate, metastasise, and evade immune surveillance. MYCN amplified NB have distinct protein and gene expression alterations,

when compared with MYCN non-amplified NB, suggesting that MYCN amplification is associated with activated mitochondrial oxidative and glycolytic metabolism, and glutamine synthesis (M. G. Vander Heiden et al., 2009). Thus, NB stage and MYCN amplification status influences prognosis, with stage 1 patients having a five-year survival rate greater than 95%, compared to 40-50% in patients with stage 4 disease with MYCN amplification.

NB metabolic profile is characterised by aerobic glycolysis and reduced mitochondrial oxidative metabolism (M. G. Vander Heiden et al., 2009). For cancer cells, including NB, to enable a high proliferative rate, they undergo a metabolic shift, where their glucose uptake is increased allowing for increased glycolytic rates at the expense of more efficient energy production by oxidative phosphorylation (OXPHOS). This process is known as the Warburg effect or aerobic glycolysis. Despite the paradoxical nature of this process, enhanced glycolysis is needed to generate the intermediates used in biosynthetic pathways important for cancer cell proliferation (Aminzadeh et al., 2015).

NBs show a high rate of lactic acid production with a low rate of oxygen consumption. This demonstrates the switch from mitochondrial OXPHOS to glycolysis (Levy et al., 2012). NB tumours do not contain lower numbers of mitochondria (Rene' G Feichtinger et al., 2010; Lyser, 1974), but rather have reduced production of succinate dehydrogenase (Rene' G Feichtinger et al., 2010). Dysregulation within mitochondria, such as alterations in chromosome 1p36 observed in approximately 20-40% of primary NBs (Carén et al., 2007), the deletion of the long arm of chromosome 11 (Guo et al., 1999), gain of chromosome 17 (Plantaz et al., 1997), and MYCN amplification (Brodeur et al., 1984), are associated with NB tumour cell metabolic reprogramming (Aminzadeh et al., 2015). The high rates of lactic acid efflux and low rate of oxygen consumption are thought to confer a selective advantage for cancer cells by facilitating rapid proliferation, immune evasion, pro-tumorigenic activity, and resistance to altered mitochondrial metabolism. Lactate-mediated acidification of the tumour microenvironment results in enhanced tumour invasiveness and reduced immune responses (Singer et al., 2011). It also acts as an oncometabolite, upregulating the transcriptional activity of key oncogenes, transcription

factors, and other genes involved in the regulation of the cell cycle and proliferation (San-Millan et al., 2019).

The ketogenic diet (KD) leads to an acute increase in both serum ketone body concentration and mitochondrial oxidation of ketone bodies. The primary ketone bodies are β-hydroxybutyrate (β-OHB) and Acetoacetate (AcAc). Acetyl CoA, a product of ketone body metabolism can be integrated into the Krebs cycle (Morscher et al., 2015). Due to mutations in the mitochondrial DNA and enzymes of cancer cells, they do not have the ability to metabolise ketone bodies for energy production (Isidoro et al., 2004). Fasting and the KD have been shown to reduce blood glucose and slow cancer progression in animal models (Maroon et al., 2013; Morscher et al., 2015). Glucose deprivation combined with the KD in cancer cells has been linked to elevated mitochondrial reactive oxygen species (ROS) and subsequent oxidative toxicity in cancer cells (Allen et al., 2013; Aykin-Burns et al., 2009). A phase 1 trial investigating co-radiation and KD therapy in head and neck cancer xenografts, demonstrated that mice subjected to both radiation therapy and the KD, had a slight improvement in their tumour growth rate and survival compared to mice that only underwent radiation therapy (Ma et al., 2021). In a study involving ovarian cancer, phosphoglycerate kinase 1 (PGK1) was identified as being highly expressed [30]. Inhibiting PGK1 with NG52 reduced cell migration and cell invasion abilities. These data highlight the potential of anti-glycolytic therapies in cancer (Gou et al., 2021). Investigations into ketolytic gene expression showed that ACAT1 and BDH1 were significantly correlated with the mRNA level of ND2 in oral squamous cell carcinoma tumours (Yousefi et al., 2022).

The objective of this study was to investigate the expression of glycolytic and ketolytic genes in primary NB tumours. Studying cancer genomes may link gene expression with tumour predictors and patient survival. The R2 platform is a user-friendly oncogenomics analysis and visualization website hosting gene expression data from many tumour datasets. The glycolytic genes, *HK2*, *GAPDH* and *ENO1* were chosen as they mediate early, mid, and final stages of glycolysis, respectively, and the three ketolytic genes *BDH1*, *OXCT1* and *ACAT1* were chosen as they mediate ketolysis. These genes were analysed for MYCN amplification, survivability, and tumour stage progression in three NB datasets, containing

the highest numbers of tumour samples in the R2 platform. Glycolytic genes were associated with poor survivability, yet ketolytic gene expression was more associated with pre-metastatic cancer and better event free survivability.

3.3. Methods

3.3.1. NB dataset selection

The gene expression of glycolytic enzymes *HK2*, *GAPDH*, and *ENO1* were analysed in the context of MYCN amplification, INSS tumour stage progression and event free survival (Table 3.2). Gene expression was used as a proxy for enzyme activity. Glycolytic pathway activity was investigated by analysing the expression of the *HK2*, *GAPDH* and *ENO1* genes. The expression levels of *BDH1*, *OXCT1* and *ACAT1* genes were used to investigate ketolytic pathway activity (Table 3.2). Three NB datasets were chosen based on data set size. The R2 Genomics Analysis and Visualisation Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) facilitated the storage of tumour and genetic datasets and allowed analysis and visualisation of the data in multiple formats. The data was extracted from the platform for statistical analysis.

Table 3.2. Genes, Proteins, Functions, and Implications in NB. The glycolytic and ketolytic genes used in this study, their corresponding proteins, function, and implications in NB.

Glycolytic Genes	**Enzyme**	**Function**	**Implications in NB**
HK2	Hexokinase 2	Phosphorylates glucose to give glucose-6-phosphate (Bian et al., 2022).	HK2 expression and activity levels are increased in metastatic NB variants compared with local variants. Upregulation of HK2 strengthens cellular resistance (Botzer et al., 2016)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Oxidises glyceraldehyde 3-phosphate molecules and reduces NAD- to form NADH (Shestov et al., 2014).	Inhibition of GAPDH in NB induced apoptosis. GAPDH is important in NB cell survival (Nakazawa et al., 1997)

		Phosphorylates oxidised glyceraldehyde 3-phosphate to give 1,3-bisphosphoglycerate (Lazarev et al., 2020).	
ENO1	Enolase 1	Catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Ma et al., 2023)	ENO1 upregulation can inhibit NB cell proliferation and induce apoptosis. ENO1 has a strong dose-dependent inhibitory effect on NB tumour cell growth (Ejeskär et al., 2005).
Ketolytic Genes	**Enzyme**	**Function**	**Implications in NB**
BDH1	β-OHB-Dehydrogenase	Converts β-OHB to acetoacetate (Puchalska & Crawford, 2017).	Under expressed in glioma tumours (Maeyama et al., 2021)
OXCT1	3-Oxoacid-CoA Transferase	Catalyzes the transfer of the CoA moiety from succinyl-CoA to acetoacetate, producing acetoacetyl-CoA (Grünert et al., 2021)	Under expressed in glioma tumours. (Maeyama et al., 2021)
ACAT1	Acetyl-CoA Transferase	Catalyses the condensation of two acetyl-CoAs to make acetoacetyl-CoA. Involved in reverse reaction, breaking down acetoacetyl-CoA into two molecules of	Knockdown of mitochondrial ACAT1 expression using chlorogenic acid leads to NB cell differentiation, displaying antitumour activity. (You et al., 2023)

		acetyl-CoA. (Abdelkreem et al., 2019; Fukao et al., 1990)	

NB datasets with information pertaining to MYCN status, event free survival, INSS stage and gene expression patterns were selected. Those with the largest sample sizes were chosen for analysis. Studies that contained highly specific patient cohorts were excluded. All samples with information relating to gene expression and the parameters of interest were included in the analysis. The three datasets selected were Asgharzadeh (n = 249), Cangelosi (n = 786), and Kocak (n = 649). INSS tumour stage was classified into subgroups: stage 1, stage 2, stage 3 and stage 4. Stage 4S is applicable to children under one years of age. Samples belonging to this stage, characterised by a favourable prognosis and spontaneous tumour regression, and those that lacked staging, were omitted from analysis.

The Kocak dataset was composed of 649 primary tumour samples, obtained at the time of diagnosis prior to cytotoxic therapy. The dataset included 153 stage 1 tumours, 113 stage 2, 91 stage 3, 214 stage 4 and 78 stage 4s tumours. It was compiled by Kocak et. al., 2013, to investigate the relationship between Hox-C9 and spontaneous regression in NB (Kocak et al., 2013). The dataset can be found at the GEO accession tag GSE45547.

The Cangelosi dataset was an amalgamation of four separate datasets. In each dataset, tumour samples were taken at the time of diagnosis before cytotoxic therapy. The four datasets included were RNA-seq498 (available at the GEO accession tag GSE62564), Agilent709 (available at ArrayExpress E-MTAB-1781), Agilent262 (GEO accession tag GSE120572) and Affymetrix 413 (a combination of six sub-cohorts). The Cangelosi dataset included 786 tumour samples. This included 143 stage 1, 125 stage 2, 105 stage 3, 320 stage 4, 92 stage 4s tumours, and 1 non-assigned tumour. A more detailed description of the formation of this dataset can be found in the original paper, Cangelosi et al., 2020 (Cangelosi et al., 2020).

The Asgharzadeh dataset was composed of 249 tumour samples, related to the TARGET analysis of NB gene expression. It included 30 stage 1, 1 stage 3, 216 stage 4, and 2 unknown tumour samples. The R2 internal identifier for this dataset is ps_avgpres_targetnrbl249_huex10t.

The relationship between pathway activity and event free survival was investigated by generating Kaplan Meier (KM) plots on the R2: Genomics Analysis and Visualisation Platform. The median gene expression level was used as the cut off modus. The R2 platform tested significance between low and high gene expression groups in KM event free survival probability plots using a log rank test and reported the significance as a Chi-squared value. Values were considered significant when $*P < 0.05$. The Asgharzadeh data set was derived from 168 months of data. For the Kocak data set, 88 data sets analysed event free survival probability derived from 216 months of data. The Cangelosi data sets were derived from over 240 months of data.

Cox proportional hazards regression was subsequently used to estimate the effect of age $<18^{th}$ months / >18 months, MYCN status and stage (1 vs 4), on event free survivability for each ketolytic and glycolytic gene tested. This analysis was performed using the R2: Genomics and Analysis Visualisation Platform and generated high/low hazard ratio values, and significances.

3.3.2. Data investigation and transformation

Gene expression data relating to MYCN amplification status and INSS stage was imported into GraphPad Prism (version 9). The R2: Genomics and Analysis Visualisation Platform generated the expression data with a log2 transformation. The log2 transformed data was subjected to a Kolmogorov Smirnov normality test and visualised by generating histogram plots. It was found that the log2 transformed data often failed the normality test. The R2 database assumes Gaussian distribution of all datapoints and therefore performs a two-tailed *t*-test with a parametric (Welch's) correction between two groups. To confirm this, the raw and log2 data was assessed for distribution normality. A large proportion of datapoints for every gene in all datasets did not fit the Gaussian data distribution pattern. A reason for this may be that primary human cancer cells display gene expression patterns

that are not normally distributed and express a complex, heavy tailed phenotype (Marko & Weil, 2012). One of the most effective ways to account for the difference is by using non-parametric analytics (de Torrente et al., 2020; Marko & Weil, 2012). The data distribution was subsequently investigated. Data were non-parametric, allowing use of the Mann-Whitney U test to compare medians. The assumptions of the Mann-Whitney U test were confirmed using QQ plots.

3.3.3. Gene expression and MYCN amplification status

A Mann-Whitney U test was performed in GraphPad Prism. This test can be used to identify a significant difference between the medians of two groups, if it is known that both groups have a similar distribution. To achieve these conditions, the untransformed data was used. P values were considered significant when $*P < 0.05$.

3.3.4. Gene expression and INSS stage

INSS tumour stage was classified into subgroups: stage 1, stage 2, stage 3, and stage 4. Stage 4S is applicable to children under one years of age. Samples belonging to this stage, characterized by a favourable prognosis and spontaneous tumour regression, and those that lacked staging, were omitted (Table 3.3). A Kruskal-Wallis test with a post-hoc Bonferroni- multiple comparisons test was performed in GraphPad prism. To further compare stage 1 and stage 4, a Mann-Whitney U test was then performed. P values were considered statistically significant when $*P < 0.05$.

3.3.5. Figure and graph generation

KM curves were generated on the R2: Genomics Analysis and Visualisation Platform. All other graphs were generated using GraphPad Prism. To visualise fold changes, gene expression data were presented as a mean log2 transformation of the raw data.

3.4. Results

The impact of glucose and ketone bodies on NSC metabolism was explored in Chapter Two of this thesis. This chapter investigated glycolytic and ketolytic gene expression in NB. The SH-SY5Y cell line, a common cell line used in neurological research, is derived from NB. Therefore, the expression of glycolytic and ketolytic genes in NB may be indicative of gene expression in the SH-SY5Y cell line and other NSC lines. Given NB

genomic datasets are cancer-derived datasets, analysis of ketolytic and glycolytic gene expression was carried out in relation to MYCN oncogene amplification, stage, and prognosis. Therefore, the data generated applies to both neurological and oncology research.

The expression of glycolytic genes *HK2*, *GAPDH*, and *ENO1* were analysed in the context of MYCN amplification, INSS tumour stage progression and overall event free survival using KM plots. To generate KM data, gene expression was categorised into distinct high and low expression groups (Table 3.3).

Table 3.3. Kaplan-Meier event-free survival probability sample size (n value) of each dataset compared in Kaplan-Meier survival probability curves.

Dataset	**High expression (n)**	**Low expression (n)**	**Omitted (n)**
Kocak	237	239	173
Cangelosi	384	385	17
Asgharzadeh	123	124	2

MYCN status was classified into two subgroups: MYCN amplified and MYCN non amplified. Samples that lacked MYCN status data were omitted from analysis (Table 3.4).

Table 3.4. MYCN status sample size. This table presents the sample size (n value) of the MYCN status of each dataset.

Dataset	**MYCN amplified (n)**	**MYCN non-amplified (n)**	**Omitted (n)**
Kocak	93	550	6
Cangelosi	153	629	4
Asgharzadeh	68	175	6

The number of tumours in each stage for each data set were collated. Samples belonging to stage 4S which are characterized by a favourable prognosis and spontaneous tumour regression, as well as those lacking staging, were omitted (Table 3.5).

Table 3.5. INSS stage sample sizes. Table presents the sample size (n value) of the 5 INSS stages in each dataset.

Dataset	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 4s (omitted)**	**Omitted**
Kocak	153	113	91	214	78	0
Cangelosi	143	125	105	320	92	1
Asgharzadeh	30	0	1	216	0	2

3.4.1. HK2

HK2 expression was increased in MYCN amplified tumours in all data sets tested (****P < 0.0001 for all data sets) (Fig. 3.1, A). *HK2* expression was increased in stage four tumours in all datasets (****P < 0.0001 Cangelosi and Kocak) (Fig. 3.1, B). High *HK2* expression was correlated with poorer event free survival (****P < 0.0001 for Kocak in both datasets) (Table 3.6). The Asgharzadeh dataset was omitted from this analysis, due to lack of data.

