University of Alberta

The effects of glucose-induced metabolic injury on microglia activity and survival

by

Sara Mahmoud Kenawy

A thesis submitted to the Faculty of Graduate Studies and Research

In partial fulfillment of the requirements for the degree of

Master of Science

Centre for Neuroscience

Spring 2013

Edmonton, Alberta

Dedication

I dedicate this work to my beloved parents, both of whom believed in me and constantly supported me and nurtured my love for science even when I am miles away from home. You provided me with love and motivation all through my life; I am blessed to have you as parents. Special thanks to my loving husband Mohammed and my sweet little daughter Jasmine; having you in my life really completes me and I could not have accomplished anything if you were not part of my life, filling it with countless moments of joy and bliss.

Abstract

Glucose is the sole fuel for the brain in normal physiological conditions. Absence of glucose or its presence in high concentrations has been shown to be harmful to neurons. Microglia, the innate immune cells of the brain, are the first line of defense against changes in the CNS environment and their activity influences neuronal survival to a great extent. The effects of glucose-induced metabolic injury on microglia are unclear. Thus, the aim of this study was to investigate how hyperglycemia and hypoglycemia affect microglial phagocytotic activity, survival and secretory profile.

Results showed that glucose concentration significantly affected microglia release of pro-inflammatory cytokines and growth factors. Microglia phagocytic activity was decreased at high and low glucose concentrations as compared to normal. Interestingly, microglia deprived of glucose showed better survival, exhibited increased ramification and a more quiescent phenotype where microglia released significantly less pro-inflammatory cytokines and growth factors as compared to controls.

Acknowledgement

First and foremost I would like to acknowledge my supervisor Dr Kathryn Todd. Thank you for having faith in me all throughout the past two years. Your constant encouragement and relentless motivation kept me on the right tract and helped me grow both scientifically and personally. You are beyond doubt the best mentor a grad student can hope for.

I owe many thanks and appreciation for my advisory committee members, Dr Baker and Dr Winship for being extremely helpful and supportive all through my graduate studies. Your guidance and advice truly enriched my experience in the lab and boosted my methodologies and ways of thinking. Special thanks to Gail Rauw, Kim Paw and Matthew Churchward for technical assistance and scientific advice. Your innovative ways and patience opened the door for the rest of us to achieve excellence in our scientific endeavors.

I am very grateful to my friends and colleagues at the Todd lab. Your presence created a work environment that comprised both fun and passion for science. Special thanks to senior students who coached me at the launch of my career Kam, Sam and Shakib. Many thanks to Comfort, Andrea and Gomathi for being such supportive coworkers and pleasant companions. I am truly lucky to have you all as friends.

Finally I would like to express my deepest appreciation for the NRU support team Ashley and Kim. You made our lives so much easier.

I would also like to acknowledge the Canadian Institute of Health Research and The Davey Brain Fund for financially supporting the Centre for Neurosciences.

Table of Contents _Toc341444857

List of abbreviations

AGEs advanced glycated end products

AKG alpha-ketoglutarate

AMP adenosine monophosphate

AMPKA adenosine monophosphate kinase activation protein

ATP adenosine triphosphate

AMP adenosine monophosphate

Aβ beta amyloid protein

BBB blood-brain barrier

BCA bi-cinchoninic acid

BDNF brain derived neurotrophic factor

BSA bovine serum albumin

CNS central nervous system

DAB diaminobenzidine

DAG diacylglycerol

DAPI 4',6-diamidino-2-phenylindole

DIC differential interference contrast microscopy

DM diabetes mellitus

DMEM Dulbecco's Modified Eagle Medium

DMSO dimethyl sulfoxide

DR diabetic retinopathy

EDTA ethylenediaminetetraacetic acid

EGTA ethylene glycol tetraacetic acid

ELISA enzyme linked immunosorbent assay

FBS fetal bovine serum

GABA gamma-aminobutyric acid

GD glucose-deprived

GE glucose-excited neurons

GI glucose-inhibited neurons

GLUTs glucose transporters

HG high glucose

HPA hypothalamic pituitary adrenal axis

HPLC high performance liquid chromatography

HRP horseradish peroxidase

IBA-1 ionizing binding calcium channel protein

IGF-1 insulin growth factor one

IL-1β Interlukin 1 beta

LDL low density lipoprotein

LPS lipopolysaccharide

MAP mitogen activated protein kinases

MCP-1 monocyte chemo-attractant protein 1

MMP matrix metalloproteinase

MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF- Kβ nuclear factor kappa beta

NG normal glucose

NMDA N-methyl-D-aspartate

NO nitric oxide

NP neuropathic pain

P/S penicillin/ streptomycin

PBS phosphate-buffered saline

PI3K phosphoinisitol 3 kinase

PKC phosphokinase C

RAGEs receptors for advanced glycated end products

ROS reactive oxygen species

TBS tris-buffered saline

TCA tricarboxylic acid cycle

TMB tetramethylbenzidine

TNF-α tumor necrosis factor alpha

WHO World Health Organization

µL microliter

µm micrometer

µM micromolar

hr hour

kd kilodaltons

L Liter

M Molar

mg milligram

min minute

mM millimolar

nM nanomolar

°C degrees Celsius

List of Figures

Chapter 1

1. INTRODUCTION: Role of glucose in the brain

1.1 Brain energy metabolism and glucose utilization:

The brain is an organ that consumes a great deal of energy. Although the brain accounts for less than 2% of a person's weight, it consumes 20% of the body's energy. Glucose is the sole fuel for the brain under physiological conditions [1]. While most tissues are flexible in their choice of substrates and can use them interchangeably according to their availability, the brain derives almost all of its energy from the aerobic oxidation of glucose. Energy consumption by the brain is not accounted for by mechanical work as with muscle or by osmotic work as with the kidney. Instead, energy consumption by the brain is crucial for maintaining and restoring ionic gradients associated with synaptic transmission and neuronal firing [1, 2]. Electrical potentials and active reuptake of neurotransmitters, especially excitatory neurotransmitters such as glutamate, consume considerable amounts of brain energy. Some synthetic processes account for significant energy use as well [3]. The normal, conscious human brain consumes 19 to 33 mmol of glucose/100 g/min. The vast majority of glucose, both in the basal and activated states of the brain, is consumed through oxidative metabolism to produce carbon dioxide and water or is used in very small amounts in the synthesis of chemical constituents of the brain; at most 8-10 % is anaerobically degraded [2].

The brain is a complex organ composed of a variety of cells, each having different metabolic needs; however, every brain cell is capable of using

glucose because all of them possess glucose transporters (GLUTs) and glycolytic and tricarboxylic acid (TCA) cycle enzymes. Astrocytes are the only type of brain cell that have the unique ability to synthesize glycogen from glucose; in other words they are able to store glucose in the form of glycogen [4].

The utilization of substrates other than glucose such as glycogen, lactate, acetate, and pyruvate can also take place in the brain. These substrates can be metabolized by astrocytes to provide physiologically significant amounts of energy in addition to that derived from glucose. The use of these alternative substrates only occurs to a limited degree during abnormal circumstances such as starvation and hypoglycemia [5].

1.2 Role of the blood-brain barrier and glucose transporters in glucose utilization

Because endogenous levels of glucose and glycogen are relatively low in the brain, the brain relies on a continuously sustained supply of glucose from the blood; however, due to the water-soluble, polar nature of glucose, it does not readily cross the lipid bilayer of cell membranes [6]. Thus, all cells that rely on glucose for their metabolism must have a mechanism for facilitating its entry.

The brain is also isolated from the systemic circulation by the blood-brain barrier (BBB), which is composed of endothelial cells joined by tight junctions. The BBB forms a neurovascular unit that protects the brain from circulating neurotoxic substances while maintaining nutrients and ions in the brain at levels necessary for neuronal function [7].

Delivery of glucose from the blood to the brain requires transport across the endothelial cells of the BBB and into the neurons and glia. It is now generally accepted that glucose enters the brain via a carrier-mediated, facilitated diffusion process. The facilitative glucose transporter proteins mediate this process [8].This family of transporters includes seven proteins named GLUT 1-7. The predominant cerebral glucose transporters are GLUTs 1 and 3. GLUT1 is detected at high concentrations in the endothelial cells of the BBB and is also present in brain parenchyma, primarily in glia [9]. GLUT3 is found in neurons, and GLUT5 has been localized exclusively to microglia, the resident immune cells of the brain. The rest of the transporter family, GLUTs 2, 4, 6 and 7, have also been detected in the brain but at lower levels of expression and confined to more distinct regions [10]. For instance GLUT2 is found in the retina and muller cells of the brain, and GLUT4 has been found to localize in certain brain regions such as the hypothalamus, pituitary, cerebellum, and olfactory bulb. GLUT 4 has received much attention in research due to the vital and central role it plays in glucose homeostasis. This transporter is a special type of receptor as it acts as an insulin-responsive glucose transporter. Binding of insulin to its receptor induces the translocation of GLUT4-containing vesicles to the plasma membrane, and greatly enhances the glucose transport capacity of the cell [11].

1.3 Glucose homeostasis in the brain

Unlike other organs like the liver and to a lesser extent the kidneys the brain cannot produce glucose by gluconeogenesis which is the generation of glucose from non-carbohydrate carbon substrates such as lactate, glycerol, and some amino acids. Lacking the enzymes involved in gluconeogenesis the brain depends solely on the glucose derived from the blood and crossing the BBB. Thus it is highly important to maintain a constant blood glucose level, particularly for the central nervous system, which has no endogenous glucose supply [12].

Plasma glucose concentrations are normally maintained between 3.0–5.6 mM, but can vary between 2mM and 10mM or higher in pathological conditions. Within the brain however, cerebrospinal fluid (CSF) and interstitial brain glucose levels are approximately 30% of those found in the blood. Glucose concentrations have a tighter range and much lower variations (0.5–2.5 mM) in the CSF as compared to plasma glucose levels [13].

The brain regulates energy homeostasis by responding to circulating signals of nutrient status and responds by adjusting food intake accordingly. Thus, under normal conditions the brain senses peripheral energy status and responds by regulating the balance between energy intake, outflow and storage in order to maintain energy homeostasis within a fairly tight range. In response to this peripheral feedback, the brain also modulates the metabolic functions and insulin sensitivity of a variety of

tissues including liver, pancreas, skeletal muscle, and adipose tissue [14, 15].

This regulation process is complex and involves coordination between numerous systems and a number of interconnected brain regions including the hypothalamus, amygdala and brainstem in addition to brain regions associated with autonomic regulation and reward pathways such as the nucleus accumbens [16, 17].

Peripheral feedback about circulating glucose levels is maintained by a system of plasma glucose-sensing neurons present in the portal and mesenteric veins, carotid body and intestine. These cells deliver information about blood glucose concentrations just before blood enters the liver from the gut. This information is conveyed to the brain through the vagus nerve and spinal cord to the hind brain and the hypothalamus[15].

In these brain regions some neurons also possess a specialized ability that allows them to act as glucose sensors and alter their firing rates with fluctuating glucose concentrations. These neuronal glucose sensors are located in many areas of the brain such as the hypothalamus, nucleus solitaris and amygdala. They can be divided into two groups of glucose sensing neurons those which respond to decreases in interstitial glucose below 2.5 mM and those which respond to increases above 5 mM. They act as important entry points to provide the brain with information about circulating glucose levels [18].

These glucose-sensing neurons are further divided into glucose-excited (GE) neurons and glucose-inhibited (GI) neurons; GE neurons increase their action potential frequency with increases in brain glucose levels, while GI neurons are neurons that decrease their action potential frequency when brain glucose levels increase. Multiple subtypes of GE and GI neurons may exist and utilize different glucose sensing strategies. For example, almost all GE neurons utilize the adenosine triphosphate (ATP)-sensitive potassium channel to sense glucose and energy levels [19]. GI neurons depend on the activity of adenosine monophosphate-activated protein kinase (AMPK) which is a protein that is activated by an increase in the AMP/ATP ratio. Activation of AMPK leads to an array of diverse functions including the phosphorylation and closure of chloride channels, leading to the depolarization of GI neurons [20].

The brain gathers this information from the glucose-sensing neurons to influence the behavioral, autonomic, and motor functions that regulate metabolism [21, 22].

1.4 Neuro-endocrine regulation of glucose usage

One of the major mechanisms by which the brain maintains glucose balance is through the neuro-endocrine system. Through regulating the release of gluco-regulatory hormones like insulin, glucagon, catecholamines, cortisol and growth hormone, the brain keeps glucose levels tightly regulated. These hormones are secreted from organs in the periphery like the pancreas (e.g. insulin, glucagon), adipose tissue (e.g.

leptin) or the gastrointestinal tract (e.g. ghrelin or cholecyctokinin) and bind to receptors located in the CNS such as receptors located in the hypothalamus. The hypothalamus in turn sends back signals to the periphery that control food intake (appetite), hepatic glycogenolysis and intestinal gluconeogenesis, and plays a key role in modulating insulin sensitivity of the liver. Thus this cross talk between the CNS and the endocrine system is one of the major regulators of glucose homeostasis [23, 24].

The major hormone that is involved in the control of glucose homeostasis is insulin. It is synthesized and secreted into the plasma by pancreatic β-cells and transferred by a transporter-regulated mechanism through the BBB to the brain. However, the brain is capable of synthesizing insulin growth factor 1 (IGF-1) locally. Insulin and IGF-1 bind to tyrosine kinase receptors and produce numerous biological responses in the brain such as modulation of neuronal growth, survival, differentiation, gene expression, protein synthesis, synapse formation, and plasticity and most importantly regulate metabolism [25, 26]. Impaired insulin signaling has been recently found to be involved in the development of dementia, a new theory called type3 diabetes mellitus (DM) [27]. Insulin resistance and hyper-insulinemia have deleterious effects in the CNS. Impaired insulin signaling produces many pathological changes in the CNS such as tauopathy, β amyloid accumulations, inflammation, oxidative stress, impaired calcium homeostasis and changes in neurotransmitter levels.

Many clinical conditions can cause insulin resistance as part of their pathology such as Type 2 DM, obesity and metabolic syndrome and therefore these conditions may result in a corresponding increase in the prevalence of dementia [28].

1.5 Examples of pathological conditions of abnormal glucose levels

Any disturbances in the aforementioned regulatory mechanisms may lead to energy imbalance or energy failure and can lead to metabolic alterations such as insulin resistance and defective glucose uptake by the cells. That can eventually lead to hyperglycemia. Hyperglycemia in turn has been shown to cause neuronal damage and vascular abnormalities like atherosclerosis [29]. Chronic hyperglycemia leads to advanced atherosclerosis and elevated blood pressure and can eventually lead to stroke and metabolic syndrome. Metabolic syndrome is a clinical condition characterized by hyperglycemia, obesity and hyper-insulinemia as an indicator of insulin resistance and dyslipidemia, all of which can lead to chronic inflammation and eventually the disruption of CNS function and even dementia [29]. In the following section two examples of pathological conditions that involve glucose intolerance will be discussed.

1.5.1 Diabetes mellitus (DM)

One of the most prevalent disorders that involve disturbances of glucose homeostasis is DM. Hyperglycemia is one of the major consequences of DM; however treatment of DM with anti-diabetic drugs or insulin can lead

to episodes of hypoglycemia as well. Thus diabetes can be regarded as an example of both hyper- and hypoglycemic brain injury.

DM is a very common metabolic disorder. According to the Canadian Diabetes Association; DM affects 246 million people all over the world. Its incidence is increasing rapidly due to changes in life style, and it is estimated that by the year 2030 this number will almost double.

For at least 20 years, diabetes rates in North America have been increasing substantially. The rate of DM increases with age so, it is expected that the numbers of diabetic patients might grow as the elderly population increases in number. The World Health Organization (WHO) expects that deaths due to diabetes will increase in the next 10 years by more than 50% [30]. Diabetes is a systemic disease that results from insulin deficiency or insufficiency. It is classified into type 1 DM and type 2 DM. Type 1 DM is an autoimmune disease that results in the destruction of β-cells of the pancreas whereas type 2 DM is a complex disease that results from progressive development of insulin resistance leading to hyperglycemia. Both types can cause serious complications throughout the body and thus diabetes and its complications have become a major concern and a major cause of morbidity and mortality. Uncontrolled diabetes can lead to peripheral neuropathy, diabetic encephalopathy and cerebrovascular disease including stroke. To date, diabetes-induced peripheral neuropathy has been the focus of much research while the cellular mechanisms involved in the development of CNS complications in

DM such as diabetic encephalopathy have received much less focus and therefore are relatively poorly understood [31].

Diabetic encephalopathy is one of the emerging diabetic complications that affect the CNS, leading to decreased cognitive functions and neurobehavioral deficits. It affects the processes of learning, memory, problem solving and motor speed. Diabetic patients also suffer from somatic symptoms, sleep disturbances, compulsions, and depressive moods [32].

The effects of diabetes on the brain are immense, and anatomic alterations that have been identified in the diabetic brain include generalized brain atrophy, mostly in the subcortical regions. Magnetic resonance imaging has also demonstrated that patients with DM have hippocampal and amygdala atrophy when compared to control subjects. White matter lesions are also a feature of the diabetic brain. However, the nature of these lesions remains elusive; it has been hypothesized that they could represent demyelination [33].

Gliosis, which is the activation of brain glia cells, has also been observed in diabetic brains. The activation of microglia in specific brain areas has been shown to be involved in initiating and propagating numerous diabetic complications that affect the nervous system such as diabetic peripheral neuropathy and diabetic retinopathy [34, 35].

Changes in the microvasculature of the diabetic brain have been reported as well. The increased permeability of the BBB by mechanisms which involve hyperglycemia-induced activation of matrix metallo proteinases (MMPs) has been proposed. These enzymes are capable of digesting the extracellular matrix and can contribute to compromising the BBB; they are expressed by numerous cells in the CNS like neurons , microglia, astrocytes and endothelial cells of the BBB [36].

Diabetic encephalopathy also induces neurochemical changes in the brain, such as altered concentrations of neurotransmitters like nor-epinephrine, serotonin and dopamine in certain brain regions [35, 37].

The exact pathophysiology of cognitive dysfunction and dementia in diabetic encephalopathy is not completely understood, but according to the theory of type3 DM, it is likely that hyperglycemia, vascular abnormalities, and insulin resistance play significant and interconnected roles. Interactions between all of these events will amplify the harmful effects of each individual event and worsen insulin resistance more and more, causing more damage to neurons [28].

1.5.2 Ischemic stroke and post-ischemic glucose intolerance

The other example of a pathological condition that involves disturbances in glucose homeostasis is ischemic stroke. Stroke is one of the leading causes of death and disability worldwide. Ischemic stroke, the most common form of a stroke, is caused by a blockage of a cerebral blood vessel. Thus blood flow is either reduced or restricted, leading to reduced

flow of oxygen and nutrients including glucose; thus ischemic stroke can be considered as a clear example of hypoglycemia.

It is well known that hyperglycemia or diabetes potentially exacerbate the neuronal damage observed following ischemic stroke and that glucose intolerance is a prominent risk factor for stroke especially ischemic and embolic types of stroke [38]. Recently it has been shown that glucose intolerance is also a consequence of ischemic and embolic stroke [39].

Furthermore, numerous clinical studies reported higher plasma glucose and insulin levels in the systemic circulation following stroke in patients who were not previously suffering from or diagnosed with DM. In addition, 27% – 37% of patients admitted to hospital with stroke have been reported to have to have glucose intolerance for 3 months after the initial stroke, and approximately one-third of these cases developed DM; so ischemic stroke can be also regarded as an example of hyperglycemic brain injury [39].

Several mechanisms explaining hyperglycemia after ischemic stroke have been proposed. One of these mechanisms is the release of ischemic stroke–related cytokines which have been shown to activate the hypothalamus–pituitary–adrenal axis, leading to increased serum glucocorticoid levels and the activation of the sympathetic autonomic nervous system, leading to catecholamine release and resulting in excessive glucose production and insulin resistance [40].

