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Insulin signaling and microglia in the young and aged brain

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DOI:
[10.33612/diss.183132348](https://doi.org/10.33612/diss.183132348)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Branco Haas, C. (2021). *Insulin signaling and microglia in the young and aged brain*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.183132348>

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Insulin signaling and microglia in the young and aged brain

Clarissa Branco Haas

Most of the research described in this PhD dissertation was conducted at the Biomedical Sciences of Cells and Systems Department, Moleculair Neuroscience, University Medical Center Groningen, University of Groningen, the Netherlands.

Printed by	Ipskamp Printing
Cover Design	Clarissa Branco Haas, Jenna Wingfield
On the cover	Front cover: representation of the brain as a colorful cloud with several microscopy images generated by the PhD candidate during the PhD studies. Colorful cloud and front cover art prepared using www.canva.com . Back cover: representation of a diverse group of people admiring a diverse population of brain cells.
Financial support	University Medical Center Groningen University of Groningen Research School BCN UMCG De Cock foundation

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Insulin signaling and microglia in the young and aged brain

PhD thesis

to obtain the degree of PhD at the
 University of Groningen
 on the authority of the
 Rector Magnificus Prof. C. Wijmenga
 and in accordance with
 the decision by the College of Deans.

This thesis will be defended in public on

Wednesday 3 November 2021 at 16.15 hours

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Abbreviations

Term	Fullname
AD	Alzheimer's disease
Akt	protein kinase B
APP	amyloid precursor protein
ATP	adenosine triphosphate
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
CA1	cornu Ammonis 1
CA3	cornu Ammonis 3
CNS	central nervous system
CoQ	coenzyme Q
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
CSF-1R	colony stimulating factor 1 receptor
DG	dentate Gyrus
ERK	extracellular signal-regulated kinase
ETS	electron transfer system
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FOXO	forkhead family of transcription factors
GSK3- β	glycogen synthase kinase 3 beta
HRR	high resolution respirometry
IGF-1	insulin growth factor-1
IGF-1R	insulin growth factor-1 receptor
IL	interleukin
iNOS	nitric oxide synthase
IR	insulin receptor
IFR8	interferon regulatory factor 8
IRS	insulin receptor substrate
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide

NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NO	nitric oxide
OXPHOS	oxidative phosphorylation
PBMC	peripheral blood mononuclear cells
PD	Parkinson`s disease
PI3K	phosphatidylinositol 3-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
syn	synuclein
TCA	tricarboxylic acid
TGF	transforming growth factor
TLR4	toll-like receptor 4
TNF	tumor necrosis factor

Preface

Since the discovery that insulin affects the central nervous system (CNS), many advances had been seen in the field. The characterization of insulin function in the hippocampus and its effects in learning and memory processing were key factors for opening a new perspective for treatment of neurodegenerative disorders such as Alzheimer's disease. Insulin signaling resistance in the aging brain and insulin signaling in astrocytes are more recent findings that broaden the perspective of the role of insulin in the brain. Insulin emerges as part of a complex network that involves not only neurons but also glia cells. Brain insulin signaling functions can be altered or lost during life. This complexity is increased by the fact that insulin signaling, as in the periphery, causes cellular metabolism adaptations in the brain. During the studies that generated this thesis new molecular and cellular mechanisms of insulin signaling in the hippocampus and its effects in microglia and neuroinflammation of both young and aged brains were identified. New insights into insulin effects in microglia metabolism followed by a new strategy to access microglia oxygen metabolism and mitochondrial function were also objects of study. In this introduction the historical and current overview of insulin, and its function and resistance in the brain is presented. Brain aging, oxygen metabolism, neuroinflammation and insulin signal in glial cells are also conceptualized and summarized.

CHAPTER 1

General introduction and outline of the thesis

INSULIN

In 1923, Frederick Banting and John Macleod received the Nobel Prize for the discovery of insulin, a finding with extensive medical and societal impact (Bliss 1997). Since its discovery, insulin signals, and its mechanisms of action have been the subject of countless studies. Insulin is essential for human life in both the peripheral and nervous tissues (Ghasemi, Haeri et al. 2013). Insulin is one of the most important anabolic hormones identified and because insulin signaling has diverse effects on several cellular processes, it is classified as a pleiotropic hormone (Fulop, Larbi et al. 2003). The primary function of insulin signaling in the periphery is the regulation of blood glucose levels, indirectly modulating lipogenesis, protein synthesis and cellular proliferation (Wood 2006). Muscle, liver and adipose tissue have the highest sensitivity to insulin, a property that is strongly related to the metabolic function of insulin in peripheral tissues (Wood 2006). Once the insulin produced in the pancreas reaches the various tissues, it affects cellular bioenergetics, survival, and development, demonstrating its essential role for the organism functioning as a whole.

CNS Insulin signaling

The first findings of insulin effects in the CNS are regarding focal and diffuse lesions observed in the diabetic brain, which were called, diabetic encephalopathy. Such findings were the first evidence connecting peripheral insulin signaling to alterations in the brain (Alex, Baron et al. 1962, Grunnet 1963, Chowers, Lavy et al. 1966, Reske-Nielsen, Lundbaek et al. 1966). Meanwhile, the detection of immunoreactive insulin in dog cerebrospinal fluid (CSF) (Margolis and Altszuler 1967) challenged the idea previously proposed that insulin could not cross the blood brain barrier (BBB) (Haugaard, Vaughan et al. 1954). Further, the binding of insulin to hypothalamus and other brain tissue areas was demonstrated in rats using radioactive insulin (van Houten, Posner et al. 1979, Baskin, Woods et al. 1983). It was followed by the observation that insulin binding in the brain was much higher in the cellular membrane if compared to mitochondria or nuclei (Marks, Maddison et al. 1988). Additionally, Marks and colleagues showed that Insulin Receptor (IR) mRNA was most abundant in the olfactory bulb, cerebellum, hippocampus, pyriform cortex, choroid plexus and arcuate nucleus (Marks, Porte et al. 1990), which are areas primarily related to

learning and memory, providing new clues on brain insulin signaling and its connection to memory processing and cognition.

Epidemiological studies revealed that diabetic patients have a three times higher risk to develop dementia and neurodegenerative disorders, such as Alzheimer's disease (AD) (Ott, Stolk et al. 1999, Steen, Terry et al. 2005, McNay, Ong et al. 2010). Experimental studies demonstrated that the hypothalamus and brain regions related to learning and memory processing, such as the hippocampus, cortex and amygdala, are enriched in IR (Heni, Kullmann et al. 2015). Considering all these findings it was hypothesized that insulin signaling affects cerebral function and that insulin signaling resistance affects and compromises cognitive processing. Although several clinical trials reported that intranasal insulin treatments were protective for neurological diseases, such as AD, dementia, stroke, Parkinson's disease (PD) and multiple sclerosis (MS), the cellular and molecular mechanisms of action of insulin in the CNS are far from understood (Avgerinos, Kalaitzidis et al. 2018).

The source of insulin in the CNS is still a debated subject. The most prevalent hypothesis is that most of the insulin in the brain is derived from the blood. In fact, insulin can cross the BBB, facilitated by a transport system that is triggered by IR expressed in endothelial cells localized in the surface of blood vessels (Blazquez, Velazquez et al. 2014, Gray, Meijer et al. 2014). This system is saturated independently and at lower levels of insulin when compared to the levels that are necessary to maintain normal glucose blood homeostasis (Banks, Jaspan et al. 1997a, Banks, Jaspan et al. 1997b). In addition, it was also demonstrated that the brain produces a small amount of insulin which exact source is not defined (Kuwabara, Kagalwala et al. 2011), but what triggers this insulin production or if differs in function compared to peripheral insulin is still unknown.

For several years, the commonly held view was that CNS energy metabolism was not dependent on insulin signaling (Schulinkamp, Pagano et al. 2000), but we now know that cerebral insulin signaling is required for the control of nutrient uptake at the cellular level in the brain (Gerozissis 2008, Garcia-Caceres, Quarta et al. 2016). Although the important role of insulin in regulating brain energy substrate consumption, its function goes beyond energy metabolism, and is also implicated in cellular pathways in the brain related to neurotrophic support and cognition (van der Heide, Ramakers et al. 2006, Muller, Gnoatto et al. 2011, Haas, Kalinine et al. 2016).

In the CNS, insulin has the highest affinity to IR, but it can also bind to the insulin growth factor-1 receptor (IGF-1R), illustrated in Figure 1 below depicting the insulin pathway and biological actions of this signal in the brain (Fernandez and Torres-Aleman 2012). When insulin binds IR, the IR substrates (IRS) downstream of mainly IRS type 1 (IRS-1), are phosphorylated, triggering complex signal transduction cascades involving PI3K – Akt and RAS – ERK.

Figure 1. Insulin/IGF-1 signaling pathways and cellular responses. Insulin and insulin-like growth factor 1 (IGF-1) bind to structurally related tyrosine kinase receptors. Canonical insulin and IGF-1 signaling are mediated through PI3K–AKT and ERK cascades, promoting phosphorylation of downstream proteins that results in protein activation/inhibition regulating critical processes of cellular survival and proliferation. Increased mTOR phosphorylation triggers protein translation and autophagy, inhibitory phosphorylation of GSK3β and FOXO controls apoptosis, inhibition of FOXO phosphorylation and further activation of superoxide dismutase (SOD) controls oxidative stress; AKT phosphorylation is also related to ERK phosphorylation, gene transcription and regulation of cell proliferation (Fernandez and Torres-Aleman 2012). IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; ERK: RAS extracellular signal-related kinase; GSK3β: glycogen synthase kinase 3. Original figure created with BioRender.com.

The PI3K – Akt pathway leads to the activation of mTOR and FOXO and the inhibition of GSK3- β culminates in the regulation of protein transcription, apoptosis, autophagy, and oxidative stress. On the other hand, the RAS – ERK pathway regulates gene transcription and cellular proliferation (Figure 1). The balance between and modulation of both pathways regulates cell survival and cell death, underscoring the importance of this signaling cascade in the CNS (Fernandez and Torres-Aleman 2012).

Brain insulin signaling resistance

The decrease in the cellular plasma membrane fluidity related to the limited movement of lipids in the cellular membrane culminate in modifications of IR internalization, re-expression and degradation (Duarte, Moreira et al. 2012). Such modifications are related to the disruption of insulin signaling, that characterizes the phenomena called insulin resistance. The main regions affected by insulin resistance in the brain are hippocampus, cortex and the choroid plexus and it has been associated with pathological chronic neuroinflammation and loss of cognitive function (De Felice and Ferreira 2014, Heni, Kullmann et al. 2015). Those alterations are also prominent in neurodegenerative disorders; in 2005, Steen and colleagues termed brain insulin resistance in AD patients as diabetes type 3 (Steen, Terry et al. 2005). Previously, we demonstrated that IGF-1 transport from the periphery to the brain as well as IGF-1R expression was increased in aged mice. Surprisingly, these alterations did not result in increased IGF-1 function in the brain (Muller, Fernandez et al. 2012). These observations indicated that IGF-1 signaling was resistant during aging which also occurs in the insulin signaling pathway. The mechanisms that underlie brain insulin resistance are poorly understood and their elucidation is relevant as potential targets for the treatment of neurodegenerative disorders. This topic is also addressed in the research reported in chapter 2 of this thesis (Haas, Kalinine et al. 2016).

Brain insulin signaling and oxygen metabolism

Oxygen is essential for the survival of eukaryotic organisms and the demand for oxygen varies depending on tissue characteristics and functions in the human body. In humans, the brain uses approximately 20% of the total oxygen (Gandhi and Abramov 2012). Most of this oxygen is consumed by mitochondria

during oxidative phosphorylation for ATP production, the main energy carrying molecule in the cells. Besides ATP production, mitochondrial oxidative phosphorylation (respiratory chain) has an important role in cellular function, and all respiratory enzymatic complexes (I-V) work to maintain an efficient and tightly regulated ATP production. In the brain, mitochondrial oxygen metabolism plays an important role in tissue homeostasis (Waldbaum and Patel 2010). Four critical elements related to mitochondrial oxygen metabolism in the brain are summarized in Figure 2.

Figure 2. Mitochondrial oxygen metabolism is implicated in various processes in the brain tissue and is critical for tissue homeostasis. Original figure created with BioRender.com.

The cellular and molecular mechanisms that connect mitochondrial oxygen metabolism to the regulation of these four elements are still under investigation. Neurons are profoundly dependent on mitochondrial function to establish membrane excitability and neurotransmission. Chemical synapses microenvironment are highly dense in mitochondria. Calcium handling and buffering and ATP production activities are affected by the mitochondrial electron transfer system and oxygen consumption (Vos, Lauwers et al. 2010). Electrons that leak from the electron transport chain or respiratory chain from complexes I or III during normal oxidative respiration can result in the formation of the superoxide anion and further reactive oxygen species (ROS) (Watts, Pocock et al. 2018) which are important to maintain several redox-dependent cellular pathways and at high levels promote cellular oxidative damage (Yin, Boveris et al. 2014). H_2O_2 (hydrogen peroxide) represents the most stable form of ROS which makes

its measurement a good parameter for studying ROS biology and mitochondrial metabolism. Low to intermediate levels of H_2O_2 are involved in the regulation of redox-sensitive signaling and transcription such as the mitochondrial ROS-dependent phosphatase inactivation as seen in the MAPK and PI3K-Akt pathways (Handy and Loscalzo 2012).

The relation between oxygen metabolism and IR activity is well established in the literature for peripheral tissues, where controlled ROS levels serve as a second messenger in the insulin cascade activation by targeting mainly protein tyrosine phosphatases (PTP), which are rapidly oxidized, blocking their activity (Goldstein, Mahadev et al. 2005, Goldstein, Mahadev et al. 2005). In this fashion, ROS reverse the inhibitory effect of PTPs on insulin signaling (Meng, Buckley et al. 2004). The role of insulin-induced H_2O_2 in the autophosphorylation of CNS IR was demonstrated for cerebellar granular neurons and in the hypothalamus (Pomytkin, Costa-Nunes et al. 2018). Also, insulin decreased ROS production in neuronal cultures and an insulin receptor signaling is necessary to regulate mitochondrial respiratory complex III H_2O_2 production in the synaptosome (Muller, Haas et al. 2013). On the other hand, the exact mechanisms that couple mitochondrial/extramitochondrial ROS production to insulin signaling in glia cells are still unresolved. This topic was studied with a focus on microglia in the research presented in this thesis.

AGING

The worldwide population is living longer; while in the 40's average life expectancy was between 45 - 47 years, in both developed and upcoming countries, life expectancy has increased to 75 - 77 years (Driscoll, Hong et al. 2008, Malta, Abreu et al. 2016). Also, the number of centenarians (>100 years of age) is progressively increasing all over the world (Eisenstein 2012). Research in gerontology has shown that death is being delayed because people are reaching very old age in better health (Vaupel 2010). People are living longer because humans are being able to extensively manipulate the multifactorial secondary outcomes related to aging.

Cell senescence decreases the ability of a tissue to recover after an injury or pathogenic event (He and Sharpless 2017). Although, initially, cell senescence was mainly attributed to endogenous cellular genetic instability, evidence showed

that it is mostly triggered by high levels of environmental stressors (He and Sharpless 2017) that influence the cell epigenetically (Lopez-Otin, Blasco et al. 2013). Exposure to toxic agents, poor nutrition, sedentarism, pollution and UV light are some examples of environmental stressors, which, when controlled can lead to beneficial adaptative responses (Calabrese and Mattson 2017). The possibility to have a better control on which mechanisms trigger and are involved in the aging process at the cellular level is changing the perspective of longevity. Even if lifestyle changes towards a healthier state is still a challenge in the 21st century, there is an awareness that better habits can influence longevity, what have an impact in society. Beyond lifestyle changes, the next challenge is the discovery of more efficient external manipulations, such as pharmacological approaches that can prevent, delay, or result in healthier standards of aging.

The aging CNS

Natural aging, intrinsic to all living beings, is accompanied by cellular senescence in the CNS, and remarkably related to alterations in memory and motor performance (Yeoman, Scutt et al. 2012). The brain during aging is significantly susceptible to develop a range of neurodegenerative disorders as AD (Scheltens, Blennow et al. 2016) and stroke (Krishnamurthi, Feigin et al. 2013). The distinction between the effects of normal aging from pathology that compromises brain function is still a challenge (Hedden and Gabrieli 2004, Mattson and Arumugam 2018). The progressive loss of neuronal function in the frontal cortex and hippocampus during aging are strongly associated with irreversible cognitive alterations (Morrison and Baxter 2012). Besides morphological changes, neurochemical brain adaptations are also part of the long process of aging, including modifications in energy metabolism and mitochondrial function (Hoyer 2004, Yin, Boveris et al. 2014, Mattson and Arumugam 2018), oxygen metabolism and neuroendocrine signaling (Muller, Zimmer et al. 2012, Dickstein, Weaver et al. 2013, Haas, Kalinine et al. 2016). Even though, in the last decade, there was significant scientific progress in the field, the cellular and molecular mechanisms responsible for aging-associated cognitive impairment are still not completely understood and the discovery of pharmacological treatments for neurodegenerative disorders is still a big challenge to overcome (Lopez-Otin, Blasco et al. 2013, Gutchess 2014).

The cognitive deficits related to neurodegeneration that are observed

in aging are widely related to changes in the cellular expression of neurotrophic factors. Neurotrophic factors or neurotrophins are molecules that support and mediate signals related to development, differentiation, maintenance and plasticity of brain function throughout life (Tapia-Arancibia, Aliaga et al. 2008). The main neurotrophic factor involved in cognition is brain-derived neurotrophic factor – BDNF (Budni, Bellettini-Santos et al. 2015). Decreased BDNF signaling, mainly in the hippocampus, is associated to decreased neurogenesis and to disruptions in the structural and functional CNS plasticity. The term plasticity was proposed by (Thoenen 1995) as “ the capacity of neurons or glial cells to improve or to depress the synaptic efficacy through biochemical or morphological changes in a dynamic fashion”. Neurogenesis, defined as an endogenous process that involves coordinated proliferation, differentiation, and migration of neural precursor cells (Eriksson, Perfilieva et al. 1998), is modulated by microenvironmental homeostasis and a key process that contributes to brain development and to young and adult brain plasticity (Shohayeb, Diab et al. 2018). Interestingly, whether neurogenesis occurs in the human adult brain was debated for many years (Sorrells, Paredes et al. 2018), but currently, adult human neurogenesis is a widely accepted phenomenon (Boldrini, Fulmore et al. 2018, Moreno-Jimenez, Flor-Garcia et al. 2019).

Successful neurogenesis is dependent on the neurogenic niche composition of several intrinsic factors, such as hormones, trophic, neurogenic inflammatory (Xanthos and Sandkuhler 2014), and vascular factors (Shohayeb, Diab et al. 2018). During aging, the number of progenitor cells is decreased as well as neuronal differentiation and neurogenesis (Lee, Clemenson et al. 2012). This is mostly attributed to the changes in the neurogenic niche mostly caused by a slow long-term disbalance in its composition. The combination of necrotic and apoptotic factors together with a chronical proinflammatory molecules slow-release by glial cells had bring the idea that neurodegeneration and chronic neuroinflammation are key contributors to the decrease on neurogenesis, synaptic plasticity and further tissue function during aging in the CNS which mechanisms are yet to be understood (Fan and Pang 2017).

Energy metabolism dysfunction, neurodegeneration, insulin resistance, lack of neurogenesis, telomere attrition, epigenetic alterations, proteostasis disbalance, chronic neuroinflammation, intercellular communication disbalance and genomic instability are biological factors that contribute to aging (Lopez-Otin, Blasco et al. 2013) (Figure 3). In the present studies of this thesis, five of

these aspects were explored and are highlighted in red in the figure number 3.

Figure 3. Physiological contributory factors for CNS aging. The factors that were studied in this thesis are highlighted in red. Original figure created with BioRender.com.

NEUROINFLAMMATION

Inflammation is a response of the immune system to protect the organism from internal or external challenges such as microorganisms that can enter and change the homeostasis of the body triggering infection, tissue injury and illness. This response is orchestrated by a very well-coordinated action involving a plethora of cells and molecules such as cytokines. Neuroinflammation, the inflammatory responses that are centralized within the brain and spinal cord, acts to maintain CNS homeostasis and has been extensively studied during the last decade providing cues for the understanding of several physiological processes

and neuronal/behavioral disorders in the CNS (DiSabato, Quan et al. 2016).

For a long time, neuroinflammation was considered as a deleterious or toxic process in the brain, but nowadays it is also viewed as strategically intrinsic mechanisms important for the cerebral tissue integrity (Medzhitov 2008, DiSabato, Quan et al. 2016). Glial cells, mainly microglia, are the main players in neuroinflammation. In fact, microglia, together with other glial cell types (astrocytes and oligodendrocytes), neurons and circulating leukocytes work in cooperation to orchestrate the neuroinflammatory response that differs in many aspects when compared with peripheral inflammation (Medzhitov 2008).

An important paradigm shift is now happening in the neuroinflammation research field. Neuroinflammation was considered as an important response in the CNS mostly in the presence of abnormal stimuli, such as pathogens, toxins, mechanic traumas and neurodegeneration. Currently, evidence is accumulating re-classifying neuroinflammation as a process continuously present in the brain to maintain homeostasis by the cellular exchange of inflammatory molecules, such as cytokines and chemokines (DiSabato, Quan et al. 2016, Fan and Pang 2017). Further investigations have shown that glia activation, mostly microglia, contributes to microenvironmental recovery after harmful stimuli or even after ordinary disturbances, such as synapse formation or elimination (Xanthos and Sandkuhler 2014, Jassam, Izzy et al. 2017, Masgrau, Guaza et al. 2017).

Acute neuroinflammation

Before the emergence of the terminology neuroinflammation, glial reactivity was commonly used to describe the accumulation, changes in morphology and activity of glial cells in response to CNS injury (Streit, Mrazek et al. 2004). The notion of “glial reactivity” became categorized in acute and chronic neuroinflammation, terminology, currently, widely adopted. Acute neuroinflammation is the cellular response to an acute insult in the brain that activates glial cells, even before they adopt the “activated” morphology (Parakalan, Jiang et al. 2012, Vinet, Weering et al. 2012). Acute neuroinflammation can be triggered by detrimental insults accompanied by the BBB disruption or, even, by physiological intensification of neuronal activity (Streit, Semple-Rowland et al. 1998, Streit, Mrazek et al. 2004). In both cases, the neuroinflammatory response is beneficial and contributes to the recovery of microenvironmental homeostasis, supporting normal neuronal activity. It would be interesting and valuable to

develop a new terminology in the literature to differentiate between both situations and the wide range of neuroinflammatory states and activities.

Chronic neuroinflammation

Chronic neuroinflammation is characterized by the continuous release of inflammatory molecules from the insult that went through the acute inflammation stage where the inflammation for unknown reasons is not controlled or resolved (Acarin, Paris et al. 2002). This phenomenon is related to the maintenance of cerebral disease triggered by an infection, such as encephalitis (Garden 2002), and also to the etiopathology of neurodegenerative disorders, such as dementia, Alzheimer's and Parkinson's disease (Akiyama, Barger et al. 2000). Chronic neuroinflammation is a process that is intrinsic to aging, but the exact stage in life and what exact are the causes of chronic neuroinflammation are still unknown.

