METABOLIC HORMONES AND PHYSICAL ACTIVITY AS REGULATORS OF BRAIN GLIAL CELL FUNCTIONS AND NEUROIMMUNE STATUS

by

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**METABOLIC HORMONES AND PHYSICAL ACTIVITY AS REGULATORS OF BRAIN GLIAL CELL FUNCTIONS AND NEUROIMMUNE STATUS**

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Abstract

Alzheimer’s disease (AD), the most prevalent form of dementia worldwide, is characterized by several cellular hallmarks including chronic neuroinflammation. Glial cells, the immune and support cells of the brain, become activated in response to pathological formations in the AD brain and neuroinflammation occurs as a result. Persistent activation of glial cells can result in chronic neuroinflammation, which can be damaging to brain tissue. The goal of my PhD research project was to elucidate the cellular mechanisms by which certain non-neuronal factors increase the risk of developing AD, such that new treatment options for AD might be identified. My research focused on two risk factors for developing AD: type 2 diabetes mellitus (T2DM) and sedentary (SED) lifestyles. The overarching hypothesis of my thesis is that the metabolic hormone dysregulation as well as reduced physical activity, contribute to AD pathogenesis through neuroimmune mechanisms. I focused on three specific hypotheses: 1) insulin, a metabolic hormone that has reduced functionality in T2DM, regulates the neuroinflammatory response of glia; 2) the incretin hormones glucagon like peptide (GLP)-1 and glucose dependent insulinotropic polypeptide (GIP), which also become dysregulated in T2DM, control several aspects of glial cell function; and 3) physical activity (PA) has an impact on the neuroimmune status of the brain, which is regulated in a monocyte chemoattractant protein (MCP)-1-dependent manner. Using in vitro cell culture techniques, I demonstrate that glial cells express the insulin receptors. I show that insulin could have anti-inflammatory properties in the brain and may protect against glia-mediated neurotoxicity, while GLP-1 and GIP exhibit anti-apoptotic, antioxidant and trophic effects on glial cells. Utilizing in vivo murine studies, I demonstrate that PA can modify glial cell activation and regulate the expression of immune cytokines in the brain. Additionally, I reveal that the neuroimmune status-modifying activity of PA relies partially on MCP-1. I have discovered that insulin, GLP-1, GIP and PA regulate specific aspects of neuroinflammation. Therefore, targeting neuroinflammation may represent viable AD treatment options, which should be explored in future clinical studies.

A version of the work presented in chapter 2 has been published: [Spielman, L.J.], Bahniwal, M., Little, J.P., Walker, D.G., Klegeris, A. (2015) Insulin Modulates In Vitro Secretion of Cytokines and Cytotoxins by Human Glial Cells. Current Alzheimer’s Research. 12: 684-693. As lead contributor for this primary research article, I completed all of the laboratory work, except for the data contained in figure 2.2, which was performed by M. Bahniwal. I analyzed and interpreted the data, made conclusions regarding the significance of this work and prepared the manuscript, with editorial comments from M. Bahniwal, J.P. Little, D.G. Walker and A. Klegeris.

A version of the work presented in chapter 3 has been submitted for publication with the European Journal of Cell Biology: [Spielman, L.J.], Gibson, D.L., Klegeris, A. Incretin Hormones Regulate Microglia Oxidative Stress, Survival and Expression of Trophic Factors. Submitted September 2016. I initiated the concept behind this project and decided on appropriate laboratory techniques. I conducted all of the laboratory work, analyzed the results and interpreted the significance. The analysis of trophic factor secretion, however, was performed by Eve Technologies. I prepared this manuscript with editorial comments from D.L. Gibson and A. Klegeris.

A version of the work presented in chapter 4 has been submitted for publication with Molecular and Cellular Neuroscience: [Spielman, L.J.], Estaki, M., Ghosh, S., Gibson, D.L.,

As the lead contributor, I researched the literature and performed all the laboratory work, except for the handling of live animals and animal sacrifices, which were performed at the University of British Columbia (UBC) Vancouver as well as the analysis of serum cytokines, which was performed by Eve Technologies. I analyzed and interpreted the data and prepared the manuscript, with editorial comments from M. Estaki, D.L. Gibson, S. Ghosh and A. Klegeris.

Dr. Andis Klegeris’ laboratory is licensed under the UBC Clinical Research Ethics Board application number H10-00202 as well as the UBC Biosafety Permit B14-0058; and I have completed the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS2: CORE) training.

I also co-authored the following papers during my PhD study:


v. Schindler, S.M., [Spielman, L.J.], Pointer, C.B., Slattery, W.T., Bajwa, E.,
Neuroinflammation and Modifiable Risk Factors of Alzheimer’s Disease,
Parkinson’s Disease and Amyotrophic Lateral Sclerosis. In ‘Neuroinflammation

vi. Schindler, S.M., [Spielman, L.J.], Bajwa, E., Slattery, W.T., Harris, D.B.,
Responses: Implications for Central Nervous System Functions. In ‘Microglia:
Physiology, Regulation and Health Implications’. Giffard, E.R. (Ed.), pp.113-156,

vii. [Spielman, L.J.], Klegeris, A. (2014) Insulin and Incretins in Neuroinflammation
and Neurodegeneration. Immunoendocrinology. 1: e391.

Resistance as the Possible Link between Obesity and Neurodegeneration. Journal
of Neuroimmunology. 273: 8-21.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Aβ</td>
<td>amyloid beta protein</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>BDH</td>
<td>N-(1-napthyl)ethylenediamine dihydrochloride</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
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<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CBS</td>
<td>calf bovine serum</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>central nervous system</td>
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<td>cyclic adenosine monophosphate responsive element binding protein</td>
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<td>cerebrospinal fluid</td>
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<td>DAPI</td>
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<td>DNA</td>
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IGF  insulin-like growth factor
IGF-1R  insulin-like growth factor 1 receptor
IL  interleukin
INSR  insulin receptor
INSRA  insulin receptor isoform A
INSRB  insulin receptor isoform B
IRS  insulin receptor substrate
JNK  Jun-N terminal kinase
LOX  lipoxygenase
LPS  lipopolysaccharide
LSD  least significant difference
M1  anti-inflammatory state of mononuclear phagocytes
M2  pro-inflammatory state of mononuclear phagocytes
MAP2  microtubule associated protein 2
MAPK  mitogen-activated protein kinase
MCP  monocyte chemoattractant protein
MFI  mean fluorescence intensity
MHC  major histocompatibility complex
MIP  macrophage inflammatory protein
mRNA  messenger ribonucleic acid
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
(or tetrazolium salt)
n.d.  not detected
NADPH  nicotinamide adenine dinucleotide phosphate
NeuN  neuronal nuclei marker
NFT  neurofibrillary tangle
NFκB  nuclear factor kappa b
NGF  nerve growth factor
NO  nitric oxide
NO₂⁻  nitrite
NOS  nitric oxide synthase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NSE</td>
<td>neuron specific endonuclease</td>
</tr>
<tr>
<td>NSERC</td>
<td>Natural Sciences and Engineering Research Council of Canada</td>
</tr>
<tr>
<td>NTC</td>
<td>no template control</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>ONOO⁻</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PA</td>
<td>physical activity</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKI</td>
<td>protein kinase A inhibitor</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>Pro</td>
<td>Pro(3)GIP (glucose-dependent insulinotropic polypeptide receptor antagonist)</td>
</tr>
<tr>
<td>proNGF</td>
<td>pro nerve growth factor</td>
</tr>
<tr>
<td>RET</td>
<td>rearranged during transfection</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SED</td>
<td>sedentary</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homologous and collagenous protein</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with 0.1% tween</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin-related kinase</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
</tr>
<tr>
<td>UBCO</td>
<td>University of British Columbia Okanagan</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VWR</td>
<td>voluntary wheel running</td>
</tr>
<tr>
<td>Wort</td>
<td>wortmannin (PK3K inhibitor)</td>
</tr>
<tr>
<td>ZO</td>
<td>zonula occluden</td>
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</tbody>
</table>
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Dedication

This thesis is dedicated to the individuals
who have donated pieces of their bodies and brains to science,
and to the scientists before me who have been responsible for creating cell lines;
Without you this research would not be possible.
Chapter 1 Introduction

1.1 Overview of the Brain

The human brain is the most sophisticated, energetically active and plastic organ in the human body. It is composed of an elaborate network of neuronal circuitry and glial cells. Glial cells, which include oligodendrocytes, astrocytes and microglia, perform protective and supportive roles towards neurons in the central nervous system (CNS) \(^1\). Combined, these cells form different regions of the brain, defined by their anatomical locations and primary functions. The brain tissue is bathed in cerebrospinal fluid (CSF), which is rich in ions, enzymes, nutrients and hormones \(^2\). Encompassing the brain and CSF are three protective layers of meninges (connective tissue): dura matter, arachnoid matter and pia matter \(^3\). Beyond the layers of meninges lays the skull, a thick bone that encapsulates the brain and forms a tough barrier between the brain and the outside elements. Together, the brain and spinal cord form the CNS, which is distinctly separate from the rest of the human body by way of the blood brain barrier (BBB).

1.1.1 The Blood Brain Barrier

The BBB is a complicated and dynamic neurovascular structure that tightly regulates the passage of molecules into and out of the CNS. The BBB is made up of four principal components: endothelial cells, pericytes, the secreted basal lamina and astrocytes \(^4,5\). The endothelial cells, which line the blood vessels and surround blood plasma, provide a boundary between the blood and the interstitial fluid of the brain. The endothelial cells produce a layer of extracellular matrix called the basal lamina, which provides an anchoring and signaling site for interactions between cells of the BBB \(^4\). Pericytes, which are contractile cells positioned within the basal lamina, aid in the regulation of CNS homeostasis by communicating with endothelial cells and astrocytes via direct physical contact, as well as paracrine signaling \(^4\). Astrocytes, a subtype of glial cell, provide an additional layer of physical protection between the periphery and brain by extending their astrocytic feet, towards the endothelial cells, trapping the basal lamina and pericytes between the astrocytes.
and the endothelial cells. Together, these cellular units work to regulate the passage of electrolytes, nutrients, hormones, cytokines, as well as various other proteins and waste between the peripheral circulatory system and the CNS (Fig. 1.1) \(^5\).

**Figure 1.1 Structure of the Blood Brain Barrier.**

1.1.2 Neurons

Neurons are highly specialized, excitable cells that respond to and transmit electrical and chemical signals throughout the CNS as well as between the CNS and periphery. Neurons are typically polarized cells, as their structure includes a central cell body that connects the dendritic and axonal protrusions, which are located at opposing ends of the cell \(^6\). Signaling from one neuron to another generally occurs via synapses, which are tiny gaps (approximately 20 nm) \(^7\) between the axon terminal of one neuron and the dendrite of a neighboring neuron. In the synapse, chemical signals, called neurotransmitters, are released from one cell and diffuse to the next cell. Upon neurotransmitter interaction with receptors on the dendrites of subsequent neurons, the propagation of an electrical signal, called an action potential, may take place if sufficient neuronal excitation occurs. An action potential, which is initiated through the opening and closing of ion channels, propagates down the
length of the neuronal axon until reaching the axon terminals, where signal transduction occurs, and neurotransmitters are released from that neuron. Thus, action potentials and neurotransmitters create a cascading and branching transfer of information between cells that forms the basis of all thoughts, emotions and regulation of involuntary and voluntary movement and functions 8-10.

1.1.3 Glial Cells

In addition to the neuronal cells, glial cells are at least equally abundant in the CNS, with some researchers asserting that glial cells outnumber neurons by 10 fold 11,12. Glial cells represent the supportive and immune cells of the brain and play a critical role in regulating brain homeostasis 13. Glial cells are separated into three main cellular subtypes: oligodendrocytes, astrocytes and microglia, each of which have distinct and essential regulatory functions in the CNS.

1.1.3.1 Oligodendrocytes

Oligodendrocytes have a lipid-rich plasma membrane, which wraps around the axon of neurons to create a protective coating known as the myelin sheath 14. Oligodendrocytes play a vital role in maintaining axonal integrity and increasing the speed of action potential transmission 13,15. In addition to electrically insulating neurons, oligodendrocytes upregulate and cluster sodium channel expression along neuronal axons at the nodes of Ranvier (gaps in the myelin sheath), which further enhances saltatory nerve conduction 16,17. Oligodendrocytes also modulate neuronal growth, as their presence has been shown to enhance both axonal diameter as well as length 18,19. Like other types of glial cells, oligodendrocytes provide neurons with essential growth and trophic factors including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and insulin-like growth factor (IGF)-1 18,20,21.
1.1.3.2 Astrocytes

Astrocytes have a vast number of homeostatic and regulatory roles in the CNS. Astrocytes provide physical support to, as well as help regulate, the tight junctions of endothelial cells along the BBB; thus, astrocytes stringently control the passage of molecules into and out of the CNS. Astrocytes have the ability to both positively and negatively regulate tight junctions between the endothelial cells of the BBB. Paul et al. (2014) reported that astrocytic secretion of the chemokine monocyte chemoattractant protein (MCP)-1 resulted in the down-regulated distribution of the tight junction molecule claudin-5 along the BBB, thereby demonstrating that astrocytes are capable of increasing permeability of the BBB during inflammatory events in the CNS. Both in vitro murine studies and in vivo murine MCP-1 knockout experiments have revealed that astrocyte-derived MCP-1 enhances permeability of the BBB 17 fold by means of reduced expression of the tight junction proteins occludin, claudin-5, and zonula occludens (ZO)-1 and 2. Additionally, in a non-inflammatory state, astrocytes can positively regulate the expression of tight junction proteins in the BBB, as it has been demonstrated that absence of astrocytes leads to a significant reduction in claudin-5 and ZO-1 along the BBB in rats. The lack of astrocytes and resulting reductions in tight junction proteins correlated with increased passage of molecules across the BBB.

In addition, astrocytes can regulate the passage of nutrients across the BBB by upregulating expression of glucose transporter (GLUT) 1 by endothelial cells of the BBB. GLUT1 facilitates the transport of glucose from the blood to the brain, where glucose is catabolized as the primary source of nutrients for neurons and glial cells. Furthermore, astrocytes can convert acetyl-coenzyme A to the ketone bodies acetone, β-hydroxybutyrate and acetoacetate, which can then be used by neurons as a source of energy when no glucose is available. Additionally, it has been determined that astrocytes metabolize glucose and supply neurons with lactate, the breakdown product of glucose, by way of the lactate transporters; this occurs particularly in hypoxic conditions and glucose-and-oxygen-deprived conditions. Astrocytes also regulate the passage of a number of amino acids into the brain by positively regulating the expression of the L-system amino acid transporter along the BBB.
which transports large neutral amino acids from the extracellular space to the cytoplasm of the cell.

Astrocytes participate in the regulation of blood flow to the brain, CNS osmoregulation and the removal of neurotoxic molecules from the CNS. Astrocytes regulate cerebral blood flow by stimulating both the relaxation and contraction of smooth muscles surrounding vascular endothelium via calcium (Ca$^{2+}$)-mediated mechanisms. By controlling vasodilation and vasoconstriction, astrocytes can regulate blood flow to specific brain areas. This is of particular importance since inadequate blood flow and ischemic conditions have a wide range of potential adverse consequences including the activation of glial cells and death of brain tissue.

Astrocytes play a critical role in the regulation of neuronal plasticity and synaptic remodelling. Several studies have demonstrated that the presence of astrocytes induces neuronal differentiation, the formation and strengthening of synapses between neurons, enhanced synaptogenesis and long term potentiation, as well as enhanced synaptic activity both in vitro and in vivo. In addition to positively regulating synapse formation, astrocytes participate in synaptic pruning by phagocytosing low-activity synapses.

Astrocytes play a crucial role in the recycling of neurotransmitters. Following synaptic transmission, astrocytes take up neurotransmitters from the synaptic cleft by way of transporters such as the excitatory-amino-acid transporter (critical for clearance of glutamate), the sodium-coupled neutral amino acid transporter (critical for clearance of glutamine and other small, neutral amino acids) and other transporters. Following reuptake by astrocytes, the neurotransmitters have several potential fates, one of which is their recycling through various biochemical pathways and delivery to the neuron, where they can be reused for future synaptic transmission. Moreover, astrocytes provide neurons with several key chemical signals required for survival and response to the CNS environment. Astrocytes not only supply critical growth factors such as BDNF, GDNF and nerve growth factor (NGF), but also signal to neurons by releasing neurotransmitters as well as by secreting cytokines and chemokines during inflammatory events.
1.1.3.3 Microglia

Microglia represent the innate immune cells of the CNS. These cells, too, play a critical role in CNS homeostasis with specialized roles including phagocytosis, antigen presentation and release of cytokines and cytotoxins. Microglia regulate brain homeostasis during health, but also during the initiation and subsequent resolution of inflammation. Under normal physiological conditions, microglia are in their resting state, during which they are still highly active as they continually patrol their surroundings for foreign invaders and pathological stimuli by extending their processes and protrusions. During this surveillance process, microglia engage in repeated and transient interactions with neighboring neurons, which facilitates synaptic remodeling and elimination. Microglia aid in the formation of learning-dependent synapses. The depletion of microglia in mice results in reduced synaptic remodeling and diminished learning and memory capabilities. It is proposed that this decline in neuronal structure and cognitive function may be due to abolished microglia-derived BDNF supply. In a healthy brain, the microglia-neuron communication helps to maintain microglia in their quiescent state and occurs by direct interactions between the cluster of differentiation (CD)200 receptor on neurons and the CD200 ligand on microglia. This phenomenon has been demonstrated by experiments using CD200-deficient mice. Microglia in CD200-deficient mice exhibited an activated phenotype, as evident by their amoeboid shape, as well as an increase in cell surface expression of CD11b and CD45.

Despite what is known regarding microglia in a healthy brain, the role of microglia in disease and inflammation is better understood. During infection or injury, microglia become activated and initially exhibit pro-inflammatory characteristics, sometimes referred to as an M1 phenotype. Such activation of microglia induces morphological and physiological changes, including modification of the microglia cell shape from ramified to amoeboid-like, alterations in cell membrane receptors expression, as well as changes in their secretory profile. Upon activation, microglia expression of surface molecules including complement receptors, major histocompatibility complex (MHC) and other immune receptors, such as CD11a, CD40 and CD80 molecules, is significantly upregulated. In a state of infection or injury, microglia produce and secrete several chemokines such as interleukin (IL)-8 and...
MCP-1, which function to recruit other immune cells to the site of injury \textsuperscript{58,59}. In an attempt to neutralize the stimulating agent, microglia also release a variety of non-specific cytotoxic molecules including: pro-inflammatory cytokines, such as IL-6, interferon (IFN)-\(\gamma\) and tumor necrosis factor (TNF)-\(\alpha\); excitatory amino acids, such as glutamate and serine; several reactive oxygen species (ROS), including superoxide (\(O_2^\bullet^-\)); reactive nitrogen species (RNS), including nitric oxide (NO) and peroxynitrite (ONOO\(^-\)); as well as proteases, such as matrix metalloproteinases and cathepsins \textsuperscript{60}. When the acute inflammation begins to resolve, some of the microglia begin to change from the M1, to an M2 phenotype. In the M2 state, microglia release several anti-inflammatory cytokines, such as IL-4, IL-10 and transforming growth factor \(\beta\), in aims of repealing the inflammation following removal of the stimuli. Microglia secrete several growth and trophic factors, such as BDNF, GDNF and NGF, in attempts to repair tissue damage and resolve the inflammation \textsuperscript{58,60}. Thus, microglia are essential in the protection of the brain against pathogenic invaders and damaging stimuli.

However, under long-term pathological conditions, when the stimulating agent cannot be removed by immune efforts, microglia are known to change from an activated to a dysregulated and over-activated state; such a pro-inflammatory state is also known as microgliosis \textsuperscript{58}. Over-activation of microglia leads to the superfluous secretion of the aforementioned cytotoxic factors, and consequently results in the activation of other microglia, which can cause significant death to adjacent cells including neurons and glial cells. Therefore, microgliosis has been implicated as a major contributing factor in several forms of brain disease, particularly neurodegenerative diseases, which are partially characterized by chronic neuroinflammation \textsuperscript{58} (Fig. 1.2).
1.2 The Immune System

The human immune system is a collection of host defense mechanisms targeted against harmful stimuli, such as invading pathogens, damaged tissue or noxious substances. The immune system is vastly complicated and is made up of several different cell types, organs and physiological processes, which may become activated depending on the site of initiation and the type of triggering stimuli. One of the primary functions of the immune system is generation of inflammation. The ultimate goal of the inflammatory process is to rid the body of any harmful substances, and subsequently repair any associated tissue damage in order to return the body to its previous homeostatic state 61-64.
1.2.1 The Peripheral Immune System

Upon introduction of the inflammatory stimuli, the body’s immune system responds rapidly. Tissue mast cells stimulate blood vessel vasodilation, as well as increased blood vessel permeability. This increase in blood vessel permeability allows extravasation of leukocytes from the blood vessels to the site of the immune reaction. Neutrophils, which are multinucleated granular leukocytes, phagocytose the invading pathogen and necrotic cellular debris in addition to releasing cytokines and chemokines. Secreted chemokines include IL-8, MCP-1 and macrophage inflammatory protein (MIP), which are chemoattractant molecules that summon other immune cells to the site of inflammation. Upon arrival or activation, macrophages aid in the phagocytic process, while simultaneously secreting a variety of pro-inflammatory cytokines, particularly IL-1β, IL-6 and TNF-α, which in combination have a synergistic effect that magnifies the inflammatory response. IL-1β, IL-6 and TNF-α increase blood vessel permeability and induce synthesis of acute-phase response proteins, such as C-reactive protein. Thus, the process of acute inflammation is self-perpetuating; however, as the stimulating agents are removed and the injury is repaired, the inflammation becomes self-resolving and the body once again returns to a homeostatic state.

1.2.2 The Brain Immune System

Each organ system of the human body has its own resident immune cells, which are designed specifically to protect and ward off toxic stimuli introduced into that system. Thus, the CNS, too, has a specific host defense mechanism, which primarily relies on the actions of the BBB, the recently discovered brain lymphatic system, microglia and astrocytes, and to a lesser extent, the immune cells of the periphery, such as T cells. As mentioned in section 1.1.1., the BBB plays a vital role in separating the CNS from the periphery, and in doing so, the BBB protects the brain and spinal cord from many elements in the periphery that may be damaging to the CNS. However, the most recognized participants in brain immunity are the microglia, the resident macrophages of the CNS. As detailed in section 1.1.3.3, microglia undergo a variety of morphological and physiological changes in response to the events of
infection, trauma, ischemia or neurodegeneration. These changes in microglia phenotype and function allow for rapid extermination of microorganisms, removal of toxic agents, limitation of tissue damage and cellular necrosis, as well as restoration of brain homeostasis.

1.2.3 Chronic Neuroinflammation and Oxidative Stress

In the event of chronic neuroinflammation, the typical immune response and removal of pernicious substances becomes dysregulated and endures due to persistence of the initial stimuli. Under these circumstances of chronic inflammation, the effects of microglia changes from beneficial to damaging, as continuous and excessive amounts of cytokines, ROS and proteases are persistently secreted into the intercellular space. Certain cytokines are known to have toxic effects on surrounding cells; for example, TNF-α is a potent inducer of apoptosis. When present in large quantities, such as during the events of chronic inflammation, ROS can be highly destructive to cells. Since many of these secreted cytotoxic substances are non-specific in nature, healthy neighboring cells, including other glial cells and neurons, suffer collateral damage as a result of the misdirected and unremitting immune attack. As such, chronic neuroinflammation and chronic oxidative stress have been implicated in the propagation of several neurodegenerative brain diseases, including multiple sclerosis, Huntington’s disease, Alzheimer’s disease (AD) and Parkinson’s disease (PD), to name a few.

