Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

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SINALIZAÇÃO DA INSULINA NO CÉREBRO: ALTERAÇÕES NEUROQUÍMICAS, COGNITIVAS E NEUROINFLAMATÓRIAS ASSOCIADAS AO ENVELHECIMENTO.

Tese de Doutorado

Clarissa Branco Haas

Porto Alegre, Setembro de 2017.

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(Orientador)

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“Os experimentos da vida nunca caberão em um artigo científico.”

(“Life experiments never will fit into a scientific paper”)

- Clarissa Branco Haas (Groningen, 2017)

“A saudade é uma dor que fere nos dois mundos.”

- Chico Xavier

“O conhecimento nos faz responsáveis”

- Che Guevara

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SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada em três partes principais: I, II e III, que contém nove tópicos. A parte I é formada pelos tópicos: (1) Introdução e (2) Objetivos; a parte II contém os três capítulos experimentais (tópicos 3, 4 e 5) desta tese, redigidos em formato de artigo científico já publicado ou em preparação para publicação em periódicos internacionais, e por esse motivo foram redigidos em inglês, sendo que cada um contém introdução, materiais e métodos, discussão e bibliografia específica, e a parte III, que apresenta os seguintes tópicos: (6) Discussão, (7) Conclusão, (8) Perspectivas e (9) Referências.

O tópico número 1 (Introdução) apresenta o embasamento teórico utilizado para o racional utilizado bem como desenvolvimento das perguntas científicas que deram origem aos experimentos que são apresentados nos capítulos experimentais dessa tese (tópicos número 3, 4 e 5). Os experimentos que deram origem aos 2 primeiros capítulos experimentais foram realizados no Departamento de Bioquímica, ICBS, UFRGS e os experimentos que deram origem ao terceiro capítulo experimental foram realizados no Departamento de Neurociências, Sessão de Fisiologia Médica, UMCG, Universidade de Groningen na Holanda. O tópico número 6 (Discussão) contém um apanhado geral dos resultados, bem como a interpretação dos mesmos, e é seguida do tópico número 7 (Conclusão) e 8 (Perspectivas) que descreve as perguntas científicas, a serem respondidas futuramente, geradas a partir dos resultados apresentados no presente trabalho.

O último tópico número 9 (Referências) contém a lista de referências usadas na introdução e na discussão. Os Anexos I, II, III, IV e V são artigos científicos publicados durante o período da tese e que são relacionados diretamente com o assunto da mesma.

LISTA DE ABREVIATURAS

AA: Ácido Araquidônico

Akt: Proteína cinase B (PKB), do inglês "V-Akt Murine Thymoma Viral Oncogene"

Arg-1: Arginina -1

BDNF: Fator Neurotrófico Derivado do Encéfalo, do inglês "Brain-Derived Neurotrophic Factor"

BHE: Barreira Hemato – Encefálica (que em inglês é "Blood Brain Barrier" – BBB)

BrdU: 5-Bromodeoxiuridina

CA1: Cornu Amonis 1 (Subregião Hipocampal)

CA3: Cornu Amonis 2 (Subregião Hipocampal)

COX: Cicloxigenase

DM: Diabete Mellitus

DNA: Ácido Desoxirribonucleico

ERK: Kinases reguladas por sinal extracelular, do inglês "extracellular signal-regulated kinases"

EROS: Espécies Reativas de Oxigênio, do inglês, "Reactive Oxygen Species" (ROS)

FOXO: do inglês "Forkhead Box"

GD: Giro Denteado, em inglês "Dentate Gyrus" (DG) (Subregião Hipocampal)

GFAP: Proteína Glial Fibrilar Ácida, do inglês "Glial Fibrillary Acidic Protein"

GSK3-β: Glicogênio cinase 3, do inglês "Glycogen Synthase Kinase 3"

HIV: do inglês "Human Immunodeficiency Virus"

ICV ou (i.c.v.): Intracerebroventricular

IGF-I: Fator de Crescimento Semelhante à Insulina tipo I, do inglês "Insulin Growth Factor-I"

IL-10: Interleucina - 10

IL-1β: Interleucina - 1β

IL-6: Interleucina - 6

IRS: Substrato do Receptor de Insulina, do inglês “Insulin Receptor Substrate”

LPS: Lipopolissacarídeo

mTOR: Proteína Alvo da Rapamicina em Mamíferos, do inglês “Mammalian Target of Rapamycin”

NeuN: Antígeno Neuronal Nuclear, do inglês “Neuronal Nuclear Antigen”

NF-κB: do ingles “Nuclear Factor kappa-light-chain-enhancer of activated B cells”

NGF: do ingles “Nerve growth factor”

PGE: Prostaglandina E

PGH2: Prostaglandina H2

PI3K: Fosfatidilinositol 3 Kinase, do inglês “Phosphatidylinositol 3 – Kinase”

RI: Receptor de Insulina

RNA: Ácido Ribonucleico

SNC: Sistema Nervoso Central, do inglês, “Central Nervous System”

TNF-α: Fator de Necrose Tumoral

TrkB: Receptor de BDNF do inglês “Tyrosine – Related Kinase B”

TXA2: Tromboxano A2

PARTE I

RESUMO

O envelhecimento, processo iminente a todo ser vivo, no SNC é caracterizado por alterações como, por exemplo, a neuroinflamação crônica, que estão associadas a processos de neurodegeneração e ao aumento da incidência de doenças neurológicas ligadas ao surgimento de demência. A insulina, o hormônio anabólico mais importante descoberto até hoje, tem sua sinalização como processo vital que está presente desde bactérias até a espécie humana e desde os tecidos periféricos até o SNC. Mesmo a sinalização cerebral de insulina sendo um tema bem definido na literatura, pouco se sabe sobre a sua função em células da glia, principalmente astrócitos e microglia, componentes chaves do processo de neuroinflamação. A neuroinflamação foi considerada, por muitos anos, tóxica ao SNC, mas atualmente evidências importantes têm sido encontradas sugerindo que processos pró-inflamatórios são primariamente benéficos ao cérebro ou encéfalo e podem assumir papel tóxico à medida que se tornam crônico. Assim, considerando o papel da insulina no SNC, bem como o aumento da expectativa de vida da população mundial que acarreta o aumento dramático da incidência de doenças neurodegenerativas, foi investigada, na presente tese a relação da sinalização fisiológica de insulina com processos cognitivos, neurotróficos e neuroinflamatórios e também a resistência na sinalização da mesma causada pelo envelhecimento cerebral. Foi demonstrado que a administração intracerebroventricular de insulina melhora a cognição de animais jovens, mas o mesmo não ocorre no envelhecimento. A nível celular e molecular, foi visto um distúrbio na conexão da sinalização de insulina e BDNF, bem como na ativação microglial e sinalização pró-inflamatória da insulina que parecem estar comprometidos no envelhecimento. Além disso, foi observado que a microglia é sensível à sinalização direta de insulina via PI3K e que essa sinalização microglial é adaptada e sofre mudanças na vida adulta. Em conjunto com a literatura, foi demostrado por esta tese que existe uma ruptura de paradigmas na interpretação dos processos neuroinflamatórios, que deixam de ser vistos somente como um fator tóxico ao cérebro, mas também como um artifício elementar de adaptação do SNC aos diversos estímulos que as células nervosas recebem durante o curso da vida, desde o nascimento até o envelhecimento.

Palavras-chave: Insulina – Microglia – SNC – Envelhecimento – Memória – Neuroinflamação – ROS – Neurogênese

ABSTRACT

Aging is a process that is found in all living being in the CNS. It is characterized by modifications, such as chronic neuroinflammation, which are associated with neurodegeneration and represent a risk factor for neurological diseases. Insulin is the most important anabolic hormone ever discovered. Insulin signaling represents an essential process that is present from bacteria to humans and from the periphery to the brain. Insulin signaling in the CNS is a well-defined topic in the literature. Most of the knowledge regarding brain insulin signaling still report findings in neurons and little is known about insulin function in glia, especially astrocytes and microglia that are key players of neuroinflammation. Neuroinflammation has been considered a toxic factor to the CNS, however, in the last few years, important evidences have been found that proinflammatory processes are primarily beneficial and may play a toxic role as soon as they become chronic. Thus, considering the role of insulin in the CNS, as well as the increased populational life spam worldwide, the present thesis investigated the relation of physiological insulin signaling and the brain insulin signaling caused by aging in cognition, neurochemistry and neuroinflammation. We showed that insulin intracerebroventricular administration improved the cognition of young animals, but the same was not observed in aging. At the cellular and molecular level, we found a disruption in the connection of insulin and BDNF signaling. We also show that a microglial activation and pro-inflammation triggered by insulin in young brain appear to be lost during aging. In addition, it was observed that microglia is sensitive to direct insulin signaling via PI3K and that this microglial signaling suffers adaptations and changes during life. Together with the recently changes in the literature, the findings of this work demonstrate that there is a rupture of paradigms in the interpretation of neuroinflammatory processes, which are no longer seen only as a toxic factor to the brain, but also as an smart adaptation of the CNS to the various stimuli that brain cells receive during the course of life, from birth to aging.

Key words: Insulin - Microglia - CNS - Aging - Memory - Neuroinflammation - ROS – Neurogenesis

1. INTRODUÇÃO

1.1 Envelhecimento

A população mundial está envelhecendo. Enquanto em 1940 a expectativa de vida era de somente 47 anos de idade, em países desenvolvidos, hoje as pessoas vivem em média até 77 anos de idade (Driscoll et al., 2008). No Brasil, esse aumento foi similar, passando de 45,5 para 75,5 anos, respectivamente (Malta et al., 2016). Além disso, programas de controle de natalidade têm promovido a diminuição de nascimentos em países desenvolvidos ou em desenvolvimento. Isso também é um fator contribuinte para o envelhecimento da população mundial. Consequentemente ao aumento da expectativa de vida, a incidência de doenças ligadas à senescência celular também aumentam como, por exemplo, a incidência de câncer (Malta et al., 2016).

1.1.a Envelhecimento do SNC

A nível central, o envelhecimento natural iminente a todo o ser vivo, é caracterizado por alterações no SNC que podem ser ilustradas pelo declínio na memória, alterações na visão, perda de audição progressiva, diminuição da coordenação motora e da força muscular. Diferentemente das alterações normais, doenças neurodegenerativas são caracterizadas por uma progressiva e lenta perda neuronal específica, levando a déficits neurológicos substanciais (Ziv and Melamed, 2010). A perda neuronal progressiva no córtex frontal e hipocampo, durante o envelhecimento, têm sido fortemente associados a prejuízos cognitivos (Morrison and Baxter, 2012). Além das alterações morfológicas, alterações neuroquímicas também estão presentes ao longo do processo de envelhecimento, incluindo prejuízos no metabolismo da glicose (Hoyer, 2004), alterações na função mitocondrial (Yin et al., 2014),

aumento na geração de EROS e a diminuição da sinalização neuroendócrina (Dickstein et al., 2013; Haas et al., 2016; Muller et al., 2012b).

O envelhecimento acarreta o aumento da incidência de doenças neurológicas ligadas ao surgimento de demência, como as doenças de Alzheimer e Parkinson, que têm sido alvo de intensas investigações nos últimos anos. Apesar do imenso progresso científico neste campo, durante a última década, os mecanismos celulares e moleculares cerebrais responsáveis pelos prejuízos cognitivos associados ao envelhecimento, ainda não foram totalmente estabelecidos (Gutchess, 2014; Lopez-Otin et al., 2013). Assim sendo, o envelhecimento é um reconhecido fator de risco para desenvolvimento de doenças neurodegenerativas e prejuízos cognitivos. Coincidentemente a sinalização de insulina diminui com o envelhecimento e está associada a declínio cognitivo e processos inflamatórios.

Os déficits cognitivos que são observados no envelhecimento são relacionados a alterações neurofisiológicas, como, a diminuição do fluxo sanguíneo no cérebro, neurodegeneração, neuroinflamação crônica e diminuição da sinalização cerebral de insulina. A expressão de fatores neurotróficos, sendo o principal deles o BDNF, parece ser um marcador importante que tem sua produção diminuída a partir das alterações neurofisiológicas encontradas no envelhecimento (Budni et al., 2015). A diminuição dos níveis de BDNF, principalmente no hipocampo, está associada também a prejuízos na neurogênese, que é um processo chave para a plasticidade sináptica no cérebro adulto, sendo que no envelhecimento ocorre a diminuição do número de células progenitoras e da diferenciação neuronal (Lee et al., 2012). Processos como o de neurogênese são altamente regulados pelo nicho celular que, no caso do envelhecimento, se encontra alterado devido a presença de neurônios em degeneração que expressam antígenos de membrana, e ainda neuroinflamação crônica.

1.2 Insulina

No início da década de 1920, o cirurgião canadense Frederick Banting juntamente com Charles Best, jovem estudante na época, realizaram os experimentos que finalmente concretizaram uma que seria parte das mais dramáticas descobertas científicas de todos os tempos e receberam o prêmio Nobel, em 1923, pela "descoberta da insulina". No final do ano de 1922, a insulina que estava presente nos extratos de pâncreas bovino já era experimentada em humanos e com uma rapidez surpreendente, mas também compreensível devido à demanda na época, em 1923 o extrato era produzido em larga escala pela Companhia Farmacêutica Eli Lilly, tendo um impacto imensurável na vida dos pacientes que sofriam com as consequências devastadoras da Diabetes Mellitus (DM) (Bliss, 1997). Desde sua descoberta, a insulina vem sendo objeto de pesquisa e, hoje, sabemos que a sinalização desse hormônio é um processo vital que está presente desde bactérias até a espécie humana, desde os tecidos periféricos até o SNC (Ghasemi et al., 2013).

A insulina é considerada o hormônio anabólico mais importante identificado até hoje e o seu efeito bioquímico primário é manter os níveis sanguíneos fisiológicos de glicose e ainda estimular a lipogênese, síntese proteica e proliferação celular (Wood, 2006). Praticamente todos os tecidos corporais são sensíveis à sinalização de insulina; músculo, fígado e tecido adiposo apresentam maior sensibilidade à insulina se comparados aos outros tecidos periféricos (Wood, 2006). Além disso, a insulina é considerada um hormônio pleiotrópico pela sua múltipla atividade sobre diversas cascatas de sinalização, regulando processos metabólicos, como a utilização dos nutrientes pelas células, e também processos tróficos e de sobrevivência celular (Fulop et al., 2003).

1.2.a. Sinalização Cerebral da Insulina

Os primeiros relatos científicos dos efeitos cerebrais da DM são dos anos 1960, 40 anos após a descoberta da insulina, e mostram lesões focais e difusas no cérebro de pacientes diabéticos. Tal fenômeno foi denominado de Encefalopatia Diabética (Alex et al., 1962; Chowers et al., 1966; Grunnet, 1963; Reske-Nielsen et al., 1966). Desde então, uma sequência de descobertas, como o "binding" da insulina no cérebro (Baskin et al., 1983; van Houten et al., 1979), seguida da identificação da propagação do sinal da insulina a nível subcelular (Marks et al., 1988) e tecidual, (Marks et al., 1990) contribuíram para os avanços em relação aos seus efeitos e propiciaram um melhor entendimento acerca das suas propriedades biológicas.

Estudos epidemiológicos reportaram que pacientes diabéticos apresentavam problemas de memória e tinham três vezes mais chances de desenvolver Doença de Alzheimer (DA) (McNay et al., 2010; Ott et al., 1999; Steen et al., 2005). Além disso, relatos experimentais demonstraram que o hipotálamo e as regiões cerebrais relacionadas com memória e aprendizado, como hipocampo, córtex e amigdala, são as mais ricas em receptores de insulina (RIs) (Fernandez and Torres-Aleman, 2012; van der Heide et al., 2006). Desta maneira, foi possível propor que a sinalização de insulina via RIs afeta a função cerebral, e que, assim como na periferia, ela pode se tornar resistente e afetar processos cognitivos. Hoje, tratamentos utilizando insulina pela via intranasal com o objetivo de melhorar a memória e retardar o avanço de processos neurodegenerativos em pacientes com patologias neurológicas que levam à demência, como por exemplo, a DA, se encontram em fase final de testes (Craft et al., 2017).

A origem da insulina no SNC é considerada uma questão controversa na literatura, e a hipótese mais aceita, atualmente é que mesmo existindo alguma produção de insulina no

cérebro (Kuwabara et al., 2011), a maior parte da insulina utilizada pelo mesmo é obtida da corrente sanguínea e atravessa a Barreira Hematoencefálica (BHE) mediada por um sistema de transporte sinalizado por RIs localizados nas células endoteliais dos vasos sanguíneos que irrigam o SNC (Blazquez et al., 2014). Esse sistema de transporte da insulina pela BHE é saturável a níveis menores e independentes daqueles necessários para regular a homeostase da glicose sanguínea (Banks et al., 1997a; Banks et al., 1997b). Ainda, anteriormente se acreditava que as células cerebrais não eram dependentes da sinalização da insulina para captação de glicose (Schulingkamp et al., 2000), conceito que atualmente tem sido refutado. Entretanto, o que tem sido consenso atualmente é que as funções da insulina no cérebro vão muito além do metabolismo da glicose, e estão relacionadas com sinalizações que culminam na regulação de processos cerebrais mais complexos como, por exemplo, síntese proteica, suporte neurotrófico e, aprendizado e a memória.

Uma vez no SNC, a insulina tem maior afinidade pelos seus RIs, mas também pode se ligar nos receptores do fator de crescimento semelhante a insulina (IGF-1R), o que exemplifica os mecanismos cooperativos entre insulina e IGF-1 no cérebro (Blazquez et al., 2014). Como pode ser visualizado na figura 1, adaptado de (Blazquez et al., 2014), ao se ligar no seu receptor, a insulina desencadeia, primeiramente, a fosforilação dos substratos dos receptores de insulina (IRSs) que, por sua vez inicia a ativação de cascatas de fosforilação que, em diversos pontos, divergem para promover o efeito da insulina em processos biológicos celulares variados. As cascatas de sinalização intracelular desencadeadas pelos RIs cerebrais tem suas respostas disparadas principalmente pela fosforilação do IRS-1, que quando fosforilado tem sua sinalização bifurcada em duas vias principais, PI3K – Akt e RAS – ERK. A cascata da via PI3K – Akt promove a ativação e inibição de proteínas como mTOR, GSK3-β e FOXO, que estão relacionadas com a regulação da transcrição proteica, apoptose, autofagia e estresse oxidativo, enquanto que a cascata RAS – ERK regula processo de

transcrição gênica e proliferação celular. O controle fino dessas duas cascatas, PI3K – Akt e RAS – ERK, está intimamente relacionado com o controle dos processos de sobrevivência e morte celular (Fernandez and Torres-Aleman, 2012; van der Heide et al., 2006).

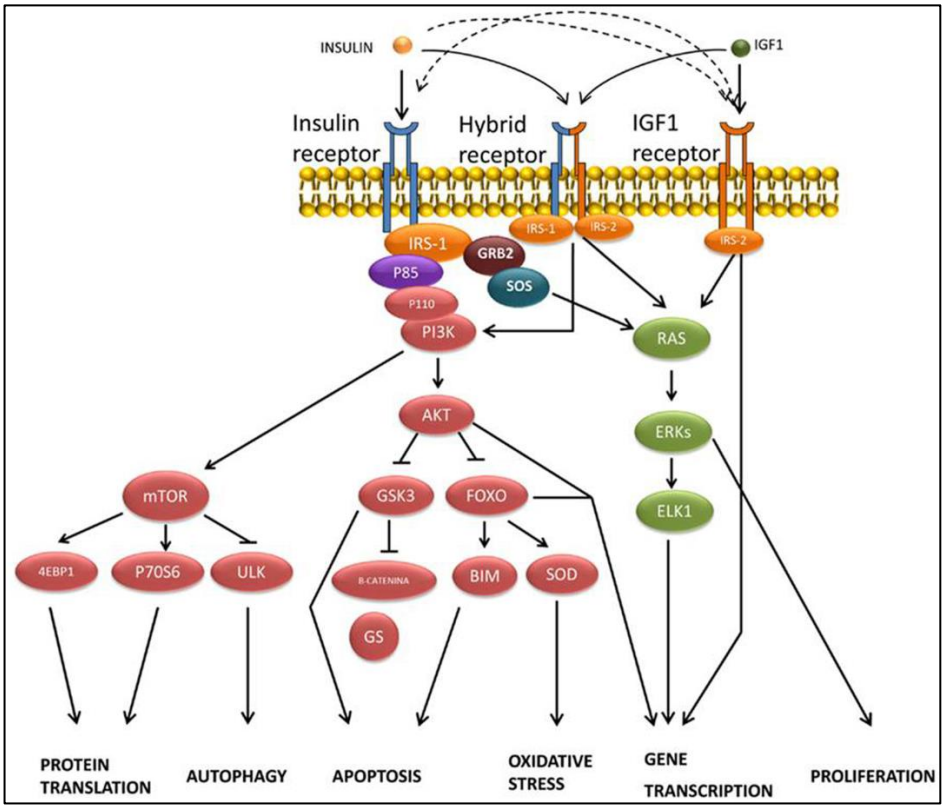

Figura 1. Cascata de sinalização de insulina no cérebro e suas ações biológicas, adaptado de (Blazquez et al., 2014).