Another mechanism that might explain post ischemic glucose intolerance is the effect of ischemic stroke on the BBB. Ischemic stress is reported to alter the expression of the tight junction–associated proteins and induce up-regulation of MMPs particularly, MMP-2 and MMP-9. The compromised BBB can lead to changes in glucose transport system and lead to hyperglycemia [41].

Furthermore, recent findings in the literature and in clinical studies suggest that this period of glucose intolerance following ischemia might be the result of a developing insulin resistance or reduced insulin sensitivity. This was supported by the finding that the expression levels of insulin receptors, especially in brain areas that are most affected by stroke like the cortex and the hypothalamus, are reduced following stroke. It was also found that the serum insulin levels were significantly higher in stroke animals as compared to control animals in several in vivo animal studies; this post ischemic hyperinsulinemia also fits in with the hypothesis of a developing insulin resistance following stroke [42].

After cerebral ischemia, brain derived neurotrophic factor (BDNF) expression levels are significantly decreased in the brain. Apart from the fact that BDNF is a member of the neurotrophin family and has potent neuroprotective effects against brain injury and stroke, it is also important to note that current studies on animal models of obesity and diabetes revealed that BDNF is an important regulator of glucose metabolism in peripheral organs like the liver, skeletal muscles and adipose tissue [43]. It

has been also shown that BDNF reduces both food intake and blood glucose levels and that these effects of BDNF depend not only on its action in reducing food intake, but also that BDNF improves hyperglycemia and insulin resistance through enhancing insulin signaling. The hypothalamus is thought to be the target site through which BDNF performs its metabolic actions. Thus functional loss of BDNF during stroke may decrease insulin sensitivity and cause insulin resistance which can be another explanation of the observed phenomenon of insulin resistance following ischemic stroke [44, 45].

The effects of a pre-existing hyperglycemia and glucose intolerance on the recovery and survival after ischemic stroke have been very well studied in the literature and it is widely accepted that glucose intolerance worsens the outcome of stroke. However, the fact that stroke itself induces hyperglycemia and glucose intolerance calls for further investigations to find out if this is a normal protective mechanism to reduce damage of the brain during stroke or merely one of the harmful consequences of stroke that will exacerbate the damage.

These two pathological conditions that involve glucose intolerance and many others like obesity and metabolic syndrome emphasize the significance of the brain metabolic state on the outcome of and recovery from brain disorders and reveal the importance of glucose homeostasis on neuronal fate and survival.

1.6 Mechanisms of hyperglycemia-induced neuronal injury

Hyperglycemia is the increase in glucose levels in the blood and in the interstitial fluid. The threshold for plasma glucose levels before diagnosis of DM is 8 mM. Hyperglycemia is a common type of metabolic injury that results from defective regulatory mechanisms of glucose homeostasis. Examples of some pathological conditions that results in hyperglycemia are DM, gestational diabetes, metabolic syndrome and disturbances of pituitary and adrenal glands.

Uncontrolled chronic or acute hyperglycemia leading to the accumulation of high levels of glucose in the extracellular fluid is one of the key causal factors for the development of various complications in a number of organs such as the kidneys as in diabetic nephropathy and the retina as in diabetic retinopathy [46].

Hyperglycemia-induced metabolic and vascular complications also affect the cerebrovascular system and the peripheral and central nervous systems as well. Hyperglycemia-induced peripheral neuropathy and hyper-algesia are common complications that affect the peripheral nervous system. Increased risk of stroke, seizures, diabetic encephalopathy and cognitive deficits are examples of hyperglycemia-induced complications that affect the CNS [47].

Extensive research has shown that hyperglycemia can harm the CNS and cause neuronal injury and death, primarily through mechanisms related to the metabolic and oxidative state of the cell. One of the main pathways of hyperglycemia-induced neuronal injury is glucose flux through the polyol pathway[48].

Metabolism of glucose by the polyol pathway involves the reduction of glucose to sorbitol by aldose reductase enzyme. This reaction uses NADPH as the hydrogen donor; following that, the sorbitol produced is oxidized to fructose by the enzyme polyol dehydrogenase. Normally, metabolism of glucose by this pathway constitutes a very small percentage of total glucose utilization; however, during periods of hyperglycemia, intracellular glucose levels increase significantly, leading to increased metabolism of glucose by the polyol pathway [49].

The increase in sorbitol levels formed through this reaction leads to osmotic changes promoting cell dysfunction and nerve conduction impairment as in the case of diabetic-induced cataracts. Furthermore, consumption of the important coenzyme NADPH in the aldose reductase reaction results in the shifting of this coenzyme from other important physiological reactions such as the recycling of glutathione (a powerful antioxidant), and this can lead to tilting the balance of oxidants/antioxidants to the oxidative side [50].

NADPH is also involved in the synthesis of nitric oxide (NO) from arginine. Nitric oxide is the key vasodilator in the microcirculation; therefore, a shift

in coenzyme availability might decrease NO synthesis and promote vasoconstriction and poor blood supply [29].

Another mechanism by which hyperglycemia causes neuronal and vascular injury is the increased flux of glucose through the hexosamine biosynthesis pathway[51]. Normally glucose is converted by glucokinase into glucose-6-phosphate which enters the normal glycolytic pathway, where the majority of glucose is metabolized to pyruvate; a small quantity of glucose-6-phosphate is converted by glucose-6-phosphate isomerase to form fructose-6-phosphate, the majority of which enters glycolysis and the tricarboxylic acid (TCA) cycle for the support of basic anabolic processes. However, 2–5% of the fructose-6-phosphate enters the hexosamine biosynthesis pathway where fructose 6-phosphate combines with the amino acid glutamine to form glucosamine 6-phosphate. The rate-limiting enzyme controlling this step is glutamine/fructose-6-phosphate aminotransferase. Glucosamine 6-phosphate is further metabolized to UDP-N-acetylglucosamine [52].

During hyperglycemia the increased flux of glucose through the hexosamine biosynthesis pathway and the production of high levels of UDP-N-acetylglucosamine produce a defect in insulin-stimulated glucose transport, resulting in insulin resistance, possibly through the activation of protein kinase C (PKC). Activation of PKC has been shown to phosphorylate insulin receptors and decrease their activity, which has

downstream effects on signal transduction and glucose transporter translocation, causing impaired glucose transport [53].

In hyperglycemia, the increase in PKC activity through increasing the synthesis of diacylglycerol (DAG) and enhancing the metabolism of glucose to DAG precursors through glycolysis can also take place [54]. PKC hyperactivity promotes hypertension and atherogenesis and contributes to vascular abnormalities observed in hyperglycemic condition as well [55].

The accumulation of advanced glycated end products (AGEs) is also an important mechanism by which hyperglycemia induces vascular and neuronal complications. This mechanism involves the accelerated nonenzymatic modification of proteins by glucose and other sugars to form AGEs. The reactive dicarbonyl group from sugars reacts with protein amino groups and leads to the formation of multiple, reactive species, collectively named AGEs [56].This glycation reaction, which is also termed Millard's reaction, was first detected in diabetic patients for hemoglobin. The discovery of the glycated form of hemoglobin has revolutionized the monitoring and study of patients with diabetes [57].

There are a few general mechanisms by which AGE formation may cause pathologic changes. For example, rapid intracellular AGE formation can directly alter protein structure and function in tissues. Also, AGEs can lead to inflammation and apoptosis through interactions with AGE-specific cellular receptors [58]. AGEs have specific receptors called the RAGE

receptors; they are multiligand receptors which serve as receptors for amyloid-β peptide (Aβ) on neurons, microglia, astrocytes, and cells of vessel walls. Increased expression of RAGE is observed in conditions as DM and Alzheimer`s disease.

The activation of RAGE receptors initiates many signaling pathways, some of which can eventually lead to increased oxidative stress and inflammation. Also these receptors play important and regulatory roles in mediating β-amyloid-related harmful effects observed in Alzheimer`s disease. RAGE can also activate microglia, the innate immune cells of the brain. This activation eventually leads to the activation of nuclear factor NF-Kβ, leading to increased release of pro-inflammatory cytokines and neuronal apotosis [59-61].

While each of the previously mentioned pathways may be injurious to neurons alone, collectively they cause an imbalance in the mitochondrial redox state of the cell by leading to excessive formation of reactive oxygen species (ROS) and can eventually lead to a vicious cycle where each component amplifies the harm produced by the other components. Thus hyperglycemia is one of the predominant types of metabolic injury that affect the CNS. A summary of the biological events that can lead to neuronal injury in hyperglycemia are shown in the following diagram [62].

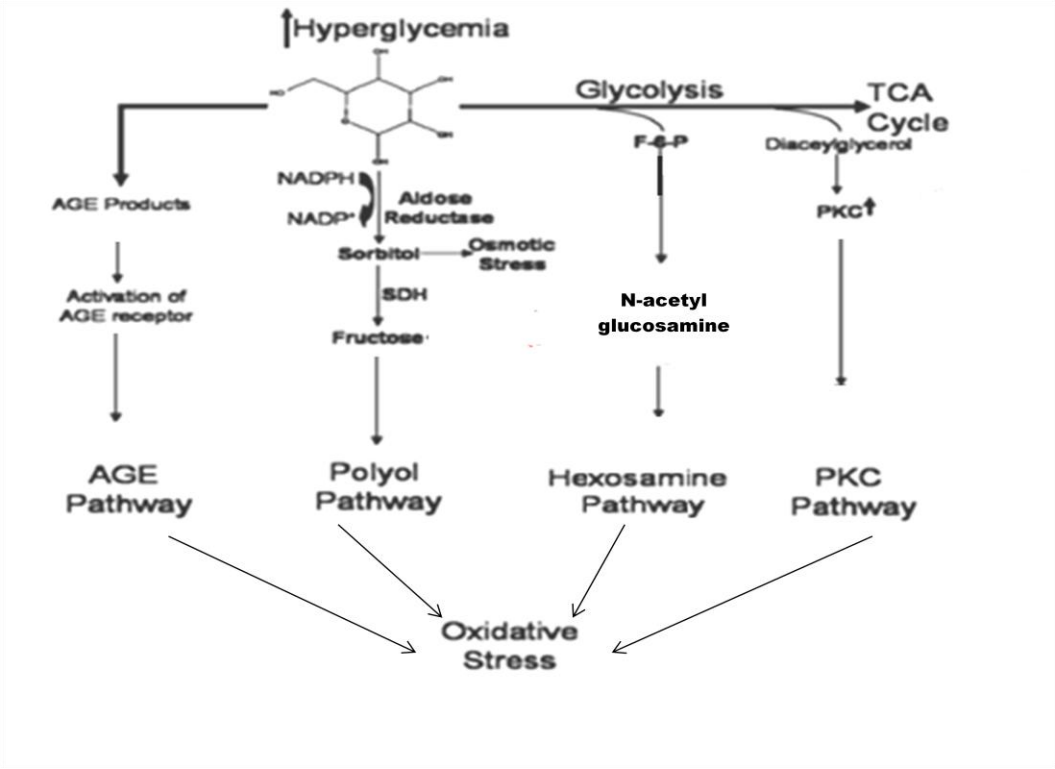


Figure 1: Hyperglycemia activates the detrimental pathways of AGE, polyol, hexosamine and PKC pathways which lead to redox imbalance and further oxidative stress. This diagram is adapted from *(Edwards, J.L., et al.2008).*

1.7 Mechanisms of hypoglycemia-induced neuronal injury

Hypoglycemia is another type of metabolic injury that is opposite to hyperglycemia and can be described as inadequate levels of glucose. This type of metabolic injury is commonly associated with different pharmacological and therapeutic approaches to achieve euglycemia (normal blood glucose levels) during the treatment of diseases like DM. Hypoglycemia is most commonly observed with insulin and oral hypoglycemic agents [63, 64]. Also in conditions that involve a reduced blood supply to the brain like ischemic stroke, inadequate glucose and nutrient levels reaching the CNS is one of the major outcomes.

Depriving the brain of glucose, leading to severe hypoglycemia, can cause brain damage and long-term impairments in learning and memory. Initially hypoglycemia was considered to kill neurons by depriving them of glucose. However, currently it is now widely accepted that hypoglycemia kills neurons actively rather than just by starvation. When blood glucose falls to a range of 2.5 mM to 1 mM, sudden energy failure occurs,and that leads to the activation of hypothalamic pituitary adrenal axis (HPA). Subsequently symptoms due to the release of adrenaline by the sympathetic nervous system start to show up, indicative of the body`s attempt to counteract the falling blood glucose levels. Some of these symptoms are restlessness, hypertension, sweating, anxiety, hunger and palpitation [65-67].

Hypoglycemia constitutes a unique metabolic brain insult. Vast differences have been noted between the mechanism of neuronal injury induced by hypoglycemia and neuronal damage caused by other forms of neuronal injury such as hyperglycemia and ischemic brain insults. Hypoglycemic neuronal damage is primarily caused by excitotoxicity leading to an increase in intracellular calcium and neuronal apoptosis. Hypoglycemia also causes disruption of the normal glucose flux into the TCA cycle, resulting in a lowering of all glycolytic intermediates, such as lactate and pyruvate. However excess oxaloacetate, accumulates due to its inability to interact with sufficient decarboxylated pyruvate to form citrate. The resulting increase in oxaloacetate leads to the production of excess

aspartate, an excitatory neurotransmitter that can be released into the tissues from cells. In addition, cells in hypoglycemia also become leaky due to energy failure and defective membrane transport mechanisms. The leakiness and general inability of cells to maintain amino acids within results in an increase of glutamate levels in the extracellular space as well, although glutamate increases to a lesser degree than aspartate. The aspartate and glutamate bind to NMDA receptors to open ion channels permeable to calcium and can cause excitotoxicity and neuronal death. GABA levels similarly increase in the extracellular space, but its inhibitory effect is insufficient to prevent the effects induced by the excitatory amino acids released [68]. Brain areas that are markedly affected by hypoglycemia are the cerebral cortex, hippocampus and caudate nucleus; however the brainstem and the cerebellum are relatively spared in hypoglycaemia, probably owing to a very efficient glucose transporter system in the cerebellum and the fact that brainstem nuclei can remarkably continue protein synthesis even at very low glucose levels [69]. Another biochemical alteration that occurs in hypoglycemia is the development of tissue alkalosis. The first cause of tissue alkalosis is increased ammonia production as the cells catabolize protein and deaminate amino acids. Ammonia is a very strong base and it drives up cellular pH quickly. The second reason for alkalosis in hypoglycemia is lactate deficiency due to the decreased glycolytic flux in hypoglycemia.

Lactic acid is a normally acidifying agent. This hypoglycaemic alkalosis contrasts with the acidosis generated in cases of brain ischemia [70].

During severe hypoglycaemia, the brain attempts to maintain its energy state by oxidation of substrates other than glucose such as proteins and fatty acids and also exogenous molecules which still circulate the blood in hypoglycemia like glycerol, lactate, and ketone bodies. The main molecule in determining the cellular energy state (ATP) is reduced, and levels of AMP, are increased [71].

1.8 Brain cell populations and the role of glia in health and disease

The brain is a complex organ that comprises numerous cell types. Apart from neurons which are the brain cells that are involved in conveying and processing electrochemical signals, there are nonneuronal cell types named glial cells. These cells mainly perform supportive and regulatory functions in the CNS, but they play a variety of other critical roles in the brain including development, facilitating neuronal transmission, brain metabolism, maintenance of the extracellular environment, regulation of neuronal repair after injury, formation of the BBB, and regulating the inflammatory response in the brain. Glial cells greatly outnumber neurons in the CNS and their activation seems to mediate many pathological conditions. Glial cells can be classified as macroglia and microglia [72].

Macroglia include cells like oligodendrocytes and astrocytes. Astrocytes are the most abundant type of microglia. They are characteristic star-shaped glial cells in the brain and spinal cord. They perform many

functions, including biochemical support of endothelial cells which form the BBB; they have numerous projections that anchor neurons to the BBB. They regulate the external chemical environment of neurons by removing excess ions and recycling neurotransmitters released during synaptic transmission and they also have a principal role in the repair and scarring process of the brain and spinal cord following traumatic injuries [73].

Microglia are the other class of glial cells. Microglia cells represent 5–10% of CNS glial cells and they act as a first line of defense against pathogen invasion by generating innate immune responses through recognizing, sequestering and processing antigens. They are mesodermal in origin. Microglia enter the brain during early development and transform into a branched, ramified morphological phenotype in the mature brain. They are found almost evenly distributed throughout the CNS [74]. This ramified phenotype is also called the resting form of microglia, but this term is not the best term to describe this morphology of microglia and a better term would be surveying microglia since these cells exhibit high motility of their processes and are constantly surveying the CNS for any signs of threat or danger. After a pathological event, these cells undergo a transformation in which they acquire an amoeboid morphology, and they gain the capacity to migrate, proliferate and phagocytose [75]. Although response to threats and pathogens is considered the primary function of microglia, it is also important to mention that microglia play essential roles in the normal physiological conditions of the CNS as well; it has been shown that

microglia are crucial in neurogenesis and synaptic pruning during early development.

The role of microglia in the healthy and diseased brain has gained a lot of attention during the last decade, and whether microglia activation is harmful to the brain remains controversial. Microglia can be perceived as a double edged weapon that can sometimes be harmful if overactivated for long periods of time and some other times essential for recovery of the brain. Microglia are known to exert both inflammatory and anti-inflammatory effects. Activated microglia are capable of producing trophic molecules, such as BDNF, glutamate transporters, and antioxidants, but also molecules that can be potentially neurotoxic, such as NO and pro-inflammatory cytokines [76].

Recently growing evidence demonstrates that microglia play a pivotal role in the development, regulation and progression of brain diseases and disorders. However, the exact role of microglia in the healthy and injured CNS remains an exciting and novel area of research, and the role played by microglia in response to hyperglycemic and hypoglycemic brain metabolic injuries is yet to be clearly shown [77].

Although the effects of hyper- and hypoglycemia on neurons have been very well studied in the literature, very few studies have addressed microglial response to these types of brain metabolic injury. Most of what we know now about how microglia will respond to changes in glucose

levels is derived from in vivo studies of metabolic disorders like DM. The activation of microglia in DM and DM-induced complications such as diabetic retinopathy (DR) and neuropathic pain (NP) is a marked phenomenon [34, 77]. However very few studies have investigated the effects of high or low glucose levels on microglia behavior directly by employing an in vitro culture system where microglia are subjected to varying levels of glucose. It is now widely accepted that changes in microglia secretory profiles of pro- or anti-inflammatory cytokines can greatly influence neuronal fate and survival [78]. Investigating changes in microglia secretory profiles in response to varying glucose levels may provide us with a possible link between the marked microglia activation and neuronal injury in conditions of glucose intolerance. In the following section a summary of microglia behavior during some of the prevalent diabetic complications will be discussed.

1.9 Microglia in diabetic-induced complications

Diabetic neuropathy and retinopathy are two major and prevalent complications of diabetes. Microglia have been shown to play important roles in the development and the progression of these diabetic complications.

Neuropathic pain (NP) is a chronic condition that is characterized by increased pain perception of noxious stimuli, or in other words hypersensitivity to normally innocuous stimuli. Neuropathic pain is often resistant to existing pharmacological treatments such as non-steroidal

anti- inflammatory drugs and opioids. It generally occurs following injury to peripheral nerves or CNS following trauma, surgery, cancer, infection or diabetes [79].

Diabetes mellitus is the leading cause of neuropathy worldwide. The severity of diabetic neuropathy is proportional to the duration of diabetes and is increased with improper control of blood glucose levels. Almost 30% of diabetic patients will develop diabetic neuropathy. Symptoms of NP include paresthesia, numbness and burning [80]. Compelling evidence from extensive research has shown that the development of NP involves abnormal excitability of the nervous system resulting from multiple functional and anatomical alterations following peripheral nerve injury such as impairment of sensory nerve conduction velocity, micro-angiopathy, axonal degeneration, Schwann cell pathology or demylenation [81].