Brain glial cells play an important role in oxidative stress regulation. Oxidative stress is defined as an imbalance between the production of ROS/RNS and antioxidant defenses, which can result in considerable cellular damage. The brain is especially susceptible to oxidative stress for a number of reasons, including the high metabolic rate of oxygen (O₂) in the brain (20% of all O₂ in the body is consumed in the brain), as well as the larger quantities of phospholipids present in the brain, which are particularly susceptible to peroxidation in the presence of high levels of ROS. ROS can be produced in the brain during routine processes, such as a result of electron transport chain activity, during phagocytosis and respiratory burst, due to prostaglandin synthesis, following physical exercise, during inflammation, as well as succeeding exposure to environmental pollutants. However, ROS and RNS can also be intentionally produced in small quantities by the body in order to
perform a number of necessary physiological functions. For example, O$_2^•$-, a well-defined ROS molecule, is produced due to the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which operates as a defense mechanism against invading pathogens $^{83,84}$. Additionally, NO, a well-defined RNS molecule, is produced by nitric oxide synthase (NOS) enzymes, and has important biological roles in regulation of vasodilation, immune system defenses and neuronal activity $^{85-87}$. However, when in excess, O$_2^•$-, can combine with NO to form ONOO•, which is known to cause immense cellular damage by way of lipid peroxidation, protein oxidation and nitration, as well as the induction of apoptosis and necrosis $^{88}$. Thus, when quantities of ROS and RNS are in excess, oxidative stress can produce significant damage to tissues of the body including the brain $^{82}$.

Glial cells, particularly microglia, help regulate ROS levels in the brain through antioxidant enzymes. In general terms, antioxidant enzymes are proteins that defend against oxidative stress by neutralizing ROS $^{82}$. There are a number of well-characterized antioxidant enzymes that are produced by microglia, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) $^{81,89-91}$. SOD is known primarily for combing O$_2^•$- and hydrogen ion (H$^+$) in a dismutation reaction to form hydrogen peroxide (H$_2$O$_2$) $^{92}$. In the presence of high H$_2$O$_2$ concentrations, CAT acts catalytically to break down H$_2$O$_2$ into water (H$_2$O) and O$_2$, thereby protecting cells against the toxic effects of H$_2$O$_2$ $^{93,94}$. GPx functions as an antioxidant enzyme by reducing H$_2$O$_2$ to H$_2$O, while simultaneously oxidizing glutathione (GSH) to produce glutathione disulfide (GSSG) $^{95}$. Together, with the aid of other antioxidant enzymes, these proteins function to convert excess amounts of the damaging ROS and RNS to the essential H$_2$O and O$_2$ molecules (Fig. 1.3). Therefore, microglia produce these crucial antioxidant enzymes to protect the brain by reducing the presence of the damaging ROS and RNS (Fig. 1.3). However, throughout various forms of brain disease, chronic inflammation and oxidative stress outweighs the capability of the glial cells to defend against these toxic events, which leads to further brain damage and propagation of brain disease.
1.2.4 Brain Trophic Factors

BDNF, GDNF and NGF are produced and secreted by several cell types in the brain, including neurons, oligodendrocytes, astrocytes and microglia. It has been demonstrated that transcription of BDNF, GDNF and NGF is regulated by cyclic adenosine monophosphate responsive element binding protein (CREB) and nuclear factor kappa B (NFκB). However, a variety of physiological factors influence this regulation. For example, it has been shown that NO and O₂⁻ negatively regulate BDNF transcription in motor neurons by way of reduced CREB and NFκB activation. Trophic factor expression can be upregulated following certain lifestyle modifications, such as an increase in exercise as well as a decrease in caloric intake. These trophic factors are transcribed as prepro-neurotrophins, which are converted to pro-neurotrophins in the Golgi apparatus, and are subsequently packaged in secretory vesicles. Within the secretory vesicles, further processing of select pro-neurotrophins takes place through the activity of proconvertases, resulting in the secretion of both pro-neurotrophins and mature neurotrophins from the cell into the extracellular space. ProBDNF, proNGF and proGDNF are secreted by various cell types and are subsequently cleaved in the extracellular space. Serine protease plasmin and selective matrix metalloproteinases cleave both proBDNF and proNGF to form mature...
neurotrophins, while the mechanism of the extracellular cleavage of proGDNF has yet to be elucidated. Extracellular secretion of these trophic factors by neurons occurs rapidly following neuronal depolarization, while it has been demonstrated that trophic factor secretion by microglia is induced following their activation. For example, IFN-\(\gamma\) induces secretion of trophic factors by microglia both in vivo and in vitro. It has been proposed that this secretion of trophic factors by microglia plays a pivotal role in glia-mediated neuroprotection during infection and disease.

BDNF and NGF, which belong to the neurotrophin family of trophic factors, produce their beneficial effects by forming homodimers and interacting with the tropomyosin-related kinase (Trk) family of receptors, including TrkA, TrkB, and TrkC. BDNF and NGF have been shown to bind to TrkA, TrkB, and TrkC; however, BDNF preferentially binds to and activates TrkB, while NGF has preference for TrkA. Binding to Trk receptors activates the downstream signaling cascades of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC). BDNF and NGF also bind to and activate the p75 neurotrophin receptor (p75NTR), which leads to activation of the NFκB and Jun-N terminal kinase (JNK) pathways. Interestingly, Trk receptor activity is partially governed by the p75NTR on several levels. For example, interaction between p75NTR and Trk receptors promotes the activation of Trk receptors by their preferred neurotrophins and hinders the activation of Trk receptors by non-preferred neurotrophins. It has been suggested that the correct ratio of Trk receptors to p75NTR is essential for appropriate cellular responses to trophic factors. Additionally, it has been shown that the p75NTR inhibits the ubiquitination of Trk receptors following the binding of NGF to the TrkA receptor. This reduces the internalization and degradation of TrkA, which permits for further activation and subsequent signaling through this pathway.

GDNF, on the other hand, belongs to the GDNF family of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily of trophic factors, and operates primarily through the GDNF receptor alpha 1 (GFRα1), as well as the rearranged during transfection (RET) receptor tyrosine kinase. Since GFRα1 expression has been shown to be reduced in GDNF null mice, it is believed that the expression of GFRα1 is regulated by the presence of GDNF. Similar to activation of the Trk receptors or p75NTR by BDNF and NGF, activation of the GFRα1/RET cascades by GDNF results in the induction of MAPK, PI3K, PLC and NFκB
pathways, thereby producing similar outcomes for all three trophic factors $^{100,107}$.

Activation of the BDNF, NGF and GDNF downstream signaling pathways leads to a number of cellular consequences, primarily due to the upregulation of pro-survival and synaptic plasticity genes, such as CREB $^{118}$. These trophic factors produce many beneficial effects in the CNS, including promotion of neuronal cell survival, cellular differentiation, neurite outgrowth, regulation of synapse formation and remodeling, regulation of long-term potentiation and depression, as well as regulation of apoptosis and oxidative stress $^{53,96,100,119-122}$. The cellular responses induced by these trophic factors have been shown to impact overall human brain function in a variety of ways, including improved memory and learning capabilities, reduced feelings of anxiety and moderation of the relationship between life stressors and depression $^{123-125}$. Since BDNF, GDNF and NGF regulate so many critical CNS functions, deficit of these trophic factors has been implicated in the induction of a number of brain diseases, including AD $^{100,116}$.

In fact, significant evidence suggests that alterations in the levels of brain trophic factors play a pivotal role in the progression of AD $^{126-129}$. It has been established that AD brains contain significantly less BDNF and GDNF than healthy brains (up to 62% and 50% reduction, respectively) $^{107,130}$. Other studies have demonstrated that hippocampal levels of the NGF precursor molecule proNGF in post mortem tissue of AD patients can be up to 41% higher than in control brain tissue $^{131}$. It has also been determined that serum concentrations of BDNF, GDNF and NGF are reduced by 21%, 58% and 58%, respectively, in AD patients compared to healthy subjects, which correlates with a 37% decrease in memory capabilities as determined by the Mini-Mental State Examination $^{132}$. Therefore, discovering mechanisms that could be used enhance the expression of these crucial trophic factors in the brains of AD patients has the potential to yield a beneficial effect on the clinical outcomes for individuals suffering from this devastating neurodegenerative disease.

1.3 Neurodegenerative Diseases

Dementia is an umbrella term that refers to several brain diseases characterized by progressive neuronal loss and decline in cognitive, behavioral or motor function. Currently, there are nearly 47 million people who suffer from dementia worldwide $^{133}$. As the global
population continues to age, the incidence of dementia is expected to rise, since age is the most significant risk factor for developing dementia\(^\text{134}\). In fact, it has been projected that the prevalence of neurodegenerative diseases will rise to an alarming 132 million cases by the year 2050\(^\text{133}\); thus, dementia arguably represents the greatest medical concern of the 21st century\(^\text{135-137}\). Dementia includes a number of neurodegenerative diseases ranging from amyotrophic lateral sclerosis (ALS) to PD and AD. Each of these specific forms of dementia have unique symptoms that correlate to distinct pathological features and affected brain regions. For example, ALS is a motor neuron disease that accounts for approximately four to eight percent of all dementias, and is characterized by the progressive spread of limb weakness, cognitive dysfunction and emotional incontinence\(^\text{138}\). PD, which affects an estimated seven million people globally\(^\text{139}\), is characterized by filamentous \(\alpha\)-synuclein deposits, which form insoluble Lewy bodies, and the selective loss of dopaminergic neurons, primarily in the substantia nigra\(^\text{140-142}\). These pathological alterations result in an array of movement-related symptoms, ranging from rigidity of muscles, slowed movement of voluntary muscles, resting tremors, unstable balance and changes in speech patterns\(^\text{140,143}\). Thus, neurodegeneration, which can take many forms due to a variety of causes, represents a tremendous burden to both those suffering from these diseases and those who act as caregivers, as well as to the entire global population, as these diseases generate a tremendous socioeconomic burden on our health care systems\(^\text{133}\).

**1.4 Alzheimer’s Disease**

AD, the most common form of dementia, currently affects upwards of 34 million people worldwide\(^\text{144}\). This disease primarily affects the elderly, as the age of onset for sporadic (non-familial) AD is typically over 65\(^\text{133}\). AD is characterized by progressive deterioration in cognitive and functional abilities, as well as changes in mood and personality, which have been attributed to two pathological hallmarks: extracellular amyloid beta (A\(\beta\)) plaques and intracellular neurofibrillary tangles (NFTs)\(^\text{145,146}\). A\(\beta\) plaques form as a result of overproduction as well as reduced clearance of irregular A\(\beta\) protein. A\(\beta\) peptides cluster together to form oligomers and fibrils, and eventually aggregate as plaque deposit\(^\text{147}\). NFTs, on the other hand, form when the cytoskeletal microtubule filaments composed of tau
become hyperphosphorylated. This hyperphosphorylation causes the microtubules to destabilize and as a result the neuronal cytoskeleton comes apart, causing the neurons to shrivel and shrink due to these intracellular fibrils. As the neurons wither, they become disconnected from one another, hindering neuronal communication, and in many cases, the neurons become necrotic as a result \[148\]. Even though Aβ plaques and NFTs are the defining features of AD, they may or may not represent the root causes of this disease, as there are presently several theories that attempt to explain the pathological phenomena behind the neurodegeneration that occurs in AD brains.

1.4.1 Hypotheses of Alzheimer’s Disease

There are currently several highly related hypotheses that attempt to explain the pathogenesis of AD including: The Cholinergic Hypothesis, the Amyloid Hypothesis, the Tau Hypothesis, the Oxidative Stress Hypothesis, the Trophic Factor Hypothesis and the Inflammation Hypothesis.

1.4.1.1 The Cholinergic Hypothesis of Alzheimer’s Disease

The Cholinergic hypothesis of AD hinges on evidence that there is significant loss of cholinergic neurons in the hippocampus, basal forebrain and frontal cortex of AD patients \[149,150\]. This loss of cholinergic neurons results in a significant decline in acetylcholine (ACh) levels in the AD brain and is proposed to underlie the cognitive deficit and memory loss that define AD. Moreover, three out of the four AD medications that are currently available in Canada are acetylcholinesterase inhibitors, demonstrating that targeting aspects of ACh signaling, at the least, provides a somewhat viable treatment option for reducing AD progression \[149,151\]. However, the limited efficacy of these drugs leads many experts to believe that there are alternate root causes for the cognitive decline observed in AD.
1.4.1.2 The Amyloid Hypothesis of Alzheimer’s Disease

The Amyloid Hypothesis states that the neurodegeneration observed in AD is due to pathological levels of Aβ deposition in the brain, which occurs as a result of increased Aβ production and reduced Aβ clearance\textsuperscript{152}. The main evidence to support this hypothesis is the discovery of mutations in several genes responsible for the processing of amyloid precursor protein, which lead to elevated levels of toxic Aβ oligomers and plaques\textsuperscript{152,153}. However, this theory has several caveats, as the levels of Aβ deposition in the AD brain do not always correlate well with cognitive decline, and Aβ deposition occurs in the brains of all individuals, not just those with AD, albeit to a much lesser extent in healthy subjects\textsuperscript{152,153}.

1.4.1.3 The Tau Hypothesis of Alzheimer’s Disease

The Tau Hypothesis of AD states that the dementia observed in AD is due to the hyperphosphorylation of tau protein, leading to the accumulation of NFTs. This hypothesis is supported by evidence showing that levels of NFT deposition positively correlates with the intensity of cognitive decline in AD\textsuperscript{154}. However, NFTs are not unique to AD, as these formations are found in several other forms of dementia, including Down’s syndrome, ALS and Pick’s disease\textsuperscript{155-157}. Thus, NFTs alone cannot explain the specific cognitive decline observed in AD patients.

1.4.1.4 The Oxidative Stress Hypothesis of Alzheimer’s Disease

Mounting evidence indicating oxidative stress as the driving force behind AD pathogenesis has led to the Oxidative Stress Hypothesis of AD. It has been reported that, when compared to healthy individuals, patients with AD demonstrate increased lipid peroxidation, as indicated by amplified detection of 4-hydroxy-2-nonenal (HNE)-pyrrole and acrolein, particularly surround NFTs\textsuperscript{158,159}. Compared to non-demented controls, protein and DNA oxidation has also been demonstrated in the AD brain, as exemplified by an approximately 23% increase in brain protein-carbonyl content\textsuperscript{160}, a well-defined marker of
protein oxidation, as well as 38% increase in 8-OH-adenine and a 36% increase in 8-OH-guanine, well defined markers of DNA oxidation. It has also been demonstrated that Aβ peptides cause intracellular accumulation of free radicals, as the presence of Aβ peptides has been shown to trigger a two-fold increase in neuronal H₂O₂ quantities.

1.4.1.5 The Trophic Factor Hypothesis of Alzheimer’s Disease

The Trophic Factor Hypothesis of AD is an emerging theory relying on evidence that there are alterations in the levels of brain trophic factors, which are critical to growth and survival of brain cells. It has been established that AD brains contain significantly less BDNF and GNDF than healthy brains (up to 62% and 50% reduction, respectively). Other studies have demonstrated that hippocampal levels of the NGF precursor molecule pro nerve growth factor (proNGF) in post mortem tissue of AD patients can be 41% higher than in control brain tissue. Other studies have determined that serum concentrations of BDNF, GDNF and NGF are reduced (by 21%, 58% and 58%, respectively) in AD patients compared to healthy counterparts, which correlated with a 37% lower memory capabilities as determined by the Mini-Mental State Examination.

1.4.1.6 The Inflammation Hypothesis of Alzheimer’s Disease

The Inflammation Hypothesis of AD states that it is not the pathological formations observed in AD that cause the neurodegeneration, but rather the brain’s persistent and cyclical immune reaction to these formations that results in the symptoms associated with AD. This hypothesis is supported by animal and clinical studies that demonstrate significantly higher activation of astrocytes and microglia in the brain regions with the highest concentrations of Aβ plaques and NFTs (primarily the frontal cortex and hippocampus). The reactive gliosis surrounding these formations coincides with a significant increase in expression of pro-inflammatory cytokines, chemokines and proteolytic enzymes in AD patients. Studies investigating inflammation in the post-mortem brains of AD patients have demonstrated that there is a significant upregulation in cyclooxygenase (COX)-1 and COX-2 (up to two fold), as well as an increase in 12-lipoxygenase (LOX) and 15-LOX.
expression (two and 1.5 fold, respectively)\textsuperscript{167}. This upregulation in COX and LOX expression could in turn lead to upregulated production of numerous inflammatory mediators, including prostaglandins, prostacyclins, thromboxanes and leukotrienes\textsuperscript{168}. Furthermore, AD brains also exhibit a three-fold increase in IL-1β and a 2.5-fold increase in TNF-α concentrations compared to control subjects\textsuperscript{167,169}.

Of the several hypotheses of AD, the Inflammation Hypothesis is rapidly gaining increasing recognition. However, since currently available drugs offer limited relief to those suffering with AD, and there is a tremendous failure rate in clinical trials searching for effective AD medications, perhaps targeting neuroinflammation may offer an alternative strategy. This idea is supported by research showing that long-term use of non-steroidal anti-inflammatory (NSAID) medications, reduces the risk of developing AD (as much as a 0.32 relative risk), compared to individuals who do not consume NSAIDs\textsuperscript{170}. However, clinical trials assessing the effectiveness NSAID drugs in the treatment of AD have yet to be successful\textsuperscript{171,172,173}. Additionally, steroidal anti-inflammatory drugs, such as dehydroepiandrosterone, have failed in AD clinical trials as well\textsuperscript{174}. Perhaps some of these clinical failures may be due to our limited understanding of how these drugs interact with peripheral and central immune systems in vivo. Thus, preventative strategies may currently represent the best options for reducing the prevalence of AD.

1.4.2 Risk Factors for Developing Alzheimer’s Disease

There are several identified risk factors for developing AD. Some risk factors are non-modifiable, and are therefore completely unavoidable, such as age and genetic susceptibility. However, there are several risk factors for developing AD that are potentially modifiable, including type 2 diabetes mellitus (T2DM), midlife hypertension, midlife obesity, physical inactivity, smoking and low education\textsuperscript{134,175}. It has been suggested that introducing deliberate lifestyle changes could alter these modifiable risk factors, thereby reducing an individual’s consequent risk of developing AD. In fact, one meta-analysis projected that up to 50 percent of all cases of AD could potentially be eliminated if the population prevalence of these lifestyle-related risk factors were eradicated\textsuperscript{134}.  

\[ \text{expression (two and 1.5 fold, respectively)} \textsuperscript{167} \]
1.4.2.1 Insulin Signaling and Related Risk Factors for Developing Alzheimer’s Disease

T2DM, one of the most significant and modifiable risk factors for developing AD, is primarily driven by dysregulated production and/or action of one small 51 amino acid peptide hormone: insulin. Insulin is produced and secreted predominantly by pancreatic β-cells\textsuperscript{176}. It is primarily known for its metabolic role in the regulation of blood glucose levels following the ingestion of food, by signaling various tissues, primarily the liver, muscle and adipose tissue to take up glucose and store it as glycogen\textsuperscript{177-180}. The metabolic role of insulin extends beyond regulation of glucose uptake, however, as insulin is known to down-regulate gluconeogenesis, enhance glycogen synthesis, increase adiposity by promoting lipid synthesis and inhibit lipolysis as well as fatty acid esterification\textsuperscript{181-183}. Thus, insulin is a critical hormone for the maintenance of metabolic homeostasis in the body.

Insulin resistance is a condition in which the cells of the body have a reduced response to insulin, even in the presence of elevated concentrations of this hormone. Typically, insulin resistance results from excessive carbohydrate intake, leading to amplified insulin secretion by the pancreatic β-cells in a compensatory effort to normalize rising blood glucose levels. Chronic high blood-glucose levels coupled with chronic hyperinsulinemia can lead to a decreased response to insulin by cells, otherwise known as insulin resistance. However, if carbohydrate intake is in excess of tolerance over extended period of time, the pancreatic β-cells can eventually become less functional and, thereby, produce less insulin. Although detrimental, insulin resistance is not recognized as a disease state. However, when there is a mismatch between insulin produced and demand, insulin resistance can lead to the development of T2DM\textsuperscript{143}.

The loss of insulin signaling in the body has devastating consequences, since insulin is critical to several biological processes. The insulin signaling cascade is initiated upon the binding of insulin to, and the activation of, the insulin receptor (INSR). The INSR is a tetrameric receptor tyrosine kinase\textsuperscript{184}. Upon activation, the INSR interacts with the Src homologous and collagenous protein (Shc), or insulin receptor substrate (IRS)-1 or IRS-2, which in the CNS appear to be redundant proteins, as they seem to be activated interchangeably\textsuperscript{185,186}. In both the periphery and CNS, activation of IRS-1/2 initiates the
PI3K-dependent pathway, which is essential for regulating cell survival, synaptic signaling, cytokines, chemokines, growth factors and transcription of apoptosis regulators, as well as the activation of glycogen synthase. If the INSR activates the Shc receptor substrate, however, the MAPK pathway becomes engaged, which leads to the regulation of cell growth, proliferation and differentiation, the promotion of cytokine transcription and the regulation of protein translation. Thus, the insulin signaling cascade induces an array of diverse and complex cellular responses.

Similar to its role in the periphery, insulin in the brain promotes glucose uptake by glial cells, to be used as a source of energy. However, insulin also has neuromodulatory properties, as evident by the associated increase in serotonin synthesis and inhibition of norepinephrine re-uptake by pre-synaptic neurons. Additionally, insulin plays a pivotal role in CNS cell survival and synaptic plasticity, as it inhibits the activation of apoptosis-inducing peptides, promotes the outgrowth of neurites, enhances synapse formation and mediates neuronal growth and differentiation; thereby, insulin has a significant impact on learning and memory.

Dysregulation of insulin signaling, such as in T2DM, has been implicated in AD onset and pathogenesis. In fact, some experts even believe AD to be a direct consequence of brain insulin resistance, nicknaming AD “Type 3 Diabetes”. Research has shown that activation of insulin signaling pathways are impaired in AD brains, which is likely due to decreased insulin levels in the brains of AD patients. In a healthy brain, insulin signaling blocks the formation of Aβ plaques and NFTs; however, in an AD brain, the insulin signaling cascade is diminished, which instead promotes the formation of Aβ plaques and NFTs.

Chronic peripheral hyperinsulinemia, such as that observed in T2DM individuals, leads to down-regulated transport of insulin into the brain, which ultimately results in a decrease in brain insulin levels and signaling. Moreover, studies show that INSR are upregulated on cells surrounding Aβ plaques; this is likely a compensatory mechanism due to low insulin availability in AD brains. As previously mentioned, AD individuals have elevated CNS TNF-α concentrations, which inhibit tyrosine kinase activity of the INSR, thus further downregulating the insulin signaling cascade and, in turn, reducing the down-stream cell survival and neuromodulatory effects. Since, insulin signaling is critical for cell
survival, neuronal plasticity, learning, memory and inhibition of Aβ plaques and NFT formation, the reduction of insulin in AD brains can contribute significantly to the pathogenesis of AD (Fig. 1.4)\textsuperscript{145,214,215}. Consequently, it is not surprising that T2DM may increase one’s risk of developing AD by up to 50 percent\textsuperscript{134}.

**Figure 1.4** Effects of Type 2 Diabetes Mellitus and Dysregulated Insulin Signaling on the Pathogenesis of Alzheimer’s Disease.

Although the effect of insulin on neurons has been studied extensively, the effect of insulin on glial cells is relatively unexplored.

**1.4.2.2 Incretin Signaling and Related Risk Factors for Developing Alzheimer’s Disease**

In addition to insulin, two highly related metabolic hormones have also recently been implicated as molecules that likely contribute to the development of AD. Those are the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Following nutrient ingestion, the small peptide hormones GLP-1
amino acids) and GIP (42 amino acids) are secreted by intestinal endocrine cells into the blood stream. These hormones navigate to the pancreas, where they stimulate the production and secretion of insulin, making the indirect interactions between insulin and incretins vital to the regulation of energetic homeostasis in the body 177-179.

The GLP-1 and GIP signaling cascades intersect frequently, and the biological outcomes resulting from incretin hormone receptor stimulation are accordingly very similar. GLP-1 binds and activates the seven-transmembrane G-protein coupled GLP-1 receptor (GLP-1R), while GIP binds and activates the seven-transmembrane G-protein coupled GIP receptor (GIPR) 216,217. Binding of incretins to these receptors leads to downstream activation of either the PI3K or the protein kinase A (PKA) pathway, which induces a subsequent range of physiological effects 217. GLP-1 and GIP signaling through the PI3K and PKA pathways leads to the transcriptional regulation of inflammatory mediators including IFN-β, IL-6, inducible NO synthase, MCP-1 and TNF-α. Additional regulatory effects initiated through these pathways include inhibition of apoptosis and upregulated transcription of growth regulators, such as cyclin D1, TNF receptor superfamily member 6 and TNF receptor superfamily member 6 ligand 217-221.