O nosso grupo de pesquisa vêm, há mais de 10 anos, pesquisando a sinalização de insulina nos processos de aprendizado e memória e os mecanismos moleculares que impactam estes processos. Assim, demonstramos que o exercício físico aumenta a sinalização de insulina no hipocampo de camundongos e também a memória espacial dos mesmos (Muller et al., 2011), que a insulina regula a produção mitocondrial de Espécies Reativas de Oxigênio (EROS) (Muller et al., 2012b), e que essa sinalização está reduzida no envelhecimento cerebral

(Muller et al., 2012a; Muller et al., 2013). As investigações sobre o papel da insulina no cérebro jovem e a resistência à sinalização desta no envelhecimento foram objeto de estudo desta tese. Entre outros resultados, demonstramos que a administração farmacológica de insulina diretamente no cérebro, desencadeia diferentes respostas neurotróficas e cognitivas em ratos jovens e velhos (Haas et al., 2016).

1.2.b. Resistência à Sinalização Cerebral da Insulina

Mudanças na sinalização desencadeadas pelos RIs durante o processo de envelhecimento cerebral podem surgir devido aos níveis aumentados de colesterol e diminuição da fluidez da membrana plasmática que prejudica os movimentos laterais dos lipídeos de membrana, ou, ainda, por modificações na internalização, re-expressão ou degradação do mesmo pelo proteossoma (Duarte et al., 2012). A essas alterações também tem sido associadas à diminuição do número de RIs cerebrais e sua capacidade de ligação no hipocampo, córtex e plexo coroide associadas ao surgimento de processos neurodegenerativos. Além disso a diminuição da quantidade dos RIs está relacionada com déficits no aprendizado e memória encontrados durante o envelhecimento (De Felice and Ferreira, 2014; Heni et al., 2015). De fato, a presença marcante dessa resistência ao sinal da insulina foi denominada Diabetes tipo 3 por De la Monte e seu grupo de pesquisa em 2005 (Steen et al., 2005). Resultados anteriores, do nosso grupo de pesquisa, mostraram que o cérebro envelhecido apresenta mecanismos adaptativos, embora não efetivos, que envolvem aumento dos receptores, mas diminuição da sinalização pelo IGF-I, hormônio com efeitos semelhantes à insulina (Muller et al., 2012a). A busca por mecanismos que estão inibidos e defeitos nas vias de sinalização da insulina, no cérebro, quando os receptores de insulina se encontram resistentes aos sinais enviados pela

mesma são de extrema importância para que novos alvos farmacológicos e assim potenciais terapias sejam descobertos.

1.3. Neuroinflamação

A neuroinflamação é um processo que visa manter a homeostase do microambiente cerebral. O processo neuroinflamatório é estrategicamente importante para a defesa e integridade do tecido nervoso (Medzhitov, 2008). Nele, as células imunes, vasculares e neuronais, residentes do SNC ou não, incluindo neurônios, glia (astrócitos, microglia, oligodendoglia), macrófagos, linfócitos e leucócitos infiltrados, atuam de forma complexa e coordenada. A neuroinflamação é exacerbada quando estímulos novos, anormais, externos e internos atingem o cérebro, como por exemplo, infecções por microrganismos patogênicos, toxinas, traumas mecânicos e degeneração tecidual (Medzhitov, 2008). Nesse momento, as células que compõem a glia mudam seu fenótipo e são "ativadas", fenômeno que foi considerado tóxico ao SNC por muitos anos e que atualmente vêm sofrendo uma quebra de paradigma devido a achados importantes de que a ativação das células gliais, principalmente da microglia, contribui beneficamente para a recuperação do microambiente após estímulos nocivos ao SNC ou até mesmo quando estes não são encontrados (Jassam et al., 2017; Masgrau et al., 2017; Xanthos and Sandkuhler, 2014).

As reações inflamatórias no SNC diferem em diversos aspectos das respostas inflamatórias de outros tecidos, sendo notável a presença predominante da resposta imune inata (Medzhitov, 2008), uma vez que a presença da BHE reduz muito a permeabilidade dos vasos e microvasos cerebrais, tornando muito mais difícil ativar cascatas do complemento que recrutam células do sistema imune adaptativo, como leucócitos, para o tecido nervoso. Com exceção dos linfócitos T, que em condições severas, conseguem se infiltrar rapidamente pela BHE. Assim, os mecanismos de sinalização dos processos neuroinflamatórios são baseados no

controle da produção e liberação, bem como troca, de citocinas e quimiocinas pelas células envolvidas nesse sistema (Xanthos and Sandkuhler, 2014).

1.3.a. Neuroinflamação Aguda

Antes da terminologia "neuroinflamação" ser amplamente adotada pelos neurocientistas, o termo comumente usado para descrever o acúmulo de células gliais, mais precisamente de microglia e astrócitos, que ocorria em resposta imediatamente após injúrias no SNC, era "reatividade glial" (Streit et al., 2004). O avanço nas investigações dos processos inflamatórios no SNC possibilitou ampliar a visão acerca deste tema, e, hoje, o termo "reatividade glial" é visto como uma resposta muito exacerbada e incomum (Streit et al., 2004), sendo que a classificação e estudo da neuroinflamação como aguda e crônica, mesmo em situações fisiológicas, ganhou espaço definitivo na literatura.

Atualmente, tem se atribuído um sentindo mais amplo à resposta inflamatória aguda, sendo esta classificada como a ativação das células gliais, que poderia ocorrer ainda antes de adaptações morfológicas (Parakalan et al., 2012; Vinet et al., 2012), em resposta a insultos neuronais ou até mesmo ao aumento da atividade neuronal, sem o rompimento da BHE e sem a infiltração de leucócitos. Á longo prazo, essa resposta neuroinflamatória aguda pode ser benéfica ao SNC, uma vez que contribui para manter a homeostase tecidual e trabalha no suporte do aumento das atividades neuronais normais como ocorrem, por exemplo, em flutuações na atividade sináptica (Streit et al., 2004; Streit et al., 1998).

1.3.b. Neuroinflamação Crônica

A resposta neuroinflamatória crônica compreende a contínua liberação de mediadores químicos inflamatórios a partir do insulto inicial (Acarin et al., 2002) e é mais relevante no contexto do desenvolvimento de doenças no SNC, não somente aquelas que envolvem a

entrada e permanência de patógenos ao cérebro, como a encefalite crônica em portadores do vírus HIV (Garden, 2002), mas também aquelas relacionadas à instalação de processos neurodegenerativos permanentes, como Doença de Alzheimer, Parkinson e Huntington (Akiyama et al., 2000). No envelhecimento cerebral, mesmo não se tratando de uma patologia, a neuroinflamação crônica é encontrada, e até o presente momento não foram encontrados os fatores desencadeantes desse fenômeno bem como os benefícios da instalação de processo crônicos de inflamação no cérebro.

1.3.c. Microglia

Microglia é a denominação dada ao grupo de macrófagos residentes do SNC que, mesmo sendo classificadas como macrófagos, possuem origem diferenciada (saco vitelino) (Ginhoux et al., 2013), e suas funções especializadas, que vão muito além das funções imunes, fazem destas um tipo celular único (Aguzzi et al., 2013). Assim, a microglia é composta por células altamente sensíveis ao microambiente e altamente plásticas, que apresentam uma assinatura molecular diferente dos monócitos periféricos e de outros tipos de macrófagos teciduais (Butovsky et al., 2014). A Microglia foi identificada há quase um século por Del Rio-Hortega, que, por estudos histológicos, identificou formas ramificadas que ficaram conhecidas como microglia resting e formas ameboides, conhecidas como microglia ativada (sempre associada à doença) (Kettenmann et al., 2011; Kettenmann et al., 2013). É compreensível que na época a classificação da microglia tenha sido limitada à sua morfologia, mas, atualmente, técnicas mais refinadas têm demonstrado que essa classificação teria sido equivocada e que a microglia ativada (ameboide) apresenta funções tróficas importantes em ambas as condições normal e patológica (Hellwig et al., 2013). Um exemplo seria a microglia no período pós-natal que apresenta a morfologia ameboide, mas é extremamente importante para moldar as estruturas sinápticas do cérebro adulto (Schafer et al., 2012).

No cérebro maduro, onde essas células variam não só sua morfologia, mas a sua resposta molecular quantas vezes forem necessárias, as funções microgliais conhecidas até o momento são: (i) funções imunológicas fagocitárias: que compreendem a captação e digestão de patógenos e de fragmentos celulares e (ii) funções não imunológicas: que vão desde o monitoramento do microambiente, passando pelo controle da integridade e função das sinapses, bem como fagocitose sináptica, quando necessário, até produção e liberação controlada de fatores tróficos (BDNF, IGF-1, NGF) que são importantes para o suporte da neurogênese e da homeostase cerebral (Morris et al., 2013). No envelhecimento, elas assumem uma morfologia diferenciada, que é induzida pelo microambiente nervoso cronicamente inflamado, chamada "primed". A microglia primed tem ramificações mais curtas e em menor número e apresentam reatividade exacerbada a estímulos imunológicos exógenos, bem como dessensibilização a estímulos regulatórios endógenos (Norden and Godbout, 2013; Raj et al., 2014).

No desempenho de qualquer uma das suas funções, a microglia, ao ser ativada, apresenta aumento na cascata de sinalização da COX, que é a principal cascata de sinalização microglial e também a melhor descrita até o momento. Essa sinalização que pode ser desencadeada por inúmeras moléculas, como, por exemplo, IL-1β, TNF-α, IL-6, LPS, promove e mantém o estado neuroinflamatório (Hoozemans et al., 2011). Quando ativada, essa cascata fosforila e ativa o gene da transcrição da cascata enzimática do ácido araquidônico (AA) via NF-κβ (Rao et al., 2012). O AA, por sua vez, é metabolizado a PGH2 pela COX-2. Por sua vez, a PGH2 é convertida a PGE2 pela PGE sintase de membrana ou citosólica, ou pela tromboxano sintase à TXA2 (Hoozemans et al., 2011). Outras moléculas importantes para a sinalização e fisiologia da microglia são os EROS que participam na sinalização da ativação da microglia (Bordt and Polster, 2014). Estas são responsáveis por sinalizar a translocação do NF-κβ do citosol para o núcleo, além de serem mensageiros secundários à sinalização das interleucinas. Coincidente

aos macrófagos, e diferente da maioria dos outros tipos celulares, a NADPH oxidase ou NOX são fontes extremamente importantes de EROS juntamente com a mitocôndria nas células microgliais.

1.3.d. Sinalização da Insulina em Células Gliais

Embora atualmente a pesquisa e, assim, as publicações sobre as propriedades da insulina no cérebro tenham aumentado exponencialmente, a informação sobre a sinalização de insulina sobre células gliais ainda é escassa. A primeira publicação que considerou o tema data de 1989 (Shemer et al., 1989) e reporta que em culturas gliais mistas (contendo microglia, astrócitos e oligodendrócitos) o receptor de insulina é fosforilado, ou seja, funcional na presença da mesma. Ao longo dos anos, a maior parte do avanço nesse tema foi em relação aos astrócitos, nos quais a sinalização de insulina foi mostrada em astrócitos em amostras oriundas de cérebro humano e em roedores, sendo que em roedores, foi visto que essa sinalização astrocitária no hipotálamo é responsável pelo controle da oferta de glicose aos neurônios, bem como controle da homeostase energética também na periferia (Garcia-Caceres et al., 2016; Garwood et al., 2015; Spielman et al., 2015). Em relação à microglia, foi demonstrado ainda que a microglia expressa IRs e que esses são sensíveis e apresentam respostas que modulam a atividade microglial (Adzovic et al., 2015; Mamik et al., 2016; Spielman et al., 2015).

Desta maneira, considerando a importância da microglia para os processos de sobrevivência das células neurais e para a função cerebral neste conjunto de investigações, direcionamos nossos esforços para entender as diferenças na sinalização de insulina em ratos velhos e jovens, e, particularmente, as possíveis respostas da microglia quando desafiadas com insulina.

2. OBJETIVOS

2.1. Objetivo Geral

O objetivo geral da presente tese foi investigar indicadores celulares, moleculares, inflamatórios e comportamentais (memória espacial) associados à sinalização da insulina no cérebro de ratos no curso do envelhecimento e suas potenciais diferenças com animais jovens. Ainda, investigamos particularmente o fenótipo da microglia em reposta a insulina *in vitro* (cultura primária de microglia) e *ex vivo* (camundongos adultos).

2.2. Objetivos específicos

2.2.a. Investigar se a administração farmacológica i.c.v. de insulina é capaz de melhorar a memória espacial em ratos jovens e velhos.

2.2.b. Investigar marcadores celulares e moleculares das cascatas de insulina e de sinalização da memória no hipocampo de ratos submetidos ao tratamento farmacológicos de administração i.c.v. de insulina.

2.2.b.i. Investigar a expressão dos componentes da cascata de sinalização da insulina no hipocampo de ratos jovens e velhos tratados com insulina i.c.v.

2.2.b.ii. Investigar efeitos neurotróficos da insulina no hipocampo de ratos jovens e velhos submetidos ao tratamento com insulina i.c.v.

2.2.b.iii. Avaliar os efeitos da insulina sobre a expressão de sinaptofisina, BDNF e receptor de BDNF (TrkB) no hipocampo de ratos jovens e velhos submetidos ao tratamento com insulina i.c.v.

2.2.c. Investigar se a administração farmacológica i.c.v. de insulina é capaz de modular a resposta neuroinflamatória no hipocampo de animais jovens e velhos.

2.2.c.i. Verificar se a insulina modifica a quantidade de microglia e sua ativação no hipocampo de ratos jovens e velhos submetidos ao tratamento com insulina i.c.v.

2.2.c.ii. Verificar se a insulina modula a expressão de marcadores neuroinflamatórios (Nf-κβ, COX-2, IL-1β e IL-6) no hipocampo de ratos jovens e velhos submetidos ao tratamento com insulina i.c.v.

2.2.c.iii. Investigar se o efeito da insulina na expressão de marcadores neuroinflamatórios é transitório ou prolongado.

2.2.d. Avaliar se a microglia é sensível à insulina, e se sua resposta é diferente em microglia oriunda de cultura primária e do cérebro adulto.

2.2.d.i. Avaliar o efeito da insulina na produção de EROS, função mitocondrial e citotoxicidade em microglia neonatal obtida por cultura primária.

2.2.d.ii. Investigar o efeito da insulina sobre a produção das interleucinas IL-1β, IL-6 e TNF-α em microglia neonatal obtida por cultura primária.

2.2.d.iii. Investigar o efeito do tratamento com insulina intranasal em camundongos adultos sobre a expressão genética dos marcadores inflamatórios IL-1β, IL-6 e TNF-α, IL-10, Arg-1 e COX-2.

PARTE II

3. CAPÍTULO I

Brain Insulin Administration Triggers Distinct Cognitive and Neurotrophic Responses in Young and Aged Rats

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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 rats, but not in 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 rats, but not in 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 presynaptic 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 tropomyosin receptor kinase B (TrkB) receptors in the hippocampus of young rats, but not of aged rats. Our data therefore indicate that aged rats can have normal IR downstream protein expression but failed to mount a BDNF response after challenge in a spatial memory test. In contrast, young rats showed insulin-mediated TrkB/BDNF response, which paralleled with improved memory performance.

Keywords Insulin · Brain · Hippocampus · Neurogenesis · Spatial Memory · BDNF

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Introduction

Aging is a major risk factor for the development of brain disorders related to cognitive impairment and dementia [1–3]. 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 [4, 5]. Although the majority of brain regions are affected by aging, neurons from the hippocampus have shown remarkable vulnerability to death [1]. 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 [6, 7].

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 [8, 9]. 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 [10–12]. However, deficient insulin control of this pathway can cause changes in the neurotrophic support and a loss of neural cells [13]. 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 [14].

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) [15, 16] to improve synaptic plasticity and cognitive function [17]. 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 [18]. Thus, pharmacological strategies to increase these neurobiological 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 [13], 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 BNDF 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-h light/12-h dark cycle with free access to food and water. The rats (five 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).

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 μL of insulin (20 mU) (Humulin R® Lilly, Indianapolis, USA) or vehicle (NaCl 0.9 %) (5 min/1 μL/min) for five consecutive days. Immediately 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 analyses and hippocampal BDNF quantification, while another group (n=6–8 per group) was challenged in the Morris water maze (MWM) task and then euthanized 24 h later to analyze the BDNF levels in the hippocampus (Fig. 1a).

Spatial Memory—MWM Task

We conducted the MWM task following the same protocol used in [19]. 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 5 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 four trials per day separated by a minimum of 12 min each one. Each trial lasted up to 60 s, and the rats were allowed to rest on the hidden black platform for 20 s. 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 four trials per animal/day/group and was used as an indicator of learning. The second, namely test phase (1 day), is an indicator of memory retention. A probe test was performed without the platform 24 h after the 5 days of training, and the time spent in the target quadrant was measured. 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 Blotting

The Western blot assay was performed according to Muller [7]. 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

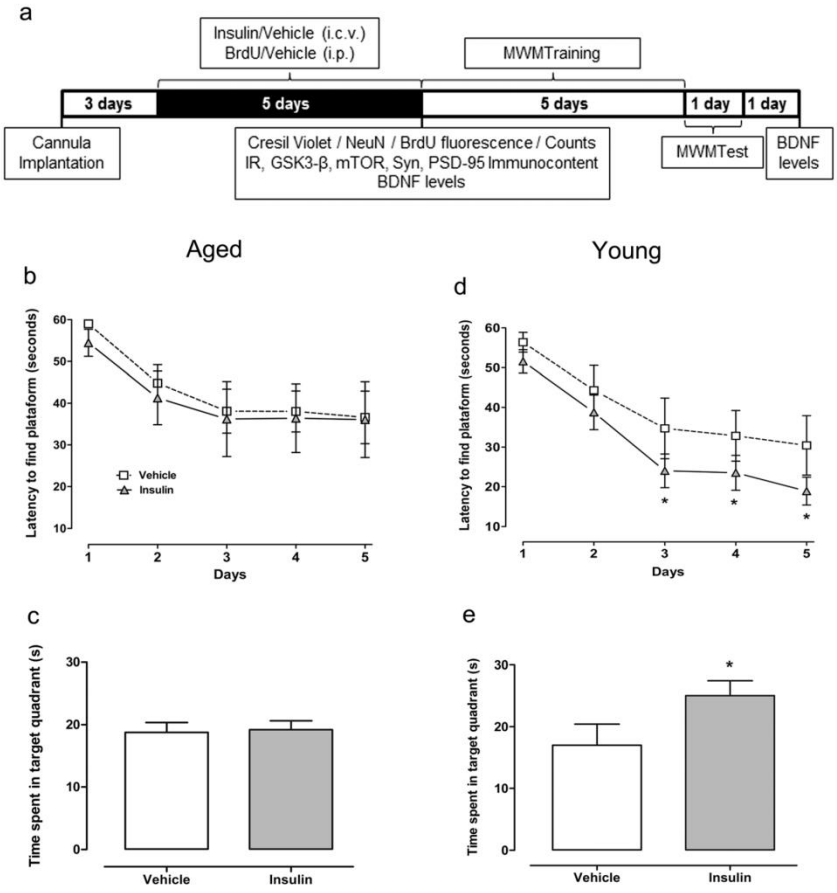


Fig. 1 Insulin i.c.v. administration did not improve spatial memory performance of aged rats. Diagram of experimental design (**a**). The hippocampal-dependent memory of aged rats was tested in the Morris water maze (MWM) task. After 5 days of insulin i.c.v. administration, the animals were submitted 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 of the training protocol, and increased the time spent in the target quadrant during the test day (**e**) ($*p<0.05$), (mean±SEM)

$CaCl_2$, 1 mM $MgCl_2$, 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×*g*, 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 (IR) β (1:1000) (CST, Danvers, USA), phospho-GSK-3β (p-GSK3β^{Ser9}) (1:1000) (CST, Danvers, USA), GSK3β (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), tropomyosin receptor kinase B (TrkB) (1:1000) (Abcam, San 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 ImageJ software (Rockville, USA).