MITOCHONDRIAL RESPIRATION AND CELLULAR GLYCOLYSIS

A set of metabolic reactions and a complex connection between them and their products compose cellular respiration. These metabolic reactions convert chemical energy from oxygen molecules or nutrients into adenosine triphosphate (ATP), and then release waste products (Schmidt-Rohr 2020). Cellular respiration is composed of mitochondrial respiration and cellular glycolysis. Glycolysis is not efficient for ATP production because only 2 ATP molecules per glucose molecule are generated, whereas mitochondrial respiration produces 36 ATP molecules per glucose molecule (Yetkin-Arik, Vogels et al. 2019). Mitochondrial respiratory capacity varies depending on cell origin and tissue localization, influencing the dynamics of energy production and metabolism. (Artyomov, Sergushichev et al. 2016). In this thesis mitochondrial respiration was studied in the context of macrophage biology focusing on microglia.

MICROGLIA

The terminology “glia” was introduced in mid-19th century and defined “the nerve glue”, at that time, being specifically classified as astrocytes by Santiago Ramón y Cajal. Later microglia were identified as a different cell population by Del Río-Hortega when its morphological variations were characterized (Sierra,

de Castro et al. 2016). Glial cells count 50% of the brain cells in humans, primates, and rodents (Herculano-Houzel, Mota et al. 2006). Microglia, astrocytes, oligodendrocytes, and oligodendrocyte precursor cells (NG2 cells) are the most studied glial cell types. Tanycytes and radial cells are also glial cells, which functions are less understood (Smedlund and Hill 2020). Microglia are the CNS resident macrophages. Distinct from tissue macrophages, microglia originate from the yolk sac (Ginhoux, Greter et al. 2010). Bone-marrow-derived monocytes do not contribute to the mature microglial pool (Aguzzi, Barres et al. 2013). Microglia are tailored to the CNS and their functions go beyond classical macrophage properties, making microglia a unique cell type population (Aguzzi, Barres et al. 2013).

Microglia can adopt a range of phenotypes, ranging between highly ramified, also called resting or surveilling, and amoeboid, or activated cells (Kettenmann, Hanisch et al. 2011, Kettenmann, Kirchhoff et al. 2013). Microglia are very sensitive to microenvironmental changes, are extremely plastic and have a different molecular signature, when compared to peripheral monocytes and other tissue macrophages types (Butovsky, Jedrychowski et al. 2014). Over time, activated microglia were associated with CNS disease, a concept that has been changing over the last decade, since more refined techniques and the emergence of new markers allowed the study of microglia beyond morphological alterations. This new concept indicated that activated microglia, depending on the situation, can present trophic functions that are essential for both healthy and diseased brain (Hellwig, Heinrich et al. 2013). During brain development, microglia have an amoeboid morphology, and contribute to refining synaptic structures and connections in the developing brain (Schafer, Lehrman et al. 2012). The microglial lifetime and functional outcomes in different situations are represented in Fig. 4.

Microglia functions can be separated into immune and non-immune activities. Immune microglial functions are mainly related to the phagocytic activity against synapses, cells, cellular fragments, and pathogens. Microglia non-immune functions include micro-environmental monitoring, control of synaptic integrity and function and production of neurotrophic factors (BDNF, IGF-1, NGF) that support neurogenesis and brain homeostasis (Morris, Clark et al. 2013). During aging, in both rats and mice, microglia can become sensitized or primed, phenomenon that is induced by chronic neuroinflammation. Primed microglia present fewer and shorter processes, are less ramified and display an

exacerbated response to (exogenous) immune stimuli (Norden and Godbout 2013, Raj, Jaarsma et al. 2014).

Figure 4. Schematic summary of microglial lifetime. Development, maturation, aging and the functional changes influencing onset, severity and progression of neurodegeneration in AD and PD; yellow charts show contributory factors for each phenotype; blue charts show characteristics of adult and primed microglia based on (Spittau 2017) with modifications. Original figure created with BioRender.com.

The molecular pathway triggered by the binding of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, to its receptor, TLR4, is one of the best characterized cellular pathways related to microglia activation. This pathway is activated via MAPKs phosphorylation, COX-2 activation and NF- κ B translocation from the cytosol to the nucleus culminating in the high expression of pro-inflammatory interleukins, such as IL-1 β , TNF- α , IL-6 (Wang, Xiong et al. 2015). Recent technological advances, mainly using robust multi and single-cell genome and epigenetic analysis of microglia ex-vivo have helped clarifying and characterizing how complex microglia populations can be not only at

the steady state, but also depending on the disease context, age and on the cerebral localization (Galatro, Holtman et al. 2017, Eggen, Boddeke et al. 2019, Alsema, Jiang et al. 2020, Kracht, Borggrewe et al. 2020). This genetic and epigenetic heterogeneity is now in the process to be validated as functional heterogeneity at the protein level. Microglia activation also involves metabolic adaptations especially because phagocytic activity is exacerbated and also because they re-structure their cytoskeleton upon migration (Artyomov, Sergushichev et al. 2016).

Microglia oxygen metabolism

Microglia are phagocytic cells that require a special oxygen metabolism and bioenergetics program for correct activity (Artyomov, Sergushichev et al. 2016, O'Neill and Pearce 2016, Curi, de Siqueira Mendes et al. 2017, Van den Bossche, O'Neill et al. 2017, Correa-da-Silva, Pereira et al. 2018, Liu and Ho 2018). Most studies on peripheral macrophage metabolism have focused on macrophage polarization primarily using LPS for pro-inflammatory activation or IL-4 for anti-inflammatory or alternative activation *in vitro* (Van den Bossche and Saraber 2018). LPS-stimulated macrophages express inducible nitric oxide synthase (iNOS) and produce nitric oxide (NO) from arginine, accompanied by increased glycolysis and reduced mitochondrial respiration (OXPHOS) and increased ROS production. That metabolic shift shows the role of mitochondria as a key factor facilitating the pro-inflammatory response, since this higher release of ROS is important to phagosome digestion together with ROS generated by NADPH oxidase enzymes or NOX (Bordt and Polster 2014). ROS also induce the translocation of NF- κ B from the cytosol to the nucleus and acts as second messengers of interleukin signaling. The bioenergetic regulation of microglia was reviewed, highlighting that microglia express the genetic tools required for both glycolytic and oxidative energy metabolism (Zhang, Chen et al. 2014, Ghosh, Castillo et al. 2017). Surprisingly, it was reported that the quantitative expression of genes involved in oxidative metabolism is comparable or higher in microglia than in neurons and astrocytes (Ghosh, Castillo et al. 2017). During the research presented in thesis, microglia oxygen metabolism was also addressed, and an experimental model to evaluate oxygen metabolism in primary microglia using a high resolution respirometer was developed.

Glial insulin signaling

Insulin signaling in glia is a topic that is receiving growing attention. From the first publication, in 1989, where (Shemer, Adamo et al. 1989) reported that glial insulin receptors take one minute to be phosphorylated *in vitro*, progress regarding this subject was almost exclusively obtained in rodents and human astrocytes (Garwood, Ratcliffe et al. 2015, Spielman, Bahniwal et al. 2015, Garcia-Caceres, Quarta et al. 2016, Fernandez, Hernandez-Garzon et al. 2017, Cai, Xue et al. 2018). It was demonstrated that at low nanomolar concentrations, insulin upregulates the secretion of IL-6 and IL-8 in human astrocytes (Spielman, Bahniwal et al. 2015). Additionally, rodent hypothalamic astrocytes depend on insulin signaling to control glucose availability to neurons (Garcia-Caceres, Quarta et al. 2016). Another interesting finding not long ago was that a cooperative mechanism between insulin and IGF-1 can stimulate glucose uptake by astrocytes, which could explain how the brain uses glucose without changes in circulating insulin levels (Fernandez, Hernandez-Garzon et al. 2017). The first evidence that insulin signaling in astrocytes can influence behavior was recently published. It was showed that IR expression in astrocytes is related to anxiety- and depressive-like behaviors in mice through astrocytic ATP exocytosis and increased purinergic signaling on dopaminergic neurons (Cai, Xue et al. 2018). Insulin signaling in other types of glia cells such as oligodendrocytes are still to be uncovered.

Microglia insulin signaling

Microglia insulin signaling and its molecular or behavioral outcomes are understudied. So far, it is known that microglia express IGF-1 and IRs and that receptors are sensitive to insulin in human microglial cell lines (Spielman, Bahniwal et al. 2015) the molecular mechanisms of rodent primary microglia or secondary outcomes of this signal related to interleukin production or behavior are still poorly defined. In the experiments reported in this thesis, insulin signaling and functional outcomes of insulin were widely explored in rodent microglia *in vivo* and *in vitro*. Complementarily, the paradox of insulin signaling in the young brain and insulin resistance during aging in the context of neurotrophic signaling (Haas, Kalinine et al. 2016) and neuroinflammation (Haas, de Carvalho et al. 2020) were also objects of study in the present thesis.

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Outline of the thesis

The main goal of the research presented in this thesis was to delineate the function of insulin in the brain in relation to learning and memory, neuroprotection, neuroinflammation and microglia physiology and metabolism in both young and aged brain. In the experimental chapters 2 and 3, young and aged rats were compared with the aim to better understand the cellular and molecular mechanisms and outcomes of insulin signaling/resistance, and, in chapters 4 and 5, microglia metabolism was studied regarding insulin signaling and mitochondrial oxygen metabolism.

In **chapter 2**, we investigated if the aging brain would be sensitive to a pharmacological intracerebroventricular (i.c.v.) treatment with insulin and if it would improve spatial memory in aged Wistar rats. Also, the effect of insulin resistance on the mechanistic connection of insulin signaling and the downstream signal BDNF was investigated.

Second, knowing that chronic neuroinflammation is a component of aging in the CNS and that insulin signaling/resistance is still poorly understood in glia cells, in **chapter 3**, it was hypothesized that i.c.v. injections with insulin would affect neuroinflammation in young hippocampus and have a role in insulin signaling resistance during aging. Another hypothesis, that was confirmed was that murine primary microglia are sensitive to insulin.

In **chapter 4**, aiming to better understand the effect of insulin signaling on metabolism, cell survival and pro-inflammatory cytokine production, murine primary microglia were challenged *in vitro* and *in vivo* with insulin.

Since there were only limited established techniques in the literature to analyze oxygen and mitochondrial metabolism in microglia, in **chapter 5**, a technical protocol using high resolution respirometry (HRR) analysis was developed to analyze oxygen metabolism and mitochondrial physiology in murine primary microglia.

Finally, in **chapter 6**, the findings reported in this thesis were summarized, discussed in the context of recent literature, and future perspectives were defined. The focus of this general discussion is to emphasize the connection between insulin signaling and neuroinflammation.

CHAPTER 2

Brain insulin administration triggers distinct cognitive and neurotrophic responses in young and aged rats

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Abstract

Aging is a major risk factor for cognitive deficits and neurodegenerative disorders, and impaired brain insulin receptor (IR) signaling is mechanistically linked to these abnormalities. The main goal of this study was to investigate whether brain insulin infusions improve spatial memory in aged and young rats. Aged (24 months) and young (4 months) male Wistar rats were intracerebroventricularly injected with insulin (20 mU) or vehicle for five consecutive days. The animals were then assessed for spatial memory using a Morris water maze. Insulin increased memory performance in young but not aged rats. Thus, we searched for cellular and molecular mechanisms that might account for this distinct memory response. In contrast with our expectation, insulin treatment increased the proliferative activity in aged but not young rats, implying that neurogenesis-related effects do not explain the lack of insulin effects on memory in aged rats. Furthermore, the expression levels of the IR and downstream signaling proteins such as GSK3- β , mTOR and pre-synaptic protein synaptophysin were increased in aged rats in response to insulin. Interestingly, insulin treatment increased the expression of the brain-derived neurotrophic factor (BDNF) and TrkB receptors in the hippocampus of young but not aged rats. Our data therefore indicate that aged rats can have normal IR downstream proteins expression, but failed to mount a BDNF response after challenge in a spatial memory test. In contrast, young rats showed insulin-mediated BDNF response, which paralleled with improved memory performance.

Key words: insulin; brain; hippocampus; neurogenesis; spatial memory; BDNF

Introduction

Aging is a major risk factor for the development of brain disorders related to cognitive impairment and dementia (Hofer, Berg et al. 2003, Ojo, Rezaie et al. 2012, Dorszewska 2013). The progressive age-related neuronal loss along with the disruption of neural systems that participate in different aspects of neural transmission, particularly in the frontal cortex and hippocampus, has been implicated in the deterioration of cognitive function (Whitwell and Jack 2005, Morrison and Baxter 2012). Although the majority of brain regions are affected by aging, neurons from the hippocampus have shown remarkable vulnerability to death (Hofer, Berg et al. 2003). Particularly in the dentate gyrus (DG), the main site of neurogenesis in the hippocampus, there is a decreased capacity to generate newborn neurons. Among the aged-related mechanisms driving these alterations is the decreased neuroendocrine and neurotrophic signaling (Porte, Baskin et al. 2005, Muller, Fernandez et al. 2012).

Accordingly, impaired neuronal insulin receptor (IR) response, namely central insulin resistance, activates key pathophysiological mechanisms potentially leading to Alzheimer's disease (AD) and cognitive deficits (de la Monte and Wands 2005, Talbot, Wang et al. 2012). It is currently recognized that through the brain IR, insulin regulates a variety of downstream signaling proteins, including PI3K, GSK3- β , and mTOR, that participate in the regulation of neuronal death and survival (Stein and Murphy 2012, Muller, Haas et al. 2013, Werner and LeRoith 2014). However, deficient insulin control of this pathway can cause changes in the neurotrophic support and a loss of neural cells (Stern, Chen et al. 2014). In contrast, intracerebral insulin delivery in cognitively impaired rodents or intranasal administration in AD patients is able to partially restore the IR signaling, thereby improving the mechanisms of cell survival and memory formation (de la Monte 2013).

Interestingly, in the brains of young rodents, the insulin/IGF-I system appears to work in a cooperative manner with brain-derived neurotrophic factor (BDNF) (Trejo, Piriz et al. 2007, Takach, Gill et al. 2015) to improve synaptic plasticity and cognitive function (Ye, Tai et al. 2012). Hence, the reduced IR signaling that occurs with aging might result in a parallel decay in BDNF levels, ultimately contributing to decreased synaptic plasticity and memory deficits (Mattson, Maudsley et al. 2004). Thus, pharmacological strategies to increase these neuro-

biological factors might slow the progression of cellular and functional deficits associated with aging. However, whereas insulin administration to the brain has been shown to improve hippocampal-dependent memory and other functional outcomes in young rodents (Stern, Chen et al. 2014), little is known with respect to the brain of aged rodents, which is assumed to have lower IR responsiveness.

The main goal of this study was to investigate whether brain insulin infusions improve cognitive function through IR signaling, mediating neuronal proliferation and BDNF expression in aged rats. We found that insulin did not improve spatial memory in aged rats but was able to improve memory in young rats, which paralleled with increased BDNF levels.

Material and Methods

Animals

Male Wistar rats (n=48) were housed in plastic cages in a temperature-controlled room under a 12 hour light/12 hour dark cycle with free access to food and water. The rats (5 per cage) were assigned to one of the following groups: young-vehicle (4 months old, n=12), young-insulin (4 months old, n=12), aged-vehicle (24 months old, n=12) or aged-insulin (24 months old, n=12). Animal use was approved by UFRGS Local Ethic Committee for Animal Experimentation (CEUA); project number #26376.

Surgical procedure

The rats were anesthetized by an intraperitoneal (i.p.) injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). A 27-gauge 9-mm guide cannula was unilaterally placed 0.9 mm posterior to the bregma, 1.5 mm right of the midline and 2.6 mm ventral to the superior surface of the skull through a 2 mm hole made in the cranial bone. On the third day post-surgery, the rats displayed normal food intake and water consumption as well as spontaneous locomotion.

Experimental design

Three days after surgery, the animals received a daily intracerebroventricular (i.c.v.) infusion of 5 μ L of insulin (20 mU) (Humulin R[®] Lilly, Indianapolis, USA) or vehicle (NaCl 0.9%) (5 min/1 μ L/min) for five consecutive days. Imme-

diately after each i.c.v. infusion, the animals received an i.p. injection of BrdU (25 mg/kg) (Sigma, St. Louis, USA). A group of rats (n=4-6 per group) was sacrificed after the last injection of insulin for immunohistochemistry and Western blotting analysis, and hippocampal BDNF quantification, while another group (n=6-8 per group) was challenged in the Morris water maze (MWM) task and then euthanized twenty-four hours later to analyze the BDNF levels in the hippocampus (Fig. 1a).

Spatial memory - Morris Water Maze task

We conducted the MWM task following the same protocol used in (Muller, Gnoatto et al. 2011). The apparatus consisted in a black circular pool (200 cm in diameter) with water temperature of 21 ± 1 °C. The pool was located in a well-lit white room with several visual stimuli hanging on the walls to provide spatial cues. During five days of training, the rats (n=6-8, animals per group) learned to escape from the water by finding a hidden rigid black platform submerged approximately 1 cm below the water surface in a fixed location. The MWM task has two parts. The first, namely training phase (5 days), is an indicator of memory acquisition, where each animal was submitted to 4 trials per day separated by a minimum of 12 min each one. Each trial lasting up to 60 s and the rats were allowed to rest on the hidden black platform for 20s. Failing to find the platform in 60 s, the animal was placed on the platform and allowed to rest for 20 s. The result is the average of 4 trials per animal/day/per group and was used as an indicator of learning. The second, namely test phase (1 day), which is an indicator of memory retention. A probe test was performed without the platform twenty-four hours after the five days of training, and the time spent in the target quadrant was measured [17]. Videos were obtained and analyzed using the N-Maze program. Twenty-four hours after the MWM task, the animals were euthanized, and the brain hippocampus was dissected for BDNF quantification.

Western Blot

The Western blot assay was performed according (Muller, Fernandez et al. 2012). The right hippocampus (n= 4-6, animals per group) was dissected and homogenized in a lysis buffer (1 % NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10 % glycerol, 1 mM CaCl₂, 1 mM MgCl₂, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 g/ml aprotinin, and 0.1 % phosphatase inhibitor cocktails I

and II, Sigma, St. Louis, USA). The homogenate was centrifuged (13,000 xg, 10 min), and the supernatant was collected. Next, 10 µg of protein from the right hippocampus homogenate was separated by electrophoresis on a 10 % polyacrylamide gel and electrotransferred to PVDF membranes as previously reported with some modifications [16]. Insulin Receptor β (IR) (1:1000) (CST, Danvers, USA), Phospho-GSK-3 β (p-GSK3 β Ser9) (1:1000) (CST, Danvers, USA), GSK3 β (1:1000) (CST, Danvers, USA), m-TOR (1:1000) (CST, Danvers, USA), Cleaved Caspase 3 (1:500) (CST, Danvers, USA), Caspase 3 (1:500) (CST, Danvers, USA), TrkB (1:1000) (Abcam, S. Francisco, USA), β -Actin–Peroxidase (β -Act) (1:40000) (Sigma, St. Louis, USA), Synaptophysin (Syn) (1:1000) (Sigma, St. Louis, USA), and PSD-95 (1:1000) (Sigma, St. Louis, USA) primary antibodies together with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000) (GE, Little Chalfont, UK) secondary antibodies were used to detect downstream insulin signaling targets. Visualization was performed using an image quant LAS 4000 (GE, Little Chalfont, UK) and quantified using Image J software (Rockville, USA).

Immunohistochemistry

The left hemispheres of the euthanized rats were used for immunohistochemistry analysis (n=4-6 per group) (Muller, Gnoatto et al. 2011). These hemispheres were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 h, transferred to a 30 % sucrose solution in PBS and then maintained in PBS 0.1 M with Sodium Azide 0.1 % at 4 °C. Coronal sections (50 µm) were obtained using a Vibratome (Leica, Wetzlar and Mannheim, Germany) and stored in PBS 0.1 M with Sodium Azide 0.1 % at 4°C. One of 8 random series was collected for the analyses. To conduct cresyl violet staining, the sections were immersed in cresyl violet (Sigma, St. Louis, USA) during 20 minutes and then washed with flowing tap water. Next, the slices were dehydrated with 70 %, 90 %, and 100 % EtOH, soaked in a 50:50 EtOH:xylene mixture, and stored in 100 % xylene. Finally the slides were mounted with Permount. To perform the immunofluorescence studies, first the sections were permeabilized for 15 min with Triton X-100 in PBS 0.1 M and blocked for 1 h with a blocking buffer (BSA 2 %, Triton X-100, TBS). After this, the tissue sections were incubated overnight with primary antibody NeuN (1:500) (Millipore, Billerica, USA) to stain neuronal nuclei. Then, after three washes with PBS 0.1 M, the sections were incubated with the second-

ary antibody Alexa Fluor 594 (1:1000) (Invitrogen, Carlsbad, USA) for 2 h and DAPI (0.0001%) (Santa Cruz, Dallas, USA) for 25 min at 4°C. After 24 h, all of the sections were mounted with FluorSave Reagent (Calbiochem, San Diego, USA). To determine the number of neuronal nuclei per mm², 6 slices/animal were photographed using a confocal fluorescence microscopy system (Olympus FV1000, Shinjuku, Japan) and analyzed using Image Pro Plus 6.0 software. We used the mean of nuclei counts of three ROIs (100µm²) and multiplied the result by 100 (total 1 mm²). To determine the neuronal nuclei fluorescence per image, we used the mean fluorescence of three ROIs using Image J software.

BrdU assay

The BrdU assay was performed as described previously (Bekiari, Giannakopoulou et al. 2015), with some modifications. The dorso – ventral coronal (from -3.2 to -5.6) hippocampal sections obtained to perform immunohistochemistry as previously described were used to perform the BrdU assay. Four slices per animal (one slice of 12 random series) were first incubated in HCl 0.1M for 30 min at 65°C, washed three times with PBS 0.1 M and incubated in Boric acid 0.3 M for 15 min at room temperature. After three washes, the sections were incubated overnight with anti-BrdU (1:200) (Sigma, St. Louis, USA) and anti-NeuN (1:500) (Millipore, Billerica, USA) followed by two hours incubation with Alexa Fluor 488 and 594 (Millipore, Billerica, USA 1:1000). After 24 h, the sections were mounted with FluorSave Reagent (Calbiochem, San Diego, USA). The mean of BrdU-positive cells counted manually by three blinded researchers (hilus, subgranular zone (SGZ) and granular zone (GCL) of dentate gyrus) was used.

BDNF ELISA Assay

The right hippocampus was homogenized in the lysis buffer used for Western blotting (n=6-8 animals per group). The BDNF level was measured through commercially available ELISA kit. All methodological procedure followed the manufacturer's instructions (ref.DY248, R&D systems, Minneapolis, USA). Firstly, the 96-well microplate was coated with 100 µL per well of the "capture antibody". After overnight incubation at room temperature samples were added (100µg) as well as serial dilutions of BDNF standard (170 ng/mL) dilutions and reagent diluent (1% BSA5 in PBS). After 2h 100 µL of the "detection antibody" that is the biotinylated mouse anti-human BDNF antibody was added.

Subsequently streptavidin-HRP, “substrate solution” (1xH₂O₂; 1x Tetramethylbenzidine) and “stop solution” (2N H₂SO₄) were added. The optical density of each well was assessed setting the microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, USA) at 450 nm. The results were analyzed using SoftMax Pro® Software (SpectraMax M5; Molecular Devices, Sunnyvale, USA).