Although the effects of these hormones have been primarily studied in the periphery, several studies now highlight crucial roles for GLP-1 and GIP in the CNS. These incretin hormones are known to cross the BBB by diffusion 222, and GLP-1 is also known to be synthesized by neurons in the brainstem 223. Within the CNS, GLP-1 promotes neuronal growth and cellular proliferation, stimulates neurite outgrowth, and inhibits neuronal apoptosis; thus, GLP-1 has a positive growth-regulating effect in the brain 224. Furthermore, GLP-1 has verified immunomodulatory effects in the CNS, as demonstrated by in vitro murine studies showing that GLP-1 reduces the secretion of IL-1 from activated microglia and astrocytes 225. A separate in vivo murine study has shown that intraperitoneal injections of GIP derivatives decrease microglia activation 226. Another study demonstrated that intraperitoneal injections of the GIP analogue, D-Ala³GIP, reduced expression of 8-oxoguanine, a marker of oxidative stress by up to two fold in the cortex and the hippocampus of AD mice 227. These data indicate that the incretin hormones are probable immune-modifying agents in the CNS, with established effects on both neuronal and glial cells.
When insulin resistance occurs, such as in T2DM, secretion of GLP-1 is also diminished, while biological response to GIP is reduced. Thus, T2DM may be a risk factor for AD due to reduced incretin signaling in the brain. GLP-1 and its enzyme-resistant analogs have been used in several animal studies, and have been shown to improve neuroplasticity, protect against Aβ plaque deposition and prevent memory loss. GIP demonstrated similar effects, as the GIP analog D-Ala2GIP reduced Aβ plaque load and astrocyte activation, as well as minimized oxidative stress in mice brains (Fig. 1.5).

Although some discoveries and advances have been made that establish the role that dysregulation of GLP-1 and GIP may play in the promotion of AD, most studies have focused on the effects of these incretins on neurons. The effects of GLP-1 and GIP on glial cells and neuroinflammation remain understudied. Therefore, the full effects of these incretin hormones on brain activity has yet to be determined.

**Figure 1.5** Effects of Type 2 Diabetes Mellitus and Dysregulated Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotropic Polypeptide Signaling on the Pathogenesis of Alzheimer’s Disease.
1.4.2.3 Physical Activity and Related Risk Factors for Developing Alzheimer’s Disease

In addition to aberrant insulin and incretin signaling, lack of physical activity (PA) has emerged as another important risk factor for developing AD. PA has been defined as a consistent routine of body movement that burns calories, while exercise, a subcategory of PA, is defined as a planned, structured and repetitive activity designed to enhance muscular tone or endurance. Both PA and exercise have been shown to significantly reduce the risk of AD in both animal and human studies.

Although the relationship between leading a sedentary (SED) life and the development of AD is highly complex and not completely understood, it is well established that introduction of PA provides an effective protective mechanism against the onset and progression of neurodegeneration. Leading a SED life can often lead to obesity, which is associated with chronic systemic low-grade inflammation in the periphery. This low-grade chronic inflammation may contribute to neurological disorders through elevated peripheral levels of the inflammatory cytokines IL-1β, IL-6 and TNF-α, which can readily cross the BBB. In addition to the upregulation in pro-inflammatory cytokine levels, overweight individuals often exhibit altered and dysregulated insulin signaling, including lowered CNS insulin levels, altered INSR distribution, reduced INSR activation and inhibited downstream insulin signaling pathways, all of which enhance Aβ plaque deposition and NFT formation in AD. This illustrates the overlapping mechanisms by which T2DM, obesity and SED behavior may promote the cellular hallmarks of AD.

However, it has been shown that introduction of PA can reduce the hazard ratio of developing AD to 0.24. Thus, performing regular PA is highly beneficial in reducing the risk of developing AD.

Several murine studies have highlighted that voluntary wheel running (VWR), a form of PA in mice, results in significant improvements in learning and memory, which correlated with a two-fold increase in hippocampal neurogenesis in the brains of physically active mice. Other studies have discovered that introduction of PA (VWR) or exercise (forced treadmill running) can enhance brain expression of BDNF, vascular endothelial growth factor (VEGF) and IGF-1, and increase learning and memory in healthy rodents, while reducing...
markers of oxidative stress. This evidence remains true when studying the effects of PA and exercise in mouse models of AD. The introduction of exercise to a presenilin 2 mutant mouse model of AD resulted in significantly lower Aβ deposition in the cortex and hippocampus, compared to mice not exposed to exercise. Other murine studies, using NSE/htau23 AD mice, demonstrated that the introduction of exercise can reduce the number of activated microglia 2.7 fold and minimize the activation of astrocytes nine fold. Moreover, randomized controlled clinical trials have revealed that exercise can slow the progression of AD, as evident from the exercise group displaying greater memory capacity, as well as improved cognitive and executive function when compared to the SED group. Since the currently available drugs targeted against AD progression have demonstrated limited efficacy, perhaps the introduction of regular PA may serve not only as a valuable preventive option against the development of AD, but also as a possible disease modifying strategy.

Although several studies have determined that PA is beneficial to the brain by way of increasing brain levels of trophic factors, serotonin and dopamine, very few studies have looked at the glial cell and neuroimmune-modifying potential of PA, particularly in non-diseased and non-aged models. Therefore, it is possible that PA provides benefits to the brain through glial cell-mediated mechanisms that have yet to be discovered.

1.5 Cell Culture Models

Many of the experiments in this thesis utilized immortalized cell lines to model cells of the CNS. The cell lines utilized were derived from cancerous tissue, or were experimentally created; thus, these cell lines constitute a sub-population of cells that bear some form of mutation, which permits their survival for extended periods of time in vitro. Cell lines are invaluable for research in cell biology as they are inexpensive, readily available, have a rapid proliferation rate, are easy to maintain and can be used for high throughput assays. However, the mutations that are characteristic features of these cell lines, results in a deviation of such cells from their primary counterparts in terms of morphology, gene expression, secretome and/or function. The use of primary cells, which are obtained directly from human or animal tissue, have limitations as well, including difficulty...
obtaining these cells, slow proliferate rate (or no proliferation) and limited life span, all of which limit the routine use of primary cells in majority of the experiments described in this thesis. Since primary cells may represent native tissue cells more accurately than cell lines, they are therefore considered to be better models of in vivo cell behavior. Significant findings that were discovered using cell lines have been confirmed in this thesis using primary cells.

The experiments in this thesis utilized the U-118 MG and the U-373 MG human astrocytoma cell lines, as models of astrocytes, in polymerase chain reaction (PCR) experiments. Both U-118 MG and U-373 MG cells were originally derived from glioblastoma tissue extracted from middle-aged Caucasian males. It has been demonstrated that U-373 MG and U-118 MG express specific astrocyte markers, comparable to their primary astrocyte counterparts, such as glial fibrillary acidic protein (GFAP), glutamate transporter (GLT)-1 and excitatory amino acid transporter 1 (EAAT1). These cells are commonly used in cell culture studies as models of human astrocytes.

Human THP-1 promonocytic cells and murine BV-2 microglial cells were used as models of microglia. Human THP-1 cells were originally derived from a 1-year old male with acute monocytic leukemia. THP-1 promonocytic cells possess receptors that are associated with microglia, including complement receptor 1 and CD11b, and carry out functions characteristic of microglia cells, including cytokine secretion and phagocytosis. Murine BV-2 microglia arose from primary murine microglia that were immortalized after infection with a v-raf/v-myc recombinant retro-virus. When compared to their primary counterparts, BV-2 cells possess a number of similar receptors, secrete the same cytokines, elicit a comparable response to stress and inflammation, and trigger the activation of other glial cells. THP-1 and BV-2 cells are commonly used as representatives of microglia cells in cell culture studies.

The SH-SY5Y human neuroblastoma cell line, as well as the NSC-34 murine motor neuron-like hybrid cell line were used as models of neuronal cells. Human SH-SY5Y cells originate from neuroblastoma tissue from a 4-year old female. These cells possess short fine cell processes, and express markers of neuronal cells including, microtubule associated protein 2 (MAP2), neuron specific endonuclease (NSE) and dopamine transporter (DAT). It has been repeatedly demonstrated that SH-SY5Y cells are an effective model when studying glia-neuron interactions. NSC-34 mouse motor neuron-like hybrid cells were
created from the fusion of motor neuron enriched embryonic mouse spinal cord cells with mouse neuroblastoma. These cells resemble their primary neuronal counterparts by expressing neuronal nuclei marker (NeuN) and synaptophysin. Furthermore, NSC-34 cells have been identified as a valid model for the study of neurodegeneration.

Although these cell lines are invaluable for studying the contribution of particular cell populations to health, homeostasis and disease, isolated cells in mono-culture do not always behave in the same manner as they would in co-culture or in vivo, where multiple cell types are in continuous contact and communication with each other. This is of particular relevance when studying metabolic effects in cell culture reductionist models, as metabolism represents multi-cell, multi-system holistic processes. Nonetheless, studying the discrete aspects of metabolism in particular cell types can help elucidate the mechanisms responsible for integrated biological outcomes.

1.6 Research Hypotheses and Aims

The aim of my thesis work was to investigate the possible mechanisms by which T2DM and a SED lifestyle may lead to the increased risk of AD. Since T2DM, SED behaviour and AD are all associated with varying levels of chronic inflammation, I decided to investigate the potential mechanisms by which T2DM and SED behavior may augment certain features of neuroinflammation observed in the brains of AD individuals. More specifically, I investigated the ways in which aspects of T2DM and SED behavior lead to upregulated glial cell-mediated neuroinflammation, which is a prominent pathological feature of AD.

The overarching hypothesis of my thesis is that the metabolic hormone dysregulation as well as reduced physical activity, contribute to AD pathogenesis through neuroimmune mechanisms.

The first specific hypothesis of my thesis, which is addressed in chapter 2, is that insulin, a metabolic hormone that has reduced functionality in T2DM, regulates the neuroinflammatory response of glia.
To address this hypothesis, I focused on the following specific objectives:

1) Determine whether glial cells have the molecular machinery (receptors and receptor substrates) necessary to detect and respond to insulin.

2) Establish whether insulin regulates the secretion of cytokines by glial cells.

3) Determine whether insulin can protect neurons against glial cell-mediated cytotoxicity.

4) Examine whether IGF-1, a hormone that induces a cell signaling cascade nearly identical to insulin, has a similar effect to insulin on glial cell-mediated cytotoxicity.

The second specific hypothesis, which is addressed in chapter 3, is that the incretin hormones GLP-1 and GIP, which also become dysregulated in T2DM, control several aspects of glial cell function.

To address this hypothesis, I focused on the following specific objectives:

1) Determine whether glial cells express the receptors, which are necessary to detect and respond to GLP-1 and GIP.

2) Establish whether GLP-1 and GIP can regulate microglial cell survival.
   a. Define which microglial receptors are engaged by GLP-1 and GIP.
   b. Examine which microglial intracellular signaling pathways are activated by GLP-1 and GIP.

3) Assess whether GLP-1 and GIP have an impact on the expression of brain trophic factors by microglia.
   a. Determine which microglial intracellular signaling pathways are activated by GLP-1 and GIP to regulate trophic factor expression by glial cells.
   b. Determine whether GLP-1 and GIP have an impact on trophic factor secretion by microglia.

4) Determine whether GLP-1 and GIP have an impact on oxidative stress in microglial cells by examining how these hormones impact intracellular ROS levels, secretion of RNS and expression of antioxidants.

5) Define whether GLP-1 and GIP have an impact on microglial cell-mediated cytotoxicity towards neurons.
The **third specific hypothesis**, which is addressed in chapter 4, is that voluntary PA affects the neuroimmune status of the brain, and that the neuroimmune responses to PA are regulated in an MCP-1-dependent manner.

To address this hypothesis, I focused on the following specific objectives:

1) Determine whether PA has an impact on brain cytokine expression.
   
   a. Investigate whether PA-induced changes in brain cytokine expression are affected by genetic deletion of MCP-1.

2) Determine whether PA impacts glial cell activation markers.
   
   a. Establish whether PA-induced glial cell activation is affected by genetic deletion of MCP-1.

3) Determine whether PA has an impact on immune-regulating receptors in the brain, similar to what has been observed by others in peripheral tissues.

4) Determine whether the brain cytokine expression following PA is similar to changes in serum cytokine levels following PA.
Chapter 2 Insulin Modulates In Vitro Secretion of Cytokines and Cytotoxins by Human Glial Cells

2.1 Overview

AD is the most common form of dementia worldwide. T2DM has been implicated as a risk factor for AD. Since T2DM is a peripheral inflammatory condition, and AD brains exhibit exacerbated neuroinflammation, we hypothesized that inflammatory mechanisms could contribute to the observed link between T2DM and AD. Abnormal peripheral and brain insulin concentrations have been reported in both T2DM and AD. The neurotrophic role of insulin has been described; however, this hormone can also regulate inflammatory responses in the periphery. Therefore, we used in vitro human cell culture systems to elucidate the possible effects of insulin on neuroinflammation. We show that human astrocytes and microglia express both isoforms of the INSR as well as the IGF-1 receptor (IGF-1R). They also express IRS-1 and IRS-2, which are required for propagation of insulin/IGF-1 signaling. We show that at low nanomolar concentrations, insulin could be pro-inflammatory by upregulating secretion of IL-6 and IL-8 from stimulated human astrocytes and secretion of IL-8 from stimulated human microglia. This effect dissipates at higher insulin concentrations. In contrast, insulin at a broader concentration range (10 pM – 1 μM) reduces the toxicity of stimulated human microglia and THP-1 monocytic cells towards SH-SY5Y neuronal cells. These data show that insulin may regulate the inflammatory status of glial cells by modulating their select functions, which in turn can influence the survival of neurons contributing to the observed link between T2DM and AD.

2.2 Background

AD is a devastating neurodegenerative condition, which affects more than 34 million people worldwide, and currently has no cure. Glia, the immune and supporting cells of the brain, become chronically activated in AD brains in response to the accumulation of Aβ plaques and NFTs, which are two of the key pathological hallmarks of AD.
Several studies have shown impairments in insulin signaling in AD brains, presented as lowered insulin concentrations, altered INSR distribution, a decrease in INSR activity, and changes in IRS-1 and IRS-2 protein level or activity. Altered brain insulin signaling may be of particular importance to AD pathogenesis since insulin, through activation of the PI3K pathway, blocks the formation of Aβ plaques and NFTs. Insulin also directs glucose uptake into the CNS, promotes neuronal survival by inhibiting apoptosis and regulates learning and memory.

Insulin resistance and T2DM have been identified as risk factors for AD. T2DM affects more than 347 million people worldwide, and the prevalence of T2DM is on the rise, which in turn could lead to increased incidence of AD. T2DM is a complex metabolic disorder characterized by hyperglycemia due to functional insulin resistance and lowered brain insulin concentration due to downregulated insulin transport systems across the BBB.

Recent studies have shown that administering insulin directly to the brain via the intranasal route can alleviate symptoms of mild cognitive impairment. Studies exploring the cellular and molecular mechanisms behind the CNS activity of insulin have mainly focused on its effects on neuronal cells. Even though insulin is known to regulate peripheral inflammatory reactions, very few studies have investigated the effects of insulin on the inflammatory status of the brain, including effects of insulin on glial cells.

We hypothesized that glial cell lines, as well as primary human glia, express functional INSRs and associated signaling molecules, which could modulate pro-inflammatory responses of glial cells including 1) secretion of pro-inflammatory cytokines and 2) their cytotoxic activity. We demonstrate that cultured human microglia and astrocytes express INSRs and that treatment with insulin leads to complex physiological responses of glial cells including a bell-shaped concentration-dependent upregulation of IL-6 and IL-8 secretion, and a decrease in microglia and monocytic cell mediated toxicity towards SH-SY5Y neuroblastoma cells.
2.3 Materials and Methods

2.3.1 Materials

Human recombinant insulin (expressed in yeast), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), tetramethylethylenediamine (TEMED) and triton X-100 were obtained from Sigma Aldrich (Oakville, ON, Canada). Bromophenol blue and GelGreen stain were obtained from Van Waters and Rogers International (Mississauga, ON, Canada). Human recombinant IFN-γ, IL-1β, IL-6 and TNF-α, as well as enzyme-linked immunosorbent assay (ELISA) development kits for MCP-1, IL-6 and IL-8 were purchased from PeproTech (Rocky Hill, NJ, USA). GoTaq Green Master Mix, Aurum ribonucleic acid (RNA) extraction kit and iScript complementary DNA (cDNA) synthesis kit were purchased from Bio-Rad (Mississauga, ON, Canada). All other reagents were obtained from ThermoFisher Scientific (Ottawa, ON, Canada).

2.3.2 Cell Culture

The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human neuroblastoma SH-SY5Y cell line was a gift from Dr. R. Ross, Fordham University, NY. Human primary astrocytes were obtained from epileptic patients undergoing temporal lobe surgery. The specimens were from normal tissue overlying the epileptic foci. The Clinical Research Ethics Board for Human Subjects of the UBC approved the use of human brain materials. Human astrocyte cultures were prepared following previously published protocols and allowed to reach confluence.

Primary human microglia were obtained from post-mortem brain tissues according to established protocols. Tissues were donated to the Banner Sun Health Research Institute (Sun City, AZ, USA) through their Brain and Body Donation Program with informed consent and the approval of the Banner Research Institutional Review Board. Cells were cultured in Dulbecco’s modified Eagle medium: nutrient mixture F-12 Ham (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) (F10 media), penicillin (100 U/ml) and
streptomycin (100 μg/ml) in T-75 flasks incubated at 37°C in humidified 5% CO₂ and 95% air atmosphere.

2.3.3 Ribonucleic Acid Extraction and Reverse Transcription Polymerase Chain Reaction

Total RNA extraction was performed using the Aurum mini kit, according to the protocol provided by the supplier (Bio-Rad). RNA was quantified using an Eppendorf Biophotometer Plus (Thermo Scientific) spectrophotometer. RNA purity values were within the acceptable optical density (OD) \( \frac{260}{280} \) range of 1.8 to 2.0. All RNA samples were stored at -80 °C. One μg of each RNA sample was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad) as per the manufacturer’s instructions. The resulting cDNA was amplified in a total volume of 25 μl (12.5 μl GoTaq Green Master Mix, 5.5 μl nucleotide-free water, 5 μl primers and 2 μl cDNA) using primers specific for the IGF-1R, IRS-1, IRS-2 and the two insulin receptor isoforms INSRA and INSRB. In addition, a primer pair that recognized both isoforms of INSR was used. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as the housekeeping gene. Table 2.1 lists the reverse transcriptase polymerase chain reaction (RT-PCR) sequences of primers used in this study. Amplification of cDNA included the following steps: Denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, with the final extension step lasting 5 min. PCR products were separated on a 12% polyacrylamide gel, stained with 1x GelGreen nucleic acid stain and visualized in the Fluorchem Q image analysis cabinet (Cell Biosciences, Santa Clara, USA).
Table 2.1 Sequences of Primers Used in Insulin Receptor and Insulin Substrate Reverse Transcriptase Polymerase Chain Reaction Experiments.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INSR (both isoforms)</td>
<td>GCT GGA TTA TTG CCT CAA AGG</td>
<td>TGA GAA TCT TCA GAC TCG AAT GG</td>
<td>75</td>
</tr>
<tr>
<td>INSRA</td>
<td>TTT TCG TCC CCC GGC CAT</td>
<td>CCA CCG TCA CAT TCC CAA C</td>
<td>63</td>
</tr>
<tr>
<td>INSRB</td>
<td>TTT CGT CCC CAG AAA AAC CTC T</td>
<td>CCA CCG TCA CAT TCC CAA C</td>
<td>98</td>
</tr>
<tr>
<td>IRS-1</td>
<td>GCC TAT GCC AGC ATC AGT TT</td>
<td>TTG CTG AGG TCA TTT AGG TCT TC</td>
<td>95</td>
</tr>
<tr>
<td>ISR-2</td>
<td>TGA CTT GTC CCA CTT</td>
<td>CAT CCT GGT GAT AAA GCC AGA</td>
<td>77</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>GAG CAG CTA GGA GGG AAT TAC</td>
<td>AAG TTC TGG TTG TCG AGG A</td>
<td>48</td>
</tr>
<tr>
<td>G3PDH</td>
<td>CCA TGT TCG TCA TGG GTG TGA ACC A</td>
<td>GCC AGT AGA GGC AGG GAT GAT GTT C</td>
<td>215</td>
</tr>
</tbody>
</table>

2.3.4 Enzyme Linked Immunosorbent Assay

ELISAs for IL-6, IL-8 and MCP-1 were performed as per the manufacturer’s instructions (PeproTech). To induce cytokine secretion by glial cells, combinations of cytokines at previously determined concentrations were used: human astrocytes were stimulated by a combination of IFN-γ (150 U/ml) plus IL-1β (100 U/ml). For microglia, a combination of IL-6 (1 ng/ml), TNFα (2 ng/ml) and IL-1β (0.1 ng/ml) was used. The detection limits were experimentally determined to be 0.2 ng/ml, 0.06 ng/ml and 0.02 ng/ml for the IL-6, IL-8 and MCP-1 ELISA, respectively.

2.3.5 Cytotoxicity of Primary Human Microglia and Human THP-1 Monocytic Cells towards SH-SY5Y Neuronal Cells

The cytotoxicity experiments were performed as previously described. Briefly, human monocytic THP-1 cells were added to 24-well plates at a concentration of 5 x 10^5 cells/ml, in 0.9 ml of DMEM-F12 medium containing 5% FBS (F5 media), while human microglia were used at 7.5 x 10^4 cells/ml, in 0.9 ml F5 media. To achieve secretion of cytotoxins, cells were exposed to a combination of three different cytokines IL-6 (1 ng/ml),...
TNF-α (2 ng/ml) and IL-1β (0.1 ng/ml) in the absence or presence of a range of insulin concentrations (10 pM - 1 μM). Following 48 h incubation, 0.5 ml of cell-free supernatants were collected for ELISA and 0.4 ml of cell-free supernatants were transferred to 24-well plates containing SH-SY5Y cells. The neuronal cells had been plated 24 h earlier, at a concentration of 2 x 10⁵ cells/ml, in 0.4 ml F5 media. Following 72 h incubation, SH-SY5Y neuronal cell viability was assessed by the MTT assay.

2.3.6 Cell Viability Assay

The MTT assay was performed as previously described. In this assay, MTT is converted to a purple formazan dye by metabolically active cells. The viability of SH-SY5Y cells was determined by adding MTT (0.5 mg/ml) to the SH-SY5Y cell cultures. Following 1-2 h incubation, the dye was solubilized by adding an equal volume of SDS/DMF (20% sodium dodecyl sulphate, 50% N,N-dimethyl formamide, pH 4.7) solvent to each well and measuring absorbance at 570 nm using a spectrophotometer. MTT data are calculated as (O.D._sample – O.D._media) / (O.D._control – O.D._media) x 100%. Where O.D._sample represents the absorbance measurement in each well, O.D._media represents the absorbance measurement in the wells containing media only and O.D._control represents to the absorbance measurement of the cells which received no treatment.

2.3.7 Statistical Analyses

Data obtained were analyzed using randomized-block design analysis of variance (ANOVA), followed by Fisher’s least significant difference (LSD) post-hoc test, as previous studies of similar nature have utilized these statistics. Data are presented as means ± standard error of the mean (SEM). Significance was established at P < 0.05.
2.4 Results

2.4.1 Expression of Insulin Receptors, Insulin-Like Growth Factor-1 Receptor and Insulin Receptor Substrates in Primary Human Glia and Human Glia-Like Cell Lines

Prior to testing the effects of insulin on astrocytes and microglia, it was first established that glial cells express INSRs and insulin signaling molecules. RT-PCR experiments showed that the human monocytic THP-1 and astrocytic U-373 MG cell lines (Fig. 2.1A) as well as human primary microglia and astrocytes (Fig. 2.1B) express both INSR isoforms, IRS-1, IRS-2 and the IGF-1R. G3PDH, which was used as the housekeeping gene, was also detected in all samples studied. Expression levels of some of the genes varied among the different cell types as evident by faint bands for INSRA in U-373 MG and primary human astrocytes.
Figure 2.1 Polyacrylamide Gels Showing Expression of Insulin Receptors, Insulin Receptor Substrate 1, Insulin Receptor Substrate 2 and Insulin-Like Growth Factor 1 Receptor by Human Monocytic THP-1 Cells (A; Left), Human Astrocytoma U-373 MG Cells (A; Right), Primary Human Astrocytes (B; Left) And Primary Human Microglia (B; Right). Total RNA was extracted from the respective cells, converted to cDNA and amplified by RT-PCR using gene-specific primers. Expression patterns of messenger RNAs (mRNAs) by different cell types are representative of three independent experiments. NTC (no template control) is shown for PCR performed with the INSR primers in the absence of cDNA.