Immunohistochemistry

The left hemispheres of the euthanized rats were used for immunohistochemistry analysis (n=4–6 per group) [19]. 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 eight random series was collected for the analyses. To conduct cresyl violet staining, the sections were immersed in cresyl violet (Sigma, St. Louis, USA) for 20 min 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 secondary 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 square millimeter, six 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 ($100\mu m^2$) and multiplied the result by 100 (total 1 mm^2). To determine the neuronal nuclei fluorescence per image, we used the mean fluorescence of three ROIs using ImageJ software (Rockville, USA).

BrdU Assay

The BrdU assay was performed as described previously [20], 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 0.1 M 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 2-h 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 were 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 procedures 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 (100 μg) as well as serial dilutions of BDNF standard (170 ng/mL) dilutions and reagent diluent (1 % BSA5 in PBS). After 2 h, 100 μL of the "detection antibody" that is the biotinylated mouse anti-human BDNF antibody was added. Subsequently, streptavidin-HRP, "substrate solution" (1xH_2O_2: 1x tetramethylbenzidine), and "stop solution" (2N H_2SO_4) 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±SEM. 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, c). By contrast, insulin significantly increased the performance of young animals in both the acquisition and retention phases of MWM (probe trial) (Fig. 1d, 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), p-GSK3β^{ser9} (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 p-GSK3β^{ser9} (Fig. 2f), but not of IR (Fig. 2e), mTOR (Fig. 2g), or cleaved caspase-3 (Fig. 2h) when compared with young-vehicle.

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 consecutive injections of insulin). The representative images showed that vehicle-treated 24-month-old

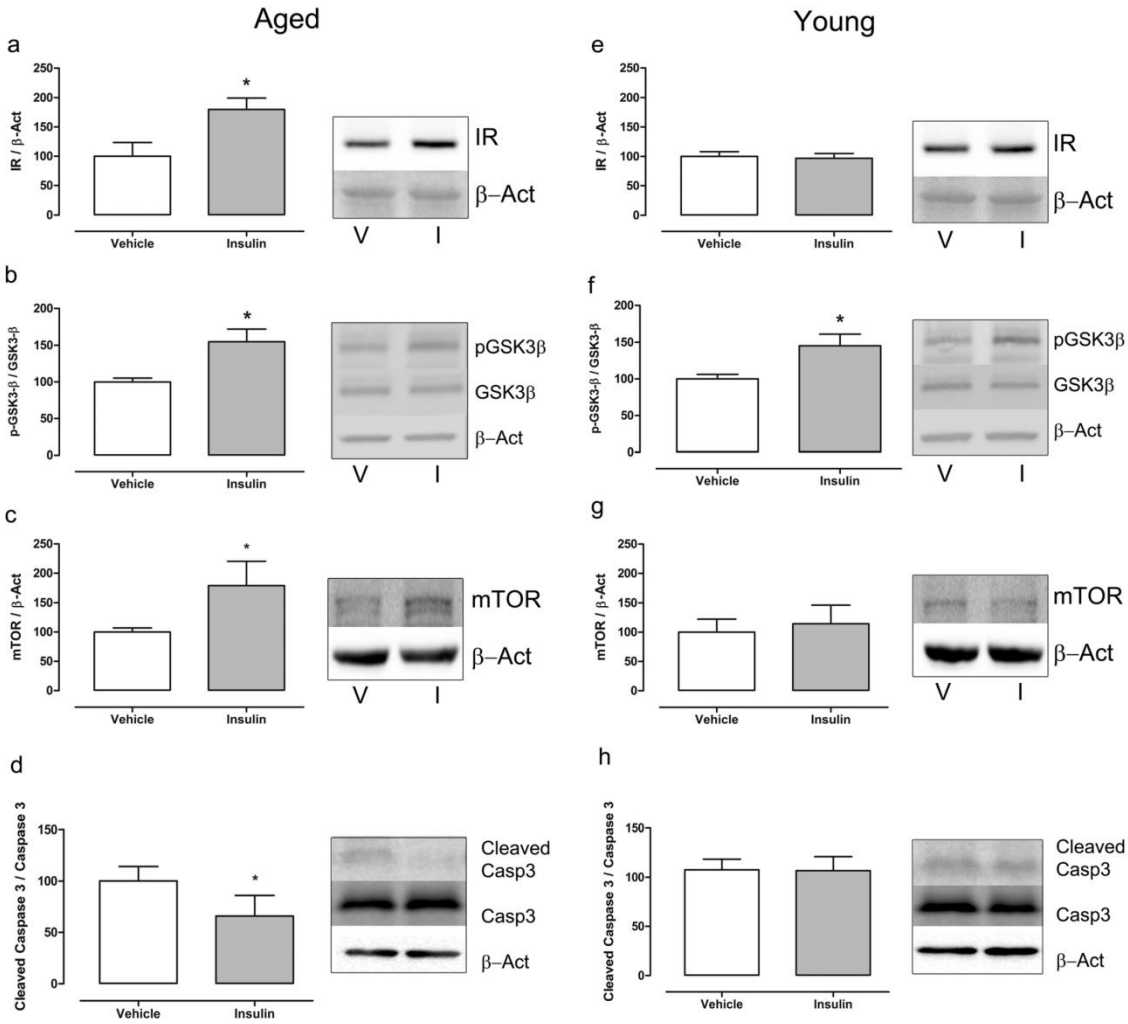
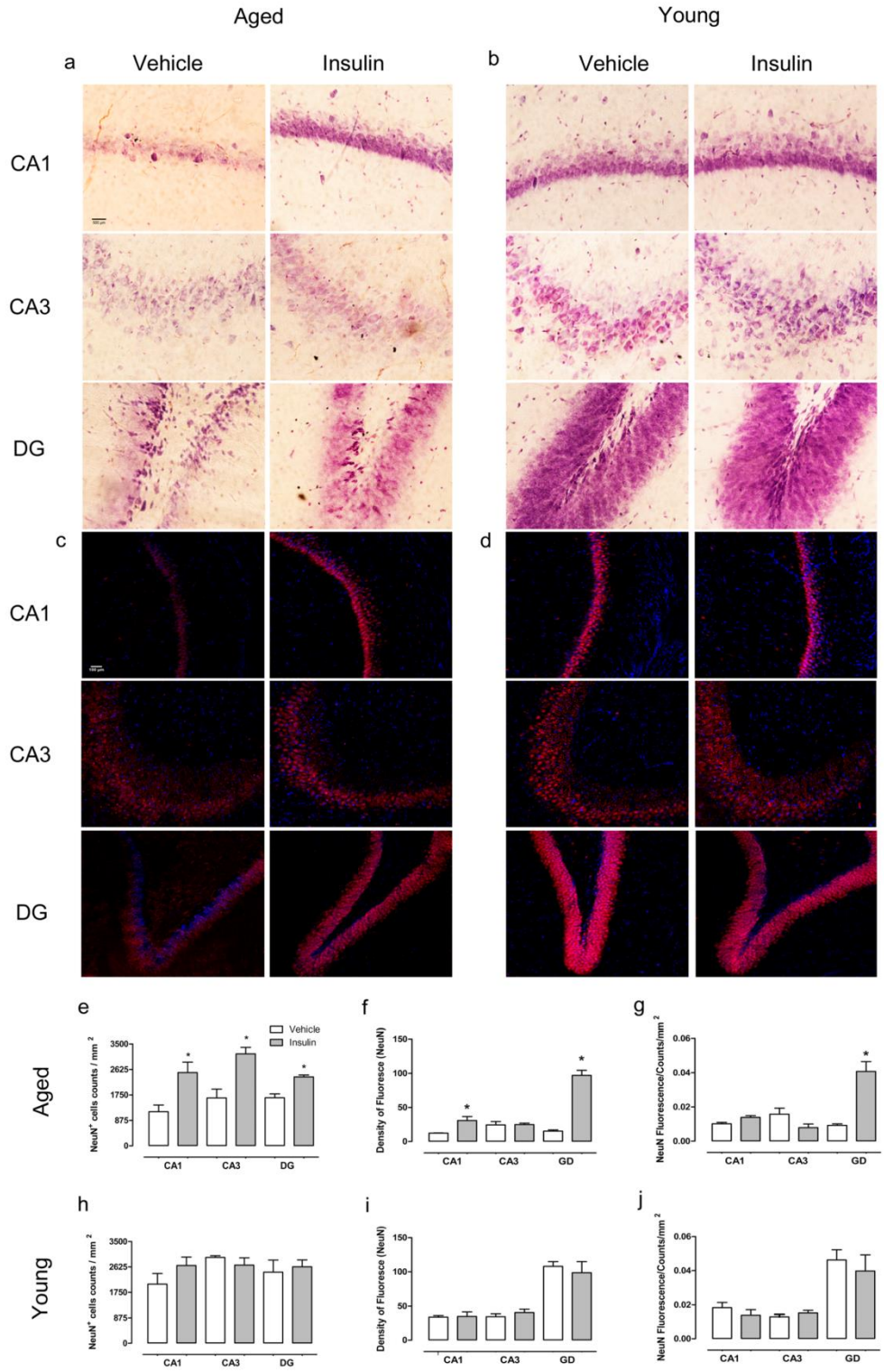


Fig. 2 Insulin administration increased the expression of insulin receptor (IR) and downstream proteins and decreased apoptotic marker expression of aged rats. Aged rats that received i.c.v. insulin injections for 5 days showed an increased expression of the downstream hippocampal signaling proteins compared with the vehicle controls: IR expression (**a**) (*$p<0.05$), phosphorylated leve p-GSK3β^{Ser9}. (**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 (**d**) and mTOR (**e**) were not significantly affected, whereas the phosphorylated level of p-GSK3β^{Ser9}. (**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±SEM)

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 rats (Fig. 3a), but not of 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 of 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 and Table 1). 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 of young rats (Fig. 3j).

Given the prominent neurotrophic effects of insulin in the DG and the critical role of this hippocampal subfield in


Aged
Young
a
Vehicle
Insulin
b
Vehicle
Insulin
CA1
CA3
DG
c
d
CA1
CA3
DG
e
f
g
Aged
Vehicle
Insulin
NeuN+ cells counts / mm 2
3500
2625
1750
875
0
CA1
CA3
DG
Density of Fluoresce (NeuN)
150
100
50
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CA1
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GD
NeuN Fluorescence/Counts/mm 2
0.06
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◀ **Fig. 3** Insulin-induced neurotrophic effect in the CA1, CA3, and DG hippocampal subregions of aged rats. The rats were subjected to an i.c.v. injection with vehicle or insulin for 5 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*=500 μm. 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*=100 μm. Insulin increased NeuN-positive counts/mm^2 in the hippocampal subregions 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/mm^2 ratio in the DG of aged rats (**g**) (*p<0.05). Insulin did not significantly increase NeuN-positive counts/mm^2 (**h**), NeuN density of fluorescence (**i**), or NeuN fluorescence/NeuN counts/mm^2 ratio in the hippocampal regions of young rats (**j**) (mean± SEM). *CA1* cornu ammonis1, *CA3* cornu ammonis 3, *DG* dentate gyrus

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 Presynaptic 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 presynaptic terminals, and the postsynaptic density protein 95 (PSD-95), a marker of postsynaptic terminals. Insulin increased the expression of Syn in the hippocampus of aged rats (Fig. 5a), but not of 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).

Table 1 Summary of insulin effects in aged and young rats

	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 post-MWM	–	↑

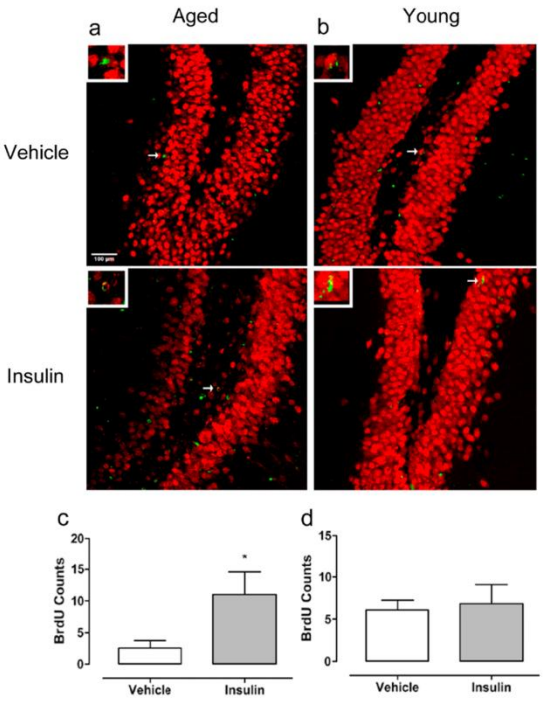

Fig. 4 Insulin i.c.v. injection induced neurogenesis in the hippocampal dentate gyrus (DG) of aged rat. Representative images of BrdU and NeuN staining in aged (**a**) and young (**b**) rats in the DG of the hippocampus (n= 4–6 per group). Insulin significantly increased the number of BrdU-positive counts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

Insulin i.c.v. Infusion Modulates the BDNF Levels and the Immunocontent of Its TrkB in the Hippocampus of Young Rats, But Not of Aged Rats

The BDNF expression and insulin signaling is proposed to interact to improve learning and memory function in young [15], and we further evaluated this assumption in aged rats. Five days of insulin treatment did not increase BDNF levels in both young and aged rats (Fig. 6a, b, pre-MWM). Similarly, we found that after 5 days of insulin administration, there was no increment in the TrkB expression in aged rats (Fig. 6c, pre-MWM). In contrast, insulin increased TrkB expression in young rats (Fig. 6d, pre-MWM). Considering that insulin improved the spatial memory performance in the MWM paradigm, we investigated whether BDNF levels and insulin interact to facilitate this effect (post-MWM). Insulin did not increase BDNF levels in the hippocampal homogenates of the aged rats (Fig. 6e) post-MWM. Conversely, insulin significantly increased hippocampal BDNF levels in young rats compared with the young vehicle group (Fig. 6f) post-MWM.

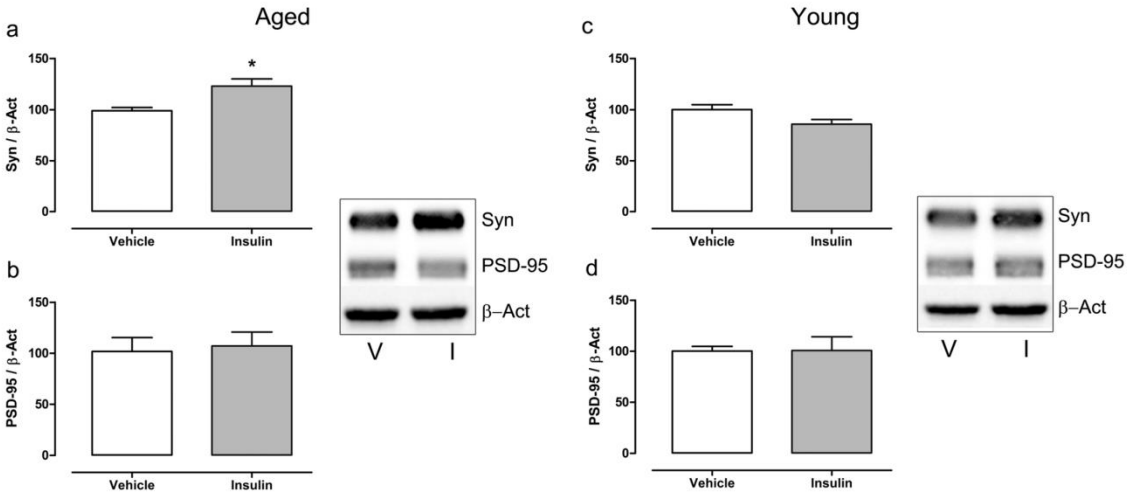

Fig. 5 Insulin i.c.v. administration increased synaptic plasticity in the hippocampus of aged rats. To evaluate synaptic plasticity, we analyzed two synaptic proteins, synaptophysin (Syn) and PSD-95. Aged rats showed increased expression of the presynaptic Syn marker (**a**) (*$p<0.05$), but not the 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±SEM)

Discussion

Our results demonstrated that 5 days of i.c.v. insulin administration increased spatial memory performance in young rats, but not in 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-3_{ser9}, 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 neurodegenerative processes and memory deficits [9, 21]. 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 administration 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 antiapoptotic effects [8, 22]. When insulin binds to IR, it promotes the phosphorylation and inhibition of GSK3-β, thereby the proapoptotic intracellular signaling mediated by GSK3-β is inhibited [23]. 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 antiapoptotic protein mTOR and the decreased proapoptotic 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 [5]. 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 [24]. Furthermore, Lind and collaborators demonstrated that NeuN fluorescence is linked to neuronal functionality and constitutes a proxy of healthy status of postmitotic neurons [25]. 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 [26]. In addition, the survival of new neurons correlated with increased spatial learning in one study [27] and with worsening results in another study [28].

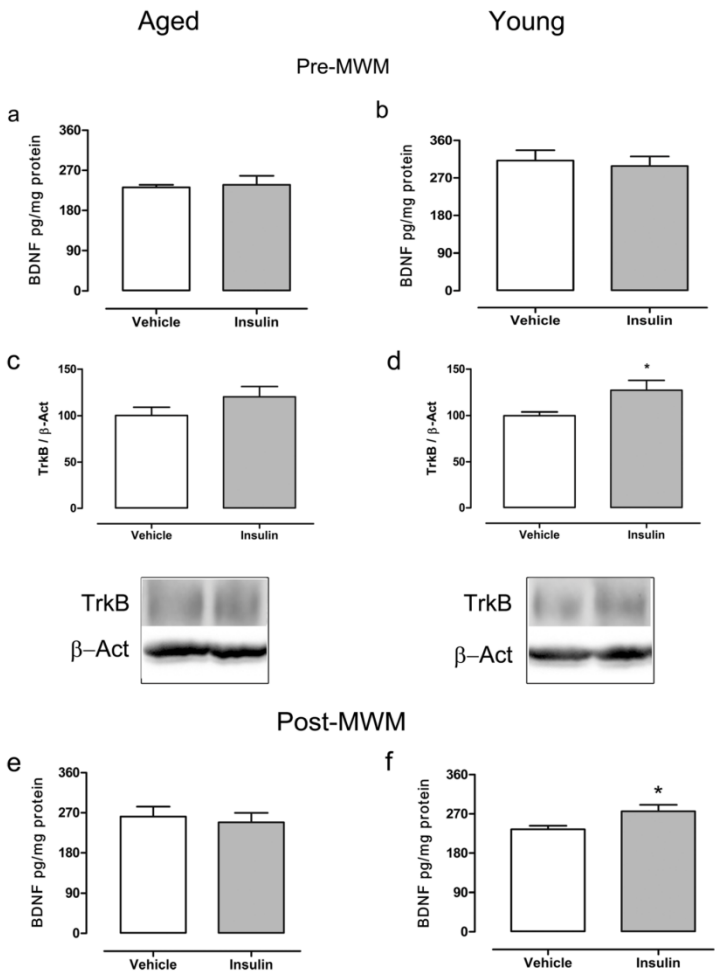

Fig. 6 Insulin i.c.v. administration did not improve hippocampal BDNF and TrkB levels of aged rats. The hippocampal BDNF (**a**) and TrkB (**c**) levels were assessed just after insulin treatment of aged rats and did not respond to insulin administrations. Aged rats did not respond to insulin administrations on BDNF levels after water maze task (**e**). Similarly, BDNF did not 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±SEM)

Furthermore, although adult neurogenesis and the incorporation of newborn neurons into hippocampal circuits seems to improve spatial memory formation [29], disorders in cognitive functions were not observed in rodent strains whose neurogenesis was ablated with antimitotic drugs or irradiation [30, 31]. 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 [32]. In aged rats, we found that insulin increases the presynaptic protein synaptophysin but had no significant effect on the postsynaptic 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 [5] 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, GABAr, and glutamate receptors/transporters that could influence BDNF synthesis [33, 34]. 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 adaptions, 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 [14].

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.

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Compliance with Ethical Standards The animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee 22535 of the Federal University of Rio Grande do Sul (UFRGS), Brazil.

Competing Interests There are no conflicts of interest to declare.