Statistical Analysis

Data were analyzed by Student’s t-test, with the exception of MWM data, which were analyzed by repeated measures one-way analysis of variance (ANOVA) followed by Tukey post-hoc. Data were presented as mean ± S.E.M. P values < 0.05 were considered statistically different.

Results

Insulin i.c.v. infusions did not improve spatial memory performance of aged rats

First, we evaluated whether insulin administration improves spatial memory performance in aged and young rats. Insulin did not improve the spatial memory performance of aged animals, neither in acquisition (training) nor in the retention phase (probe) (Fig. 1b and c). By contrast, insulin significantly increased the performance of young animals in both the acquisition and retention phases of MWM (probe trial) (Fig. 1d and e).

Insulin i.c.v. infusions increased downstream insulin signaling in hippocampus of aged rats

To determine whether insulin infusions impact hippocampal insulin pathway, we performed Western blot analysis. We found that insulin increased the expression of IR (Fig. 2a), pGSK3-βser9 (Fig. 2b) and mTOR (Fig. 2c), as well as decreased cleaved caspase 3 (Fig. 2d) in aged rats relative to that of aged-vehicle rats. In young-insulin rats, there was an increase in the immunocontent of pGSK3-βser9 (Fig. 2f) but not of IR (Fig. 2e), mTOR (Fig. 2g) or cleaved caspase 3 (Fig. 2h) when compared with young-vehicle.

Figure 1. Insulin i.c.v. administration did not improve spatial memory performance of aged rats.

Diagram of experimental design (a). The hippocampal-dependent memory of aged rats was tested in the Morris Water Maze (MWM) task. After five days of insulin i.c.v. administration, the animals were submitted to the MWM. In aged rats, neither the latency to find the platform during the training days (b) nor the time spent in the target quadrant during the test day (c) was changed by insulin. Young rats injected with insulin decreased the latency to found the platform (D) (insulin < vehicle, * $p < 0.05$) from third to fifth day training protocol, and increased the time spent in the target quadrant during the test day (E) (* $p < 0.05$), (Mean $\pm$ SEM).

Insulin i.c.v. infusions induced neuronal proliferation in the hippocampus of aged rats

The neurotrophic role of insulin was first evaluated in the hippocampus using the neuronal nuclear marker cresyl violet (24 h after the 5 consecu-

Figure 2. Insulin administration increased the expression of insulin receptor (IR) and downstream proteins and decreased apoptotic marker expression of aged rats. Aged rats that received i.c.v. insulin injections for five days showed an increased expression of the downstream hippocampal signaling proteins compared with the vehicle controls: IR expression (a; $*p < 0.05$), phosphorylated levels of pGSK3-BetaSer9 (b; $*p < 0.05$) and mTOR expression (c; $*p < 0.05$) and a decreased expression of apoptotic marker cleaved Caspase 3 protein (d; $*p < 0.05$). The expression of proteins involved in the insulin signaling pathway showed a characteristic profile in young rats administered an i.c.v. injection of insulin: IR (e) and mTOR (g) were not significantly affected, whereas the phosphorylated level of pGSK3-BetaSer9 (f; $*p < 0.05$) was increased compared with the vehicle controls. The cleaved Caspase 3 expression was not affected by treatment with insulin (h) (Mean $\pm$ SEM).

tive injections of insulin). The representative images showed that vehicle-treated 24-month-old rats had apparently less neuronal density in the CA1, CA3 and DG hippocampal subfields (Fig. 3a). Also, insulin i.c.v. administration seemed to increase the nuclear neuronal staining in the hippocampal CA1, CA3 and DG subfields of aged (Fig. 3a) but not young rats (Fig. 3b). Similarly, insulin increased the number of NeuN-positive neurons in hippocampal CA1, CA3 and DG subfields of aged rats (Fig. 3c, e) but not young rats (Fig. 3d, h).

NeuN fluorescence intensity is usually associated with preserved functional integrity [22,23]. Our data show that insulin increases the fluorescence of NeuN in the CA1 and particularly the DG of aged rats (Fig. 3f) but had no statistically significant effects in young rats (Fig. 3i). However, in young rats, the fluorescence of DG is higher than that of the other hippocampal areas (Fig. 3i). Furthermore, by analyzing the ratio between the fluorescence of NeuN and NeuN counts, we found that insulin increased NeuN fluorescence/counts in the DG of aged rats (Fig.3g) but not in young rats (Fig.3j).

Given the prominent neurotrophic effects of insulin in the DG and the critical role of this hippocampal subfield in neurogenesis during adulthood [24], we then investigated whether the increase in the number of NeuN-positive cells and NeuN fluorescence induced by insulin might be linked to newborn neurons. Indeed, insulin administration significantly increased the number of BrdU-positive cells in the DG of aged rats when compared with vehicle-treated aged rats (Fig. 4a, c). By contrast, insulin did not significantly affect the fraction of BrdU-positive cells in the DG of the young rats (Fig. 4b, d).

Insulin i.c.v. infusions increased pre-synaptic plasticity in the hippocampus of aged rats

We also investigated whether insulin signaling was capable of modulating the hippocampal immunocontent of two markers of synaptic plasticity: synaptophysin (Syn), a marker for pre-synaptic terminals, and the postsynaptic density protein 95 (PSD-95), a marker of post-synaptic terminals. Insulin increased the expression of Syn in the hippocampus of aged rats (Fig. 5a) but not young rats (Fig. 5c). PSD-95 expression was not altered in response to insulin infusion in aged or young rats compared with their respective vehicle controls (Fig. 5b, c).

Insulin i.c.v. infusion modulates the BDNF levels and the immunocontent of its receptor (TrkB) in the hippocampus of young rats but not aged rats

The BDNF expression and insulin signaling is proposed to interact to improve learning and memory function in young (Takach, Gill et al. 2015) we further evaluated this assumption in aged rats. Five days of insulin treatment did not increase BDNF levels in both young and aged rats (6a and b, preMWM). Similarly, we found that after five days of insulin administration there was no increment in the TrkB expression in aged rats (6c, preMWM). In contrast, insulin increased

Figure 3. Insulin induced neurotrophic effect in the CA1, CA3 and DG hippocampal sub regions of aged rats. The rats were subjected to an i.c.v. injection with vehicle or insulin for five days. Representative images obtained with cresyl violet staining of the hippocampal regions CA1, CA3 and DG of aged (a) and young rats (b). Scale bar = 500um. Representative images using NeuN immunostaining and DAPI staining of the CA1, CA3 and DG of the hippocampus of aged (c) and young rats (d). Scale bar = 100um. Insulin increased NeuN-positive counts/mm² in the hippocampal sub regions of aged animals (e; *p<0.001) but increased the NeuN density of fluorescence only in the CA1 and DG (f; *p<0.0001). Similarly, insulin injection increased the NeuN fluorescence/NeuN counts/mm² ratio in the DG of aged rats (g; *p<0.05). Insulin did not significantly increase NeuN positive counts/mm² (h), NeuN density of fluorescence (i) or NeuN fluorescence/NeuN counts/mm² ratio in the hippocampal regions of young rats (j); (Mean±SEM)

Figure 4. Insulin i.c.v. injection induced neurogenesis in the hippocampal dentate gyrus (DG) of aged rat. Representative images of BrdU and NeuN staining in aged (a) and young (b) rats in the DG of the hippocampus (n=4-6, per group). Insulin significantly increased the number of BrdU-positive counts in the DG of aged (c; *p<0.05) but not young rats (d); (Mean ± SEM); Scale bar = 100µm. Insert in the left top indicate a higher magnification image of BRDU.

TrkB expression in young rats (6d, preMWM). Considering that insulin improved the spatial memory performance in the MWM paradigm, we investigated whether BDNF levels and insulin interact to facilitate this effect (postMWM). Insulin did not increase BDNF levels in the hippocampal homogenates of the aged rats (Fig. 6e) postMWM. Conversely, insulin significantly increased hippocampal BDNF levels in young rats compared with the young vehicle group (Fig. 6f) post MWM.

Figure 5. Insulin i.c.v. administration increased synaptic plasticity in the hippocampus of aged rats. To evaluate synaptic plasticity, we analyzed two synaptic proteins, synaptophysin (Syn) and PSD-95. Aged rats showed increased expression of the pre-synaptic Syn marker (a; * $p < 0.05$) but not the postsynaptic marker PSD-95 (b). Insulin did not cause significant changes in the expression of Syn (c) or PSD-95 (d) in the hippocampus of young rats (Mean $\pm$ SEM).

Discussion

Our results demonstrated that five days of i.c.v. insulin administration increased spatial memory performance in young but not aged rats. In addition, insulin administration increased the proliferation of newborn neurons, BrdU-positive neurons, in the hippocampal DG subfield of aged rats. At the molecular level, these effects were accompanied by an increased expression of IR, pGSK-3ser9, mTor, and Syn in the hippocampus; however, such responses had no significant impact on BDNF expression or spatial memory performance. In contrast, the administration of insulin increased BDNF and TrkB expression in the hippocampus and spatial memory in young rats without causing significant increments of newborn neurons in the hippocampal DG. Based on these findings, we suggest that a disruption between neurotrophic IR signaling and BDNF expression in the hippocampus could account for the distinct memory profile induced by insulin in young and aged rats.

The mechanisms coordinated by IR signaling are impaired similarly in peripheral and central tissues due to aging. One could argue that brain insulin resistance, which precedes cognitive deficits, is a contributory mechanism to neu-

Figure 6. Insulin i.c.v. administration did not improve hippocampal BDNF and TrkB levels of aged rats. The hippocampal BDNF (a) and TrkB (c) levels were assessed just after insulin treatment of aged rats and did not respond to insulin administrations. The same occurred with BDNF levels after water maze task (e). Similarly, BDNF didn't increase when evaluated just after insulin injection (b). Opposed to this, there was an increased TrkB expression just after insulin injection in young rats (d; * $p < 0.05$). BDNF level was also increased when assessed after water maze task in young rats in response to insulin administrations (f; * $p < 0.05$); (Mean $\pm$ SEM).

rodegenerative processes and memory deficits (Steen, Terry et al. 2005, Talbot, Wang et al. 2012). Accordingly, insulin administration constitutes a promising strategy for activating IR pathways, which are impaired in the hippocampus of aged rats. Given that the cellular and molecular brain responses to insulin admin-

istration were more prominent in aged rats, we assume that insulin is a feasible neuroprotective strategy against neuronal loss, albeit it is not related to the improvement of memory performance.

Actually, insulin signaling in the brain tissue is associated with several neurotrophic and anti-apoptotic effects (de la Monte and Wands 2005, De Felice, Lourenco et al. 2014). When insulin binds to IR, it promotes the phosphorylation and inhibition of GSK3- β , thereby the pro-apoptotic intracellular signaling mediated by GSK3- β is inhibited (Doble and Woodgett 2003). In the brain of aged rats the increased immunocontent of IR in response to insulin likely represents a molecular biomarker of neurotrophic effect. Moreover, the increased phosphorylation of GSK3- β induced by insulin administration in both young and aged rats, along with the increased expression of the anti-apoptotic protein mTOR and the decreased pro-apoptotic cleaved caspase 3 only in aged rats, implies that aged animals are still capable of responding to exogenous sources of insulin.

Studies in humans and animal models suggest that the cognitive decline associated with aging is more likely to be a consequence of the alteration in synaptic function in the hippocampus rather than a consequence of neuronal loss (Morrison and Baxter 2012). Our results showed that the cerebral administration of insulin increased the NeuN fluorescence/counts mostly in the DG of the aged rats. Mullen and colleagues characterized NeuN as a maturing and differentiated neuronal-specific nuclear protein (Mullen, Buck et al. 1992). Furthermore, Lind and collaborators demonstrated that NeuN fluorescence is linked to neuronal functionality and constitutes a proxy of healthy status of post-mitotic neurons (Lind, Franken et al. 2005). Here, we demonstrated that although insulin induced neurogenesis in the DG of aged animals, there was no direct impact on spatial memory performance. Actually, neurogenesis in the DG declines dramatically with age, which could represent the cause of age-related impairment in hippocampal-dependent memory; however, this statement remains highly controversial (Merrill, Karim et al. 2003). In addition, the survival of new neurons correlated with increased spatial learning in one study (Drapeau, Mayo et al. 2003) and with worsening results in another study (Bizon and Gallagher 2005). Furthermore, although adult neurogenesis and the incorporation of newborn neurons into hippocampal circuits seems to improve spatial memory formation (Kee, Teixeira et al. 2007), disorders in cognitive functions were not observed in rodent strains whose neurogenesis was ablated with anti-mitotic drugs or irradiation (Shors, Townsend

et al. 2002, Saxe, Battaglia et al. 2006). Therefore, there is not a consensus on the casual relationship between neurogenesis and cognitive impairment.

Cognitive function including memory formation and acquisition requires a complex collaborative and dynamic interaction between regions of the brain required for maintaining neuronal networks (Rosenzweig and Barnes 2003). In aged rats, we found that insulin increases the pre-synaptic protein synaptophysin, but had no significant effect on the post-synaptic marker PSD95, which suggests that insulin is not able to increase synaptic connectivity in the aging hippocampus. It has been proposed by Morrison and Baxter (Morrison and Baxter 2012) that the degree to which a decrease in the capacity for the generation of new neurons has a primary role in age-related impairments in hippocampal-dependent memory remains elusive and requires further investigation. Accordingly, our results shed light on the existence of signaling network in the hippocampus linking insulin, BDNF levels and spatial memory performance. We found that i.c.v. injection of insulin in the brain increased hippocampal BDNF levels and spatial memory in young rats but not in aged rats, implying that this network is necessary for learning and memory mechanisms, but due to brain aging, this connection is missing in older rats. "Our results did not clarify the precise mechanism enrolled in the increased BDNF levels, since the IR activation, in both neurons and astrocytes, modulates a plethora of neurotransmitter receptors and enzymes, such as CREB, MAPK, GABA_A, glutamate receptors/transporters that could influence BDNF synthesis (van der Heide, Ramakers et al. 2006). These issues were not addressed in this work, and deserve future investigations.

Whereas clinical approaches have supported the use of intranasal insulin for halting or rescuing memory deficits in patients with AD, this present experimental study sheds lights on cellular and molecular adaptations, even in the apparent absence of memory deficits of aged rats. These benefits may delay the progression of brain aging, potentially delaying the onset of neurodegenerative disorders (de la Monte 2013).

Our data, therefore, suggest that insulin-mediated effects on spatial memory do not involve neurogenesis but may be mediated via alterations in TrkB and BDNF levels in young animals. Despite showing normal IR signaling, aged rats fail to mount this TrkB and BDNF response.

	Aged Rats	Young Rats
Spatial Memory (MWM)	-	↑
IR	↑	-
pGSK3B	↑	↑
mTOR	↑	-
NeuN⁺ cells	↑	-
Cleaved caspase 3	↓	-
BrdU counts	↑	-
Syn	↑	-
BDNF pre MWM	-	-
TrkB pre MWM	-	↑
BDNF pos MWM	-	↑

Table 1. Summary of insulin effects in aged and young rats

Acknowledgments

We thank Prof. Dr. Harm Kampinga from University of Groningen, for his generous scientific contributions to this work. This work was supported by grants from CNPq (Grant No. 401645/2012-6), FAPERGS, and CAPES.

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CHAPTER 3

Insulin activates microglia and increases COX-2/IL-1 β expression in young but not in aged hippocampus.

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*Chapter published in Brain Research (2020) 1741:146884
DOI: 10.1016/j.brainres.2020.146884*

Graphical Abstract

Abstract

Brain insulin resistance and neuroinflammation are known to increase with age. Insulin exerts metabolic roles on neurons and astrocytes, but its effects on microglia is unclear. In this study we investigated whether insulin affected microglia in the hippocampus of young and aged rats. We injected intracerebroventricular (i.c.v.) insulin (20 mU) or vehicle for five days and evaluated microglial inflammatory markers in the hippocampus of young (3 months) Wistar rats. Increased microglial activation (Iba-1⁺CD68⁺ cells) and COX-2/IL-1 β levels in the hippocampus were found. Since the aged brain is an experimental model for brain insulin resistance and chronic neuroinflammation we submitted aged rats (22 months) to i.c.v. insulin/vehicle administration and found no significant increase in Iba-1⁺CD68⁺ microglia or COX-2/IL-1 β levels. To further investigate whether insulin triggered transient or persistent proinflammatory responses, young rats were evaluated eight-days after the last insulin injection. Microglia were persistently activated, and COX-2 levels remained elevated in the hippocampus, which paralleled increased spatial memory performance in the Morris Water Maze behavioral task. To determine if microglia were directly responsive to insulin, primary microglia were challenged with insulin and increased Akt Ser473 phosphorylation, a protein activated by the insulin receptor, was detected. These data suggest that microglia in the hippocampus integrate insulin signaling and neuroinflammatory responses and that this signal is disrupted during chronic inflammation. In our concept, the disruption between microglia activation by insulin signaling is a new pathological mechanism behind insulin resistance in the aging brain.

Key words: insulin; microglia; aging; memory; hippocampus; neuroinflammation

Introduction

Microglia are a specialized and dynamic population of central nervous system (CNS) parenchyma-resident macrophages that are able to detect tissue damage and (micro) environmental variations (Hanisch and Kettenmann 2007). Although, microglia are the primary innate immune cells in the brain, their CNS-tailored functions go beyond their well-characterized roles in neuroinflammation (Salter and Beggs 2014). Microglia also have important functions in non-immune brain physiology and are reported to contribute to learning and memory (Parkhurst, Yang et al. 2013). Other important microglia functions are trophic neuronal support, removal of cellular debris, synaptic pruning, injury repair and homeostatic support for living neurons, which is crucial for the neural circuit remodeling and synaptic plasticity (Salter and Beggs 2014, Galatro, Holtman et al. 2017, Janssen, Dubbelaar et al. 2017).

Microglia sense disturbances in brain homeostasis and turn out to be “activated”, a phenotype characterized by morphological changes, e.g. process retraction and thickening, and upregulation of a broad spectrum of cellular membrane surface antigens, such as CD68 (O’Neil, Witcher et al. 2018). Microglial activation is accompanied by expression of several intracellular molecules including the COX enzyme family followed by intracellular and extracellular expression of cytokines (Akundi, Candelario-Jalil et al. 2005). Neuroprotective functions of COX-2 are already described, which include contributions to learning and memory processing (Teather, Packard et al. 2002, Bazan 2006, Choi, Aid et al. 2009). Although substantial amount of evidences had identified both IL-1 β and IL-6 as important cytokines with potential neuroprotective functions (Seibert and Masferrer 1994, Tak and Firestein 2001, Frakes, Ferraiuolo et al. 2014, Borsini, Zunszain et al. 2015, Gruol 2015, Takemiya, Fumizawa et al. 2017) indicating that proinflammatory cytokines responses are not only detrimental to the brain (Biber, Owens et al. 2014), IL-1 β and IL-6 neurophysiological and neuroprotective signals still need further scrutiny.

During aging, microglia adopt a more activated and sensitized state phenotype referred to a “primed”, resulting in an exaggerated inflammatory response to an inflammatory stimulus (Perry and Holmes 2014, Raj, Jaarsma et al. 2014). Aging is associated with persistent changes in the brain microenvironment, and chronic neuroinflammatory stimulation has been proposed as a key factor asso-

ciated with microglial dysfunction and a mechanism for inducing the age-related functional deficits (Holtman, Raj et al. 2015, Karch and Goate 2015, Norden, Muccigrosso et al. 2015, Spittau 2017). However, although aged microglia may adopt an undesirable phenotype, the mechanistic effectors of this reprogramming are not well comprehended.

Insulin has diverse functions in the CNS, including neurotrophic signaling (Bomfim, Forny-Germano et al. 2012, Rhea, Salameh et al. 2019). Brain insulin administration improved memory in young but not in aged rats, which was related with a disrupted connection between both insulin and BDNF pathways in the aged hippocampus (Haas, Kalinine et al. 2016). Accordingly, experimental rodent models and studies with aged patients have consistently demonstrated a strong mechanistic association between deficient insulin signaling, cognitive disruption and Alzheimer's disease (Talbot, Wang et al. 2012, Ghasemi, Haeri et al. 2013, Lopez-Otin, Blasco et al. 2013, Gutchess 2014, Steculorum, Solas et al. 2014). The role of insulin in neurons has been extensively investigated, but only recently its role in glial cells has received the necessary attention. For instance, insulin signaling in hypothalamic astrocytes controls the availability of glucose to neurons, which is crucial for the regulation of whole body energy homeostasis (Garcia-Caceres, Quarta et al. 2016). Recently, it was demonstrated that insulin receptor (IR) and upstream proteins (IRS-1 and IRS-2) are expressed in human primary astrocytes and microglia supporting the notion that insulin may directly bind to and influence microglia activity (Spielman, Bahniwal et al. 2015), but, it is still unclear the downstream microglial insulin signaling (Akt-GSK3- β), how insulin affects microglia activation *in vivo*, and, similarly as occurs in aged neurons (Rodriguez-Rodriguez, Sandebring-Matton et al. 2017), if primed microglia are resistant to insulin.

In order to address the effect of insulin on microglia *in vivo*, we performed insulin infusions directly into the brain of young and aged rats and analyzed their activation, and COX-2, IL-6 and IL-1 β expression in the hippocampus. To detect a direct insulin-mediated response by microglia, we performed *in vitro* experiments using primary microglia cultures.

Material and Methods

In vivo experiments

In vivo experiments were performed using 3 and 22-month-old male Wistar rats under approval of the UFRGS Local Ethic Committee for Animal Experimentation (CEUA); project number: #26376. Wistar rats were housed in groups of maximum five animals, in a room with controlled temperature, under a 12 h light/dark cycle and with free access to food and water.

Glucose Tolerance Test – GTT

Wistar rats (3 and 6 months) were used for glucose tolerance test (GTT) evaluation. Glucose homeostasis was determined as previously described (Muller, Cammarota et al. 2008) using the glucose tolerance test (GGT), which was performed prior to animal sacrifice. A concentrated glucose solution (2 mg/g i.p.) was injected into mice fasted for 12 h, and blood was collected from a small puncture on the tail at 0, 30, 60, and 120 min after injection. Blood glucose level was measured with glucometer (AccuChek Active, Roche Diagnostics, USA), and the area under the curve was used to compare the glucose tolerance among groups.

Cerebrospinal fluid sampling

Before the surgery for cannula implantation the anesthetized rats were placed in the stereotaxic equipment (Insight, São Paulo, Brazil) and 100-120 μ L of cerebrospinal fluid (CSF) was collected from the cisterna magna with an insulin syringe similar as previously described by (Oses, Muller et al. 2019). Samples contaminated with blood cells were discarded.

Surgery and intracerebroventricular treatments

First animals were submitted to surgery for cannula implantation. Animals were anesthetized using intraperitoneal (i.p.) ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in stereotaxic equipment (Insight, São Paulo, Brazil). A cannula (27-gauge 9-mm) was positioned according to the following coordinates: 0.9 mm posterior from the bregma, 1.5 mm right of the midline and 2.6 mm ventral to the superior cranial surface. Animals were left to recover for 2 days, and on the third day post-surgery, when the rats already displayed normal food and water consumption as well as spontaneous locomotion the intracerebroven-

tricular (i.c.v.) treatment was started. Young and aged rats received i.c.v. insulin 20 mU (Humulin R®; Lilly) or vehicle (NaCl 0.9%) once a day for five consecutive days injected through the cannula implanted in the fourth ventricle. After i.c.v. administrations, rats were randomly allocated in two different groups: i) Twenty-four hours after the last insulin administration, a group of both young and aged rats were euthanized by decapitation and the brain samples were collected for further analysis; and ii) After 8 days of the last insulin administration a second group comprising only young rats were euthanized and brain samples were also processed for further analysis.