2.4.2 Effects of Insulin on Secretion of Pro-Inflammatory Cytokines by Primary Human Astrocytes

Once we established the expression of the mRNA for INSRs in human astrocytes, we tested the effects of insulin on the secretion of two well-studied pro-inflammatory cytokines, IL-6 and IL-8, by primary human astrocytes, since the upregulation of these cytokines has been well established in AD, as well as in T2DM. Unstimulated astrocytes do not secrete detectable levels of these two cytokines, and addition of insulin alone (1 pM – 1 μM) to astrocytes in absence of inflammatory stimuli did not induce secretion of detectable levels of IL-6 or IL-8 (data not shown); therefore, insulin (1 pM – 1 μM) was added to human astrocytes that were stimulated with a combination of IFN-γ (150 U/ml) and IL-1β (100
U/ml). Insulin had a bell-shaped concentration-dependent effect on IL-6 secretion by astrocytes; 1 nM was the only concentration of insulin that significantly enhanced the release of IL-6 by stimulated primary human astrocytes (Fig. 2.2A, P = 0.003, Fisher’s LSD post-hoc test). The effect of insulin on IL-8 secretion by human astrocytes closely resembled its effect on IL-6 secretion described above. Statistically significant enhancement of IL-8 secretion was observed at 1 and 10 nM concentrations only (Fig. 2.2B, P = 0.01 at both concentrations, Fisher’s LSD post-hoc test). Secretion of IL-6 or IL-8 could not be detected in supernatants of human astrocytes treated with insulin (1 pM – 1 μM) alone (data not shown).
Figure 2.2 Interleukin 6 (A) and Interleukin 8 (B) Secretion by Primary Human Astrocytes Stimulated with Interferon γ (150 U/ml) Plus Interleukin 1β (100 U/ml) in the Presence of a Range of Insulin Concentrations (Shown on the Abscissa) for 48 h. Data (means ± SEM) from 11 independent experiments are presented. The detection limits of ELISAs are shown as dotted lines. * P < 0.05, **P < 0.01 different from cells stimulated in the absence of insulin according to the randomized block design ANOVA (F and P values indicated on the figure), followed by Fisher’s LSD post-hoc test.

2.4.3 Effects of Insulin on Secretion of Pro-Inflammatory Cytokines by Primary Human Microglia

Unstimulated human microglia secreted low, but detectable levels of IL-8 and MCP-1 (Fig. 2.3). Microglial secretion of both these cytokines was enhanced significantly by the combination of IL-6 (1 ng/ml), TNF-α (2 ng/ml) and IL-1β (0.1 ng/ml) (Fig. 2.3, P < 0.01 for IL-8 and MCP-1, Fisher’s LSD post-hoc test). Due to limited availability of primary human microglia, only three concentrations of insulin were tested (10 pM, 1 nM and 100 nM).
Primary human microglia stimulated in the presence of 10 pM and 1 nM insulin showed a statistically significant enhancement in IL-8 release compared to the cells stimulated in the absence of insulin (Fig. 2.3A, $P < 0.01$ at both concentrations, Fisher’s LSD post-hoc test). Insulin, when added to stimulated primary human microglia at 1 nM and 100 nM, caused a statistically significant reduction in MCP-1 secretion compared to cells stimulated in the absence of insulin (Fig. 2.3B, $P < 0.01$ at both concentrations, Fisher’s LSD post-hoc test). Effects of insulin alone on human microglia cytokine secretion were not studied since secretion of IL-6 and MCP-1 by the microglia-like human THP-1 cells was not detected after their exposure to insulin (1 pM – 1 μM, data not shown). Furthermore, as described above, insulin alone also did not induce detectable secretion of cytokines in primary human astrocytes.
Figure 2.3 Interleukin 8 (A) and Monocyte Chemoattractant Protein 1 (B) Secretion by Primary Human Microglia Stimulated with a Combination of Interleukin 6 (1 ng/ml), Tumor Necrosis Factor α (2 ng/ml) and Interleukin 1β (0.1 ng/ml) in the Absence or Presence of Three Different Insulin Concentrations (Shown on the Abscissa) for 48 h. Data (means ± SEM) from three to four independent experiments are presented. The detection limits of the corresponding ELISAs are shown as dotted lines. ** P < 0.01, different from cells stimulated in the absence of insulin; ## P < 0.01, different from unstimulated cells; randomized block design ANOVA, followed by Fisher’s LSD post-hoc test. F and P values for stimulated cells according to the randomized block design ANOVA are indicated on the figure.

2.4.4 Toxicity of Stimulated Primary Human Microglia and THP-1 Monocytic Cells Towards SH-SY5Y Neuronal Cells: Effects of Insulin

Our previous studies have shown that supernatants from unstimulated human microglia and human monocytic THP-1 cells are not toxic to the human neuronal SH-SY5Y
cells; however, stimulation of monocytic cells with select combinations of endogenous cytokines induces cytotoxic secretions from these cells \(^{276,278}\). In this study, we used a combination of IL-6 (1 ng/ml), TNF-α (2 ng/ml) and IL-1β (0.1 ng/ml) to induce human microglial toxicity towards SH-SY5Y neuronal cells in the absence or presence of three different concentrations of insulin (Fig. 2.4). After microglia were stimulated for 48 h at 37 °C, their supernatants were transferred to SH-SY5Y cells. Following 72 h incubation, SH-SY5Y cell viability was assessed by the MTT assay. Although not statistically significant, insulin showed a concentration-dependent trend towards reducing the toxicity of microglia supernatants with the limited numbers of observations made (Fig. 2.4).

**Figure 2.4 Viability of Human Neuronal SH-SY5Y Cells Treated with Supernatants from Primary Human Microglia that had Been Left Unstimulated or were Stimulated with a Combination of Interleukin 6 (1 ng/ml), Tumor Necrosis Factor α (2 ng/ml) and Interleukin 1β (0.1 ng/ml) in the Absence or Presence of Three Different Insulin Concentrations (Shown on the Abscissa).** After 48 h, supernatants from microglia were transferred onto SH-SY5Y neuronal cells. Following 72 h incubation, the viability of SH-SY5Y cells was determined by the MTT assay. Data (means ± SEM) from five independent experiments are presented. F and P values for stimulated cells according to the randomized block design ANOVA are indicated on the figure.

The above experiment was repeated by using human THP-1 monocytic cells as a microglia model. In this experiment, THP-1 cells were incubated with different concentrations of insulin in the absence (open bars, Fig. 2.5) or presence (closed bars, Fig. 2.5) of stimulating cytokine cocktail. Insulin on its own did not induce cytotoxicity of THP-1 cells. However, insulin significantly inhibited toxicity of cytokine-stimulated THP-1 cells.
towards SH-SY5Y cells at all concentrations studied (closed bars, Fig. 2.5). Adding insulin directly to SH-SY5Y cells at the time of transfer of supernatants from stimulated THP-1 cells had no effect on neuronal cell viability (data not shown). We used ANOVA to analyze these data (Fig. 2.5), as we were primarily interested in the effects of insulin in the presence of inflammatory stimuli. However, we have included data showing the viability of SH-SY5Y cells in absence of stimuli for visual reference.

**Figure 2.5 Insulin Reduces Toxic Secretions from Stimulated Human Monocytic THP-1 Cells.** THP-1 cells were either stimulated with a combination of IL-6 (1 ng/ml), TNF-\(\alpha\) (2 ng/ml) and IL-1\(\beta\) (0.1 ng/ml) or left unstimulated in the absence or presence of a range of insulin concentrations (shown on the abscissa). After 48 h, supernatants from THP-1 cells were transferred onto SH-SY5Y neuronal cells. Following 72 h incubation, the viability of SH-SY5Y cells was determined by the MTT assay. Data (means ± SEM) from five independent experiments are presented. ** P < 0.01, different from cells stimulated in the absence of insulin; # P < 0.05, different from unstimulated cells; randomized block design ANOVA, followed by Fisher’s LSD post-hoc test. F and P values for both stimulated and unstimulated cells according to the randomized block design ANOVA are indicated on the figure.

2.4.5 Toxicity of Stimulated THP-1 Monocytic Cells Towards SH-SY5Y Neuronal Cells: Effects of Insulin-Like Growth Factor 1

Supernatant transfer experiments were also conducted to establish the effect of IGF-1 on the toxicity of THP-1 cells towards SH-SY5Y cells (Fig. 2.6). Experiments were performed exactly as described above for Fig. 2.5, except that IGF-1 was added to THP-1 cells instead of insulin. The addition of IGF-1 to unstimulated THP-1 cells caused small, but statistically significant, increase in toxicity of THP-1 cell supernatants towards SH-SY5Y
cells according to the Fisher’s LSD post-hoc test. However, IGF-1 did not enhance or inhibit cytotoxicity of stimulated THP-1 cells. We used ANOVA to analyze these data (Fig. 2.6), as we were primarily interested in the effects of IGF-1 in the presence of inflammatory stimuli. However, we found that IGF-1 only had an effect on cells which were not stimulated. We have included data showing the viability of SH-SY5Y cells in presence of stimuli for visual reference.

**Figure 2.6 Effect of Insulin-Like Growth Factor 1 on the Toxicity of THP-1 Cells towards SH-SY5Y Cells.** THP-1 cells were either stimulated with a combination of IL-6 (1 ng/ml), TNF-α (2 ng/ml) and IL-1β (0.1 ng/ml) or left unstimulated in the absence or presence of a range of IGF-1 concentrations (shown on the abscissa). After 48 h, supernatants from THP-1 cells were transferred onto SH-SY5Y neuronal cells. Following 72 h incubation, the viability of SH-SY5Y cells was determined by the MTT assay. Data (means ± SEM) from four independent experiments are presented. **P < 0.01, different from unstimulated cells in the absence of IGF-1; # P < 0.05, different from unstimulated cells; randomized block design ANOVA, followed by Fisher’s LSD post-hoc test. F and P values for both stimulated and unstimulated cells according to the randomized block design ANOVA are indicated on the figure.

2.5 Discussion

Insulin has been shown to have diverse CNS effects, which include the promotion of neuronal and astrocyte survival by inhibiting apoptotic signaling cascades; regulation of synaptic plasticity, by affecting the balance between long-term depression and long-term potentiation, facilitation of neurite outgrowth and synapse formation; and regulation of learning and memory.
Insulin binds and activates either of the two INSR isoforms (INSRA or INSRB), as well as IGF-1R. IRS-1 and IRS-2 are interchangeable insulin signaling molecules, which are critical for perpetuating the insulin signal within the cell after receptor activation. IRS-1/2 are substrates engaged by all three receptors: INSRA, INSRB and IGF-1R. Thus far, insulin CNS signaling has been mainly studied in neurons and astrocytes. The presence of INSR and IGF-1R has been shown in these two cell types in the rat brain. A recent study demonstrated INSR and IRS expression by human astrocytes, and further showed that incubating human astrocytes with insulin led to a dose-dependent increase in Akt phosphorylation and PI3K activation. Treatment of human neurons with insulin or IGF-1 leads to activation of the INSR and downstream signaling molecules. These studies demonstrate that insulin signaling is functional in human astrocytes and neurons. Here we confirmed and extended these studies by demonstrating the expression of mRNAs encoding INSRA, INSRB, IGF-1R, IRS-1 and IRS-2 in human primary microglia and astrocytes as well as in two cell lines commonly used as surrogates of these glial cell types. We also confirmed expression of all of the above mRNAs by human neuronal SH-SY5Y cells (data not shown). These data strongly suggest the presence of the cellular machinery necessary for insulin signaling in human astrocytes and indicate for the first time that microglia functions could also be modified by insulin, similar to neurons and astrocytes. Presence of these receptors in human glial cells could be confirmed through the use of various protein detection methods.

Secretion of IL-6, IL-8 and MCP-1 by glial cells was studied, since these cytokines play an important role in regulating the CNS inflammation. IL-8 and MCP-1 are chemotactic molecules, which recruit microglia to the site of injury during inflammation, while IL-6 has been shown to initiate and perpetuate the chronic neuroinflammatory cycle in several inflammatory diseases. Glial secretion of these cytokines has been established by previous studies; while hyperglycemia has been shown to enhance secretion of IL-6, IL-8, MCP-1 and TNF-α by rodent microglia. Astrocytes secrete a number of pro-inflammatory cytokines including IL-8 and MCP-1. Clinical relevance of these cytokines has also been demonstrated by a recent study demonstrating that IL-6, IL-8 and MCP-1 are increased up to seven fold in T2DM patients compared to the control group without T2DM. Furthermore, Aβ, which accumulates in AD brains, enhances secretion of IL-6 from
astrocytes and clinical studies have shown that IL-6, IL-8 and MCP-1 are increased between four and seven fold in the brains of AD patients compared to non-demented controls.

IL-6 and IL-8 secretion by primary human astrocytes was studied after their stimulation with a combination of IFN-γ and IL-1β in the presence of a range of insulin concentrations (1 pM – 1 μM). Secretion of both cytokines showed a bell-shaped insulin concentration dependence. Insulin at 1 nM concentration caused maximum enhancement of IL-6 secretion by stimulated primary human astrocytes, while 1 nM and 10 nM insulin concentrations led to maximal secretion of IL-8 by human astrocytes. Similar bell-shaped insulin responses have been observed before for INSR activation in primary rat adipocytes, as well as for mitogenic effects of insulin on fibroblasts. Reduced efficacy of insulin at higher concentrations could be due to INSR binding sites becoming saturated, causing stabilization of pre-bound insulin, which in turn may lead to negative-cooperativity between receptor sites. This phenomenon has been shown to be responsible for a bell-shaped insulin response curve in hamster kidney cells.

Insulin was most effective at modulating astrocyte secretions at the 1 – 10 nM range. Such concentrations can be observed in serum, especially in T2DM and pre-diabetic patients, but are quite high for the CNS, where insulin concentrations are typically 10 – 100x lower compared to circulating insulin. Moreover, insulin levels are further decreased in the CNS of T2DM patients due to downregulated insulin transport across the BBB. However, increased inflammation, as is seen in T2DM and obesity, can cause disruptions to the BBB, leading to free passage of small circulating molecules, including insulin, into the CNS. The BBB often becomes compromised in neurodegenerative diseases, including AD and PD, potentially leading to local insulin passage. Although CNS insulin concentrations do not change dramatically, the ever-fluctuating peripheral insulin concentrations can still have a direct effect on astrocytes, which form tight associations with the BBB and are often the first cells to sample constituents entering CNS from the peripheral blood. Additionally, intranasal insulin is typically administered in the order of mid nM concentrations. Therefore, the concentrations of insulin that were effective on human astrocytes in this study could be representative of CNS conditions characterized by high insulin concentrations.
At low concentrations (~10 pM), insulin has been shown to exhibit anti-inflammatory effects on peripheral blood mononuclear cells including reduced production of ROS and nuclear factor kappa B activation; however, at high concentrations, insulin exhibits pro-inflammatory effects on activated macrophages. Since microglia belong to the mononuclear phagocyte system, we hypothesized that insulin may have similar immunomodulatory effects on this cell type. When primary human microglia were stimulated with a combination of endogenous cytokines (IL-6, TNF-α and IL-1β) in the presence of a range of insulin concentrations (10 pM – 100 nM), their secretion of IL-8 was enhanced in the presence of 10 pM and 1 nM insulin exhibiting a bell-shaped insulin response curve similar to the effect of insulin on cytokine secretion by astrocytes. On the contrary, MCP-1 secretion by stimulated human microglia was inhibited by insulin in a concentration-dependent manner; this effect was statistically significant at higher insulin concentrations of 1 nM and 100 nM. 10 pM insulin is within the normal range for CNS insulin concentrations. One nM and higher insulin concentrations could model a disrupted BBB, as can be the case during inflammation or therapeutic intervention by, for example, intranasal administration of insulin.

Stimulated microglia secrete a number of molecules, including glutamate, ROS, quinolinic acid and proteases, which are capable of inducing neuronal death. Previous studies have demonstrated that activated glial cell supernatants are toxic towards neuronal cells. Since insulin is known to be neuroprotective, we hypothesized that some of this protective activity of insulin could be due to its ability to reduce cytotoxic secretions by microglia. Human microglial toxicity towards neuronal SH-SY5Y cells was induced by a combination of IL-6, TNF-α and IL-1β. With increasing concentrations of added insulin (10 pM – 100 nM), there was a trend towards a decrease in microglia supernatant toxicity. Although this trend did not reach statistical significance with the numbers of observations made in the case of primary microglia, similar anti-neurotoxic activity of insulin was observed by using human monocytic THP-1 cells as a model of microglia. Similar to microglia experiments, stimulation of THP-1 cells in the presence of insulin reduced the toxicity of their supernatants towards SH-SY5Y cells (Fig. 2.5); and in this case, the protective effect was statistically significant. It is important to note that insulin treatment of microglia and THP-1 cells had no effect on their viability (data not shown).
Furthermore, adding insulin directly to SH-SY5Y cells at the time of transfer of cytotoxic supernatants had no effect on neuronal cell viability, which ruled out the possibility that the protective effects observed were due to insulin being carried over to neuronal cells with monocytic cell supernatants.

Since insulin and IGF-1 can signal through each other’s receptors and demonstrate highly overlapping signaling cascades, we also tested IGF-1 as an inhibitor of monocytic THP-1 cell cytotoxic secretions. IGF-1 (10 pM – 1 μM) did not affect viability of SH-SY5Y cells exposed to supernatants from stimulated THP-1 cells; however, IGF-1 on its own induced small but significant cytotoxicity of THP-1 cells (Fig. 2.6). Therefore, the effects of insulin and IGF-1 on human monocytic THP-1 cell and possibly microglial cytotoxicity appear quite different. Similar discrepancies between the effects of insulin and IGF-1 have been described for other cell types including; keratinocytes, adipocytes, fibroblasts and hepatocytes.

Our results indicate that in addition to its effects on cell viability, neuronal plasticity, synaptic signaling, learning and memory, insulin may play an important role as a modulator of neuroimmune responses of glial cells. Similar to its actions on the peripheral cells, effects of insulin on glial cells appear to be complex. First, the effects of insulin on the secretion of some of the cytokines follow a bell-shaped concentration dependence, which means that insulin may be effective only within a narrow concentration range. Second, insulin has contrasting effects on cellular functions in different cell types. Our study demonstrates that insulin enhances secretion of certain cytokines (IL-6 by human astrocytes, IL-8 by human astrocytes and microglia), while inhibiting secretion of others (MCP-1 by human microglia and cytotoxins by monocytic cells). Even though the in vitro cell culture models used in this study cannot accurately represent cellular interactions in the CNS, the data obtained indicate that two of the main CNS glial cell types may have a capacity to respond to changing CNS insulin concentrations.

The effects of insulin on neuroinflammation in vivo remain to be elucidated, but one possible interpretation of the data obtained could be that insulin at low concentrations exhibits overall pro-inflammatory properties by enhancing glial secretion of inflammatory cytokines IL-6 and IL-8, while at higher concentrations it has anti-inflammatory properties due to suppressing cytotoxic activity of microglia and inhibition of MCP-1 secretion. MCP-1
is responsible for immune cell recruitment during inflammation both in the periphery and CNS; it plays a key role as regulator of neuroinflammation. Furthermore, MCP-1 secretion by microglia is known to have negative effects on neurogenesis. It is possible for brain insulin concentrations to reach nanomolar concentrations used in this study. This could be a result of disrupted BBB or an outcome of intranasal insulin therapy, which is currently under investigation as a possible treatment for AD.

It is important to note that, although we examined the effect of predetermined concentrations of exogenously added insulin on select glial cell function, FBS, which is added to the cell culture media is also known to contain small amounts of insulin (typically 30 pM, according to the supplier of FBS). Therefore, the concentration of insulin in cell culture medium used in this study (5% FBS) can often reach 1.5 pM. Regardless of the presence of this small excess amount of insulin, which was introduced by addition of FBS, the data presented show that addition of insulin to cultured cells can affect their neuroimmune functions in a concentration-dependent manner.

2.6 Chapter Conclusion

Once considered strictly a peripheral hormone, insulin has now been shown to be vitally important for normal brain function. Malfunctions in the insulin signaling cascades can contribute to a variety of brain diseases including AD. Recent success of intranasal insulin in the alleviation of mild cognitive impairment highlights the significance of insulin in the CNS. Our data indicate that both astrocytes and microglia should be considered, along with different neuronal populations, as the probable CNS targets of insulin. Glial cells can have overall neuroprotective or adverse effects on neurons depending on the environment they are exposed to. The balance between these opposing activities is regulated by specific mediators, such as cytokines. Our data indicate that insulin also could participate in regulating glial cell functions in the CNS and that its effect could vary significantly depending on its extracellular concentration. This information should be considered when exploring and optimizing insulin-based therapies in animal models of CNS diseases, and future clinical trials.
Chapter 3 Incretin Hormones Regulate Microglia Oxidative Stress, Survival and Expression of Trophic Factors

3.1 Overview

The incretin hormones GLP-1 and GIP are primarily known for their metabolic function in the periphery. GLP-1 and GIP are secreted by intestinal endocrine cells in response to ingested nutrients. Both GLP-1 and GIP stimulate the production and release of insulin from pancreatic β cells as well as exhibit several growth-regulating effects on peripheral tissues. GLP-1 and GIP are also present in the brain, where they provide modulatory and anti-apoptotic signals to neurons. However, very limited information is available regarding the effects of these hormones on glia, the immune and supporting cells of the brain. Therefore, we set out to resolve whether primary human microglia and astrocytes, two subtypes of glial cells, express the GLP-1R and GIPR, which are necessary to detect and respond to GLP-1 and GIP, respectively. We further tested whether these hormones, similar to their effects on neuronal cells, have growth-regulating, antioxidant and anti-apoptotic effects on microglia. We show for the first-time expression of the GLP-1R and the GIPR by primary human microglia and astrocytes. We demonstrate that GLP-1 and GIP reduce apoptotic death of BV-2 microglia through the binding and activation of the GLP-1R and GIPR, respectively, with subsequent activation of the PKA pathway. Moreover, we reveal that incretins upregulate BV-2 microglia expression of BDNF, GDNF and NGF in a PI3K- and PKA-dependent manner. We also show that incretins reduce oxidative stress in BV-2 microglia by inhibiting the accumulation of ROS and release of NO, as well as by increasing the expression of the antioxidant GPx1 and SOD1. We confirm these results by demonstrating that GLP-1 and GIP also inhibit apoptosis of primary murine microglia, and upregulate expression of BDNF by primary murine microglia. These results indicate that GLP-1 and GIP affect several critical homeostatic functions of microglia, and could therefore be tested as a novel therapeutic treatment option for brain disorders that are characterized by increased oxidative stress and microglial degeneration.
3.2 Background

The incretin hormones, GLP-1 and GIP, are secreted primarily from intestinal L and K cells, respectively. These hormones are best known for their role in stimulating the production and secretion of insulin from pancreatic β cells following the ingestion of nutrients. However, the role of incretins extends beyond strictly metabolic functions. It has been demonstrated that incretins also play a role in regulating growth and mediating inflammation by peripheral cells. Both GLP-1 and GIP promote cellular proliferation and inhibit apoptosis of pancreatic β cells by activating the PKA and PI3K pathways. GIP has been shown to induce the secretion of IL-6 and IL-1β from adipose tissue, while GLP-1 reduces the secretion of IL-6, TNF-α and MCP-1 by adipocytes. Interestingly, GLP-1 and GIP can freely cross the BBB by simple diffusion, and they are also produced by neuronal and glial cells locally in the brain. In the CNS of mice, GLP-1 promotes neuronal growth and proliferation, induces neurite outgrowth, reduces oxidative stress and inhibits neuronal apoptosis, whereas GIP derivatives have been shown to reduce apoptosis of neurons and induce hippocampal neuron progenitor cell proliferation. Since these incretins behave as important neuroprotective signaling molecules in the CNS, they have been suggested as promising pharmacological agents for the treatment of several brain disorders, including AD and PD.