Recently it has been shown that glial cells, mainly microglia, play a critical role in the mediation of diabetic pain neuropathy. Microglia activation, migration and release of pro-inflammatory substances in the dorsal horn with the development of pain hypersensitivity have been demonstrated in several animal models. The release of molecules such as ATP, monocyte chemoattractant protein (MCP-1), or MMP9 following nerve injury leads to the activation of microglia. Activation of microglia can promote the release of numerous inflammatory mediators such as IL-1β, IL-6, and TNF-α and ROS such as NO contributing to nerve hyper-excitability. Furthermore, activated microglia have the ability to phagocytose myelin which can be

another mechanism for promoting neuropathy and altering nerve conduction velocity [82]. Also activated microglia are capable of secreting BDNF, but recent data suggest that microglial BDNF is not neurotrophic in the spinal cord, but rather results in the sensitization of neurons to excitatory stimuli and results in further hyper-excitability of spinal cord neurons and neuropathic pain [83, 84].

Diabetic retinopathy (DR) is a complication of long-term diabetes and the main cause of blindness in young adults. Microglial cells are located in the inner part of the retina, around vessels. These cells become activated and migrate in the subretinal space in DR [85]. The activation of microglia induced by hyperglycemia has been associated with the early development of DR. Cytokines, released by activated microglia were shown to contribute to neuronal cell death in the retina. Indeed the increased oxidative stress during hyperglycemia has been shown to activate microglia and their release of inflammatory cytokines in the retina through increasing the activity of molecules such as NF- Kβ and PKC. Also hyperglycemia can lead to microglial activation in DR through AGE-RAGE interaction as previously mentioned in the introduction. Activated microglia contribute to neuronal cell degeneration and compromise the BBB, and thus the degree of microglia activation may influence the extent of retinal injury [34, 86].

1.10 Summary and thesis objectives

Owing to the fact that glucose is the sole fuel of the brain under normal circumstances, the effects of hyperglycemia and hypoglycemia on neurons have been very well studied in the literature. However, the effects of these types of metabolic injury on the non-neuronal populations of the CNS such as glial cells have received much less attention and are therefore relatively poorly understood. Glial cells greatly outnumber neurons in the CNS and they are crucial to neuronal survival owing to the supportive and protective functions they provide to neurons. In addition to that, their activation seems to mediate many pathological conditions. Microglia cells represent 5–10% of CNS glial cells and they act as a first line of defense against pathogen invasion due to their ability to become activated, proliferate and migrate towards and phagocytose invading pathogens. It is now widely accepted that microglia play critical roles in the development, regulation and progression of numerous brain diseases and disorders due to the fact that microglia activation greatly influences neuronal fate. Microglia are known to exert both inflammatory and anti-inflammatory effects and thus their activation can be either deleterious or beneficial to neurons depending on the type and severity of brain injury [87]. However, the role of microglia in the healthy and injured CNS remains controversial. Recent studies have demonstrated the activation of microglia in the spinal cord, retina and hypothalamus in uncontrolled hyperglycemic conditions. Also microglia have been shown to contribute to

the development and the progression of NP, one of the major complications of DM [88, 89]. The exact behavior of microglia in response to metabolic injuries like hyper- and hypoglycemia is yet to be clearly shown; such findings will increase our knowledge about the role played by these immune cells in conditions that involve glucose intolerance like DM, obesity, metabolic syndrome and post ischemic glucose intolerance.

Activated microglia release a variety of neuromodulating and neuroactive substances that can affect neuronal fate like ROS such as NO, a molecule that can interact with oxygen to form the highly toxic molecule peroxynitrite which is a powerful oxidant and nitrating agent that can cause harm to neurons by resulting in lipid peroxidation, protein and enzyme oxidation and even DNA damage. Thus, studying the release of ROS from microglia in response to hyper- or hypoglycemia is the first objective of this project [34, 90].

Microglia can also release IL-1β and TNFα which are pro-inflammatory cytokines that can participate in mediating the inflammatory state observed in the central and peripheral nervous systems during many neurodegenerative and pathological brain conditions, including DM and stroke. Through acting on their respective receptors and signaling pathways these cytokines can cause neuronal injury [86]. Studying the release of IL-1β and TNFα from microglia in response to hyper- or hypoglycemia is the second objective of this project.

Furthermore, activated microglia can release growth factors like BDNF a molecule that plays important roles in proliferation, differentiation and survival of neurons and is currently recognized as a major participant in the regulation of food intake, insulin signalling and maintenance of metabolic homeostasis [43, 91]. BDNF has been also shown to be an important molecule for the development of diabetic neuropathy [83]. Measuring the release of BDNF from microglia in response to hyper- or hypoglycemia is the third objective of this project.

Recent studies demonstrate that the development of diabetes-induced neuropathy involves an imbalance between the excitatory amino acid glutamate and the inhibitory amino acid gamma-aminobutyric acid (GABA). In addition, alpha-ketoglutarate (AKG), which is an intermediate of the TCA cycle is converted into glutamate by mitochondrial glutamate dehydrogenase and thus glucose and its intermediate AKG are considered precursors of glutamate. In addition, hypoglycemic brain injury involves excitotoxicity through increased extracellular levels of glutamate as mentioned above [84]. Glial cells like microglia are involved in the recycling of glutamate by reuptake mechanisms. The fourth objective of this project is to determine microglial release of glutamate in response to different glucose concentrations in an attempt to understand how these cells can contribute to excititoxicity in hyper- or hypoglycemic conditions.

In addition, changes in the protein levels of MMPs, specifically MMP-2 and MMP-9, in microglia cultures in response to hyperglycemia were of a

particular interest since these enzymes are capable of digesting the extracellular matrix and can contribute to compromising the BBB, which is frequently observed in conditions of hyperglycemia such as DM [92]. Measuring the protein levels of MMP-2 and MMP-9 in cell lysates of microglia cultures in response to hyperglycemia or hypoglycemia is the fifth objective of this project.

Determining the effect of hyper- and hypoglycemia on microglia survival and phagocytic activity is the final objective of this project. Survival or death of microglia can directly and indirectly affect neuronal survival; survival of microglia can affect neuronal fate depending on the surviving phenotype of microglia and whether the surviving microglia are activated and deleterious or quiescent and supportive to neurons. Phagocytic activity of microglia is one of the most important functions of these immune cells which enables them to constantly maintain a clean and healthy environment in the CNS by removing dead cells and debris and engulfing invading pathogens. Changes in phagocytic activity of microglia have been shown to contribute in the development and the progression of many CNS disorders like Alzheimer's disease, Parkinson's disease and Prion disease. In these neurodegenerative disorders the defective phagocytic activity of microglia can lead to the accumulation of toxic proteins and exacerbate the progression of the disease. Alternatively, in other conditions such as multiple sclerosis the excessive phagocytic

activity of microglia contributes to the demylination process and the progression of the disease [93, 94].

Based on the above mentioned material, the hypothesis for this project was that hyperglycemia and hypoglycemia will activate microglia such that they produce molecules that are potentially harmful to neurons.

Chapter 2:

Materials and Methods

2.1 Primary microglial cultures

Mixed brain cultures were prepared from the brains of postnatal one day old Sprague-Dawley rats according to the method described by Lai and Todd [95]. Brains were dissected and the meningies were carefully removed. Dissected brains were dissociated by incubation in 0.25% trypsin-EDTA for enzymatic digestion in a water bath at 37^{o}C for 25 min, and then mechanically triturated in DMEM/F-12 with10% FBS and 2% penicillin-streptomycin, to obtain a cell suspension. The cell suspension was then plated on poly-L-lysine-coated 12-well plates. After 14 days microglia were isolated by incubation of the mixed cultures with 0.25% trypsin-EDTA in DMEM/F-12 in a ratio of 1:3 for 25 min to detach all other cells in the mixed cultures except for microglia. Then trypsin was deactivated by treating the cells with DMEM/F-12 with 10% FBS. A final media change was made the following day and the cells were kept overnight in DMEM/F-12 and 2% penicillin-streptomycin.

2.2 Glucose treatments

Isolated microglia cultures were grown for 24 hrs in three glucose concentrations dissolved in DMEM/F-12 with 2% FBS: glucose-deprived (GD, 0 mM glucose), normal glucose (NG, 17.5 mM glucose) or high glucose (HG, 30 mM glucose). Lipopolysaccharide (LPS) was used as a positive control for activating microglia at 1 µg/ml either in NG, HG or GD media.

2.3 Immunocytochemistry

Pure microglia cultures were fixed with formaline for 15 min and then the cells were washed three times with PBS and blocked and permeablized with 10% horse serum and 0.1% Triton X-100 for an hour, washed three times with PBS and incubated overnight at 4^{o}C with antibody against ionized calcium binding adaptor molecule 1(Iba-1) in a concentration of (1:1000) as a specific marker for microglia. Next day the cells were washed again with PBS and incubated with the appropriate biotinylated secondary antibody (1:200) for 2 hrs. DAB (diaminobenzidine, Biomeda), was used for chromogenic detection of microglia and for examining their morphology.

2.4 BCA protein assay

Media in the culture wells was aspirated out and collected for other experiments. The cells attached to the plates were washed three times with PBS and then lysed using lysis buffer. Protein concentrations in the cell lysates were determined by the bicinchoninic acid (BCA) protein assay. A mixture (190 µl) containing BCA solution and copper bisulphate (Fisher) in ratio of 50:1 was added to 10 µl of the cell lysates and a serial dilution of standards prepared from albumin to reach a final volume of 200 µl. After 30 min incubation at 37^{o}C, the plates were read at 562 nm on a plate reader.

2.5 NO assay

Nitrite levels in the media were determined using the Griess reaction. The collected culture media were first incubated with sulphanilamide (in 3 M HCl stock, final concentration 0.25%) and then N-naphthylethylenediamine (final concentration 0.005%) was added to the mixture. The final mixture was read on a plate reader at 540 nm.

2.6 Enzyme linked immuno-sorbent assay

Assays using ELISA kits for IL-1β and TNF–α were performed according to the manufacturer's protocol. Plates (96-well) were coated with the appropriate capture antibody and were allowed to stand overnight at $4^{o}C$. They were then blocked with the ELISA diluents (1% bovine serum albumin (BSA) in PBS) for 1 hr. After blocking, the samples and standards diluted in ELISA diluent were added and incubated for 2 hr. The biotinylated detection antibodies were then added to the mixture and incubated for 1 hr, followed by incubation with HRP-conjugated strepavidin (in ELISA diluent) for half an hour. After each step a washing step was performed by washing the plates 3 times in PBS. Finally, the chromagen tetramethylbenzidine (TMB) was added to develop color. Upon development of the chromagen, 1.8 N sulphuric acid was added to stop the reaction, and the plates were read on a plate reader at 450 nm.

2.7 BDNF competitive ELISA

The original protocol for this assay was modified from the actual manufacturers' protocol. Basically 100 µl of standard were mixed with bicarbonate buffer, and the standard was used to make a serial dilution. Samples (100 µl) and standards were added to uncoated 96-well plates. The plates were wrapped with parafilm and incubated with goat anti-BDNF overnight at 4 ^{o}C. The next day samples and standards were aspirated out and the plates were washed three times with PBS. The plates were then blocked with the ELISA diluent 1% BSA in PBS for 2 hrs. After blocking, plates were incubated with horseradish peroxidase (HRP)-conjugated IgG for 30 minutes. The chromagen tetramethylbenzidine (TMB, Sigma, 0.027 % in 0.82 % sodium acetate, 0.36 % citric acid, and 40% methanol) was added to develop color. Upon development of the chromagen, 1.8 N sulphuric acid was added to stop the reaction, and the plates were read on a plate reader at 450 nm.

2.8 Western blots

After the 24 hour treatment, the cells were harvested and lysed in ice-cold lysis buffer containing 50 mM Tris, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 150 mM NaCl, 0.8% Triton X-100, and 0.2% sodium dodecyl sulphate (SDS). The cell lysates were boiled for 10 min with sample buffer that consists of 62.5 mM Tris-HCl (pH 6.8), 7.5% glycerol, 2% SDS, 15 nM Bromophenol blue and 1% β-mercaptoethanol and subjected to SDS-electrophoresis using 12% polyacrylamide electrophoresis gels and transferred to polyvinylidene fluoride (PVDF)

membranes. The membranes were blocked with a 10% skimmed milk solution in Tris-buffered saline (TBS) containing 0.1% Tween (TBS-T). After blocking the membranes were washed for 10 min with TBS-T three times. The membranes were incubated with primary antibodies against MMP-2 and MMP-9 overnight at 4^{o} C, the next day the membranes were washed again with TBS-T three times for 10 min each and incubated with the appropriate horseradish peroxidase conjugated secondary antibodies for 2 hrs at room temperature, and developed with an ECL chemiluminescence kit. For caspase 3 and cleaved caspase the same procedure was followed but using 15% polyacrylamide electrophoresis gel instead, and the developing of membranes for cleaved caspase was performed using ECL plus chemiluminescence kit. To ensure total protein levels were equal in all samples, loading volumes were adjusted according to their measured protein concentrations from the BCA assay and actin was used as a loading control in all samples.

2.9 MTT assay

Cell survival was measured by the MTT (thiazolyl blue tetrazolium bromide) assay that measures mitochondrial activity. Cells were incubated with 0.5 mg of MTT/ml in PBS for 1 hr, solubilized with dimethyl sulfoxide (DMSO), and then read on a plate reader at 540 nm.

2.10 Phagocytosis assay

Phagocytic activity of microglia was determined by measuring their uptake of 1.0 µm carboxylate modified fluorescent latex beads. Live cells were

incubated with fluorescent latex beads for 2 hrs after treatment, and the cells were then washed three times with ice-cold PBS. The cells were fixed followed by washing three times with PBS and stained with DAPI nuclear stain. Cells were observed under fluorescence and DIC (differential interference contrast) microscopy and the number of cells with beads inside was counted and results were expressed as the % of cells with beads to the total number of cells.

2.11 High performance liquid chromatography (HPLC)

Measurement of glutamate release in the media of treated microglia was carried out using HPLC. The media were collected and the proteins in the media were precipitated out by methanol (2:1 volume methanol:sample); the remaining supernatants were then subjected to analysis using a Waters Symmetry C18 Column coupled with a guard column containing the same stationary phase. The supernatants were subjected to derivatization by o-phthaldialdehyde to produce a fluorescent derivative prior to running through mobile phases. Mobile phase A, adjusted to pH 6.2 with NaOH, contained 900 ml 0.08 M NaH_2PO4, 240 ml methanol, 20 ml acetonitrile, and 10 ml tetrahydofuran (THF). Mobile phase B, adjusted to pH 6.2 with NaOH, contained 1340 ml 0.04 M NaH_2PO4, 1110 ml MeOH, and 60 ml THF. The temperature of the column was held at 30^{o}C and the sample cooler was kept at 4 ^{o}C. The mobile phase initial gradient was set at 80% A and 20%B. The flow rate was adjusted to 0.5 ml/minutes. The gradient changed to 10%B and 90% A over approximately 48 minutes. At 50 minutes the flow rate reached 0.8ml/min

and the final conditions were 100% B. The detector used was a Waters 2474 fluorescence detector with an excitation wave length set at 344 nm and emission wave length set at 433 nm.

2.12 Statistical analyses

The levels of significance were calculated using Graph Pad Prism soft ware. In cases where multiple comparisons were required, a two way analysis of varience (ANOVA) was used. To compare between only two groups the Student`s t-test was employed. The data are expressed in the form of means ±SEM. Groups were considered significantly different if the p value <0.05.

Chapter 3

Results section

3.1 The effect of glucose levels on microglial release of NO

In this experiment microglia release of NO in response to exposure to different glucose levels (HG = 30mM, NG= 17.5mM, GD= 0mM) for 24 hours was determined. LPS was used to activate microglia (1µg/mL). Microglia release of NO was increased in the HG condition when compared to the glucose-deprived condition. There was no significant difference observed between HG and NG groups. All LPS-treated groups exhibited a significant increase in NO release as compared to the NG group.

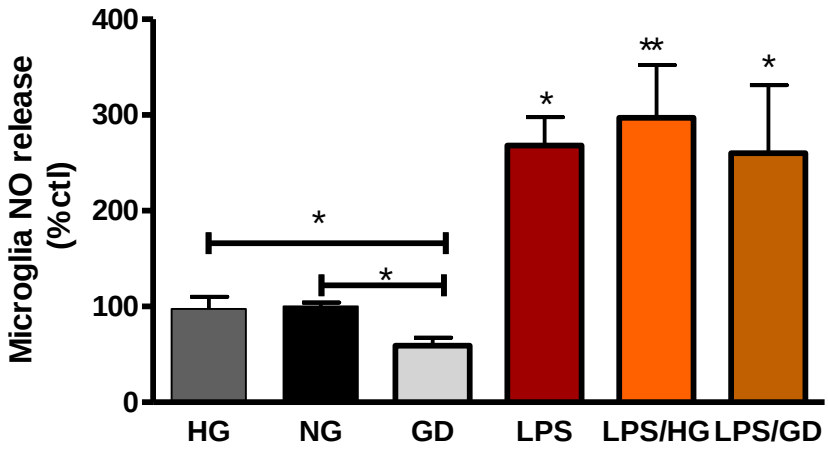


Figure 2: MIcroglial NO release at different glucose levels.Nitrite levels were determined and normalized to protein levels in each well. Data are expressed as % control, and histograms represent the means ± SE. Number of repetitions for this experiment is 6 times; n (number of wells)=14. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.2 The effect of glucose levels on microglia release of IL 1-β

Microglia release of IL 1-β in response to exposure to different glucose levels for 24 hrs was determined. Microglia release of IL 1-β was decreased in the GD condition when compared to the normal glucose condition. There was no significant difference in the release of IL 1-β between HG and NG. All LPS treated groups exhibited a significant increase in IL 1-β release. LPS induced increase of IL 1-β was reduced in the glucose deprived group**.**

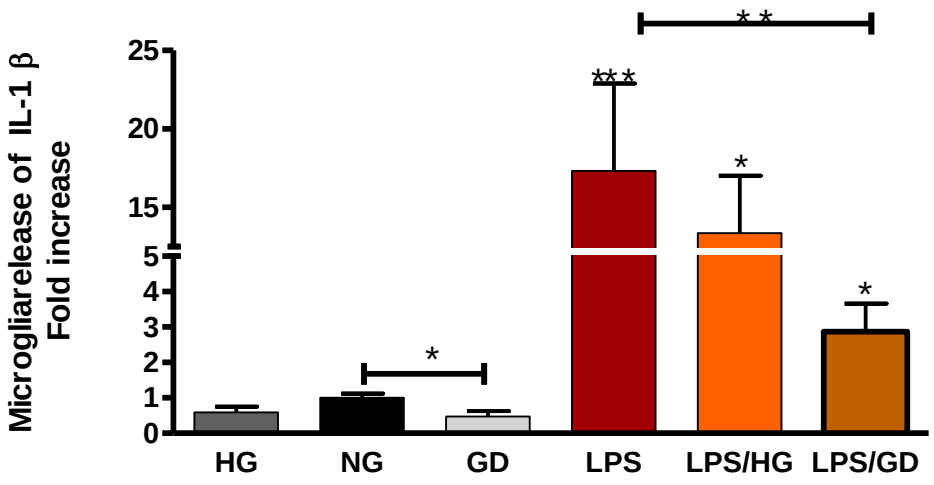


Figure 3: Microglia IL-1β release in different glucose levels. Primary microglia cultures were treated with different glucose concentrations for 24 hours. Levels of IL 1-β in media were determined and normalized to protein levels in each well. Data are expressed as fold increase compared to control (NG), and histograms represent the means ± SE. number of repetitions for this experiment is 5 times; n=14. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated

3.3The effect of glucose levels on microglial release of TNFα

Microglial release of TNF-α was decreased in the GD condition when compared to the normal and high glucose conditions. There was no significant difference in the release of TNF-α between HG and NG. All LPS treated groups exhibited a significant increase in TNF-α release. LPS induced increase of TNF-α was reduced in the GD group.