Immunohistochemistry and Confocal Microscopy

Immunohistochemistry was conducted as previously described (Haas, Kalinine et al. 2016). After euthanasia, brains were collected to perform immunohistochemistry. After the brain fixation in 4% paraformaldehyde, in 0.1 M phosphate buffer, at pH 7.4 for 24 h, they were transferred to PBS and cut into coronal sections (50 μ m) with a Vibratome (Leica, Buffalo, USA). Of every eight slices, one was collected and permeabilized for 15 min. (Triton X-100 in PBS 0.1 M) and blocked (BSA 2 %, Triton X-100, TBS) for 1 h. Primary antibodies Iba-1 (1:1000; #019-19741; Fujifilm WAKO, Richmond, USA) and CD68 (1:500; #MCA341R; AbD Serotec, Hercules, USA) were incubated overnight, followed by the secondary antibodies Alexa Fluor 594/488 (1:1000; #A32731; #A32744; Invitrogen, Waltham, MA) for 2 h at room temperature. Nuclei marker DAPI (0.0001%; #28718-90-3; Santa Cruz Biotech., Dallas, USA) was used for 25 min at 4°C. Pictures were captured by the entire thickness Z stack for analysis. After images acquisition using confocal microscope (Olympus FV1000, Shinjuku, Japan) they were analyzed using Image J® software, (NIH, Bethesda, USA). The number of microglia cells expressing both Iba-1 and CD68 were quantified from a total of six slices per animal covering the 3 hippocampal subregions CA1, CA3, and DG. Three regions of interests (ROIs) (100 μ m²) per hippocampal subregion were placed always at the same position for all images. The average of cell counts by the three ROIs per subregion per slice was multiplied by 10, and the results were expressed in cells/mm². For expressing the results in Figures 1 and 3, The percentage of CD68-positive microglia over total microglial cells was calculated as previously described (Peng, Xiao et al. 2014) taking in account the total number of microglial cells that varies depending on the individual animal. For that we calculated the

percentage of cells displaying Iba-1/CD68 colocalization (activated microglia)/mm² by the total number of cells expressing Iba-1 (total microglial number)/mm² per animal. We analyzed such parameters for each hippocampal subregion (CA1, CA2 and DG). Total number of cells used for percentage of CD68-positive microglia calculation can be found in the supplementary tables 1 and 2 regarding results on Figure 1 and supplementary tables 3 and 4 regarding results on Figure 3.

Western Blot

For *in vivo* experiments hippocampal tissue were homogenized in lysis buffer (EDTA 2mM, protease and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, USA) 0.001%, Tris HCl 50 mM, pH 7.4, glycerol 10 % and Triton X-100 1%) and centrifuged (15000 g/10 min). For *in vitro* experiments, microglia were washed twice in PBS and a non-denaturing cell lysis buffer (CST, Danvers, MA) was used accordingly the manufacturer's instruction. Resultant protein suspensions were measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and further diluted in sample buffer (SDS 10%, glycerol 40% and bromophenol blue 0.25%; Sigma, St. Louis, USA) to a final concentration of 1 μ g/ μ L. 20-40 μ g protein were separated by weight using electrophoresis SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) in transfer buffer (25 mM Tris, 150 mM glycine, 20% v/v methanol). Blocking procedures were performed according to the primary antibodies' datasheets using nonfat dry milk (Bio-Rad, Hercules, CA) or BSA (Sigma, St. Louis, USA). After blocking, membranes were washed 2 times with Tween-TBS and once with TBS and the primary antibodies p-AktSer473 (1:1000; #4060; CST, Danvers, MA), Akt (1:2000; #2920; CST), p-GSK3- β Ser9 (1:1000; #9323, CST), GSK3- β (1:1000; #9315, CST), COX-2 (1:1000; #160112, Cayman, Ann Arbor, USA), β -Actin (1:5000-1:10000; #Ab6276, Abcam, Cambridge, MA), were incubated overnight at 4 °C. After primary antibody incubation, membranes were washed 3 times, and the HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1:5000; # NA9310; # NA9340, GE, Little Chalfont, UK) were incubated 2 h at room temperature. Proteins of interest were immunodetected by chemiluminescence using both Image Quant LAS 4000 (GE, Little Chalfont, UK) or ChemiDocMP® (Bio-Rad, Hercules, CA). Protein expression was quantified using both ImageLab 5.2.1® (Bio-Rad, Hercules, CA) and Image J® (Rockville, USA). The immunocontent of each target

protein was divided by the constitutive protein present in each sample and after that normalized by the relative expression of the target in the control group (%).

ELISA Assays

For hippocampus homogenates (prepared using the same protocol described in the Western Blot section) IL-1 β and IL-6 (#RAB0278 #RAB0311; Sigma, St. Louis, USA) kits were used. The protocols were performed according to the instructions of the manufacturer. Samples and standards were assessed in duplicates and the coefficient of variation was less than 5% for both assays. For CSF insulin levels an ELISA Insulin kit (Millipore Ref# EZRMI-13K) was used and the technical procedures were performed accordingly to the manufacturer instructions. For measurements using ELISA in hippocampus homogenates the results were corrected by the concentration of total protein which was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

In vitro experiments

Male and female C57Bl/6 mice pups were used to perform experiments *in vitro*. C57Bl/6 pups were provided by the Central Animal Facility, CUMC, University of Groningen. All experiments were performed according to the experimental animal guidelines of the University of Groningen, The Netherlands and approved by the local IACUC.

Microglia Cell Culture and Treatments

Primary neonatal microglia culture was performed according (Schaafsma, Zhang et al. 2015) with modifications. C57Bl/6 mice of postnatal day P0-P2 (female and male) were sacrificed by decapitation and the forebrains were removed from the skull and maintained in Dissection Medium (DM) – Hanks Bovine Salt Serum (HBSS; Lonza), 45% D-(+)-Glucose (Sigma, St. Louis, USA), 1M HEPES (Lonza), 100 U/mL Penicillin/100 mg/mL Streptomycin (Sigma, St. Louis, USA). Meninges, brain stem and cerebellum were separated, and the forebrains were minced and washed with DM. Next, a chemical dissociation step was performed, the minced tissue was incubated in 1x DNase (Sigma, St. Louis, USA) - 2.5 % trypsin (Lonza, Maastricht, The Netherlands) - DM, during 25 min, 37 °C, mixing gently every 5 min. The chemical dissociation was stopped by addition of heat inactivated (HI) 20% Fetal Bovine Serum (FBS – Gibco, Waltham, USA)

containing 1x DNase and 0.1 mg/mL trypsin inhibitor (Sigma, St. Louis, USA) in DM. Tissue was then followed by a quickly wash using 10% HI FBS with 1x DNase in DM following by a mechanical dissociation using glass pipette until a cell suspension was obtained. The cell suspension was washed in microglia medium – MM (4.5 mg/mL glucose – 0.584 mg/mL L-glutamine –Dulbecco's Modified Eagle Medium (DMEM – Gibco), 10% HI FBS (Gibco), 1 mM sodium pyruvate (Lonza), 100 U/mL Penicillin-100 mg/mL Streptomycin) and centrifuged (10 min.; 960 rpm; 12 °C). The cell pellet was re-suspended in MM and plated in T75 culture flasks (1.5 brains/flask) that where cultured at 37 °C in a humidified atmosphere at 5% CO₂. In the day after, the medium was refreshed and then the medium changes were done every 3-4 days. After 7-10 days, 33% L929 (ATCC, Manassas, USA) fibroblast-conditioned medium containing M-CSF was added to the microglia medium in order to stimulate microglia proliferation. After 11-15 days of culture, when cells reached 100% confluence, microglia were obtained by mitotic shake-off using an orbital shaker (1 h; 150 rpm; 37 °C). After seeding, microglia were cultured in 50:50 MM supplemented with post shake-offs medium. The microglia primary cell culture purity was accessed by Western Blot for Iba-1 microglial marker (1:1000; #019-19741 Fujifilm-WAKO; Neuss, Germany) compared to neuronal β -III-Tubulin (1:5000; #ab7751; Abcam, Cambridge, UK), astrocytes GFAP (1:1000; #Z033420-2; Agilent; Dako, Santa Clara, CA) and oligodendrocytes MBP (1:1000; #AB980; Sigma-Millipore) markers (Sup. Fig.1). Insulin, wortmannin and DMSO (Sigma) were used to treat the cells and respective concentrations and incubation times can be found in the figure legends. Cells were always submitted to drug treatments at a maximum of 2 days after the shake-off.

Statistical Analysis

Analysis between 4 groups with two variances (treatment and age) were performed by two-way Analysis of Variance (ANOVA) followed by multiple comparisons Tukey posthoc test. For 2 groups analysis unpaired Student's t-test was used. GraphPad Prism® Software was used for statistical analysis and graphs preparation. Data were presented always as average $\pm$ S.E.M. P values $\leq$ 0.05 were considered statistically significant. Exact p values can be found in the respective result descriptions.

Results

Insulin triggers microglia activation in the young but not in the aged hippocampus.

To evaluate the insulin effects on microglia *in vivo*, we performed Iba-1/CD68 co-staining (Fig. 1a) to assess microglia activation in the hippocampus. We found that after five days of i.c.v. insulin treatment, microglia of young rats were more activated in CA1 ($p=0.022$) (Fig. 1b) and CA3 ($p=0.009$) (Fig. 1c) hippocampal subregions when compared to controls. No significant alterations were detected in the DG ($p=0.165$) (Fig. 1d). During aging, CNS develops resistance to insulin signaling and increased neuroinflammation (Haas, Kalinine et al. 2016, Raj, Yin et al. 2017). Next, we performed the same treatment in aged rats with insulin to assess whether aged (primed) microglia might be resistant to insulin challenges. Interestingly, insulin did not further increase the number of already present activated microglia. (Fig. 1b-d). As expected, and in conjunction with previous observations (Ojo, Rezaie et al. 2015, Solano Fonseca, Mahesula et al. 2016), naïve aged rats showed significantly increased microglial activation in the hippocampus in comparison to young rats - CA1 ($p=0.001$) (Fig. 1b) and DG ($p=0.002$) (Fig. 1d).

Insulin increases COX-2 and IL-1 β expression in the hippocampus of young rats.

Trying to elucidate the neuroinflammatory molecules that would link to microglia activation in young rats, we determined COX-2, IL-1 β and IL-6 expression levels in the hippocampus. Insulin significantly increased COX-2 levels in young rats ($p=0.003$), an almost 3-fold increase compared to the young vehicle group was observed. In aged animals, we did not detect an increase in COX-2 levels in response to insulin (Fig. 2a, b). In young animals, this profile was accompanied by increased levels of IL-1B ($p=0.038$) (Fig. 2c) but not IL-6 (Fig. 2d). In contrast, the level of IL-6 level was decreased in aged animals after insulin treatment ($p=0.031$) (Fig. 2d).

Microglia remain activated after 8 days of insulin treatment in young rats.

Next, we determined the persistence of the neuroinflammatory response to insulin in young Wistar rats. Young rats were subjected to the same protocol of 5-days insulin (i.c.v, 20 mU) and analyzed microglia activation 8 days after the

last administration (Fig. 3a). We found that microglia were persistently activated in the CA3 (p=0.015) (Fig. 3c), but not in the CA1 region (p>0.05) (Fig. 3b). To determine the neurological impact of insulin and if this treatment was neuroprotective during this 8 day-period, we performed a Morris water maze test, as previously described by us and others (Adzovic, Lynn et al. 2015, Haas, Kalinine et al.

Figure 2. Insulin increases COX-2 and IL-1 β in young rats hippocampus. (A) Representative images of western blot detection of COX-2 expression in young and aged animals' hippocampus. (B) Quantification of western blot detection of COX-2 expression (n=5-6 animals per group). (C) ELISA analysis of interleukin 1 β (IL-1 β) in young and aged (n=7-10 animals per group) Wistar rats' hippocampus. (D) ELISA analysis of interleukin 6 (IL-6) in young and aged rats (n=5-6 animals per group). Results are expressed as average $\pm$ SEM. * $p \leq 0.05$; ** $p \leq 0.01$; ns: $p > 0.05$.

2016). We confirmed that relative to vehicle, insulin treatment increased spatial memory performance in young rats ($p=0.020$) (Sup. Fig. 2).

COX-2 but not IL-1 β expression persists after 8 days of insulin treatment.

To investigate if the prolonged CA3 microglial activation (Fig. 3) is accompanied with a persistent increased expression of COX-2 and IL-1 β , we measured these inflammatory markers 8 days after the last insulin treatment. COX-2 levels remained increased in the hippocampus as previously observed (Fig. 1) when compared to vehicle ($p=0.0409$) (Fig. 4a, b). The increased IL-1 β levels observed after 5 days of insulin treatment (Fig. 2c) returned to baseline levels at day 8 post-administration (Fig. 4c), and IL-6 levels were not significantly altered (Fig. 4d).

Figure 3. Microglia activation triggered by insulin is persistent in CA3 hippocampal subregion after 8 days of insulin treatment. (A) Confocal microscopy representative images of microglia - Iba1 (green), activated microglia - CD68 (red) and nuclear - DAPI (blue) markers in both young and aged vehicle/insulin treated animals in the sub regions Scale bar = 25 μ m. (B) Microglial activation (Iba1+CD68+ cells/Iba1+ cells) in CA1 (n=4 animals per group). (C) Microglia activation (Iba1+CD68+ cells/Iba1+ cells) in CA3 (n=4 animals per group). (D) Microglia activation (Iba1+CD68+ cells/Iba1+ cells) in DG (n=4 animals per group). Results are expressed as average $\pm$ SEM. *p $\leq$ 0.05; **p $\leq$ 0.01; ns: p>0.05.

Primary microglia cells are responsive to insulin

To determine if microglia are responsive to insulin, we used primary microglia cell culture and analyzed the phosphorylation and activation of proteins AKT and GSK3- β , which are downstream signals of the insulin receptor. Cells exposed to insulin (1000 ug/mL) for 30 min displayed increased AKT phosphorylation (p=0.003) (Fig. 5a), but GSK3- β phosphorylation was not found altered (Fig. 5b). Incubation with the PI3K inhibitor wortmannin prevented insulin-induced AKT phosphorylation (Sup. Fig. 1), confirming that, insulin receptor downstream signals occur via PI3K and AKT in microglia.

Figure 4. Hippocampal COX-2 persists increased after 8 days of insulin treatment.

(A) Representative images of COX-2 immunodetection in the hippocampus. (B) COX-2 expression level (n=6-7 animals per group) acquired from the western blot analysis. (C) Interleukin 1 β (IL-1 β) and (D) interleukin-6 levels were measured in the hippocampal homogenates of young and aged Wistar rats' through ELISA assays (n=7-8 animals per group). Results are expressed as average $\pm$ SEM. *p=0.05; **p=0.01; ns: p>0.05.

Discussion

Our findings imply that the inflammatory response to insulin is different between young and aged microglia. This notion is supported by the increased number of CD68-positive microglia in the CA1 and CA3 hippocampal subfields of young but not aged rats in response to insulin. Complementary, the analysis of the inflammatory markers associated with microglia highlighted a concomitant higher expression of COX-2 and IL-1 β in the hippocampus of young rats, and these elevated COX-2 were sustained until at least 8 days after insulin administration. Aged rats did not respond to insulin challenges with microglial activation and increased hippocampal levels of IL-1 β /COX-2 as was observed in young animals, suggesting a complementary mechanism associated with brain insulin resistance during aging process. Also, we confirmed in primary microglia cultures,

Figure 5. Insulin triggers AKT phosphorylation in primary microglia. Qualitative analysis of insulin pathway proteins by western blot. (A) p-Akt-Ser473 and Akt immuno-detection in primary microglia stimulated with insulin (1000 ng/mL) or vehicle (PBS) for 30 minutes (n= 6-7 replicates per group) (B) p-GSK3- β Ser9 and GSK3- β immuno-detection in primary microglia stimulated with insulin (1000 ng/mL) or vehicle (PBS) for 30 minutes (n= 6-7 replicates per group).

that insulin activates the phosphorylation of AKT (Fig. 5 and Sup. Fig. 1), which signals downstream of the insulin receptor (Fernandez and Torres-Aleman 2012). These cellular cultures were generated from neonatal mouse pups, suggesting that microglia in young mice and rats can respond to insulin and that this responsiveness is lost or perturbed with aging.

Microglia are a highly ramified cell type in close contact with synapses, supporting an ideal microenvironment for synaptic network connectivity (Wu, Dissing-Olesen et al. 2015). Here, the activation of microglia, particularly in the CA1 and CA3 subregions of young rats, suggests that insulin influences

the expression profile of inflammatory mediators and spatial memory processing, which is a function highly dependent on the hippocampus. We found that the pattern of protein expression in microglia of young rats after insulin treatment was accompanied by increased performance in the acquisition and test phases of the Morris Water Maze (MWM) task (Sup. Fig. 2) corroborating an association between microglia phenotypes and memory function (Haas, Kalinine et al. 2016). Although the positive effects of both COX-2 and IL-1 β on memory were already described by others (Teather, Packard et al. 2002, Bazan 2006, Goshen, Kreisel et al. 2007, Donzis and Tronson 2014, Takemiya, Fumizawa et al. 2017), our results primarily connect this signature with insulin mediating higher brain functions, likely through microglia. Also, it was previously demonstrated that COX gene expression in the hippocampus of 24 months-old rats was not altered (Aid and Bosetti 2007) corroborating with our finding that COX-2 protein levels are not changed in 22 months-old. However, it should be taken into consideration that neither the referred study nor our study provided evidences to discard possible abnormalities in COX enzymatic activity due to aging.

The resulting response of microglia to insulin challenges seems to be impaired during aging, which is coincident with the decline in the peripheral and central insulin signaling previously described (Steculorum, Solas et al. 2014), moreover, animal models of diabetes showed decreased insulin levels in CSF (Nameni, Farhangi et al. 2017, Nameni, Hajiluian et al. 2017). Also, despite controversies, it was already demonstrated that the brain produces a small amount of insulin, albeit is still unknown the exact function and whether this synthesis might contribute to extracellular levels within a brain affected by aging (Aid and Bosetti 2007). Adult rats manifest impaired glucose control during the early periods of the aging process likely associated with deficits in the peripheral insulin signaling (Arnold, Arvanitakis et al. 2018). We found a resistant profile in the GTT in 180 days old rats compared to 60 days old expressed by a higher peak of blood glucose levels after the i.p. administration of a sucrose load (Sup. Fig. 3A) and a significantly higher area under the curve (AUC) (Sup. Fig. 3B). The levels of insulin in the CSF of fed animals were not changed in aged rats (22 months old), compared to young ones (3 months old) (Sup. Fig. 4A), however the insulin level on hippocampus were decreased on aged animals compared to young (Sup. Fig. 4B) pointing a possible impair on insulin delivery or activation on hippocampus of the aged animals.

Insulin, serves as a hub for several physiological roles underlying cell survival (Bruning, Gautam et al. 2000) and control of hypothalamic glucose support from astrocytes to neurons (Garcia-Caceres, Quarta et al. 2016). Besides, insulin has been previously proposed to integrate peripheral and central immune signals (De Felice and Ferreira 2014), and not coincidentally, insulin resistance has been connected with increased neuroinflammation and aged-related abnormalities (Lopez-Otin, Blasco et al. 2013, Steculorum, Solas et al. 2014). Our study shows that at basal conditions, the microglia in aged animals are more activated than in young ones, which is in agreement with others (Shoham, Linial et al. 2019) and with the sustained inflammation hypothesis in the course of aging (Wyss-Coray and Mucke 2002). Also, when insulin was injected in aged rats there was neither hippocampal microglia activation nor increased IL-1 β production, but a decreased in IL-6 hippocampal levels. Although we showed that primary microglia are responsive to insulin and leads to increase IL-1 β production, we cannot rule out that in our *in vivo* experiments this cytokine originates from other cells in the CNS. However, this limitation does not negate the possibility that microglia might be enrolled in neuroprotection even at activated state and expressing pro-inflammatory molecules, albeit persistent inflammation represents a substrate for unfavorable neurological outcomes (Saijo and Glass 2011, Heneka, Kummer et al. 2014). Importantly, long-term i.c.v. infusions of insulin in young rats prevented neuroinflammatory and memory impairment outcomes induced by lipopolysaccharide (LPS) (Adzovic, Lynn et al. 2015) reinforcing its direct importance as a neuroinflammatory mediator. Although the relationship between hippocampal neuroinflammation, microglia phenotypes and cognitive decline during aging is largely discussed in the current literature in both experimental and clinical studies (Tangestani Fard and Stough 2019), here the absence of a functional endpoint is a limitation for future clinical interpretations of the present results.

Also, as previously conceptualized by others, neuroinflammation is a result of integrated reactions that take place in astrocytes and microglia but further involves a concomitant exchange of signals with neurons (Tjalkens, Popichak et al. 2017). Remarkably, i.c.v. insulin infusion diffuses through the brain tissue and may activate IR signals in neuronal and astrocytic cells thereby impacting microglia physiology, or alternatively intrinsic microglial mechanisms may be affected by the insulin administration and propagate effects to the neighbor cells, or both (Butterfield, Di Domenico et al. 2014). Although this particular question is still

unsolved, our present findings bring subsidies to further investigate the interplay between insulin and the triad astrocyte-neuron-microglia mediating neuroinflammatory responses. Another question rises from the immunological bidirectional communication between periphery and brain. It is known that peripheral inflammatory cytokines, such as IL-6, IL-1 β , TNF- α , cross the blood brain barrier and cause increased expression of transcriptional factor NF κ - β within the brain cells accompanied by locally increments in the pro and anti-inflammatory effectors (Fishel, Watson et al. 2005). Conceptually exacerbated production of pro-inflammatory mediators oppose central and peripheral insulin signaling and may be a risk factor of neurodegeneration and cognitive problems (Akiyama, Barger et al. 2000). However, these studies often use strategies to exacerbate the global inflammatory status with LPS injections and diet-induce obesity and DM type 2. We already showed that “normal aged rats” did not respond to insulin infusions (same strategy used in the present study) with increased spatial memory performance, which predicts a lack of association between the reported microglial phenotypes and inflammatory markers with spatial memory outcomes. This assumption is in line with the effects of insulin in young rats.

In conclusion, insulin signaling caused a persistent microglia activation in the hippocampus of young rats, together with increased expression of inflammatory molecules COX-2 and IL-1 β , and microglia were sensitive to insulin via the AKT signaling pathway. In aged brains, an apparent lack of responsiveness to insulin was detected, indicative of aging-associated insulin resistance of the CNS. In our concept, the rupture between the neuroinflammatory insulin signaling in the hippocampus and memory processing is a new pathological mechanism behind insulin resistance and cognitive decline in the aging brain.

Acknowledgements

This work was supported by the Brazilian grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq #426796/2016-0 and #141100/2013-3, CNPq – Instituto Nacional de Neurociência Translacional – INNT #465346/2014-6, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul – FAPERGS – PRONEX #16/2551-0000499-4. This study was also funded by the Abel Tasman Talent Program – Graduate School of Medical Sciences sponsorship and Jan Kornelis de Cock Stichting grant awarded to CBH from

the University of Groningen - The Netherlands.