To date, most research on the CNS effects of GLP-1 and GIP has focused on their interaction with neuronal cells, with very few studies investigating the effect of these hormones on glia. We hypothesized that since GLP-1 and GIP inhibit apoptosis of neurons, as well as reduce peripheral inflammation and oxidative stress, these incretins could also be indirectly neuroprotective through the regulation of glial cell functions. We demonstrate that several different glial cell types express the GLP-1R and GIPR. We observed that both GLP-1 and GIP inhibit TNF-α-induced apoptosis of BV-2 murine microglia and THP-1 human monocytic cells, upregulate the expression of BDNF, GDNF and NGF by BV-2 cells and reduce oxidative stress in BV-2 microglia. Microglia play a significant role in regulating brain homeostasis by continuously surveying their surroundings for environmental cues of toxins and pathogenic molecules, and also by providing neurons and other cells with chemical support in the form of nutrients and trophic factors. Therefore, GLP-1 and GIP,
which are already being investigated for their potential therapeutic use in brain diseases, such as AD and PD, may protect the brain not only by providing direct benefits to neurons, but also by preventing microglial degeneration and protecting neurons through microglia-mediated mechanisms.

3.3 Materials and Methods

3.3.1 Materials

SensoLyte Homogeneous AMC Caspase -3/7 Assay Kit was purchased from AnaSpec (Freemont, California, USA). Exendin(9-39), a GLP-1R antagonist and Pro(3)GIP, a GIPR antagonist, were purchased through BioLynx (Brockville, ON, Canada). GoTaq Green Master Mix, Aurum Total RNA extraction kit and iScript cDNA synthesis kit were purchased from Bio-Rad (Mississauga, ON, Canada). DAKO rabbit anti-GFAP antibodies, GIP and PI3K inhibitor wortmannin were purchased through Cedarlane Labs (Burlington, ON, Canada). PCR primers for \( \beta \)-actin, CAT, GLP-1R, GIPR, GAPDH, GPx1 and SOD1 were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). GLP-1 was purchased from PeproTech (Rocky Hill, NJ, USA). The following antibodies were purchased from Santa Cruz Biotechnology (San Jose, CA, USA): rabbit anti-mouse BDNF, GDNF, NGF and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies. Acrylamide/bis (29:1, 30% solution), bovine serum albumin (BSA), calf bovine serum (CBS), DMEM-F12, diethanolamine, glycine, penicillin/streptomycin/amphotericin B solution, DMF and SDS were purchased from ThermoFisher Scientific (Ottawa, ON, Canada). Ammonium persulfate, dichlorofluorescin diacetate (DCFHDA), DMSO, ExtrAvidin alkaline phosphatase, fluoroshield with 4’,6-diamidino-2-phenylindole (DAPI), goat serum, Hoechst 33324, lipopolysaccharide (LPS), MTT, N-(1-napthyl)ethylenediamine dihydrochloride (BDH), phosphoric acid, phosphatase substrate tablets, propidium iodide, sodium nitrite, sulfanilamide, TEMED and triton X-100 were obtained from Sigma Aldrich (Oakville, ON, Canada). Bromophenol blue, GelGreen stain, methanol, phosphate buffered saline (PBS) tablets and PKA inhibitor (PKI) were obtained from Van Waters and Rogers International (Mississauga, ON, Canada). Rabbit anti-ionized Ca\(^{2+}\) binding adaptor molecule.
1 (IBA-1) antibodies were purchased from WAKO (Irving, CA, USA).

3.3.2 Cell Culture

The human monocytic THP-1 cells (microglia model), the human U-118 MG and U-373 MG astrocytoma cells were obtained from the ATCC (Manassas, VA, USA). The murine BV-2 microglia cell line was a gift from Dr. G. Garden (Center on Human Development and Disability, University of Washington, Seattle, WA, USA). The human neuroblastoma SH-SY5Y cell line was a gift from Dr. R. Ross (Fordham University, NY, USA). The murine NSC-34 neuronal cells were a gift from Dr. A. Milnerwood (Centre for Applied Neurogenetics and Brain Research Centre, UBC, Vancouver, Canada). cDNA from primary human microglia were obtained from Kinsmen Laboratory of Neurological research, UBC. Human microglia had been extracted from epileptic patients undergoing temporal lobe surgery. The specimens were from normal tissue overlying the epileptic foci. The use of human brain materials was approved by the UBC Clinical Screening Committee for Human Subjects. Primary murine microglia were extracted from adult C57BL/6 mouse brains as previously described except that dispase II was omitted from the cell dissociation medium. Mice were purchased from Charles River and housed at the UBC Okanagan campus under protocols approved by the animal care committee. Microglia in resulting glial cell cultures were immunohistochemically identified as IBA-1 positive and GFAP negative cells. Cells were cultured in DMEM-F12 supplemented with 10% CBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and 250 ng/ml amphotericin B (F10 media) in T-75 flasks incubated at 37°C in humidified 5% CO₂ and 95% air atmosphere.

3.3.3 Ribonucleic Acid Extraction and Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted using the Aurum Total RNA mini kit, according to the protocol provided by the supplier (Bio-Rad). RNA was quantified and RT-PCR was performed as previously described. The RT-PCR primers used in this study are listed in table 3.1. Amplification of cDNA and visualization of amplification products were performed as previous described.
Table 3.1 Sequences of Primers Used in Incretin Receptor Reverse Transcriptase Polymerase Chain Reaction Experiments.

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<th>Target Gene</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
<th>Product Size (bp)</th>
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<td>GCC GCC TGA ACA AAC TCA AG</td>
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3.3.4 Cell Death Assay

Viability of THP-1 and BV-2 cells was monitored using a live/dead cell staining technique. Simultaneous use of two fluorescent dyes allowed for a two-colour differentiation between the population of live and dead cells. The first dye, propidium iodide, which fluoresces red, permeates cells with disrupted cell membranes and was therefore used to detect dead cells. The second dye, Hoechst 33324, which fluoresces blue, was used as the counterstain to detect total cells present (live and dead), as this dye permeates all cell membranes.

Briefly, THP-1 cells were added to immunohistochemistry slides at a concentration of 2 x 10^5 cells/ml in 0.2 ml of F5 media. THP-1 cells were placed in the incubator for 30 min, after which they were exposed to GLP-1 (5 μg/ml) or GIP (5 μg/ml). These concentrations of GLP-1 and GIP were chosen based on our preliminary data and previously published research 377,378. These concentrations of GLP-1 and GIP are higher than typical serum GLP-1 and GIP concentrations, which range from approximately 20 pmol/ml to 400 pmol/ml 379. After 30 min, TNF-α (100 ng/ml) was added to select wells. The cell density and timing of cell stimulation were selected based on previous experiments of similar nature 277,309,313,380.
After 30 min, TNF-α (100 ng/ml) was added to select wells. Vehicle control wells received 5 μl milliQ water. Following 24 h incubation, the F5 media were removed, the cells were centrifuged at 450 g for 7 min and resuspended in 0.2 ml PBS. Propidium iodide (20 μg/ml) and Hoechst 33324 (1 μg/ml) were added to the cells, followed by incubation in the dark for 5 min. Cells were centrifuged as before to remove the dyes and resuspended in PBS for imaging.

The BV-2 cell viability assay was performed exactly as described for THP-1 cells above, except BV-2 cells were placed in the incubator for 24 h to adhere to the well surface following the initial seeding step. Since BV-2 cells are adherent, the media or PBS-dye solutions covering the cells were simply suctioned off and replaced with fresh PBS without having to centrifuge and resuspend the cells.

To determine which receptors mediate the anti-apoptotic effect of GLP-1 and GIP, 1 nM of the GLP-1R antagonist, exendin(9-39), or 1 nM of the GIPR antagonist, Pro(3)GIP, was added to the cell culture media 20 min prior to the addition of the incretins and stimulating agents. In order to determine which specific intracellular signaling pathways were activated by GLP-1 and GIP to produce the anti-apoptotic effect, 10 nM of the PI3K inhibitor, wortmannin, or 10 nM of the PKA inhibitor, PKI, were added to the cell culture media 20 min prior to the addition of the incretins and stimulating agents. Viability of primary murine microglia was assessed exactly as described above for the BV-2 cells, except primary microglia were seeded at a density of 0.5 x 10^5 cells/ml in 0.15 ml of F5 medium.

Images of cells were acquired using Olympus fluorescence microscope and analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA, USA) at excitation/emission 536/617 nm for propidium iodide and 350/470 nm for Hoechst 33324. Three separate images (biological replicates) were taken of each well and cell counts from the three images were averaged to create one N value. Cell numbers were counted using ImageJ software (National Institute of Health) and % of dead cells was calculated.

3.3.5 Caspase-3/7 Assay

THP-1 and BV-2 cells were seeded in 96-well plates and stimulated exactly as described above (section 2.4). Caspase-3/7 activity was assessed using the AnaSpec.
SensoLyte Homogeneous AMC Caspase-3/7 assay kit according to the manufacturer’s instructions. Fluorescence intensity was measured on GloMax-Multi+ Microplate Multimode Reader (Promega, Maddison WI, USA) at excitation/emission 354/442 nm.

3.3.6 Immunofluorescence Assay

The immunofluorescence assay was used to quantify the relative expression of trophic factors by BV-2 cells\textsuperscript{381,382}. Cells were added to immunohistochemistry slides at 2 x 10\textsuperscript{5} cells/ml in 0.2 ml of F5 media, and incubated for 24 h. In the case of primary murine microglia, cells were seeded at a concentration of 0.5 x 10\textsuperscript{5} cells/ml in 0.15 ml of F5 media. After 24 h, 0.2 ml fresh F5 media were added, and cells incubated for 30 min, following which GLP-1 and/or GIP (5 μg/ml) were added to select wells. Vehicle control wells received 5 μl milliQ water. Following treatment with the incretins or their vehicle solution, cells were incubated for 24 h and then assayed for the presence of BDNF, GDNF and NGF. 1 nM wortmannin or 1 nM PKI were added to the culture media 20 min prior to the addition of GLP-1 and GIP. Subsequently, cells were washed with PBS and fixed with ice cold methanol, following which the cells were air dried and washed with PBS three times. Cells were incubated in blocking solution containing 10% goat serum in PBS for 20 min, and washed again with PBS. Cells were incubated with rabbit anti-mouse primary antibodies at optimum dilutions (GDNF 1:50, BDNF 1:50, NGF 1:50) in blocking solution containing 1.5% goat serum in PBS for one h, following which they were washed with PBS three times for 5 min each. Cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:100) for 45 min in blocking solution containing 1.5% goat serum in PBS. Finally, cells were washed three times with PBS, and 100 μl of fluoroshield with DAPI were added to each well. Immunofluorescence was detected using Olympus fluorescence microscope and analyzed using MetaMorph at excitation/emission 350/470 nm for DAPI and 490/525 nm for FITC (BDNF, GDNF, NGF). Two images of DAPI stained nuclei (corresponding to two biological replicates) and two corresponding images of FITC stained trophic factor (corresponding to two biological replicates) were taken of each treatment well. The two images of DAPI stained nuclei were used to determine the cell counts of each treatment type. The two images of the FITC stained trophic factor (either BDNF, GDNF or
NGF) were used to determine the average immunofluorescence per cell for each treatment type. Overlay images were also produced. Immunofluorescence values and cell counts were determined by ImageJ software.

3.3.7 Brain-Derived Neurotrophic Factor Secretion Measurements

BV-2 microglia cells were stimulated as described in section 2.6. After 24 h incubation, cell-free supernatants were collected and stored at -20 °C. BDNF concentrations in supernatant samples were measured by Eve Technologies (Calgary, Canada) using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Assay detection limits were reported to be 0.1 pg/ml.

3.3.8 2’,7’-Dichlorodihydrofluorescein Diacetate Assay for Detection of Reactive Oxygen Species

The 2’,7’-Dichlorodihydrofluorescein Diacetate (H2DCFDA) assay was used to monitor the intracellular production of ROS in BV-2 microglia. Cell membranes are permeable to DCFHDA, which is rapidly converted to dichlorofluorescin (DCFH) by intracellular esterases. This conversion of DCFHDA to DCFH traps the compound within the cell, where it can then be oxidized by intracellular ROS to dichlorofluorescein (DCF), a detectable fluorescent product. BV-2 cells were seeded into 96-well plates at a concentration of 2 x 10^5 cells/ml in 0.2 ml of F5 media. Intracellular production of ROS was induced by adding TNF-α (5 ng/ml). GLP-1 or GIP at various concentrations (200 ng/ml – 5 μg/ml) were added to the cells, followed by TNF-α 20 min later. Vehicle control wells received 5 μl milliQ water. Following 24 h incubation, cells were washed with PBS three times, resuspended in 0.2 ml PBS containing 1 μM DCFHDA and incubated in the dark for 1 h. ROS production was estimated using the FLUOstar Omega microplate reader (BMG Labtech, Nepean, ON, Canada) excitation/emission 485/535 nm.
3.3.9 Detection of Reactive Nitrogen Species by the Griess Assay

The secretion of NO by BV-2 cells was monitored using the Griess assay, which quantifies nitrite (NO$_2^-$), the stable product of NO breakdown$^{384}$, that is released from the cells. When combined with the Griess reagent, NO$_2^-$ changes the culture media solution from colourless to pink, which can be measured spectrophotometrically. BV-2 cells were seeded into 96-well plates at a concentration of 2 x 10$^5$ cells/ml, in 0.2 ml of F5 media. To induce NO secretion, LPS (0.5 μg/ml) was added to BV-2 cells. A range of GLP-1 or GIP concentrations (200 ng/ml – 5 μg/ml) was added to the cells immediately prior to stimulation with LPS. Vehicle control wells received 5 μl milliQ water. Following 24 h incubation, 50 μl of BV-2 culture media from each well were transferred into a new 96-well plate. Sodium nitrite standards (0.1 - 40 μM) were prepared using F5 media. Following the addition of cell supernatants and sodium nitrite standards to the 96-well plate, equal volumes of Griess reagent (1% sulfanilamide, 0.1% BDH, 2.5% phosphoric acid) were added to the wells, and absorbance at 570 nm was immediately measured by FLUOstar Omega microplate reader.

3.3.10 Quantitative Polymerase Chain Reaction

Cells were left untreated or treated with GLP-1 or GIP (5 μg/ml) for 24 h. RNA was extracted and converted to cDNA as described in section 2.3. qPCR was performed and analyzed as previously described$^{385}$, using primers listed in table 3.2.

**Table 3.2 Sequences of Primers Used in Antioxidant Enzyme Quantitative Polymerase Chain Reaction Experiments.**

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<th>Product Size (bp)</th>
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3.3.11 Microglia-Mediated Cytotoxicity Assay

The cytotoxicity experiments were performed as previously described \(^{277,309,313,380}\). Briefly, BV-2 or NSC-34 cells were added to 96-well plates at a concentration of \(2 \times 10^5\) cells/ml in 0.2 ml F5 media. To induce the secretion of cytotoxins, BV-2 cells were exposed to a combination of murine IFN-\(\gamma\) (200 U/ml) plus LPS (0.5 \(\mu\)g/ml), in the absence or presence of a range of GLP-1 or GIP concentrations (1 ng/ml – 5 \(\mu\)g/ml). Incretins were added to BV-2 cells 30 min prior to stimulation with IFN-\(\gamma\) and LPS. Following 24 h incubation, NSC-34 media were replaced with 150 \(\mu\)l of cultured media from BV-2 cells. Following 72 h incubation in the BV-2 cultured media, NSC-34 neuronal cell viability was assessed using the MTT assay.

3.3.12 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay

The MTT assay was performed as previously described \(^{314,315,375}\). This assay measures cell viability by assessing the conversion of MTT to a purple formazan dye by metabolically active cells. MTT (0.5 mg/ml) was added to the NSC-34 cell cultures. Following 1 h incubation, an equal volume of 20\% SDS/50\% DMF (pH 4.7) solvent was added to each well. The plates were incubated for another 3 h to allow for complete solubilization of the MTT dye followed by absorbance measurement at 570 nm using the FLUOstar Omega microplate reader. MTT data are calculated as \((\text{O.D.}_{\text{sample}} - \text{O.D.}_{\text{media}}) / (\text{O.D.}_{\text{control}} - \text{O.D.}_{\text{media}}) \times 100\%\). Where \(\text{O.D.}_{\text{sample}}\) represents the absorbance measurement in each well, \(\text{O.D.}_{\text{media}}\) represents the absorbance measurement in the wells containing media only and \(\text{O.D.}_{\text{control}}\) represents the absorbance measurement of the cells which received no treatment.

3.3.13 Statistical Analyses

Data obtained were analyzed using one-way ANOVA, followed by Dunnett’s or Tukey’s post-hoc test. Data are presented as means \(\pm\) SEM. Significance was established at \(P < 0.05\).
3.4 Results

3.4.1 The Incretin Receptors, Glucagon-Like Peptide 1 and Glucose Dependent Insulinotropic Polypeptide, are Expressed by Brain Cell Types

To determine if glial cells express the incretin receptors GLP-1R and GIPR, which are required for the cellular response to GLP-1 and GIP, respectively, we performed RT-PCR experiments. We found that primary human microglia, THP-1 human monocytes (microglia model), primary human astrocytes, SH-SY5Y human neuronal cells, BV-2 murine microglial cells and NSC-34 murine neurons express the GLP-1R and GIPR, while human U-118 MG astrocytes only express the GLP-1R, and human U-373 MG astrocytes only express the GIPR (Fig. 3.1). Previous studies have demonstrated GLP-1R and GIPR expression at both the mRNA and protein level by murine microglia, astrocytes and neurons, as well as by human neurons. Here, we demonstrate, for the first time, the expression of GLP-1R and GIPR mRNA by primary human microglia and astrocytes. Expression of GLP-1R and GIPR proteins by primary human glia could be confirmed in future studies.

It is important to note that while GLP-1R was detected in all human microglia samples studied, the expression of GIPR by human microglia was not consistent; thus, GIPR mRNA was detected in microglia prepared from two different individuals, but it was absent in microglia prepared from three other individuals. GAPDH, which was used as the housekeeping gene, was detected in all samples studied.
Figure 3.1 Expression of Incretin Receptor Messenger Ribonucleic Acid by Various Cell Types. Polyacrylamide gels showing expression of GLP-1R, GIPR and GAPDH (housekeeping gene) by primary human microglia, THP-1 human monocytes (microglia model), SH-SY5Y human neurons, primary human astrocytes, U-118 MG astrocytes, U-373 MG astrocytes, BV-2 murine microglia and NSC-34 murine neurons. Total RNA was extracted from the respective cells, converted to cDNA and amplified by RT-PCR using gene-specific primers. The mRNA expression patterns by the different cell types are representative of three to five independent experiments. All negative controls prepared by omitting RT or mRNA showed no amplified products (data not shown).

3.4.2 Glucagon-Like Peptide 1 and Glucose Dependent Insulinotropic Polypeptide Inhibit Apoptosis of Microglia via Activation of the Protein Kinase A Pathway

Since GLP-1 and GIP are known to inhibit apoptosis in several peripheral cell types, as well as in CNS neurons, we hypothesized that GLP-1 and GIP may have anti-apoptotic effects on microglia as well. Addition of TNF-α at high concentration (100 ng/ml) increased BV-2 cell death measured 24 h later by approximately 300% above control (Fig. 3.2A). Exposure of BV-2 cells to GLP-1 (5 μg/ml) or GIP (5 μg/ml) for 30 min, prior to the addition of TNF-α, reduced TNF-α-induced BV-2 cell death. Combining GLP-1 and GIP did not lead to an enhanced protective effect when compared to treatment with individual incretins (Fig 3.2A). The decrease in BV-2 cell death was due to a reduction in apoptosis as...
indicated by the decline of caspase-3/7 activity in THP-1 cells following treatment with GLP-1 and GIP (Fig. 3.2F).

Similar to murine BV-2 cells, exposure of human THP-1 monocytic cells to TNF-α (100 ng/ml) increased cell death approximately 300% above control (Fig. 3.2G). Treatment of THP-1 cells with GLP-1 or GIP prior to incubation with TNF-α significantly inhibited microglia cell death (Fig. 3.2G), as well as reduced the activity of caspase-3/7 in THP-1 cells (Fig. 3.2L). We also confirmed that GLP-1 and GIP significantly inhibited TNF-α-induced death of primary microglia extracted from adult mice brains (Fig. 3.3).
Figure 3.2 *Incretins Inhibit Apoptosis of BV-2 Murine Microglia Cells and THP-1 Human Monocytic Cells.* Live/dead staining with propidium iodide (red) and Hoechst 33324 (blue) was used to measure viability of BV-2 cells (A-E) or THP-1 cells (G-K) treated with TNF-α in the absence and presence of GLP-1 or GIP (shown on the abscissa, A and G). Corresponding images of control cells (B, H), and cells exposed to TNF-α (C, I), GLP-1 plus TNF-α (D, J) and GIP plus TNF-α (E, K) are shown (blue = all cells, pink = dead cells). Caspase-3/7 activity was also measured and is presented relative to cells not treated with vehicle, incretins or TNF-α (F, L). Data (means ± SEM) from five to eight independent experiments are presented. *P < 0.05, **P < 0.01 different from control cells treated with vehicle only [v. control]; # P < 0.05, ## P < 0.01 different from cells treated with TNF-α, according to one-way ANOVA followed by Tukey’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.
Figure 3.3 Incretins Inhibit Primary Murine Microglia Cell Death. Staining with fluorescent dyes was used to measure viability of primary murine microglia treated with TNF-α in the presence or absence of GLP-1 or GIP (shown on the abscissa). Data (means ± SEM) from six independent experiments are presented. ** P < 0.01, different from cells treated with vehicle only [v. control]; ## P < 0.01, different from cells treated with TNF-α, according to one-way ANOVA followed by Tukey’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.

To confirm that the observed effects of incretins on microglial cell survival were mediated by their interaction with the specific incretin receptors, we used specific antagonists of the GLP-1R and GIPR. Figure 3.4A and 3.4B illustrate that the cell death inhibitory effects of GLP-1R and GIP were attenuated by specific antagonists of their corresponding receptors. The GLP-1R antagonist exendin(9-39) had significant effect on the cell death inhibition by GLP-1 and not GIP, while GIPR antagonist Pro(3)GIP had a significant effect on cell death by GIP but not GLP-1. The addition of exendin(9-39) or Pro(3)GIP to BV-2 cells in the absence of TNF-α and incretins had no effect on viability of BV-2 cells (data not shown).

To determine which intracellular signaling pathways GLP-1 and GIP activate to inhibit microglia cell death, we used the PI3K pathway inhibitor wortmannin and the PKA pathway inhibitor PKI to demonstrate that the regulation of BV-2 cell death occurred via activation of the PKA, but not the PI3K, pathway (Figs. 3.4C and 3.4D). The addition of wortmannin or PKI to BV-2 cells in absence of other stimuli did not induce death of BV-2 cells (data not shown). In summary, GLP-1 and GIP activate their respective receptors and work through the PKA pathway to inhibit BV-2 microglia death (Figs. 3.4E).
Figure 3.4 Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotrophic Polypeptide Inhibit BV-2 Microglia Cell Death by Engaging the Glucagon-Like Peptide 1 Receptor and Glucose-Dependent Insulinotrophic Polypeptide Receptor, Respectively, and Activating the Protein Kinase A Pathway. Staining with fluorescent dyes was used to measure viability of BV-2 murine microglia treated with the GLP-1R antagonist exendin(9-39) [Ex] or the GIPR antagonist Pro(3)GIP [Pro] (A, B), the PI3K inhibitor wortmannin [Wort] or the PKA inhibitor [PKI] (C, D) prior to exposure of BV-2 cells to TNF-α in the presence of GLP-1 (A, C) or GIP (B, D). Data (means ± SEM) from six to 12 independent experiments are presented. ** P < 0.01, different cells treated with vehicle only [v. control]; # P < 0.05, ## P < 0.01, different from cells treated with TNF-α plus GLP-1 (A, C) or GIP (B, D), according to one-way ANOVA followed by Tukey’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.
3.4.3 The Effect of Glucagon-Like Peptide 1 and Glucose Dependent Insulinotropic Polypeptide on the Expression of Trophic Factors by Microglia

Previous studies have demonstrated that GLP-1 and GIP have neuroprotective properties, while separate studies have shown that engagement of the corresponding GLP-1R and GIPR by these hormones leads to the downstream activation of CREB in peripheral tissues. CREB is a transcription factor with many targets and is known to induce expression of several growth and trophic factors. Thus, we hypothesized that GLP-1 and GIP could potentially be neuroprotective through microglial-specific upregulation of neurotrophic factors. Exposure of BV-2 microglia to GLP-1 (5 μg/ml) or GIP (5 μg/ml) for 24 h increased the expression of BDNF, by approximately 1.7 fold when compared to the unstimulated cells treated with vehicle only (Fig. 3.5B). Exposure of BV-2 cells to GLP-1 (5 μg/ml) or GIP (5 μg/ml) similarly increased the expression of two other trophic factors: GDNF by approximately 1.5 to 1.7 fold (Fig. 3.5D) and NGF, by approximately 1.6 fold (Fig. 3.5F). Exposure of cells to a combination of GLP-1 and GIP did not result in a statistically significant synergistic effect on trophic factor expression by BV-2 cells, even though there was a small increase in BDNF and NGF levels when cells were exposed to both incretins simultaneously. We also confirmed that GLP-1 and GIP could enhance trophic factor expression, by primary adult murine microglia, as indicated by a three- to 3.2-fold upregulation in BDNF expression by these cells following treatment with GLP-1 and GIP, respectively (Fig. 3.5H).
**Figure 3.5** Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotropic Polypeptide Upregulate Trophic Factor Expression by BV-2 Microglia as well as by Primary Murine Microglia. Following 24 h incubation with GLP-1 (5 μg/ml) or GIP (5 μg/ml), BDNF (A, G), GDNF (C) and NGF (E) expression by BV-2 microglia (A – F) and primary murine microglia (G, H) was measured by using immunofluorescence labeling. Mean fluorescence intensity (MFI) of the trophic factor expression (green) per cell (nuclei = blue) is shown relative to values obtained from control cells which did not receive incretins or vehicle (B, D, F, H). Data (means ± SEM) from six to eight independent experiments are presented. * P < 0.05, ** P < 0.01, different from vehicle control [v. control], according to one-way ANOVA followed by Dunnett’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.