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4. CAPÍTULO II

Insulin *in vivo* increases microglial activation and proinflammatory outcomes in the hippocampus of young but not aged rats

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Manuscrito em preparação

Insulin *in vivo* increases microglial activation and proinflammatory outcomes in the hippocampus of young but not aged rats

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Abstract

Insulin signaling has been described as a key pathway involved in learning and memory processing in hippocampus. During aging insulin signaling decreases and inflammation increases. Such alterations may initiate in early adult life thereby contributing for the cumulative cell damage overtime and aged-related cognitive deficits. However, a key question has been to identify subtle brain alterations in the prodromal phase. It was proposed that brain insulin resistance is an early pathological event that precedes memory deficits. Also, although insulin receptors in astrocytes regulate neuronal metabolism and brain function, the impact of insulin signaling on hippocampal microglia phenotype and neuroinflammatory responses associated to hippocampal dependent memory function still needs elucidation. Also, though disruptions of the immune system trigger impairments in learning and memory, the link between insulin, neuroinflammation and memory function in young and aged brain is still unclear. To address this gap we injected insulin (20 mU) intracerebroventricular (i.c.v) once a day, during 5 days, and evaluated short-term (24h after last insulin administration) neuroinflammatory outcomes in young and aged Wistar rats. Long-term (9 days after last insulin administration) outcomes were evaluated only in young rats. At short-term, insulin increased microglia number/activation (CA3 subfield), and the hippocampal expression levels of NF-κB/ COX-2/IL-1β in young rats promptly 24h after five days administration. In contrast to young rats, insulin was not able to produce similar increments in the hippocampus of aged-rats. Eight days after insulin, the cellular and molecular markers of neuroinflammation remained elevated in young rats and paralleled with increased spatial memory function in the Morris water maze (MWM). The present data strongly suggests that microglia integrates insulin signaling and neuroinflammatory responses in the hippocampus to promote learning and memory processing in young but not aged rats. IL-1β is produced by activated microglia during neuronal activity and plays a critical role in regulating synaptic transmission. The insulin signaling is disrupted in aged hippocampus and appears to affect the proinflammatory effects induced by insulin, and finally the capacity of aged brain to process new memories.

Key words: Insulin –Microglia – Brain – Aging – Memory – Hippocampus

1. Introduction

Aging is an intriguing biological process that provides molecular and cellular substrates for the decreasing on physiological integrity, and depending on the contribution of genetic and environmental factors, place individuals at increased risk of cognitive deficits and neurodegenerative disorders (Haas et al., 2016; Lopez-Otin, Galluzzi, Freije, Madeo, & Kroemer, 2016; Muller, Fernandez, et al., 2012; Muller, Zimmer, et al., 2012). An important characteristic of these brain abnormalities that follows aging is the time-dependent accumulation of cellular damage, triggered by the rupture or exhaustion of the neurobiological mechanisms designed to maintain functional brain homeostasis (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013). The mechanistic components leading to aged-associated neuronal dysfunction and cognitive deficits are likely initiated early in adult life, and include exaggerated and prolonged neuroinflammation, coupled with deficient neuroendocrine signaling particularly in the hippocampus, a brain region involved in learning and memory formation and consolidation, in which these cumulative alterations, overtime, may render neurons more vulnerable to neurodegeneration (Barrientos, Frank, Watkins, & Maier, 2012; Barrientos, Hein, Frank, Watkins, & Maier, 2012; Grabert et al., 2016; Janssen et al., 2017; Morrison & Baxter, 2012; Steculorum, Solas, & Bruning, 2014).

Actually, aging is associated with changes in the neuroinflammatory environment, and recent studies have implicated microglial dysfunction as a contributory factor in age-related neurodegenerative disorders (Karch & Goate, 2015; Spittau, 2017). Microglia are a specialized and dynamic population of macrophages resident in the central nervous system (CNS) parenchyma that are able to detect tissue damage and environmental variations through their branched projections (Hanisch & Kettenmann, 2007). Moreover, beyond the well-characterized roles of microglia in the immune response, current studies are still trying to

expand our knowledge regarding their non-immune functions in the aged brain physiology and pathology (Salter & Beggs, 2014), as the case of adult mice microglia contribution in learning and memory mechanisms (Parkhurst et al., 2013). However, as primary innate immune cells in the brain, microglia also provide trophic support for injury repair, and removal of debris and even living neurons, which is crucial for the neural circuit remodeling and organizing synapses (Galatro et al., 2017; Janssen et al., 2017; Salter & Beggs, 2014). Importantly, once microglia senses any disturbance in the homeostasis of the brain microenvironment it becomes "activated", a process characterized by morphological changes and up regulation of a broad spectrum of intracellular neuroactive molecules, like cellular membrane surface antigens, such as CD11b and CD68, cytoplasmic cyclooxygenase (COX) enzyme and the nuclear transcription factor NF-κB pathway, considered as an important regulator of proinflammatory gene expression of the cytokines IL-1β and IL-6 (Frakes et al., 2014; Norden, Muccigrosso, & Godbout, 2015; Perry & Holmes, 2014; Seibert & Masferrer, 1994; Tak & Firestein, 2001). Remarkably, the phosphorylation of NF-κB subunits control many aspects implicated in the molecular interactions, degradation and transcription activity of NF-κB dimers (Christian, Smith, & Carmody, 2016). Emerging evidences highlight the interplay between systemic aged-related comorbidities, such as type-2 diabetes mellitus and the exaggerated responses of microglia chronically sensitized by previous challenges, namely microglia primed. Also, it has been proposed that type-2 diabetes mellitus heightens the risk for the development of Alzheimer's disease (Talbot et al., 2012).

Therefore, it has been a realistic conjecture that resistance to insulin/IGF-I signaling also reaches the brain tissue and may compromise its function. Thus, beyond the former belief as being a peripheral hormone, insulin is also now recognized by the diverse functions within the brain, including neurotrophic signaling (Bomfim et al., 2012). Recently, we have demonstrated that brain insulin administration improved memory in young but not in aged

rats, which was related with decreased capability to increase BDNF expression in the aging hippocampus (Haas et al., 2016). It is known, so far, that insulin receptors (IRs) are highly expressed in brain regions involved in cognitive functions, like cortex, amygdala and hippocampus, and not surprisingly, during aging the decreasing IR signaling may pave the way for the development of neurological abnormalities (Talbot et al., 2012). Accordingly, experimental rodent models and studies with aged patients have consistently demonstrated a strong mechanistic association between deficient insulin signaling, cognitive disruptions and Alzheimer's disease (Ghasemi, Haeri, Dargahi, Mohamed, & Ahmadiani, 2013; Gutchess, 2014; Lopez-Otin et al., 2013; Steculorum et al., 2014; Talbot et al., 2012). On the other hand, that deficient insulin signaling, which follows normal aging process, represents an adaptive response attempting to minimize cell proliferation, decreasing metabolism and extending lifespan (de la Monte, 2013; Lopez-Otin et al., 2013; Steculorum et al., 2014) .

Whereas the role of IRs in neurons has been extensively investigated, only recently the insulin signaling in glial cells has received attention. According to (Garcia-Caceres et al., 2016), insulin signaling in hypothalamic astrocytes controls the availability of glucose to neurons while the ablation of astrocytic IRs compromise mitochondrial function and hypothalamic neuronal connectivity, which is crucial for the regulation of whole body energy homeostasis. Also, IR and downstream proteins (IRS-1 and IRS-2) are expressed in microglia supporting the perception that insulin may directly bind to its receptor and influence microglia activity (Spielman, Bahniwal, Little, Walker, & Klegeris, 2015). Importantly, intracerebroventricular infusion of insulin in young rats prevented neuroinflammatory and memory impairment outcomes induced by lipopolysaccharide (LPS) injection (Adzovic et al., 2015).

In spite of the fact that insulin signaling is related with learning and memory and it becomes resistant over time, there is still a considerable gap in the understanding of the relationship between its signaling and the expression of inflammatory markers in the hippocampus of young and aged rats. Also, it is unclear whether a short-term burst of insulin in a normal brain may impact microglial profile and would contribute to memory performance in a hippocampal dependent task. We therefore injected insulin directly into the brain of young and aged rats to uncover microglial proliferation, proinflammatory and memory outcomes.

2. Materials and Methods

2.1 Animals

Experiments were performed using 3 and 22 month-old male Wistar rats under approval of the Local Ethic Committee for Animal Experiment (CEUA-UFRGS) project number: #26376. The 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.

2.2 Surgery

Intracerebroventricular insulin injections were administrated through a cannula that was implanted in the fourth ventricle by stereotaxic surgery. Rats were anesthetized using intraperitoneal (i.p.) ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and placed in stereotaxic equipment (Insight, São Paulo, Brasil). 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 thereafter the i.c.v. treatment with insulin was started.

2.3 Experimental Design

In both experimental protocols performed: (i) Short-term protocol and (ii) Long-term protocol all animals received intracerebroventricular Insulin 20mU (Humulin R®; Lilly) or vehicle (NaCl 0.9%) once a day for five consecutive days.

i) Short-term protocol: one day after the last insulin treatment, both young and aged animals were euthanized and the brain samples were collected for further assays. Detailed short-term protocol experimental design is graphically represented in the figure 1A.

ii) Long-term protocol: one day after the last insulin treatment, young animals were submitted to 8 days Morris Water Maze behavioral test to evaluate spatial memory. Twenty-four hours after the test phase day, the animals were euthanized and brain samples were collected. Detailed long-term experimental design is graphically represented in the figure 1B.

2.4 Immunohistochemistry

Immunohistochemistry was conducted as previously described (Haas et al., 2016). After euthanasia, brains were collected to perform fluorescent staining in the hippocampus in both short (Figure 1A) and long-term (Figure 1B) protocols. After the brains fixation in 4% paraformaldehyde, in 0.1 M phosphate buffer, at pH 7.4 for 24 h, they were transferred to a 30% sucrose solution in PBS. Brains were cut into coronal sections (50 μm) with a Vibratome (Leica) and stored in PBS 0.1 M with sodium azide 0.1 % at 4° C. 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 Iba1 (1:1000; WAKO; #019-19741) and CD68 (1:500; AbD Serotec; #MCA341R) were incubated overnight to stain microglia markers, followed by the secondary antibodies Alexa Fluor 594/488 (1:1000) (Invitrogen) for 2 h at room temperature. Nuclei marker DAPI (0.0001%) (Santa Cruz, Dallas, USA) was used

for 25 min at 4° C. The cells quantification was performed by six slices per animal using confocal microscopy (Olympus FV1000, Shinjuku, Japan) and analyzed using Image J® software (Rockville, USA). Cells were counted from 3 region of interests (ROIs) (100 μm^2)/image. The average of cell counts by the 3 ROIs per slice was multiplied by 100, and the result was expressed in cells/mm^2.

2.5 Western Blot

Animals were euthanized and the right hippocampi were collected to perform a Western Blot assay according to (Haas et al., 2016) with some modifications. Each hippocampus was homogenized in a lysis buffer (EDTA 2 mM, 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). Supernatant was collected and protein was diluted in a sample buffer (10 % SDS, glycerol 40 % and bromophenol blue 0.25%; Sigma, St. Louis, USA) to a final concentration of 1 µg/µL. 10-50 µg of protein was separated by weight in a 10 % polyacrylamide gel and electro-transferred to nitrocellulose membranes (GE, Little Chalfont, UK). Blocking procedures were performed according to the primary antibodies datasheets, provided by correspondent manufacturers. Next, primary antibodies COX-2 (1:1000; Cayman; #160112), p-Nf-κB^{Ser468} (1:1000; CST; #3039s), Nf-κB (1:1000; CST; #4764s) were incubated overnight followed by 2 h incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000) (GE, Little Chalfont, UK) secondary antibodies. Imunodetection was made by chemoluminescence using Image Quant LAS 4000 (GE, Little Chalfont, UK) and the protein expression was quantified using Image J® software (Rockville, USA).

2.6 ELISA Assays

Cytokines IL-1β (Sigma; #RAB0278) and IL-6 (Sigma; #RAB0311) were measured in the hippocampus homogenates (prepared using the same protocol described in the Western Blot section) using ELISA commercial kits. 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.

2.7 Morris Water Maze (MWM) Task

After treatment with insulin, young rats submitted to the long-term protocol had their spatial memory evaluated by Morris Water Maze task that was performed accordantly with (Haas et al., 2016; Muller et al., 2011). A black circular swimming pool was used (200cm diameter) with controlled water temperature (21± 1°C). Spatial cues were provided through black geometric signals that were hung on the white walls. The test consists of two phases: training and test. During the training phase (7 days) spatial memory acquisition was evaluated by challenging the animals to find a hidden platform submerged 1 cm below the water level in a fixed location. Each animal was exposed to 4 trials/day with a minimum interval of 12 min.; animals had a maximum of 60 s to find and 20 s to rest on the platform. When animals failed to find the platform, within 60 s, they were placed there by the operator and allowed to rest for the same 20 s. Twenty-four hours after the training phase animals were submitted to the test phase. During the test phase, spatial memory retention was evaluated by quantification of the time spent in the quadrant where the platform had been previously positioned. Videos of both training and test phases were analyzed by Any-Maze® software (Stoelting). Twenty-four

hours after the end of the MWM task animals were euthanized and brain samples were collected for further analysis.

2.8 Statistical Analysis

Data collected from the short-term protocol (4 groups; 2 variables – age and treatment) were analyzed by two-way Analysis of Variance (ANOVA) followed by multiple comparisons Tukey *post hoc* test. The data collected from the long-term protocol were analyzed using three different strategies: (i) to analyze MWM data, we performed the repeated measurements two-way Analysis of variance (ANOVA) followed by multiple comparisons Tukey *post hoc* test; (ii) when 3 groups were analyzed we used one-way Analysis of Variance (ANOVA) followed by multiple comparisons Tukey *post hoc* test, and (iii) when 2 groups were analyzed unpaired Student's t-test was used. Data were presented as mean ± S.E.M. p values < 0.05 were considered statistically different. Detailed significant p values can be found in the respective figure legends.

3. Results

3.1 Insulin triggers microglia proliferation and activation in the hippocampus.

In order to investigate if microglia have their number and activation affected by insulin administration we evaluated Iba-1 positive cells counts in the hippocampus of young and old rats submitted to the short-term protocol (Figure 1A). A representative image is depicted in the (Figure 2B). Similarly to (Cerbai et al., 2012) we found that aged rats had significant less microglia number in CA1 and CA3 subfields of hippocampus when compared to young animals (Figure 2B and 2C). Insulin administration significantly increased the microglia

number in CA3 area of young and CA1/CA3 areas of aged rats hippocampus (Figure 2B and 2C). There were no significant changes in the microglia number in the DG of young or aged animals (Figure 2D).

To evaluate microglia activation in the hippocampus we performed Iba-1 and CD68 co-staining and quantified the cells that have both proteins co-localized (Figure 2A). Aged rats showed significant increased microglial activation in CA1, CA3 and DG compared to young rats (Figure 2E-G). Previous works already showed such age-related profile (Ojo, Rezaie, Gabbott, & Stewart, 2015; Solano Fonseca et al., 2016). Insulin significantly increased microglial activation in the CA1 (Figure 2E) and CA3 (Figure 2F) subfields in young animals compared to their vehicle counterparts. Insulin did not cause significant activation of microglia in aged animals (Figure 2E-G).

3.2 Insulin increases NF-κB and COX-2 expression in the hippocampus of young rats.

Since NF-κB and COX-2 are considered classical neuroinflammatory markers (Kaltschmidt, Widera, & Kaltschmidt, 2005) we evaluated their expression levels in young and aged rats after the short-term protocol (Figure 1A). We found that total NF-κB level was increased whilst the phosphorylation at Ser^{468} was decreased in aged rats compared to young (Figure 3A, 3B and 3D). Insulin treatment did not significantly affect total NF-κB expression (Figure 3A, 3B and 3D) but decreased the phosphorylation status at Ser^{468} only in young rats (Figure 3B and 3D). Similarly, insulin significantly increased COX-2 in young rats (Figure 3C and 3D).

3.3 Insulin increases hippocampal concentrations of IL-1β in young rats and decreases IL-6 in aged rats.

Next, we also investigated the concentrations of IL-1β and IL-6 in hippocampal homogenates of young and aged rats after the short-term protocol (Figure 1A). Both cytokines IL-1β and IL-6 are involved in learning and memory formation, and proinflammatory signaling (Donzis & Tronson, 2014). Our results demonstrated that insulin significantly increased IL-1β levels in young (Figure 4A) while decreased IL-6 levels in aged rats (Figure 4B).

3.4 Insulin improves spatial memory and increases microglial proliferation/activation in the hippocampus of young rats submitted to the long-term protocol.

We next tested; i) whether the increased concentrations of hippocampal proinflammatory markers as well as the microglial adaptations caused by five days insulin administration in young animals were transitory or prolonged, and ii) additionally, we tested whether inflammatory adaptations increases the performance of young rats in a spatial learning and memory task (please see Figure 1B). Five days of i.c.v. insulin significantly decreased the latency to find the platform at day 7 of the training session (Figure 5A) and increased the time spent in the target quadrant in the test day (day 8) (Figure 5B). This finding confirmed that insulin increases spatial memory in young rats as previously demonstrated by our research group and others (Adzovic et al., 2015; Haas et al., 2016). Insulin increased microglia number in the CA1 and CA3 compared to vehicle (Figure 5C-E) while a significant activation was observed only in the CA3 subfield (Figure 5H). It seems that the effects of insulin on number/activation of microglia may last some days (8 days) but is likely hippocampal area-dependent. Also, insulin did not cause significant increase in the microglia number/activation in the DG (Figure 5F and 5I).

3.5 Insulin increases COX-2 expression in the hippocampus of young rats submitted to the long-term protocol.

The total NF-κB, p-NF-κB^{Ser468}, COX-2, IL-1β, IL-6 levels were re-assessed in total hippocampal tissue homogenates after the long-term protocol experiments (Figure 1B). The effects of insulin on NF-κB expression and p-NF-κB^{Ser468} did not persist after 8 days of the insulin last day administration (Figure 6A, 6B and 6D). However, COX-2 immunocontent remained increased in consequence of previous insulin administration compared to vehicle (Figure 6C and 6D).

Intriguingly, both IL-1β (Figure 6E) and IL-6 (Figure 6F) levels of young rats submitted to long-term protocol were increased after the MWM task when compared to young rats submitted to the short-term protocol. However, after the long-term protocol, insulin did not cause significant changes on both IL-1β and IL-6 levels when compared to young vehicle group (Figure 6E and 6F).

4. Discussion

In this work we showed that five days of i.c.v. insulin in rats caused short- and long-term increase in microglial activation in the CA3 subfield, expression levels of IL-1β and Cox-2 that facilitates memory performance in the classical paradigm of MWM task. At short-term, following five days of insulin challenges, aged rats were not able to orchestrate similar increments as did young animals, which may be accounted to the previously described aged-associated spatial memory deficits.

Spatial memory deficit is a common phenotype displayed by aged rats and although the mechanisms behind such deficit have multiple components, an important challenge has

been to unravel signals able to integrate this broad variety of contributory factors. The insulin/IGF-1 system, which is now recognized to work similarly in the peripheral and CNS tissues, serves as a hub for several physiological roles underlying cell survival (Bruning et al., 2000). For instance, it was recently demonstrated that IRs in astrocytes control the access to and utilization of glucose by POMC neurons of hypothalamus (Garcia-Caceres et al., 2016). Also, we have demonstrated that IRs trough downstream PI3K pathway regulate mitochondrial production of hydrogen peroxide by brain synaptosome and neuronal cultures (Muller et al., 2013). Together, these findings bring into light an integrative functional cellular model in which beyond neurons, non-neuronal cells particularly glial cells are also under the influence of insulin signaling (Steculorum et al., 2014). Remarkably, decreased insulin/IGF-1 signaling, coupled with increased inflammation were nominated as active participants in the aged-related abnormalities (Lopez-Otin et al., 2013; Steculorum et al., 2014). Altogether, increased proinflammatory phenotype, type-2 diabetes mellitus and AD are archetypal of the integration between peripheral and central of insulin resistance. Besides, insulin/IGF-1 signaling seems to integrate peripheral and central immune signals (De Felice & Ferreira, 2014).

An important concern regarding age-associated alterations is the propensity to develop cognitive deficits and neurodegenerative disorders. However, the behavioral phenotypes and neurological symptoms merely represent an end stage of the accumulation of cellular damage overtime. The key question has been to identify neurobiological markers of subtle brain alterations in the prodromal phase (Jack et al., 2013). It was demonstrated that brain insulin resistance, illustrated by the increased phosphorylation of IRS-1^{Ser616} and IRS-2$^{Ser636/639}$ in the hippocampus of postmortem patients, precedes cognitive deficits and is not necessarily related with type-2 diabetes mellitus (Talbot et al., 2012). Based on the belief that early neurobiological alterations may provide the clues for mechanistic insights, we targeted basal

neuroinflammation of rats challenged with insulin. Therefore, we injected insulin i.c.v. and look at the microglia number/activation and expression of molecular markers of inflammation to detect distinct responses in the hippocampus of young and aged rats. Because we demonstrated that insulin i.c.v did not increase spatial memory of aged rats (Haas et al., 2016) we tested only young rats in the MWM task.