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Supplementary Material

Hippocampal Subregion	Young Vehicle	Young Insulin	Aged Vehicle	Aged Insulin
CA1	382 $\pm$ 53	494 $\pm$ 59	426 $\pm$ 97	298 $\pm$ 58
CA3	430 $\pm$ 59	574 $\pm$ 30	414 $\pm$ 65	344 $\pm$ 72
DG	462 $\pm$ 57	522 $\pm$ 100	452 $\pm$ 50	390 $\pm$ 59

Supplementary Table 1. Iba-1⁺ cells per experimental group.

Hippocampal Subregion	Young Vehicle	Young Insulin	Aged Vehicle	Aged Insulin
CA1	158 $\pm$ 36	312* $\pm$ 61	311* $\pm$ 74	187 $\pm$ 39
CA3	205 $\pm$ 23	376* $\pm$ 55	316 $\pm$ 55	254 $\pm$ 57
DG	219 $\pm$ 11	339* $\pm$ 38	389* $\pm$ 45	305 $\pm$ 27

Supplementary Table 2. Iba-1⁺ CD68⁺ cells per experimental group. Mean $\pm$ SEM. *p $\leq$ 0.05 (Young Insulin > Young Vehicle); #p $\leq$ 0.05 (Aged Vehicle>Young Vehicle).

Hippocampal Subregion	Young Vehicle	Young Insulin
CA1	350 $\pm$ 13	537* $\pm$ 43
CA3	263 $\pm$ 25	411* $\pm$ 44
DG	497 $\pm$ 74	632 $\pm$ 59

Supplementary Table 3. Ilba-1+ cells per experimental group. Mean $\pm$ SEM. *p $\leq$ 0.05 (Young Insulin > Young Vehicle).

Hippocampal Subregion	Young Vehicle	Young Insulin
CA1	125 $\pm$ 30	262* $\pm$ 15
CA3	120 $\pm$ 21	273* $\pm$ 23
DG	340 $\pm$ 57	479 $\pm$ 40

Supplementary Table 4. Ilba-1+ CD68+ cells per experimental group. Mean $\pm$ SEM. *p $\leq$ 0.05 (Young Insulin > Young Vehicle).

Supplementary Figure 1. Insulin signaling inhibition prevents insulin dependent AKT phosphorylation in primary microglia. Western Blot figure comparing the expression of Akt-Ser473 phosphorylation in primary microglia in response to 30 min. insulin (100 ng/ml and 1000ng/ml) with or without a 30 min. pre-incubation of wortmannin (100 nM).

Supplementary Figure 2. Insulin treatment improves spatial memory in young rats. Water Maze behavioral task performance in Wistar rats after insulin i.c.v. treatment. (A) Morris Water Maze training phase (7 days); seconds to find the platform per day (n=6 animals per group). (B) Morris Water Maze test phase (80 day); time in seconds spent to the animal to find the hidden platform (n=6 animals per group). Results are expressed as mean $\pm$ SEM. * $p \leq 0.05$.

Supplementary Figure 3. Glucose tolerance test (GTT) in 3-months and 6-months old mice. The blood glucose homeostasis profile after an i.p. load of sucrose was different between aged and young rats (A), and along with the calculation of area under the curve (AUC) indicates 180 days old rats have preliminary signs of insulin resistance. Results are expressed as mean $\pm$ SEM. * $p \leq 0.05$.

Supplementary Figure 4. Cerebrospinal fluid CSF and hippocampal homogenates insulin levels in young and aged rats. Insulin measurements performed in the cerebrospinal fluid (CSF) (A) ($p=0.11$) collected from the cisterna magna and hippocampal homogenates (B) ($p=0.007$) of aged rats (22 months) relative to young rats (3 months). Results are expressed as mean $\pm$ SEM. * $p\leq 0.05$.

Supplementary Figure 5. Microglia primary cell culture purity. Western Blot figure comparing the expression of microglia (Iba-1), astroglia (GFAP), Oligodendrocyte (MBP) and neuron (β -Tubulin) markers between primary microglia and brain homogenate.

CHAPTER 4

The effects of Insulin on murine microglia metabolism and cytokine expression *in vitro* and *in vivo*

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Manuscript in preparation

Abstract

Microglia are the tissue-resident macrophages of the CNS, with specialized roles that are shaped by their neural microenvironment and go beyond immunological functions. Insulin is a hormone with well-recognized functions in the brain. Physiological IR signaling is associated with brain health whereas central resistance to insulin effects is mechanistically linked with cognitive deficits and neurodegenerative disorders. Recently, it was reported that IR in astrocytes of the hypothalamus are targeted by insulin and regulate whole body energy homeostasis. Although IR pathway is well described in neurons and more recently in astrocytes, it is unclear whether it produces functional responses in microglial cells. Here, we show that insulin inhibited microglial peroxide production, increased mitochondrial MTT activity in primary microglia and increased IL-1 β specifically after an LPS challenge. Conversely, intranasal insulin administration in adult mice increased the expression of the pro-inflammatory cytokine TNF- α . These data indicate that insulin elicits different responses in microglia *in vitro* versus *in vivo*.

Key words: insulin signaling; primary microglia; intranasal insulin; reactive oxygen species

Introduction

The central nervous system (CNS) is supported by the continuous exchange with and supply of metabolites, energy substrates and hormones from the periphery. Insulin is a well-characterized hormone which functions in the CNS are mostly related to energy metabolism and neurotrophic functions (Fernandez and Torres-Aleman 2012). In the brain, insulin regulates important cellular functions such as survival, gene transcription, protein translation, oxidative stress cell proliferation (Fernandez and Torres-Aleman 2012) and learning and memory processing (Muller, Gnoatto et al. 2011, Kleinridders, Ferris et al. 2014, Haas, Kalinine et al. 2016). Over the last 30 years, great advances have been made regarding insulin signaling in the brain, but only a small part pertains to insulin signaling in glial cells.

The expression of functional IRs and downstream proteins had been described in astrocytes *in vitro* and *in vivo* by (Garwood, Ratcliffe et al. 2015). Further studies had shown how the modulation of these receptor affects the brain function. Insulin signaling works as glucose sensors regulating brain glucose uptake in the mice hypothalamus (Garcia-Caceres, Quarta et al. 2016). Additionally, specific depletion of IRs in astrocytes results in delayed puberty and testosterone production deficiency (Manaserh, Chikkamenahalli et al. 2019). In the other hand, the effects of insulin on microglia *in vitro* and *in vivo* is less understood. Studies available had focused on the insulin neuroinflammatory effects in the brain related mainly to microglia activation and interleukin production by the whole brain tissue or tissue from specific brain areas such as hippocampus (Adzovic, Lynn et al. 2015, Mamik, Asahchop et al. 2016, Haas, de Carvalho et al. 2020). Trying to address direct effects of insulin in microglia (Haas, de Carvalho et al. 2020), we showed that primary microglia are sensitive to insulin via PI3K signal and Akt phosphorylation *in vitro*, but the sensitivity of adult microglia to insulin *in vivo* and the biological outcomes of such signaling are important questions still to be addressed.

Microglia are CNS-resident innate immune cells with specialized roles that are shaped by their cellular microenvironment (Aguzzi, Barres et al. 2013). A key characteristic of activated microglia is the high phagocytic activity presented by this phenotype (Fu, Shen et al. 2014). Phagocytosis is a reactive oxygen species (ROS)-dependent process, being the membrane bound NADPH oxidase enzyme

and the oxidative phosphorylation in the mitochondria the main sources of ROS in innate immune cells (monocytes and macrophages) (Bordt and Polster 2014). The high efficiency of ROS production and further effective phagocytosis are important for eliminating pathogens and for synapse and cellular debris removal (Biswas and Mantovani 2012). In addition to its role in phagocytosis, a signaling pathway is regulated by ROS in microglia, involving nuclear translocation of NF- κ B, where it acts as a transcriptional activator of cytokine genes such as IL-1 β , IL-6 and TNF- α (Biswas and Mantovani 2012).

Since most neural cells are sensitive to neuroendocrine signaling from the periphery, and the fact that insulin may integrate various signals and functional properties in the brain, in this study we analyzed the microglial response to insulin. *In vitro*, we investigated insulin effects on H₂O₂ production, related to cell metabolic activity/NADPH oxidase activity and interleukin gene (IL-1 β , IL-6 and TNF- α) expression. *In vivo*, we investigated insulin effects on gene expression of pro- and anti-inflammatory genes (IL-1 β , IL-6, TNF- α , IL-10, Arg-1 and COX-2).

Material and Methods

Animals

Male and female C57Bl/6 mice pups (P0 - P2) and C57Bl/6 adult mice (3 - 4 months) obtained from UMCG Central Service for Laboratory Animals (CDP) University of Groningen were used to perform *in vitro* and *in vivo* experiments, respectively. All experiments were performed according to the experimental animal guidelines of the University of Groningen, The Netherlands. When applicable, the animals were group housed under standard conditions in a 12/12h light/dark cycle with food and water available ad libitum.

Primary Microglia Culture

Primary neonatal microglia culture was performed according (Schaafsma, Zhang et al. 2015) with modifications. C57Bl/6 mice were sacrificed, and the brains maintained in dissection medium (DM) – Hanks bovine salt serum (HBSS; Lonza), 45% D-(+)-Glucose (Sigma, St. Louis), 1 M HEPES (Lonza), Penicillin 100 U/mL Streptomycin 100 mg/mL (Sigma, St. Louis). Meninges, brain stem and cerebellum were removed, and the rest of the CNS was minced and washed once in dissection medium. Next, a chemical dissociation step was performed where

the minced tissue was incubated in 1 x DNase (Sigma, St. Louis) - 2.5% trypsin (Lonza) - dissection medium, during 25 min, 37 °C, mixing gently every 5 min. The chemical dissociation was stopped by addition of 20% Fetal Bovine Serum (FBS – Gibco) – 1x DNase – 0.1 mg/mL trypsin inhibitor (Sigma, St. Louis) – dissection medium to the tissue followed by a quickly washing using 10% FCS – 1 x DNase in DM. Then, a mechanical dissociation was performed in the same medium (10% FCS – 1 x DNase - DM) using glass pipette until a cell suspension was obtained. Next, the cell suspension was washed in 25 mL of microglia medium – MM - (4.5 mg/mL glucose – 0.584 mg/mL L-glutamine – Dulbecco's Modified Eagle Medium (DMEM – Gibco), 10% FBS (Gibco), 1 mM sodium pyruvate (Lonza), Penicillin 100 U/mL - Streptomycin 100 mg/mL) and centrifuged (10 min.; 960 rpm; 12 °C). The cell pellet was re-suspended in MM and plated in T75 culture flasks (1.5 brains/flask) that where cultured at 37°C in a humidified atmosphere at 5% CO₂. In the day after, the medium was refreshed and then the medium changes were done every 3-4 days. After 7-10 days of mixed glial culture, 33% L929 fibroblast-conditioned medium was added to the microglia medium to stimulate microglia proliferation. L929 fibroblast-conditioned medium contains M-CSF that is obtained by addition of normal microglia medium (without 1mM sodium pyruvate) to 80% confluent L929 cells (L-929 cell line; ATCC, Manassas), 2 days cultivation and collection of the conditioned medium that is filtered and sterilized. After 11-15 days of culture, when the cells reached 100% confluence, microglia were obtained by mitotic shake off using an orbital shaker (1 h; 150 rpm; 37°C). After seeding, microglia were cultured in 50:50 microglia medium supplemented 50% with the medium collected from the mixed glial culture after the shake offs. Purity of the primary microglia cell culture isolated and cultivated under the present protocol was previously demonstrated (Haas, de Carvalho et al. 2020).

Microglia Treatments

All *in vitro* experiments were performed using insulin from bovine pancreas suitable for cell culture (Sigma). Cells were always submitted to drug treatments after a maximum of 3 days after the shake off. Insulin (1000ng/mL) 3h or 24h incubation in normal 50:50 medium followed or not by 30 min Apocynin (Millipore) at final concentrations of 10µM and 100µM, alternatively only Apocynin was added using the same concentrations.

Amplex Red Assay

The cellular release of H_2O_2 was assessed by the Amplex Red® oxidation method that was carried out according to (Muller, Haas et al. 2013) with modifications. Primary microglia were plated (20,000 cells/well of a black 96-well plate), treated and 50 μ M Amplex Red and 0.1 units/mL horseradish peroxidase in Krebs-Ringer HEPES buffer (Sigma) pH 7.4. Fluorescence was monitored kinetically at excitation (530/525 nm) and emission wavelengths (590/535 nm), at a sensitivity set of 50 in a Synergy H1 microplate reader (Biotek), every 90 s during 30 min, 37°C. Results between 5 and 25 min were analyzed in RFU/sec and the basal rate of H_2O_2 formation in the control group (average of values) was calculated as 100% and the values from treated groups were calculated related to control group basal rate.

MTT assay

The MTT assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl Blue Tetrazolium Blue - MTT; Sigma, St. Louis, USA). Primary microglia were plated (20,000 cells/well of a 96-well plate), received the proper treatments, as described above, and, were incubated with MTT during 2 h, at 37 °C, 5% CO₂. MTT is converted in dark blue water-insoluble formazan by mitochondrial dehydrogenases of living cells. Then, microglia were lysed in dimethyl sulfoxide (DMSO; Millipore) to release the formazan and detected by absorbance at a wavelength of 570nm. MTT assay measures cellular metabolic activity as an indicator of cell metabolic activity (Mosmann 1983).

LDH Cytotoxicity Assay

For cytotoxicity evaluation a commercial kit was used to access lactate dehydrogenase (LDH) cytosolic enzyme activity (Pierce™ LDH Cytotoxicity Assay Kit; Thermo) and the instructions from the manufacturer were followed. Primary microglia were plated (20,000 cells/well of a 96-well plate), the cells received the proper treatments, 3 wells were treated with the Lysis Buffer 1x, 45 minutes before the assay was started. This treatment with lysis buffer provides a positive control, once plasma membrane damage releases LDH into the cell culture media (maximum LDH activity). Three wells containing only culture media (background) and 3 wells containing ultrapure sterile (negative control) water were used as internal controls. Next, 50 μ L of cell culture media was collected from each well and the

assay was performed. The extracellular LDH in the media can be detected by enzymatic reactions that result in a red formazan that can be measured at 490nm. To calculate % Cytotoxicity the equation below was used where the average of absorbance of the cells not treated with any drug (control group) was used as spontaneous LDH activity.

$$\% \text{ Cytotoxicity} = (\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity} \times 100) / (\text{Maximum LDH activity} - \text{Spontaneous LDH activity})$$

ELISA

ELISA was used to detect interleukins IL1- β , IL-6 and TNF- α in the primary microglia cell culture supernatants. For IL1- β detection, ATP (1mM) was added 15min. before the collection of the medium, to promote the release of IL1- β from the cell inflammasome. The ELISA assays were performed according to the manufacturer's instructions (Mouse TNF-Alfa ELISA MAX™ Deluxe, Biolegend, Cat# 430906; Mouse IL-1 β ELISA MAX™ Deluxe, Biolegend, Cat# 432606 and Mouse IL-6 ELISA MAX™ Deluxe, Biolegend, Cat#431306).

Intranasal insulin administration to adult mice

Intranasal (i.n.) insulin administration was performed as described in (Hanson, Fine et al. 2013). Awake animals were used to avoid confounding effects of repeated anesthesia. First, mice (3-4 months age) were habituated to handling for a period of two weeks before the onset of intranasal administration. Each mouse received 2 i.n. insulin administration sessions per day. They were held with their neck parallel to the floor while a volume of 3 μ l of liquid was administered each time at 45-degree angle. Insulin was slowly ejected from the micropipette forming a small droplet with about 3 μ l. Immediately after mouse inhales this small drop, new drop was ejected in the other nostril 2-3 sec later. Mice were then kept in this position for 15 s. This procedure was repeated two times per session. A total of 1.75U (60 μ g) /24 μ l was delivered per day divided over the two sessions, one in the early morning and another one in the late afternoon. During treatment body weight and blood glucose levels were measured. Mice were sacrificed 12 h after the 7th day (last session) of insulin injection, and these brains were collected, and microglia were isolated as described below.

Adult Mice Acute Isolation of Microglia Enriched - Cell Suspensions

The acute isolation of microglia enriched - cell suspension was performed in accordance with (Vainchtein, Vinet et al. 2014) with modifications. First, the mice submitted to the intranasal insulin treatment were perfused with 0.9% saline under isoflurane anesthesia and the brains were isolated and collected in medium A: HBSS (Gibco) supplemented with 7.5 mM HEPES (Lonza) and 0.6% glucose (Sigma-Aldrich). Subsequently, the forebrains were minced and mechanically dissociated using a glass tissue homogenizer followed by 3 diameters of glass pipette to achieve a single cell suspension. The preparation was, still, filtered using a 70- μ m cell strainer (BD FALCON). Then, cells were centrifuged at 200 rcf, at 4 °C, for 10 min, and the pellet was gently resuspended in 75% Percoll (GE Healthcare) and a layer of 25% Percoll, followed by a layer of PBS, was added and the gradients were centrifuged at 800 rcf, 4 °C, 10 min. Afterwards, the myelin layer (between PBS and 25% Percoll layer) was removed and discarded and the layer containing the fraction enriched in microglia, between 25% and 75% Percoll, was carefully collected. The microglial enrichment of the preparation was confirmed by Western Blot for the Iba-1 marker.

Quantitative Real-Time PCR (qPCR)

RNA was extracted from microglia enriched - cell suspensions using the RNeasy Micro kit (Qiagen) according to the manufacturer's protocol. Total RNA concentrations were measured using a Nanodrop (ND1000) and reverse transcription was performed with a reaction mixture containing Revert Aid TM M-MuLV Reverse Transcriptase, Ribolock TM RNase Inhibitor, M-MLV buffer. The quantitative PCR reaction contained iQTM SYBR Green Supermix (Bio-Rad) and was performed in 384 well plates (Applied Biosystems) using an ABI7900HT machine (Applied Biosystems). The primers were all designed with Primer designer v3.0, Primer Express software or NCBI Primer-Blast and ordered from Bi-olegio (The Netherlands). The genetic expression was calculated by normalization to the GAPDH gene levels. See Supplemental Table 1 for primer information.

Statistical analysis

The data containing more than 2 groups were analyzed by one-way Analysis of variance (ANOVA) followed by multiple comparisons test Tukey. Body weight and Blood Glucose data (Figure SF2) were analyzed using one-way Anal-

ysis of Variance (ANOVA) followed by multiple comparisons test Tukey when 2 groups were analyzed we used unpaired Student's t-test. Data were presented as mean $\pm$ S.E.M. p values < 0.05 were considered statistically different.

Results

Insulin decreases ROS production in microglia *in vitro*

Since insulin has important effects on ROS, specially hydrogen peroxide metabolism in the brain (Muller, Zimmer et al. 2012, Muller, Haas et al. 2013), and microglia are cells with high phagocytic capacity in which ROS are an important component of phagocytosed particles degradation because of its microbicidal activity (Dupre-Crochet, Erard et al. 2013), we first determined if insulin treatment altered ROS production in microglia *in vitro*. Stimulation of microglia with insulin (1000 ng/mL) for 24 h, but not 3 h, decreased H_2O_2 production (Figure 1A) ($p=0.0033$). We next investigated possible mechanisms by which insulin regulated microglial H_2O_2 metabolism. The source of ROS can be mitochondrial or extra-mitochondrial. When extra-mitochondrial the main source of ROS is NADPH oxidase (Dupre-Crochet, Erard et al. 2013). Apocynin (Apo) was used to inhibit NADPH oxidase in microglia. Apocynin alone decreased H_2O_2 production when cells were exposed to 100 μ M ($p=0.041$), but not 10 μ M of this inhibitor (Figure 1B). Twenty-four hours pre-incubation with insulin alone decreased H_2O_2 production to a similar level as Apo ($p=0.0264$), insulin pre-incubation combined with Apo at both 10 μ M ($p=0.0442$) and 100 μ M ($p=0.0173$) concentrations also decreased H_2O_2 levels (Figure 1B). Because apocynin was not able to decrease even more H_2O_2 production after a pre-treatment with insulin it is possible that insulin is acting in decreasing extra-mitochondrial ROS source probably direct inhibiting NADPH oxidase.

Insulin increases microglia cell metabolic activity *in vitro*

To better understand the biological outcome of insulin inhibition on ROS production in microglia, MTT assays were performed as an indication of metabolic activity measurement. Interestingly, 100 μ M of the NADPH oxidase inhibitor apocynin alone ($p<0.0001$) increased MTT conversion/cellular metabolism, and 24 h of 1000 ng/mL insulin alone ($p<0.0001$) also increased MTT conversion. 24 h pre-incubation of insulin with 10 μ M and 100 μ M apocynin in the last 30 min

Figure 1. Insulin effects on ROS (H_2O_2) production in primary microglia. H_2O_2 production by primary microglia was assessed using Amplex Red dye Assay; fluorescence (RFU) emitted from the dye per second. (A) Fluorescence units from primary microglia treated with insulin for 3h or 24h. (B) RFU measurement from control primary microglia pretreated with insulin for 24h followed by NADPH oxidase inhibitor Apocynin (Apo) for 30 min as indicated. Results are depicted as mean $\pm$ S.E.M. Individual dots indicate technical and biological replicates of 3-4 independent experiments.

also significantly increased MTT activity ($p < 0.0001$) (Figure 2A). Interestingly, 30 min of 10 μ M apocynin together with insulin statistically increased MTT activity ($p < 0.0001$) whereas 30 min of 10 μ M apocynin alone had no significant effect. Since MTT activity reports on mitochondrial metabolism, we next checked if the treatments would be toxic to the microglia cells by measuring LDH release to the cell culture medium which is indicative of cell lysis. Neither Apocynin nor insulin were found to have toxic effects on microglia (Figure 2B).

LPS increases IL-1 β production in microglia treated with insulin *in vitro*

A prominent function of microglia is cytokine production, which is driven by many mechanisms such as ROS signaling. Next it was determined if insulin alters pro-inflammatory cytokine production by in microglia in dependent of secondary signals from other cell types. Cells treated with insulin (30 min or 3 h) did not cause significant differences in the concentrations of secreted IL-6, TNF- α or IL-1 β (Figures 3A-C). As expected, LPS stimulation (3 h or 24 h) significantly induced secretion of these three cytokines ($p < 0.0001$) (Figures 3A-F). Even a longer exposure to insulin (24 h) did not result in increased secretion of the cytokines tested (Figures 3D-F) in the cell culture medium. However, when cells were challenged with LPS for 3 h after 24 h insulin pretreatment, IL-1 β levels were

Figure 2. The effect of insulin on cell metabolic activity and cytotoxicity in primary microglia. Cells were treated with Apocynin (Apo) for 30 min in the absence or presence of insulin for 24h as indicated. (A) Cell metabolic activity, through mitochondrial function, was assessed in MTT assays. (B) Potential cytotoxicity of insulin and Apo treatments were assessed using LDH activity assays. Results = mean $\pm$ S.E.M. Individual dots indicate technical and biological replicates of 3-4 independent experiments.

significantly increased compared to a 3 h LPS treatment alone ($p < 0.0001$) (Figure 3D). This effect seemed selective for IL-1 β as no changes in IL-6 and TNF- α levels were observed (Figure 3E and 3F).