The specific PI3K and PKA inhibitors (wortmannin and PKI) were used to demonstrate that the GLP-1- and GIP-induced upregulation of BDNF, GDNF and NGF was mediated by these pathways (Fig. 3.6). GLP-1 and GIP were shown to increase trophic factor expression by microglia in a PI3K- and PKA-dependent manner in all cases, except for GIP-induced expression of BDNF, which was not inhibited by the addition of wortmannin or PKI. Addition of the inhibitors alone did not have a toxic effect on the BV-2 cells as determined by the MTT cell viability assay (data now shown).
Figure 3.6 Effects of Phosphatidylinositol 3 Kinase and Protein Kinase A Inhibitors on Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotropic Polypeptide-Induced Upregulation of Trophic Factor Expression. Immunofluorescence technique was used to measure GLP-1- (A, C, E) or GIP (B, D, F)-induced expression of BDNF (A, B), GDNF (C, D) and NGF (E, F) by BV-2 murine microglia pre-treated with the PI3K inhibitor wortmannin [Wort] or the PKA inhibitor [PKI]. Data (means ± SEM) from six to 12 independent experiments are presented. * P < 0.05, ** P < 0.01 different from cells treated with GLP-1 or GIP alone, according to one-way ANOVA followed by Dunnett’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.

Since the growth factors are secreted by microglia into the surrounding medium, we also studied the effects of incretins on the concentration of one of the growth factors in the
supernatants of BV-2 microglia cells. Fig. 3.7 demonstrates that exposure of BV-2 cells to GLP-1 or GIP resulted in a trend towards increased secretion of BDNF (P = 0.23, according to one-way ANOVA); however, this effect was not statistically significant with the number of observations made.

**Figure 3.7** The Effect of Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotropic Polypeptide on Secretion of Brain-Derived Neurotrophic Factor by BV-2 Microglia. Following 24 h incubation with GLP-1 (5 μg/ml) or GIP (5 μg/ml), BDNF secretion by BV-2 microglia measured using multiplex analysis. Data (means ± SEM) from five independent experiments are presented. No significance according to one-way ANOVA followed by Dunnett’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.

3.4.4 The Effect of Glucagon-Like Peptide 1 and Glucose Dependent Insulinotropic Polypeptide on Oxidative Stress of BV-2 Microglia

Previous studies have shown that GLP-1 and GIP upregulate expression of the antioxidant enzyme, heme oxygenase 1 by endothelial cells. We sought to determine whether these incretins affect the microglial expression of ROS, NO and antioxidant enzymes. Addition of a sub-toxic concentration of TNF-α (5 ng/ml) resulted in an approximately four- to five-fold increase in intracellular ROS production by BV-2, as detected by the H₂DCFDA assay (Figs. 3.8A and 3.8B). Pre-exposure of BV-2 cells to GLP-1 (Fig. 3.8A) or GIP (Fig. 3.8B), significantly reduced intracellular levels of ROS in TNF-α-treated cells. TNF-α at 5 ng/ml, or incretins alone, did not affect viability of BV-2 cells, which was monitored by the MTT assay (data not shown).
Stimulation of BV-2 cells with LPS (0.5 μg/ml) for 24 h resulted in a 12-fold increase in their NO secretion as measured by the NO$_2^-$ accumulation in the cell culture medium. The addition of GLP-1 prior to stimulation with LPS significantly reduced the NO secretion in a concentration-dependent manner (Fig. 3.8C); however, addition of GIP prior to stimulation with LPS had no effect on the secretion of NO by BV-2 cells (Fig. 3.8D). LPS or incretins at the concentrations used, did not affect viability of BV-2 cells monitored by the MTT assay (data not shown).

Since GLP-1 and GIP have been shown to reduce oxidative stress in peripheral tissues $^{393,394}$, qPCR was used to determine whether GLP-1 and GIP upregulate three antioxidant enzymes, GPx1, SOD1 and CAT, which play distinct roles in the reduction of cellular oxidative stress. Treatment with GLP-1 upregulated expression of GPx1 by 1.8 fold and SOD1 by 1.6 fold, but had no effect on the expression of CAT by BV-2 murine microglia (Figs. 3.8E-G). Treatment with GIP also upregulated expression of GPx1 by 1.8 fold, with a trend towards significantly increased expression of SOD1 ($P = 0.20$, according to one-way ANOVA followed by Dunnett’s post-hoc test), but had no effect on upregulation of CAT by BV-2 murine microglia (Figs. 3.8E-G).
Figure 3.8 The Effect of Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotropic Polypeptide on Intracellular Reactive Oxygen Species, Nitrite Secretion and Antioxidant Enzyme Expression by BV-2 Microglia. BV-2 cells were left unstimulated, or stimulated with TNF-α (A, B) or LPS (C, D) in the presence or absence of GLP-1 or GIP (shown on the abscissa). Following 24 h incubation, relative abundance of ROS in BV-2 cells was measured using the H2DCFDA assay (A, B), and NO2- secretion was detected by the Griess assay (C, D). Prior to qPCR analysis, BV-2 cells were left untreated, or treated with GLP-1 (5 μg/ml) or GIP (5 μg/ml). Following 24 h incubation, qPCR was performed using gene-specific primers for GPx1 (E), SOD1 (F) and CAT (G). Gene expression levels are presented relative to the housekeeping gene β-actin. Data (means ± SEM) from seven to 12 independent experiments are shown. * P < 0.05, different from control, # P < 0.05, different from cells incubated with TNF-α only (A, B) or LPS only (C, D), according to one-way ANOVA followed by Dunnett’s post-hoc test. P and F values for the main effects of the one-way ANOVA are also shown.
3.4.5 The Effect of Glucagon-Like Peptide 1 and Glucose Dependent Insulinotropic Polypeptide on Microglia-Mediated Cytotoxicity

Since we demonstrated that GLP-1 and GIP modulate several different microglia functions, we hypothesized that incretins could indirectly affect neuronal survival through glia-mediated mechanisms. Transfer of supernatants from BV-2 cells that had been stimulated with IFN-γ plus LPS onto mice neuronal NSC-34 cells significantly reduced the neuronal cell viability. Instead of utilizing TNF-α, a cytokine used in previous experiments, IFN-γ plus LPS were chosen as stimulants in these experiments, since we determined in preliminary studies that TNF-α alone did not induce BV-2 microglia neurotoxicity towards NSC-34 cells, and previous reports have demonstrated that a combination of IFN-γ plus LPS induce glial cell-mediated neurotoxicity276,277,380. Exposure of BV-2 cells to GIP prior to stimulation with IFN-γ plus LPS reduced BV-2 microglia-mediated toxicity towards NSC-34 cells in a concentration dependent manner (Fig. 3.9B). Exposure of BV-2 cells to GLP-1, however, did not induce similar neuroprotective effect (Fig. 3.9A). GIP did not have direct protective effects on NSC-34 cells since adding this incretin to neuronal cells at the time of their exposure to cytotoxic supernatants from stimulated BV-2 cell did not increase neuronal cell viability (data not shown).
Figure 3.9 The Effects of Glucagon-Like Peptide 1 and Glucose-Dependent Insulinoctropic Polypeptide on Microglia-Mediated Cytotoxicity. BV-2 murine microglia were left unstimulated, or stimulated with IFN-γ plus LPS in the presence or absence of a range of GLP-1 (A) or GIP (B) concentrations (shown on abscissa). Following 24 h incubation, cultured media from BV-2 cells were transferred on to NSC-34 cells. After 72 h incubation, the viability of NSC-34 cells was determined by the MTT assay. Data (mean ± SEM) from eight to 10 independent experiments are presented. # P < 0.05, different from cells stimulated with IFN-γ plus LPS in the absence of GLP-1 or GIP, according to one-way ANOVA followed by Dunnett’s post-hoc test. P and F values for the main effects of one-way ANOVA are also shown.

3.5 Discussion

GLP-1 and GIP have recently entered the research spotlight as possible neuroprotective agents, which may have the potential to slow down the progression of neurodegenerative diseases. Analogues of these hormones, including liraglutide and (Val8)GLP-1-Glu-PAL, which are already approved for the treatment of T2DM, are currently undergoing clinical trials for the treatment of AD, and have been indicated as potential treatment options for PD. Both AD and PD are partially characterized by enhanced brain cell death, glial cell dysregulation and high levels of oxidative stress. GLP-1 and GIP could be beneficial in AD and PD due to their anti-apoptotic, antioxidant and cell growth regulating effects on neurons. However, the full potential of GLP-1 and GIP action has not yet been recognized since very few studies have researched the effects of these incretins on glia.
Here we show for the first time that the GLP-1R and GIPR are present on primary human microglia and astrocytes, and that these incretins could act through their specific receptors to reduce microglial cell death in a PKA-dependent manner. This is of particular significance since Aβ plaques, one of the cellular hallmarks of AD, induce degeneration and apoptosis of microglia. Additionally, apoptosis of microglia is a prominent feature accompanying dopaminergic neuronal cell death seen in PD. Microglia have been shown to become chronically activated in AD and PD, and such overactivation leads to microglial apoptosis, which reduces the number and efficacy of microglia present in the brains of individuals suffering from these pathologies. Therefore, less microglia are available to perform their essential homeostatic and reparative functions, such as supplying neurons with nutrients, growth and repair signals, as well as performing phagocytosis of necrotic cells. Ultimately, the loss of functional microglia in the AD and PD brains leads to exaggerated disease pathology. It is interesting to note that the two astrocytic cell lines, U-118 MG and U-373 MG, differentially expressed the GLP-1R and GIPR; human U-118 MG astrocytes only expressed the GLP-1R, whereas human U-373 MG astrocytes only expressed the GIPR. It is possible that both these astrocyte cell lines express the two incretin receptor types studied; however, expression could not be detected in all cases by the methods used. Alternatively, the unusual pattern of incretin receptor expression by these two astrocytoma cell lines may be due to genomic modifications that are inherently present in cancerous cell lines. It is also possible that the observed patterns of GLP-1R and GIPR expression are due to the undifferentiated state of U-118 MG and U-373 MG cells. Expression of both of these receptors may appear when these cells are differentiated and become more similar to primary astrocytes.

Additionally, neurons rely on the action of glia to protect them and provide them with nutrients, neurotransmitters and survival signals. However, it has yet to be demonstrated that GLP-1 and GIP regulate these functions of glia. We show that GLP-1 and GIP enhance microglial expression of BDNF, GDNF and NGF. Measurements of BDNF in BV-2 cell culture supernatants indicated that these incretins may also induce increased secretion of the growth factors, which are critical for neuronal survival. For example, BDNF has been shown to protect neurons from infection- and injury-induced damage. Age-related deficiency in brain BDNF levels in mice can lead to impaired learning and memory.
GDNF has been proven to be neuroprotective in ischemic injury to the brain \(^\text{128}\). Significant decline in the brain GDNF levels has been shown to induce excessive glutamate release, leading to excitotoxicity of dopaminergic neurons in mice \(^\text{406}\). Similarly, NGF is particularly important in regulating neuronal plasticity and promoting survival of cholinergic neurons in the cerebral cortex, hippocampus, basal forebrain and hypothalamus \(^\text{407}\). Furthermore, it has been demonstrated that AD brains exhibit significant reductions in BDNF concentrations (up to 62\%) \(^\text{130}\), while PD brains have shown declines in GDNF levels \(^\text{408}\), which may partially explain the neuronal degeneration observed in the brains of individuals with AD and PD. Our data indicate that the use of GLP-1 and GIP, or their analogues, in the treatment of AD and PD could be beneficial by increasing the levels of trophic factors supplied to neurons by microglia, which are critical for regulating normal neuronal function and survival \(^\text{126-129}\).

Oxidative stress, which is defined as an imbalance between damaging ROS and antioxidant defenses, is known to produce considerable tissue injury and enhance the progression of several diseases, including AD \(^\text{80}\). The brain is an organ rich in phospholipids, which are particularly susceptible to peroxidation as a result of high levels of ROS. It has been demonstrated that malondialdehyde and HNE, two biomarkers of lipid peroxidation, are significantly increased in the brains of AD patients \(^\text{409}\). This has been further confirmed by researchers who showed that protein carbonyls, HNE and 3-nitrotyrosine (oxidative stress markers), were elevated in the brains of individuals with cognitive decline and memory impairment, and that these markers were correlated with the levels of hippocampal Aβ deposition \(^\text{410}\). Furthermore, Namioka et al. (2016) demonstrated that the increased levels of oxidative stress observed in AD patients were associated with an increase in physical frailty, putting elderly patients at greater risk for injury and death \(^\text{411}\). We demonstrate that GLP-1, in particular, may be beneficial in reducing the pathological and symptomatic changes in AD associated with increased oxidative stress, since GLP-1 reduced intracellular ROS, decreased NO secretion and upregulated expression of the antioxidant enzymes GPx1 and SOD1 by microglia. Therefore, in addition to reducing microglia degeneration, GLP-1 may act as a potential therapeutic agent by targeting the enhanced oxidative stress observed in the brains of AD patients.

We demonstrate that in addition to their previously described direct protective actions on neurons, GLP-1 and GIP may be indirectly neuroprotective by modifying several aspects
of microglial cell function including regulation of cell death, upregulation of trophic factor expression and reduction of oxidative stress (Fig. 3.10). These changes in microglia function are of particular importance since microglia-mediated cytotoxicity, as a result of dysregulated microglial activation, could be damaging to neurons and represents a prominent pathological feature in both AD and PD. Since both GLP-1 and GIP are secreted by intestinal cells, their secretion is altered in such diseases as irritable bowel disease, Crohn’s disease and Colitis. Intriguingly these diseases have been indicated as potential risk factors for developing neurodegenerative diseases and this link may be at least partially due to reduced signaling of incretins to neurons and glia.

Figure 3.10 Overview of the Pathways Affected by Glucagon-Like Peptide 1 and Glucose-Dependent Insulinotropic Polypeptide that Leads to the Increase in Trophic Factor Expression, Upregulation of Antioxidant Enzyme Expression and Reduction in Cell Death of Microglia. AC, adenylyl cyclase; BDNF, brain-derived neurotrophic factor, cAMP, cyclic adenosine monophosphate; GDNF, glial cell-line derived neurotrophic factor; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1R, glucagon-like peptide 1 receptor; GPx1, glutathione peroxidase 1; NGF, nerve growth factor; PKA, protein kinase A; PI3K, phosphoinositide 3-kinase; SOD1, superoxide dismutase 1.
3.6 Chapter Conclusion

The incretin hormones, GLP-1 and GIP, have been shown to be neuroprotective by providing direct benefits to neurons \(^{224,368-371}\) and now, to microglia. Since microglia are critical in regulating brain homeostasis and mounting a response to physiological and pathological stressors, it is essential that when studying the potential of treatment options for neurological diseases, the effects of the treatment on microglia do not go unrecognized. This study demonstrates that GLP-1 and GIP could be neuroprotective by inducing trophic factor expression, reducing apoptosis and upregulating antioxidant enzyme expression by microglia. Several CNS diseases, such as AD, PD, schizophrenia and major depressive disorder are characterized, at least partially, by chronic oxidative stress and microglia degeneration \(^{78,79}\), therefore, the full beneficial effects of GLP-1 and GIP are only just being discovered. GLP-1 and GIP may represent a promising new avenue for treatment of several CNS disorders, which are in desperate need of viable treatment options.
Chapter 4 The Effects of Voluntary Wheel Running on Neuroinflammatory Status: Role of Monocyte Chemoattractant Protein 1

4.1 Overview

The health benefits of exercise and PA have been well researched and it is widely accepted that PA is crucial for maintaining health. One of the mechanisms by which exercise and PA exert their beneficial effects is through peripheral immune system adaptations. To date, very few studies have looked at the regulation of neuroimmune reactions in response to PA. We studied the effect of VWR on pro- and anti-inflammatory cytokine levels, patterns of glial cell activation and expression of toll-like receptor (TLR) 4 in the brains of female C57BL/6 mice. By using homozygous MCP-1 null mice, we investigated the role of this key immunoregulatory cytokine in mediating VWR-induced neuroinflammatory responses. We demonstrated that, compared to their SED counterparts, C57BL/6 mice exposed for seven weeks to VWR had increased levels of pro- and anti-inflammatory cytokines, markers of glial cell activation and TLR4 expression in the brain. Comparison of serum and brain cytokine concentrations revealed that the alterations in brain cytokine levels could not be explained by the peripheral effects of PA. We propose that the modified neuroimmune status observed in the VWR group represents an activated immune system, as opposed to a suppressed immune system in the SED group. Since MCP-1 knockout mice displayed differing patterns of pro- and anti-inflammatory brain cytokine expression and glial activation when compared to their wild-type counterparts, we concluded that the effects of VWR on neuroimmune reactions are modulated by MCP-1. These identified immunomodulatory effects of PA in the brain could contribute to the observed positive relationship between physically active lifestyles and a reduced risk for a number of neurodegenerative diseases that possess a significant neuroinflammatory component.

4.2 Background

It is generally understood that PA is important for maintaining a healthy body. The benefits of both PA (any bodily movement involving the contraction of skeletal muscle that
increases energy expenditure above the basal level) and exercise (a subcategory of PA that involves deliberate, structured and repetitive activity aimed towards enhanced muscular tone or endurance abilities) are well known. PA and exercise enhance cardiovascular endurance, increase muscular strength, boost metabolism, improve signaling of various hormones and decrease adiposity. Research has highlighted the overall anti-inflammatory effect that exercise and PA produces on the body when compared to a SED lifestyle.

Some of the key immune-modulating effects that regular exercise and PA have in the periphery include a reduction in circulating pro-inflammatory cytokines, like TNF-α and MCP-1, with an increase in anti-inflammatory cytokines including IL-10. These cytokines and chemokines are capable of crossing the BBB, and can serve as a means of communication between the peripheral and CNS immune system. There are a number of additional ways in which the CNS and peripheral immune system communicate, including the recently discovered (and still somewhat debated) CNS lymphatic system, extravasation of immune cells across the BBB and secretion of cytokines and chemokines by brain endothelial cells. PA and exercise may alter some or all of the above mechanisms involved in the peripheral to CNS immune system communication.

Exercise can also transform adipose tissue resident macrophages from an M1 to an M2 state. The mechanism behind these phenomena is not fully understood, but is thought to involve the downregulated expression of TLR4 on several cell types, including monocytes, adipocytes, myocytes and hepatocytes, leading to a significant reduction of inflammation. Overall, while much is known about the effects of PA and exercise on peripheral immunity, the effects of PA on the brain immune system are relatively unknown.

PA and exercise can have several beneficial effects on functions of the CNS, such as improved mood and mental health, as well as enhanced memory and cognitive function. Exercise is known to enhance neuronal release of the neurotransmitters serotonin and dopamine. Exercise has also been associated with enhanced long-term potentiation and neurogenesis, as well as higher expression of CNS cell survival signals, like BDNF, GDNF and VEGF. However, only limited information is available regarding the effects of exercise and PA on glia, the immune and helper cells of the brain.

In this study, we used VWR mice to examine the effects of PA on brain cytokines...
production and glial cell activation. We also studied the role that MCP-1 plays in these responses, since previous research has shown that MCP-1 mediates cross talk between the peripheral immune system and the immune system of the brain \textsuperscript{23,434}. MCP-1, which is capable of crossing the BBB \textsuperscript{435}, is produced by many cell types in the periphery as well as the CNS, including smooth muscle cells, fibroblasts, monocytes, astrocytes and microglia \textsuperscript{436}. This immune cytokine is upregulated during pathological events in both the periphery and the CNS, including arthritis, cardiovascular disease, multiple sclerosis and AD \textsuperscript{436}. More specifically, MCP-1 has been shown to increase permeability of the BBB \textsuperscript{23} by downregulating the expression of the tight junction proteins ZO-1 and occludin by brain endothelial cells \textsuperscript{437}. This leads to increased extravasation of immune cells across the BBB \textsuperscript{438,439} and facilitates increased crosstalk between the peripheral and CNS immune systems.

Our results demonstrate that VWR upregulates expression of several pro- and anti-inflammatory cytokines in the brain, stimulates glial cell activation and induces changes in CNS TLR4 expression. We also demonstrate that the neuroimmune-modulatory effects of VWR in the CNS are at least partially dependent on MCP-1. Furthermore, we demonstrate that the changes in brain cytokine levels in response to VWR appear to be independent of changes in circulating peripheral cytokine levels, indicating that PA may have unique and brain-specific immune-modulatory roles.

4.3 Materials and Methods

4.3.1 Materials

Ammonium persulfate, DMSO, ExtrAvidin alkaline phosphatase, phosphatase substrate tablets, protease inhibitor cocktail, sodium deoxycholate, TEMED and Triton X-100 were obtained from Sigma Aldrich (Oakville, ON, Canada). Bromophenol blue was obtained from Van Waters and Rogers International (Mississauga, ON, Canada). Murine ELISA development kits for MCP-1, IL-4, IL-10, TNF-\(\alpha\) and IFN-\(\gamma\) were purchased from PeproTech (Rocky Hill, NJ, USA). Acrylamide/bis (29:1, 30% solution), BSA, CBS, DMEM-F12, diethanolamine, glycine, hydrochloric acid, penicillin/streptomycin, Pierce BCA protein assay kit, SDS and SuperSignal West Pico enhanced chemiluminescent (ECL)
substrate were purchased from ThermoFisher Scientific (Ottawa, ON, Canada). Precision Plus Protein Kaleidoscope ladder was purchased from Bio-Rad (Mississauga, ON, Canada). DAKO rabbit anti-GFAP antibodies were purchased through Cedarlane (Burlington, ON, Canada). Rabbit anti-IBA-1 antibodies were purchased from WAKO (Irving, CA, USA). Rabbit anti-actin antibody, anti-TLR4 and anti-signal transducer and activator of transcription (STAT) 1 antibodies were purchased from SantaCruz Biotechnology (San Jose, CA, USA). Horseradish peroxidase (HRP) labelled goat anti-rabbit antibody was purchased from Cell Signaling (Orange County, CA, USA).

4.3.2 Mice

Female C57BL/6 mice (termed MCP-1+/+) and female MCP-1 knockout (MCP-1−/−) mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Mating pairs were set up to breed in house at the Modified Barrier Facility at the UBC. All procedures involving the care and handling of the mice were approved by the UBC Committee on Animal Care, under the guidelines of the Canadian Council on the Use of Laboratory Animals. Mice were housed individually in “shoebox” style cages (Columbus Instruments) in a temperature-controlled room (22 °C) on a 12 h light/dark cycle with access to food and water ad libitum. At the age of six weeks, mice were randomly divided into two groups: 1) VWR (with access to free running wheel (wheel diameter of 10.16 cm, interior diameter of 9.2 cm, wheel width of 5.1 cm), Columbus Instruments), as a model for PA and 2) SED (with no access to free running wheel). All mice were placed in their respective cages for three days prior to ‘day 1’ of the experiment to acclimate to the wheels and solitude. Mice were housed in these conditions for seven weeks. There were four experimental groups in total: MCP-1+/+ SED (N = 8), MCP-1+/+ VWR (N = 8), MCP-1−/− SED (N = 3) and MCP-1−/− VWR (N = 7). Only three animals were available for the MCP-1−/− SED group.