The present results show a potential direct modulation of insulin in the microglia of the hippocampus. At resting state, we showed that the microglia number is lower in aged compared to young rats; meanwhile the activation status is higher in aged rats. After five days of insulin challenges, activated microglia were increased only in young rats. Remarkably, the effects of aging and insulin on microglia number/activation are likely dependent of IRs present in microglia. However, considering that IGF-1 receptor, a cognate of IR, is expressed in microglia as well as microglia scan their synaptic domains and sense changes in neuronal activity, both conjectures are plausible (Kettenmann, Kirchhoff, & Verkhratsky, 2013). Actually, the effects of insulin on microglia were different among the hippocampal subregions CA1, CA3 and DG. This finding can be explained by the fact that microglia have different subpopulations with diverse features in both young and aged brains (Grabert et al., 2016; Hanisch, 2013). Actually, the neurons of the hippocampal subregions also have heterogeneous populations that probably connects with microglia expressing specific proteins (Lee, Yoganarasimha, Rao, & Knierim, 2004). In addition to the short-term microglial responses to insulin, which was particularly more prominent in the CA3 (increased number and activation), young rats also displayed a parallel increase in the hippocampal tissue expression of the pro-inflammatory markers total Nf-κB, COX-2, and IL-1β. Intriguingly, insulin did not influence the expression of COX-2 and IL-1β in aged rats. Remarkably, there was a prolonged increase in the microglia number/activation in the CA3 subfield, and in the expression levels of COX-2 and IL-1β, that parallels with increased spatial memory performance. Although, we were not

able to demonstrate that these proinflammatory markers increase exclusively due to microglia activation, seminal conceptual works now recognize the importance of microglia in shaping synapse of normal brain thus enabling synaptic connectivity and normal memory function (Kettenmann et al., 2013). Therefore, it seems that insulin is necessary for a proper microglia functioning, and in opposite; decreased microglia responsiveness to insulin may result in cognitive deficits.

Actually, for years it was assumed that microglial proliferation and activation, as well as the expression of pro-inflammatory interleukins, such as IL-1β and IL-6, could be only detrimental for the brain, always acting as toxic signals designed to kill cells (Biber, Owens, & Boddeke, 2014). However, that concept is outdated and many studies have shown that neuroinflammation is primarily beneficial to the brain structure and function. Even in a pro or anti – inflammatory state, this complex mechanism aims to promote CNS homeostasis (Biber et al., 2014). Microglia are a highly ramified cell type that surrounding synapses, supporting the ideal microenvironment for the cellular network that is critical for the healthy functioning of the CNS (Wu, Dissing-Olesen, MacVicar, & Stevens, 2015). The present findings reinforce the interplay between insulin signaling and glial cells mediating brain functions. As stated before, insulin signaling in astrocytes has already been shown (Garcia-Caceres et al., 2016; Garwood et al., 2015), but the literature concerning microglia is still quite restricted (Mamik et al., 2016). In reality, it is still unclear whether normal insulin signal acts directly in the microglia of young rats, or alternatively insulin orchestrates the activity of neurons, astrocytes and microglia at individual synapses to produce a normal memory function.

Currently, there is increasing interest relative to learning and memory mechanisms and neuroinflammatory signaling. Indeed, pro-inflammatory interleukins, such as IL-1β, was demonstrated to exert key roles in learning and memory processing (Donzis & Tronson, 2014;

Goshen et al., 2007). Conversely, Adzovic and colleagues reported that 28 days i.c.v. insulin treatment displayed anti-inflammatory effects against LPS-induced pro-inflammatory outcomes in young rats (Adzovic et al., 2015). In contrast, here insulin showed prominent pro-inflammatory effects that pave the way to facilitate memory processing, e.g, increase neuronal/synaptic plasticity and neurogenesis (Aimone, Deng, & Gage, 2011), that are missed during the course of aging.

In conclusion, insulin signaling caused short-term and prolonged microglia activation in the CA3 subfield of young rats' hippocampus. Insulin, also, increased the expression of pro-inflammatory markers NF-κB, COX-2, and IL-1β in the young brain. Aged rats were not able to react to insulin in the same manner in terms of microglia activation and pro-inflammatory markers. Therefore, the insulin signaling is disrupted in aged hippocampus and appears to affect the pro-inflammatory effects induced by IR, and finally the capacity of aged brain to process new memories.

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Figure Legends:

Figure 1. Experimental design diagram. (A) Graphical representation of the short-term experimental protocol (9 days). On the day 0 both young (n=20) and aged (n=18) animals had the cannula implantation by stereotaxic microsurgery and were recovered for 3 days. After the post-operative recovery, on the 3rd day, intracerebroventricular (i.c.v.) insulin (20 mU) injections were daily performed for 5 days. Twenty-four hours after the treatment period animals were euthanized and brain samples were collected to perform immunohistochemistry for ionized calcium-binding adapter molecule 1 (Iba1) and Cluster of Differentiation 68 (CD68) proteins, Western Blot for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), phosphorylated NF-κB Ser^{468} and Cyclooxygenase 2 (COX-2) proteins, and ELISA assays for detection of Interleukins 1β (IL-1β) and Interleukin 6 (IL-6). (B) Graphical representation of long-term experimental protocol (17 days). On the day 0 young animals (n=12) had the cannula implanted by stereotaxic microsurgery and recovered for 3 days. After the post-operative recovery, on the 3rd day, daily intracerebroventricular (i.c.v.) insulin (20 mU) injections were performed for a period of 5 days. Twenty-four hours after the treatment period animals were submitted to Morris Water Maze (MWM) task. Animals were initially submitted to 7 days of the MWM training phase, and tested in the subsequent day (15th day) the test phase was performed. Twenty-four hours after the MWM test phase the animals were euthanized and brain samples were collected to perform Immunohistochemistry (Iba1/CD68), Western Blot (NF-κB/p-NF-κB^{Ser468}/COX-2) and ELISA (IL-1β/IL-6).

Figure 2. Insulin triggers microglia proliferation and activation in the hippocampus. (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 hippocampal sub regions Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3), and Dentate Gyrus (DG); Scale bar = 25μm. (B) Microglia (Iba1+ cells) counts/mm^2 in the CA1 (n=6-8 animals per group); aged animals have less microglia number compared to young vehicle animals (#; p=0.0385); there is no significant statistical difference (p>0.05) between young vehicle and young insulin groups; there is no significant difference (p>0.05) between aged vehicle and aged insulin groups. (C) Microglia (Iba1+ cells) counts/mm^2 in the CA3 (n=6-8 per group); aged animals have less microglia number compared to young vehicle animals (##; p=0.0016); insulin increased the number of microglia in both young (*; p=0.0390) and aged animals (*; p=0.0443). (D) Microglia (Iba1+ cells) counts/mm^2 in the DG (n=6-8 animals per group). There is no statistical difference in the number of microglia in DG between groups (p>0.05). (E) Microglial activation (Iba1+CD68+ cells counts/mm^2/Iba1+ cells counts/mm^2) in CA1 (n=4 animals per group). Aged animals have significantly more activated microglia in CA1 than young vehicle animals (##; p=0.0012), and insulin increases microglial activation in young animals (*; p=0.0226); there is no statistical difference between aged vehicle and aged insulin (p>0.05). (F) Microglial activation (Iba1+CD68+ cells counts/mm^2/Iba1+ cells counts/mm^2) in CA3 (n=4 animals per group). Aged animals have more activated microglia in CA3 than young vehicle animals (#; p=0.0420), and insulin increases microglial activation in young animals (**; p=0.0019); there is no statistical difference between aged vehicle and aged insulin groups (p>0.05). (G) Microglial activation (Iba1+CD68+ cells counts/mm^2/Iba1+ cells counts/mm^2) in DG (n=4 animals per group). Aged animals have more activated microglia in DG than young vehicle animals (##; p=0.0022), and insulin did not significantly increase microglial activation in the DG neither in young, nor in aged animals (p>0.05). Results are expressed as mean ± SEM.

Figure 3. Insulin increases NF-κB and COX-2 expression in the hippocampus of young rats. (A) Western blot detection of total NF-κB expression (expressed in % of control) normalized by β-Actin in the young and aged animals' hippocampi homogenates after short-term protocol procedure (n=6 animals per group). NF-κB expression is increased in aged animals when compared with young vehicle (#; p=0.0394); insulin does not affect total NF-κB expression in both young and aged rats (p>0.05). (B) Western blot detection of p-NF-κB^{Ser468} (expressed in % of control) normalized by NF-κB in the young and aged animals' hippocampi homogenates after short-term protocol procedure (n=6 animals per group). pNF-κB^{Ser468} is decreased in aged animals when compared with young vehicle animals (#; p=0.0209); insulin decreased pNF-κB^{Ser468} only in young animals (*; p=0.0196). (C) Western blot detection of COX-2 expression (expressed in % of control) normalized by β-Tubulin in the young and aged animals' hippocampi homogenates after short-term protocol. Insulin increased COX-2 expression in only in young animals (**; p=0.0033). (D) Representative images of western blot membranes with p-NF-κB^{Ser468}, total NF-κB, COX-2, β-Actin and β-Tubulin respective imunodetection. Results are expressed as mean ± SEM.

Figure 4. Insulin caused distinct effects in the hippocampal concentrations of IL-1β and IL-6 in young and aged rats. (A) ELISA analysis of interleukin 1β (IL-1β) in young (n=7-10 animals per group) and aged (n=7-10 animals per group) Wistar rats treated with vehicle/insulin 20mU, 5days, after the short-term protocol. Insulin increased IL-1β in young rats (*; p=0.0479); there were no differences between young and aged groups, or aged vehicle and aged insulin groups (p>0.05). (B) ELISA analysis of interleukin 6 (IL-6) in young (n=5-6 animals per group) and aged (n=5-6 animals per group) Wistar rats treated with vehicle/insulin 20mU, 5days, after the short-term-protocol. Insulin decreased IL-6 only in aged rats (#; p=0.0309); there were no significant differences between young and aged

groups, or young vehicle and young insulin groups ($p>0.05$) Results are expressed as mean ± SEM.

Figure 5. Previous insulin injections into the brain cause long-term effects on spatial memory and microglia number/activation in young rats. (A) In the last day of the training phase, young animals treated with insulin significantly decreased the latency to find the platform (n=6-8 animals per group) compared with young vehicle group (*; $p=0.0439$). (B) In test phase, insulin increased the time young rats spent in the target quadrant (*; $p=0.0203$) (n=6). (C) 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 Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3), and Dentate Gyrus (DG); (n=4) Scale bar = 25μm. (D) Microglial (Iba1+ cells) counts/mm^2 in the CA1 (n=4 animals per group); insulin positive effects on microglial number were still detected 8 days after treatment (*; $p=0.0286$). (E) Microglial (Iba1+ cells) counts/mm^2 in the CA3 (n=4 animals per group); insulin effects on microglial number were still detected 8 days after treatment (*; $p=0.0273$). (F) Microglial (Iba1+ cells) counts/mm^2 in the DG (n=4 animals per group); insulin had no significant effects on microglial number ($p>0.05$). (G) Microglial activation (Iba1+CD68+cells counts/mm^2/Iba1+ cells counts/mm^2) in CA1 (n=4 animals per group). Insulin effects on microglial activation were not detected 8 days after treatment ($p>0.05$). (H) Microglia activation (Iba1+CD68+ cells counts/mm^2/Iba1+ cells counts/mm^2) in CA3 (n=4 animals per group). Insulin effects on microglial activation were still detected after 8 days treatment in CA3 (*; $p=0.0159$). (I) Insulin had no significant effects on microglial activation in DG (n=4 animals per group)($p>0.05$). Results are expressed as mean ± SEM.

Figure 6. Long-term effects of insulin on neuroinflammatory markers in young hippocampus. (A) Western blot detection of NF-κB expression (expressed in % of control) normalized by β-

Actin in the young animals' hippocampus (n=6 animals per group) homogenates after the long-term protocol. The insulin effect on NF-κB expression was not observed anymore after 8 days of MWM period (p>0.05). (B) Western blot detection of p-NF-κB^{Ser468} (expressed in % of control) normalized by NF-κB in the young animals' hippocampus (n=6 animals per group) homogenates after the long-term protocol. The insulin effect on pNF-κB^{Ser468} immunocontent was not significant 8 days after MWM (p>0.05). (C) Western blot of COX-2 expression (expressed in % of control) normalized by β-Actin in the hippocampal homogenates of young animals (n=6 animals per group) allocated to the long-term protocol. Insulin effects on COX-2 expression were still present after 8 days (*; p=0.0409). (D) Representative images of western blot membranes with p-NF-κB^{Ser468}, NF-κB, COX-2, β-Actin and imunodetection. (E) ELISA analysis of interleukin 1β (IL-1β) in young rats (n=6-7 animals per group) treated with vehicle/insulin 20 mU, 5days. Insulin effects on IL-1β were no longer observed after 8 days (p>0.05). When comparing young short-term vehicle and young long-term vehicle animals it seems that MWM task increased the IL-1β levels (***; p=0.0003). (F) ELISA analysis of interleukin 6 (IL-6) in young rats (n=6-7 animals per group) treated with vehicle/insulin 20mU, 5 days. Insulin effects on IL-6 were no longer observed after 8 days (p>0.05). When comparing young short-term vehicle and young long-term vehicle animals we observed significantly increased IL-6 levels (**; p=0.0096).

Figure 1. Experimental design diagram.

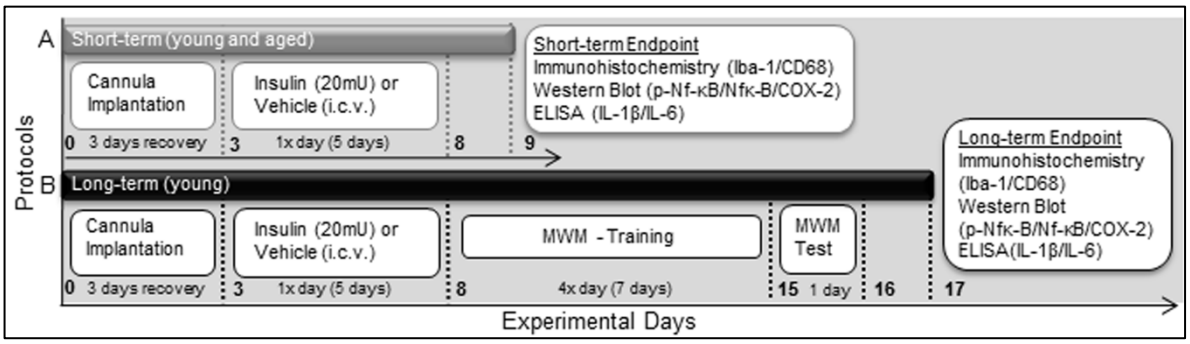
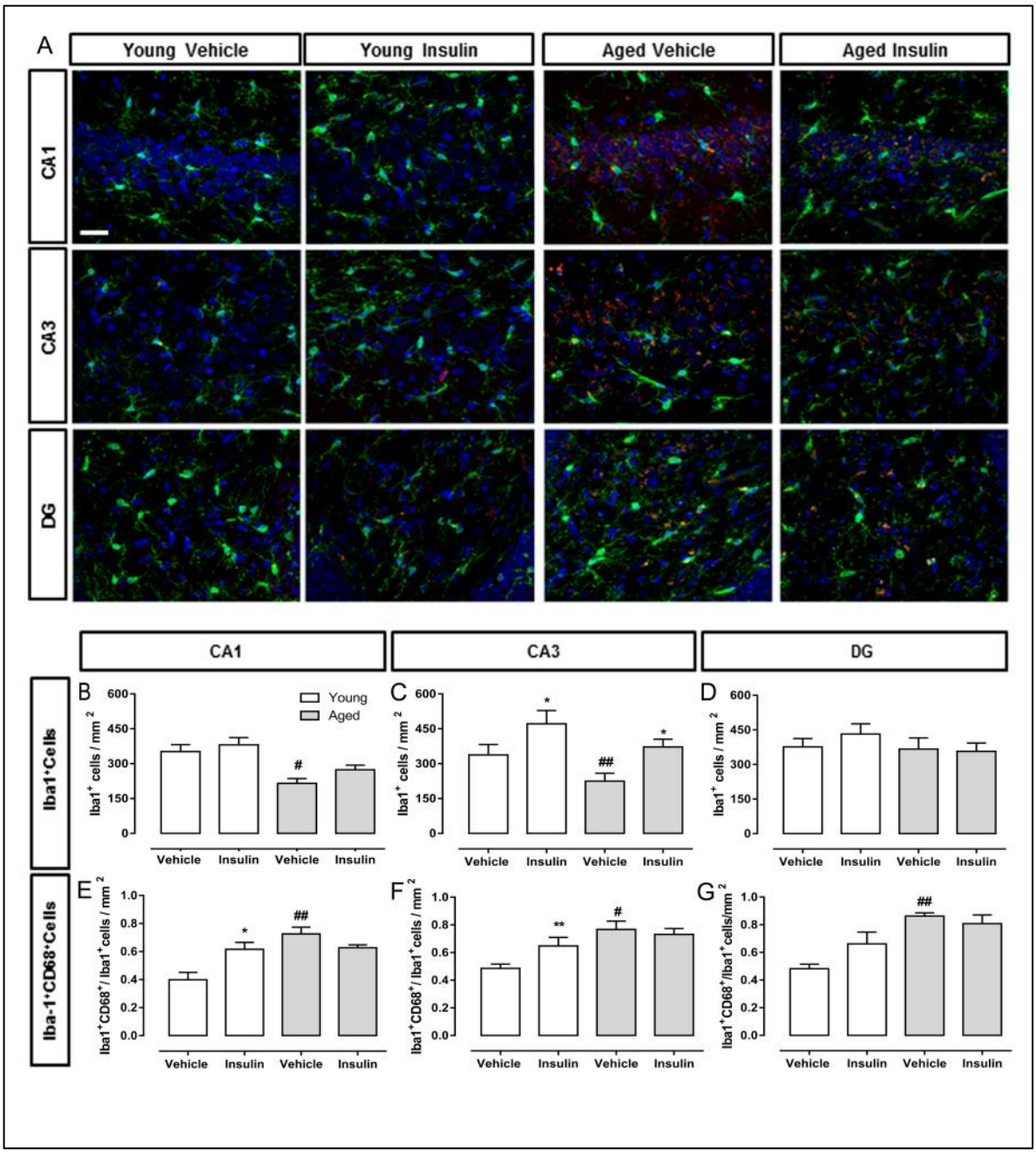


Figure 2. Insulin triggers microglia proliferation and activation in the hippocampus.



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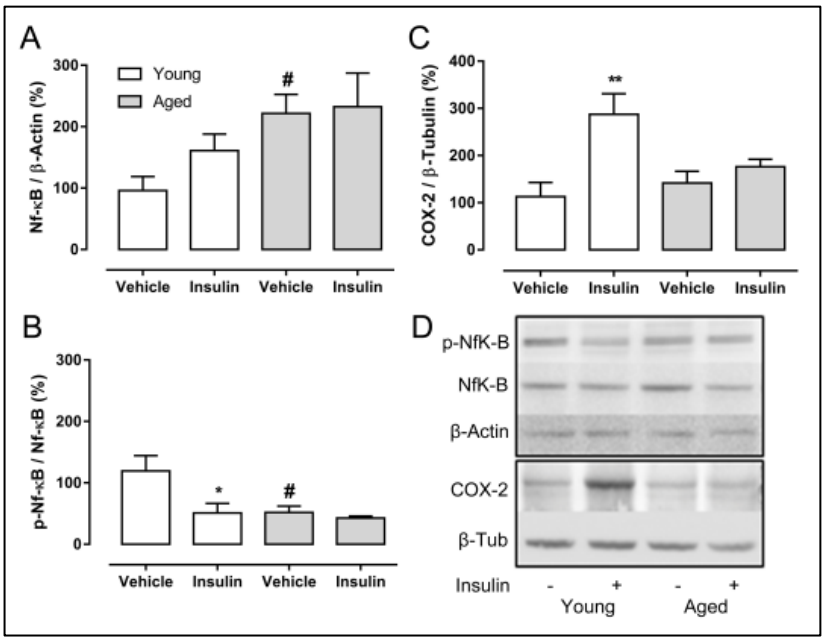


Figure 4. Insulin caused distinct effects in the hippocampal concentrations of IL-1β and IL-6 in young and aged rats.