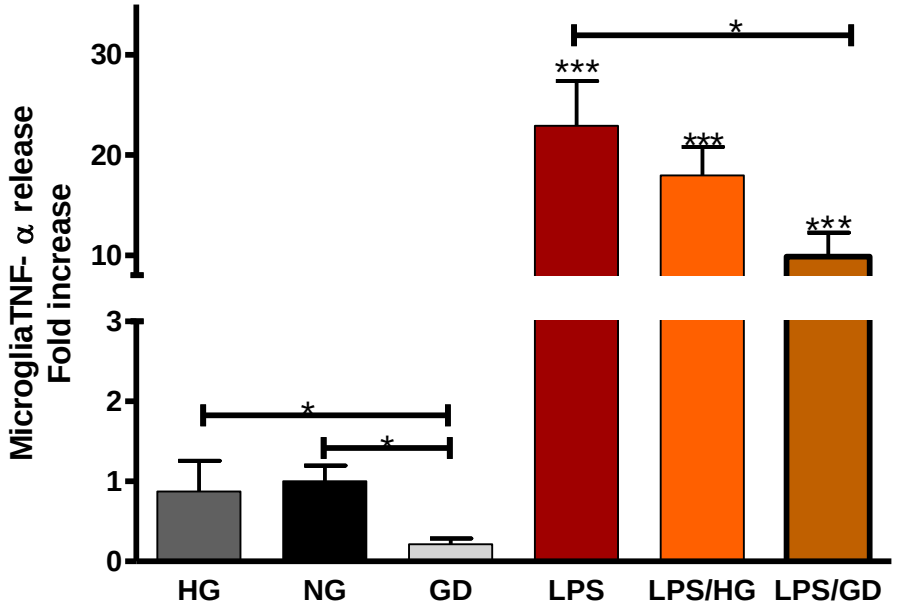


Figure 4: Microglia relese of TNF-α at different glucose levels.Primary microglia cultures were treated with different glucose concentrations for 24 hours. TNF-α levels were determined and normalized to protein levels in each well. Data are expressed as fold increase as compared to control (NG), and histograms represent the means ± SEM. number of repetitions for this experiment is 5 times; n=14. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.4 The effect of glucose levels on microglial release of BDNF

After studying the effect of glucose levels on microglial release of pro-inflammatory cytokines and ROS, we studied microglial release of the growth factor BDNF in response to exposure to different glucose levels for 24 hrs. Microglial release of BDNF was increased in the HG condition when compared to the normal glucose and glucose-deprived conditions. There was a significant decrease in the release of BDNF in the GD as compared to NG. All LPS-treated groups exhibited a significant increase in BDNF release. LPS-induced increase of BDNF was not affected by glucose deprivation.

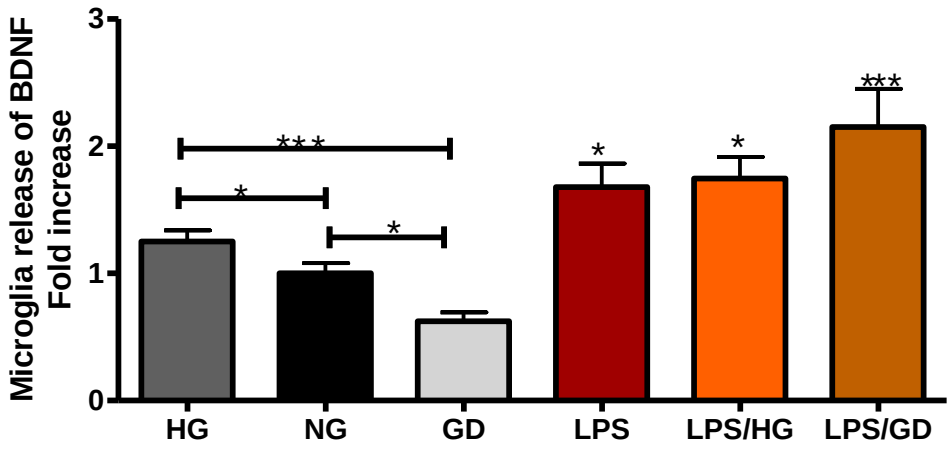


Figure 5: Microglia release of BDNF at different glucose levels.BDNF levels in microglia media were determined and normalized to protein levels in each well. Data are expressed as fold increase compared to control, and histograms represent the means ± SE. Number of repetitions for this experiment is 7 times; n=16. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.5 The effect of glucose levels on microglial release of glutamate

Microglial release of the excitatory neuro-transmitter glutamate in response to different glucose levels for 24 hrs was determined using HPLC [96]. Glucose levels had no effect on the release of glutamate in this experiment. Microglia release of glutamate was increased in all the LPS treated groups except the glucose-deprived group. Glucose deprivation blocked the LPS-induced increase in glutamate release in primary microglia cultures.

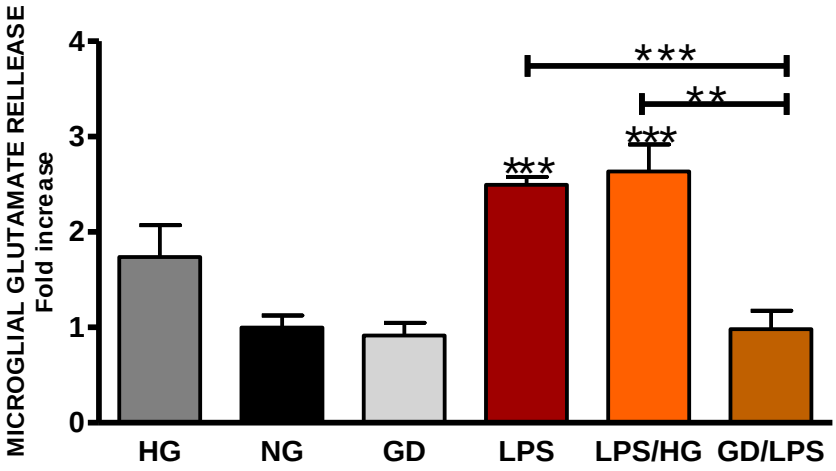


Figure 6: Microglia release of glutamate in different glucose levels. Glutamate levels were determined and normalized to protein levels in sample. Data are expressed as fold increase compared to control, and histograms represent the means ± SE (n=7). Each experiment was pooled out of 8 wells. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.6 The effect of glucose levels on MMP-2 protein levels in microglia cell lysates

Protein levels of MMP-2 were increased in the HG culture conditions including LPS-treated microglia in high glucose. Depriving microglia of glucose had no effect on protein levels of MMP-2 in the cell lysates. Treating microglia with LPS in a glucose-deprived media resulted in very low protein levels in the cell lysates; thus MMP-2 was very hard to detect in the LPS/GD group.

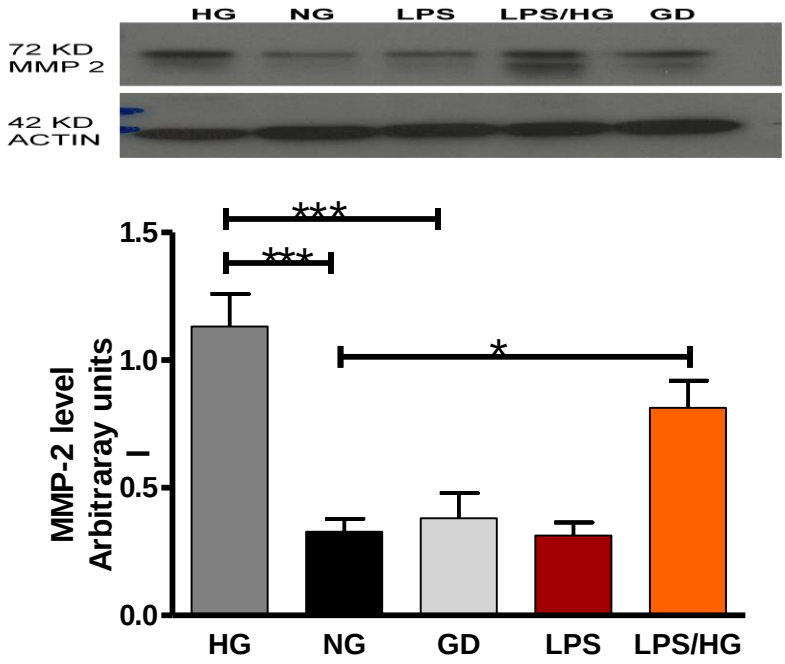


Figure 7: MMP-2 protein levels in cell lysates of microglia exposed to different glucose levels. MMP-2 protein levels in microglia cell lysates were determined and normalized to actin. Data are expressed as means ± SE. Number of repetitions for this experiment is 4 times; each experiment was pooled out of 8 wells. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.7 The effect of glucose levels on MMP-9 protein levels in microglia cell lysates

Unlike MMP-2, different glucose levels had no effect on MMP-9 protein levels in microglia cell lysates. However, in a similar manner as the previous experiment, treating microglia with LPS in a glucose-deprived media resulted in too low protein levels in the cell lysates, making MMP-9 very hard to detect in the LPS/GD group as well.

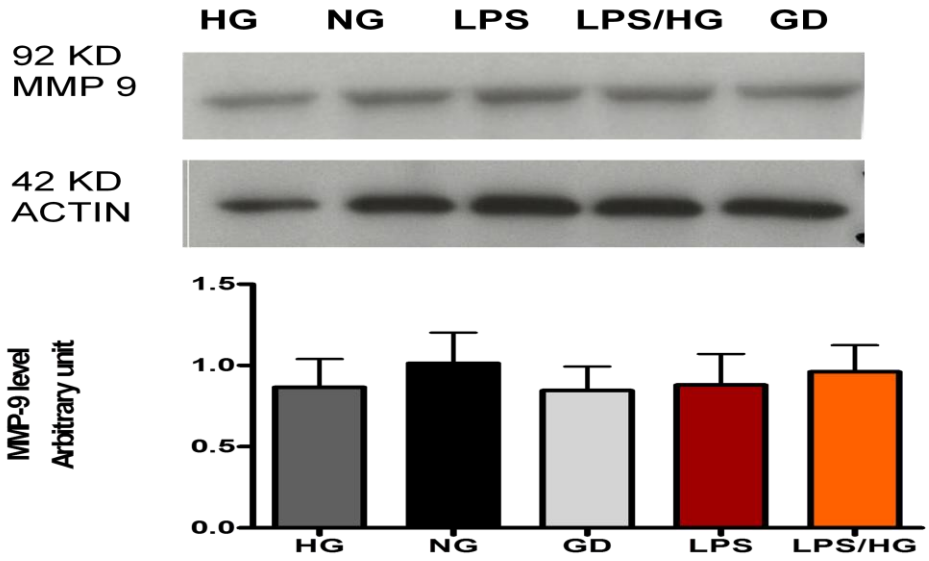


Figure 8: MMP-9 protein levels in microglia cell lysates exposed to different glucose levels. MMP-9 levels in microglia cell lysates were determined and normalized to actin. Loading volumes were adjusted according to their measured protein concentrations. Data are expressed as means ± SE. Number of repetitions for this experiment is 4 times; each experiment was pooled out of 8 wells. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.8 The effect of glucose levels on microglia survival using the MTT assay

Microglia survival was significantly increased in the GD group as compared to all other groups, while survival in the LPS-treated groups was significantly reduced as compared to normal control culture condition. Microglia survival in the glucose deprived and LPS-treated group showed the worst outcome. This may explain the low protein levels insufficient to detect MMP-2 and MMP-9 in the previous experiments. HG culture condition had no effect on microglia survival compared to normal controls.

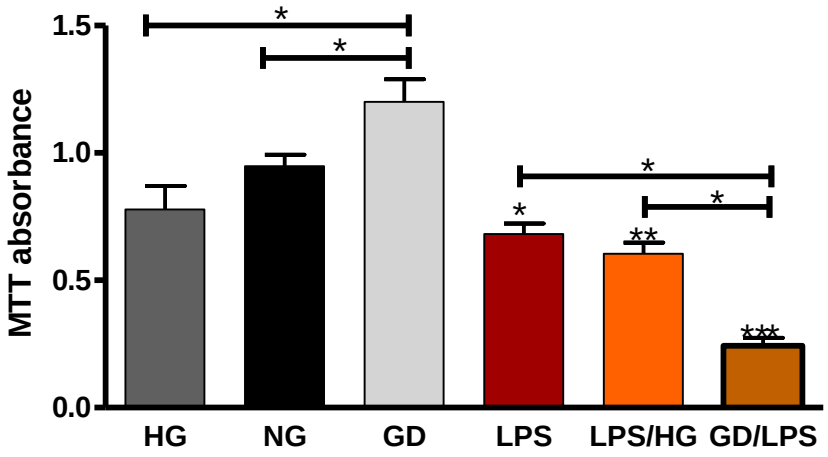


Figure 9: Microglia survival in response to exposure to different glucose levels. Primary microglia cultures were treated with different glucose concentrations for 24 hours. Microglia survival was measured by the MTT assay. Data are expressed as means ± SE. Number of repetitions for this experiment is 5 times; n=20. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.9 The effect of glucose levels on microglia survival by measuring caspase 3 protien levels in cell lysates using WB

In order to corroborate results obtained from the MTT viability assay we decided to measure microglia expression of the pro- and the cleaved form of caspase 3 as an indication of apoptotic cell death. Protein levels of caspase 3 were significantly reduced in the GD group in comparison to all other groups. No significant changes in the expression levels of the pro form of caspase 3 were observed between the remaining groups. Protein levels in the LPS/GD group were too low to detect caspase 3 levels in the cell lysates and thus this group was excluded in this experiment.

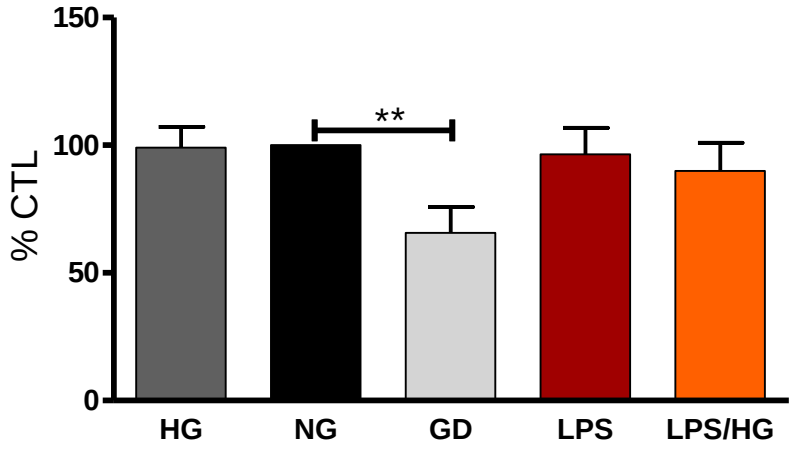


Figure 10: Microglia expression of caspase 3 in response to exposure to different glucose levels. Caspase 3 levels in microglia cell lysates were determined and normalized to actin. Data are expressed as % control, and histograms represent the means ± SE. Number of repetitions for this experiment is 4 times; each experiment was pooled out of 8 wells. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.10 The effect of glucose levels on microglia survival by measuring cleaved caspase 3 protein levels in cell lysates

In a similar experiment protein levels of cleaved caspase 3 were determined in cell lysates of primary microglia cultures exposed to varying glucose levels for 24 hrs. Microglia expression of cleaved caspase 3 was significantly lower in the GD group in comparison to all other groups, indicating better survival of microglia in this group. Also significant increases in the expression levels of the cleaved form of caspase 3 were observed in all LPS-treated groups indicating more death in these groups.

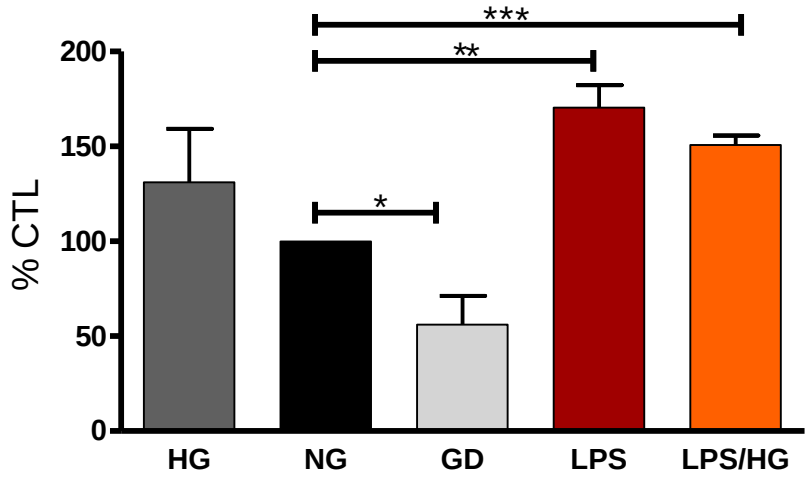
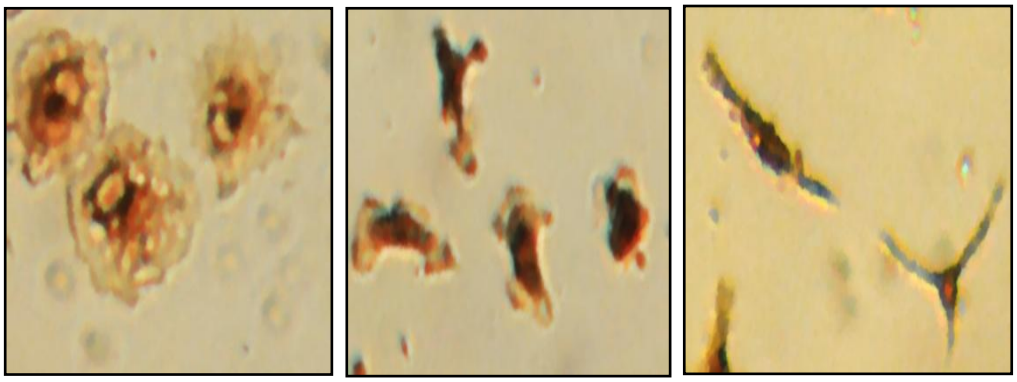


Figure 11: Microglia expression of cleaved caspase survival in response to exposure to different glucose levels. Microglia expression of cleaved caspase 3. Data are expressed as % control, and histograms represent the means ± SE. Number of repetitions for this experiment is 4 times; each experiment was pooled out of 8 wells. The denotations *, ** and *** represents a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.11The effect of glucose levels on microglial morphology

Microglia cultures exposed to different glucose levels levels (HG = 30mM, NG= 17.5mM, GD= 0mM) were fixed and stained against Iba-1 using DAB as a chromogen. The three morphological forms (ramified, amoeboid and spherical) were counted and expressed as a percentage of the total cell number. N=4 (number of wells/treatment condition); for each well 11 random images were taken under a 10x microscope and subjected to counting and analysis.

Figure 12: Different morphological forms of microglia



Spherical Amoeboid Ramified

3.11.1 Microglia treated with different glucose concentrations for 24 hrs

LPS treatment significantly increased microglia exhibiting a spherical form across groups Figue13, (panel B, D, F), indicating microglia activation as expected. LPS treatment in the GD group resulted in a fewer number of cells with a shrinking apoptotic morphology (panel F). GD (panel E) showed more cells with a ramified morphology.