3.4.2. GAPDH

GAPDH expression was increased in MYCN amplified tumours in all three datasets (****P < 0.0001 Cangelosi, Kocak, ***P < 0.001 Asgharzadeh) (Fig. 3.1, C). *GAPDH* expression was increased in stage four tumors in all datasets (****P < 0.0001 in Asgharzadeh, Cangelosi, and Kocakdata sets) (Fig. 3.1, D). High *GAPDH* expression was correlated with poorer event free survival probability in two datasets (****P < 0.0001 in Cangelosi, and ***P < 0.001 in the Kocak dataset) (Table 3.6).

3.4.3. ENO1

ENO1 expression was increased in MYCN amplified NB in all datasets (****P < 0.0001 in Cangelosi and Kocak, *P < 0.05 in Asgharzadeh) (Fig. 3.1, E). *ENO1* expression was increased in stage four tumours in four datasets (****P < 0.0001 in Cangelosi, Kocak, *P < 0.05 in the Asgharzadeh dataset). High *ENO1* expression was correlated with poorer event free survival probability in two datasets (****P <0.0001 in Cangelosi, **P < 0.01 in Kocak) (Fig. 3.1, F) and (Table 3.6).

Ages under and over 18 months, stage 1 and 4 tumours, and MYNC status were included in Cox regression survival analysis with respect to outcome and glycolytic gene expression in each data set. *HK2*, *GAPDH* and *ENO1* were identified as significant risk factors with respect to patients under and over 18 months, in stage 1 and 4 metastases, and in MYNC non amplified tumours in Kocak and Cangelosi datasets.

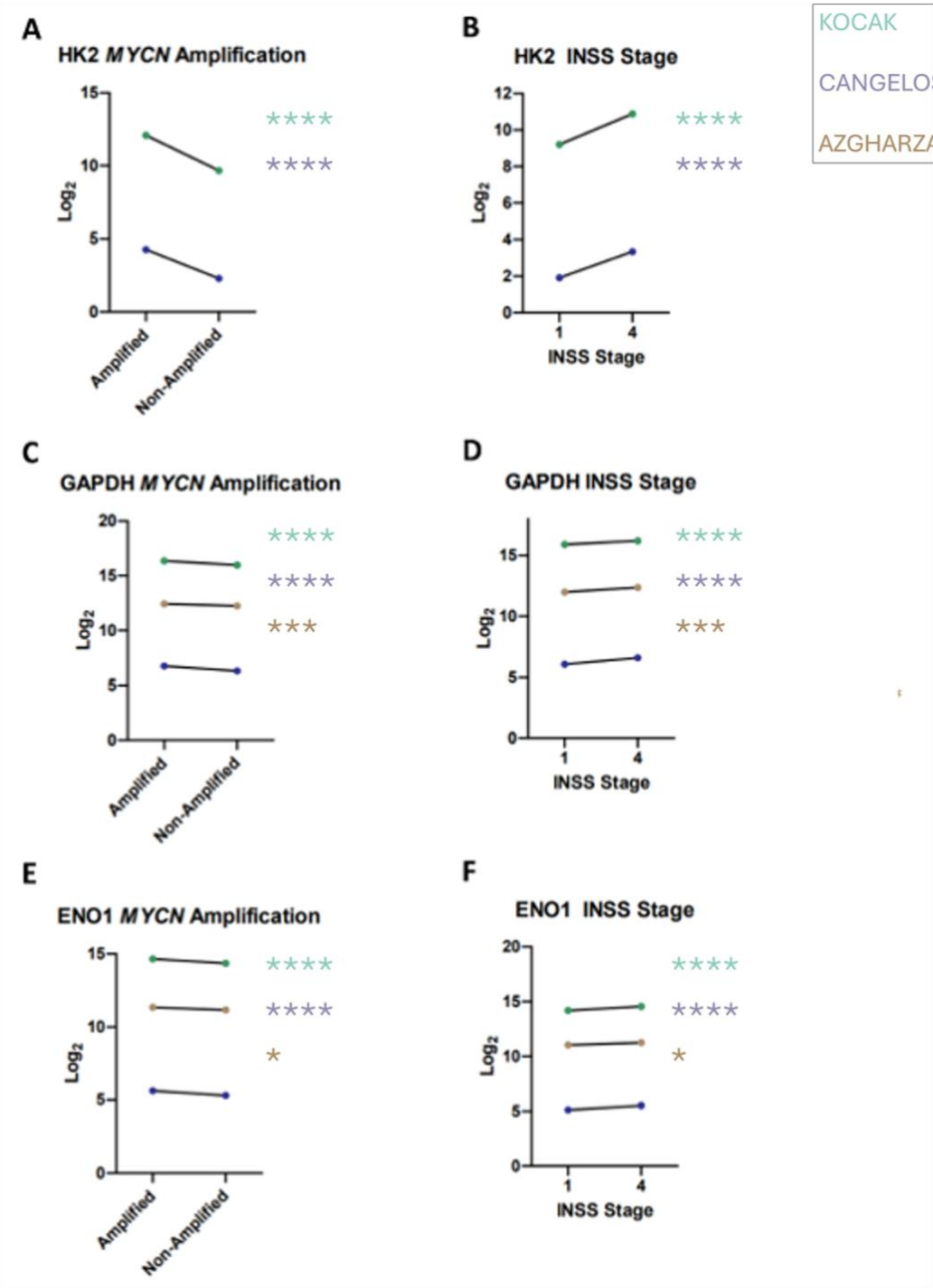


Figure 3.1. HK2, GADPH, and ENO1 expression increased in MYCN amplified tumours. (A) *HK2* expression in MYCN and non-MYCN amplified NB. (B) *HK2* expression in stage 1 and 4 NB. (C) *GAPDH* expression in MYCN and non-MYCN amplified NB. (D) *GAPDH* expression in stage 1 and 4 NB. (E) *ENO1* expression in MYCN and non-MYCN amplified NB. (F) *ENO1* expression in stage 1 and 4 NB. **P* < 0.05. ***P* < 0.01. ****P* < 0.001. *****P* < 0.0001.

Table 3.6. Correlations of HK2, GADPH, and ENO1 with event free survival probability. Glycolytic genes HK2, GAPDH, and ENO1 from the three datasets demonstrating outcome, and P-value.

Gene	Dataset	Outcome	P Value
HK2 Expression	Kocak	High is worse	$p = 1.19 \times 10^{-12}$
	Cangelosi	High is worse	$p = 7.29 \times 10^{-23}$
	Asgharzadeh	Not available	Not available
GAPDH Expression	Kocak	High is worse	$p = 8.29 \times 10^{-10}$
	Cangelosi	High is worse	$p = 1.45 \times 10^{-20}$
	Asgharzadeh	ns	P = 0.353
ENO1 Expression	Kocak	High is worse	$p = 3.20 \times 10^{-07}$
	Cangelosi	High is worse	$p = 1.71 \times 10^{-17}$
	Asgharzadeh	ns	p = 0.148

3.4.4. Expression of ketolytic genes in NB

The gene expression of ketolytic enzymes *BDH1*, *OXCT1*, and *ACAT1* were analysed in the context of MYCN amplification, INSS tumour stage progression and overall event free survival.

3.4.5. BDH1

BDH1 expression was increased in MYCN amplified NB in all datasets (****$P < 0.0001$ in Cangelosi and Kocak, ***$P < 0.001$ in Asgharzadeh) (Fig. 3.2, A). *BDH1* expression was unchanged in stage 4 tumours in all datasets (Fig. 3.2, B). High *BDH1* expression was correlated with poorer event free survivability in two datasets (****P <0.0001 in Cangelosi, **P <0.01 in Kocak) and unchanged in the Asgharzadeh dataset (Table 3.7).

3.4.6. OXCT1

OXCT1 expression was increased in MYCN amplified NB in all datasets (*$P < 0.05$ in Asgharzadeh, Cangelosi and Kocak) (Fig. 3.2, C). *OXCT1* expression was decreased in stage 4 NB in all datasets (****$P < 0.0001$) (Fig. 3.2, D). There was no significant difference in event free survival in any of the datasets (Table 3.7).

3.4.7. ACAT1

ACAT1 expression was increased in MYCN amplified NB in all datasets (****$P < 0.0001$ in Asgharzadeh and Cangelosi, **$P < 0.01$ Kocak) (Fig. 3.2, E). *ACAT1* expression was decreased in stage 4 NB in all datasets (****$P < 0.0001$) (Fig. 3.2, F). Low *ACAT1* expression was correlated with poorer event free survivability in two datasets (****$P < 0.0001$ in Cangelosi, **$P < 0.01$ in Asgharzadeh) (Table 3.7).

Ages under and over 18 months, stage 1 and 4 tumours, and MYNC status were included in Cox regression survival analysis with respect to outcome and ketolytic gene expression in each data set. OXCT1, and ACAT1 were generally not identified as significant risk factors in patients under and over 18 months, in stage 1 and 4 metastases, and in MYNC non amplified tumours, in the Kocak and Cangelosi datasets.

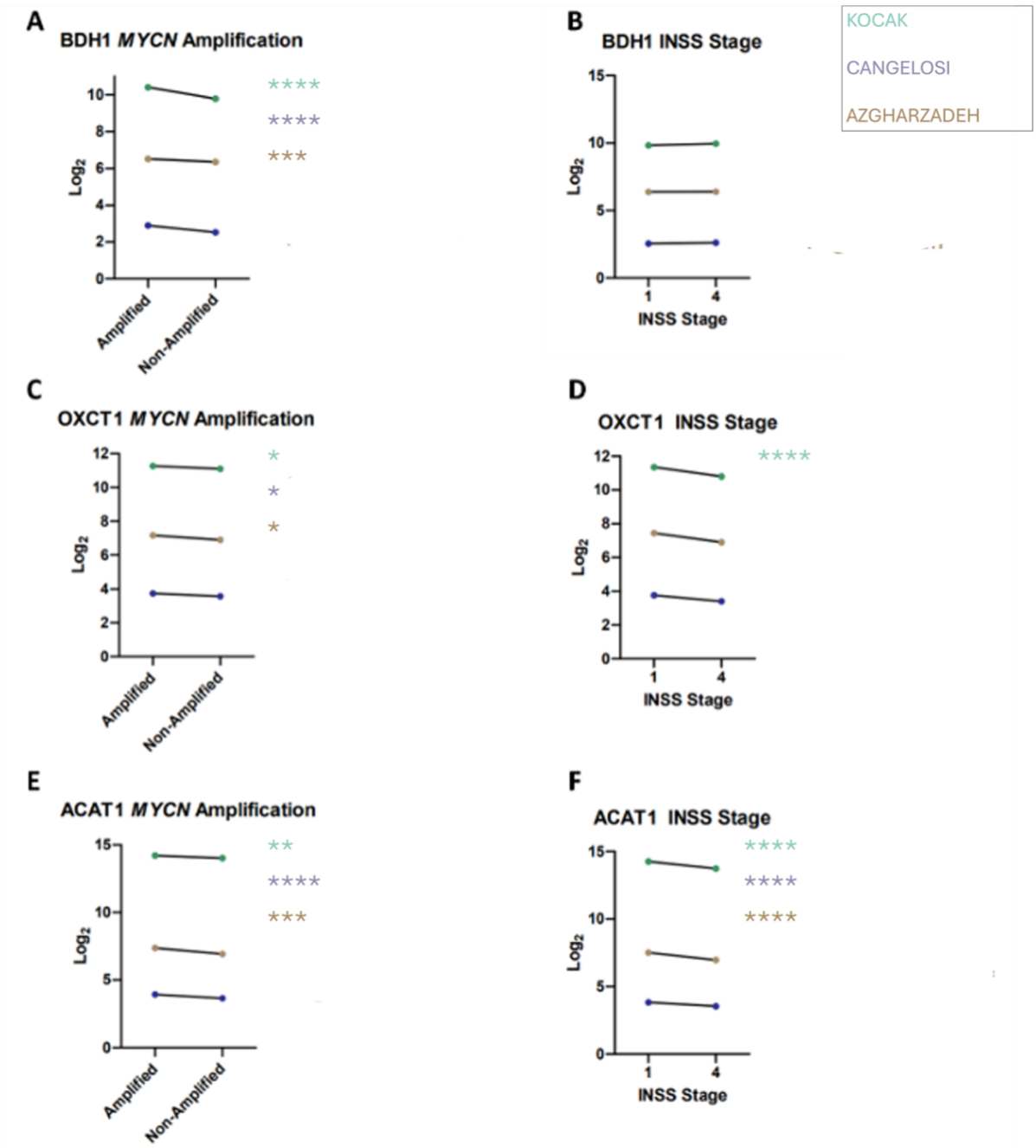


Figure 3.2. BDH1, OXCT1, and ACAT1 expression increased in MYCN amplified tumours. (A) *BDH1* expression in MYCN and non-MYCN amplified NB. (B) *BDH1* expression in stage 1 and 4 NB. (C) *OXCT1* expression in MYCN and non-MYCN amplified NB. (D) *OXCT1* expression in stage 1 and 4 NB. (E) *ACAT1* expression in MYCN and non-MYCN amplified NB. (F) *ACAT1* expression in stage 1 and 4 NB. **P* < 0.05. ***P* < 0.01. ****P* < 0.001. *****P* < 0.0001.

Table 3.7. Correlations of BDH1, OXCT1, and ACAT1 with event free survival probability. Ketolytic genes BDH1, OXCT1, and ACAT1 from the three datasets demonstrating outcome, and P-value.

Gene	Dataset	Outcome	P Value
BDH1 Expression	Kocak	High is worse	$p = 5.16 \times 10^{-04}$
	Cangelosi	High is worse	$p = 1.33 \times 10^{-6}$
	Asgharzadeh	ns	p = 0.188
OXCT1 Expression	Kocak	ns	p = 0.272
	Cangelosi	ns	p = 0.116
	Asgharzadeh	ns	P = 0.537
ACAT1 Expression	Kocak	ns	$p = 4.85 \times 10^{-03}$
	Cangelosi	Low is worse	$p = 5.49 \times 10^{-5}$
	Asgharzadeh	ns	p = 0.0818

3.5. Discussion

This study aimed to elucidate the role of genomics in understanding the metabolic processes of ketolytic and glycolytic metabolism in NB. The findings of this study shed light on the association between gene expression profiles, MYCN amplification, INSS stage progression, and event-free survivability in NB. Elevated levels of *HK2*, *GAPDH*, and *ENO1* were found to be linked to MYCN amplification, INSS stage progression, and poorer event-free survivability. Additionally, the expression of *BDH1*, *OXCT1*, and *ACAT1* showed an association with MYCN amplification, although *OXCT1* and *ACAT1* expression levels were not correlated with INSS stage progression or poorer survivability. Therefore, MYCN amplification was associated with increased expression of glycolytic genes, contributing to tumour cell proliferation and survival, and poorer patient outcomes (Matthew G. Vander Heiden et al., 2009). MYCN amplification was associated with a lower expression of ketolytic genes. Cox regression analysis revealed *HK2*, *GAPDH* and *ENO1* genes as risk factors with respect to outcomes in stage 1 and stage 4 tumours, and in ages under and over 18 months where data sets were available, while ketolytic genes were generally not associated with poorer outcomes with respect to age or metastases.

Our results correlate with those generated by Applebaum *et al.* In their investigation of transcriptome datasets, including those from NB patients and derived cell lines, they identified nine genes that are upregulated in hypoxia including *HK2* and *ENO1*. The expression levels of these genes were associated with poor patient outcome in three independent NB cohorts (Applebaum et al., 2016).