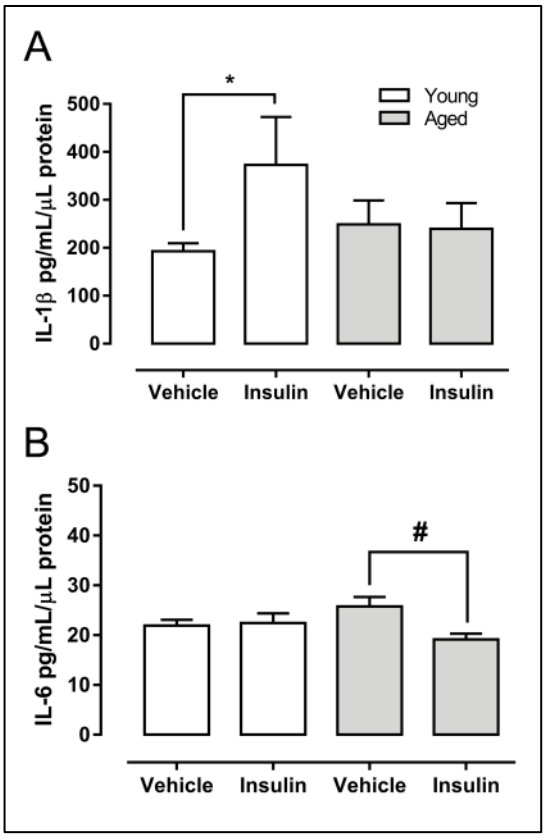


Figure 5. Previous insulin injections into the brain cause long-term effects on spatial memory and microglia number/activation in young rats.

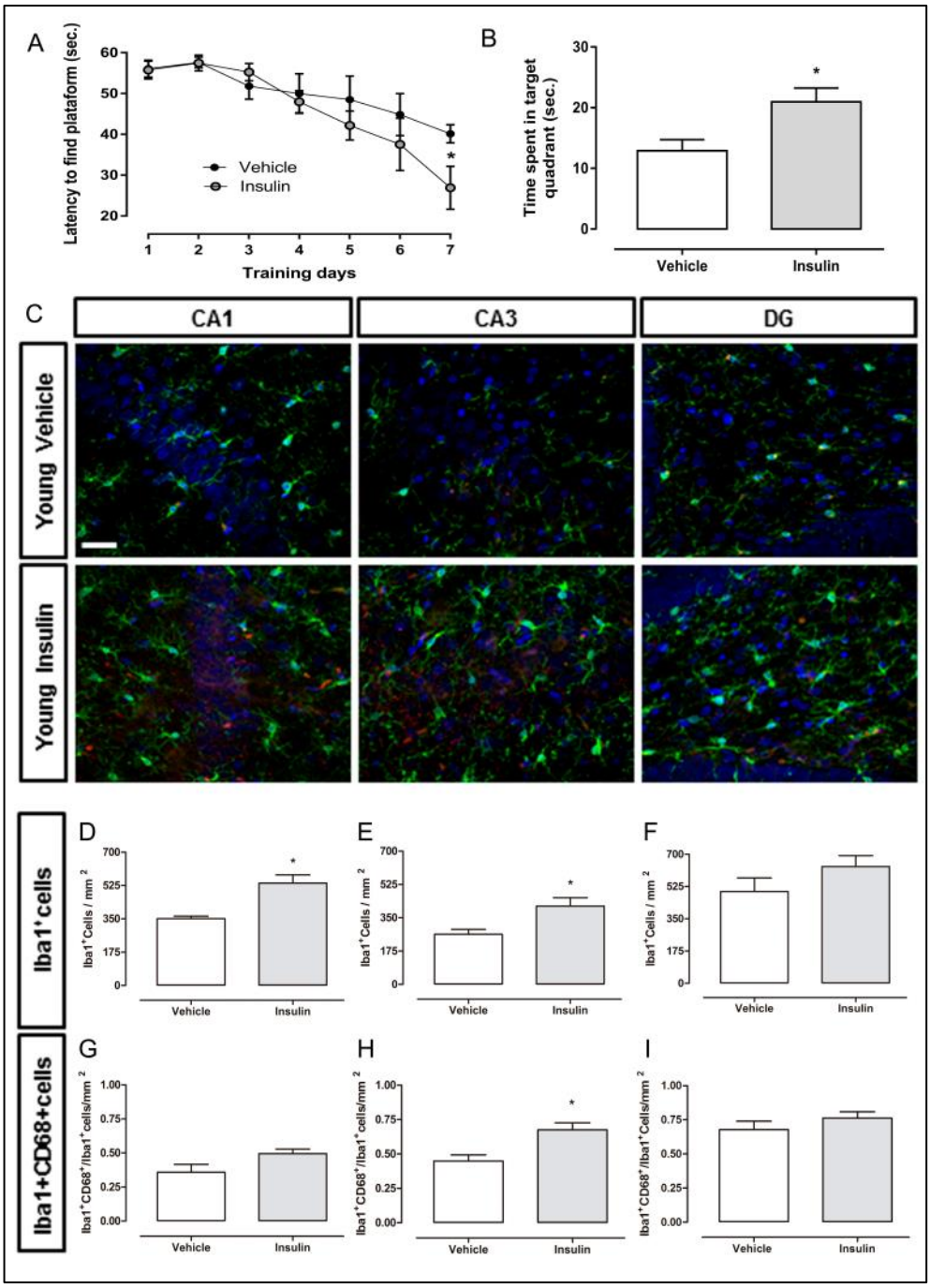
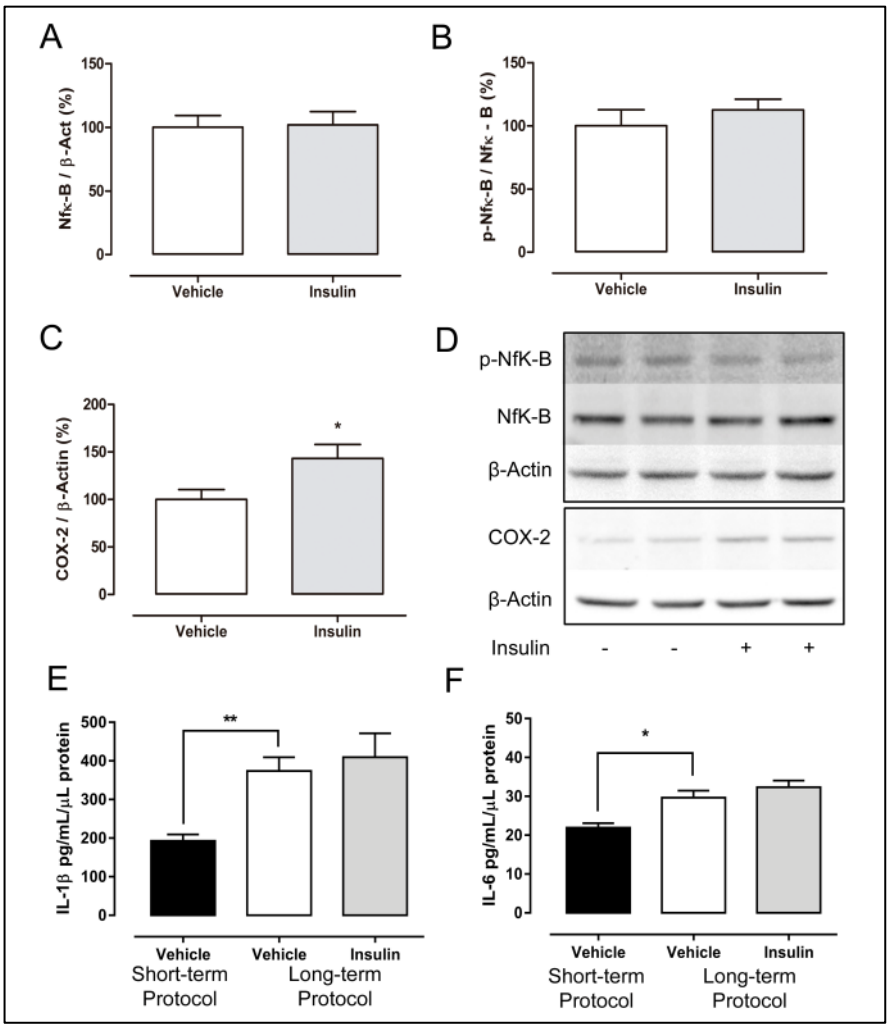


Figure 6. Long-term effects of insulin on neuroinflammatory markers in young hippocampus.



5. CAPÍTULO III

Microglial Responsiveness to Insulin: Different Insulin Effects in Newborn and Adult Microglia

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Manuscrito em preparação

Microglial Responsiveness to Insulin: Different Insulin Effects in Newborn and Adult Microglia

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Abstract

Microglia are the brain-specific macrophages and there is a consensus that they are a unique type of macrophage, with specialized roles that go beyond neuroimmunology. Insulin is a hormone with well-recognized functions in the brain through insulin receptors (IRs) downstream signaling proteins. Physiological IRs 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 IRs in astrocytes of hypothalamus are targeted by insulin and regulate whole body energy homeostasis. However, although IRs signaling is well described in neurons and now astrocytes, we have little information about whether it produces functional responses in microglial cells. Here we show an important conceptual findings regarding insulin signaling in microglia. We show that insulin increase Akt phosphorylation in primary microglial cells and this activation is inhibited by a classic insulin pathway inhibitor Wortmanin (PI3K inhibitor). We also found that insulin provides an inhibitory effect on ROS production and increases cell viability in primary microglia. We also observed that insulin triggers IL-1β production/release when the microglia were exposed to an inflammatory challenge with LPS. Conversely, an *in vivo* intranasal insulin treatment in adult mice increases genetic expression of the pro-inflammatory cytokine TNF-α, showing that insulin signaling has different roles in both newborn and adult microglia.

Key words: Insulin signaling – Microglia – Primary microglia – Intranasal insulin – ROS

1. Introduction

Central Nervous System (CNS) continuously exchanges metabolites, energetic substrates and hormones, such as insulin, with the periphery. Insulin is a hormone with well-characterized signals in the brain, mostly related to neurotrophic effects and learning and memory processing in the adult brain. In neurons, insulin acts in cooperation with IGF-I through the binding to insulin receptors (IRs) and thus activating downstream IRS-1>PI3K>AKT pathway, which in consequence activates/inactivates downstream proteins, such as GSK3-β, mTOR and FOXO (Fernandez & Torres-Aleman, 2012). These signals are necessary to regulate brain functions such as death or survival, gene transcription, protein translation and oxidative stress (Fernandez & Torres-Aleman, 2012). Alternatively to this pathway, insulin can also, act through ERK kinases promoting cell proliferation. Great progress has been done in the past 30 years regarding insulin signaling in the brain. Unfortunately, only a small part of it concerns glial cells, particularly microglia. Astrocytes express functional IRs and downstream proteins (Garwood et al., 2015) that work as glucose sensor by the regulation of brain glucose uptake in the hypothalamus (Garcia-Caceres et al., 2016). Since insulin signaling in astrocytes seems to be more related with brain energy metabolism, until the present moment, literature, in terms of microglia, has mostly reported the outcomes of insulin treatments on microglia relative to activation state and the expression of neuroinflammatory proteins markers (Adzovic et al., 2015; Mamik et al., 2016). Therefore, it is important to pursue a better understanding regarding a possible IRs signaling in microglia and the meaning of this signal to microglial physiology.

Although microglia are the brain-specific macrophages, it is a consensus nowadays that they are a unique type of macrophage, with specialized roles that go beyond neuroimmunology. Microglia behavior is dependent on how the molecular machinery of the

cells, in the brain, sense and adapt themselves to the environmental challenges, working to promote brain homeostasis (Aguzzi, Barres, & Bennett, 2013). A transcriptome study reported that both mouse and human microglia have a specific molecular signature that distinguishes microglia from peripheral monocytes and different types of specific tissue-macrophages (Butovsky et al., 2014). Interestingly, microglia varies its functional patterns in newborn and adulthood. In the first days of life, brain is still under extensive maturation and synaptic pruning is highly activated (Schafer et al., 2012), microglia are highly recruited amoeboid cells that sculpt postnatal neural circuits playing a key role in the synapses phagocytosis. Microglia, in the second postnatal week, assume a ramified morphology and, although they are still involved in synaptic pruning, other functions may arise from this morphology and thus microglia adapt themselves to even subtle changes in the microenvironment changing their expression of proteins and projections, and also their physiology (Schafer et al., 2012; Stephan, Barres, & Stevens, 2012; Stevens et al., 2007).

The microglial adaptions to the micro-environmental challenges during life, maintains a key characteristic of these cells which is a highly phagocytic cell-type mostly seen in an activated state (Fu, Shen, Xu, Luo, & Tang, 2014). Phagocytosis is a Reactive Oxygen Species (ROS)-dependent process since the phagosome, that contains the engulfed particle that needs to be removed, is digested by ROS. The innate immune cells (monocytes and macrophages) have their main sources of ROS in the NADPH oxidases enzymes (mostly localized in the cell membrane) and in the mitochondria (Bordt & Polster, 2014). Indeed, the high efficiency of ROS production is important not only for eliminating pathogens but also for synapses and cellular debris phagocytosis purposes (Biswas & Mantovani, 2012). Besides the role in phagocytosis, a complex signaling pathway may be regulated by ROS in microglia, such as the induction of Nf-κB translocation from the cytosol to the nuclei, where it acts as a

second messenger for cytokines responses, such as IL-1β, IL-6 and TNF-α (Biswas & Mantovani, 2012).

Given the majority of neural cells are sensitive to neuroendocrine signaling from the periphery, and the fact that insulin may integrate various signals and functional properties, in the present study we addressed whether primary microglia from mice were responsive to insulin challenges. We also assessed both postnatal and adult microglia inflammatory outcomes. In the primary microglia (postnatal), we investigated insulin effects on H_2O_2 related to cell viability/NADPH oxidase activity and interleukins (IL-1β, IL-6 and TNF-α) expression. Also, in the adult microglia we investigated insulin effects on gene expression of neuroinflammatory markers (IL-1β, IL-6, TNF-α, IL-10, Arg-1 and COX-2).

2. Material and Methods

2.1 Animals

Male and female C57Bl/6 mice pups (P0 - P2) and C57Bl/6 adult mice (3 - 4 months) were used to perform *in vitro* and *in vivo* experiments. All experiments were performed according to the experimental animal guidelines of the University of Groningen, The Netherlands. When applicable, the animals were housed in groups under standards species conditions in a 12/12h light/dark cycle with food and water available ad libitum.

2.2 Primary Microglia Culture

Primary neonatal microglia culture was performed according (Schaafsma et al., 2015) with modifications. C57Bl/6 mice of postnatal day 0-2 mice (female and male pups) were sacrificed by decapitation and the brains were removed from the skull and maintained in dissection medium – 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 dissected and the forebrain was minced and washed once in dissection medium. Next, a chemical dissociation step was performed where the minced tissue was incubated in 1x DNAse (Sigma, St. Louis, USA) - 2.5 % trypsin (Lonza) - dissection medium, during 25 min, 37 ºC, 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, USA) – dissection medium to the tissue followed by a quickly washing using 10% FCS – 1x DNAse - dissection medium. Then, a mechanical dissociation was performed in the same medium (10% FCS – 1x DNAse - dissection medium) using glass pipette until a cell suspension was obtained. The cell suspension, afterwards, was washed in 25 mL of microglia medium (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), 100 U/mL Penicillin-100 mg/mL Streptomycin) and centrifuged (10 min.; 960 rpm; 12 ºC). The cell pellet was re-suspended in microglia medium and plated in T75 culture flasks (1.5 brains/flask) that where cultured at 37 ºC in a humidified atmosphere at 5% CO_2. 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 in order to stimulate microglia proliferation. L929 fibroblast-conditioned medium contains M-CSF that is obtained by addition of normal microglia medium (without 1 mM sodium pyruvate) to 80% confluent L929 cells (L-929 cell line; ATCC, Manassas, USA), 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 ºC). 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. The microglial purity of the primary cell culture preparations was accessed by Western Blot for Iba-1 (Supplementary Figure 1).

2.3 Microglia Treatments

All experiments with primary microglia were performed using Insulin from bovine pancreas suitable for cell culture (Sigma). Cells were always used after a maximum of 3 days after the shake off.

For the experiments conducted to test microglial sensitivity to insulin (Figure 1), 3 types of treatment were performed: (i) 30 min Ins + FBS: simple 30 min incubation in normal 50:50 medium containing 10% FBS and LPS (Sigma) 100 ng/mL was added 3h in normal 50:50 medium containing 10% FBS as positive control, (ii) 30 min Ins - FBS: primary cells

that were cultured in 50:50 medium had their medium changed for a microglia medium without 10% FBS during 2h hours prior to the insulin incubation and, then, a simple 30 min insulin incubation was performed in the same way using the microglia medium without 10% FBS; 3 h LPS (Sigma) 100ng/mL was added as positive control, (iii) 24 h Ins + FBS: simple 30 min incubation in normal 50:50 medium containing 10% FBS and LPS (Sigma) 100 ng/mL was added 24h, as positive control, and (iiii) 30 min Ins – FBS ± Wort: primary cells that were cultured in 50:50 medium had their medium changed for a microglia medium without 10% FBS during 2 h prior to the insulin incubation. Wortmanin 100 nM (Sigma) diluted in DMSO (Sigma) 0.0001% and only DMSO 0.0001% were added 30 min prior to insulin, then, a simple 30 min insulin incubation was performed in the same way using the microglia medium without 10% FBS.

For the experiments conducted to test insulin effects in ROS production (Figure 2), 2 types of treatment were performed: (i) 3 h or 24 h insulin (1000ng/mL) incubation in normal 50:50 medium containing 10% FBS and (ii) 24 h insulin incubation in normal 50:50 medium containing 10% FBS followed or not by 30 min Apocynin (Millipore) at final concentrations of 10 μM, 100 μM and 1 mM, alternatively only Apocynin was added using the same concentrations.

For the experiments conducted to test insulin effects in cell viability and cytotoxicity (Figure 3), 24 h insulin incubation in normal 50:50 medium containing 10% FBS followed or not by 30 min Apocynin (10 μM, 100 μM and 1 mM), alternatively only Apocynin was added using the same concentrations.

2.4 Western Blot

After cellular cultivation and treatments, microglia were washed twice in PBS and a non-denaturing cell lysis buffer (CST) was used accordantly the manufacturer's datasheet. Microglia lysates (protein suspensions) were diluted in sample buffer (10% SDS (Bio-Rad); 40% glycerol; 0.25% bromophenol blue; Sigma, St. Louis, USA) and the next steps to perform western blots were performed as (Haas et al., 2015). Samples were separated by weight using SDS–PAGE electrophoresis gel and were transferred to a nitrocellulose membrane equilibrated with transfer buffer (25 mM Tris, 150 mM glycine, 20% v/v methanol). Membranes were blocked in (5%) nonfat dry milk (Bio-Rad) or (3%) BSA (Sigma, St. Louis, USA) both diluted in T-TBS (Tween-20) 2 h at room temperature. After blocking, membranes were washed 2 times with T-TBS and once with TBS and the primary antibodies p-Akt^{Ser473} (1:1000; CST; #4060), Akt (1:2000; CST; #2920), p-Erk1/2$^{Thr202/Tyr204}$ (1:1000; CST; #9101), Erk-1 (1:1000; CST; #9102), p-GSK3-β^{Ser9} (1:1000; CST; #9323), GSK3-β (1:1000; CST; #9315), β-Actin (1:5000-1:10000; Abcam; #Ab6276), IR (1:1000; CST; #3020), Iba1(1:1000; Wako; #019-19741), GFAP(1:2000; Dako; #Z033420-2), MBP(Chemicon; #AB980) and β-III-Tubulin (1:10000; Abcam; #Ab7751) were incubated overnight at 4 ºC. After primary antibody incubation, membranes were washed 3 times, as previously described, and the horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000) secondary antibodies (GE, Little Chalfont, UK) were incubated 2 h at room temperature. After 3x washing, the imunodetection of proteins of interest was made by chemoluminescence using. ChemiDocMP® imager (Bio-Rad) and protein expression was quantified by ImageLab 5.2.1® software (Bio-Rad).

2.5 Amplex Red Assay

The cellular release of H_2O_2 was assessed by the Amplex Red® oxidation method that was carried accordantly to (Muller et al., 2013) with modifications. Primary microglia were plated (20,000 cells/well of a black 96-well plate), treated and 50 μM Amplex Red and 0.1 units/mL horseradish peroxidase in Krebs-Ringer HEPES buffer (Sigma) pH 7.4. The 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 seconds during 30 min, 37ºC. 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 the basal rate.

2.6 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 ºC, 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 570 nm.

2.7 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, as described above, and, 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 µL 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 490 nm. 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.

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

2.8 ELISA

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

2.9 Adult Mice Intranasal Insulin Treatment

The intranasal (i.n.) insulin awake administration was performed accordantly (Hanson, Fine, Svitak, & Faltesek, 2013). 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. administration sessions per day. They were held with their neck parallel to the floor while a volume of 3 μl of liquid was administered each time at 45-degree angle. Insulin was slowly ejected from the micropipette forming a small droplet with about 3 μl. 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 μg) /24 μl was delivered per day divided between the two sections, 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 section) of insulin injection, and these brains were collected and microglia were isolated as described below.

2.10 Adult Mice Acute Isolation of Microglia Enriched - Cell Suspensions

The acute isolation of microglia enriched - cell suspension was performed in accordance with (Vainchtein 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-µm cell strainer (BD FALCON). Then, cells were centrifuged at 200 rcf, at 4 ºC, 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 ºC, 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 cell culture preparations was accessed by Western Blot for Iba-1 (Supplementary Figure 1).