It is important to note that previous research determined that housing mice individually can have an impact on certain aspects of neuroinflammation; however, it was necessary to house mice in solitude to preclude competition for access to the free running wheel, and to prevent fighting associated with social ranking, which can produce a considerable stress response. To determine whether the mice were, in fact, performing
PA, wheel rotation were counted at 1 h intervals by magnetic switches interfaced to a computer using Windows software. However, due to technical errors beyond our control, wheel rotation numbers were collected for only the first two weeks of the seven-week experimental period, and therefore, there is no wheel data available for weeks three through seven. SED mice were intentionally housed in absence of a locked free running wheel, since studies have demonstrated that rodents climb and play on locked wheels 442, providing a form of PA. Following the seven-week period, mice were anesthetized with isoflurane, blood was collected via cardiac puncture and mice were sacrificed by cervical dislocation. Mice brain tissue and serum were collected and stored at -80 °C.

4.3.3 Brain tissue Dissection and Protein Extraction

Brain tissues were dissected as previously described 443. The frontal cortex, cerebellum and hippocampal areas were separated under the dissecting microscope. Brain sections were placed into microtubes containing 500 μl radioimmunoprecipitation assay (RIPA) buffer with 5 μl of protease inhibitor cocktail. The brain sections were homogenized, incubated on ice for 15 min and then centrifuged at 32,800 x g at 4 °C for 15 min. Protein concentrations in all samples were measured by the Pierce BCA protein kit as per the manufacturer’s instructions. Working stock of protein (1 mg/ml protein) in RIPA buffer was prepared and stored at -20 °C.

4.3.4 Enzyme-Linked Immunosorbent Assay

ELISAs for MCP-1, TNF-α, IL-10, IL-4 and IFN-γ were performed as per the manufacturer’s instructions (PeproTech), and the detection limits were experimentally determined to be 0.9, 0.1, 0.6, 0.1 and 0.5 pg/g of wet tissue, respectively.

4.3.5 Western Blots

100 μg of frontal cortex mice brain protein was denatured in 1x loading buffer at 70 °C for 5 min and ran on a 12% SDS polyacrylamide gel. Protein samples were transferred for
5 h at 60 V onto a nitrocellulose membrane, which was blocked with 5% BSA in tris-buffered saline with 0.1% tween (TBST), and incubated with primary antibodies at optimal dilutions (GFAP 1:20,000; IBA-1 1:2,000; TLR4 1:500; STAT1 1:1,000; or actin 1:500) in 3% BSA/TBST overnight. Membranes were washed with TBST for 10 min 3x and incubated with secondary anti-rabbit antibody (1:10,000) in 3% skim milk powder/TBST for 2 h. Membranes were washed again as before, incubated in ECL reagent for 5 min, imaged using Fluorchem Q image system and analyzed with ImageJ software (National Institute of Health, USA). A common protein sample “MCP-1+/+ SED 1” was run on each gel to serve as a visual indicator of proper sample loading, protein transfer and antibody binding efficiency. Chemiluminescence values obtained from this common control sample band were used to normalize chemiluminescence values obtained from bands on different blots.

Subsequently, intensities of individual bands were normalized against the level of actin, the housekeeping gene, which was measured in the same samples after stripping of the membranes. Western blotting guidelines described by Taylor and Posch (2014) were followed to minimize errors associated with variation in sample loading and protein transfer.

4.3.6 Serum Cytokine Measurements

Cytokine concentrations in mouse serum samples were measured by Eve Technologies (Calgary, Canada) using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and a Milliplex Mouse Cytokine / Chemokine kit (Millipore, St. Charles, MO, USA). Assay detection limits were reported to be 0.1 pg/ml.

4.3.7 BV-2 Cell Culture Experiments

The murine BV-2 microglia cell line was obtained from Dr. G. Garden (Center on Human Development and Disability, University of Washington, Seattle, WA, USA). Cells were cultured in DMEM-F12 supplemented with 10% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in T-75 flasks incubated at 37°C in humidified 5% CO2 and 95% air atmosphere. BV-2 cells were added to 24-well plates at a concentration of 5 x 10⁵ cells/ml in 0.9 ml of DMEM-F12 containing 5% CBS. To model the effects observed in vivo, anti-
murine MCP-1 antibody was added to the BV-2 cells in culture 30 min prior to the addition of 100 μM H$_2$O$_2$, which was used to model oxidative stress associated with PA. Following 48 h incubation, supernatants were collected for ELISA.

4.3.8 Statistical Analysis

Data obtained were analyzed using Shapiro-Wilk normality test, Student’s unpaired t-test and two-way ANOVA, followed by Fisher’s LSD post-hoc test. Average wheel running measurements were log transformed, since daily running averages were not normally distributed for all groups (MCP-1$^{+/+}$ VWR group Shapiro-Wilk normality test, P = 0.02). Data are presented as means ± SEM. Significance was established at P < 0.05. Since the effect of VWR and MCP-1 on brain cytokine levels and glial activation marker expression was the primary objective of this study, a two-way ANOVA was used instead of other statistical tests. Fisher’s LSD post-hoc test was utilized due to unequal sample sizes.

4.4 Results

4.4.1 The Effects of Monocyte Chemoattractant Protein 1 on Voluntary Wheel Running Behavior in Mice

We found there was no significant difference in VWR activity between MCP-1$^{+/+}$ VWR mice and MCP-1$^{-/-}$ VWR mice over the first two weeks of the study (P = 0.23). Thus, this evidence indicates the MCP-1 does not affect VWR behavior in mice (Fig. 4.1).
Figure 4.1 Average Wheel Revolutions per Day in Voluntary Wheel Running Groups of Monocyte Chemoattractant Protein 1<sup>+/+</sup> and Monocyte Chemoattractant Protein 1<sup>-/-</sup> Mice. Data points represent the log transformed daily average number of wheel revolutions per individual mouse in weeks one and two (horizontal bars represent means ± SEM). Differences between the VWR groups during weeks one and two were not statistically significant, according to Student’s unpaired t-test (P = 0.23).

4.4.2 The Effects of Voluntary Wheel Running on Brain Cytokine Levels are Influenced by Monocyte Chemoattractant 1

Changes in the levels of five different cytokines in three different areas of the brain were measured (Fig. 4.2), and summarized in Fig. 4.3. Concentrations of MCP-1, TNF-α, IL-10 and IL-4 were elevated in most of the brain regions from MCP-1<sup>+/+</sup> VWR mice compared to MCP-1<sup>+/+</sup> SED mice. Similarly, the MCP-1<sup>-/-</sup> VWR mice displayed elevated brain concentrations of IL-4 compared to the MCP-1<sup>-/-</sup> SED group, but the MCP-1<sup>-/-</sup> mice did not show upregulation of IL-10 and TNF-α levels. IFN-γ expression in MCP-1<sup>-/-</sup> mice was also upregulated in response to VWR, which was different from MCP-1<sup>+/+</sup> mice.
Figure 4.2 Concentrations of Monocyte Chemoattractant Protein 1 (A), Tumor Necrosis Factor α (B), Interleukin 10 (C), Interleukin 4 (D) and Interferon γ (E) in Three Different Brain Regions of Monocyte Chemoattractant Protein 1+/− and Monocyte Chemoattractant Protein 1−/− Mice Following Exposure to Seven Weeks of Sedentary or Voluntary Wheel Running Conditions. Data (means ± SEM) from three to eight mice are presented. The detection limits of the ELISAs are shown as dotted lines; n.d. = not detected. a P < 0.05 compared to the respective brain section from MCP-1+/− SED mice; b P < 0.05 compared to the respective brain section from MCP-1+/− VWR mice; c P < 0.05 compared to the respective brain section from MCP-1−/− SED mice, according to the two-way ANOVA followed by Fisher’s LSD post-hoc test P and F values for the main effects of two-way ANOVA are also shown. Post hoc analyses were not performed with values below the detection limit.

![Figure 4.2](image)

Figure 4.3 Overview of the Changes in the Brain Cytokine Levels of Monocyte Chemoattractant Protein 1+/− and Monocyte Chemoattractant Protein 1−/− Mice in Response to Voluntary Wheel Running.

4.4.3 The Effects of Voluntary Wheel Running on Astrocytes and Microglia: The Role of Monocyte Chemoattractant 1

Once we discovered that VWR increased the expression of both pro- and anti-inflammatory cytokines in the brains of MCP-1+/− mice, and that MCP-1−/− mice had a differing pattern of brain cytokine expression compared to MCP-1+/− mice, we sought to investigate the activation status of the brain glial cells, which could be the source of these brain cytokines. Western blot analysis confirmed that VWR enhanced expression of GFAP,
an astrocytic cell activation marker in the frontal cortex of MCP-1+/+ mice (Fig. 4.4).

However, VWR did not cause activation of microglia, as evident by the lack of statistically significant upregulation in brain IBA-1 expression in MCP-1+/+ VWR mice compared to MCP-1+/+ SED mice (P = 0.17, Fisher’s LSD post-hoc test) (Fig. 4.4). The MCP-1−/− mice had lower levels of both GFAP and IBA-1, compared to MCP-1+/+ mice. There was no detectable difference in glial cell activation markers between the MCP-1−/− VWR and MCP-1−/− SED mice. We recognize that it is beneficial to perform immunohistochemistry techniques in addition to western blotting to distinguish glial cell activation and proliferation, both of which can lead to increase in glial markers; however, we only had access to mice brain protein, and not to mice brain slices, which are required for immunohistochemical analysis.

**Figure 4.4** Representative Bands of Glial Fibrillary Acidic Protein, Ionized Calcium Binding Adaptor Molecule 1 and Actin Expression in the Frontal Cortex of Sedentary and Voluntary Wheel Running Groups of Monocyte Chemoattractant Protein 1+/+ and Monocyte Chemoattractant Protein 1−/− Mice (A). Chemiluminescence analysis of GFAP (B) and IBA-1 (C) protein expression levels (means ± SEM) from three to five mice normalized against those of actin. a P < 0.05 compared to MCP-1+/+ SED mice; b P < 0.05 compared to MCP-1+/+ VWR mice, according to two-way ANOVA followed by Fisher’s LSD post-hoc test. P and F values for the main effects of two-way ANOVA are also shown.

4.4.4 The Effects of Voluntary Wheel Running on Toll-Like Receptor 4 Expression in the Brains of Mice: The Role of Monocyte Chemoattractant Protein 1

Since the anti-inflammatory effects of PA in the periphery could involve systemic decreases in the expression of TLR4, we investigated whether the observed immune-modifying effects of VWR in the brain were associated with changes in the TLR4 levels. Western blot analysis revealed that, contrary to what has been previously observed in the periphery, TLR4 expression was increased in MCP-1+/+ VWR mice compared to their
SED counterparts (Fig. 4.5). Although the same trend was observed in MCP-1+/− mice, it did not reach statistical significance (P = 0.11, Fisher’s LSD post-hoc test).

**Figure 4.5** Representative Bands of Toll-Like Receptor 4 and Actin Expression in the Frontal Cortex of Sedentary and Voluntary Wheel Running Groups of Monocyte Chemoattractant Protein 1+/− and Monocyte Chemoattractant Protein 1−/− Mice (A). Chemiluminescence analysis of TLR4 protein expression levels (means ± SEM) from three to four mice normalized against those of actin (B). a P < 0.05 compared to MCP-1+/− SED mice; b P < 0.05 compared to MCP-1+/− VWR mice, according to two-way ANOVA followed by Fisher’s LSD post-hoc test. P and F values for the main effects of two-way ANOVA are also shown.

4.4.5 The Effects of Voluntary Wheel Running on Signal Transducer and Activator of Transcription 1 Expression in the Brains of Mice: The Role of Monocyte Chemoattractant Protein 1

Since STAT1 is significantly and transiently upregulated in myocytes following exercise, and has been associated with infiltration of immune cells across the BBB, we sought to investigate whether STAT1 expression was altered in the brain following VWR. We found that total STAT1 expression in the frontal cortex of either MCP-1+/− or MCP-1−/− mice was not affected by VWR (Fig. 4.6). However, MCP-1−/− mice had significantly lower levels of total STAT1, compared to MCP-1+/− mice regardless of the PA status.
Figure 4.6 Representative Bands of Signal Transducer and Activator of Transcription 1 and Actin Expression in the Frontal Cortex of Sedentary and Voluntary Wheel Running Groups of Monocyte Chemoattractant Protein 1+/+ and Monocyte Chemoattractant Protein 1−/− Mice (A). Chemiluminescence analysis of STAT1 protein expression levels (means ± SEM) from three to four mice normalized against those of actin (B). a P < 0.05 compared to MCP-1+/+ SED mice; b P < 0.05 compared to MCP-1+/+ VWR mice, according to two-way ANOVA followed by Fisher’s LSD post-hoc test. P and F values for the main effects of two-way ANOVA are also shown.

4.4.6 Levels of Serum Cytokines in Voluntary Wheel Running and Sedentary Mice

Since the observed changes in brain cytokine levels could be due to altered levels of peripheral blood cytokines caused by VWR, we analyzed mice serum levels of the pro- and anti-inflammatory cytokines studied. No significant differences in serum concentrations of TNF-α, IL-10, IL-4 or IFN-γ, except for between MCP-1+/+ SED and MCP-1−/− VWR for IL-4 (P = 0.02) were detected between any of the mice groups (Table 4.1). Thus, our data indicate that neither VWR, nor the presence or absence of MCP-1 had a detectable effect on the peripheral levels of these cytokines.
Table 4.1 Serum Cytokine Levels in Sedentary and Voluntary Wheel Running Groups of Monocyte Chemoattractant Protein 1+/+ and Monocyte Chemoattractant Protein 1−/− Mice a,b,c.

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1+/+ SED</td>
<td>1.75 ± 0.96</td>
<td>1.48 ± 0.43</td>
<td>0.55 ± 0.27*</td>
<td>n.d.</td>
</tr>
<tr>
<td>MCP-1+/+ VWR</td>
<td>1.81 ± 0.74</td>
<td>1.23 ± 0.53</td>
<td>0.42 ± 0.04</td>
<td>n.d.</td>
</tr>
<tr>
<td>MCP-1−/− SED</td>
<td>1.05 ± 0.32</td>
<td>0.32 ± 0.33</td>
<td>0.27 ± 0.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>MCP-1−/− VWR</td>
<td>1.37 ± 0.33</td>
<td>0.77 ± 0.24</td>
<td>0.21 ± 0.02</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a Data are expressed as (means ± SEM) from 3-4 mice
b n.d. = not detected
c Main effects of two-way ANOVA for TNF-α: PA P = 0.81, F = 0.06; MCP-1 P = 0.46, F = 0.58; for IL-10: PA P = 0.82, F = 0.05; MCP-1 P = 0.07, F = 3.89; for IL-4: PA P = 0.28, F = 1.26; MCP-1 P = 0.02, F = 7.79
*significantly different from MCP-1−/− VWR

4.4.7 Secretion of Cytokines by Cultured BV-2 Mouse Microglia in Response to Reduced Monocyte Chemoattractant Protein 1 Levels

Cell culture studies were used to confirm the role of MCP-1 as a modulator of glial cell cytokine secretion. H2O2 was chosen as the microglial stimulus, since it mimics the production of ROS that follows PA. The effect of PA on the brain redox balance is far more complex than simply applying H2O2 to cultured brain cells; nevertheless, we chose this model since H2O2 has been shown to be produced by mitochondria in rodent brains (60-350 pmol/min/mg of protein) and its concentration increases following exercise. H2O2 was applied to cultured cells on its own in the absence of inflammatory stimuli, additional oxidative stressors or anti-oxidant compounds that are known to be enhanced in the brain following PA. This was done in order to keep the experimental system as simple as possible for confirming the role of MCP-1 in regulating the cytokine release by cells in response to oxidative stress. The addition of 100 μM H2O2 to mouse BV-2 microglial cells significantly enhanced their secretion of MCP-1 (Fig. 4.7A) and IL-4 (Fig. 4.7B), similar to the increases observed in the brains following VWR (Figs. 4.2A and 4.2D).

Neutralizing MCP-1 by use of anti-murine MCP-1 antibodies significantly reduced the levels of not only MCP-1, but also IL-4 in the BV-2 cell supernatants. Thus, microglial IL-4 secretion could be modified by reducing extracellular levels of MCP-1.
TNF-α, IL-10 and IFN-γ by BV-2 cells was also studied, but no induction by 100 μM H₂O₂ was observed for these cytokines (data not shown).

**Figure 4.7** Secretion of Monocyte Chemoattractant Protein 1 (A) and Interleukin 4 (B) by BV-2 Cells that were left Untreated or Stimulated with 100 μM Hydrogen Peroxide in the Presence or Absence of Two Concentrations of Anti-Murine Monocyte Chemoattractant Protein 1 Antibody for 48 h. Data (means ± SEM) from four independent experiments are presented. The detection limits of the corresponding ELISAs are shown as dotted lines. a P < 0.05, compared to untreated cells exposed to vehicle solution only; b P < 0.05, compared to cells stimulated with H₂O₂ in the absence of antibody, according to one-way ANOVA followed by Fisher’s LSD post-hoc test. P and F values for the main effect of one-way ANOVA are also shown.

4.5 Discussion

We chose to investigate the frontal cortex, hippocampal area and cerebellum, since these areas of the brain contribute to the moderation of PA and exercise behavior \(^{450-452}\). More specifically, increased activation of neurons in the frontal cortex has been positively correlated with motivation to run \(^{453}\). A study by Basso and Morrell (2015) confirmed these findings by showing that inactivating neurons of the frontal cortex led to an approximate 40% decrease in wheel running activity in mice, further indicating that the frontal cortex plays a role VWR behavior of rats \(^{454}\). The cerebellum, which is the motor control center of the brain, was an obvious choice for this study, since this brain region plays a major role in motor movement and motor memory \(^{451,455}\).

The hippocampus, which is known for its role in learning and memory, has also been shown to be highly affected by PA. Exercise has been shown to increase the total number of
hippocampal neurons and their activation, as well as the hippocampal production of GDNF, BDNF and several synaptic proteins such as synapsin 1, synaptophysin and postsynaptic density protein-95. It has been suggested that the increased activation of hippocampal neurons during PA implicates them in modulating motor behavior, as demonstrated by Rhodes et al. (2005), who found that a three-fold increase in neuronal activation in the hippocampus led to a 10-fold increase in distance run by the rats. This notion is also supported by Bender et al. (2015), who showed that hippocampal theta oscillations regulate locomotion speed and variability in rodents, and that the hippocampus may be involved in motivation, arousal and motor program selection associated with PA.

We chose to investigate the potential effects of PA on neuroimmune status by use of VWR, since it has been previously demonstrated by many studies that alternate forms of exercise, such as treadmill running, produce a substantial stress response, elevate neuroinflammation and induce neuronal damage. Other studies have demonstrated that although high-impact forced exercise (treadmill running) in mice can lead to improved physical performance, mice exposed to this form of PA demonstrated significant impairment to memory. Thus, many researchers prefer VWR over treadmill running as a model of PA, as this form of PA is less stressful and arguably more neuroprotective. However, this viewpoint is not supported by all researchers, as many other studies have found neuroimmune benefits following forced treadmill exercise. Additionally, some exercise experts are not in favor of VWR as a model of PA, as this form of PA typically only reports ‘total dose’ of exercise, and does not report about pattern or intensity of exercise. Lerman et al. (2002) compared a number of PA-related parameters, including the running distance, duration and average speed across seven different rodent strains exposed to both VWR and treadmill exercise. This study demonstrated that when subjected to VWR, C57BL/6 mice (the strain of mice used in this study), ran at an average speed of 25 m/min, while C57BL/6 mice exposed to treadmill stress were able to run at average maximum speeds of 22 m/min, demonstrating that this strain of mice may perform better when exposed to VWR, compared to treadmill running.

Recent research highlights that moderate exercise and PA have potent anti-inflammatory activities in the periphery, including increased serum levels of IL-10, and
decreased serum levels of IL-6 and TNF-α. However, very few studies have looked at the effects of PA on the immune status of the brain. We investigated CNS levels of several pro- and anti-inflammatory cytokines to establish the cytokine response in the brain following VWR. We showed that VWR induced an overall increase in pro-inflammatory (MCP-1 and TNF-α) and anti-inflammatory (IL-10 and IL-4) cytokines in the frontal cortex, hippocampus and cerebellum of MCP-1+/+ VWR mice. We propose that this overall increase in brain cytokine levels represents activation of the neuroimmune system in the VWR group, as opposed to the suppressed immune system found in SED mice. An activated immune system is more proficient at recognizing and responding appropriately to endogenous stimuli and pathogenic invaders. To better model a SED environment and prevent mice from using objects placed in the cage for climbing and other forms of PA, animals were purposefully housed in the absence of a locked running wheel or toys; however, the lower level of environmental enrichment for animals housed in the absence of running wheel may have contributed to the observed difference in brain immune characteristics between PA and SED groups.

Our data, showing that VWR leads to increases in select brain cytokines levels, are similar to previous findings of Nichol et al. (2008), who reported that Tg25765 mice models of AD had increased levels of IFN-γ and MIP-1α in the hippocampus following exercise. However, our study revealed that VWR did not increase levels of brain IFN-γ, which is typically associated with a robust pro-inflammatory environment, thereby indicating that the increase in brain cytokine levels in MCP-1+/+ VWR mice does not necessarily represent a pro-inflammatory environment but could indicate glial cell activation making them more proficient at protecting the CNS.

We also investigated the role that MCP-1 plays in mediating VWR-induced neuroimmune responses. We focused on MCP-1, since it is upregulated during inflammatory events in the CNS and has also been shown to regulate tight junctions at the BBB, and is therefore critical in mediating cross talk between the peripheral and central immune systems. We showed that MCP-1−/− VWR mice displayed elevated brain levels of IL-4 compared to the MCP-1−/− SED group, similar to what was observed in MCP-1+/+ VWR mice. However, MCP-1−/− VWR mice lost the ability to induce IL-10 compared to their SED counterparts, which was different from what was observed in MCP-1+/+ VWR mice.
data indicate that brain cytokine responses to VWR are at least partially regulated by MCP-1. In contrast to MCP-1+/+ mice, MCP-1−/− VWR mice displayed a significant increase in IFN-γ, a powerful pro-inflammatory cytokine, when compared to their SED counterparts, indicating that lack of MCP-1 mediates a different inflammatory response in the brain.

These changes in brain cytokine levels in response to VWR suggest that glial cells may become activated in response to PA in an MCP-1-mediated manner. Activation of glial cells is critical for the maintenance of CNS homeostasis under normal physiological conditions, as well as during acute tissue damage and other pathological events. Astrocyte and microglial activation is most commonly measured as upregulated expression of GFAP and IBA-1, respectively; therefore, we used these proteins as indicators of glial cell activation. Astrocytes, but not microglia became activated in response to VWR. These results are consistent with previous research, which indicated that PA enhances astrocyte activation. Other studies have demonstrated an overall decline in microglia activation following PA in aged mice, as well as declined activation of both astrocytes and microglia in mouse models of AD upon introduction of PA. A great deal of research has shown that glial cells behave differently with age and in individuals with neurological diseases; however, our study was specifically designed to investigate the neuroimmune-modifying effects of PA in non-aged, non-diseased animals to avoid these potential confounders. Since previous research has shown that PA reduces the chronic glial cell over-activation associated with age and neurodegenerative disease, and our data demonstrate that glial activation is enhanced in VWR mice compared to their SED counterparts, it could be suggested that regular/moderate PA maintains the activation of glia within a homeostatic range, thus achieving an overall beneficial effect. One interpretation of these data is that the neuroimmune responses to VWR may be contingent on MCP-1. A complementary interpretation is that MCP-1−/− mice represent a different neuroimmune phenotype altogether, since MCP-1−/− SED mice had lower baseline expression of GFAP, IBA-1 and STAT1, but expressed higher levels of TNF-α, compared to MCP-1+/+ SED mice. It may not be the lack of MCP-1 that directly changes the neuroimmune responses to PA, but rather, the lack of MCP-1 may change how the brain develops, which leads to different levels or types of glia, thus indirectly causing a different response when animals are exposed to PA. Therefore,
MCP-1 could be modulating the neuroimmune responses to VWR through direct or indirect mechanisms.