The results of this study are in line with previous research regarding the significance of metabolic reprogramming, particularly aerobic glycolysis in tumour progression. Aerobic glycolysis is a hallmark of the metabolic reprogramming that contributes to tumour progression. In NB, the mechanisms regulating this function are largely unknown. Thus, therapeutic interventions are largely unsuccessful in metastatic NB. Unlike aerobic glycolysis, the ketolytic pathway utilizes OXPHOS (Morscher et al., 2015; Vidali et al., 2015). NB OXPHOS metabolism is characterized by a reduction in oxidative rate, believed to originate in defective electron transport chain enzyme activity, and a reduction in mitochondrial DNA copy number resulting in a reduced capacity to generate ATP (R. G. Feichtinger et al., 2010). Downregulation of OXPHOS is associated with poor clinical outcome among some cancers and correlates with a metabolic signature characteristic of the Warburg effect in invasive and metastatic tumours (Ashton et al., 2018). As such OXPHOS has become a therapeutic target in cancer treatment including breast cancer, melanoma, and glioma. For example, metformin, a first-line pharmacological treatment for type 2 diabetes, reduces hepatic gluconeogenesis decreasing mitochondrial respiration chain activity and ATP production, which in turn inhibits proliferation and induces apoptosis in cancer cells including NB (Ben Sahra et al., 2010).

The findings of this study also align with previous studies demonstrating that NB tumours show a reduction in OXPHOS (R. G. Feichtinger et al., 2010) and a concordant reduction in their capacity to metabolise ketone bodies in the presence of sufficient glucose (Morscher et al., 2015). Ketone bodies are metabolised via OXPHOS (Barry et al., 2018). Two of the three ketolytic genes *OXCT1* and *ACAT1*, were not associated with a poor prognosis whereby their expression was not integral to survival expectation or low expression led to reduced survivability.

MYCN is detected in approximately 50% of high-risk NB cases, although it is unclear when its amplification is initiated and in which cells (Otte et al., 2020). Previous genomic datasets demonstrated MYCN amplification as an oncogenic driver in NB, by increasing glycolytic and OXPHOS metabolic capacities to meet increasing energy demands (Oliynyk et al., 2019). As such, MYCN amplification is a key independent prognostic factor for identifying NB tumour progression and outcomes (Otte et al., 2020). Our data shows its amplification in each of our glycolytic and ketolytic datasets, emphasizing its potential significance in NB metabolism. Likewise, Cox regression analysis revealed that *BDH1* and *OXCT1,* and *HK2* and *ENO1* are non-significantly associated with poorer outcomes in MYNC amplified tumours.

A limitation of this study was the variation in the size of the datasets; however, this research contributes to the understanding of NB metabolism by utilizing genomics to investigate glycolytic and ketolytic pathways. The elevated expression of specific genes (*HK2*, *GAPDH*, and *ENO1*) was associated with MYCN amplification, INSS stage progression, and poorer event-free survivability in high-risk NB. The expression of *BDH1*, *OXCT1*, and *ACAT1* was also linked to MYCN amplification; however, *OXCT1* and *ACAT1* expression levels were not correlated with INSS stage progression or poorer survivability. These findings validate the glycolytic gene expression profiles in metastatic and MYCN-amplified high-risk NB tumours and suggest the potential use of these genes in prognosis prediction. Furthermore, this study highlights the reliability and utility of the R2 database as an oncogenomic tool for NB research. Future investigations should focus on the role of latter phase ketolytic genes in prognosis as they may be correlated with improved outcomes in NB patients. Proteomic analysis of these glycolytic and ketolytic factors as they relate to NB should also be explored. Additionally, in vitro experiments using NSC cell lines may also be conducted, building on the genetics basis of NSC metabolism identified in this study.

4. Chapter Four.

The Impact of Glucose Deprivation & Ketone Body Supplementation on SH-SY5Y & NE4C Cells

4.1. Abstract

This chapter examines the impact of glucose deprivation on proliferating cell growth, metabolic viability, phenotype, and glycolytic activity. This study also investigates the impact of β-OHB supplementation on cell survival, health and metabolism in glucose deprived conditions. NB SHSY5Y and neuroepithelial NE4C cell lines were selected to model metabolic influence on cell growth. Both cell lines exhibited reduced density, viability, and glycolysis under glucose deprivation. NE4C cells showed increased sensitivity to glucose deprivation, when compared to SH-SY5Y cells. β-OHB supplementation influenced metabolic activity by reducing glycolysis, indicating a potential shift toward OXPHOS, but failed to significantly improve cell density or viability. Phenotypic analysis showed that glucose deprivation and ketone body supplementation did not induce spontaneous differentiation in SHSY5Y and NE4C cells, maintaining their NSC phenotype. These findings highlight the role of glucose in NSC metabolism and suggest that β-OHB may partially compensate for glucose deprivation by altering energy pathways; however, it does not fully rescue deficits in growth and viability. This research provides insights into the metabolic responses of NSCs to nutrient deprivation, demonstrating the interplay between glycolysis and mitochondrial function. These data highlight the need for further investigation into mitochondrial function and metabolic adaptations in NSCs under stress, offering potential avenues for therapeutic approaches to target neuro-degenerative and neurodevelopmental conditions.

4.2. Introduction

The human brain relies heavily on a constant supply of glucose as its primary energy source (Alherz et al., 2021; Jády et al., 2016). The significant role of glucose in maintaining neuronal function highlights the need to understand the cellular and molecular consequences of glucose availability on neural cells. NSC lines, including the human-derived NB, SH-SY5Y cell line, and the murine neuroepithelial NE4C cell line serve as valuable models for studying the impact of metabolic alterations on neural cell health and viability (Gardiner & Freeman, 2016; Xiong et al., 2020). This research aims to investigate the impact of glucose restriction and deprivation on these cell lines, focusing on cellular growth, metabolic viability, metabolic activity, and the potential ameliorative effects of ketone body supplementation.

Neural cells exhibit high metabolic activity, relying primarily on glucose for their energy requirements. Cerebral glucose is metabolised through glycolysis to produce ATP, and further metabolism through OXPHOS in mitochondria provides the energy needed for the functioning of mature neurons. Under normal conditions, glucose availability is regulated to ensure a continuous supply to the brain. However, in various pathological conditions including cerebral ischemia, neurodegenerative and developmental diseases, and prolonged fasting, glucose availability may become compromised. Understanding how neural cells adapt to such alterations in their energy supply is crucial in identifying pathological events and therapeutic strategies (Molloy & Barry, 2024).

The SH-SY5Y and NE4C cell lines are effective in vitro models for examining neuronal behaviour under various metabolic conditions. SH-SY5Y cells are commonly used in neurobiological research due to their ability to differentiate into neuroblastic cells[53]. NE4C cells exhibit characteristics of NPCs, making them suitable for studying early neural development and differentiation[59]. Therefore, both cell lines allow for the investigation of cellular responses to glucose restriction and deprivation.

This research aims to determine whether SH-SY5Y and NE4C cells can grow without glucose and to assess the duration required to reach confluency under glucose-restricted conditions. It is hypothesised that these cells will grow at a slower rate in glucose-

restricted and glucose-deprived conditions compared to regular glucose conditions. Glucose restriction and deprivation are also expected to decrease cellular density and metabolic viability.

Phenotypic changes in SH-SY5Y and NE4C cells subjected to glucose restriction and deprivation will also be evaluated. It is hypothesised that glucose restriction and deprivation will not lead to spontaneous differentiation in these cell lines. The impact on cellular glycolysis will also be examined, with the hypothesis that glucose deprivation will decrease glycolytic activity in both cell lines. Investigating these phenotypical changes is essential for elucidating the metabolic adaptations of neuronal cell lines under metabolic restrictions.

Ketone bodies, including β-OHB, are alternative neuronal energy sources produced during prolonged fasting or carbohydrate restriction. These molecules cross the BBB and compensate for reduced glucose availability (Achanta & Rae, 2017; Barry et al., 2018; Cotter et al., 2013; Cunnane, Courchesne-Loyer, Vandenberghe, et al., 2016). This research will investigate the impact of β-OHB supplementation on SH-SY5Y and NE4C cells cultured under regular glucose (RG), glucose-restricted (LG), and glucose-deprived (GF) conditions. It is hypothesised that β-OHB supplementation may enhance cell density, metabolic viability, and modulate glycolysis in these cells. Insights from these investigations may demonstrate the potential of ketone bodies in supporting neuronal health and function following metabolic alterations.

These experiments aim to understand the cellular and metabolic responses of neuronal cells to glucose availability. The findings may have implications for developing strategies to protect neuronal health in conditions such as neurodegenerative and neurodevelopmental diseases. The potential therapeutic role of ketone body supplementation will be determined, providing insights into alternative energy sources to support brain function under nutrient deprivation. However, the adverse effects of such supplementation in the context of cancer metabolism should also be considered based on the results identified in Chapter Three of this thesis.

By addressing these objectives to test these hypotheses, this research aims to contribute to the understanding of neuronal metabolism under glucose restriction and deprivation. The results will inform further research on the impact of metabolic manipulation on neuronal health.

4.3. Aims and Hypotheses

4.3.1. Aims

i. Determine whether SH-SY5Y and NE4C cells can grow without glucose and the time needed to reach confluency.
ii. Assess the effects of glucose restriction and deprivation on SH-SY5Y and NE4C cell density, viability, and glycolysis.
iii. Evaluate the phenotype of SH-SY5Y and NE4C cells under glucose-restricted and deprived conditions.
iv. Examine the impact of ketone body supplementation on SH-SY5Y and NE4C cells during glucose restriction and deprivation.

4.3.2. Hypotheses

i. SH-SY5Y and NE4C cells will grow more slowly without glucose.
ii. Glucose deprivation/restriction will reduce cell density, viability, and glycolysis.
iii. Glucose deprivation/restriction will not induce spontaneous differentiation in SH-SY5Y and NE4C cells.
iv. Ketone supplementation will improve density, viability, and reduce glycolysis under glucose-deprived/restricted conditions.

4.4. Materials and Methods

4.4.1. SH-SY5Y & NE4C Cell Culture, Subculturing, and Media Change

The SH-SY5Y and NE4C cell lines were used for all experiments. All procedures were performed under sterile conditions using appropriate aseptic techniques described below. Cells were grown and maintained in an incubator (GS Gold 48L) at 37°C in 5% CO_2. Frozen cell lines were recovered from liquid nitrogen storage and seeded in a sterile laminar flow hood (Telstar Bio IIA Class II cabinet). Cells were transferred to a sterile T25

flask containing 5 ml of regular medium (RM) (Table 4.1). RM served as the basic growth medium for daily cell culture and maintenance before transferring and seeding into cell culture plates. The flask was stored in an incubator overnight with the cap slightly unscrewed to allow adequate gaseous exchange. The following day, the RM was replaced to remove dead cells and the remaining freezing agent, dimethyl sulfoxide (DMSO). The media in the flask was changed every two days by removing 3 ml of old RM and replacing with 3 ml of fresh RM. The SH-SY5Y and NE4C cell lines doubled approximately every 24 hours.

Cell viability and confluency were checked daily using a light microscope (Olympus CKX53 light microscope). Cells were ready for subculturing at approximately 70% confluency. For subculturing, the media was aspirated off, and 3 ml of 1X trypsin/EDTA was added to detach the adherent cells. The flask was incubated for 3 minutes. After incubation, the trypsin was inspected for cloudiness, indicating the detachment of cells. Then, 1 ml of RM was added to the flask and pipetted multiple times to ensure complete detachment of cells and to break up cellular clumps. Two new T25 flasks, each containing 5 ml of fresh RM, received 2 ml of cell suspension. The flasks were then incubated in the manner outlined above. All reagents were warmed in a water bath at 37°C before use.

Table 4.1. Components of regular medium

Component	**% of total volume of media**
DMEM/F12	88%
FBS	10%
Penicillin-Streptomycin	1%
Sodium pyruvate	1%

4.4.2. Aseptic Technique

Sterility was maintained using 70% ethanol. 100% ethanol (Sigma Aldrich) was diluted with sterile distilled water to yield a 70% ethanol solution. Nitrile-free gloves were worn during experiments. Experiments requiring a sterile field were conducted in the laminar

flow hood. Before introduction to the hood, all materials and gloved hands were sprayed with 70% ethanol. After completing experiments, all materials were removed from the hood. The waste container was removed, emptied, and cleaned with 70% ethanol before being returned to the hood. The entire surface of the hood was then cleaned with 70% ethanol. Finally, the hood was turned off and the protective sash lowered. The hood was exposed to ultraviolet light for 1 hour to ensure sterility for future experiments.

4.4.3. Cell Seeding, Counting, and Plating

Experimental seeding was carried out when the cells reached approximately 70% confluency. The cell media was aspirated, and the flasks were washed with 10 ml of Hank's balanced salt solution (HBSS). Next, 3 ml of trypsin was added to the flask and incubated for 3 mins. The trypsin was neutralised with 1 ml of RM, and the cell suspension was transferred to a 50 ml Falcon tube. The flask was further washed with 6 ml of HBSS, which was then added to the Falcon tube. The Falcon tube was centrifuged for 3 mins at 550 g. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of glucose-free Dulbecco's modified eagle medium (DMEM). A sample was taken from the suspension for cell counting. 10 µl of cell suspension was diluted with 90 µl of glucose-free DMEM. Then, 12 µl of this solution was combined with 12 µl of trypan blue. 10 µl of this solution was then added to a haemocytometer. Using a light microscope, the number of cells in each of the four (4x4) quadrants was counted, excluding cells on the peripheral lines. The counts from the four quadrants were averaged and multiplied by two to adjust for the trypan blue dilution. This number was then multiplied by 10^4 to account for the haemocytometer dilution. This number was then multiplied by 10 to account for the dilution with glucose-free DMEM. The volume of cell suspension required to achieve a seeding density of 5,000 cells per well was calculated using the following equation:

$$\frac{Number\ acquired\ by\ accounting\ for\ dilutions\ factors}{1000} = \frac{5000}{x}$$

$$x = volume\ cell\ solution\ per\ well$$

4.4.4. Preparation of β-OHB Stock

To prepare 10 ml of 100 mM β-OHB stock, 126 mg of (R)-(−)- 3-Hydroxybutyric acid sodium salt (Sigma) was dissolved in 10 ml of glucose free DMEM. The solution was vortexed and then sterile-filtered. A sterile syringe (Norm-Ject), a sterile 0.22 µM syringe filter (Ultracruz), and a sterile needle (Sterican) were sprayed with 70% ethanol and placed into the laminar flow hood. Using the needle and syringe, the β-OHB solution was drawn up. The sterile filter was then attached to the syringe, and the solution was filtered into a 15 ml Falcon tube. The prepared β-OHB stock was stored in the fridge until ready to use. When added to media groups, the β-OHB concentration of 10 mM was obtained. This concentration was chosen based on the results revealed in the SH-SY5Y cell line by Alherz et al. (Alherz et al., 2021).

4.4.5. Cell Culture Plating

Experiments were conducted in 96-well plates (Appendix 1). A plate was labelled appropriately with the date of seeding, cell line type (SH-SY5Y or NE4C), researcher's initials, and the treatment groups; regular glucose (RG), low glucose (LG), glucose-free (GF), and β-OHB supplemented groups (RGβ, LGβ, GFβ) (Table 4.2 A and B). The calculated volume of cell suspension was added to each well, followed by 200 µl of the corresponding media preparations. Then, each of the wells was inspected using light microscopy, to ensure the presence of cells. The plates were subsequently incubated for two days. After incubation, a media change was performed. The media groups were warmed in a 37°C water bath and sprayed with 70% ethanol before being placed into the laminar flow hood. Using a multichannel pipette, 100 µl of media was removed from each well and replaced with 100 µl of fresh media. The plates were re-examined using microscopy to confirm cells were present. The plates were incubated for a further two days for a total growth time of 96 hours (h).

Table 4.2.A. Components of media groups

Component	Regular Glucose (RG) (5 mM)	Low Glucose (LG) (1 mM)	Glucose Free (GF) (0 mM)
DMEM/F12	9,000 µl	9,000 µl	9,000 µl
FBS	1,000 µl	1,000 µl	1,000 µl
Penicillin-Streptomycin	100 µl	100 µl	100 µl
Sodium pyruvate	100 µl	100 µl	100 µl
L-glutamine	100 µl	100 µl	100 µl
Glucose	45 µl	9 µl	0 µl

Table 4.2.B. Components of media groups supplemented with β-OHB.