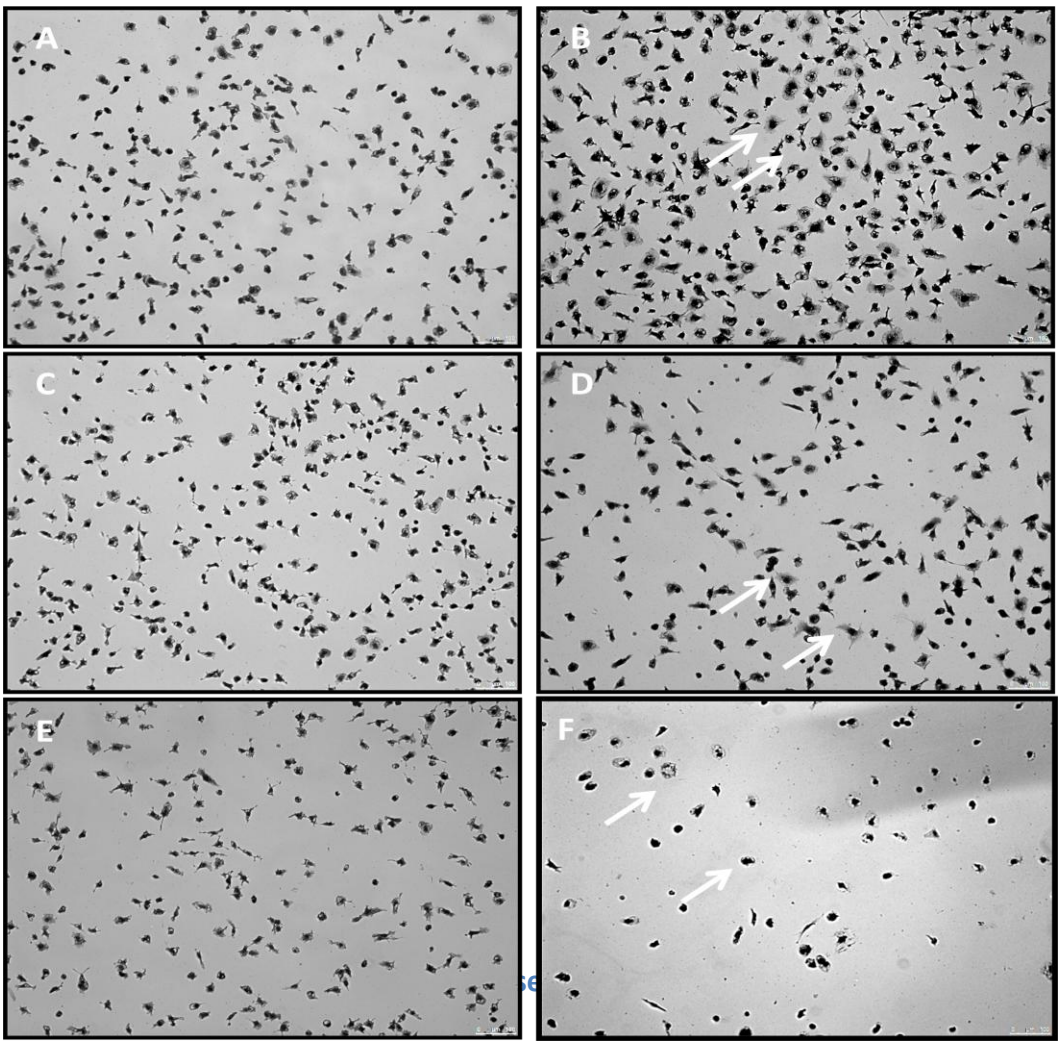


Figure 13: Microglia morphology in different glucose levels for 24 hrs. Microglia treated with different glucose levels for 24 hrs (0, 17.5, 30 mM) were immune-stained against Iba-1 and observed for their morphology. A=NG, B=NG/LPS, C=HG, D=HG/LPS, E=GD, F=GD/LPS. White arrows point out the spherical morphology of microglia upon activation with LPS.

3.11.2 Quantification of microglial morphological changes in response to different glucose concentrations for 24hours

Quantification of the results in Figure 13 revealed that the majority of microglia (> 50%) exhibit an amoeboid morphology in normal culture conditions and in normal glucose concentration, as shown in Figure 14 A. However, microglia deprived of glucose showed a more ramified morphology compared to all other groups, as shown in Figure 14c. All LPS-treated microglia exhibited a more spherical morphology as a sign of activation, as shown in Figure 14D. LPS treatment induced cell death and reduced the total number of cells in the glucose deprived microglia significantly as compared to just LPS treatment, as shown in Figure 14D. LPS-treated microglia showed less amoeboid morphology than normal controls relative to their total number of cells due to high percentage of microglia with the spherical morphology. No significant differences were observed in the morphology of microglia between HG and NG. The results obtained from this experiment support our previous findings about the activity state of microglia in terms of cytokine and growth factor release.

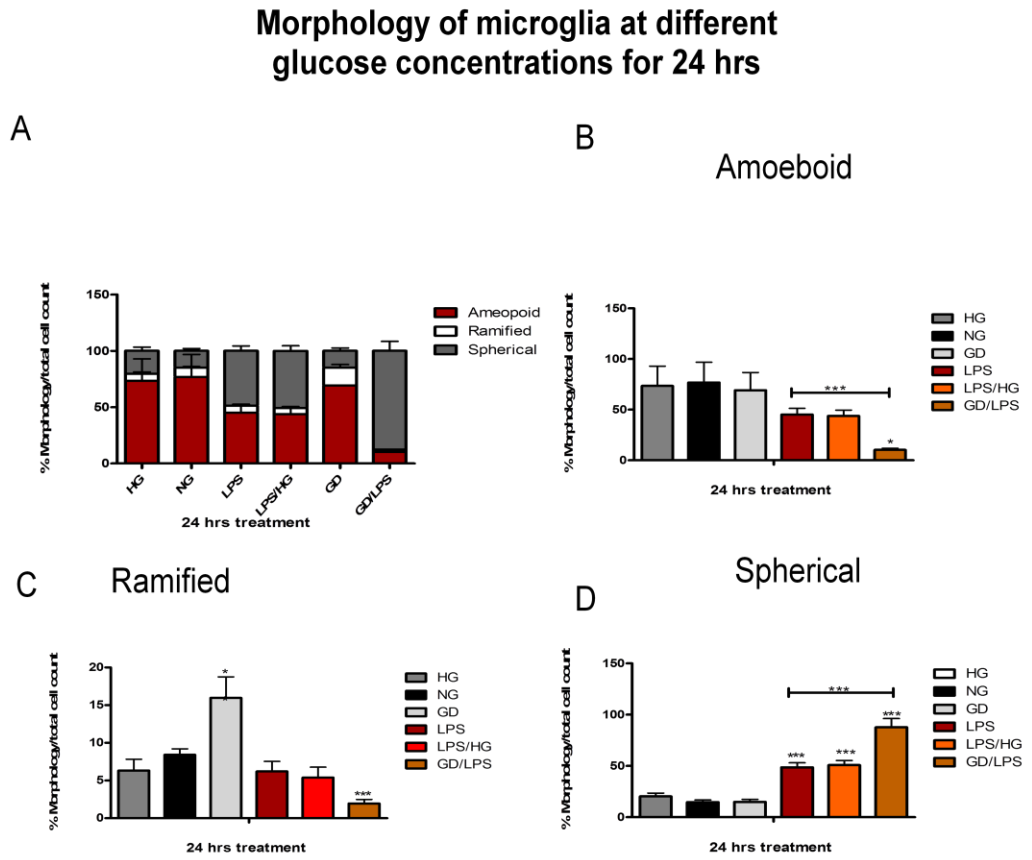


Figure 14: Quantification of microglia morphological changes in response to different levels of glucose for 24 hrs. Microglia treated with different glucose levels (0, 17.5, 30 mM) for 24 hrs were immune-stained against Iba-1 and observed for their morphology. In each treatment group the representative percentage of each morphological form within the group is shown in figure 14A. Changes in the number of microglia cells exhibiting an amoeboid, ramified, spherical morphology in each treatment group expressed as % of the total number of cells are shown in figure 14B, 14C, 14D respectively. The (*) denotes a significant difference as compared to NG. The denotations *, ** and *** represents a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.11.3 Microglia treated with different glucose concentrations for 72 hrs

In order to confirm results from the previous morphology experiment, where microglia were treated with different glucose levels for 24 hrs, we decided to repeat the experiment and extend the exposure time of microglia to treatments for 72 hrs instead.

Results obtained from this experiment replicated the previous experiment some of the results were more prominant. For example more cell death can be observed in all LPS-treated groups. This is shown in Figure 15B, C, and D. The ramified morphology of microglia in the GD group is more pronounced in this experiment as shown in Figure 15 E, suggesting that the longer microglia are grown in a media deprived of glucose the more ramified they become. The cell death in the GD/LPS condition was more pronounced as well in this experiment.

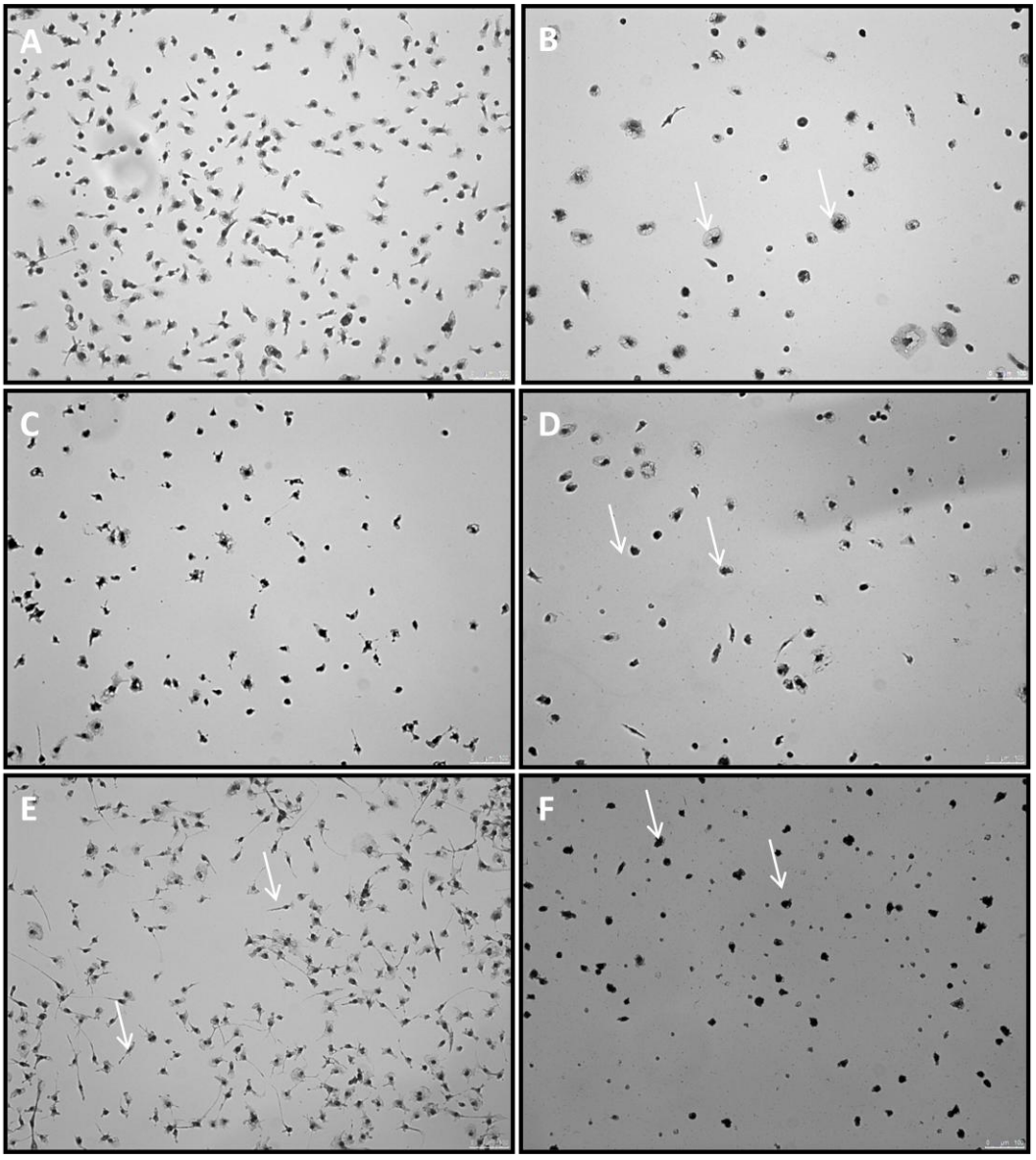


Figure 15: Microglia morphology at different glucose levels for 72 hrs.

Microglia treated with different glucose concentrations (0, 17.5, 30 mM) for 72 hrs were immune-stained against Iba-1 and observed for their morphology. A=NG, B=NG/LPS, C=HG, D=HG/LPS, E=GD, F=GD/LPS

3.11.4 Quantification of microglia morphological changes in response to different glucose concentrations for 72hours

In this experiment, changes in the morphology of microglia treated with different glucose concentrations for 72 hrs were determined.Quantification of the number of the three morphological forms of microglia and calculation of the percentage of each morphological form of microglia in each treatment group was performed. These percentages are shown in Figure 16A. In accordance with the previous experiment the ramification of microglia was increased significantly in the glucose-deprived as compared to NG cells. Depriving microglia of glucose for 72 hrs resulted in a highly ramified morphology with extended processes, as shown in Figure15 E. All LPS-treated groups of microglia became shrunken and apoptotic as expected. Microglia deprived of glucose and exposed to LPS had the least total number of cells and exhibited more cell death, as shown in Figure 16D. Unlike the previous experiment the majority of microglia in the NG control group started showing signs of activity. Microglia in the NG had about the same percentage of amoeboid and spherical forms (~50%).

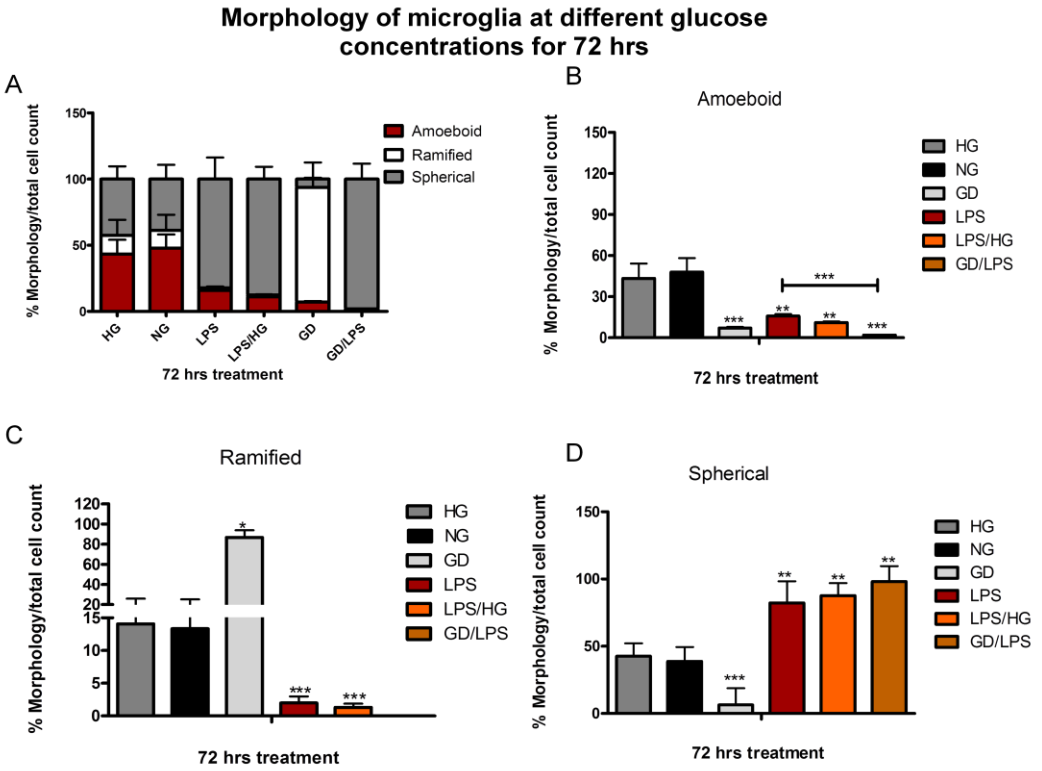


Figure 16: Quantification of microglia morphological changes in response to different levels of glucose for 72hrs. Microglia treated with different glucose concentrations (0, 17.5, 30 mM) for 24 hrs were immune-stained against Iba-1 and observed for their morphology. In each treatment group, the representative percentage of each morphological form is shown in Figure 15A. Changes in the number of microglia cells exhibiting an amoeboid, ramified, spherical morphology in each treatment group expressed as % of control are shown in Figure 15B, 15C, 15D respectively. The denotations *, ** and *** represent a P< 0.05, <0.001 and < 0.0001 respectively compared to NG unless indicated.

3.12The effect of glucose levels on microglia phagocytic activity

Phagocytic activity of microglia is one of the primary and crucial functions of these cells. The effects of glucose levels on microglial phagocytic activity have not yet been clearly shown. In this experiment the effects of exposing microglia to different glucose concentrations for 24 hrs were investigated. Microglia cultures were treated with different glucose concentrations for 24 hrs and incubated with fluorescent beads for 2 hrs before the analysis. Fixed cells were washed 3 times with PBS to remove all non-phagocytosed cells. Cells were stained with the nuclear stain DAPI and examined under a fluorescent microscope. Microglia grown in normal glucose for 24 hrs showed a high percentage of cells with beads (>60%), indicating a normal phagocytic activity, as shown in Figures 17 and 21. Microglia grown in normal glucose and LPS for 24 hrs showed spherical morphology as expected and moderate phagocytic activity (about 50%), as shown in Figures 18 and 21. However, depriving microglia of glucose for 24 hrs resulted in a very low percentage of cells with beads, indicating abnormal and disrupted phagocytic activity as compared to normal (<20%), as shown in Figures 19 and 21. In a similar manner, microglia grown in HG for 24 hrs showed less percentage of cells with beads indicating a reduced phagocytic activity as well (<40%); data are shown in Figures 20 and 21.

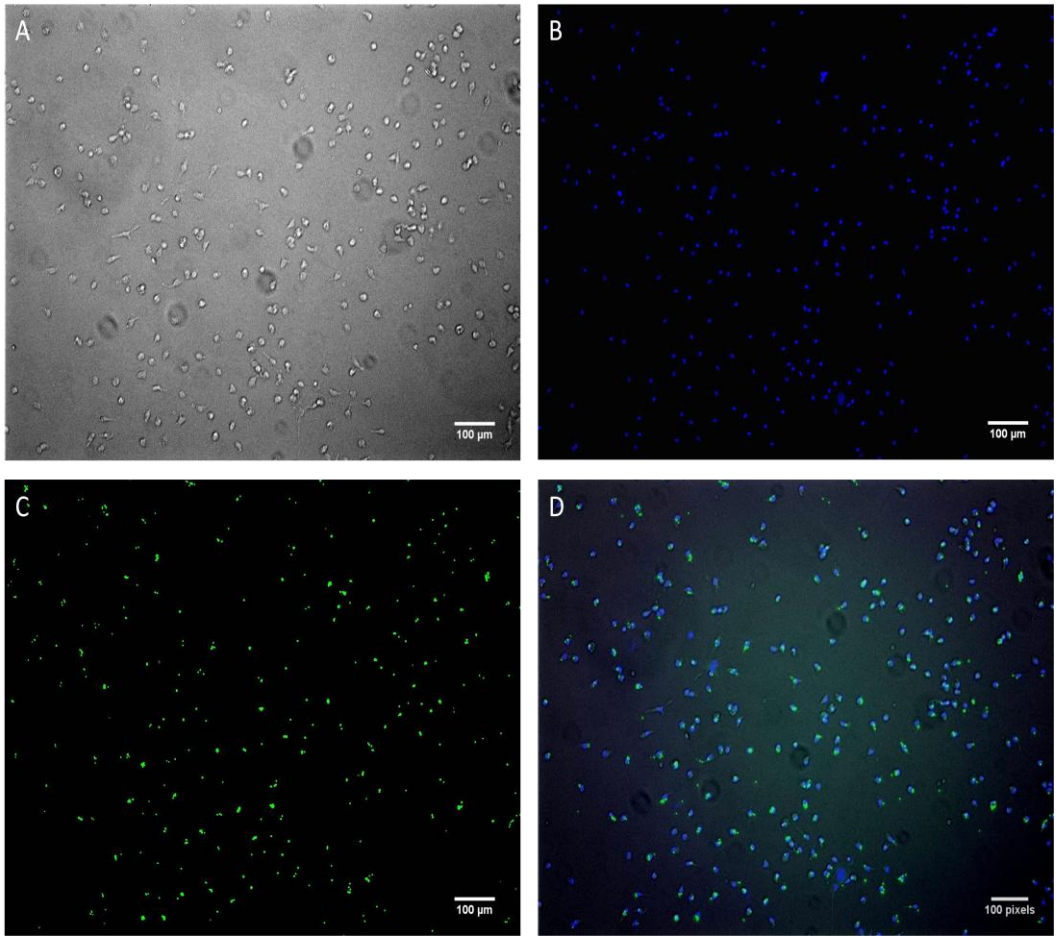


Figure 17: Phagocytic activity of microglia grown in normal glucose for 24 hrs. Microglia treated with NG (17.5 mM) for 24 and incubated with 1.0 µm fluorescent latex beads for 2 hrs were fixed and stained with DAPI nuclear stain. Cells were examined under a fluorescent microscope and with DIC microscopy. N= 4; 8 wells in total were subjected to analysis, random 21 pictures per well were taken automatically and subjected to analysis. The number of cells with beads inside was counted and results were expressed as the % of cells with beads to the total number of cells. A= microglia in DIC, B=DAPI nuclear stain of microglia, C=fluorescent beads under fluorescent microscope, D= merged image of A, B, C.