2.11 Quantitative Real-Time PCR (qPCR)

RNA was extracted from microglia enriched - cell suspensions using the RNeasyMicro 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 RevertAidTM M-MuLV Reverse Transcriptase, RibolockTMRNase 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, PrimerExpress software or NCBI Primer-Blast and ordered from Biolegio (The Netherlands). The genetic expression was calculated by normalization to the GAPDH gene levels. See Suplemental Table 1 for primer information.

2.12 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 Analysis 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 ± S.E.M. p values < 0.05 were considered statistically different. Details of p values can be found in the respective figures' legends.

3. Results

3.1 Primary microglia is sensitive to insulin via PI3K.

Firstly, we addressed the question whether mice microglia were sensitive to a direct insulin challenge without a neuronal or astroglial insulin intermediary signaling. For that we applied different treatment protocols on primary microglia cultures followed by Western Blot. Qualitative analysis of Western Blot shows that 30 min. of insulin incubation was enough to trigger AKT phosphorylation at Ser473 (Figure 1A) or without the addition of FBS (Figure 1C) to the cell culture medium. In accordance to (Schaafsma et al., 2015), we found that 3 h of LPS 100 ng/mL increases Erk1/2 phosphorylation at Thr202/Tyr204, showing that the microglia culture is properly responsive to LPS (Figure1A). On the other hand, there was no significant effect of insulin on Erk1/2 phosphorylation at Thr202/Tyr204 or GSK3-β phosphorylation at Ser9 with 30 min. treatment (Figure 1A; 1B; 1D).

The next question was if a longer insulin treatment would change Erk1/2 or GSK3- β phosphorylation and/or total Erk1/2 or GSK3-β protein levels. There were no significant differences after 24 h incubation with insulin (Figure 1E); however, as previously described by (Schaafsma et al., 2015), we found that a 24 h LPS treatment was able to increase AKT phosphorylation at Ser473. We still checked whether insulin was acting via PI3K and performed experiments incubating insulin for 30 min with and without the PI3K inhibitor Wortmanin. We found that AKT phosphorylation was inhibited by the Wortmanin 100 nM incubation (Figure 1F and 1G) showing that primary microglia are responsive to insulin, by classical insulin pathway PI3K.

3.2 Insulin decreases ROS production in primary microglia.

Since primary microglia was responsive to insulin next we addressed the question what were the outcomes of this signal for the primary microglia cell physiology. First, we analyzed ROS production using Amplex red fluorescent dye and found that during 24 h incubation but not 3 h insulin decreased ROS production with a dose of 1000 ng/mL (Figure 2A). Since the two main sources of ROS in microglia are NADPH oxidase and mitochondria, we next tried to find out the mechanism by which insulin was regulating microglial ROS metabolism. To investigate witch target insulin was acting to decrease ROS production in microglia we used apocynin to inhibit NADPH oxidase. Apocynin was incubated in primary microglia with or without insulin. As expected, apocynin decreased ROS production when cells were exposed to 100 μM and 1mM of this inhibitor (Figure 2B). Twenty-four hours pre-incubation with insulin decreased ROS production in the same level as apocynin, but when both insulin and apocynin were added to the cell culture the ROS production did not significantly decrease (Figure 2B).

3.3 Insulin increases primary microglia cell viability.

Trying to better understand how insulin was acting to inhibit ROS production in microglia, cell viability (MTT) was performed. It was observed that 100 μM and 1 mM of apocynin alone, 24 h of 1000 ng/mL insulin alone, and 24 h pre-incubation of insulin with 10 μM and 100 μM apocynin in the last 30 min significantly increased cell viability (Figure 3A). Similar effect was not seen for insulin 24 h pre-incubation of insulin together with 1mM of apocynin, and for 30 min apocynin (10 μM) incubation. Interestingly, 30 min of 10 μM apocynin together with insulin statistically increased cell viability whereas 30 min of 10 μM apocynin alone had no significant effect. In order to evaluate if apocynin treatment was toxic for microglia, a control experiment, using LDH assay was performed. Apocynin incubated alone or with insulin was not toxic to microglia (Figure 3B).

3.4 Insulin increases IL-1β levels after a LPS challenge in primary microglia

One of the most prominent functions of microglia is the cytokines production, which is driven by many mechanisms such as ROS signaling, we investigated whether insulin was regulated H_2O_2 parameter in primary microglia. Insulin incubated for 30 min or 3 h did not cause significant differences in the concentrations of IL-6, TNF-α or IL-1β in the cell supernatant (Figures 3A-C). As positive control, LPS was incubated for 3 h or 24 h and these three cytokines significantly increased implying cells were functional (Figures 3A-F). Even a longer protocol of 24 h incubation with insulin did not increase the concentrations of IL-6, TNF-α or IL-1β (Figures 3D-F). However, when cells were challenged with LPS for 3 h after 24 h insulin pretreatment IL-1β levels were increased at levels higher than the 3 h LPS treatment alone (Figure 3D). This effect seems to be selective to IL-1β since was not observed for IL-6 and TNF-α (Figure 3E and 3F)

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

To investigate inflammatory outcomes regarding insulin signaling in the adult microglia, we used 7 days *in vivo* intranasal insulin treatment in adult mice. As previously reported by others (Chen et al., 2014; Marks, Tucker, Cavallin, Mast, & Fadool, 2009; Zhang et al., 2016), intranasal is an efficient and less invasive approach to delivery insulin directly to the brain. Seven days of awoke intranasal insulin administration was able to increase selectively TNF-α genetic expression in cell suspensions enriched in microglia (Figure 5C). A set of pro- and anti-inflammatory markers, such as IL-1β (Figure 5A), IL-6 (Figure 5B), IL-10 (Figure 5D), Arg-1 (Figure 5E) and COX-2 (Figure 5F) were also evaluated and did not show significant differences induced by the treatment. During the experimental procedure, body weight (Suplemental Figure 2A) and blood glucose (Supplemental Figure 2B) were monitored and, as expected (Ramos-Rodriguez et al., 2017), there were no significant differences in that parameters.

4. Discussion

The present study shows important conceptual findings regarding insulin signaling in microglia. With repeated measurements we were able to show that 30 min insulin increased Akt phosphorylation in primary microglial cells and this activation was inhibited when a classic insulin pathway inhibitor Wortmanin (PI3K inhibitor) is incubated together with insulin. We also shed light into the implications of this signal in both postnatal and adult microglia. Firstly, investigating ROS production and cell viability in the postnatal microglia, we found that insulin provided an inhibitory effect on ROS production, but, under the same conditions, it increases cell viability in primary microglia. It was also observed that insulin triggers an important increase in the IL-1β level when microglia were exposed to an

inflammatory challenge with LPS. Secondly, an *in vivo* intranasal insulin treatment in adult mice causes controversial effects on microglia increasing the gene expression of a pro-inflammatory cytokine TNF-α.

We previously reported significant increased hippocampal BDNF level and memory performance due to insulin administration into the brain of young relative to aged rats (Haas et al., 2016). Also, we found increased concentrations of neuroinflammatory markers in hippocampus of young but not aged rats along with increased ability to perform a spatial memory task (Haas et al., 2017 – manuscript in preparation). These findings highlight the importance of brain insulin signaling and the concept that it may be disrupted due to aging contributing to aged-associated neurological deficits. Although, it is recognized that neurons and astrocytes expresses IRs, our published work was not able to separate the effects on specific cell-types in response to insulin administration Beyond the recent finding of insulin regulating particularly astrocytic glucose uptake and metabolism concomitant with POMC neurons activity, we tried to expand the effects of insulin to the microglia function (Garcia-Caceres et al., 2016). Thus, given the literature exploring the insulin signal in microglial cells is scarce (Chen et al., 2014; Mamik et al., 2016; Spielman, Bahniwal, Little, Walker, & Klegeris, 2015), we directly challenged primary microglia cell cultures from mice with insulin and brought that microglia responsiveness to insulin is dependent of PI3K pathway, at least postnatal microglia (Figure 1).

Searching for molecular outcomes associated with microglial insulin signaling we found that insulin decreased ROS production (Figures 2A and 2B), while increased cell viability (Figure 3A). This finding agrees with a previous report from our research group that insulin decreased H_2O_2 production from mice synaptosome via PI3K pathway. 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 far lower. It is possible to speculate that insulin signal is increasing the mitochondrial ATP production via mitochondrial complex V (ATP synthase) and consequently, reducing 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, having a synergic dose-dependent effect with apocynin (Figure 2B and 3A). Further experiments are necessary to better understand what is the target of insulin implicated regarding decreasing ROS production in the postnatal microglia, since NADPH oxidase possess distinct isoforms expressed in different cell compartments, and particularly NOX4 may be expressed in the mitochondria (Nayernia, Jaquet, & Krause, 2014). Indeed when assume resting state, microglia express more NOX1 (cytosol-membrane) and NOX4 (mitochondria) than NOX2 (membrane-phagosome) (Cheret et al., 2008; Li et al., 2009), which suggests that unravel specific targets may contributes to better understand the mechanisms involved.

It was intriguing, but not totally unexpected, that we found different patterns of outcomes when we compared insulin treatments in both newborn and adult microglia. Previously, we found that 5 days of intracerebroventricularly insulin injections increased IL-1β protein expression in the hippocampus of young rats (Haas et al., 2017 – manuscript in preparation). Here we found that after 7 days of i.n. insulin injections resulted in an increased microglial gene expression of TNF-α, that is also a pro-inflammatory cytokine as IL-1β. Besides the different phenotypes that are assumed by postnatal and adult microglia that were already discussed in the introduction section, it is also important to understand that insulin has different functions in postnatal and adult brain (Fulop, Larbi, & Douziech, 2003; Ghasemi, Haeri, Dargahi, Mohamed, & Ahmadiani, 2013). Indeed it is already known that TNF-α may

represent a neuroprotective signal as well as microglia adapt themselves to the microenvironment changes during the entire lifespan (Vinet et al., 2012).

In conclusion, microglia directly respond to insulin challenges via PI3K signal as well as causes distinct response in postnatal and adult microglia enriched preparations. Whether this signal impacts neuron-to-astrocytes microglia interactions and function needs to be addressed in additional studies.

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Figure Legends

Figure 1. Microglia is sensitive to insulin via PI3K phosphorylation. Qualitative analysis of insulin pathway proteins detected by Western Blot (A) Mouse primary microglia incubated 30 min with insulin in the medium containing FBS; qualitative analysis indicate that both doses of insulin (100 ng/mL and 1000 ng/mL) increased expression of p-Akt^{Ser473}/Akt; 3 h LPS (100 ng/mL), used as positive control, increased p-Erk1/2$^{Thr202/Tyr204}$/Erk; Akt and Erk1/2 total expression was not affected by insulin or LPS. (B) Mouse primary microglia incubated 30 min with insulin in the medium containing FBS; qualitative analysis indicates that p-GSK3-β ^{Ser9} and GSK3-β were not affected by insulin (30 min) or LPS (3 h) with FBS. (C) Mouse primary microglia incubated 30 min with insulin in the medium without FBS; qualitative analysis indicates that insulin (1000 ng/mL) increased Akt phosphorylation at Ser473; Akt total expression was not affected by insulin or LPS. (D) Mouse primary microglia incubated 30 min with insulin in the medium without FBS; qualitative analysis indicates that insulin (1000 ng/mL) increased Akt phosphorylation at Ser473; Akt total expression was not affected by insulin or LPS. Mouse primary microglia incubated 24h with insulin (1000 ng/mL) and LPS (100 ng/mL) in the medium containing FBS; qualitative analysis indicate that Akt phosphorylation at Ser473 after 24 h treatment with LPS (100 ng/mL); 24 h insulin did not cause significant differences in Akt phosphorylation at Ser473 and Erk1/2 at Thr202/Tyr204; any condition changed p-GSK3-β^{Ser9} or GSK3-β expression. (F) Mouse primary microglia incubated 30 min with insulin and/or wortmanin in medium without FBS in two independent experiments; qualitative analysis indicate that both doses of insulin (100 ng/mL and 1000 ng/mL) with or without Wortmanin (PI3K phosphorylation inhibitor) did not modifies IR expression; both doses of insulin (100 ng/mL and 1000n g/mL) increased Akt phosphorylation at Ser473; and insulin (1000 ng/mL) plus Wortmanin (PI3K phosphorylation inhibitor) was

not able to increase Akt phosphorylation; both insulin or Wortmanin did not significantly affect the expression of total Akt protein.

Figure 2. Insulin effects in ROS (H_2O_2) production in primary microglia. Scatter plot with bars graph of the Amplex Red assay Analysis of H_2O_2 production accessed by in mice primary microglia. (A) Primary microglia treated 3h and 24h with insulin (1000ng/mL) in microglia medium containing FBS; 24 h 100ng/mL insulin decreased H_2O_2 production when compared with control (**; p=0.0033). (B) Primary microglia treated with the NADPH oxidase inhibitor Apocynin (Apo) for 30 min together or not with 24h insulin (1000 ng/mL) pre-incubation in microglia medium containing FBS. Apocynin inhibit H_2O_2 production in the concentration of 100 μM (**; p=0.067) and 1mM (*; p=0.0391) when compared with control group; a single 24 h insulin incubation decreases H_2O_2 production in comparison with the control group (*; p=0.0310) to the same level as 100 μM and 1 mM Apocynin (100 μM and 1 mM Apo x Ins 1000 ng/mL = p>0.05). Primary microglia pre-incubated with 24 h insulin and treated 30 min with 10 μM (*; p=0.0455) or 100 μM (*; p=0.0191) Apo had decreased H_2O_2 production when compared to control group, there was no difference when these groups were compared to 24 h insulin 1000 ng/mL group and insulin did not decrease this parameter when a single 30 min 10 μM Apo was compared to 30 min 10μM Apo + Insulin (p>0.05). Results = mean ± S.E.M.

Figure 3. Insulin effects in cell viability and cytotoxicity in primary microglia. Analysis of cell viability, through mitochondrial function, accessed by MTT assay, and cytotoxicity, accessed by LDH activity assay, in mice primary microglia. Cells treated with the NADPH oxidase inhibitor Apocynin (Apo) for 30 min together or not with 24h insulin (1000 ng/mL) pre-incubation in microglia medium containing FBS. (A) Apocynin increased the conversion of MTT by functional mitochondria activity when the concentration of 100 μM and 1 mM

(****; p<0.0001) Apo was compared to the control group. The 24 h insulin incubation itself increased the conversion of MTT by functional mitochondria activity when once was compared to the control group (****; p<0.0001). Primary microglia pre-incubated with 24h insulin and treated 30 min with 10 μM or 100 μM Apo had increased MTT conversion when compared to the control group (****; p<0.0001), there was no difference when these groups were compared to 24 h insulin 1000 ng/mL group, but insulin increased this parameter when a single 30 min 10 μM Apo was compared to 30 min 10 μM Apo + Insulin (****; p<000.1). (B) None of the conditions tested changed the cytotoxicity of primary microglia when compared to the control group (p>0.05). Results = mean ± S.E.M.

Figure 4. Insulin effects in pro-inflammatory interleukins IL-1β, IL-6 and TNF-α expression and release by primary microglia. Levels of interleukins (IL-1β, IL-6 and TNF-α) accessed by ELISA assay in microglia medium containing FBS in cells with or without Insulin (100 ng/mL and/or 1000 ng/mL) or LPS (100 ng/mL). (A) None of the insulin incubation times (30 min or 3 h) caused significant changes in the IL-1β levels; LPS incubation during 3 h was used as positive control (****; p<0.0001). (B) None of the insulin incubation times (30 min or 3 h) caused significant changes in the IL-6 levels; LPS 3 h and 24 h was used as positive control (****; p<0.0001). (C) None of the insulin incubation times (30 min or 3 h) caused significant changes in the TNF-α levels; LPS 3 h and 24 h was used as positive control (****; p<0.0001). (D) Twenty-four hours of insulin treatment (1000 ng/mL) had no significant effects in the IL-1β levels (p>0.05). LPS incubation for 3 h increased IL-1β levels (****; p=0.0001), and when insulin was added 24 h previous to 3 h LPS incubation IL-1β levels displayed a higher increment even compared with single LPS treatment (LPS 3 h group x Ins + LPS 3 h group; ****; p<0.0001). (E) A 24 h insulin treatment (1000 ng/mL) kept IL-6 levels similar to controls (p>0.05), and 3 h LPS incubation increased IL-6 levels (****; p=0.0001) whereas insulin added 24 h previous to 3 h LPS incubation did not significantly

change the levels of IL-6 ($p>0.05$). (F) Twenty-four hours insulin treatment (1000ng/mL) did not affect TNF-α levels in the ($p>0.05$), and 3 h LPS did not significantly change TNF-α levels ($p>0.005$). Twenty-four hours incubation of previous to 3 h LPS did not change the levels of TNF-α ($p>0.05$) Results = mean ± S.E.M.

Figure 5. Intranasal *in vivo* insulin treatment increases TNF-α gene expression in adult microglia. *In vivo* 7 days of 1.75 U intranasal (i.n.) insulin effects in microglia in adult mice accessed by quantitative real-time PCR analysis of the RNA extracted from acute isolated microglial enriched - cell suspensions. *In vivo* 7 days intranasal i.n. insulin treatment did not affect (A) IL-1β and (B) IL-6 genes expression. (C) *In vivo* 7 days intranasal i.n. insulin increased TNF-α gene expression (*; $p=0.0321$). The treatment did not change gene expression of (D) IL-10, (E) Arg-1 and (F) COX-2.

Supplemental Figure 1. Primary microglia, total brain homogenates and microglial enriched-cell suspension expression of brain cellular markers. (A) Qualitative analysis of brain cellular markers protein expression showed that primary microglia and microglial enriched-cell suspension express much more Iba-1 (microglia marker) compared with brain homogenate showing the high purity of microglia in the primary microglia cells and the microglial enriched-cell suspension.

Supplemental Figure 2. Body weight and blood glucose monitoring during intranasal insulin treatment. (A) Body weight monitoring during 7 days of intranasal insulin treatment once a day; no difference was found in mice the body weight between control and insulin groups. (B) Blood glucose monitoring in the days 1, 2 and 7 of the 7 days insulin protocol treatment; no difference was found in the mice blood glucose between control and insulin groups.

Figure 1. Microglia is sensitive to insulin via PI3K phosphorylation.

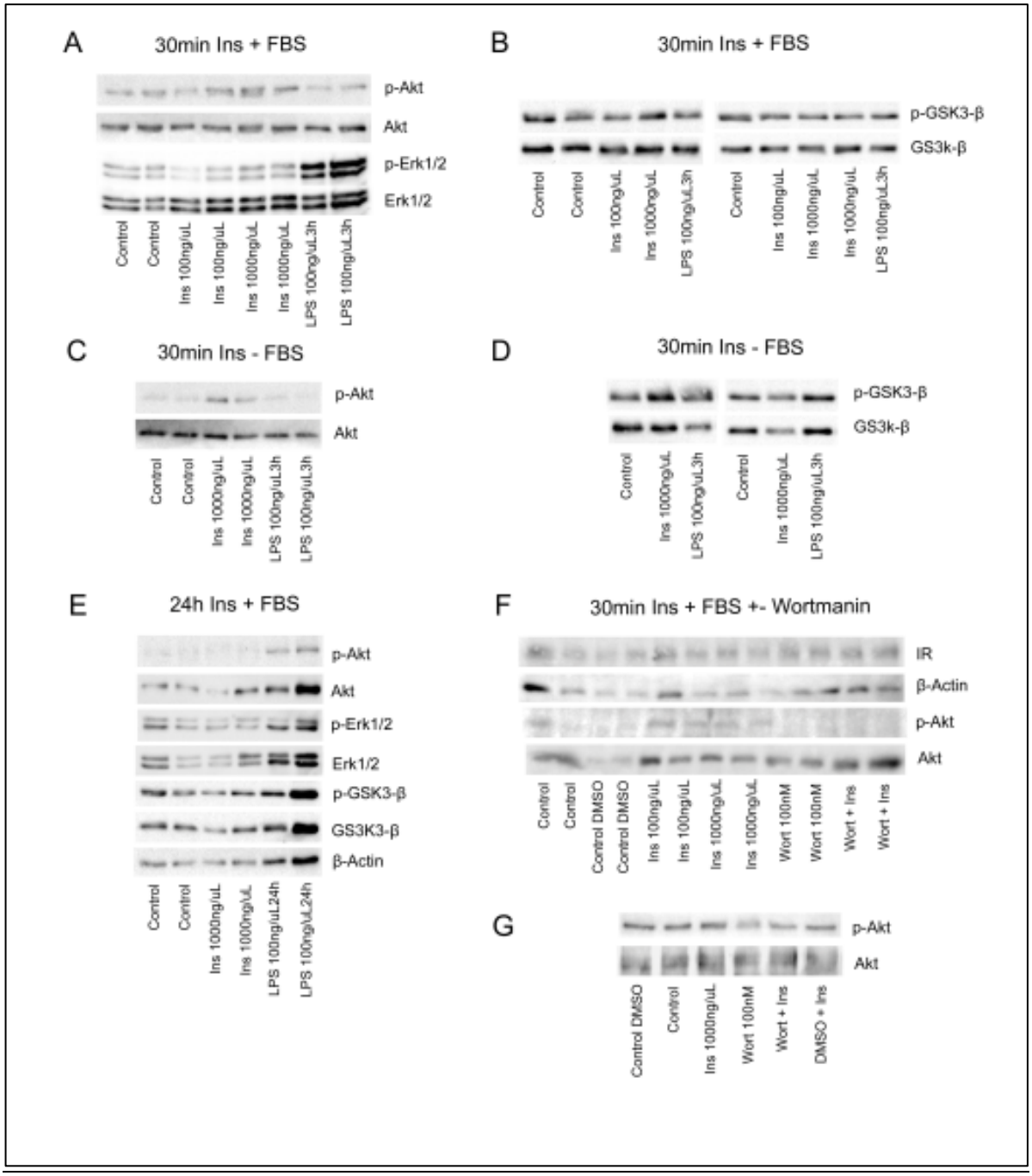


Figure 2. Insulin effects in ROS (H_2O_2) production in primary microglia.