Component	RGβ	LGβ	GFβ
DMEM/F12	8,000 µl	8,000 µl	8,000 µl
FBS	1,000 µl	1,000 µl	1,000 µl
Penicillin-Streptomycin	100 µl	100 µl	100 µl
Sodium Pyruvate	100 µl	100 µl	100 µl
L-glutamine	100 µl	100 µl	100 µl
Glucose	45 µl	9 µl	0 µl
β-OHB Stock	1,000 µl	1,000 µl	1,000 µl

4.4.6. Cell Fixation

After 96 h of growth, the plates were fixed with 4% paraformaldehyde (PFA). Using a multichannel pipette, 200 µl of media was carefully removed from all the wells. The wells were gently washed with 200 µl of HBSS, which was subsequently removed. 100 µl of 4% PFA was added to each well and left for 15 mins to fix the cells. After this time, the 4% PFA was removed, and the wells were washed with 200 µl of HBSS. The HBSS was removed and 200 µl of fresh phosphate-buffered saline (PBS) was added. Light microscopy

confirmed the presence of cells in the wells. The plates were stored in the fridge at 4°C until immunocytochemistry (ICC) was performed.

4.4.7. Immunocytochemistry

Before primary antibody staining, a blocking solution containing 5% normal goat serum (NGS) in 0.05% Triton X-100 in 1X PBS was applied for 1 h. The primary antibodies, nestin, GFAP, and βIII-Tubulin (βIII), were diluted in the blocking solution. 50 µl of this solution was added to the wells and the plates were refrigerated overnight. The following day, the primary antibodies were removed, and the wells were washed three times with 50 µl of 1X PBS, 5 mins for each wash on a plate rocker at a slow speed. The secondary antibody, FITC goat anti-mouse 488, and DAPI and actin stains were diluted in the blocking solution and added to the wells. The plates were rocked for 2 h. The cells were then washed three times with 50 µl of 1X PBS, for 5 mins each wash. Finally, 200 µl of 1X PBS was added to the wells, and the plates were stored in the fridge at 4°C.

4.4.8. Cell Imaging and Analysis

Cells were imaged using an Olympus IX81 motorized epifluorescent microscope with CellSens Dimension software (version 1.12). ImageJ Fiji (Image J 1.53t) was used for processing and analysing cell density, cell volume, process length, and process number images (Table 4.3). For each experiment, images were taken in triplicate, with three random images per well representing fields of view. The average of these triplicates was then considered as a single replicate.

Table 4.3. The Image-Based Assays

Image-Based Assay	**Method**
Cell density	DAPI stained nuclei at X4 magnification and density was measured as the percentage of the image covered by the nuclei. The average percentage was then calculated.

Cell size	The total area covered by Actin fluorescence was measured using the Analyse Particles function in ImageJ, divided by the number of cells in an image, to obtain the average size.
Process number	Approximately 10 cells per image were chosen and the number of processes per cell were counted manually. The average of all the images was calculated.
Process length	Neuronal processes of the Actin labelled cytoskeleton of cells were manually traced using ImageJ and the average length of processes in an image was calculated.

4.4.9 Cell Metabolic Viability

Thiazolyl Blue Tetrazolium Bromide (MTT) (ThermoFisher, ROI) assays were used to assess cell metabolic viability. MTT was added to each well at a concentration of 0.5 mg/ml in the culture medium and incubated for 4 h. After incubation, the culture medium was removed, and the cells were lysed in 40 µl of DMSO. The solution was repeatedly pipetted to solubilise the formazan crystals. The 96-well plate was placed on a rocker for 20 mins. Colorimetric absorbance was measured at a wavelength of 562 nm using a microplate reader (Allsheng AMR-100).

4.4.10. Glycolysis Assay

A ChromaDazzle glycolysis assay kit (AssayGenie BA0086) assessed the degree of glycolysis in the cells. Cells were plated in 96-well plates, in their respective media groups containing 10% FBS as outlined above. At 72 h, all the media was removed from the wells, and the cells were washed twice with 200 µl of warm HBSS. Media groups containing 1% FBS were then added to their respective wells and incubated for 24 h. At 96 h, a dilution of standards was prepared using 1% FBS media, and a standard provided in the ChromaDazzle glycolysis assay kit (Table 4.4). A premix was made using 10 µl of standard and 480 µl of 1% FBS media, which was further diluted with 1% FBS to make standards of known concentrations. A working reagent master mix was then prepared, based on the number of wells used for the assay. This working reagent master mix consisted of assay buffer, enzyme A and enzyme B, and NAD/MTT, all provided by the glycolysis assay kit. The media was gently removed from the wells and 95 µl of the working reagent was added

to each well, pipetting a few times to ensure mixing. The four standards were added to their wells, and 95 µl of working reagent was added to these in the same manner. Blanks of 1% FBS media were added in triplicate. The plate was wrapped in tinfoil and set on a rocker for 30 mins. At 30 mins, the plate was read at 565 nm using a microplate reader (Allsheng AMR-100).

Table 4.4. Dilutions of Standards

No	Premix + Media	L-Lactate (mM)
1	100 µL + 0 µL	10
2	60 µL + 40 µL	6
3	30 µL + 70 µL	3
4	0 µL + 100 µL	0

4.4.11. Statistical Analyses

Data are presented as the mean ± the standard error of the mean (SEM). Statistical analysis and graphs were generated using GraphPad Prism (GraphPad Prism version 10.2.3, CA USA). one-way ANOVA, two-way ANOVA, or multiple t-tests analysed statistical differences, with multiple comparison tests carried out using a Bonferroni correction. $P \leq 0.05$ was considered statistically significant.

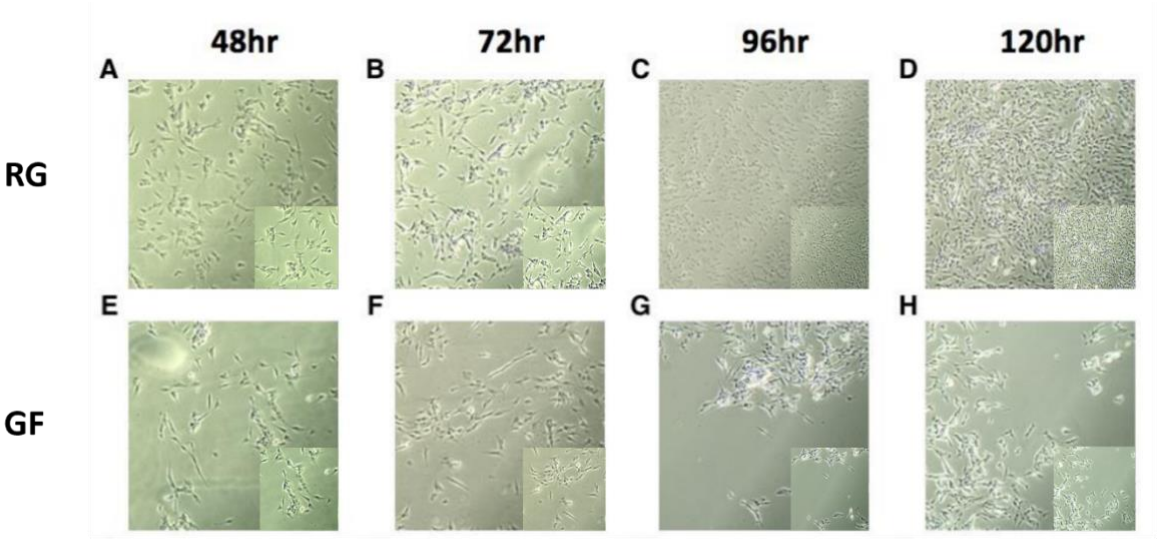
4.5. Results

4.5.1. Glucose deprived SH-SY5Y cells exhibit reduced growth

To first determine the effect of glucose deprivation on SH-SY5Y cell growth, cell density was analysed at 48, 72, 96, and 120 h and compared to RG cell culture conditions.

Density analysis of brightfield images of SH-SY5Y cells in RG conditions over 120 h showed a significant increase in cell density between 48 h (74 ± 8.02) and 96 h (227 ± 32.45) ($P \leq 0.001$), and 48 and 120 h (262.67 ± 10.97) ($P \leq 0.0001$) (Fig. 4.1, A-D, I). Brightfield images showed that cells appeared to reach confluency at 120 h (Fig. 4.1, D). Cells appeared normally distributed and healthy at 48, 72, 96, and 120 h (High magnification images). Density analysis of glucose-deprived SH-SY5Y cells showed a significant increase in cell

density between 48 (43 ± 7.09) and 120 h (135.33 ± 23.81) ($P \leq 0.05$) (Fig 4.1, E-H, I). Cell density between RG and GF conditions were significant at 96 (227 ± 32.45 and 74.76 ± 18.19, respectively) and 120 h (262.67 ± 10.97 and 135.33 ± 23.81, respectively) ($P \leq 0.05$) (Fig. 4.1, I).



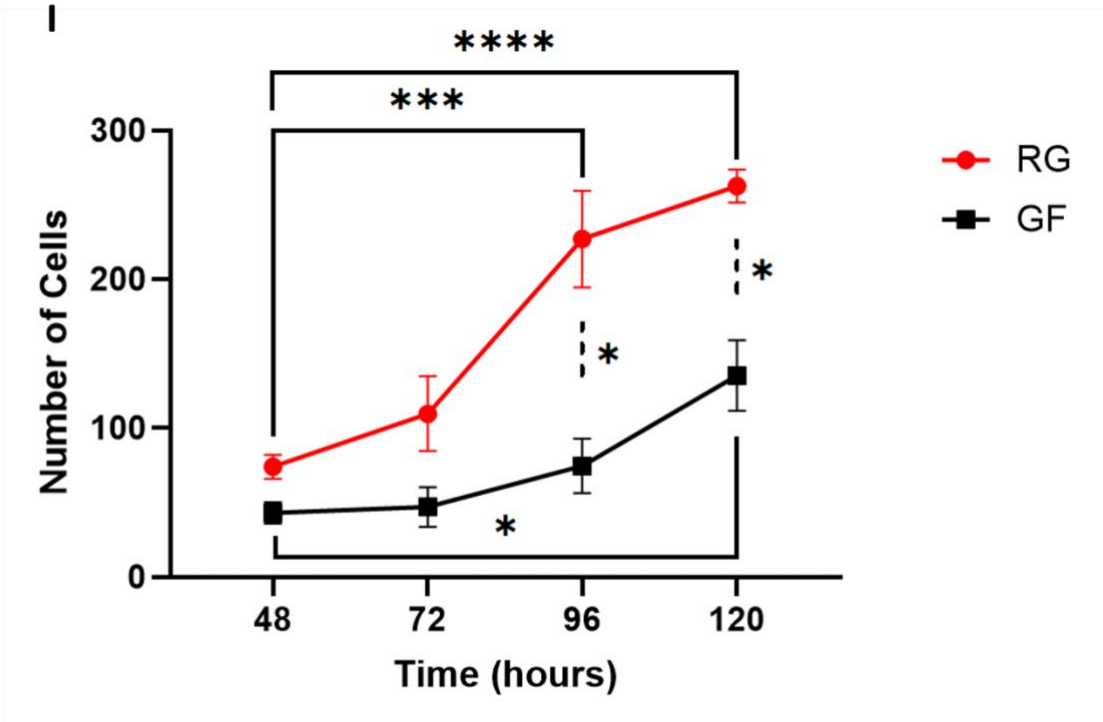


Figure 4.1. Glucose-deprived SH-SY5Y cells exhibit reduced growth. A-H. Brightfield images of SH-SY5Y cells in RG and GF conditions at 48, 72, 96, and 120 h. I. Graph depicted the of number of SH-SY5Y cells in RG and GF conditions at 48, 72, 96, and 120 h. n = 3 independently for each assay. Multiple t-test was performed to analyse the difference in density between RG and GF conditions at 48, 72, 96, and 120 h. Two-way ANOVA was performed to analyse the difference in density between the time points 48, 72, 96, and 120 for RG and GF conditions. *p ≤ 0.05. ***p ≤ 0.001. ****p ≤ 0.0001.

4.5.2. Glucose restriction and deprivation reduces SH-SY5Y cell density and metabolic viability

The 96 h timepoint was selected as the preferred time point for to prevent cell over-confluency during experimental analysis. To further test the effect of glucose deprivation on cell growth, LG culture conditions were compared to RG and GF conditions. At 96 h, a significant decrease in cell density was observed between RG (100 ± 0) and GF (36.6 ± 2.5) conditions (P ≤ 0.001) and LG (110.4 ± 8) and GF conditions (P ≤ 0.001) (Fig. 4.2, A and C).

To determine, the effect of glucose restriction and deprivation on cell metabolic viability, an MTT assay was performed and showed a significant decrease between RG (100 ± 0) and GF (51.9 ± 9.7) conditions (P ≤ 0.01) and LG (82.3 ± 2.8) and GF conditions (P ≤ 0.05) (Fig. 4.2, B).

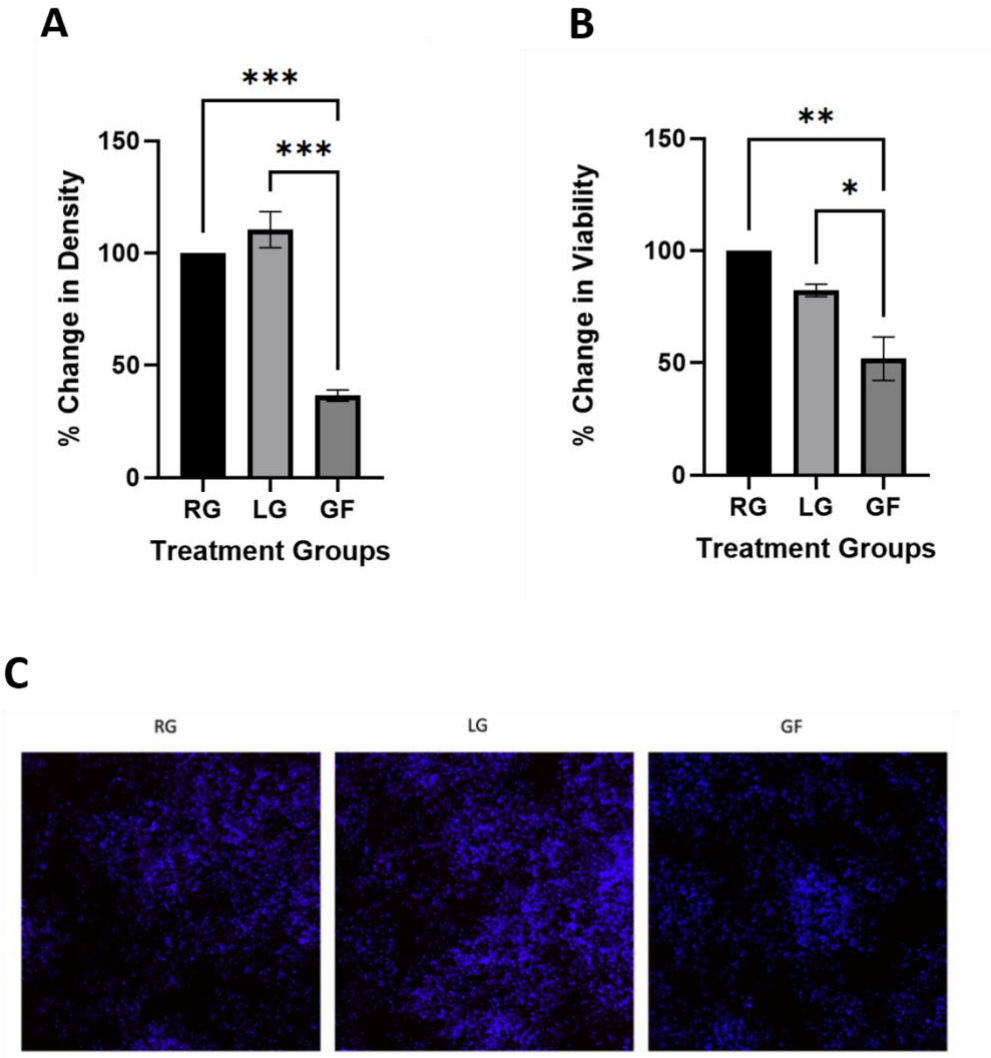


Figure 4.2. Glucose restriction and deprivation reduces SH-SY5Y cell density and metabolic viability. A. Density assay of percentage change in cellular viability of SH-SY5Y cells in RG, LG, GF conditions at 96 h. B. An MTT assay of percentage change in cell density of SH-SY5Y cells in RG, LG, GF conditions at 96 h. C. DAPI labelled nuclei for cell density analysis in RG, LG, and GF conditions at 96 h. n = 3 independently for each assay. One-way ANOVA was performed. *P ≤ 0.05. **P ≤ 0.01. ***P ≤ 0.001.