Intranasal insulin treatment increases TNF- α gene expression in microglia *in vivo*

To investigate the effect of insulin signaling in adult microglia, adult mice were intranasally treated with insulin for 7 consecutive days. As previously reported by others (Marks, Tucker et al. 2009, Chen, Zhao et al. 2014, Zhang, Dai et al. 2016), intranasal administration is an efficient and less invasive approach compared to intracerebroventricular injections to deliver insulin directly into the brain. Seven days of intranasal insulin administration selectively increased TNF- α gene expression in microglia ($p = 0.0321$) (Figure 4C). A set of pro- and anti-inflammatory markers, such as IL-1 β (Figure 4A), IL-6 (Figure 4B), IL-10 (Figure 4D), Arg-1 (Figure 4E) and COX-2 (Figure 4F) were also evaluated but no significant differences were observed after insulin treatment. During the experimental procedure, body weight (Supplementary Figure 1A) and blood glucose (Supplementary Figure 1B) were monitored and, as expected (Ramos-Rodriguez, Sanchez-Sotano et al. 2017), no significant differences in these parameters were observed. In short, insulin administration *in vivo* triggers a pro-inflammatory reaction in microglia comparable to what was observed *in vitro* (Figure 3D) in microglia after an LPS challenge and after intracerebroventricular administration as

Figure 3. The effect of insulin on the production of pro-inflammatory interleukins IL-1 β , IL-6 and TNF- α by primary microglia. (A-C) Microglia were treated with insulin (both 100ng/mL and 1000 ng/mL) for 30 min or 3 h and IL-1 β (A), IL-6 (B) and TNF- α (C) levels in cell culture medium were determined by ELISA. (A-C) Microglia were stimulated with LPS (100 ng/mL) for 3h or 24h (positive control). (D-F) Microglia were treated with insulin (1000 ng/mL) for 24h, with LPS (100 ng/mL) for 3h or for both treatments combined and IL-1 β (D), IL-6 (E) and TNF- α levels (F) were determined in cell culture medium. Results = mean $\pm$ S.E.M. Individual dots indicate technical and biological replicates of 3-4 independent experiments.

described in chapter 3.

Discussion

Here, we presented the response of microglia to insulin *in vitro* and *in vivo*. *In vitro* studies showed that insulin inhibits ROS (measured by concentrations of hydrogen peroxide) production, and under the same conditions, increases cell metabolic activity. It was also observed that insulin further increased IL-1 β levels after microglia activation by LPS. Similarly, an *in vivo* intranasal insulin treatment, in adult mice, also triggered a pro-inflammatory reaction increasing TNF- α gene expression in microglia.

The availability of studies addressing the relation of insulin signaling in the brain and neuroinflammation/microglia is scarce. In an *in vivo* approach, microglia function was evaluated after insulin treatment protocols in animal models of neurological diseases such as Alzheimer's disease (Chen, Zhao et al. 2014) and HIV infection-associated neurocognitive disorders (Mamik, Asahchop et al. 2016) where the insulin treatment presented a neuroprotective anti-inflammatory

effect under disease conditions. The effects of insulin signaling under physiological conditions is even less explored. Some progress has been obtained in evaluating insulin signaling in microglial cell lines. In agreement with our findings, it was previously described that insulin triggers a pro-inflammatory effect in microglial cell line. Insulin promoted secretion of IL-8 (Spielman, Bahniwal et al. 2015) in a human microglial cell line and reduced ROS production in a mouse microglia cell

Figure 4. Intranasal *in vivo* insulin administration in adult mice increased TNF- α gene expression in microglia. Mice received 1.75 U intranasal (i.n.) insulin on 7 consecutive days. Twelve hours after the last insulin administration, microglia were isolated, RNA extracted and analyzed by RT-qPCR for the following target genes: IL-1 β (A), IL-6 (B), TNF- α (C) TNF- α , (D) IL-10 (E) Arg-1 and (F) COX-2. Results = mean $\pm$ S.E.M. Individual dots indicate biological replicates.

line pre-stimulated with LPS (Brabazon, Bermudez et al. 2018).

We previously reported that direct administration of insulin into the brain increased memory performance in young but not in aged rats. Hippocampal BDNF levels significantly increased in young rats after insulin treatment (Haas, Kalinine et al. 2016). In addition, an increase in neuroinflammatory markers in the hippocampus of young rats was observed along with increased performance in a spatial memory task (Haas, de Carvalho et al. 2020). These findings highlight the importance of brain insulin signaling and support the notion it might be disrupted during aging and hence contributing to aged-associated neurological deficits.

Investigating the molecular outcomes associated with microglial insulin signaling we observed that insulin decreased H_2O_2 production (Figures 1A and 1B), accompanied by increased cell metabolism by MTT activity indicating increased cell metabolic activity (Figure 2A). These findings agree with our previous findings that insulin decreased H_2O_2 production in mice synaptosome via the PI3K pathway (Muller, Haas et al. 2013). The fact that insulin would be affecting cell proliferation and it would be responsible for the effects observed could be considered, but it is unlikely to be the case, since primary microglia don't proliferate after separate from astrocytes and plated if the ramified phenotype is desired for the studies (Floden and Combs 2007) what was the case in the present experiments.

When mitochondria are actively producing ATP through the oxygen consumption coupled with normal electron transport by the mitochondrial complexes, the extent of ROS production is much lower. Insulin signaling might increase mitochondrial ATP production via mitochondrial complex V (ATP synthase) and therefore reduce mitochondrial ROS production in primary microglia. The co-incubation of insulin with the NADPH oxidase inhibitor, apocynin, suggests that insulin signal also targets NADPH oxidase enzyme, as a synergistic effect of insulin with apocynin was observed (Figure 2B and 3A). NADPH oxidase possess distinct isoforms expressed in different cell compartments (Nayernia, Jaquet et al. 2014). Further experiments are necessary to determine the mitochondrial and extramitochondrial target(s) of insulin that decreases ROS production in microglia.

Remarkably, but not completely unexpected, we observed different responses on cytokine expression when comparing the effects of insulin *in vitro* and *in vivo*. Previously, we reported that 5 days of intracerebroventricularly insulin

injections increased IL-1 β protein expression in the hippocampus of young rats (Haas, de Carvalho et al. 2020). Here we observed that 7 days of intranasal insulin injections resulted in an increased microglial gene expression of TNF- α , that like IL-1 β , is also mainly considered a pro-inflammatory cytokine. TNF- α has pro, anti-inflammatory and regenerative functions, and the TNF receptor TNFR2 signal mediates the protective and regenerative effects of TNF (Dong, Fischer et al. 2016). TNF- α may exert neuroprotective signals as microglia adapt to micro-environmental changes during their lifespan (Vinet, Weering et al. 2012).

Further investigations are needed to clarify how insulin signaling is integrated in the neuron-astrocyte-microglia triad and brain function in relation to cytokine expression. An effective strategy to investigate it would be testing if insulin ICV or intranasal treatments in knockout mice for IL-1 β and TNF- α genes would show different outcomes in memory tasks performances compared to wild type mice. Another strategy would be to measure the cytokine levels in the hippocampal extracellular fluid collected by microdialysis after ICV or IN insulin treatments.

Acknowledgements

This work was financially supported by the Dutch grants Abel Tasman Talent Sponsorship, and the UMCG De Cock foundation young talents annual grant. We would like to thank Evelyn Wesseling for technical assistance with RT-qPCR experiments.

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Supplementary Material

Supplementary Figure 1. Body weight and blood glucose levels during intranasal insulin treatment. (A) Daily body weight monitoring during 7 days of intranasal insulin treatment. (B) Blood glucose levels on day 1, 2 and 7 of the 7 days insulin protocol treatment. Black points indicate control group (n=9); Grey points indicate insulin group (n=11). Results = mean $\pm$ S.E.M.

Gene Name	Accession Number	Forward primer 5'-3'	Reverse primer 5'-3'
TNF- α	NM-013693	TCTTCTGTCTACTGAACTTGG	AAGATGATCTGAGTGTGAGGG
IL-10	NM-010548	AAGGGTTACTTGGGTTGCCA	TTTCTGGGCCATGCTTCTCTG
IL-1 β	NM-001037859	CCCAAAAGATGAAGGCTGC	TGATACTGCCTGCCTGAAGC
COX-2	NM-011198.4	TCTACGGAGAGAGTTCATCCT	ATTTAAGTCCACTCCATGGCCC
Arg-1	NM-007482	CAAGACAGGGCTCCTTTCAG	TTACAGTACTCTTCACCTCCT
IL-6	NM-031168	ACAACCACGGCCTTCTCTACTT	CACGATTTCACAGAGAACAGTG

Supplementary Table 1. RT-qPCR Primers information

CHAPTER 5

High resolution respirometry (HRR) as an experimental tool to analyze oxygen metabolism and mitochondrial physiology in murine microglia

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Figure 1. Schematic representation of mitochondrial OXPHOS and the mechanism of action of substrates, uncouplers and inhibitors. The respiratory ETS is an electromechanical transmembrane gradient responsible for the oxidation of nutrients that culminate in the energy production in the form of ATP called OXPHOS. Complex I (NADH: ubiquinone oxidoreductase) uses oxygen to metabolize NADH with the reduction of CoQ. The production of electrons generates an electrochemical gradient which reduces CoQ of Complex II (succinate-CoQ oxidoreductase). Complex II oxidizes succinate from the TCA cycle. Complex III (ubiquinol-cytochrome c oxidoreductase) catalyzes the reduction of cytochrome c by oxidation of ubiquinol. Complex IV (cytochrome c oxidase) transfers electrons (e⁻) to molecular oxygen and pumping protons (H⁺) to the intermembrane space generating an electrochemical gradient. Complex V converts electrochemical H⁺ gradient energy into mechanical energy, once it promotes the chemical bond energy between ADP and phosphate to form ATP. (A) Pyruvate and Malate (Pyr/Mal) increases activity of complex I; (B) Oligomycin inhibits activity of complex V; (C) FCCP uncouples oxidative phosphorylation; (D) Antimycin-A binds to cytochrome c reductase, inhibiting the oxidative phosphorylation. Based on (Gorman, Chinnery et al. 2016) with modifications. Original figure created with BioRender.com.

Abstract

Similar to peripheral macrophages, microglia are phagocytic cells that are constantly re-structuring their actin-microtubule cytoskeleton upon surveillance and migration through the tissue milieu. Microglia express a special oxygen metabolism and bioenergetic cellular program that is still not completely understood. Such properties of microglia metabolism or brain energy/oxygen metabolism changes in CNS disorders, what can be a potential diagnostic/treatment feature. Although, microglia oxygen metabolism, shows similarities to peripheral macrophage metabolism microglia express different genes required for both glycolytic and oxidative energy metabolism which still need to be functionally validated by oxygen consumption/mitochondrial physiology studies using primary (*in vitro*) or fresh isolated microglia (*ex vivo*). This chapter presents a technical protocol using high resolution respirometry (HRR) analysis as an experimental tool to analyze oxygen metabolism and mitochondrial physiology in murine primary microglia freshly obtained from mixed glial cell cultures *in vitro*. Additionally, the HRR microglia profile was compared to similar studies that used macrophage cell lines, adult mouse primary peripheral macrophages and human peripheral blood mononuclear cells (PBMC) containing monocytes and lymphocytes.

Key words: primary microglia; mitochondria; oxygen metabolism; high resolution respirometry

Introduction

Microglia are the tissue resident macrophages of the central nervous system (CNS) with a distinct origin and different functions compared to other tissue/peripheral macrophages (Crotti and Ransohoff 2016). Macrophages are regarded as tissue “sentinels”, as they are the first site of contact with damage or pathogen-associated molecular patterns, what triggers intracellular signals and results in inflammation (Tur, Vico et al. 2017). Due to the fact that peripheral macrophages and microglia are phagocytic cells, and also they are constantly re-structuring their actin-microtubule cytoskeleton upon migration through the tissue milieu, they express a special oxygen metabolism and bioenergetic cellular program (Artyomov, Sergushichev et al. 2016, Correa-da-Silva, Pereira et al. 2017, Curi, de Siqueira Mendes et al. 2017, Geeraerts, Bolli et al. 2017, Griffiths, Gao et al. 2017, Liu and Ho 2017, Tur, Vico et al. 2017, Van den Bossche, O'Neill et al. 2017, Nagy, Fekete et al. 2018). Although peripheral macrophage metabolism is well understood, the special properties of microglia metabolism or brain energy/oxygen metabolism in physiology and potential changes in CNS disorders are much less understood (Ghosh, Castillo et al. 2017).

Over the past years, research on oxygen metabolism were primarily focused on ATP metabolism, mainly considering mitochondria as an isolated unit inside the cell and the oxidative phosphorylation as a source of ATP to support the cell energetically (Artyomov, Sergushichev et al. 2016). Recently, the role of metabolic reprogramming in macrophages in relation to their inflammatory responsiveness has been studied (Artyomov, Sergushichev et al. 2016, Netea, Joosten et al. 2016). Most studies on macrophage metabolism have focused on macrophage polarization primarily using LPS for pro-inflammatory activation or IL-4 for anti-inflammatory or alternative activation *in vitro* (Van den Bossche and Saraber 2018). LPS-stimulated macrophages express inducible nitric oxide synthase (iNOs) and produce nitric oxide (NO) from arginine, accompanied by increased ATP production by glycolysis and reduced mitochondrial respiration (Palsson-McDermott, Curtis et al. 2015). All these adaptations are accompanied by an increase in the generation of reactive oxygen species (ROS) by reverse electron transfer in the oxidative phosphorylation chain. At the same time, the increased glycolysis induces the pentose phosphate pathway, resulting in increased NADPH levels. Excess NADPH is used by NADPH oxidase, resulting in more

ROS production through extra mitochondrial oxygen consumption, preparing the whole system for an enhanced phagocytosis activity (Mullarky and Cantley 2015). Much less is known about the metabolism of IL-4 treated or alternatively activated macrophages. Arginine in macrophages challenged with IL-4 is metabolized by arginase-1 and mitochondrial respiration is increased, resulting in more efficient mitochondrial ATP production (Van den Bossche, O'Neill et al. 2017, Van den Bossche and Saraber 2018).

For many years, studies of microglia oxygen metabolism were quite scarce, recently studies using both microglia cell lines and primary microglia cultures *in vitro* added important information to this field. Microglia oxygen metabolism shows high similarity to peripheral macrophage metabolism (Orihuela, McPherson et al. 2016). Zhang and colleagues reported that microglia express the genes required for both glycolytic and oxidative energy metabolism (Zhang, Chen et al. 2014). Recently, it was reported that the expression level of genes involved in oxidative metabolism is comparable or higher in microglia than in neurons and astrocytes (Ghosh, Castillo et al. 2017). However, such findings still need to be functionally validated by oxygen consumption/mitochondrial physiology studies using primary (*in vitro*) or fresh isolated microglia (*ex vivo*).

High resolution respirometry (HRR) is a technique that uses a polarographic oxygen sensor that is sensitive to very low concentrations of oxygen (as low as 5nm) and is able to directly detect oxygen microflow consumption in real-time, in a closed chamber with no oxygen exchange with the external environment (Gnaiger 2014, Gnaiger E, Aasander Frostner E et al. 2019). Cellular oxygen metabolism and subsequent mitochondrial function are analyzed using SUIT (substrate-uncoupler-inhibitor-titration) protocols. Using HRR, mitochondrial function is measured by the ability of mitochondria to generate ATP in response to energy demand. Using intact cells, oxygen metabolism is analyzed for the entire cellular system capacity to uses oxygen for mitochondrial ATP production and for extra-mitochondrial oxidative reactions. In the case of microglia such extra-mitochondrial oxygen consumption is an important measurement. General mitochondrial oxidative phosphorylation (OXPHOS) is depicted in figure 1; inspired by (Gorman, Chinnery et al. 2016). The respiratory electron transfer chain complexes (I–V) is an electromechanical transmembrane gradient responsible for the oxidization of nutrients that culminate in the energy production in the form of ATP (Oxidative Phosphorylation - OXPHOS). Complex I (NADH: ubi-

quinone oxidoreductase) uses oxygen to metabolize NADH with the reduction of coenzyme Q (CoQ). The production of electrons at this point generates an electrochemical gradient which reduces CoQ of Complex II (succinate-CoQ oxidoreductase). Complex II oxidizes succinate from tricarboxylic acid (TCA) cycle. Complex III (ubiquinol-cytochrome c oxidoreductase) catalyzes the reduction of cytochrome c by oxidation of ubiquinol. Complex IV (cytochrome c oxidase) transfers electrons (e^-) to molecular oxygen and pumping proton (H^+) to the intermembrane space generating an electrochemical gradient. Complex V converts electrochemical proton (H^+) gradient energy into mechanical energy, once it promotes the chemical bond energy between ADP and phosphate to form ATP.

Taking the limited knowledge of microglia mitochondrial function and cellular oxygen metabolism in physiology and disease in consideration, the main goal of the experiments described in this chapter was to characterize these processes in primary mouse microglia taking advantage of high resolution respirometry (HRR) technology.

Material and Methods

Primary Microglia Cell Culture

Primary neonatal microglia culture was performed as described in chapters 3 and 4. After 11-15 days of culture, when the cells reached 80-100% confluence, microglia were obtained by mitotic shake off using an orbital shaker (1 h; 150 rpm; 37 °C). Right after shake-off cells were collected in respiration medium - RM (HBSS + 4.5 mg/mL glucose) and centrifuged (10 min.; 960 rpm; 12 °C). The cell pellet was resuspended in 1mL RM and right after introduced into the Oroboros equipment chamber.

High Resolution Respirometry

High Resolution Respirometry was measured using Oroboros equipment (O2k, Oroboros Instruments, Innsbruck, Austria) consists in polarographic oxygen sensors that are connected to temperature-controlled, continuously stirred, sealed chambers that measure oxygen concentration within the chamber over time. For such measures the Oroboros equipment (O2k) was properly calibrated using atmospheric oxygen concentration of the day and 0% oxygen concentration (obtained by addition of sodium dithionite crystals to water) as reference points.

The protocol used was performed accordingly to the manufacturer's instructions (Gnaiger 2014) and results were expressed following the MitoFit community guideline (Gnaiger E, Aasander Frostner E et al. 2019). Detailed HRR-O2k protocol illustration can be found in Figure 2 and will be detailed following. At least 1 hour prior to the experiment, the equipment was submitted to stabilization, at 37°C, using RM. Right after the shake-off, 1-3 million viable primary microglia cells were collected, diluted in RM, centrifuged and again resuspended gently in 1 mL of RM and directly placed into the O2k equipment chamber adapted to work with 1mL, 37°C, stirring speed of 750 rpm. Approximately the first ten minutes of experiment run is recorded and named Baseline. The sequence of substrates was titrated as follow and a schematic representation of the protocol is illustrated in fig. 2.

SUIT Protocol

For real-time direct oxygen fluxes analysis using intact cells, the best assay is the equivalent measurement of cell respiratory control, which reports the rate of mitochondrial ATP production and coupling efficiency (ATP-LP), the proton leak rate (LEAK), the maximum respiratory rate (ETS) and the spare respiratory capacity (SRC) (Brand and Nicholls 2011). The following SUIT protocol was used to access such rates:

- Pyruvate/Malate (Fig. 2B): Addition of Pyr/Mal (5μL each; Sigma; Pyr f.c. 5mM in H₂O; Mal f.c. 0.5mM in H₂O). Pyr/Mal pass by cellular plasmatic membrane, working as an internal control since they are integrated in the TCA cycle (Fig. 1A). In this step oxygen consumption and ATP production by mitochondria are slightly increased and oxidative phosphorylation (OXPHOS) proper function by the beginning of the experiment run can be checked.
- Oligomycin (Fig. 2C): Addition of Oligomycin (1μL; Sigma; f.c. 2.5μM in EtOH) blocks Complex V proton channel, which is necessary for oxidative phosphorylation of ADP to ATP, reducing the electron flow through the system, what abruptly decreases O₂ consumption (Fig. 1B).
- FCCP titration (Fig. 2D): The ionophore uncoupling agent Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) is a mobile ion carrier that disrupts respiratory chain coupling by promoting H⁺ influx from the inter-membrane space to the matrix, such effect triggers O₂ consumption (Fig. 1C). Titration of FCCP (1μL steps; Sigma; f.c. 0.1 mM in EtOH total f.c 0.8 mM) until

Figure 2. Schematic representation of the HRR experiment. The red line represents the Oxygen (O_2) consumption flow (pMol/(s*MillionCells)). BASELINE is the first experimental state studied that corresponds to the basal cellular mitochondrial and non-mitochondrial O_2 consumption. (A) Pyr/Mal injection increases Complex I activity, slightly increasing O_2 consumption; this step of the protocol indicates that Oxidative Phosphorylation (OXPHOS) is properly functioning using endogenous ADP that is converted to ATP. (B) Oligomycin addition is used to block Complex V and inhibit ATP production, drastically decreasing O_2 consumption, uncovering the LEAK (Proton Leak) state. LEAK: non-phosphorylating respiratory state that corresponds to the fraction of O_2 that is used for mitochondrial ROS production. (C) FCCP titration to achieve ETS state: the maximal uncoupled mitochondrial respiratory capacity, maximal oxygen consumption by OXPHOS system, increases the O_2 consumption reaching a plateau. (D) Antimycin A: disrupts the electron transportation and completely shuts down the entire OXPHOS system, uncovering the Residual Oxygen Consumption (ROX) state that corresponds to non-mitochondrial respiration. From the values obtained of this 4 states, two additional states can be calculated: ATP-LP (Baseline – LEAK) ATP production and coupling efficiency and Spare Respiratory Capacity SRC (ETS – Baseline): oxygen consumed mainly for ROS production or loss by heat when the ETS state is achieved. Original figure created with Graphpad®.

maximum oxygen consumption is reached lead to the maximum mitochondrial maximum oxygen consumption is reached lead to the maximum mitochondrial oxygen consumption capacity.

- Antimycin-A (Fig. 2E): Addition of Antimycin-A (1µL; Sigma; f.c. 2.5µM in EtOH) that binds to cytochrome c reductase, inhibiting the oxidation of ubiquinone to ubiquinol thereby disrupting the electron flow and blocking the entire system (Fig. 1D). Addition of Antimycin-A allows assessment to mitochondrial-independent cellular oxygen consumption.

Mitochondrial function and respiratory capacity states

By the titration of the substrates pointed above the following states are identified, each specific state results in a number expressed by O_2 slope/consumption (pMol/s*Million Cells). How the states are identified during an experimental run is illustrated in Fig. 2:

- BASELINE (Fig.2A): basal cellular oxygen consumption per second/

million cells.

- LEAK: After Pyr/Mal injection and evaluation of OXPHOS properly function. The injection of Oligomycin allows identification of Oxygen consumption that is not necessary for ATP production and is lost as heat or ROS.
- ATP-LP: Calculation of Baseline with LEAK subtracted, corresponds to the amount of oxygen that is used to produce ATP by the system.
- ETS (Electron Transfer State): After several steps of FCCP titration the maximum capacity of mitochondrial oxygen consumption can be identified.
- ROX (Residual Oxygen Consumption): Oxygen consumption due to extra-mitochondrial cellular oxidative reactions (e.g. extramitochondrial enzymes that consume oxygen).
- SRC (Spare Respiratory Capacity): Oxygen consumed mainly for Reactive Oxygen Species (ROS) production or lost by heat when the ETS state is achieved. Calculation of ETS value with Baseline value subtracted.

The data were subsequently collected with the application of DatLab software 6.1.0.7. To calculate normalized values in percentage as showed in Fig. 3C the BASELINE value was considered 100% for each sample and all the other values were calculated in relation to the sample respective baseline.