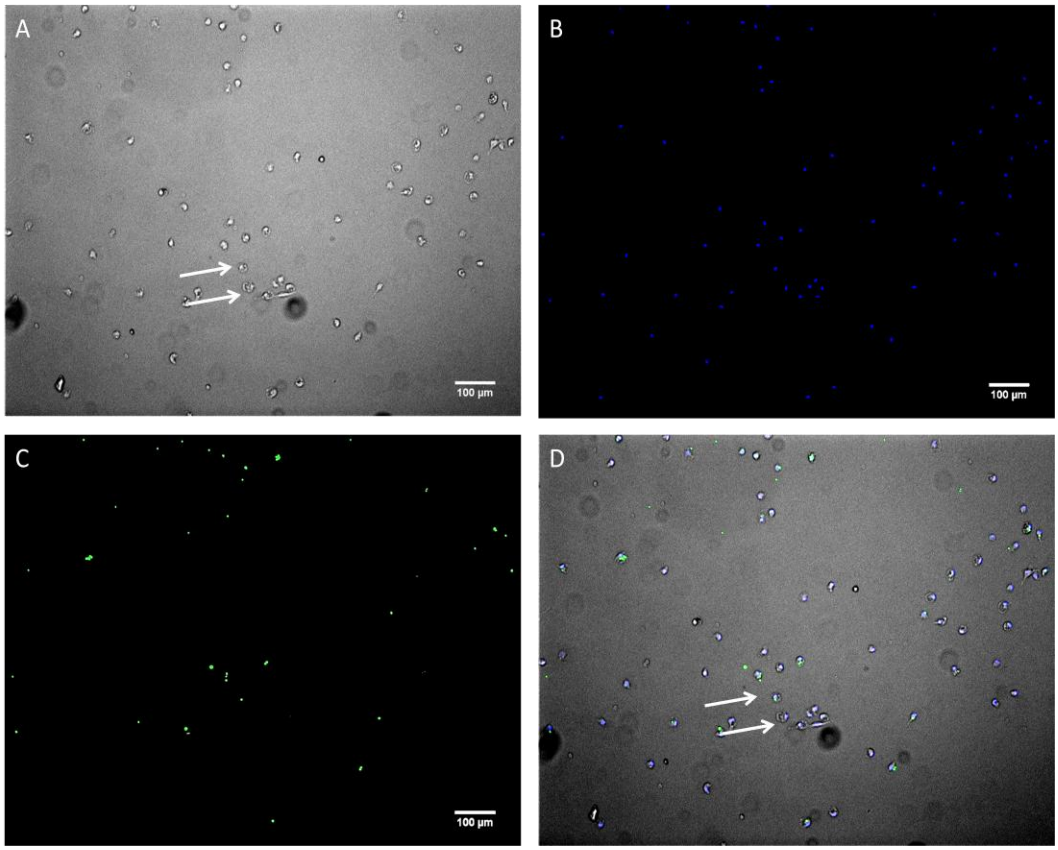


Figure 18: Phagocytic activity of microglia grown in normal glucose and LPS for 24 hrs. Microglia treated with NG and LPS for 24 were incubated with fluorescent latex beads for 2 hrs. Fixed cells were stained with DAPI nuclear stain and examined under a fluorescent microscope and DIC microscopy. N= 4; 8 wells per treatment group in total were subjected to analysis, random 21 pictures per well were taken automatically and subjected to analysis. The number of cells with beads inside was counted and results were expressed as the % of cells with beads to the total number of cells. A= microglia in DIC, B=DAPI nuclear stain of microglia, C=fluorescent beads under fluorescent microscope, D= merged image of A, B, C. White arrows point out the spherical morphology as an indication of activated microglia.

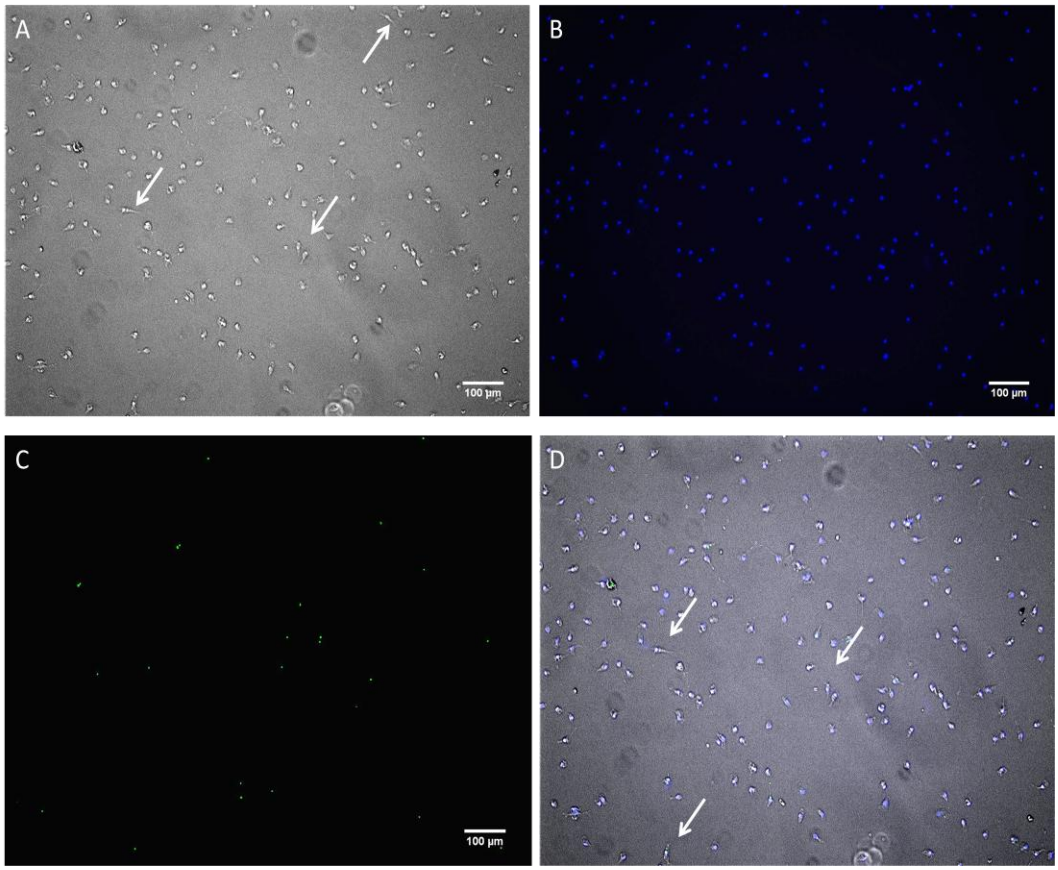


Figure 19: Phagocytic activity of microglia in glucose deprived media. Microglia treated with GD media for 24 were incubated with fluorescent latex beads for 2 hrs. Fixed cells were stained with DAPI nuclear stain and examined under a fluorescent microscope and DIC microscopy. N= 4; 8 wells in total were subjected to analysis, and random 21 pictures per well were taken automatically and subjected to analysis. The number of cells with beads inside was counted and results were expressed as the % of cells with beads to the total number of cells. A= microglia in DIC, B=DAPI nuclear stain of microglia, C=fluorescent beads under fluorescent microscope, D= merged image of A, B, C. White arrows point out the ramified morphology of microglia.

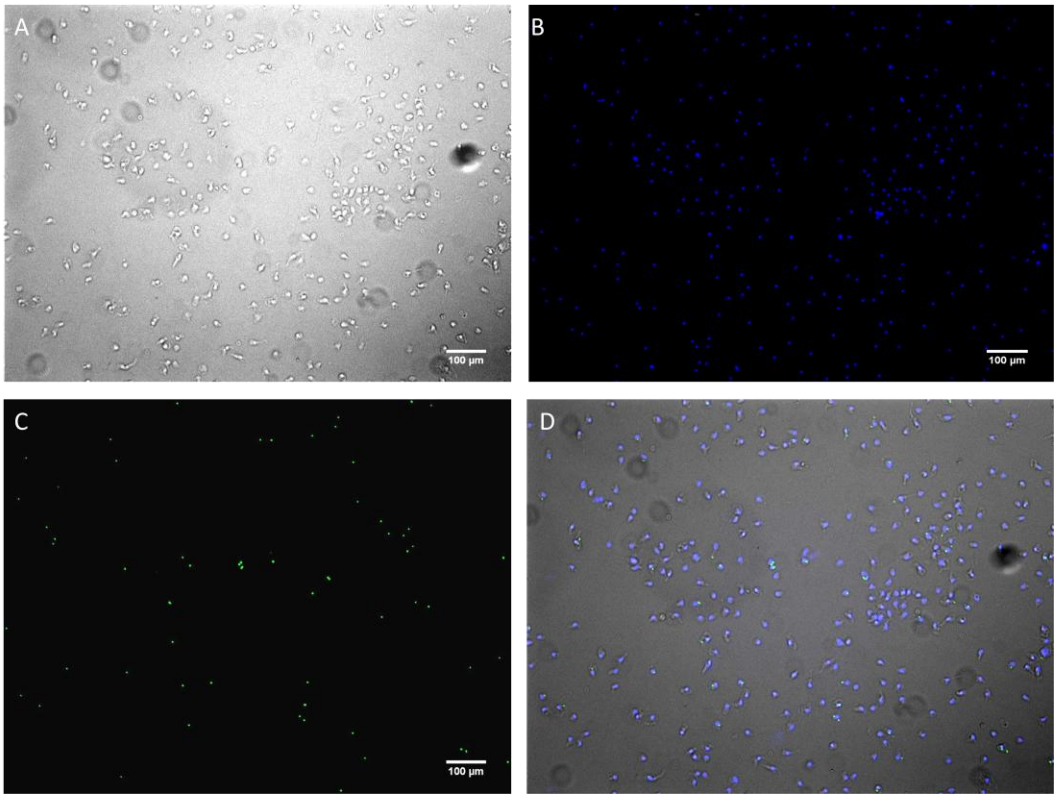


Figure 20: Phagocytic activity of microglia in high glucose. Microglia treated with HG media for 24 hrs, were incubated with fluorescent latex beads for 2 hrs. Fixed cells were stained with DAPI nuclear stain and examined under a fluorescent microscope and DIC microscopy. N= 4; 8 wells in total were subjected to analysis, and random 21 pictures per well were taken automatically and subjected to analysis. The number of cells with beads inside was counted and results were expressed as the % of cells with beads to the total number of cells. A= microglia in DIC, B=DAPI nuclear stain of microglia, C=fluorescent beads under fluorescent microscope, D= merged image of A, B, C.

3.12.1 Quantification of microglia phagocytic activity in response to different glucose levels

The number of cells with beads to the total number of cells in each treatment group was quantified. Due to extensive cell death in the GD/LPS and LPS/HG groups in the presence of the beads, those two groups were excluded from the experiment. Cell death following phagocytosis is a common observation in microglia [97].

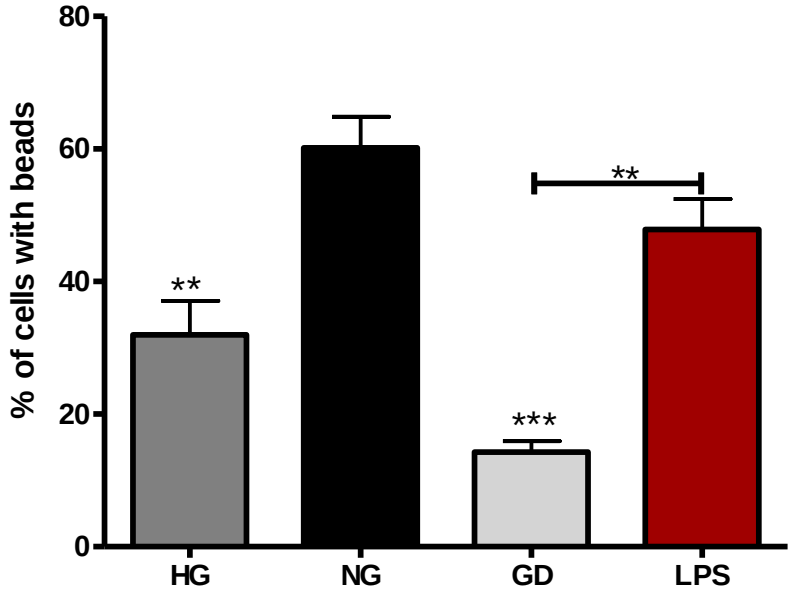


Figure 21: Effect of glucose levels on microglia phagocytic activity. The number of cells with beads was counted in Figures 17-20 and the data are expressed as the % of cells with beads to the total number of cells. The denotations ** and *** represent a P<0.001 and < 0.0001 respectively compared to NG unless indicated.

3.13 Summary of results

Results obtained from this project showed that exposing microglia to different glucose concentrations even for an acute period of time (24 hrs) affected microglia and changed their activity.

High glucose concentrations (30mM) activated microglia. Specifically, microglia in the HG condition released significantly more NO, TNFα and BDNF compared to microglia in the GD condition. Additionally, microglia in the HG condition released more BDNF than microglia in the control NG group. High glucose also resulted in increased protein levels of MMP-2 but had no effect on MMP-9 protien levels in cell lysates as measured by western blots. Subjecting primary microglia cultures to HG concentrations for 24 hrs had no effect on microglia release of glutamate and also had no effect on the morphology of microglia as compared to the control group.

Interestingly, microglia deprived of glucose (0 mM) survived better compared to the control and HG culture conditions which is shown by the MTT experiment and the caspase3 and cleaved caspase 3 western blots. Microglia deprived of glucose also released significantly reduced levels of pro-inflammatory cytokines like NO, TNF-α, IL 1-β and also the growth factor BDNF as compared to the NG group. Depriving microglia of glucose for 24 hrs also affected the morphology of microglia, resulting in an increased number of microglia exhibiting a ramified morphology.

As expected, microglia stimulated by the positive control LPS released more NO, TNF-α, IL 1-β and BDNF. LPS treatment also changed the morphology of microglia, yielding higher numbers of microglia in the

spherical form. LPS treatment also increased microglia release of glutamate. Notably, some of the LPS pro-inflammatory induced responses in the microglia cultures like increased release of TNF-α, IL 1-β and glutamate were inhibited when LPS treatment was associated with glucose deprivation. Phagocytic activity of microglia was affected by different glucose levels. High glucose and glucose deprivation decreased phagocytic activity of microglia as compared to NG. LPS treatment of microglia did not have a significant effect on microglia phagocytic activity. Data for the LPS/HG and LPS/GD groups were excluded from some experiments due to the remarkable cell death observed and the absence of any significant number of cells to be considered in the quantification process, as evident from the morphology study and the MTT study.

Data obtained from this project suggest that different glucose concentrations, whether the high glucose concentration or the absence of glucose as a source of energy, change the activity of microglia. These changes of microglia activity could influence the fate of neurons in conditions such as DM or post ischemic glucose intolerance. The data collected from this study will significantly increase our understanding of how glucose levels affect brain functioning and, especially how this will affect the behavior of microglia as important immune cells and potential therapeutic targets in pathological conditions in which the fluctuation of glucose levels is an outcome.

Chapter 4

Discussion and future directions

Discussion:

From the introduction to this thesis, it is clear that glucose is the predominant fuel for energy in the human brain. Glucose levels are maintained under tight control by a highly complex and integrated system that involves cross talk between the periphery and the CNS. This complex system includes numerous sensors of glucose levels that are distributed throughout the periphery and also in the brain and whose main function is to convey information about the energy and nutrient status of the whole body to the brain [18].

The brain is the leader that orchestrates a harmonic communication between all vital organs involved in energy metabolism like the liver, pancreas and adipose tissue. The brain controls energy homeostasis, energy consumption and energy expenditure; it is also endowed with a unique and exclusive property that enables it to remain isolated by the BBB from the rest of the circulation. Thus glucose levels in the brain are kept under a much tighter control than in the periphery [98].

Numerous in vivo and in vitro studies have demonstrated that neurons are vulnerable to changes in glucose levels. Neuronal injury or even death can be a consequence of either hyper- or hypoglycemia or even fluctuations of glucose levels. Extensive research has shown that through numerous mechanisms hyperglycemia can introduce damage to neurons, and in a

similar manner but through different mechanisms glucose deficiency or absence can also harm neurons [47, 70].

However the brain comprises other cell types in addition to neurons [99]. Glial cells are present in higher numbers than neurons and their functions are not restricted to providing support and protection of the CNS as was previously thought. These glial cells are currently recognized as one of the major contributors in the initiation and the progression of many CNS disorders like neurodegenerative disorders [72, 77].

One type of glial cell, the microglia, are the brain`s resident effector immune cells. They are capable of constantly surveying the CNS environment in a search for threats or pathogens. Once microglia encounter any type of threat to the CNS they attain a different morphology and perform either beneficial protective functions such as the release of more growth factors or deleterious functions such as the release of inflammatory cytokines and ROS. Depending on the severity or type of injury, microglia attain a deleterious or a beneficial phenotype, and depending on the extent and period of microglia activation, neuronal survival will be affected [95]. Currently it is widely accepted that microglia play fundamental roles in the development or the progression of many CNS disorders such as Parkinson`s disease, Alzheimer`s disease, and stroke and also diabetic-induced retinopathy and neuropathy [34, 78, 88, 100].

Unlike the case with neurons, the effects of different glucose levels on microglia activity have not been very well studied. Thus the goal of the study reported in this thesis was to investigate the effects of hyperglycemia and hypoglycemia on primary cultures of microglia and to determine changes in their morphology, survival and phagocytic and secretory activity. For secretory activity the effects of varying glucose concentrations on the release of pro-inflammatory cytokines like IL-1β and TNFα and the release of the growth factor BDNF was investigated. In addition, changes in microglia release of ROS like NO and the release of the excitatory amino acid glutamate were measured. Changes in protein levels of MMP 2 and 9 were also determined.

Our results show that the absence of glucose or the presence of glucose in high concentration greatly affects microglia phenotype in terms of morphology, survival and secretary profile. Although the exposure of primary microglia cultures to high levels of glucose did not significantly increase microglia activity as compared to NG, it did increase microglia activity significantly when compared to the glucose-deprived group. Microglia exposed to high glucose levels specifically increased the release of TNF-α, NO and BDNF as compared to microglia deprived of glucose; HG levels did not affect the release of IL-1β. The increase in microglia release of TNFα had been shown before by another research group who reported that HG (35 mM) cultures of microglia for 24 hrs increased their release of TNFα and GRO (the rat ortholog of human IL-8) as compared to

low glucose cultures (10mM); this research group also had similar findings with regard to the release of IL-1β. In that experiment Quan Yi and colleagues showed that high glucose increases the release of ROS and TNF-α by a mechanism that is dependent on the expression of NF-Kβ and PKC [101, 102].