4.5.3. Glucose deprivation does not alter SH-SY5Y phenotype and morphology

To determine if the reduction in cell growth, density, and metabolic viability were due to spontaneous differentiation of SH-SY5Y cells in response to glucose deprivation, cell phenotype and morphology were assessed. At 96 h in culture, all cells in RG, LG, and GF conditions expressed the NSC biomarker, nestin. The astrocytic protein GFAP was not expressed in RG, LG, and GF conditions. Likewise, βIII, a marker of mature neurons, was not expressed in RG, LG, and GF conditions (Fig. 4.3, A).

To determine if glucose deprivation reveals architectural changes typically indicative of alterations in cell health and phenotype, morphological analysis using actin to stain actin filaments and DAPI to label cell nuclei of SH-SY5Y cells in RG, LG, and GF conditions was conducted. Cell volumes showed no significant change between RG (100 ± 0) and GF (98.1 ± 11.3) conditions (Fig. 4.4, B and E). Process lengths showed no significant change between RG (100 ± 0) and GF (113.6 ± 11) conditions (Fig. 4.3, C and E). Process numbers were non-significant between RG (100 ± 0) and GF (82.7 ± 9.1) conditions (Fig. 4.3, D and E).

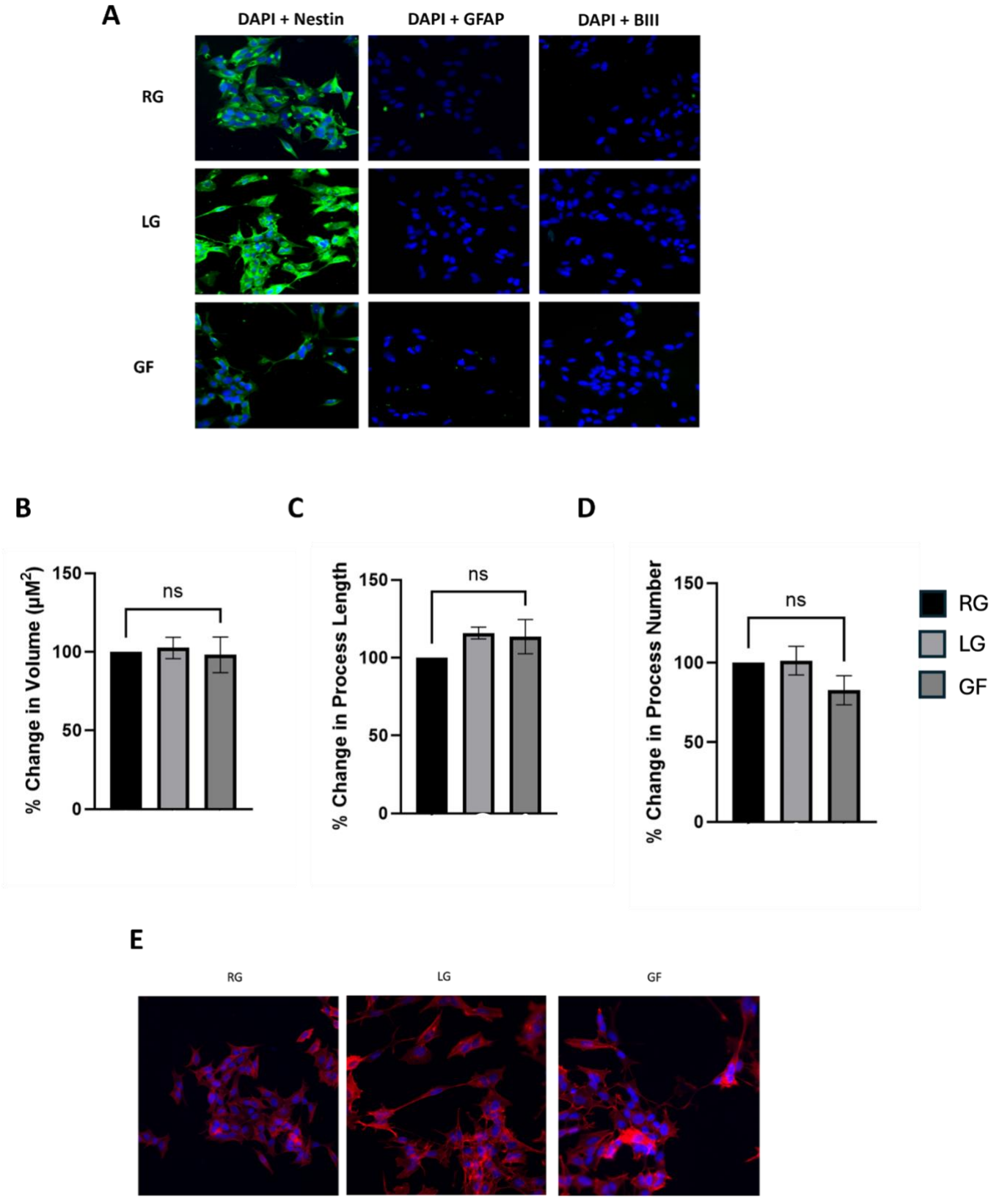


Figure 4.3. Glucose deprivation does not alter SH-SY5Y phenotype and morphology.

A. ICC assay of SH-SY5Y cells at 96 h stained with nestin, GFAP, and βIII in RG, LG, and GF conditions. B. Morphology analysis of percentage change in cell volume at 96 h in RG, LG, and GF conditions. C. Morphology analysis of percentage change in cellular process length at 96 h in RG, LG, and GF conditions. D. Morphology analysis of percentage change in

cellular process number at 96 h in RG, LG, and GF conditions. E. DAPI (blue) and Actin (red) staining of SH-SY5Y cells at 96 h in RG, LG, and GF conditions. One-way ANOVA was performed.

4.5.4. Glucose deprivation significantly reduces glycolysis in SH-SY5Y cells

To investigate whether the observed reductions in SH-SY5Y cell growth and metabolic viability under glucose restriction and deprivation were associated with a reduction in glycolytic metabolism, a glycolysis assay was conducted. At 96 h, L-lactate production of SH-SY5Y cells was significantly reduced between RG (100 ± 0) and GF conditions (54.2 ± 6) ($P \leq 0.01$), as well as between LG (113.9 ± 10) and GF conditions ($P \leq 0.001$) (Fig. 4.4).

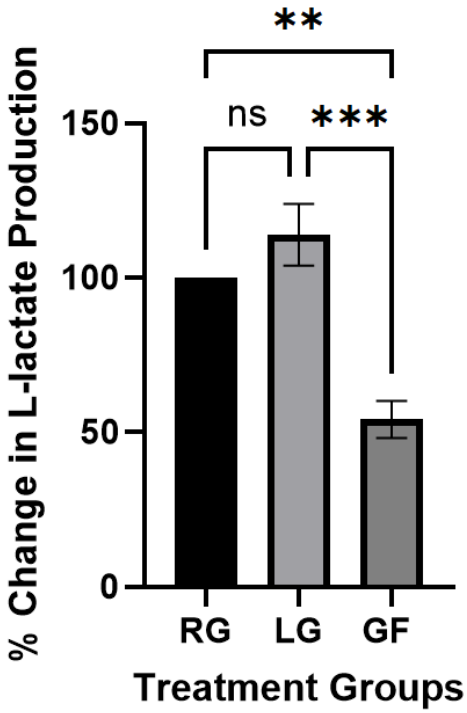


Figure 4.4. Glucose deprivation significantly reduces glycolysis in SH-SY5Y cells.
Percentage change in L-lactate production in SH-SY5Y cells at 96 h. Cells were treated with RG, LG, and GF conditions. n = 3 independently for each assay. One-way ANOVA was performed. **P ≤ 0.01. ***P ≤ 0.001.

4.5.5. Glucose restriction and deprivation reduces NE4C cell density and metabolic viability

To assess the impact of glucose deprivation on NE4C cell growth, a timepoint of 96 h was selected to avoid cell over-confluency. NE4C cells cultured in 1 mM LG culture conditions were compared with RG and GF conditions. There were no significant differences in cell density between RG (100 ± 0) and LG (176.2 ± 15.9) conditions ($P \leq 0.01$). A significant decrease was seen between RG and GF (21.2 ± 10.9) conditions ($P \leq 0.01$) (Fig. 4.5, A and C). To determine the effect of glucose restriction and deprivation on NE4C cell metabolic viability, an MTT assay was performed. The results showed a non-significant increase in viability between RG (100 ± 0) and LG (118.8 ± 10) conditions, and a significant decrease in viability between RG and GF (5.1 ± 5) conditions ($P \leq 0.001$) (Fig. 4.5, A).

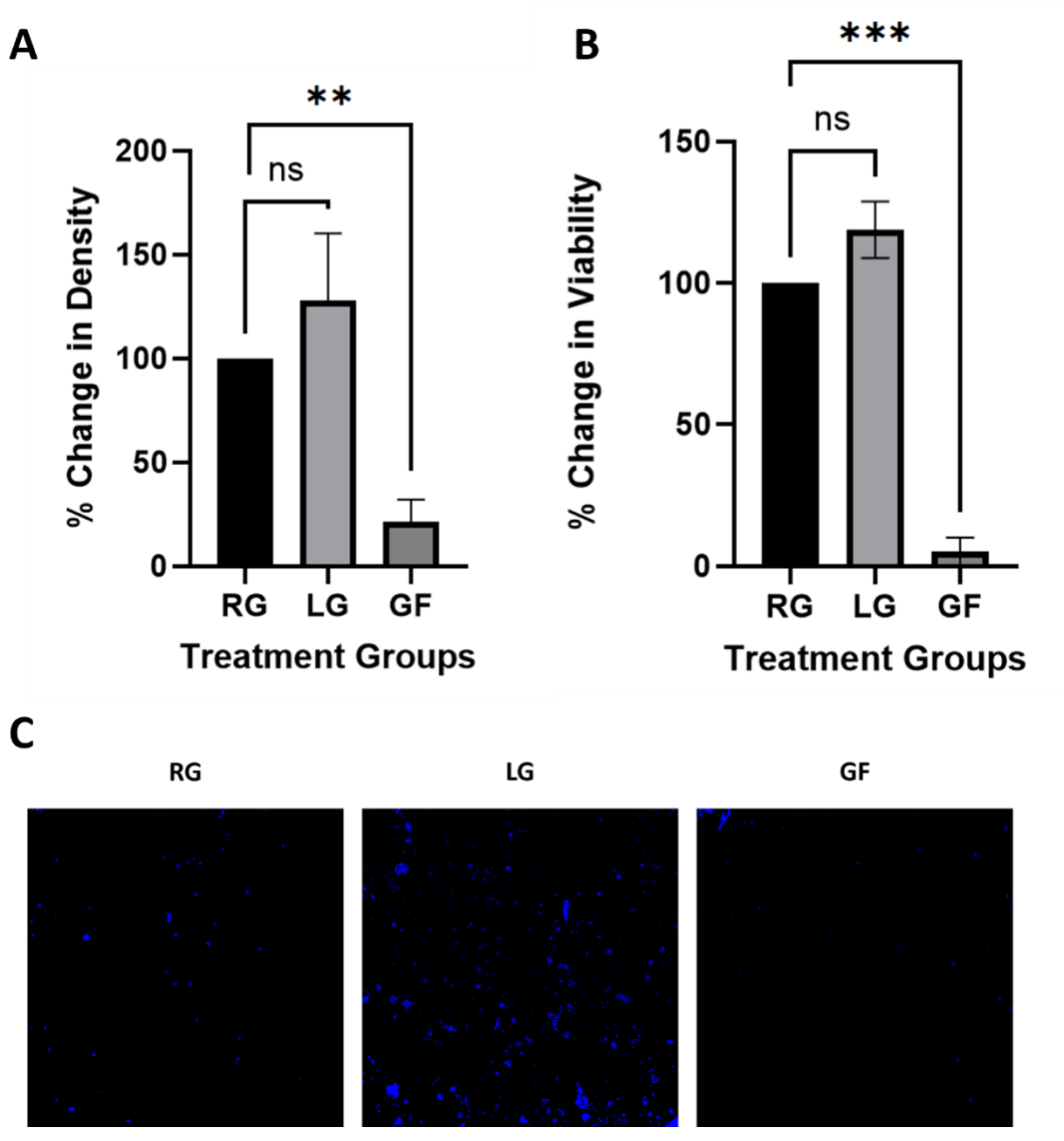


Figure 4.5. Glucose restriction and deprivation reduces NE4C cell density and metabolic viability. A. Percentage change in cell density of NE4C cells in RG, LG, and GF conditions at 96 h. B. MTT assay of NE4C cells in RG, LG, and GF conditions at 96 h. C. DAPI labelled nuclei showing NE4C cell density in RG, LG, and GF conditions at 96. n = 3 independently for each assay. One-way ANOVA was performed. **P ≤ 0.01. ***P ≤ 0.001.

4.5.6. Glucose restriction and deprivation do not alter NE4C cell morphology and phenotype

To determine if the reduction in NE4C cell growth resulted from spontaneous differentiation in response to glucose deprivation, cell phenotype and morphology were assessed. At 96 h in culture, all cells in RG, LG, and GF conditions expressed nestin. GFAP was not expressed in RG, LG, and GF conditions. βIII was not expressed in RG, LG, and GF conditions (Fig. 4.6, A). At 96 h, morphological analysis of NE4C cells in RG, LG, and GF conditions was also conducted. Cell volume showed no significant change between RG (100 ± 0) and GF (40.5 ± 18.3) conditions (Fig. 4.6, B and E). Process length showed no significant change between RG (100 ± 0) and GF (153.4 ± 40) conditions (Fig. 4.6, C and E). Process number was non-significant between RG (100 ± 0) and GF (75.8 ± 15) conditions (Fig. 4.6, D and E).