It has been suggested that the immune-modulating effect of PA in the periphery is due to the downregulation of TLR4 expression on several cell and tissue types \(^{240,422}\). TLR4 is primarily known to stimulate inflammation in response to bacterial LPS \(^{241}\). However, TLR4 triggers activation of inflammatory signaling cascades in response to a variety of other stimuli, such as heat shock proteins (HSP), high-mobility group box 1, fibrinogen and polyunsaturated fatty acids \(^{481-485}\). It has been proposed that elevated levels of HSP60, in particular, following PA may lead to cross-tolerance of TLR4, and subsequently result in the downregulation of TLR4 on the surface of several peripheral cell types \(^{481}\). We showed that the expression of TLR4 was significantly upregulated in the frontal cortex of MCP-1\(^{+/+}\) VWR mice relative to MCP-1\(^{+/+}\) SED mice, demonstrating that the CNS response to PA may be different from what is observed in the periphery.

In addition to the changes in TLR4 expression following PA, STAT1 is significantly and transiently upregulated in myocytes following exercise \(^{447}\). STAT1 has been associated with infiltration of immune cells across the BBB \(^{448}\) and total STAT1, as opposed to phosphorylated-STAT1, is positively correlated with macrophage infiltration in breast cancer tumors \(^{486}\). We hypothesized that STAT1 expression levels may increase in the brain following PA. However, we found that VWR had no effect on the expression of STAT1 in the frontal cortex of mice. We did discover, however, that STAT1 expression was significantly reduced in MCP-1\(^{-/-}\) mice regardless of PA status. STAT1 therefore represents another key signaling molecule that is regulated by PA differentially in peripheral tissues compared to the CNS. It is interesting to note that MCP-1\(^{-/-}\) mice had an overall reduction in neuroinflammation compared to the MCP-1\(^{+/+}\) mice, as evident by a decrease in both glial cell activation and total STAT1 levels in the frontal cortex. We also showed that VWR induced a neuroimmune response, and MCP-1 appeared to partially govern these reactions.

Measurements of serum cytokine concentrations showed that neither VWR nor presence or absence of MCP-1 had an effect on serum levels of TNF-\(\alpha\), IL-10, IL-4 or IFN-\(\gamma\). It could be proposed that the changes in CNS levels of cytokines can be independent from their peripheral concentrations. The observed lack of changes in peripheral cytokine levels in response to VWR differs from previous research, which demonstrate an overall increase in
circulating anti-inflammatory cytokines, and decrease in circulating anti-inflammatory cytokines with chronic exercise \(^{240,487}\). However, this discrepancy could be due to the more intense exercise regimens, such as forced treadmill running, used in most previous studies \(^{240,418,488,489}\). Previous studies utilizing MCP-1\(^{-/-}\) mice have demonstrated that this cytokine plays a critical role in controlling the serum cytokine levels, particularly pro-inflammatory cytokines \(^{490}\). Thus, an alternative interpretation to these serum cytokine level data is that the sensitivity of the serum cytokine assay was not high enough to detect the changes in serum cytokine levels in response to VWR or absence of MCP-1.

Since microglia represent a significant source of locally produced brain cytokines \(^{58,478}\), these cells could be responding to VWR by altering their secretory profile in an MCP-1-mediated manner \(^{436}\). We showed that by reducing MCP-1 concentration in cell culture medium, it was possible to reduce the secretion of IL-4 by BV-2 mouse microglial cells. These data support previous studies demonstrating that regulation of microglial cytokine secretion by MCP-1 is possible \(^{436}\). However, the in vitro data did not match perfectly the effects that were observed in vivo, which is most likely due to the more complex nature of the CNS environment, including the presence of different cell types and the BBB, and due to the mixing of acute vs. chronic exercise in the cell model.

### 4.6 Chapter Conclusion

Although the neuroimmune-modifying effects of exercise and PA are just emerging, studies indicate that PA has the ability to impact the immune system of the CNS. For example, PA can reduce over-activation of glial cells, such as during brain disease and old age \(^{476-480}\), and long-term exercise has been shown to reduce acute neuroinflammation resulting from traumatic brain injury \(^{237}\). Clinical studies indicate that leading a physically active lifestyle can reduce the risk of developing AD by up to 2.7 fold \(^{134,491}\), and PD by approximately two fold \(^{492}\), with other studies showing that resistance training can delay the progression of ALS \(^{493}\).

Our data indicate that PA may induce astrocyte activation, enhance TLR4 expression and elevate levels of pro- and anti-inflammatory cytokines in the brain. Combined, these data indicate that the immune-modulating effect achieved by PA in the brain allows for modest,
yet significant, increases in the expression of immune modifying receptors and cytokines, which ultimately may enable the immune system to mount a more rapid and effective response to endogenous or exogenous stimuli. In contrast, it is possible that in the absence of these effects, as observed in the SED group, the neuroimmune system lacks the ability to effectively respond to noxious stimuli; thus, representing an overall suppressed immune system, as has been proposed by previous studies. Our finding that MCP-1 has an impact on the neuroimmune-modifying effects of VWR opens up research avenues for developing neuroprotective strategies that focus on targeting MCP-1 and its receptors, particularly for the treatment of CNS disorders such as AD and PD, which have neuroinflammation as one of their fundamental pathological features.
Chapter 5 Conclusion

T2DM and physical inactivity are two major modifiable risk factors for the development of AD. In fact, one particular study calculated the population attributed risks for several risk factors associated with developing AD, and found that T2DM and SED lifestyles, when combined, may contribute to upwards of 15 percent of all AD cases worldwide. The same authors concluded that all seven modifiable risk factors considered in this study may contribute to as much as 50% of the AD cases worldwide\textsuperscript{134}. However, the mechanisms by which T2DM and physical inactivity contribute to the onset and progression of AD remain unclear. In this thesis, I hypothesized that neuroinflammation is partially responsible for this link. My experimental work was focused on studying several specific neuroinflammatory mechanisms by which T2DM and SED behavior may contribute to AD onset and pathogenesis.

AD is characterized by chronic neuroinflammation, increased glial cell death, reduction in trophic factor expression by glial cells, enhanced oxidative stress and an overall increase in glia-mediated toxicity, which were the cellular and molecular mechanisms I focused on during my thesis work\textsuperscript{78,167-169,398,399}. My thesis studied the \textit{in vitro} effects of three T2DM-related metabolic hormones, insulin, GLP-1 and GIP on parameters related to the activation and function of glial cells. I further explored the effects of PA on neuroimmune status of the brain \textit{in vivo}, since PA is known to be an effective preventative and disease management strategy for T2DM. Therefore, the discoveries of my thesis help to elucidate the mechanistic link between T2DM and the increased risk for developing AD.

5.1 Study Considerations and Limitations

Although the observations of chapters 2 and 3 highlight the potential of insulin, GLP-1 and GIP as neuroimmune regulating hormones, they have been made by using mainly cell lines derived from cancerous tissues (THP-1) or immortalized cell lines (BV-2). These cells are robust and have a rapid growth rate, which allows for the performance of high throughput analyses. However, there are inherent concerns when using cell lines, as these cells may differ from their primary counterparts residing in brain tissue with respect to gene or protein
The THP-1 cell line utilized in chapter 2 and 3 to model microglia are in fact promonocytic cells. THP-1 cells express receptors that are associated with microglia, including complement receptor 1 and CD11b, as well as perform functions that are characteristic of microglia cells, including cytokine secretion and phagocytosis. A study by Genin et al. (2015) confirmed that THP-1 cells can also be polarized to an M1 or an M2 phenotype, similar to that of primary human microglia. These functional similarities between THP-1 cells and primary microglia are likely due to their common myeloid lineage. However, some notable differences have been observed between THP-1 cells, particularly in their undifferentiated state utilized in this thesis work, and primary microglia. Such differences include significantly lower secretion of cytokines, such as IL-1β and TNF-α, by THP-1 cells, as well as decreased expression of peroxisome proliferator-activated receptors, when compared to primary microglia. These functional differences between THP-1 and the primary microglia that they represent, could have an impact on the neuroinflammatory effects of insulin and incretins described in chapters 2 and 3.

The murine BV-2 microglia cell line utilized in chapter 3 and 4, represent microglia that were immortalized after infection with a v-raf/v-myc recombinant retro-virus. When compared to their primary counterparts, BV-2 cells possess a number of similar receptors, including CD11b and IBA-1, secrete similar cytokines, such as TNF-α, elicit a comparable response to stress and inflammation, and prompt the activation of other glial cells. Although BV-2 cells and microglia respond in a similar manner to stimulation, such as with LPS, the BV-2 cell response is typically far less pronounced than in their primary counterparts, thus demonstrating that the cell culture experiments utilizing BV-2 cells may represent a muted version of what could be expected from primary microglia cell cultures or in vivo experiments.

This deviation from primary cells may lead to an altered physiological response in immortalized cells and thus, some results obtained using THP-1 or BV-2 cells may not represent the responses that would be typically observed with primary brain cells in vitro or in brain tissue. Therefore, I confirmed several of our observations using primary human or murine glial cells to demonstrate that these results were not restricted to the cell lines used. I demonstrated that similar effects could be observed in microglia model cells and cultured primary microglia, which more accurately represent brain microglia.
U-118 MG and U-373 MG cells were used as representatives of primary human astrocytes in PCR experiments. These cells are commonly used in cell culture studies as models of human astrocytes\textsuperscript{271}. However, they represent undifferentiated human astrocytoma cells, and therefore the detection (or lack of detection), of specific mRNA expression, may be due to genomic modifications inherent of cancerous cell lines, or due to the undifferentiated state of these cells.

Human SH-SY5Y neuronal cells and murine NSC-34 neuronal cells, have commonly been used to model primary CNS neurons, particularly in cell culture experiments that examine neuroinflammation and glia-neuron interactions\textsuperscript{277,283,286}. SH-SY5Y cells have been suggested as representative models of neurons, as they possess short fine cell processes, and express neuronal markers including, MAP2, NSE and DAT\textsuperscript{281,282}. Murine NSC-34 cells, on the other hand, resemble their primary motor neuron counterparts by expressing neuronal markers, such as NeuN and synaptophysin\textsuperscript{285}. The SH-SY5Y neuronal cells utilized in these experiments represent undifferentiated cells, which are much easier to handle than primary dopaminergic neurons, which allows more rapid data collection. However, undifferentiated SH-SY5Y exhibit a less polarized cell shape, and much higher expression of immature neuronal markers, such as NeuN, as well as much lower expression of mature neuronal markers, such as synaptophysin\textsuperscript{282,501}. Similarly, NSC-34 neuronal cells increase expression of multiple neuronal makers following their differentiation, including MAP2 and growth associated protein 43\textsuperscript{286}. Therefore, SH-SY5Y and NSC-34 may not respond quite the same as their primary or differentiated counterparts. In fact, several studies have found that SH-SY5Y and NSC-34 cells are much less responsive to change in environmental stimuli, when compared to their differentiated counterparts or primary neuronal cells\textsuperscript{501-503}. Therefore, the observations made during this thesis work by using neuronal cell cultures may represent subdued functional responses, compared to what may be observed in primary cell culture or \textit{in vivo}.

Mono-cultures of individual cell types are invaluable for studying the contributions of particular cell populations to health, homeostasis or disease; however, cells in isolation do not always express the same receptors, secrete the same substances or function the same way as they would \textit{in vivo}, where multiple cell types are in continuous contact and communication with each other\textsuperscript{287-290}. This is of particular relevance when studying the
metabolism of cells in reductionist models of cell cultures, as metabolism represents a multi-cell, multi-system, holistic process. Nonetheless, studying specific effects of certain aspects of metabolism in particular cell types can help elucidate the mechanisms behind integrated biological outcomes. Mono-cultures were chosen as the primary model system for this thesis work with the purpose of identifying specific effects of hormones on precise cell types of the brain; however, it is possible that in the CNS, where multiple cell types are in continuous contact and communication with each other, these cell types may behave in a different manner 287-290.

Furthermore, in chapter 2, I examined the effect of predetermined concentrations of exogenously added insulin on select glial cell function; however, FBS, which is added to the cell culture media is also known to contain small amounts of insulin (typically 30 pM, according to the supplier of FBS). Since the culture medium in these studies contained 5% FBS, the concentration of insulin could have reached 1.5 pM. Regardless of the presence of this small additional amount of insulin in these experiments, which was introduced by addition of FBS, the data presented in chapter 2 show that insulin can affect the neuroimmune functions of microglia-like cells, in a concentration-dependent manner.

Although the findings of chapter 4 indicate that PA may be influenced by MCP-1, this study has certain limitations that should be noted. At the time of the study, only three MCP-1−/− SED mice were available, and such a low replication number inherently reduced the reliability of these results. Additionally, to better model a SED environment, mice were housed in the absence of a locked running wheel or toys, which could have provided an alternative form of PA 442. This type of environment, however, has been associated with a lower level of environmental enrichment for the SED mice and might have caused the observed difference in brain immune characteristics between the VWR and SED groups 469.

We chose to investigate the potential effects of PA on neuroimmune status by use of VWR, since it has been previously demonstrated by many studies that more intense forms of PA, such as treadmill running, produce a substantial stress response, elevate neuroinflammation and induce neuronal damage, as well as cause significant impairment to memory 462-465. Due to these neuroimmune-related responses to high-intensity exercise, many researchers prefer VWR over treadmill running as a model of PA, as VWR is less stressful and arguably more neuroprotective 464. However, this viewpoint is not universal, as several
A study by Lerman et al. (2002) compared a number of PA-related parameters across seven different rodent strains exposed to both VWR and treadmill exercise, and found that when subjected to VWR, C57BL/6 mice (the strain of mice used in this study), ran at an average speed of 25 m/min, while C57BL/6 mice exposed to treadmill stress were able to run at average maximum speeds of 22 m/min. Therefore, C57BL/6 mice may perform better when exposed to VWR, compared to treadmill running.

Additionally, due to technical errors beyond our control, wheel rotations were counted for only the first two weeks of the seven-week experimental period, and therefore, there is no wheel data available for weeks three through seven. Although we demonstrate that the numbers of wheel rotations during weeks one and two for both the MCP-1−/− and the MCP-1+/+ groups of animals are not different, we cannot rule out that differences in wheel running between the MCP-1−/− and MCP-1+/+ animals may have appeared during weeks three to seven.

Furthermore, we recognize that in the chapter 4 experiments studying glial cell activation, it would have been beneficial to perform immunohistochemistry techniques in addition to western blotting to distinguish glial cell activation and proliferation, both of which could lead to increase in glial markers; however, we only had access to mice brain protein, and not to intact mice brain slices, which are required for immunohistochemical analysis.

5.2 Significance of Research

The experimental work described in chapter 2 addressed the hypothesis that insulin, a metabolic hormone that has reduced functionality in T2DM, regulates the neuroinflammatory response of glia. The results obtained support this hypothesis, since I was able to demonstrate that glial cells possess the receptors and receptor substrates necessary to detect and respond to insulin. Importantly, I showed that treatment of microglia with insulin could produce an overall anti-inflammatory response, by impacting the secretion of cytokines by microglia. I also demonstrated that insulin protected neurons from microglia-mediated cytotoxicity, which could take place in a pro-inflammatory environment, such as that observed in AD.
brains. I found that IGF-1, a hormone with a similar signaling cascade to insulin, did not protect against microglia-mediated neurotoxicity. Thus, I demonstrated that insulin may be neuroprotective by inhibiting glial cell-mediated neuroinflammatory mechanisms. This is of particular importance since AD brains are characterized by chronic neuroinflammation, as well as reduced insulin concentrations and defective insulin signaling. Previous studies have shown that insulin resistance in the brain contributes to AD progression by increasing the deposition of Aβ plaques and formation of NFTs. During my thesis work, I discovered a fundamentally unique mode of action for insulin in the CNS, whereby insulin regulates glial cell functions. Thus, I have shown that dysregulated insulin signaling in the brain may contribute to AD progression through glial cell-mediated mechanisms.

The experimental work described in chapter 3 addressed the hypothesis that the incretin hormones, GLP-1 and GIP, which also become dysregulated in T2DM, control several aspects of glial cell function. The results obtained in chapter 3 support this hypothesis, as I discovered that glial cells possess the receptors necessary to detect and respond to GLP-1 and GIP. I demonstrated that GLP-1 and GIP enhanced cell viability of microglia. I showed that these survival-enhancing effects were mediated through the GLP-1R and GIPR, respectively, and by subsequent activation of the PKA, but not PI3K pathway. I also showed that GLP-1 and GIP upregulated the expression of three different trophic factors by microglia, including BDNF, GDNF and NGF, all of which are critical in regulating the survival of other glia and neurons. I established that the trophic factor-inducing effect of GLP-1 and GIP were dependent on both the PI3K and PKA pathways. I revealed that GLP-1 and GIP exhibited an antioxidant effect in microglia by reducing intracellular ROS accumulation, reducing RNS secretion and upregulating expression of several antioxidant enzymes. I further demonstrated that GIP, but not GLP-1, exhibited a neuroprotective effect by reducing microglia-mediated cytotoxicity towards neurons. In this thesis, I show for the first time that GLP-1 and GIP regulate several critical aspects of glial cell function, which can in turn have a significant impact on the viability of surrounding neurons. Thus, I have provided evidence that dysregulated GLP-1 and GIP signaling may contribute to the increased risk of developing AD, since these hormones have important glial cell survival regulating functions in the brain.
The mechanisms of T2DM are multifaceted and previous studies have highlighted several aspects of this disease that may contribute to AD, including reduced brain glucose consumption, as well as enhanced deposition of Aβ plaques and formation of NFTs\textsuperscript{134,203-205}. The data described in chapters 2 and 3 of this thesis indicate that the metabolic hormone dysregulation that is characteristic of T2DM may contribute to the pathogenesis of AD through glial cell-mediated mechanisms. These findings are crucial since my research highlights the significant and severe impact that glial cell-mediated neuroinflammation can have on the pathogenesis of AD\textsuperscript{163-168}; thus, augmented neuroinflammation by way of reduced insulin and incretins in the brain could enhance AD progression by impacting glial cell function. Moreover, it is essential to understand how these hormones impact all cells of the CNS, not only neurons, especially since recent clinical trials have begun to investigate insulin and incretins as potential treatment options for AD\textsuperscript{395,504}.

In addition to learning about the effects of these hormones on neurons, understanding the effects of insulin, GLP-1 and GIP on glial cells is equally important since several compounds have been known to have a beneficial effect on one cell type and a differing or even opposing detrimental effect on the neighbouring cell type\textsuperscript{505,506}. For example, experiments utilizing mixed-cell cultures demonstrated that after a single dose of delta-9-tetrahydrocannabinol, glutamic acid decarboxylase activity, which is a marker of gamma-aminobutyric acid (GABA)ergic neurons was significantly decreased, indicating GABAergic viability was reduced. However, markers of cholinergic neurons, astrocytes and oligodendrocyte activity (viability) was not affected after one dose of delta-9-tetrahydrocannabinol\textsuperscript{505}.

Furthermore, it is essential to assess the potential glial cell-mediated effects of incretins as AD treatments, since the benefit of these hormones on neuroinflammation and glial cells may go unrecognized if the direct effect of these compounds on neurons is the only aspect examined. It has been repeatedly demonstrated that glial cell-mediated effects play a large part in the efficacy of several treatment options for brain disease\textsuperscript{290,506,507}. For example, CSF from patients with motor neuron disease was shown to be toxic to motor neurons in mono-culture, but when CSF from patients with motor neuron disease was added to neurons and glia in co-culture, it improved the survival of neurons. These results indicate that the CSF from patients with motor neuron disease may contain factors that can be harmful or
protective to neurons, and the type of response by neurons to these compounds depends on the presence of glia. Here I have demonstrated that insulin, GLP-1 and GIP may be beneficial towards neurons indirectly by way of reducing glial cell-mediated neuroinflammation. This is of particular consequence when considering the potential effect of these hormones as drug treatment options for AD, as this disease is characterized by dysregulated glial cell activation. Many experts are convinced that it is indeed the dysregulated glial cell activation that drives the pathogenesis of AD; thus, the discovery of treatment options and mechanisms that dampen the microgliosis observed in AD may prove to finally offer viable options for the treatment of AD. Future studies designed to observe the effects of insulin and incretins on whole brain neuroimmune effects would help to better the understanding of how these hormones may regulate glial cell-mediated neuroinflammation in the CNS.

The experimental work described in chapter 4 addressed the hypothesis that voluntary PA has an impact on the neuroimmune status of the brain, and that the neuroimmune responses to PA are influenced by the knockout of MCP-1. The observations made in chapter 4 partially support this hypothesis as voluntary PA in mice led to upregulated expression of both pro- and anti-inflammatory cytokines in the brain, which correlated with enhanced markers of astrocyte activation and TLR-4 upregulation in the brain. I revealed that the neuroimmune effects of VWR on the brain partially depended on MCP-1, since the brain cytokine expression and glial cell activation were differed in the brains of mice that lacked MCP-1 compared to wild-type animals. I further demonstrated that the changes in cytokine levels observed in mice brains following VWR were not consistent with the changes observed in their peripheral blood, thus, indicating that cytokine expression following PA could have been independent of peripheral cytokine responses to PA.

In chapter 4 of my thesis I showed for the first time that the neuroimmune responses to PA are partially dependent on MCP-1, either by directly regulating such responses to VWR or by inducing a completely different neuroimmune phenotype through the developmental stages of the MCP-1 knock-out animals, which subsequently respond differently to VWR. Thus, I identified that targeting aspects of MCP-1 signaling could provide beneficial neuroimmune effects that may be consistent with performing moderate PA. I also revealed that the neuroimmune effects of PA may be different from the peripheral
immune response to PA, thus demonstrating an additional critical aspect in which the peripheral immune response and brain immune response to PA differ from one another. Small, yet significant increases in markers of immune system activation following PA is consistent with immune system priming, which is beneficial in responding to future pathological events. Combined, my data from chapter 4 indicate that the moderate increases in the expression of pro- and anti-inflammatory cytokines, as well as glial activation following PA may facilitate a more effective neuroimmune response to endogenous or exogenous stimuli, consistent with neuroimmune system priming. The discovery that MCP-1 regulates the neuroimmune effects of VWR, either directly or indirectly, should encourage future research focused on targeting MCP-1 and its receptors as a potential therapeutic strategy for the treatment of AD. PA is known to reduce the risk of developing AD. Here, I have shown that perhaps PA is protective against the development of AD through neuroimmune-modulatory mechanisms.

My thesis work demonstrated that dysregulated insulin and incretin signaling observed in T2DM, as well as a lack of PA, can have prominent effects on brain glial cells and can modify neuroinflammatory responses. This PhD thesis reveals that T2DM and a SED lifestyle may contribute to the incidence of AD through glial cell-mediated neuroimmune mechanisms, which enhances the understanding of how T2DM and SED behavior contribute to the increased risk for developing AD. Understanding the cellular and biochemical mechanisms activated by metabolic hormones that are dysregulated in T2DM may help identify new potential targets for AD drug development. Additionally, since the drugs currently available for the treatment of AD offer only modest symptomatic relief, further understanding of how T2DM and physical inactivity contribute to AD may assist in the development of effective preventative strategies to reduce the incidence of AD. This thesis provides a foundation for future clinical research investigating the effects that T2DM and lack of PA may have on the neuroimmune system, and thus, may provide insight for drug development targets. Implementing future studies collecting neuroimmune data from AD patients with and without T2DM, as well as from individuals who are SED and PA, could be tremendously beneficial in understanding the relationship between these complex diseases.
References


CDC. (2015) *Division of nutrition, physical activity, and obesity,* <http://www.cdc.gov/physicalactivity/basics/glossary/>. 131


learning, memory and hippocampal neurogenesis in aged mice. *Behav Brain Res.* **272**, 131-140.


254 Yu, J. L., Ma, L., Ma, L. & Tao, Y. Z. (2014) Voluntary wheel running enhances cell proliferation and expression levels of BDNF, IGF1 and WNT4 in dentate gyrus of adult mice. *Sheng Li Xue Bao.* **66**, 559-568.


cognitive functions, and VEGF and BDNF levels in adolescent rats. Biotech Histoch. 90, 55-68.


Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. 

Arch Neurol. 69, 29-38.