It is possible that this acute exposure of microglia to high glucose was not sufficient to significantly increase their activity as compared to normal glucose culture. However extensive research revealed that AGEs are prominent inflammatory stimuli that accumulate normally in the ageing brain and abnormally in some neurodegenerative and metabolic disorders including Alzheimer's disease (AD) and DM. AGEs can activate microglia as well as neurons by acting on specific receptors for AGEs termed RAGE, leading to the production of excessive amounts of inflammatory cytokines and ROS [103]. AGE-RAGE interaction is one of the prominent mechanisms by which high glucose levels produce damage to neurons by initiating numerous pathological changes, as was previously discussed in the introduction to this thesis. However, most of the studies that addressed the AGEs–RAGE interaction in microglia have been implicated in diseases like AD and most of them focused on the effects of β-amyloidal accumulation on RAGE receptor activation [104]. In addition the majority of these studies revealed that the process of manufacturing AGEs in a laboratory setting was time-consuming, taking from 4 to 6 weeks of incubating BSA with glucose at high temperatures [105]. That suggests

that maybe if these microglia cultures were exposed to high glucose levels for longer periods of time the formation and the accumulation of AGEs would start to have an effect on RAGE receptors expressed by microglia as observed in actual clinical situations where hyperglycemia is a chronic state. Numerous clinical and animal studies of DM have incriminated AGEs and their receptors that are expressed by neurons, endothelial cells and microglia in the pathogenesis of diabetic nervous system complications including diabetic neuropathy and encephalopathy. Also, microglia have been shown to be activated in the diabetic brain, maybe through the action of AGEs on their receptors [103, 106].

Treating microglia with high levels of glucose also had some effect on the protein levels of MMPs. High glucose increased protein levels of MMP-2 but had no effect on MMP-9 protein levels in the cell lysates of microglia; normally MMP-2 and MMP-9 are secreted as inactive pro-proteins which are activated when cleaved by extracellular proteinases. MMP-2 is responsible for activating and cleaving MMP-9; however in this experiment only protein levels in the cell lysates were measured and there was no measurement of enzyme activities. The higher protein levels of MMP-2 in cell lysates suggests that more MMP-2 will be released in the media and implies a possible activation of MMP-2 and MMP-9 later on [107]. A compromised BBB due to excessive activity of MMPs, especially MMP-2 and MMP-9, is a common pathological hallmark in many neurodegenerative and brain disorders like AD. Also DM has been

associated with increased BBB permeability in some studies. Our data suggest that microglia might play a role in this through increased production of MMP-2 [108].

The increased release of BDNF in microglia cultures treated with high levels of glucose might be explained by the fact that BDNF is one of the endogenous growth factors that play major roles in maintaining metabolic homeostasis. BDNF has been shown to improve hyperglycemia and insulin resistance in diabetic mice; thus in an attempt to maintain homeostasis microglia might be producing more BDNF [43, 109].

On the other hand the increased release of BDNF in response to high glucose can also be a harmful consequence; recently it has been shown that BDNF can exacerbate NP and produce hyper-excitability of spinal cord neurons, suggesting that BDNF is one of the major contributors to NP pathology [83, 110]. This finding suggests an active part played by microglia in counteracting hyperglycemia or in exaggerating the harmful effects of high glucose on the peripheral nervous system.

An interesting finding in this study is the effect of glucose deprivation on microglia. Our results show that subjecting microglia to a culture media deprived of glucose increased microglia mitochondrial activity and reduced cell death by decreasing the expression of cleaved caspase3. That suggests that microglia did use alternative sources of energy other than glucose. Although the information about alternative sources of energy for

microglia in the literature is scant, some possible candidates include lactate, ketone bodies and free fatty acids [111]. The surprising improved survival of microglia in glucose-free media can be explained by the activation of AMP kinase activation protein (AMPKA). When microglia cultures are deprived of glucose, AMP levels are increased, which can activate AMPKA, an enzyme that plays a role in cellular energy homeostasis and is found in a number of tissues, including liver, brain, and skeletal muscles [112]. AMPKA acts as an energy sensor through detecting the ratio of ATP/AMP and acting accordingly by regulating several intracellular systems aimed at increasing the survival of the cell, including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of GLUT4 and mitochondria. Upon activation, this enzyme is capable of sending signals that regulate the energy expenditure in the cell either by stimulating energy production processes such as lipid catabolism or by inhibiting energy consuming ones such as protein, cholesterol and fatty acid synthesis [113]. AMPKA is activated in stress conditions such as when the cells are in energy crises; for instance hypoglycemia and glucose deprivation are factors that can trigger AMPKA activation [114].

In addition, AMPKA activation has been shown to affect pro-inflammatory responses of microglia. The downstream signaling pathways of this enzyme include the inhibition of NF-Kβ, the activation of which is critical for the expression of ROS and pro-inflammatory cytokines [115]. That

explains the anti-inflammatory effects of this enzyme; in numerous pathological conditions in the CNS where inflammation is a key factor, AMPKA activation produces protective anti-inflammatory effects [116]. These findings in the literature support our findings that depriving microglia of glucose results in a significant decrease in the release of inflammatory cytokines (TNF-α, IL1-β), NO and suppresses the synthesis and release of the growth factor BDNF as compared to the NG group. These findings suggest that microglia have a better survival but at the same time they are acquiring a more quiescent phenotype when deprived of glucose. That finding also explains the reason for the suppressed LPS inflammatory effects in the GD microglia group that was simultaneously treated with LPS [117]. Studies of 5-aminoimidazole-4-carboxamide riboside (AICAR), a pharmacological activator of AMPKA , revealed that AICAR also inhibits LPS-induced protein expression of TNF-α, IL1-β and also inducible nitric oxide synthase (iNOS); however the action of AICAR on iNOS was independent of AMPKA activation, which might explain the unchanged release of NO between the LPS and LPS/GD groups in our experiments [118]. These data suggest a novel method of manipulating the activity of microglia by pharmacological agents or metabolic strategies that aims at affecting AMPKA. This enzyme is currently subjected to persistent research due its potential application for treating metabolic disorders as well as its therapeutic potential for treating neuro-

inflammatory diseases where reactive microglia play an etiological role like stroke, AD, Parkinson's Disease and DM.

In conditions like hypoglycemia, excitotoxicity due to the leakage of excitatory amino acids as a result of energy failure and defective membrane transport mechanisms and the overall inability of the cells to maintain constituents is a common observation [70]. Also, in diabetic neuropathy where hyperglycemia and microglia are important players, an imbalance between excitatory and inhibitory neurotransmitters has been reported [84]. Thus it was hypothesized that different glucose levels would have an effect on the release of the excitatory amino acid glutamate. However, results obtained from this project showed no significant changes in the release of glutamate in the media of microglia upon exposure to different glucose concentrations except for the positive control (LPS)-treated groups. The inhibition of the LPS-induced release of glutamate in GD microglia can be also attributed to the activation of AMPKA. It is well known that LPS stimulates microglia to release various kinds of cytokines. Recently it has been shown that all these compounds are released sequentially and in a certain order at first TNF-α, IL-1β, then IL-6 and finally glutamate. Numerous studies have also demonstrated that glutamate release is a phenomenon that can be dependent on release of cytokines, especially TNF, IL-1b and NO [119]. Since the activation of AMPKA that is derived by glucose deprivation has been shown to reduce inflammation by reducing the expression of these cytokines, it may affect

microglial glutamate release by reducing some of the key cytokines involved in regulating microglial release of glutamate [120, 121]. Furthermore numerous studies demonstrate that the observed imbalance between excitatory and inhibitory neurotransmitters in neuropathy can be attributed to defective microglial glutamate reuptake rather than release [122].

Microglia survival experiments revealed significant survival of microglia in the glucose-deprived condition; however they also revealed significant cell death in cases where microglia were treated with LPS in glucose-deprived media. Other research groups also reported similar results when studying the effects of AMPKA activation on LPS-stimulated microglia [117]. Downstream signaling of AMPKA includes the activation of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α), a molecule that is involved in mitochondrial synthesis. Increased mitochondrial biogenesis leads to increased mitochondrial mass and values from the MTT assay may be regarded as indicators of mitochondrial mass, and the GD microglia showed significantly higher values in the MTT assay [123]. However Sag et al. have demonstrated that AMPK activity is down-regulated upon pro-inflammatory stimuli like LPS and up-regulated by anti-inflammatory cytokine stimulation like IL-10 and TGF-β. Also, over-expression or activation of AMPK results in the dampening of inflammatory responses, while inhibition of AMPK activity or expression increases the production of pro-inflammatory stimuli [124]. In

other words, glucose deprivation activated AMPKA in microglia cultures and that resulted in a significant reduction in cytokine release as compared to microglia grown in the NG condition and also produced a dampening in LPS-induced pro-inflammatory effects as compared to microglia cultures treated with LPS in the NG condition (AMPKA non-active). However treating microglia with LPS during glucose deprivation also inhibited AMPKA activity, resulting in compromised downstream signaling and reduced survival due to counteracting the normal salvage mechanism by which this enzyme acts to rescue cells in stress.

Morphology studies in this project corroborate the previously discussed findings. Explicitly, when microglia were deprived of glucose for 24 hrs they exhibited a significantly ramified morphology where they have elongated extended processes and small cell bodies as compared to the control group. No significant differences were observed between the HG group and the control group, and, as expected, the LPS-treated groups showed increased numbers of microglia in the spherical activated form. However microglia deprived of glucose and treated with LPS appeared unhealthy, shrinking and apoptotic. In order to confirm these findings the same experiment was repeated again but this time microglia cultures were exposed to different glucose concentrations for 3 days. Indeed depriving microglia of glucose increased microglia ramification significantly, and the damage induced by LPS treatment was exacerbated due to the extended exposure time in all LPS-treated groups. That was manifested by more

microglia with spherical and shrinking apoptotic morphology. Also microglia exposed to high glucose started to show signs of activation and more cells showing a spherical morphology.

As mentioned in the introduction to this thesis, phagocytosis is one of the fundamental and primary functions of microglia. Enhanced or defective microglial phagocytosis has been shown to be a major contributor in the progression of many neurodegenerative and brain disorders [78]. Our results reveal that excess glucose levels reduced the phagocytic activity of microglia; that finding is in concurrence with numerous in vivo and in vitro studies where diabetes has been shown to decrease the phagocytic activity of phagocytosing cells such as microglia [125, 126]. Also our results showed a significant decrease in the phagocytic activity of microglia when deprived of glucose. This finding seems logical due to the fact that cells deprived of glucose can be energy deficient and it is well known that the process of phagocytosis is an energy consuming process [97, 127]. However recent studies focusing on the effect of AMPKA activation on phagocytic activity of phagocytosing cells including microglia reveal that AMPKA activation increases phagocytic activity of microglia [128]. Recently, multiple pathways for activation or inhibition of AMPK have been discovered. Various stimuli including nutrients, hormones, cytokines, physiological state as well as the pathological state of the cell, have been shown to affect the activity of AMPKA [114]. In the majority of the recent studies, the activation of AMPKA was performed using

pharmacological agents such as metformine, AICAR and statins. Pharmacological inhibitors of AMPKA like compound C were also utilized to counteract the effects produced by AMPKA activators. Labuzek and his team in their experiment on the effects of metformine on AMPKA activation and phagocytic activity of microglia also relied on compound C to demonstrate that metformine-induced increase of microglial phagocytic activity is mediated through the activation of AMPKA. However there are some concerns that surround compound C as a pharmacological inhibitor of AMPKA. For example, Compound C does not inhibit AMPK activation in response to all stimuli [129, 130]. Additional investigations showed that compound C inhibits the primary transporter for the uptake of AICAR into cells, suggesting that this pharmacological inhibitor should not be used to demonstrate AMPK-dependent effects of AICAR [129]. Furthermore, in our experiments absence of glucose is thought to be the reason for the hypothetical activation of AMPKA that might explain the contradictory findings regarding phagocytic activity of microglia.

Future directions

One of the future goals of the project described in this thesis is to study the effects of conditioned media from microglia cultures subjected to varying glucose concentrations on neuronal survival. However to achieve that an in vitro models of hyperglycemia and hypoglycemia have to be established, studied and clearly understood, which is what we tried to achieve by the work presented in this thesis. Although in this project the

acute exposure of microglia to varying glucose concentrations produced significant changes in microglia activity, chronic exposure of microglia cultures to different glucose concentrations better resembles chronic conditions such as DM, and thus determining the chronic effect of hyperglycemia and hypoglycemia on microglia survival and secretory profiles is an important target. Also measuring the effects of varying glucose levels on the expression of glucose transporters, especially GLUT-5, which is an exclusive type of glucose transporter that was detected only in microglia, will be of great importance [131]. Although measuring glutamate release from microglia cultures exposed to different glucose concentrations seemed a perfectly valid area of research due to the previously mentioned reasons, measuring glutamate uptake by microglia and also the expression of microglial excitatory amino acid transporters (EAATs) in response to different glucose levels is also another compelling and important area of research. Our studies suggest a possible role of microglia in compromising the BBB through increased expression of MMP-2; however, measuring the activities of MMP-2 and MMP-9 would give a better idea about the role played by microglia in compromising the BBB in pathological conditions that involve glucose metabolic abnormalities. Furthermore, all the interesting findings about the effects of glucose absence on microglia and the possible involvement of AMPKA call for further investigations to find out about the actual protein levels of this enzyme and its activity in the presence of different glucose

concentrations. That opens the door to new pharmacological tools to manipulate microglia activity through using the available pharmacological agents to inhibit or activate this enzyme. Determining the alternative sources of energy that microglia use and ATP levels during glucose deprivation is another interesting area of research since data in the literature are somewhat inadequate and inconclusive regarding that matter. Further investigations are also required to determine whether the source of energy in microglia cultures affects their secretory profile in terms of release of cytokines. Our knowledge on the role of insulin in cognition and memory is rapidly expanding; insulin resistance and hyper-insulinemia have deleterious effects on the CNS and these effects are particularly relevant to dementia [132]. Impaired glucose utilization, mitochondrial dysfunction, reduced ATP production, and energy failure led to the hypothesis of type 3 DM, a novel theory that describes a brain form of Type 2 DM in which impaired insulin signaling is the major contributor to the development of neurodegeneration and dementia. Impaired insulin signaling produces many pathological changes in the CNS such as tauopathy, β-amyloid accumulations, inflammation, oxidative stress, impaired calcium homeostasis and changes in neurotransmitter levels, all of which are related to the pathology of dementia. Thus, determining the expression of insulin receptors in microglia and other brain cell types under different nutritional states is crucial for better understanding the so called type 3 diabetes [133, 134].

In conclusion, metabolic injury of the brain is involved in many pathological conditions, and studying the effects of various glucose concentrations on the different brain cell types like glial cells is a good approach to better understand the brain`s state in such injuries. Microglia, the resident immune cells of the brain, continue to draw the attention of the research community due to their crucial and diverse roles in developing or propagating numerous brain disorders. The role played by microglia in determining neuronal fate during different types of metabolic injury is yet to be clearly shown.

Chapter 5

References

References

1. Pellerin, L., *Food for thought: the importance of glucose and other energy substrates for sustaining brain function under varying levels of activity.* Diabetes Metab, 2011. **36 Suppl 3**: p. S59-63.
2. Rasmussen, P., M.T. Wyss, and C. Lundby, *Cerebral glucose and lactate consumption during cerebral activation by physical activity in humans.* FASEB J, 2011. **25**(9): p. 2865-73.
3. Lack, A.K., K.E. Gill, and L.J. Porrino, *Local cerebral glucose utilization in rats exposed to an enriched environment: a comparison to impoverishment.* Pharmacol Biochem Behav, 2010. **96**(4): p. 521-5.
4. Prebil, M., et al., *Astrocytes and energy metabolism.* Arch Physiol Biochem, 2011. **117**(2): p. 64-9.
5. Belanger, M., I. Allaman, and P.J. Magistretti, *Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation.* Cell Metab, 2011. **14**(6): p. 724-38.
6. Qutub, A.A. and C.A. Hunt, *Glucose transport to the brain: a systems model.* Brain Res Brain Res Rev, 2005. **49**(3): p. 595-617.
7. Dermietzel, R. and D. Krause, *Molecular anatomy of the blood-brain barrier as defined by immunocytochemistry.* Int Rev Cytol, 1991. **127**: p. 57-109.
8. Hasselbalch, S.G., et al., *Transport of D-glucose and 2-fluorodeoxyglucose across the blood-brain barrier in humans.* J Cereb Blood Flow Metab, 1996. **16**(4): p. 659-66.
9. Guo, X., M. Geng, and G. Du, *Glucose transporter 1, distribution in the brain and in neural disorders: its relationship with transport of neuroactive drugs through the blood-brain barrier.* Biochem Genet, 2005. **43**(3-4): p. 175-87.
10. Vannucci, S.J., F. Maher, and I.A. Simpson, *Glucose transporter proteins in brain: delivery of glucose to neurons and glia.* Glia, 1997. **21**(1): p. 2-21.
11. Leto, D. and A.R. Saltiel, *Regulation of glucose transport by insulin: traffic control of GLUT4.* Nat Rev Mol Cell Biol, 2012. **13**(6): p. 383-96.
12. Mithieux, G., *Brain, liver, intestine: a triumvirate to coordinate insulin sensitivity of endogenous glucose production.* Diabetes Metab, 2011. **36 Suppl 3**: p. S50-3.
13. Heikkila, O., et al., *Cerebellar glucose during fasting and acute hyperglycemia in nondiabetic men and in men with type 1 diabetes.* Cerebellum, 2010. **9**(3): p. 336-44.
14. Magistretti, P.J. and L. Pellerin, *Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging.* Philos Trans R Soc Lond B Biol Sci, 1999. **354**(1387): p. 1155-63.
15. Burcelin, R., *The gut-brain axis: a major glucoregulatory player.* Diabetes Metab, 2011. **36 Suppl 3**: p. S54-8.
16. Kalsbeek, A., et al., *Hypothalamic control of energy metabolism via the autonomic nervous system.* Ann N Y Acad Sci, 2010. **1212**: p. 114-29.
17. Lechan, R.M. and C. Fekete, *The TRH neuron: a hypothalamic integrator of energy metabolism.* Prog Brain Res, 2006. **153**: p. 209-35.

18. Burdakov, D., S.M. Luckman, and A. Verkhratsky, *Glucose-sensing neurons of the hypothalamus.* Philos Trans R Soc Lond B Biol Sci, 2005. **360**(1464): p. 2227-35.
19. Levin, B.E., *Metabolic sensing neurons and the control of energy homeostasis.* Physiol Behav, 2006. **89**(4): p. 486-9.
20. Murphy, B.A., et al., *AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons.* Am J Physiol Cell Physiol, 2009. **297**(3): p. C750-8.
21. Levin, B.E., et al., *Neuronal glucosensing: what do we know after 50 years?* Diabetes, 2004. **53**(10): p. 2521-8.
22. Levin, B.E., *Metabolic sensors: viewing glucosensing neurons from a broader perspective.* Physiol Behav, 2002. **76**(3): p. 397-401.
23. Sanchez-Lasheras, C., A.C. Konner, and J.C. Bruning, *Integrative neurobiology of energy homeostasis-neurocircuits, signals and mediators.* Front Neuroendocrinol, 2009. **31**(1): p. 4-15.
24. Blouet, C. and G.J. Schwartz, *Hypothalamic nutrient sensing in the control of energy homeostasis.* Behav Brain Res, 2009. **209**(1): p. 1-12.
25. Diamant, M., *Brain insulin signalling in the regulation of energy balance and peripheral metabolism.* Ideggyogy Sz, 2007. **60**(3-4): p. 97-108.
26. Ghasemi, R., et al., *Insulin in the brain: Sources, localization and functions.* Mol Neurobiol, 2012.
27. Craft, S., *Alzheimer disease: Insulin resistance and AD--extending the translational path.* Nat Rev Neurol. **8**(7): p. 360-2.
28. McIntyre, R.S., et al., *Brain volume abnormalities and neurocognitive deficits in diabetes mellitus: points of pathophysiological commonality with mood disorders?* Adv Ther, 2010. **27**(2): p. 63-80.
29. Bourdel-Marchasson, I., A. Mouries, and C. Helmer, *Hyperglycaemia, microangiopathy, diabetes and dementia risk.* Diabetes Metab, 2011. **36 Suppl 3**: p. S112-8.
30. Chen, L., D.J. Magliano, and P.Z. Zimmet, *The worldwide epidemiology of type 2 diabetes mellitus--present and future perspectives.* Nat Rev Endocrinol, 2011. **8**(4): p. 228-36.
31. van Dieren, S., et al., *The global burden of diabetes and its complications: an emerging pandemic.* Eur J Cardiovasc Prev Rehabil, 2010. **17 Suppl 1**: p. S3-8.
32. Brands, A.M., et al., *[Diabetic encephalopathy: an underexposed complication of diabetes mellitus].* Ned Tijdschr Geneeskd, 2003. **147**(1): p. 11-4.
33. Perantie, D.C., et al., *Prospectively determined impact of type 1 diabetes on brain volume during development.* Diabetes. **60**(11): p. 3006-14.
34. Zeng, H.Y., W.R. Green, and M.O. Tso, *Microglial activation in human diabetic retinopathy.* Arch Ophthalmol, 2008. **126**(2): p. 227-32.
35. Ahmed, N. and N. Zahra, *Neurochemical correlates of alloxan diabetes: glucose and related brain metabolism in the rat.* Neurochem Res, 2010. **36**(3): p. 494-505.
36. Wrighten, S.A., et al., *A look inside the diabetic brain: Contributors to diabetes-induced brain aging.* Biochim Biophys Acta, 2009. **1792**(5): p. 444-53.
37. Yamato, T., et al., *Diabetes mellitus decreases hippocampal release of neurotransmitters: an in vivo microdialysis study of awake, freely moving rats.* Diabetes Nutr Metab, 2004. **17**(3): p. 128-36.