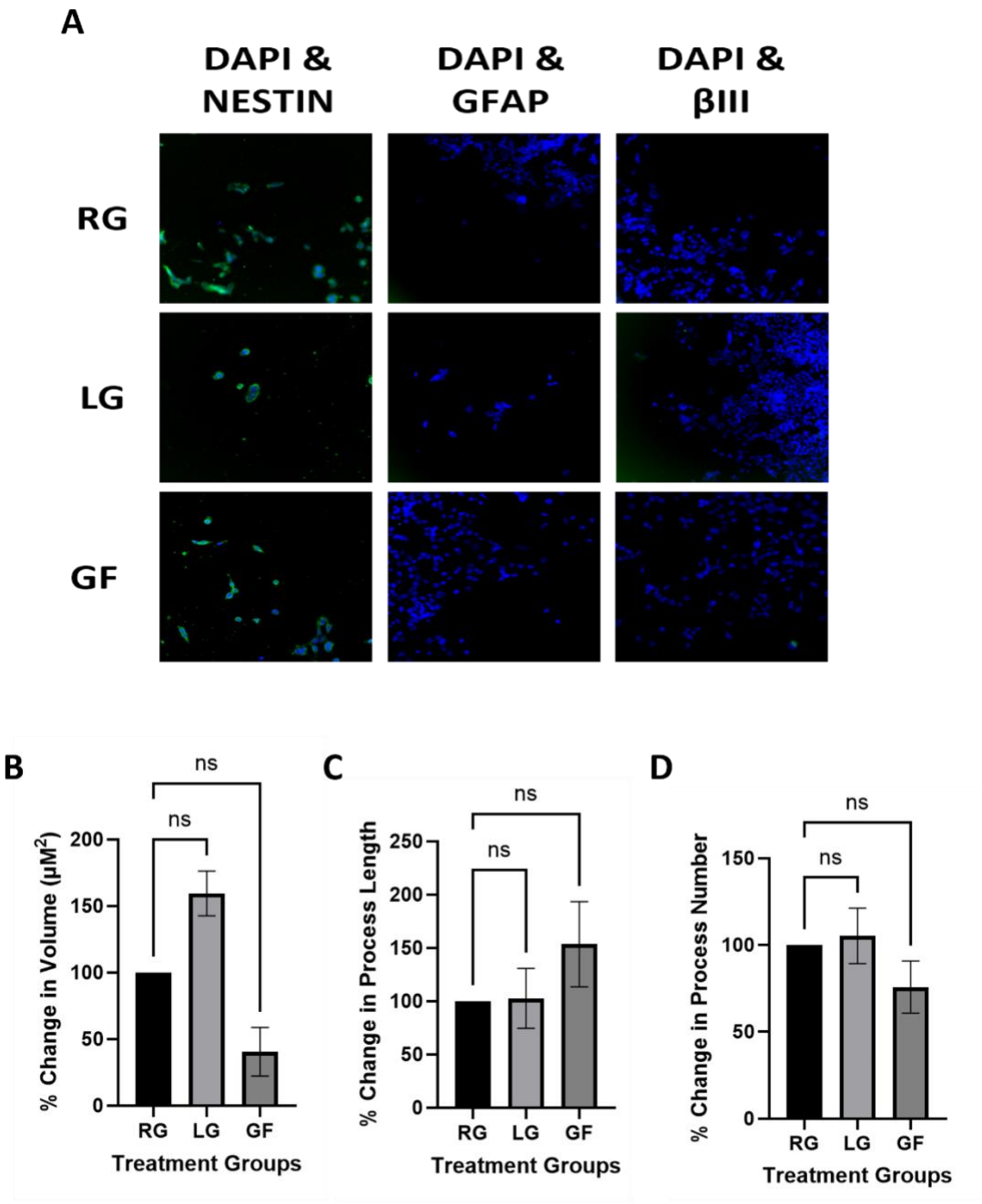

A
DAPI & NESTIN
DAPI & GFAP
DAPI & βIII
RG
LG
GF
B
ns
ns
% Change in Volume (μM²)
200
150
100
50
0
RG LG GF
Treatment Groups
C
ns
ns
% Change in Process Length
250
200
150
100
50
0
RG LG GF
Treatment Groups
D
ns
ns
% Change in Process Number
150
100
50
0
RG LG GF
Treatment Groups
E
RG
LG
GF

Figure 4.6. Glucose restriction and deprivation do not alter NE4C cell morphology and phenotype. A. ICC of NE4C cells in RG, LG, and GF conditions at 96 h. B. Percentage change in cell volume of NE4C cells in RG, LG, and GF conditions at 96 h. C. Percentage change in cell process length of NE4C cells in RG, LG, and GF conditions at 96 h. D. Percentage change in cell process number of NE4C cells in RG, LG, and GF conditions at 96 h. E. DAPI (blue) and Actin (red) staining of NE4C cells in RG, LG, and GF conditions at 96 h. One-way ANOVA was performed.

4.5.7. Glucose restriction and deprivation significantly decreases glycolysis in NE4C cells

To determine if the reduction in NE4C cell growth and metabolic viability was associated with a reduction in glycolytic metabolism following glucose restriction and deprivation, a glycolysis assay was conducted. At 96 h, the L-lactate production of NE4C cells was significantly reduced between RG (100 ± 0) and LG (75.8 ± 7) conditions ($P \leq 0.05$), and between RG and GF (19 ± 0.6) conditions ($P \leq 0.0001$) (Fig 4.7).

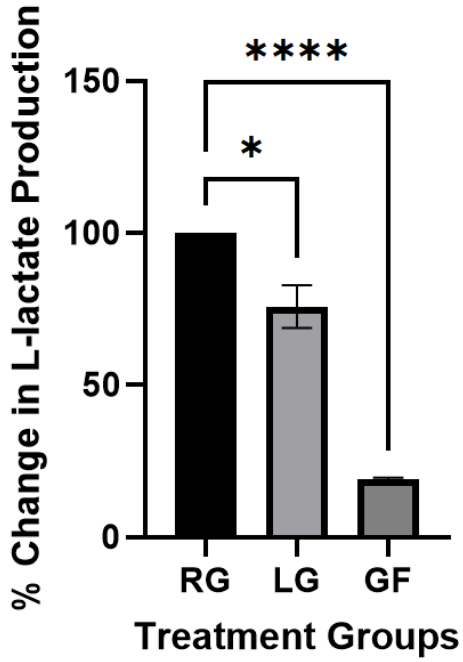
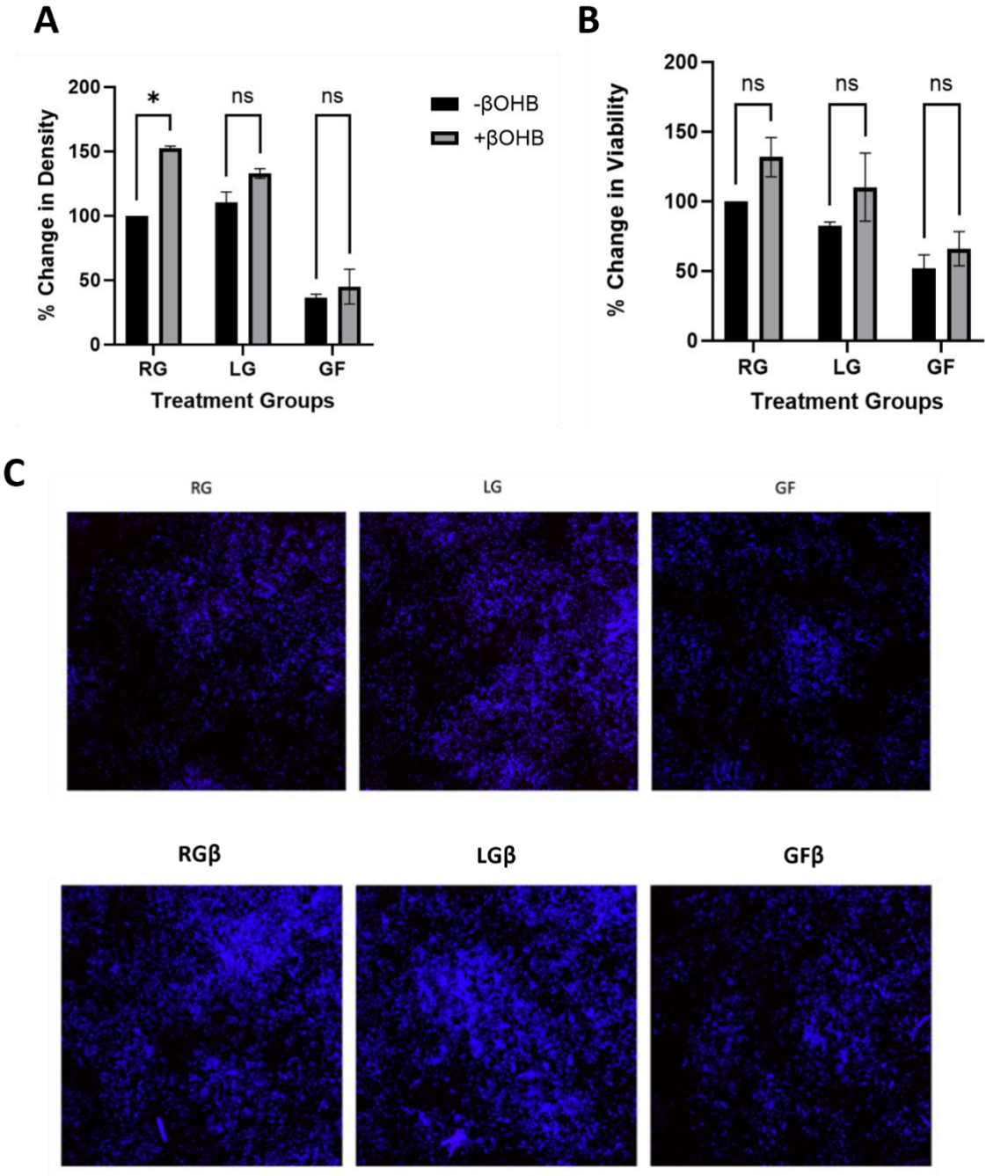


Figure 4.7. Glucose restriction and deprivation significantly decreases glycolysis in NE4C cells. Percentage change in L-lactate production in NE4C cells in RG, LG, and GF conditions at 96 h. n = 3 independently for each assay. One-way ANOVA was performed. *P ≤0.05. ****P ≤ 0.0001.

4.5.8. Ketone Supplementation minimally impacts SH-SY5Y cell density and metabolic viability

To determine the effect of β-OHB supplementation on the growth and metabolic viability of SH-SY5Y cells at 96 h, cell density analysis, and MTT assay were conducted. A cell density assay showed a significant increase in cell density between the RG (100 ± 0) and RGβ (152.6 ± 1.5) conditions ($P \leq 0.05$) (Fig. 4.8, A and C). No significant difference in cell density was observed between LG (110.4 ± 8) and LGβ (132.8 ± 3.7) conditions, and GF (36.6 ± 2.5) and GFβ (44.9 ± 13.5) conditions. At 96 h, an MTT assay showed no significant



difference in cell viability across all conditions following β-OHB supplementation. (Fig. 4.8, B).

Figure 4.8. Ketone Supplementation minimally impacts SH-SY5Y cell density and metabolic viability

A. Percentage change in cell density of SH-SY5Y cells in RG, LG, GF, RGβ, LGβ, and GFβ, conditions at 96 h. B. MTT assay of SH-SY5Y cells in RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. C. DAPI labelled nuclei showing cell density in RG, LG, GF, RGβ, LGβ, GFβ conditions at 96. n = 3 independently for each assay. Multiple t-test was performed. *P ≤ 0.05.

4.5.9. Ketone supplementation does not alter SH-SY5Y morphology and phenotype

To assess whether supplementation of culture condition media with β-OHB resulted in differentiation or morphological changes in SH-SH5Y cells, ICC analysis, and morphological analysis were conducted. At 96 h in culture, all SH-SY5Y cells in RGβ, LGβ, and GFβ conditions expressed nestin. GFAP was not expressed in RGβ, LGβ, and GFβ conditions. βIII was not expressed in RβG, LGβ, and GFβ conditions (Fig. 4.9, A). At 96 h, morphological analysis of SH-SY5Y cells cultured in RG, LG, GF, RGβ, LGβ, and GFβ conditions demonstrated no significant change in cell volume following β-OHB supplementation (Fig. 4.9, B and E). A significant decrease was observed in cell process length between RG (100 ± 0) and RGβ (84.1 ± 2) conditions (P ≤ 0.05) (Fig. 4.9, C and E). A significant increase was observed in cell process number between RG (100 ± 0) and RGβ (132.6 ± 1) conditions (P ≤ 0.05) (Fig. 4.9, D and E).

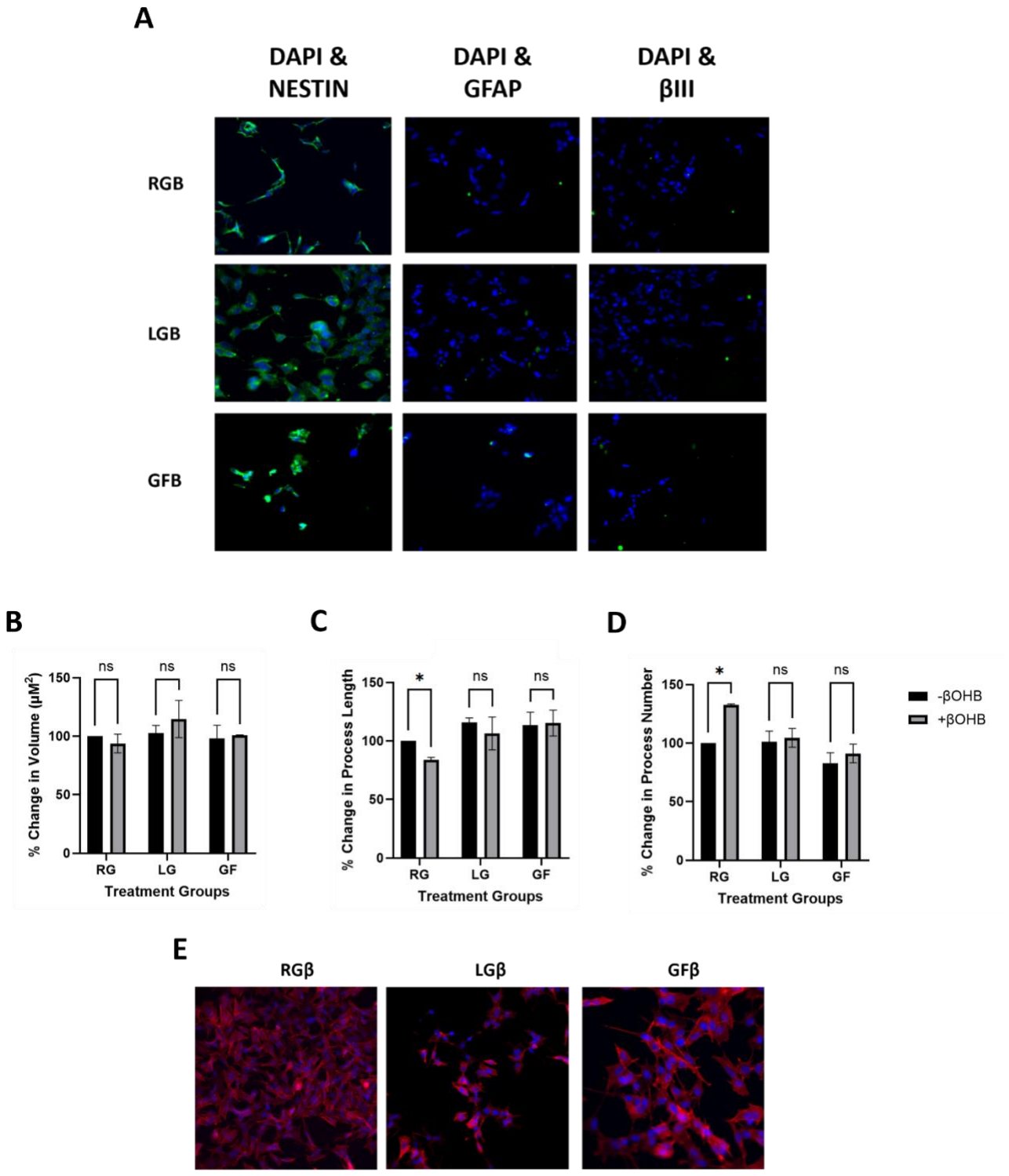


Figure 4.9. Ketone supplementation does not alter SH-SY5Y morphology and phenotype. A. ICC of SH-SY5Y cells in RGβ, LGβ, and GFβ conditions at 96 h. B. Percentage change in cell volume of SH-SY5Y cells in RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. C. Percentage change in cell process length of SH-SY5Y cells in RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. D. Percentage change in cell process number of SH-SY5Y cells in RG,

LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. E. DAPI (blue) and Actin (red) staining of SH-SY5Y cells in RGβ, LGβ, and GFβ conditions at 96 h.