Results

Primary Microglia High Resolution Respirometry Characterization

The development and characterization of real-time oxygen flow measurement protocols for intact cells are critical for research on the metabolism of specific cell types and to potentially apply such analysis to diagnosis or to study disease-induced pathologies since artefacts associated with mitochondrial isolation or cell permeabilization can be avoided (Brand and Nicholls 2011). In order to characterize the respirometry profile of intact naïve primary microglia using HRR technology, cells were harvested from mixed glial cell cultures by mitotic shake-off, placed in the equipment chamber as a cell suspension, and challenged with a set of substrates as described in details in the Methods section (Figure 1 and 2). The oxygen concentration (blue line) and the oxygen consumption (red line) inside the chamber are continuously measured.

First, it was observed that basal respiration in microglia (BASELINE) corresponds to 19.6 O₂ pMol per second per million cells (pMol/(s*MiCells) (Fig.

3B) which is similar to human peripheral blood mononuclear cells (PBMCs) (Michalak, Florczak-Wyspianska et al. 2017) and macrophage cell lines RAW 264.7/Maf-DKO (Venter, Oerlemans et al. 2014), but lower than what was previously described for adult mice peritoneal macrophages (Aflaki, Radovic et al. 2011) (Table 1). After that, addition of pyruvate/malate slightly increased oxygen consumption of microglia to 21.2 pMol/(s*MICells) (Fig. 3B); (8% more than Baseline; Fig. 3C), followed by Oligomycin injection, which dramatically decreased oxygen consumption to 4.4 pMol/(s*MICells) (Fig. 3B) or 23 % of baseline (Fig. 3C). This observation indicated that the amount of oxygen that is not necessary for ATP production (LEAK state) in microglia was very similar to what was previously described for human PBMCs (Jang, Shofer et al. 2016, Michalak, Florczak-Wys-

Figure 3. HRR real-time analysis of primary microglia (A) Representative experimental run obtained from an experiment run in the Oroboros device. Y-axes at the left side represents oxygen concentration (nmol/mL) and is depicted by the blue line; the t-axes at the right represents oxygen consumption pMol/(s*MiCells) and fluctuations, and is represented by the red line. Baseline indicates the basal primary mitochondria respiration and black arrows indicate substrate additions (Pyr/Mal - 5 mM/0.5 mM f.c.), uncoupler (8x FCCP 1 μ L titration-0.8 μ M f.c.) and inhibitors Oligomycin and Antimycin A (both 2.5 μ M f.c.). (B) Quantification of Primary Microglia HRR analysis for oxygen consumption for all states analyzed. (C) Normalized oxygen consumption quantifications in percentage calculated based on individual experiment Baseline (considered 100%). Pyr/Mal: Piruvate/Malate; Oligo: Oligomycin; ATP-LP (ATP production and coupling efficiency); Anti A: Antimycin A; ROX: Residual Oxygen Consumption SRC: Spare Respiratory Capacity. n= 2-4 per condition. Results: Mean $\pm$ SD.

pianska et al. 2017), but lower than what was previously seen for peritoneal macrophages (Aflaki, Radovic et al. 2011, Venter, Oerlemans et al. 2014) (Table 1). From Baseline and LEAK, ATP production and coupling efficiency (ATP-LP) was calculated as 14.7 pMol/(s*MiCells) (Fig. 3B), which is higher than the percentage of oxygen used by peritoneal macrophages to produce ATP (Aflaki, Radovic et al. 2011, Venter, Oerlemans et al. 2014) (Table 1).

Next, the electron transfer state (ETS), was determined, as a decrease in ETS indicates potential mitochondrial dysfunction (Brand and Nicholls 2011). For primary microglia, 8 FCCP titrations (1 μ L - 0.8 μ M f.c.) were necessary to reach a maximal ETS state, maximum mitochondrial electron transfer system capacity, corresponding to 31.9 pMol/(s*MiCells) (Fig. 3B); 174% (Fig. 3C). Such value is lower compared to all values reported for PBMCs and macrophages (Table 1). Adult peritoneal macrophages, for example, have an ETS capacity of 80 (267%) (Aflaki, Radovic et al. 2011), what is almost 3x higher than observed here in primary microglia (Table 1). From the ETS state, the SRC state was calculated to be 13 pMol/(s*MiCells) (Fig. 3B) indicating that 56% (Fig. 3C) of the oxygen consumption at ETS was spare mitochondrial respiratory capacity in primary mi-

State pMol/(s*Mi Cells)	BASELINE	LEAK	ETS	ROX	ATP-LP	SRC	Reference
Mouse Microglia (<i>In vitro</i>)	19.6 (100%)	4.4 (23%)	31.9 (174%)	2.5 (14.5%)	14.7 (77%)	13.0 (73%)	-
Human PBMCs (<i>In vitro</i>)	19.3 (100%)	6 (31%)	-	5.7 (29.5%)	13.3 (69%)	-	Michalak et. Al. 2017
Human PBMCs (<i>Ex vivo</i>)	15.7 (100%)	4.3 (22%)	36 (230%)	-	11.4 (73%)	20.3 (130%)	Jang et. al. 2017
Macroph. Cell Line (RAW 264.7/ Maf-DKO)	20 (100%)	7 (35%)	40 (200%)	4 (20%)	13 (65%)	20 (100%)	Venter et.al. 2014
Mouse Peritoneal Macroph. (<i>In vitro</i>)	30 (100%)	10 (33%)	80 (267%)	-	20 (67%)	50 (167%)	Aflaki et. al. 2011

Table 1. Table comparing different monocyte-macrophage HRR profiles. BASELINE: Basal oxygen consumption state; LEAK: state of oxygen consumption measured after Oligomycin injection that are used for ROS production and lost as heat; ETS: Electron transfer system capacity state, maximum respiratory mitochondrial capacity; ROX: Residual Oxygen Consumption obtained after Antimycin A injection; ATP-LP (ATP production and coupling efficiency): corresponds to the amount of oxygen that is used for ATP production by mitochondria; SRC: Spare Respiratory Capacity.

croglia. Interestingly, this number is lower than in other similar cellular systems (Table 1). Finally, the last state determined was ROX state, which for microglia was 2.5 pMol/(s*MiCells) (Fig. 3B);14.5% (Fig. 3C). Since the FCCP and Antimycin-A are diluted in EtOH, a control experiment was performed to check if EtOH alone would affect microglia respiration. As expected, we found that EtOH alone had no effect on O₂ flow rates (Fig. 4).

Figure 4. Representative Image of primary microglia HRR real-time control experiment using vehicle (ethanol). Representative experimental run obtained from an experiment run in Oroboros equipment. The left y-axis represents oxygen concentration (nmol/mL) and is represented by the blue line; the right y-axis represents oxygen consumption pMol/(s*MiCells) and fluctuations and is depicted by the red line. Baseline indicates the basal primary mitochondria respiration and black arrows indicate substrates (Pyr/Mal - 5 mM/0.5 mM f.c.), vehicle Ethanol 100% (8x Et 1μL titration) and inhibitors Oligomycin (2.5 uM f.c.). Et100%: Ethanol 100%.

Discussion

The present study shows the characterization of primary microglia oxygen metabolism and mitochondrial physiology with high resolution respirometry (HRR) technology. Intact primary mouse microglia were cultured *in vitro*, and analyzed with a substrate-uncoupler-inhibitor-titration (SUIT) protocol using Pyruvate/Malate, Oligomycin, FCCP and Antimycin A characterizing 6 metabolic states: Baseline, LEAK, ETS, ROX, ATP-LP and SRC. Oxygen electrodes were mainly used to study oxygen metabolism/mitochondrial function which modernized for more complex micro flow - high - throughput oxygen electrodes and equipment called respirometers (Brand and Nicholls 2011), such as the one used in the present experiments (Oroboros). Later, other technologies using multi-

well format-oxygen-dependent fluorescence quenching to monitor the oxygen/molecules concentration in the medium emerged (e.g. H-TEFA-Seahorse equipment) as an alternative mainly for analyzing cells previously cultured *in vitro*. The low time resolution and the limited number of possible substrate injections during an experimental run limits its use for sophisticated respiration analyses (Brand & Nicholls, 2011). Because, at least at our knowledge, this is the first HRR study performed with primary mouse microglia, it is not possible to compare the present findings to results from others using similar experimental models or protocols. Alternatively, we compared the present data to similar studies that used macrophage cell lines, adult mice primary peripheral macrophages and human intact peripheral blood mononuclear cells containing monocytes and lymphocytes (PBMCs). Detailed oxygen consumption values reported by others and the comparison with the present results can be found in Table 1.

The HRR profile of primary microglia was quite different from murine peripheral/peritoneal macrophages (Aflaki, Radovic et al. 2011, Venter, Oerlemans et al. 2014). This finding confirms the notion that microglia have unique characteristics compared to other macrophage types due to its origin and its complex microenvironment (Crotti and Ransohoff 2016). Although the leak of electrons from the mitochondria respiratory chain (LEAK state), the maximum mitochondrial respiratory capacity (ETS) and the spare respiratory capacity (SRC) were lower in microglia, the percentage of oxygen used for mitochondrial ATP production and coupling efficiency (ATP-LP) was slightly higher when compared to peripheral macrophages on both cell lines and adult peritoneal macrophages (Aflaki, Radovic et al. 2011, Venter, Oerlemans et al. 2014). This means that primary microglia mitochondria are more efficient in producing ATP than in their respiratory capacity or in producing ROS when compared to peripheral adult macrophages. That observation can be explained by the crucial role of microglia in energy production for CNS homeostasis maintenance and support (Lauro and Limatola 2020), especially in the newborn brain where there is a higher demand for energy. Also, a tonic extracellular ATP concentration is reported to be needed to maintain microglial ramification and surveillance (Madry, Arancibia-Carcamo et al. 2018). ATP-mediated signaling is present in many tissues, but is particularly important for microglia, the CNS innate immune cells; and in case of an insult or disturbance in the tissue, microglia will release ATP as a danger signal to recruit other immune cells to the injured site, making it important for microglia to main-

tain a high pool of intracellular ATP (Madry and Attwell 2015).

Others have evaluated microglia metabolism using technologies other than HRR (Jalin, Rajasekaran et al. 2015, Orihuela, McPherson et al. 2016, Gao, Vidal-Itriago et al. 2017, Giorgi-Coll, Amaral et al. 2017, Sarkar, Malovic et al. 2017, Wilson, Bouillaud et al. 2017, Nagy, Fekete et al. 2018). It was shown using multi-well H-TEFA technologies that similarly to macrophages, microglia switch its metabolism when activated by LPS, and nitric oxide (NO) promotes the transition from oxidative phosphorylation to glycolysis (Orihuela, McPherson et al. 2016). It was furthermore elucidated that there is a complex interplay between microglial mitochondrial impairment and inflammasome signaling dysfunction (Sarkar, Malovic et al. 2017) and, recently, (Nagy, Fekete et al. 2018) demonstrated that several oxidative energy metabolites increased the rate of basal and maximal respiration in primary microglia and BV-2 cells.

Immunometabolism studies are still limited, possibly due to their challenging nature, and the available data is somehow contradictory, probably because there is limited information on detailed protocols and studies addressing physiological naïve states. The main technical limitation is the relatively high number of cells required in these assays, combined with the fact that macrophages are highly heterogeneous and extremely sensitive to changes in their microenvironment. Either from fresh isolation of BPMCs from human blood or macrophages isolated from rodents' bone marrow or different tissues is still challenging to keep those cells in culture and expose them to different treatments *in vitro*. These limitations can be applied to our own observations and reports by others as well (Van den Bossche, O'Neill et al. 2017).

What makes microglia especially challenging to analyze by HRR is: i) their high sensitivity to *in vitro* manipulations; ii) the low yield of microglia from primary cultures or *ex vivo* isolations. Despite these limitations, microglia could be treated prior to mitotic shake off in mixed primary glial cells cultures, and right after shake-off transferred to Oroboros chambers for HRR analysis. Similarly, these microglia could be plated and submitted to multi-well H-TEFA analysis. This approach would allow simultaneous analysis by 2 different and complementary techniques, possibly increasing refinement and reproducibility of the experimental findings. Further analyses challenging microglia in culture for induction of polarized phenotypes with specific molecules, such as LPS and IL-4, and the replication of the present data by other researchers in the field are necessary ac-

tions for the complete characterization of primary microglia HRR analysis using Oroboros equipment.

In conclusion, the present data shows that it is possible to analyze primary mouse microglia cultured *in vitro*. Also, the present protocol can be used in the future for better understanding microglia oxygen metabolism and microglia physiology, and an optimized of protocol for microglia isolated from post-mortem or surgery human brain tissue samples would allow studies investigating microglia metabolism in the context of CNS disease.

Acknowledgements

This work was financially supported by the Dutch grants Abel Tasman Talent Sponsorship, and the UMCG De Cock foundation young talents annual grant. We would like to thank the availability and technical support provided at ERIBA, UMCG for using the Oroboros Equipment.

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CHAPTER 6

Thesis Summary and General Discussion

Graphical summary of the findings in experimental chapters 2, 3 and 4

Figure 1. Graphical Summary of this thesis: insulin signaling in the brain: cognitive, neuroinflammatory, and metabolic outcomes in the young and aged brain. Insulin treatment presents different outcomes in various experimental models and distinct ages. In an *in vitro* analysis using primary microglia isolated from postnatal mice (0-2 days-old) insulin affects metabolism and production of IL-1 β . Experimentation in young Wistar rats (3-4 months-old) and young mice (2-3 months-old) *in vivo* demonstrated an important inflammatory insulin signaling in the brain at the same time it affects positively spatial memory. During aging (Wistar rats 22-24 months-old), the brain cellular microenvironment is chronically inflamed, a condition that happens along with insulin resistance and neurodegeneration. Opposite of what was observed in the young brain, insulin failed to trigger neuroprotective inflammatory signals. ○: no effect. Original figure created with BioRender.com.

Thesis Summary

The role and functions of insulin in the CNS has emerged as an important topic of interest in the molecular and cellular neuroscience research field with several mechanistic hypothesis yet to be addressed. The importance of insulin signaling in processes as brain development, tissue repair, and cognition have been characterized in the last decades (Marks, Porte et al. 1990, de la Monte and Wands 2005, Ghasemi, Haeri et al. 2013, Agostinone, Alarcon-Martinez et al. 2018, Spinelli, Fusco et al. 2019). In this thesis, insulin signaling and its role in learning and memory, neurogenesis and neuroinflammation were studied in the context of the young and aged brain. In addition, insulin signaling was investigated in microglia both *in vitro* and *in vivo*.

The data reported in **chapter 2** shows that insulin, directly administered into the brain, improves the performance of young, but not aged rats in a spatial memory task. Surprisingly, insulin increased neurogenesis, insulin pathway markers, cell proliferation and synaptic markers in the aged hippocampus. One of the main neurotrophic factors associated with cognition in the brain - BDNF - was identified in young but not in aged rats in connection with intracerebroventricular injection of insulin (Haas, Kalinine et al. 2016). The disruption of the connection between insulin and BDNF downstream signals in the aged brain was proposed as a mechanism resulting in insulin signaling resistance in the aging brain.

Disruptions in insulin signaling in the hippocampus during aging regarding neuroinflammation were further investigated in **chapter 3**. It was observed that neuroinflammatory responses to insulin are different between young and aged brains. The modulation of microglial activation and IL-1 β /COX-2 expression was observed in young animals hippocampus, which was not found in aged brains, implying that microglia participate in the insulin resistance phenomena that is observed during aging. In addition, it was found that murine microglia are sensitive to insulin which activate a pathway via PI3K-Akt signaling (Haas, de Carvalho et al. 2020).

In **chapter 4** the effect of insulin signaling in microglia was explored *in vitro* and *in vivo* regarding cell viability/metabolism and cytokine production. As previously described for neurons and synaptosome preparations *in vitro* and *in vivo* (Muller, Haas et al. 2013), insulin modulated microglia oxygen metabolism *in vi-*

tro. Insulin decreased ROS production and increased mitochondrial metabolism. In microglia, insulin affects extramitochondrial ROS production, by the enzyme NADPH oxidase. An effect of insulin on lipopolysaccharide-induced IL-1 β production in microglia *in vitro* was found, what was not the case for IL-6 and TNF- α . An intranasal treatment with insulin *in vivo* resulted in an increased transcription of TNF- α , specifically in microglia.

A protocol for high throughput analysis of mitochondrial oxygen metabolism and mitochondrial physiology was standardized using high resolution respirometry (HRR). In **chapter 5**, this protocol is presented in detail. A comparison of microglia and monocytes/macrophages HRR profiles reported in the literature showed that primary microglia mitochondria are more efficient in producing ATP when compared to peripheral adult macrophages from blood in case of clinical and from peritoneum in case of experimental studies .

Finally, in **chapter 6**, the findings reported in this thesis were summarized and discussed.

General Discussion

The function of insulin in the CNS had been characterized in the past decades. This characterization first focused on insulin signaling in the body metabolism control since high expression of IR in the hypothalamus was initially observed (Baskin, Woods et al. 1983). Later, the connection of insulin signaling and other processes in the brain such as brain development, injury/repair, and cognition received attention (Marks, Porte et al. 1990) (Agostinone, Alarcon-Martinez et al. 2018, Canteiro, Antero et al. 2019, Manaserh, Chikkamenahalli et al. 2019). In the research presented in this thesis, cognition was studied in the context of insulin signaling in the young brain and resistance to insulin in the aged brain. In the first experimental chapter of this thesis (chapter 2) it was found that insulin increased spatial memory performance in young rats which was not the case in aged animals. Although insulin was able to increase neurogenesis and the expression of insulin pathway proteins, cell proliferation and synaptic markers in aged hippocampus, an increase in BDNF and BDNF receptor TrkB expression was detected in young rats after insulin treatment but not in aged rats. This data demonstrated, for the first time, a disruption in insulin and BDNF signaling in the hippocampus during aging, suggesting insulin resistance and hence loss of BDNF

signaling is an important downstream molecular mechanism of insulin resistance in the aging brain.

The ability of insulin, when injected directly to the brain, to enhance memory in young animals was previously described (Park, Seeley et al. 2000, Babri, Badie et al. 2007). It was also shown that the effect of insulin was dose dependent when insulin was injected directly in the hippocampus of rats (Moosavi, Naghdi et al. 2006) and it improved spatial memory in similar doses as showed in the present studies (Haas, Kalinine et al. 2016). The fact that insulin treatments directly into the brain did not increase cognition in aged animals was subject of interest of others (Adzovic, Lynn et al. 2015). Interestingly, IGF-2 injected into the hippocampus was able to rescue memory in aged rats (Steinmetz, Johnson et al. 2016), suggesting that even if insulin and IGF signals work in cooperation in physiology (Fernandez and Torres-Aleman 2012) it is probably a fact that this cooperation is disrupted during aging.

Studies had suggested that age associated-cognitive decline is more closely related to synaptic and neuronal network alterations than the loss of neuronal units (Burke and Barnes 2010, Morrison and Baxter 2012, Temido-Ferreira, Coelho et al. 2019). Neurons generated later in life can die in a short period of time, or are even functionally impaired meaning that they will rarely connect to the neural network (Mongiat and Schinder 2011). Alterations in the cellular microenvironment, such as chronic neuroinflammation, together with decreased neurotrophic support would be a contributing factor for the development of neurological changes during aging. These components would also explain why neurogenesis in aged hippocampus does not result in spatial memory improvements, at least in the experiments performed by us and presented in chapter 2 (Haas, Kalinine et al. 2016).

In this context, experiments were performed to address other mechanisms that could be activated by insulin promoting spatial memory improvement in young rats and that are disrupted in aged animals. In chapter 3, the role of insulin signaling in neuroinflammation and microglia physiology in both young and aged brain was investigated. The main finding was that inflammatory responses to insulin are different between microglia from young and aged rats. Unexpectedly, insulin promoted a pro-inflammatory modulation in the young hippocampus characterized by increased microglia activation and IL-1 β /COX-2 expression. These effects were not observed in aged Wistar rats. These outcomes led us to hy-

hypothesize that insulin would be acting not only on neurons but also on microglia *in vivo* to promote learning and memory. This hypothesis is supported by the fact that we did not detect microglial activation nor IL-1 β /COX-2 increased expression triggered by insulin in aged animals, where the brain is resistant to insulin.

Insulin signaling on microglia is a subject still being uncovered which limited information is available. Specially how insulin directly affects and signals in rodent primary microglia is still unresolved. For answering that question primary microglia *in vitro* were challenged with insulin. It was observed that primary microglia were responsive to insulin via PI3K-Akt signaling (Haas, de Carvalho et al. 2020). These results contribute to a better understanding of the neurobiological processes that are interrupted during the aging process, and their functional consequences in the CNS.

The sensitivity of microglia to insulin and the resistance of this signal during aging strongly suggests that primed microglia, the microglia phenotype found in aging brain, is resistant to insulin signaling at least in the hippocampus in a region-dependent fashion. The hippocampus has a heterogeneous neuronal population depending on the subregion (Lee, Yoganarasimha et al. 2004), reflecting the different functions of these subregions, but they form an integrated neuronal network to process cognition and memory. Although it is not possible to state that the pro-inflammatory alterations found after the i.c.v. insulin treatment are mediated by activated microglia, it is attractive to speculate that the neuroinflammatory insulin signal in microglia is an important factor to the neuroprotective effects of insulin on the young hippocampus previously observed and possibly lost during aging. This hypothesis is supported by the protective function of microglia already described such as the fact that microglia has a central role in originating and supporting synapses (Kettenmann, Kirchhoff et al. 2013) as well as memory function by producing BDNF (Parkhurst, Yang et al. 2013).

In contrast with this hypothesis, for many years, it had been assumed that the proliferation and activation of microglia as well as the expression of pro-inflammatory markers such as IL-1 β /IL-6, would be mostly harmful reactions to the brain, which concepts, currently, have been revisited and updated (Aguzzi, Barres et al. 2013, Biber, Owens et al. 2014, Cherry, Olschowka et al. 2014, Chen and Trapp 2016, Sochocka, Diniz et al. 2017). It is interesting how the literature developed regarding microglia activation as mentioned above. Considering that immunity serves to protect the body, why would activated microglia be exclu-

sively detrimental or disease-related? The first studies on microglia neurobiology focused on observations that were done mostly in the disease near injured sites or neurodegenerative regions where it presented mostly the active phenotype. The functions of microglia in physiology have been reviewed (Salter and Beggs 2014, Loane and Kumar 2016), and also pointed out in the present studies (Haas, de Carvalho et al. 2020). Inflammatory processes are primarily beneficial to the CNS, mostly assuming a harmful role under chronic disease adaptations (Biber, Owens et al. 2014). Even in chronic neuroinflammation it is questionable whether all activated microglia are detrimental for the CNS, because if this was the case all neural structures targeted by microglia would quickly degenerate (Chen and Trapp 2016). In our experiments, indeed, young rats presented more microglial activation and pro-inflammatory adaptations in the hippocampus after receiving insulin and also performed better in a spatial memory task, Morris Water Maze. At the same time aged rats show generally more activated microglia in their hippocampus. The molecular differences of both microglia activation and the period in life that neuroinflammation turns into a chronic phenomenon, or which intrinsic or extrinsic signals are triggering this protective or detrimental microglia activation are still topics to be investigated.