38. Feigin, V.L., et al., *Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review.* Lancet Neurol, 2009. **8**(4): p. 355-69.
39. Harada, S., W. Fujita-Hamabe, and S. Tokuyama, *Ischemic stroke and glucose intolerance: a review of the evidence and exploration of novel therapeutic targets.* J Pharmacol Sci, 2011. **118**(1): p. 1-13.
40. Rosmond, R. and P. Bjorntorp, *The hypothalamic-pituitary-adrenal axis activity as a predictor of cardiovascular disease, type 2 diabetes and stroke.* J Intern Med, 2000. **247**(2): p. 188-97.
41. Michalski, D., et al., *Interrelations between blood-brain barrier permeability and matrix metalloproteinases are differently affected by tissue plasminogen activator and hyperoxia in a rat model of embolic stroke.* Med Gas Res, 2012. **2**(1): p. 2.
42. Fujita-Hamabe, W., S. Harada, and S. Tokuyama, *[Effectiveness of metformin in prevention of development of hyperglycemia and neuronal damage caused by ischemic stress].* Yakugaku Zasshi, 2011. **131**(4): p. 533-8.
43. Rosas-Vargas, H., J.D. Martinez-Ezquerro, and T. Bienvenu, *Brain-derived neurotrophic factor, food intake regulation, and obesity.* Arch Med Res, 2011. **42**(6): p. 482-94.
44. Dave, J.A., et al., *Abnormal glucose metabolism in non-diabetic patients presenting with an acute stroke: prospective study and systematic review.* QJM, 2010. **103**(7): p. 495-503.
45. Harada, S., W. Fujita-Hamabe, and S. Tokuyama, *Ameliorating effect of hypothalamic brain-derived neurotrophic factor against impaired glucose metabolism after cerebral ischemic stress in mice.* J Pharmacol Sci, 2011. **118**(1): p. 109-16.
46. Cardoso, S., et al., *Hyperglycemia, hypoglycemia and dementia: role of mitochondria and uncoupling proteins.* Curr Mol Med, 2012.
47. Aronson, D., *Hyperglycemia and the pathobiology of diabetic complications.* Adv Cardiol, 2008. **45**: p. 1-16.
48. Lorenzi, M., *The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient.* Exp Diabetes Res, 2007: p. 61038.
49. Crabbe, M.J. and D. Goode, *Aldose reductase: a window to the treatment of diabetic complications?* Prog Retin Eye Res, 1998. **17**(3): p. 313-83.
50. Brogard, J.M., F. Caro-Sampara, and J.F. Blickle, *[Role of polyols in the development of diabetic complications. Value of aldose-reductase inhibitors].* Rev Med Interne, 1992. **13**(1): p. 69-79.
51. Beyer, A.M. and D. Weihrauch, *Hexosamine pathway activation and O-linked-N-acetylglucosamine: novel mediators of endothelial dysfunction in hyperglycemia and diabetes.* Vascul Pharmacol, 2012. **56**(3-4): p. 113-4.
52. Love, D.C. and J.A. Hanover, *The hexosamine signaling pathway: deciphering the "O-GlcNAc code".* Sci STKE, 2005. **2005**(312): p. re13.
53. Buse, M.G., *Hexosamines, insulin resistance, and the complications of diabetes: current status.* Am J Physiol Endocrinol Metab, 2006. **290**(1): p. E1-E8.
54. Koya, D. and G.L. King, *Protein kinase C activation and the development of diabetic complications.* Diabetes, 1998. **47**(6): p. 859-66.
55. Das Evcimen, N. and G.L. King, *The role of protein kinase C activation and the vascular complications of diabetes.* Pharmacol Res, 2007. **55**(6): p. 498-510.

56. Grillo, M.A. and S. Colombatto, *Advanced glycation end-products (AGEs): involvement in aging and in neurodegenerative diseases.* Amino Acids, 2008. **35**(1): p. 29-36.
57. Langendam, M., et al., *Continuous glucose monitoring systems for type 1 diabetes mellitus.* Cochrane Database Syst Rev, 2012. **1**: p. CD008101.
58. Staniszewska, M. and A. Gamian, *[Biochemical properties and clinical significance of protein glycation products].* Postepy Hig Med Dosw, 2003. **57**(2): p. 123-47.
59. Li, J., et al., *Advanced glycation end products and neurodegenerative diseases: mechanisms and perspective.* J Neurol Sci, 2012. **317**(1-2): p. 1-5.
60. Bianchi, R., I. Giambanco, and R. Donato, *S100B/RAGE-dependent activation of microglia via NF-kappaB and AP-1 Co-regulation of COX-2 expression by S100B, IL-1beta and TNF-alpha.* Neurobiol Aging, 2008. **31**(4): p. 665-77.
61. Ramasamy, R., S.F. Yan, and A.M. Schmidt, *The RAGE axis and endothelial dysfunction: maladaptive roles in the diabetic vasculature and beyond.* Trends Cardiovasc Med, 2005. **15**(7): p. 237-43.
62. Edwards, J.L., et al., *Diabetic neuropathy: mechanisms to management.* Pharmacol Ther, 2008. **120**(1): p. 1-34.
63. de Courten-Myers, G.M., et al., *Hypoglycemic brain injury: potentiation from respiratory depression and injury aggravation from hyperglycemic treatment overshoots.* J Cereb Blood Flow Metab, 2000. **20**(1): p. 82-92.
64. Vannucci, R.C. and S.J. Vannucci, *Hypoglycemic brain injury.* Semin Neonatol, 2001. **6**(2): p. 147-55.
65. Fujioka, M., et al., *Specific changes in human brain after hypoglycemic injury.* Stroke, 1997. **28**(3): p. 584-7.
66. Chan, O., et al., *Diabetes impairs hypothalamo-pituitary-adrenal (HPA) responses to hypoglycemia, and insulin treatment normalizes HPA but not epinephrine responses.* Diabetes, 2002. **51**(6): p. 1681-9.
67. Garcia de Yebenes, J. and M.A. Mena, *[Effect of insulin-induced hypoglycemia on the metabolism of monoamines].* Rev Clin Esp, 1984. **172**(4): p. 197-200.
68. Isaev, N.K., E.V. Stel'mashuk, and D.B. Zorov, *Cellular mechanisms of brain hypoglycemia.* Biochemistry (Mosc), 2007. **72**(5): p. 471-8.
69. Suh, S.W., A.M. Hamby, and R.A. Swanson, *Hypoglycemia, brain energetics, and hypoglycemic neuronal death.* Glia, 2007. **55**(12): p. 1280-6.
70. Auer, R.N., *Hypoglycemic brain damage.* Metab Brain Dis, 2004. **19**(3-4): p. 169-75.
71. Cryer, P.E., *Hypoglycemia, functional brain failure, and brain death.* J Clin Invest, 2007. **117**(4): p. 868-70.
72. He, F. and Y.E. Sun, *Glial cells more than support cells?* Int J Biochem Cell Biol, 2007. **39**(4): p. 661-5.
73. Kudo, Y., *[New development in glial cell research].* Brain Nerve, 2007. **59**(7): p. 655-67.
74. Kofler, J. and C.A. Wiley, *Microglia: key innate immune cells of the brain.* Toxicol Pathol, 2010. **39**(1): p. 103-14.
75. Vilhardt, F., *Microglia: phagocyte and glia cell.* Int J Biochem Cell Biol, 2005. **37**(1): p. 17-21.
76. Hanisch, U.K. and H. Kettenmann, *Microglia: active sensor and versatile effector cells in the normal and pathologic brain.* Nat Neurosci, 2007. **10**(11): p. 1387-94.

77. Dheen, S.T., C. Kaur, and E.A. Ling, *Microglial activation and its implications in the brain diseases.* Curr Med Chem, 2007. **14**(11): p. 1189-97.
78. Suzumura, A., *Microglia and neuronal degeneration.* Rinsho Shinkeigaku, 2011. **50**(11): p. 871.
79. Attal, N., et al., *Chronic neuropathic pain management in spinal cord injury patients. What is the efficacy of pharmacological treatments with a general mode of administration? (oral, transdermal, intravenous).* Ann Phys Rehabil Med, 2009. **52**(2): p. 124-41.
80. Veves, A., M. Backonja, and R.A. Malik, *Painful diabetic neuropathy: epidemiology, natural history, early diagnosis, and treatment options.* Pain Med, 2008. **9**(6): p. 660-74.
81. Tesfaye, S. and D. Selvarajah, *Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy.* Diabetes Metab Res Rev, 2012. **28 Suppl 1**: p. 8-14.
82. Kim, S.H., J.K. Kwon, and Y.B. Kwon, *Pain modality and spinal glia expression by streptozotocin induced diabetic peripheral neuropathy in rats.* Lab Anim Res, 2012. **28**(2): p. 131-6.
83. Coull, J.A., et al., *BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain.* Nature, 2005. **438**(7070): p. 1017-21.
84. Childers, W.E., Jr. and R.B. Baudy, *N-methyl-D-aspartate antagonists and neuropathic pain: the search for relief.* J Med Chem, 2007. **50**(11): p. 2557-62.
85. Ma, W., et al., *Microglia in the mouse retina alter the structure and function of retinal pigmented epithelial cells: a potential cellular interaction relevant to AMD.* PLoS One, 2009. **4**(11): p. e7945.
86. Ibrahim, A.S., et al., *Retinal microglial activation and inflammation induced by amadori-glycated albumin in a rat model of diabetes.* Diabetes, 2011. **60**(4): p. 1122-33.
87. Lai, A.Y., et al., *Neonatal rat microglia derived from different brain regions have distinct activation responses.* Neuron Glia Biol, 2011. **7**(1): p. 5-16.
88. Wen, Y.R., et al., *Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance.* J Formos Med Assoc, 2011. **110**(8): p. 487-94.
89. Luo, Y., C. Kaur, and E.A. Ling, *Neuronal and glial response in the rat hypothalamus-neurohypophysis complex with streptozotocin-induced diabetes.* Brain Res, 2002. **925**(1): p. 42-54.
90. Pitocco, D., et al., *Oxidative stress, nitric oxide, and diabetes.* Rev Diabet Stud, 2012. **7**(1): p. 15-25.
91. Fargali, S., et al., *Role of neurotrophins in the development and function of neural circuits that regulate energy homeostasis.* J Mol Neurosci, 2012. **48**(3): p. 654-9.
92. Zayani, Y., et al., *Abnormal circulating levels of matrix metalloproteinases and their inhibitors in diabetes mellitus.* Clin Lab, 2012. **58**(7-8): p. 779-85.
93. Neher, J.J., U. Neniskyte, and G.C. Brown, *Primary phagocytosis of neurons by inflamed microglia: potential roles in neurodegeneration.* Front Pharmacol, 2012. **3**: p. 27.
94. Deng, X. and S. Sriram, *Role of microglia in multiple sclerosis.* Curr Neurol Neurosci Rep, 2005. **5**(3): p. 239-44.

95. Lai, A.Y. and K.G. Todd, *Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury.* Glia, 2008. **56**(3): p. 259-70.
96. Odontiadis J and Rauw G , B.G., Dunn SMJ, Holt A, editors. NY: Springer., *High performance liquid chromatography General Techniques.* Hand book of Neurochemistry and Molecular Biology, 2006. **vol 18**.
97. Inman, M., *Inconspicuous consumption: uncovering the molecular pathways behind phagocytosis.* PLoS Biol, 2006. **4**(6): p. e190.
98. Lossinsky, A.S. and R.R. Shivers, *Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review.* Histol Histopathol, 2004. **19**(2): p. 535-64.
99. Kiyatkin, E.A. and M. Lenoir, *Rapid fluctuations in extracellular brain glucose levels induced by natural arousing stimuli and intravenous cocaine: fueling the brain during neural activation.* J Neurophysiol, 2012. **108**(6): p. 1669-84.
100. Lelekov-Boissard, T., et al., *Exploration of beneficial and deleterious effects of inflammation in stroke: dynamics of inflammation cells.* Philos Transact A Math Phys Eng Sci, 2009. **367**(1908): p. 4699-716.
101. Quan, Y., J. Du, and X. Wang, *High glucose stimulates GRO secretion from rat microglia via ROS, PKC, and NF-kappaB pathways.* J Neurosci Res, 2007. **85**(14): p. 3150-9.
102. Quan, Y., et al., *High glucose stimulates TNFalpha and MCP-1 expression in rat microglia via ROS and NF-kappaB pathways.* Acta Pharmacol Sin, 2011. **32**(2): p. 188-93.
103. Wang, A.L., et al., *AGEs mediated expression and secretion of TNF alpha in rat retinal microglia.* Exp Eye Res, 2007. **84**(5): p. 905-13.
104. Schmidt, A.M., et al., *The role of RAGE in amyloid-beta peptide-mediated pathology in Alzheimer's disease.* Curr Opin Investig Drugs, 2009. **10**(7): p. 672-80.
105. Rondeau, P., et al., *Thermal aggregation of glycated bovine serum albumin.* Biochim Biophys Acta, 2009. **1804**(4): p. 789-98.
106. Shaikh, S.B., et al., *AGEs-RAGE mediated up-regulation of connexin43 in activated human microglial CHME-5 cells.* Neurochem Int, 2012. **60**(6): p. 640-51.
107. Stein, V.M., et al., *Variations on brain microglial gene expression of MMPs, RECK, and TIMPs in inflammatory and non-inflammatory diseases in dogs.* Vet Immunol Immunopathol, 2011. **144**(1-2): p. 17-26.
108. Hawkins, B.T., et al., *Increased blood-brain barrier permeability and altered tight junctions in experimental diabetes in the rat: contribution of hyperglycaemia and matrix metalloproteinases.* Diabetologia, 2007. **50**(1): p. 202-11.
109. Yamanaka, M., et al., *Brain-derived neurotrophic factor (BDNF) prevents the development of diabetes in prediabetic mice.* Biomed Res, 2008. **29**(3): p. 147-53.
110. Trang, T., S. Beggs, and M.W. Salter, *Brain-derived neurotrophic factor from microglia: a molecular substrate for neuropathic pain.* Neuron Glia Biol, 2012. **7**(1): p. 99-108.
111. Kettenmann, H., et al., *Physiology of microglia.* Physiol Rev, 2011. **91**(2): p. 461-553.

112. Ramamurthy, S. and G. Ronnett, *AMP-Activated Protein Kinase (AMPK) and Energy-Sensing in the Brain.* Exp Neurobiol, 2012. **21**(2): p. 52-60.
113. Spasic, M.R., P. Callaerts, and K.K. Norga, *AMP-activated protein kinase (AMPK) molecular crossroad for metabolic control and survival of neurons.* Neuroscientist, 2009. **15**(4): p. 309-16.
114. Steinberg, G.R. and B.E. Kemp, *AMPK in Health and Disease.* Physiol Rev, 2009. **89**(3): p. 1025-78.
115. Hattori, Y., et al., *Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells.* Hypertension, 2006. **47**(6): p. 1183-8.
116. Giri, S., et al., *5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside inhibits proinflammatory response in glial cells: a possible role of AMP-activated protein kinase.* J Neurosci, 2004. **24**(2): p. 479-87.
117. Labuzek, K., et al., *AICAR (5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside) increases the production of toxic molecules and affects the profile of cytokines release in LPS-stimulated rat primary microglial cultures.* Neurotoxicology, 2009. **31**(1): p. 134-46.
118. Kuo, C.L., et al., *Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 gene expression by 5-aminoimidazole-4-carboxamide riboside is independent of AMP-activated protein kinase.* J Cell Biochem, 2008. **103**(3): p. 931-40.
119. Chen, C.J., et al., *Glutamate released by Japanese encephalitis virus-infected microglia involves TNF-alpha signaling and contributes to neuronal death.* Glia, 2011. **60**(3): p. 487-501.
120. Persson, M., et al., *Lipopolysaccharide increases microglial GLT-1 expression and glutamate uptake capacity in vitro by a mechanism dependent on TNF-alpha.* Glia, 2005. **51**(2): p. 111-20.
121. Takeuchi, H., et al., *Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner.* J Biol Chem, 2006. **281**(30): p. 21362-8.
122. Sung, B., G. Lim, and J. Mao, *Altered expression and uptake activity of spinal glutamate transporters after nerve injury contribute to the pathogenesis of neuropathic pain in rats.* J Neurosci, 2003. **23**(7): p. 2899-910.
123. Canto, C. and J. Auwerx, *PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure.* Curr Opin Lipidol, 2009. **20**(2): p. 98-105.
124. Sag, D., et al., *Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype.* J Immunol, 2008. **181**(12): p. 8633-41.
125. Maree, A.F., et al., *A quantitative comparison of rates of phagocytosis and digestion of apoptotic cells by macrophages from normal (BALB/c) and diabetes-prone (NOD) mice.* J Appl Physiol, 2008. **104**(1): p. 157-69.
126. O'Brien, B.A., et al., *A deficiency in the in vivo clearance of apoptotic cells is a feature of the NOD mouse.* J Autoimmun, 2006. **26**(2): p. 104-15.
127. Borregaard, N. and T. Herlin, *Energy metabolism of human neutrophils during phagocytosis.* J Clin Invest, 1982. **70**(3): p. 550-7.

128. Labuzek, K., et al., *Metformin increases phagocytosis and acidifies lysosomal/endosomal compartments in AMPK-dependent manner in rat primary microglia.* Naunyn Schmiedebergs Arch Pharmacol, 2009. **381**(2): p. 171-86.
129. Fryer, L.G., et al., *Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells.* Biochem J, 2002. **363**(Pt 1): p. 167-74.
130. Labuzek, K., et al., *Metformin has adenosine-monophosphate activated protein kinase (AMPK)-independent effects on LPS-stimulated rat primary microglial cultures.* Pharmacol Rep. **62**(5): p. 827-48.
131. Polito, A., et al., *Hyperglycaemia and apoptosis of microglial cells in human septic shock.* Crit Care, 2011. **15**(3): p. R131.
132. Hildreth, K.L., R.E. Van Pelt, and R.S. Schwartz, *Obesity, insulin resistance, and Alzheimer's disease.* Obesity (Silver Spring), 2012. **20**(8): p. 1549-57.
133. *Brain insulin resistance may exacerbate Alzheimer's progression. Researchers call it "Type III" diabetes.* Duke Med Health News, 2012. **18**(9): p. 4-5.
134. Steen, E., et al., *Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes?* J Alzheimers Dis, 2005. **7**(1): p. 63-80.