4.5.10. Ketone supplementation decreases glycolysis in regular glucose conditions in SH-SY5Y cells

To test whether β-OHB supplementation may result in decreased levels of glycolysis due to metabolic competition, a glycolysis assay was performed. At 96 h, a glycolysis assay examining the L-lactate production of SH-SY5Y cells cultured in RG, LG, GF, RGβ, LGβ, and GFβ conditions showed a significant decrease in glycolysis between RG (100 ± 0) and RGβ (75.3 ± 1.2) conditions ($P \leq 0.05$). No significant difference was observed between LG (113.9 ± 10) and LGβ (83.4 ± 4.9), and GF (54.2 ± 6) and GFβ (65.8 ± 1.7) conditions (Fig. 4.10).

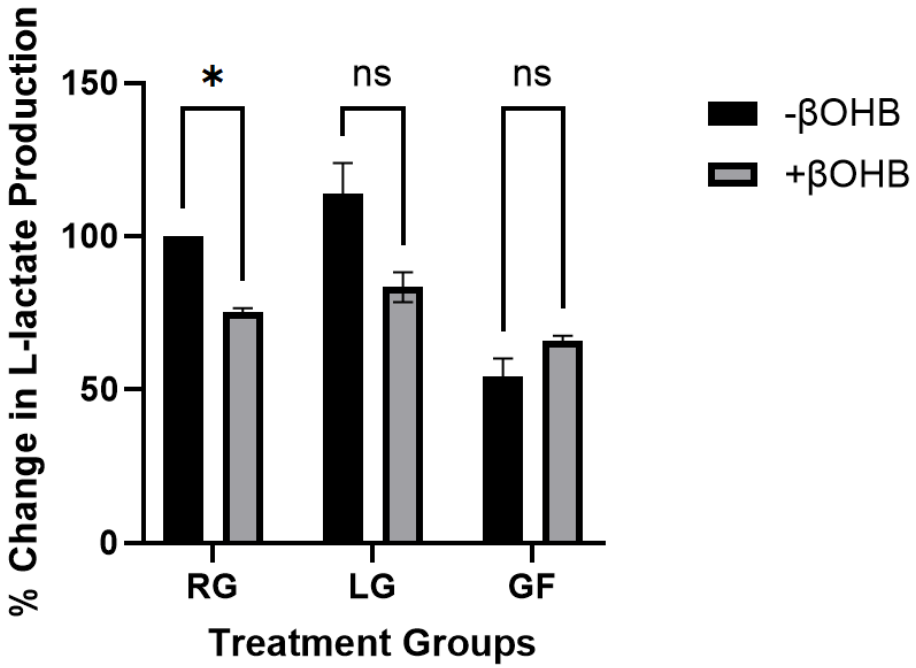


Figure 4.10. Ketone supplementation decreases glycolysis in regular glucose conditions in SH-SY5Y cells. Percentage change in L-lactate production in SH-SY5Y cells at 96 h. Cells were treated with RG, LG, GF, RGβ, LGβ, and GFβ conditions. n = 3 independently for each assay. Multiple t-test was performed. *$P \leq 0.05$.

4.5.11. The effect of ketone supplementation on the density and metabolic viability of NE4C cells

To determine the effect of β-OHB supplementation on the growth and metabolic viability of NE4C cells at 96 h, cell density analysis and an MTT assay were conducted. A cell density assay showed a significant increase in cell density between the RG (100 ± 0) and RGβ (157.7 ± 3.9) conditions ($P \leq 0.05$) and a significant decrease between LG (176.2 ± 15.9) and LGβ (67.2 ± 13.1) conditions ($P \leq 0.05$) (Fig. 4.11, A and C). At 96 h, an MTT assay showed a significant increase in NE4C cell viability between RG (100 ± 0) and RGβ (157.5 ± 10) conditions ($P \leq 0.05$) (Fig. 4.11, B).

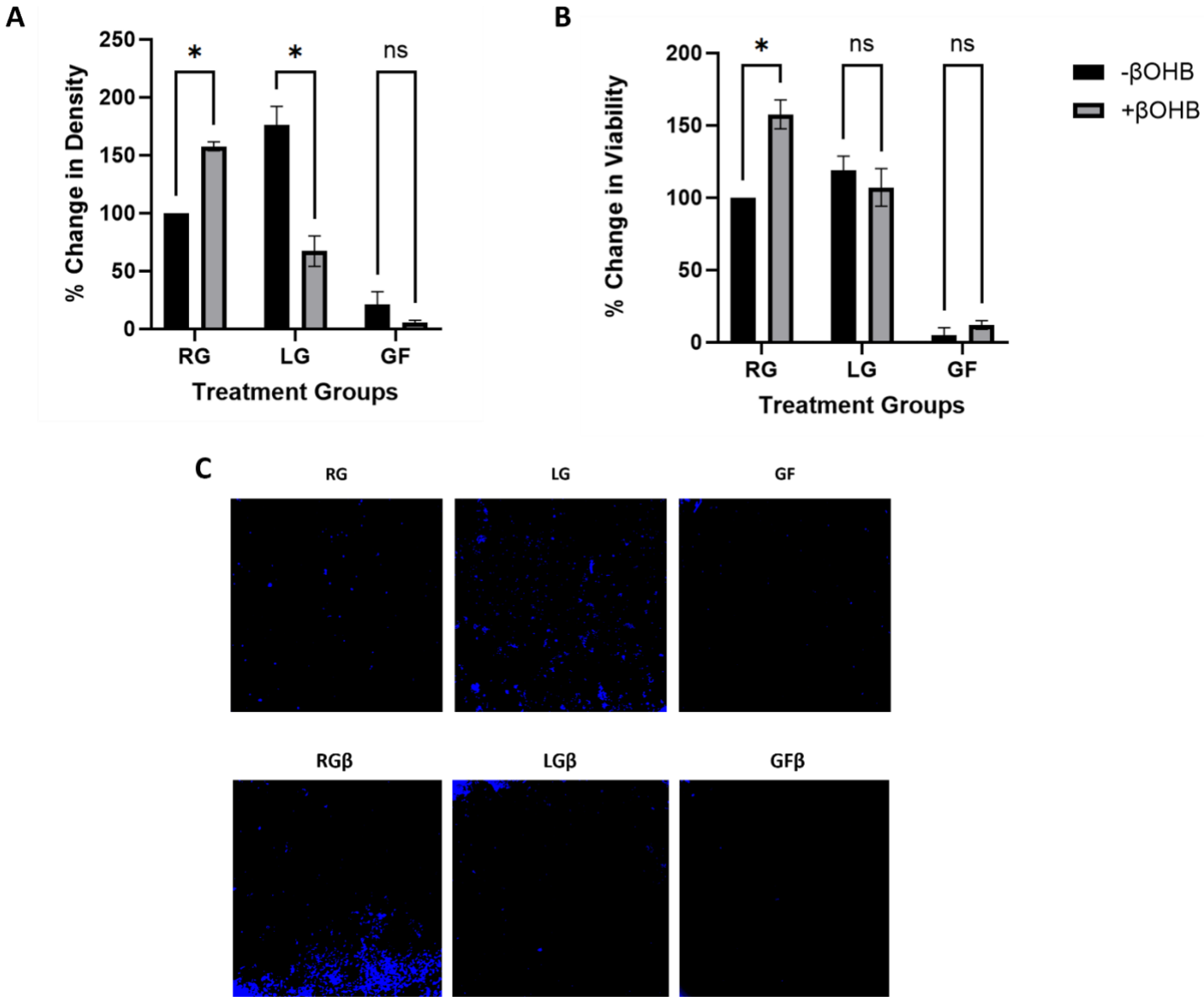


Figure 4.11. The effect of ketone supplementation on the density and metabolic viability of NE4C cells. A. Percentage change in cell density of NE4C cells in RG, LG, GF, RGβ, LGβ,

and GFβ conditions at 96 h. B. MTT assay of NE4C cells in RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. C. DAPI labelled NE4C cell nuclei showing cell density in RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. n = 3 independently for each assay. Multiple t-test was performed. *P ≤ 0.05.

4.5.12. Ketone supplementation does not alter NE4C cell phenotype and morphology

To assess whether supplementation of culture condition media with β-OHB resulted in differentiation or morphological changes in NE4C cells, ICC analysis, and morphological analysis were conducted. At 96 h in culture, all NE4C cells in RGβ, LGβ, and GFβ conditions expressed nestin. GFAP was not expressed in RGβ, LGβ, and GFβ conditions. βIII was not expressed in RβG, LGβ, and GFβ conditions (Fig. 4.12, A). At 96 h, morphological analysis of NE4C cells cultured in RG, LG, GF, RGβ, LGβ, and GFβ conditions demonstrated no significant change in cell volume following β-OHB supplementation (Fig. 4.12, B and E). Analysis of process length demonstrated no significant change following β-OHB supplementation (Fig. 4.12, C and E). Analysis of process number showed no significance following β-OHB supplementation (Fig. 4.12, D and E).

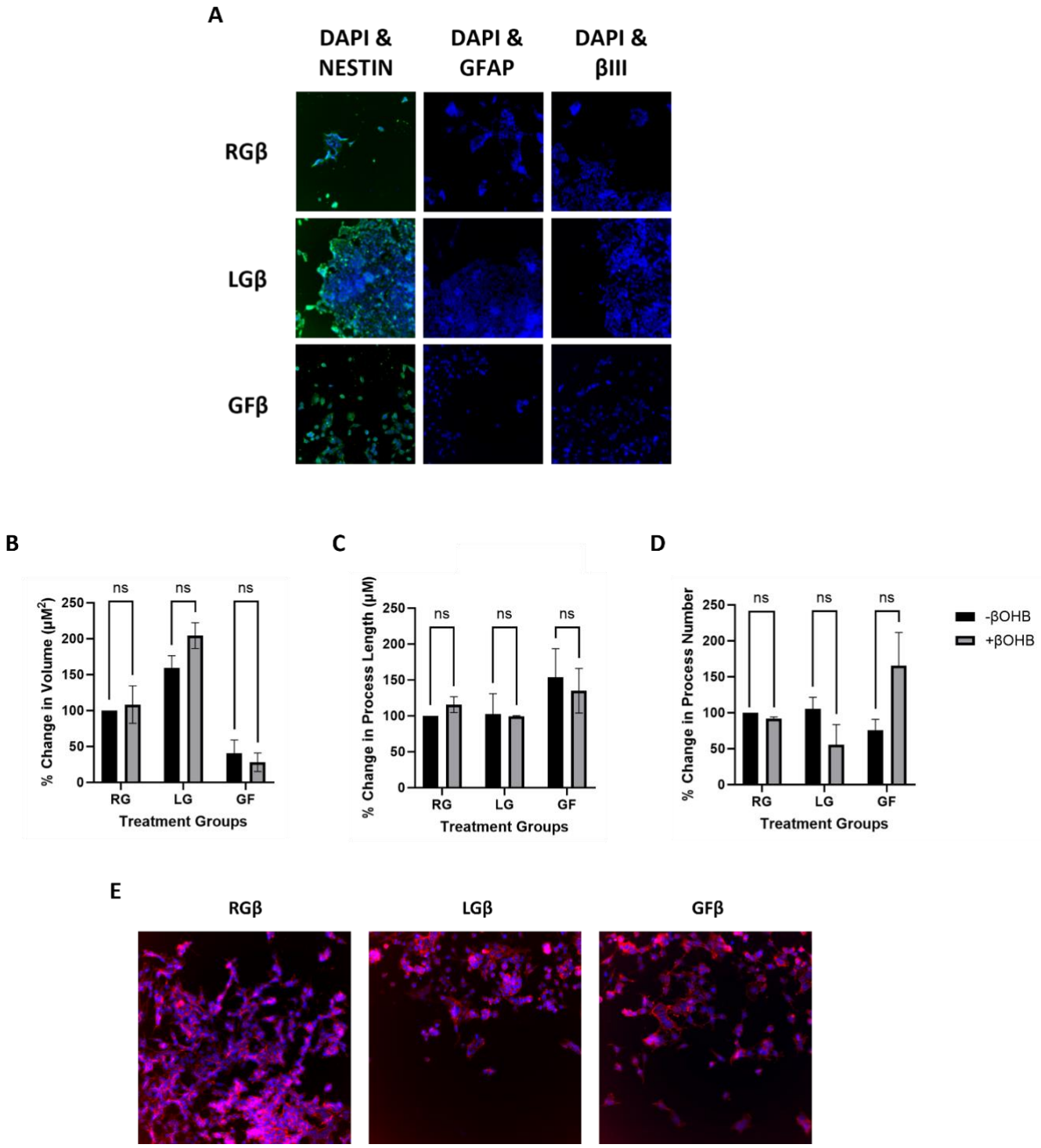


Figure 4.12. Ketone supplementation does not alter NE4C cell phenotype and morphology.

A. ICC of NE4C cells in RGβ, LGβ, and GFβ conditions at 96 h. B. Percentage change in cell volume of NE4C cells in RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. C. Percentage change in cell process length of NE4C cells RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. D. Percentage change in cell process number of NE4C cells RG, LG, GF, RGβ, LGβ, and GFβ conditions at 96 h. E. DAPI (blue) and Actin (red) staining of NE4C cells in RGβ, LGβ, and GFβ conditions at 96 h.

4.5.13. Ketone supplementation significantly decreases glycolysis in regular glucose conditions in NE4C cells

To test whether β-OHB supplementation may result in decreased levels of glycolysis, a glycolysis assay was performed. At 96 h, a glycolysis assay examining the L-lactate production of NE4C cells cultured in RG, LG, GF, RGβ, LGβ, and GFβ conditions showed a significant decrease in glycolysis in between RG (100 ± 0) and RGβ (31.2 ± 12) conditions ($P \leq 0.05$). No significant difference was observed between LG (75.8 ± 7) and LGβ (45.6 ± 20), and GF (19 ± 0.5) and GFβ (22.4 ± 1) conditions (Fig 4.13).

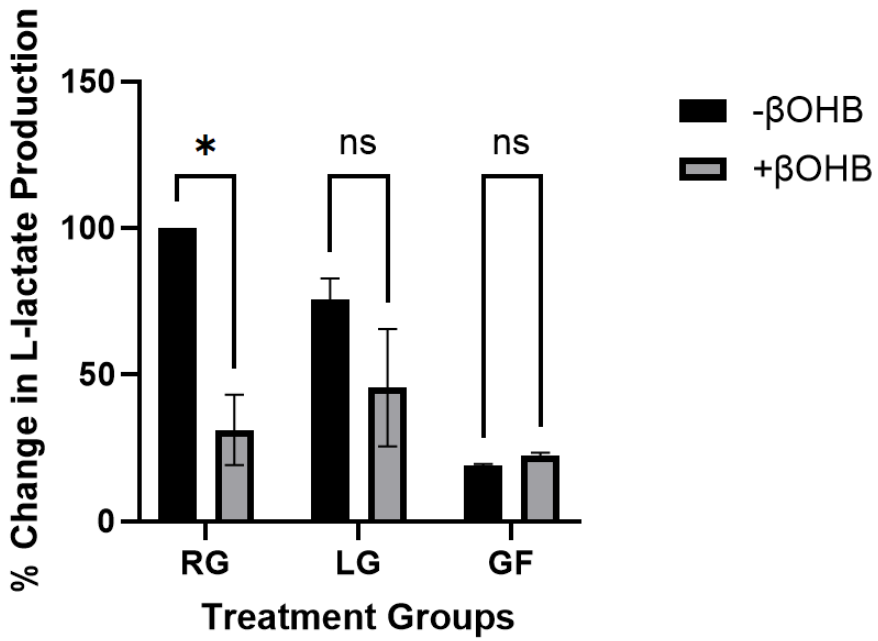


Figure 4.13. Ketone supplementation significantly decreases glycolysis in regular glucose conditions in NE4C cells. Percentage change in L-lactate production in NE4C cells at 96 h. Cells were treated with RG, LG, GF, RGβ, LGβ, and GFβ conditions. n = 3 independently for each assay. Multiple t-test was performed. *$P \leq 0.05$.