Cyclooxygenase (COX) is a main player in neuroinflammation, and COX-2 is reported to play a special role in normal neuronal function and in neurotoxicity (Minghetti 2004). Here, insulin increased COX-2 expression in the hippocampus of young rats, where this was not observed in aged rats (Haas, Kalinine et al. 2016). It was previously described that, different from COX-1, COX-2 expression is decreased in rat hippocampus during aging (Aid and Bosetti 2007). A link between COX-2 function, learning and memory and BDNF signaling had been described in diseases such as diabetes (Yang and Gao 2017), stress (Luo, Kuang et al. 2017) and AD (Chen, Zhang et al. 2013, Woodling, Colas et al. 2016) with an opposite effect compared with what we observed in physiology. Although studies on COX-2 function on learning and memory in the brain in physiology are scarce and contradictory results have been reported, COX-2 has been identified as a component mediating the consolidation of hippocampal-dependent memory (Holscher 1995, Teather, Packard et al. 2002).

Still in chapter 3 the important question whether microglia respond directly to an insulin stimulus was addressed. Even though other research groups have also studied astrocytes and microglia sensitivity to insulin (Garwood, Rat-

cliffe et al. 2015, Garcia-Caceres, Quarta et al. 2016, Mamik, Asahchop et al. 2016, Gonzalez-Garcia, Gruber et al. 2021) it was still unknown if primary rodent microglia would develop a similar profile as insulin-exposed-astrocytes. To experimentally address that question, an *in vitro* approach was used and we detected that a period as short as 30 minutes after insulin stimulation was sufficient to increase Akt phosphorylation and this effect was inhibited by a PI3K inhibitor, wortmannin. Next, we tried to unravel the molecular, cellular, and metabolic alterations triggered by insulin signaling in microglia. Taking advantage of an *in vitro* system where the effects of insulin could be observed in isolated cells, we used primary microglia as our experimental model generating the third experimental chapter of this thesis (chapter 4).

In chapter 4, the protective effect of insulin on microglia metabolism was observed. Correlating with previous findings that insulin have effects on ROS production in synaptosome via PI3K and in neurons *in vitro* (Muller, Haas et al. 2013), insulin decreased ROS production and increased cell metabolism/viability. Because in phagocytic cells, half of the ROS production occurs extracellularly by NADPH oxidase (Nayernia, Jaquet et al. 2014), the next question was if insulin would be affecting extracellular ROS production in microglia. Through pharmacological manipulation of NADPH oxidase in primary microglia it was observed that insulin affects extramitochondrial ROS production, but it was not possible to conclude if it happens by a direct or indirect effect on NADPH oxidase. More experiments are necessary to find the cellular and molecular mechanism of insulin action on microglial ROS metabolism as well as the biological outcomes of insulin effect on microglia metabolism.

In chapter 4, experiments addressing the effect of insulin on microglia using an intranasal treatment approach demonstrated the connection of insulin signaling with inflammation in the brain *in vivo*. The intranasal route of administration is an important less invasive strategy to brain-drug delivery when compared to direct brain injection. In rodents, it is already demonstrated that insulin delivered intranasally reaches the hippocampus and modulate behavior (Marks, Tucker et al. 2009). Clinical studies had also successfully demonstrated that intranasal insulin administration reaches the CSF (Born, Lange et al. 2002) with no increase in peripheral levels of insulin (Hamidovic, Khafaja et al. 2017). Molecules are generally absorbed by the respiratory epithelia along trigeminal nerve components reaching the olfactory bulb region with further dispersion to other

brain regions as cortex and hippocampus. Although specific mechanisms related to the transfer of various compounds from the nasal mucosa to the brain regions, as hippocampus, are yet to be determined, evidence suggests that this transfer happens at the perivascular spaces of cerebral blood vessels, although it is not possible to rule out intracellular transport (Lochhead and Thorne 2012).

The enhanced *TNF- α* gene expression found in microglia *ex vivo* after intranasal insulin treatment brings new insights into how the signals triggered by insulin in a complex microenvironment as in the hippocampus works. Combining this data with the observation that intracerebroventricular insulin increases IL-1 β at protein level in the hippocampus of Wistar rats in chapter 3 (Haas, de Carvalho et al. 2020) points to the mechanisms of neuroinflammatory insulin signaling which probably affects cells in a complex intercellular fashion that involves not only neurons but also microglia. A prospective investigation on potential molecular mechanisms would be proposed using strategies to silence the insulin receptor gene expression or further proteins participating in insulin signaling as Akt specifically in microglia and further evaluate animal brain function and behavior.

Modulation of learning and memory by cytokines had been explored but the exact function of this signal in cognition is yet controversial. It is known that inflammatory cytokines commonly associated with the peripheral immune system are also found in the CNS (Hopkins and Rothwell 1995, Rothwell and Hopkins 1995), but their exact sources are not well understood. IL-1 β transcription was upregulated in the brain of young Wistar rats submitted to various memory behavioral tests (Goshen, Kreisel et al. 2007, del Rey, Balschun et al. 2013, Scholz, Doidge et al. 2016). Additionally, it was also found in the CA1 hippocampus sub-region that *TNF- α* at both the gene and protein level was higher expressed after a fear conditioning memory task in rats (Scholz, Doidge et al. 2016). (Takemiya, Fumizawa et al. 2017) recently reported that protein levels of IL-1 β were increased in the hippocampus of young but not adult rats after a spatial memory task. *TNF- α* role in cognition had been reported as increased in the cerebellum of rats after a learning task (Cartford, Gemma et al. 2002), and the loss of function of this cytokine resulted in deficits in learning paradigms (Baune, Wiede et al. 2008).

Others demonstrated neuroprotective effects of pro-inflammatory cytokines such as *TNF- α* and IL-1 β (Carlson, Wieggl et al. 1999, Vinet, Weering et al. 2012, Dong, Fischer et al. 2016). We showed for the first time that insulin sig-

naling acts through pro-inflammatory signals in the hippocampus of young rats, which was reinforced by the fact that aged rats which are insulin resistant lack such neuroprotective effect when receiving insulin direct to the brain (Haas, de Carvalho et al. 2020). Future experiments injecting insulin into the mouse brain with disrupted IL-1 β /TNF- α signaling would confirm the relationship between insulin and these signals in hippocampus and differences in such components during aging. A strategy of targeting insulin/IL-1 β /TNF- α specifically in microglia *in vivo* would shed light on the mechanisms regarding the insulin resistance of primed microglia.

Next, we aimed to investigate whether insulin also affect mitochondrial ROS production and mitochondrial physiology in microglia but was hampered by a lack of established technical protocol for analyzing microglia using high resolution respirometry. A protocol was standardized using this high throughput technique, and the results are reported in the last experimental chapter of the thesis (chapter 5). A reliable protocol was developed using primary microglia direct from mixed glial primary cell culture shake-offs. It was found that primary microglia mitochondria are more efficient in producing ATP than in their respiratory capacity or in producing ROS when compared to peripheral adult peritoneal macrophages and monocytes.

As studied and discussed in chapter 4, ROS production in microglia mostly originates from extramitochondrial sources, mainly the NADPH oxidase enzyme. Also, the microglia used in this protocol were from newborn mouse where there is a higher demand for energy when compared to adult brain. A prospective and interesting experiment is to apply the described protocol to microglia enriched preparations as used in chapter 4 and compare both findings. This protocol offers a good approach to studying microglia metabolism using a very sensitive method. It would be very interesting to study human microglia metabolism (from postmortem brain or surgical specimens) in the context of the aged or diseased brain. Also, the mechanism of insulin signaling in microglia oxygen metabolism, as well as microglia mitochondria physiology can be studied in the future using this protocol.

Future Perspectives

The complexity of cellular and molecular networks of insulin signaling in the CNS is being unfolded as well as its loss of function during aging. The combination of neurotrophic, neuroinflammatory, and metabolic signals triggered in both neurons and glial cellular populations will be an object of further research and will shed light on the mechanisms that contribute to cognitive disfunction during aging. Broader access to high throughput unbiased sequencing such as bulk RNAseq and single cell RNAseq is very valuable to uncover new mechanisms related to insulin signaling in the brain. Uncovering new genetic signatures in the hippocampus or isolated microglia of young and aged mice treated with insulin injections directly into the brain for example would be an interesting experimental approach for investigating molecular mechanisms related to the brain insulin resistance phenomena.

For the study of insulin neuroinflammatory signaling an alternative experimental perspective would be the application of cytokines and other inflammatory markers screening at the protein level in hippocampal tissue, hippocampal extracellular fluid, isolated microglia, and CSF after insulin in both young and aged animals. Again, it would give new clues on inflammatory molecular mechanisms related to insulin neuroprotection and its relation to memory processing. A similar approach would be very interesting for studying the effects of insulin in microglia metabolism. Metabolomics analysis of microglia extracted from mice that received insulin would provide very valuable information. Analyzing oxygen metabolism and mitochondrial physiology in microglia cultivated *in vitro* and *ex vivo* using high throughput methods to measure oxygen consumption such as HRR in the presence of insulin is a promising perspective in the field.

Conclusion

The discovery of insulin was a revolutionizing treatment for diabetes. The insulin signaling research field has evolved tremendously getting to a point of insulin treatments being targeted to the brain and emerging as an alternative therapy for various neurological conditions, mainly neurodegenerative disorders. The exploration of neuroinflammatory hormonal signaling top down and bottom

up and the disruption of this signaling during chronic neuroinflammation and neurodegeneration as reported in these studies is an emergent research area. The presented work contributes to the general understanding of insulin signaling in the brain, mainly uncovering brain insulin neuroinflammatory signaling specific to the level of microglial cells and insulin signaling resistance during aging.

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CHAPTER 7

Nederlandse samenvatting

Nederlandse samenvatting

Sinds de baanbrekende ontdekking van insuline als behandeling voor diabetes, toonden klinische observaties aan dat diabetespatiënten een grotere kans hebben om neurodegeneratieve aandoeningen als de ziekte van Alzheimer te ontwikkelen. De rol en functies van insuline in het centrale zenuwstelsel zijn een belangrijk onderwerp van interesse in het moleculaire en cellulaire neurowetenschappelijke onderzoeksveld. De hippocampus is een belangrijk hersengebied dat betrokken is bij leer- en geheugenprocessen en een van de gebieden dat wordt beïnvloed door neurodegeneratie. De karakterisering van de functie van insuline in de hippocampus en de effecten ervan op leren en geheugenverwerking leverde een nieuw perspectief voor de behandeling van aan dementie gerelateerde neurodegeneratieve aandoeningen. Het neurodegeneratieve proces wordt gekenmerkt door progressieve atrofie en functieverlies van neuronen. Hoewel neuronen van oudsher worden gezien als de belangrijkste cellen in de hersenen, zijn gliacellen essentieel voor de functie en het in standhouden van de homeostase van de hersenen. De drie meest voorkomende soorten gliacellen zijn oligodendrocyten, astrocyten en microglia. Microglia zijn de weefselmacrofagen van de hersenen met immunologische functies en spelen een belangrijke rol bij neuroinflammatie. Ongevoeligheid voor insuline in verouderde hersenen en insuline signalering in astrocyten is een recente bevinding over de rol van insuline in de hersenen. Insuline is een belangrijk hormoon dat naast astrocyten ook neuronen beïnvloedt, en de functie van insuline kan tijdens het leven veranderen of (deels) verloren gaan. Daarnaast kan insuline, net als in de periferie, de cellulaire stofwisseling in de hersenen veranderen.

In het onderzoek beschreven in dit proefschrift, werden nieuwe moleculaire en cellulaire mechanismen van insuline signalering in de hippocampus en de effecten op microglia en neuroinflammatie in de hersenen van jonge en oude ratten bestudeerd. De effecten van insuline op het microglia metabolisme zijn onderzocht door middel van een nieuwe methode om het zuurstofmetabolisme en de mitochondriale functie van microglia te bepalen. In hoofdstuk 1 wordt een historische en actueel overzicht gegeven van het insuline onderzoeksveld, en wordt insuline (resistentie) van de hersenen geïntroduceerd. Daarnaast worden veroudering van de hersenen, zuurstof metabolisme, neuroinflammatie en insuline signalen in gliacellen samengevat. In hoofdstuk 2 worden experimentele gegevens gerapporteerd die aantonen dat rechtstreekse toediening van insuline

in de hersenen het geheugen van jonge, maar niet van oude ratten, verbetert. De belangrijkste bevinding van deze onderzoeken was dat de insulinesignalering in de hersenen is verbonden met een belangrijk en bekend eiwit (BDNF), wat verstoord raakt tijdens het ouder worden bij Wistar-ratten.

In hoofdstuk 3 zijn de effecten van insuline op neuroinflammatie onderzocht. Het is bekend dat er tijdens het ouder worden een verlies van gevoeligheid voor insuline optreedt, wat parallel loopt met chronische neuroinflammatie die nauw verwant is aan neurodegeneratie. De belangrijkste cel die verband houdt met neuroinflammatie in de hersenen is microglia, maar het effect van insuline op microglia was slecht begrepen. De resultaten van de experimenten die tot dit hoofdstuk hebben geleid, laten zien dat microglia gevoelig zijn voor insuline, zowel in cel kweken, als in de hersenen van ratten, en dat het tijdens het ouder worden de hersenen hun gevoeligheid voor insuline verliezen. Al met al zijn de gegevens gepresenteerd in hoofdstuk 2 en 3 belangrijk om te definiëren hoe insuline in de hersenen werkt, wat essentieel is voor toekomstig gebruik van insuline als therapie voor neurodegeneratieve ziekten. Hoofdstuk 4 beschrijft experimenten die het insuline-effect op microglia in muizenhersenen verder aantonen en de insuline effecten op het microglia-metabolisme bestuderen. Net als in de periferie heeft insuline ook in de hersenen een functie op de celstofwisseling. In de context van het metabolisme is het zuurstofverbruik door de cel een belangrijke speler. Zuurstof kan binnen of buiten mitochondriën worden gemetaboliseerd. De gegevens gepresenteerd in hoofdstuk 4 laten zien dat, zoals eerder beschreven voor neuronen, insuline het microglia-zuurstofmetabolisme en de productie van reactieve zuurstof soorten moduleert op zowel mitochondriaal-afhankelijke als -onafhankelijke wijze. Aanvullend in hoofdstuk 5 werd een protocol voor high-throughput analyse van mitochondriaal zuurstofmetabolisme en mitochondriale fysiologie gestandaardiseerd met behulp van hoge resolutie respirometrie (HRR). Dit protocol is een alternatief voor het bestuderen van het zuurstofmetabolisme van microglia met behulp van een zeer verfijnde en gevoelige techniek, dit is belangrijk omdat zeer kleine veranderingen in het zuurstofverbruik door de cel biologisch zeer relevant kunnen zijn. Deze nieuwe strategie kan worden gebruikt voor het bestuderen van verschillende effecten van medicijnen of moleculen in het metabolisme van microglia, evenals voor het bestuderen van microglia uit klinische monsters bij verschillende ziekten. Tot slot, in hoofdstuk 6, worden de bevindingen in dit proefschrift samengevat en besproken in de context van relevante wetenschappelijke literatuur.

APPENDICES

I. Acknowledgements

II. Curriculum vitae

I. Acknowledgements

It is with great happiness that I write the acknowledgments part of my Dutch thesis specially in challenging times as we currently see ourselves. It is important to mention that this thesis is part of an international cooperation between The Netherlands-Brazil which I was so blessed to be part of. Many efforts came together to make this collaboration successful, and I have a long list of people and institutions to acknowledge. First I would like to deeply thank to my promoters specially my main promoter **Bart Eggen** for the opportunity to conduct these studies and learn so much about microglia and science during the whole process to have this piece done. It was a challenging, rich and much valued experience. I still remember when I brought some preliminary data from Brazil in my backpack during a short visit, the data was contradicting the whole literature at the moment, and you still believed on that and thought it was an interesting finding and I could study it further in Groningen. My experience doing science together with you and all colleagues at the Neuroscience Department at the time had transformed myself as a scientist and as a person. Thank you very much. I also would like to thank my second promoter **Jon Laman** for his valued input during the way specially on how I interpretate my results and how I communicate my findings. Also, I would like to thank my third promoter of this thesis **Luis Portela** that supervised me since the beginning of my scientific carrier as a student scientist in Brazil. Roska and his team at Biochemistry Department, UFRGS paved the way and gave me the basis to follow my international scientific endeavors. I also want to acknowledge my co-promoter during my PhD studies, in Brazil, **Alexandre Muller**, that is also a very good friend. I am sure without Alexandre's mentoring I would never had believed I would go so far in my carrier always respecting my moral values and doing real science, always trying to understand what the data is telling me and not the other way around. Other than that Alexandre introduced me the amazing CNS insulin signaling topic, what I will be always grateful.

I would like to thank **Erik Boddeke, University Medical Center Groningen (UMCG) and University of Groningen (RUG)** that provided a great work and study environments as well as financial support through the Abel Tasman Young Talent Fellowship and De Cock foundation PhD grant. I also thank the **Research School of Behavioral and Cognitive Neuroscience (BCN) and Graduate School of Medical Sciences (GSMS)** for the very good training, courses, networking, conferences opportunities and for printing this thesis. I also would like to thank the assessment committee **Prof. dr. E.A. van der Zee, Prof. dr. A.M. Dolga, Prof. dr. P. Martinez Martinez** and for the availability of the opponents that will be giving me the opportunity to publicly defend this thesis.

When we (PhD students and postdocs) try to explain our “work” in science to family and friends that are not familiar with what we do is always a very difficult task. I use to say to them that PhD is not only something in between study/training/work but a complete unusual lifestyle. I must thank my family for all the support they always gave me during this crazy lifestyle, they mostly always believed in my potential more than myself. My grandmother **Wilma Branco** (*in memoriam*) inspired me to do and be anything I wanted, always an upfront mind of her time. My mother **Nara Branco**, an incredible awarded journalist who always made me visualize myself as a successful woman in any profession I would choose and my father **Joao Renato Haas** a biochemist which was always an example that a great professional is a result of a great personality and character and that there is no other way to go. Not differently, I thank my sister **Renata Haas** that as half attorney half artist shows me everyday that a bit of stubbornness is important to make us to move forward on our dreams.

When I moved to Groningen in 2015 me and **Thiago Alexandre** had a 3-4 year relationship which now became a 10-year relationship, 3-year marriage full of love and respect as it should be. Thank you my dear Thiago for believing on my dreams as impossible as they look like, and supporting me in each step on the way doesn't matter what happens. **Suzana Branco** and **Virginia Buglione**, thank you for the support and great example as well. Also, thank you **Luiz Garcia**, **Monique e Clair Alexandre e Marina Spoor** for everything.

Next, I want to acknowledge some professors that I had been lucky to have as my “informal mentors” during this journey that had roles that are not written in any of the chapters of this thesis so far. I want to specially thank **Harm Kampinga** for believing not only on me but on the Brazilian science, starting a fruitful collaboration with UFRGS many years ago. I hope I fulfilled his expectations. As a friend, I want to thank Harry to be this amazing person that I was so lucky to have close to me and gave me so much support. Thank you for loving Brazil and supporting Brazilian science! Another so important informal mentor along the way was **Antonio Galina** someone that introduced the fantastic world of mitochondria and respirometry to me and inspired me to study mitochondrial oxygen metabolism in microglia. Other than that, the best mitochondriologist I ever met and a great scientist Brazil must be proud of. A third but not less important informal mentor is **Elizandra Braganhol**, she inspires me and helps me to move forward even with all difficulties we women in science still face every day!

I would like to thank my paranympths **Claudio Tiecher** and **Maiara Muszkopf** for the friendship and all the support and help. Claudio is someone that I can discuss the connection between science and god and Maiara is someone that make me laugh even when it looks impossible. I want to say that I have two

“informal paronymph” **Marissa Dubbelaar** such a great friend that gave me so much support and help to prepare this book. I can say I would have even a second “informal paronymph” **Trix van der Sluis-Rozema**, which always made my mornings happier with her amazing smile and always kind attitude. My dear paronymphs I am grateful to all your help during the way and I miss you very much in my every-day life. I must, also, especially thank for the daily support I had in Groningen from my friends **Xiaoming Zhang, Zhuran Yin, Pong and Yang Heng**, you taught me resilience, patience and a different way to see the world! Thank you! You truly showed me that “Life experiments don’t fit into a scientific paper”.

Some other special people had helped me during my time in Groningen when experiments didn’t work and when I was missing my family, when everything looked impossible to move forward. Thank you **Eduardo Matos, Javier Villamil, Inge Holtman, Laura Kracht, Ilia Vainchtein, Chaitali Paul, Su-ping Peng and Thaiany Quevedo**. I would like to thank the dedication and the technical support, training in the lab and the help in some experiments to **Nieske Brouwer and Evelyn Wesseling**, you both are very inspiring women and I am glad I met and worked with you. I also would like to thank and acknowledge many other colleagues that contributed during my time in Groningen, **Susanne Kooistra, Michel Meijer, Hilmar van Weering, Tjalling Nijboer, Inge Zijdewind, Chairi Misrielal, Emma Gerrits, Priya Bodha, Eline Sportel, Koen van Zomeren, Rob Bakels, Thais Galatro, Corien Grit, Leroy Schreuder, Malte Borggrewe, Roeland Prak, Rianne van der Pijl**.

I would like to also thank colleagues from Brazil which collaborated in the first and second chapter of this thesis, **Afonso de Carvalho, Eduardo Kalline, Eduardo R. Zimmer, Andressa Brochier and Jean Oses**. Many incredible friends from Brazil, amazing scientists, that collaborated in my papers or not also need to be acknowledged here, **Giovana Bampi, Gisele Hansel, Samanta Loureiro, Marcelo Ganzela and Jussania Gnoato**, you are examples of scientists and human beings and I have learned so much with you, moral, ethics and respect to others always in front of any scientific/professional interest, it is very important to have you in my life to keep moving forward. I would like to acknowledge my dear friend **Jenna Wingfield** for the contribution for my book’s cover with the amazing drawing she made, she could kept exact what I wished. Thank you!

I also would like to acknowledge all friends, family and educators that somehow contributed to the achievement of this thesis which list would be too long to detail here. I am grateful for each person I met and each relationship that shaped me during this journey, and if you are using your time to read this book and this acknowledgments, **Thank you as well!**

I. Curriculum vitae

Clarissa Branco Haas was born on the 22th of February in Porto Alegre, Brazil. In 2006, She started her higher education studies at the Universidade Federal do Rio Grande do Sul (UFRGS) - Pharmacy school, Brazil with specialization in Biochemistry – Clinical Pathology. During her scientific initiation she worked as a student research assistant under supervision of L. V. Portela and A.P. Muller at the Dept. Biochemistry UFRGS, Brazil,

studying the protective mechanisms of physical exercise and insulin in the brain. After completing her Master she started her PhD at the very same laboratory in a combined program together with the Groningen University, The Netherlands, under the supervision of B.J.L. Eggen, in 2015. During the past years Clarissa worked uncovering insulin signaling mechanisms in the brain focusing on aging and neuroinflammation. Furthermore, Clarissa has secured funding, supervised several students and served as reviewer in important scientific journals. She is also involved in projects promoting open science and reproducibility/diversity in STEM. The results of her Ph.D studies are presented in this thesis.

List of Publications

Haas C.B., Lovászi M., Pacher P., de Souza, P., Pelletier J., Sévigny J., Nemeth Z., Braganhol E., Haskó G.; Extracellular ectonucleotidases are differentially regulated in murine tissues and human polymorphonuclear leukocytes during sepsis and inflammation. *Purinergic Signaling*. DOI: 10.1007/s11302-021-09819-1.(2021)

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