4.6. Discussion

The analysis of SH-SY5Y and NE4C cell lines provides valuable insights into their responses to glucose deprivation and ketone supplementation. This enhances our understanding of their physiology and may uncover potential therapeutic and further research implications.

In terms of density and viability, both SH-SY5Y and NE4C cells were negatively impacted by glucose deprivation. β-OHB supplementation yielded similar outcomes in both cell lines. SH-SY5Y cells showed a minor increase in cell density only between RG and RGβ conditions, but no improvement in their viability was observed. NE4C cells displayed differences in density between RG and RGβ conditions, and LG and LGβ conditions. However, only a significant effect was observed in viability between RG and RGβ conditions. The non-significant to minor effect of β-OHB on density and viability in these two cell lines is similar to the response of the MCF-7 breast cancer cell line reported by Maldonado et al (2021). In their study, β-OHB supplementation failed to rescue MCF-7 breast cancer cells from the effects of glucose deprivation (Maldonado et al., 2021). Both SH-SY5Y and MCF-7 cell lines are cancer-derived cell lines (Goldstein et al., 1964; Maldonado et al., 2021), thereby showing that cancer-derived cell lines preferentially utilise glucose to ketone bodies.

Phenotypical analyses revealed that both SH-SY5Y and NE4C cell lines exhibited an NSC phenotype using ICC, showing no spontaneous differentiation in response to glucose deprivation and/or ketone supplementation. Prior research conducted by Wang et al, demonstrated that short-term glucose and oxygen deprivation of primary NSCs resulted in enhanced differentiation and long-term deprivation was detrimental. However, in their study, differentiation was induced by the removal of growth factors and serum from the media (Wang et al., 2015). Therefore, we may interpret that glucose deprivation alone is not sufficient to result in spontaneous NSC differentiation. Morphological analysis of both cell lines demonstrated stability in cell volume, neurite process length, and process number following glucose deprivation and ketone supplementation.

Glycolysis assay analysis showed that both SH-SY5Y and NE4C cell lines were significantly impacted by glucose deprivation, but only NE4C cells demonstrated a significant reduction

in glycolysis following glucose restriction (LG). Supplementation with β-OHB significantly reduced glycolysis between RG and RGβ conditions in both cell lines, suggesting a potential preferential switch to OXPHOS metabolism. Studies conducted by Stoll et al (2015), demonstrated that the addition of polyunsaturated fatty acids to undifferentiated adult NSCs increased their oxygen consumption rate significantly in the presence of a 5 mM glucose concentration (Stoll et al., 2015).

Despite the reduction in glycolysis following β-OHB supplementation, which may indicate a preference for OXPHOS metabolism, no significant change in cell density and viability was observed in our results. This aligns with the growing body of literature regarding the complexity of NSC metabolism. Recent research has shifted our understanding from NSCs being solely glycolytic to exhibiting a more complex metabolic phenotype where mitochondria play an important role (Scandella et al., 2023). Work conducted by Khacho et al (2017), showcased that deletion of the mitochondrial gene for an NADH oxidase known as Aif, caused mitochondrial dysfunction, manifesting as altered NSC self-renewal, proliferation, and differentiation (Khacho et al., 2017).

Both SH-SY5Y and NE4C cell lines exhibited similar responses to glucose deprivation and ketone supplementation, though NE4C cells appeared more sensitive to glucose deprivation. Depriving NE4C cells resulted in significant reductions in cellular density, metabolic health, and glycolysis (Table 4.5). These observations are noteworthy, given the distinct origins of these cell lines. While both exhibit NSC features and may differentiate into neural cell derivatives, the SH-SY5Y cell line is an NB cancer cell line (Brodeur, 2003; Goldstein et al., 1964), and the NE4C cell line is of foetal mouse brain in origin (Demeter et al., 2005). Therefore, observations made in NE4Cs may be more reflective of NSC responses to glucose deprivation and ketone supplementation in the developing foetal brain. Future research using human foetal-derived NSC lines may demonstrate responses more representative of the developing human foetal brain. Additionally, further investigations may also be undertaken in cell lines derived from hippocampal, basal ganglia, and brainstem regions, as in vivo studies involving such cells have demonstrated that hypoglycaemia may result in detrimental effects in these areas (Cacciatore et al., 2022).

Table 4.5. Differential Impact of Glucose Deprivation on SH-SY5Y And NE4C Cell Lines

	SH-SY5Y	NE4C	Significance
Density:			
RG	100	100	
GF	36.6	21.2	
Difference	63.4	78.8	*P ≤ 0.05
Viability:			
RG	100	100	
GF	51.9	5.1	
Difference	48.1	94.9	*P ≤ 0.05
Glycolysis:			
RG	100	100	
GF	54.2	19	
Difference	45.8	81	*P ≤ 0.05

Our results in conjunction with results obtained from other studies, highlight further experimental avenues. Research undertaken by Jády et al (2016), showed that NE4Cs can use β-OHB for mitochondrial energy production (Jády et al., 2016). Assuming both NE4C and SH-SY5Y NSC cell lines use OXPHOS following supplementation with ketone bodies, an investigation into the workings of their mitochondria is warranted. Mitochondrial activity is tightly coupled with mitochondrial morphology, therefore, examining NSC mitochondrial morphology may provide insights into the functioning of these cell lines (Giacomello et al., 2020). Further experiments may elucidate the effects of glucose deprivation and ketone body supplementation on mitochondrial activity, morphology, and their susceptibility to mitochondrial dysfunction. Studies conducted by Jagečić et al (2023), demonstrated that glucose and oxygen deprivation resulted in morphological changes in NSC mitochondria during differentiation, brought about by an increase in the number of oxygen radicals (Jagečić et al., 2023). These analyses may be repeated in varying neural cell lines, to determine if the effects observed are similar or drastically different between cell lines.

In conclusion, these investigations into the responses of SH-SY5Y and NE4C NSC lines to

glucose deprivation and ketone supplementation have provided insights into their functions, highlighting the critical role of glucose in their growth and metabolic viability. Phenotypical analyses revealed the maintenance of an NSC phenotype, indicating that glucose restriction, deprivation, and ketone supplementation do not induce differentiation in NSCs. Glycolysis assay analysis highlighted significant decreases in glycolytic metabolism following glucose restriction and deprivation in these cell lines. Supplementation with β-OHB further diminished glycolytic activity in both cell lines in RG conditions demonstrating a potential switch to OXPHOS metabolism. These findings align with previous research and indicate that NSC metabolism is more nuanced than previously believed. This research demonstrates the importance of examining metabolic interventions to identify potential therapeutic and future research directions.

5. Discussion and Future Directions

This thesis aimed to explore the impact of metabolic alterations on brain development. This was investigated through a process of literature review, genomic analysis, and in vitro experiments.

The findings of this thesis are three-pronged. Firstly, a review of the literature demonstrated that glucose is the primary energy source for NSCs, integral in proliferation, differentiation, quiescent maintenance, and biosynthetic processes. However, the high energy demands of the developing brain often exceed glucose availability. Ketone bodies, generated through β-oxidation in the liver, may be used by NSCs for energy, in addition to the synthesis of lipids involved in neurodevelopment. The regulation of β-oxidation and lipogenesis plays a crucial role in NSC behaviour and fate determination. Disruptions in these metabolic pathways are associated with neurodevelopmental, neuropsychiatric, and neurodegenerative conditions. Manipulation of neural metabolism, such as placing patients on a KD, has been clinically effective in the treatment of epilepsy and has shown promise in other neurological conditions. Such therapies elicit a neuroprotective effect thereby promoting NSC proliferation and differentiation. Based on this information, further investigations into NSC metabolic pathways, as well as determining the impact of dysregulated NSC metabolism, will contribute to the understanding of neurodevelopment and direct novel treatments for neurological conditions (Molloy & Barry, 2024).

Secondly, many similarities exist between NSCs and neural crest cell-derived cancers, namely NB. NSCs exhibit a glycolytic phenotype (Jády et al., 2016). This facilitates the large biosynthetic requirements of these rapidly dividing and growing cells (Aminzadeh et al., 2015). The same is seen in NB (Aminzadeh et al., 2015). Given the similarities, and that undifferentiated SH-SY5Y cells display a neuroblast-like morphology, and differentiation of these cells results in primary neurons (Gordon et al., 2013), they are commonly used in neurobiological research (Gardiner & Freeman, 2016). Provided the significant size of datasets concerning gene expression in NB, genomic analysis of glycolytic and ketolytic gene expression in NB was conducted. The data derived from this study relating to the expression of these genes are applicable to the SH-SY5Y cell line, and potentially other NSC lines. Therefore, this analysis provides insights into the metabolism of NSCs in the context of glycolysis and ketolysis. This data suggests that increased expression of

glycolytic genes HK2, GAPDH, and ENO1 are associated with MYCN amplification, advanced INSS stage progression, and poorer event-free survivability. It also highlights the role of aerobic glycolysis in NB tumour progression. Expression of ketolytic genes BDH1, OXCT1, and ACAT1 are associated with MYCN amplification. OXCT1 and ACAT1 levels are not correlated with INSS stage progression or poorer survivability. These findings suggest that MYCN enhances both glycolytic and ketolytic capacities, though NB tumours show a reduced ability to metabolise ketone bodies and OXPHOS activity**.** Given these results, it may be hypothesised that the SH-SY5Y cell line as well as alternative NSCs including the NE4C cell line may not be highly capable of metabolising ketone bodies.

Finally, in vitro experiments using both the SH-SY5Y and NE4C cell lines derived similar results. In terms of cell density and viability, both cell lines were negatively impacted by glucose deprivation. Supplementation with β-OHB produced minor effects. Phenotypical analysis demonstrated morphological stability in both cell lines following glucose deprivation and ketone body supplementation. Glycolysis assay analysis showed that both cell lines require glucose to function adequately, as both were significantly impacted by glucose deprivation. β-OHB supplementation led to a significant reduction in glycolysis in both cell lines between RG and RGβ conditions. The reduction in glycolysis suggests a possible switch to OXPHOS metabolism in these conditions. As discussed, these findings partially align with those generated from the genomics study. It was determined that the SH-SY5Y cell line may not be highly capable of metabolising ketone bodies. Both the SH-SY5Y and NE4C cell lines were only capable of reducing glycolysis following β-OHB supplementation in conditions containing regular amounts of glucose, i.e., 5 mM. Therefore, it appears that these NSCs need glucose to utilise ketone bodies effectively. NE4C cells were also more sensitive to glucose deprivation when compared to SH-SY5Y cells, showing significant reductions in cellular density, metabolic viability, and glycolysis. Differences in sensitivity between these cell lines may be due to their differing origins. Such differences may be accounted for by the DCAF1-mediated inactivation of Rheb-mTORC1, which is triggered by glucose deprivation in cancer cells, resulting in increased survival (Li et al., 2024).

Future research may further investigate the interplay between NSC metabolism and brain homeostasis to improve therapeutic and diagnostic options in an array of neurodevelopmental and neurological conditions. The regulation of glycolysis and OXPHOS metabolism in NSCs, and their temporal and molecular mechanisms warrant increased analysis. Such research may aid the development of interventions that utilise the neuroprotective and regenerative capabilities of NSCs, particularly in ischemic injuries, TBIs, and neurological conditions. The genomics study validated the glycolytic gene expression profiles in metastatic and MYCN-amplified high-risk NB tumours, suggesting to their potential use in prognosis prediction. Investigations may focus on the role of later-phase ketolytic genes in prognosis, as they may be associated with improved outcomes. Proteomic analysis of glycolytic and ketolytic factors in relation to NB may also be studied. Additionally, genomic and proteomic studies may be conducted in NSC lines, to gather data regarding their metabolic functioning. This may be of particular interest given their nuanced metabolism in the context of glucose deprivation and ketone body supplementation. Further, the use of NSC lines derived from human foetal brains may be more reflective of NSC responses in the developing brain, and therefore shed more light on their metabolic adaptations.

5.1. Conclusion

This thesis investigated metabolic alterations in NSCs and their implications for brain development and disease. Through literature review, genomic analysis, and in vitro experiments, the important role of glucose in NSC metabolism has been demonstrated, in addition to the potential therapeutic impact of ketone body supplementation. These results emphasise the importance of glucose in NSC proliferation and differentiation, while also highlighting the compensatory role of ketone bodies during periods of reduced glucose availability. Disturbances in these governing metabolic pathways have been associated with various neurological conditions, suggesting that ketone body interventions may offer therapeutic benefits to patients. Genomic analysis of NB revealed increased expression of glycolytic genes, thereby showing a potentially reduced ability of NB tumours to utilise ketone bodies, findings that may apply to NSC cell lines. In vitro experiments demonstrated that both SH-SY5Y and NE4C cell lines need glucose to function properly and that β-OHB supplementation may decrease glycolysis, suggesting a

metabolic shift towards OXPHOS under RG conditions. These results are supporting of existing literature.

Future research may study the regulation of glycolysis and OXPHOS in NSCs, examining their molecular and temporal mechanisms. These investigations may lead to potential new therapies using the neuroprotective and regenerative effects of NSCs for varying neurological conditions. Additionally, further genomic and proteomic studies may allow for a better understanding of the metabolic functioning of NSCs, particularly in human-foetal brain-derived cell lines which may more accurately reflect in vivo conditions. These insights may allow for improved therapeutic and research strategies for a variety of neurological conditions.

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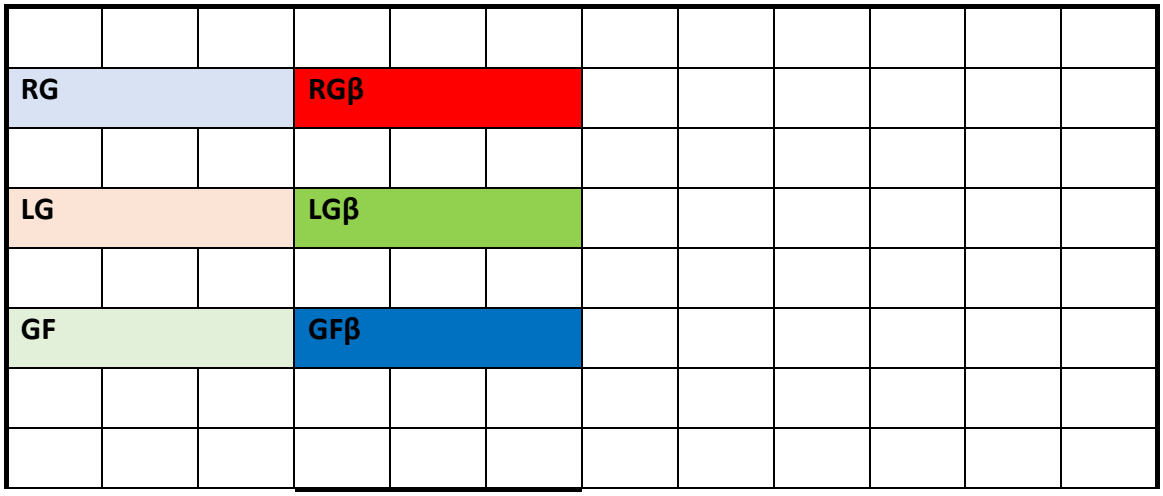
Appendix



Appendix 1. Layout of 96-well plate with media groups.