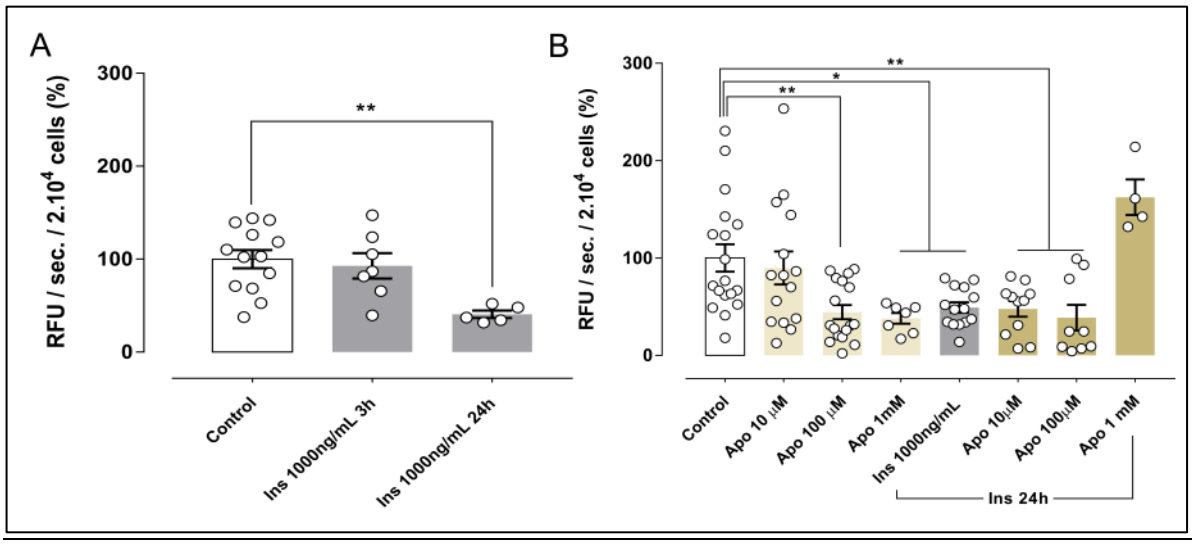


Figure 3. Insulin effects in cell viability and cytotoxicity in primary microglia.

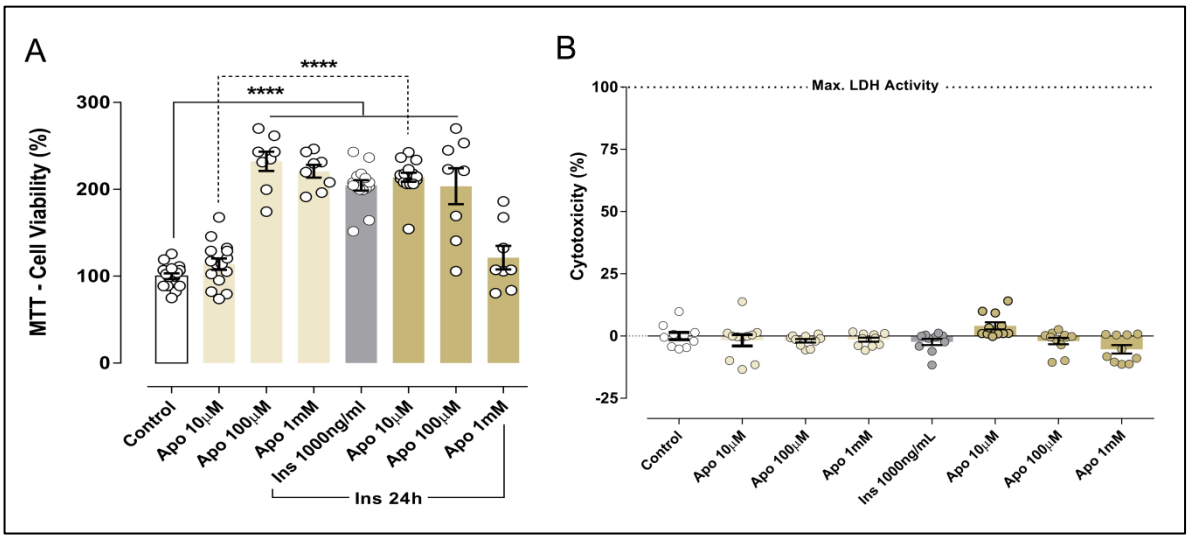


Figure 4. Insulin effects in pro-inflammatory interleukins IL-1β, IL-6 and TNF-α expression and release by primary microglia.

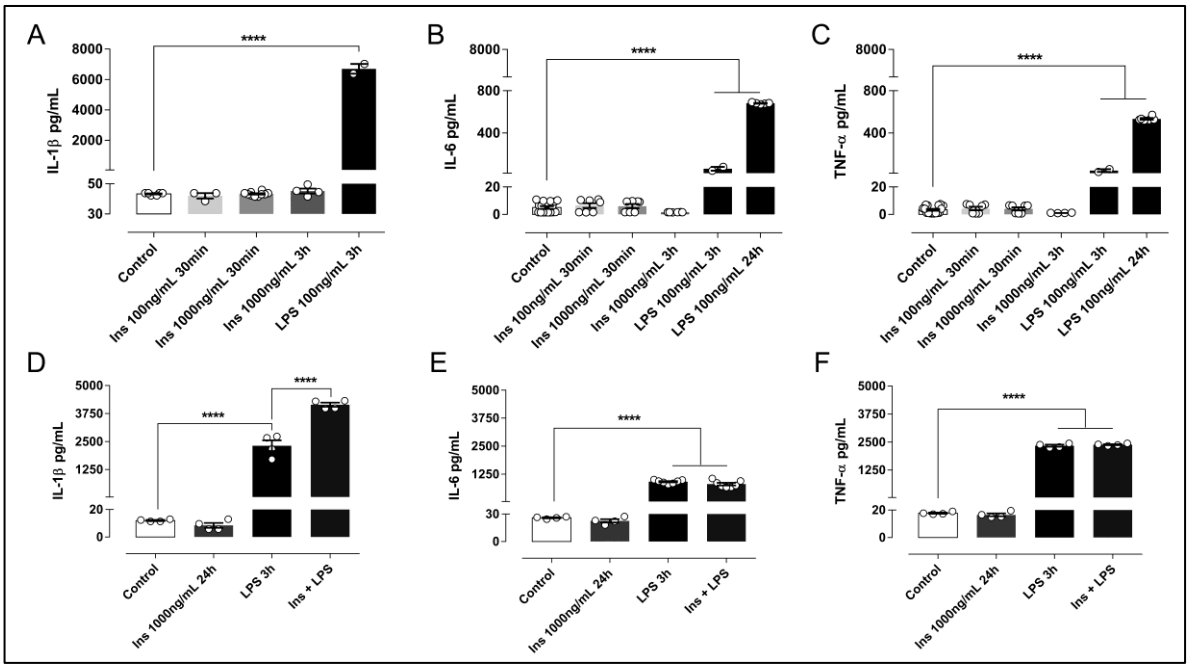
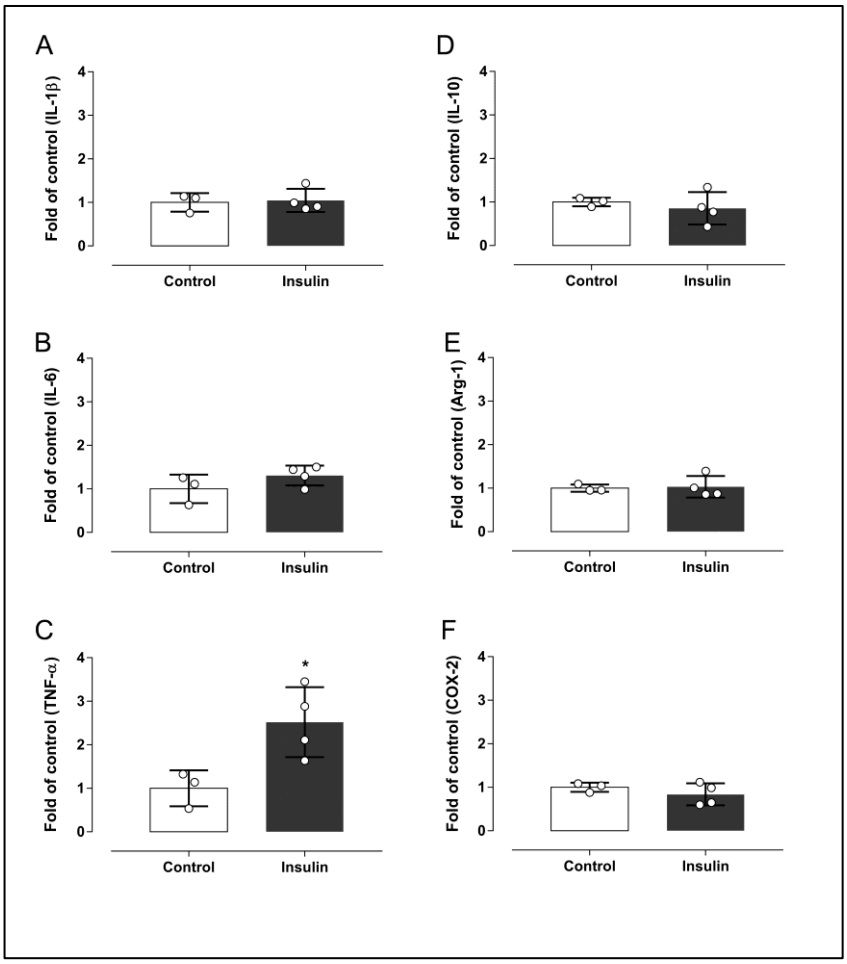
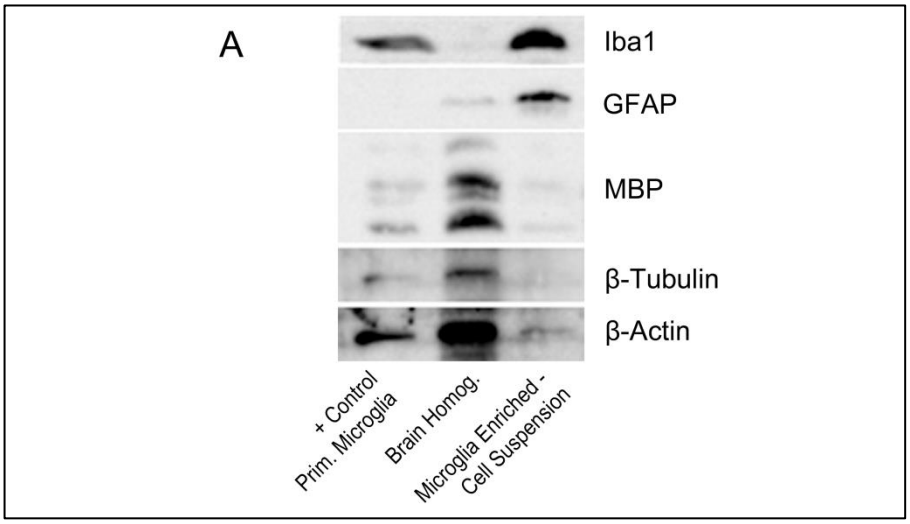


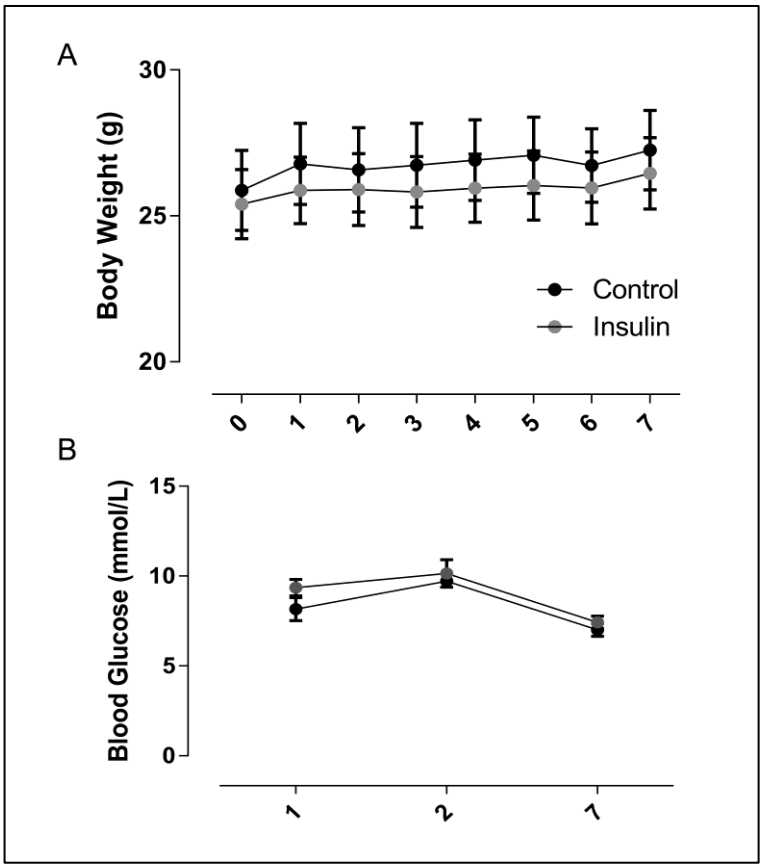
Figure 5. Intranasal *in vivo* insulin treatment increases TNF-α gene expression in adult microglia.



Supplemental Figure 1. Primary microglia, total brain homogenates and microglial enriched-cell suspension expression of brain cellular markers.



Supplemental Figure 2. Body weight and blood glucose monitoring during intranasal insulin treatment.



Supplemental Table 1. Primers Information.

Gene Name	Accession Number	Forward primer 5'-3'	Reverse primer 5'-3'
TNF-α	NM-013693	TCTTCTGTCTACTGAACTTCGG	AAGATGATCTGAGTGTGAGGG
IL-10	NM-010548	AAGGGTTACTTGGGTTGCCA	TTTCTGGGCCATGCTTCTCTG
IL-1β	NM-001037859.2	CCCAAAAGATGAAGGGCTGC	TGATACTGCCTGCCTGAAGC
COX-2	NM-011198.4	TCTACGGAGAGAGTTCATCCCT	ATTTAAGTCCACTCCATGGCCC
Arg-1	NM-007482	CAAGACAGGGCTCCTTTCAG	TTCACAGTACTCTTCACCTCCT
IL-6	NM-031168	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG

PARTE III

6. DISCUSSÃO

Considerando o papel da insulina no SNC, bem como o aumento da expectativa de vida da população mundial que acarreta o aumento dramático da incidência de doenças neurodegenerativas, nesta tese foi investigada a relação do envelhecimento com a resistência cerebral à insulina no cérebro, diminuição do suporte neurotrófico e aumento de processos neuroinflamatórios crônicos que podem ser mecanismos facilitadores para desenvolvimento de alterações neurológicas. Também foram apresentadas importantes evidências experimentais que suportam peculiaridades da sinalização cerebral da insulina. Tais resultados colaboram para um melhor entendimento dos processos neurobiológicos que são interrompidos ao longo do processo de envelhecimento cerebral, e as suas consequências funcionais. Além disso, foi demonstrada a relação da sinalização de insulina com a neuroinflamação e, ainda, que as células microgliais, macrófagos residentes do tecido cerebral, são sensíveis à insulina, e que aparentemente os Ris exercem funções diferentes dependendo das fases de neurodesenvolvimento.

No primeiro capítulo experimental, o artigo científico "Brain Insulin Administration Triggers Distinct Cognitive and Neurotrophic Responses in Young and Aged Rats.", publicado na revista Molecular Neurobiology, mostra que ratos velhos tratados com insulina intracerebroventricular não apresentam melhora na memória espacial quando comparado a animais jovens. Assim, em busca de alterações celulares que estariam contribuindo para esses efeitos distintos da insulina no envelhecimento, foi observado um resultado inesperado onde a insulina aumentou a proliferação de neurônios e a expressão de proteínas relacionadas à sua cascata de sinalização em animais velhos, mas não em jovens, o que sugere que a sinalização da insulina relacionada à proliferação neuronal e neurogênese é funcional em animais velhos. Entretanto, foi encontrada uma ruptura na conexão da sinalização de insulina com a de BDNF

nos animais velhos, uma vez que os níveis de BDNF/TrkB só foram aumentados em animais jovens após o tratamento com insulina indicando que animais velhos podem apresentar o sinal proteico a partir do IR, mas apresentam falhas na conexão com a resposta de outras sinalizações, como a do BDNF.

Estudos têm sugerido que o declínio cognitivo associado com o envelhecimento parece estar mais relacionado com alterações sinápticas do que com a perda de neurônios (Morrison and Baxter, 2012). De fato, existem diferenças eletrofisiológicas e morfológicas dos neurônios gerados na juventude e na idade adulta. Os neurônios gerados tardiamente podem ser transitórios e morrer pouco tempo depois, ou apresentarem elementos funcionais distintos ou defeituosos, implicando que após serem gerados estes não formam novas conexões e acabam morrendo (Mongiat and Schinder, 2011). Assim, alterações no nicho dessas células, como por exemplo, a neuroinflamação crônica poderiam ser a causa desta dificuldade de adaptação de novos neurônios. Nesse sentido, em busca de outros mecanismos que estariam sendo ativados pela insulina e que, consequentemente, desencadeiam ou participam da promoção da memória espacial em ratos jovens e que estariam inibidos em ratos velhos novos ensaios usando o mesmo protocolo experimental com ratos jovens e velhos foram realizados.

O segundo capítulo experimental, representado pelo manuscrito em preparação "Insulin *in vivo* increases microglial activation and proinflammatory outcomes in the hippocampus of young but not aged rats", a ser submetido, demonstra modulações neuroinflamatórias importantes no hipocampo de ratos jovens, e que não foram vistas em velhos, como, a ativação microglial na região CA3, além do aumento na expressão hipocampal de IL-1β e COX-2. Além disso, essas adaptações permaneceram após 9 dias de MWM teste para avaliação da memória espacial. Neste teste comportamental, foi novamente mostrado que animais jovens quando expostos à injeções de insulina diretamente no cérebro têm sua

memória espacial aumentada enquanto que os velhos não foram beneficiados. As alterações pró-inflamatórias causadas pela insulina mostram que o efeito da insulina não se dá apenas por neurônios e que as células gliais também participem da integração dessa resposta (Steculorum et al., 2014). Assim uma potencial modulação da insulina diretamente na microglia e a perda desta no envelhecimento poderiam sugerir que a microglia "primed" apresenta resistência à sinalização de insulina. Também deve ser considerado que o efeito da insulina variou dependendo da sub-região do hipocampo estudada.

De fato, o hipocampo apresenta populações neuronais heterogêneas dependendo da sub-região (Lee et al., 2004), e mesmo que essas populações tenham funções diferentes elas trabalham de forma integrada facilitando o processamento da memória. Também já foi relatado que a microglia apresenta diferentes populações no hipocampo e que essas também se adaptam durante o envelhecimento (Grabert et al., 2016; Hanisch, 2013). Mesmo que não seja possível afirmar com certeza que as alterações pró-inflamatórias encontradas antes e depois do teste comportamental em animais tenham sido promovidas pela microglia ativada, e de acordo com (Kettenmann et al., 2013) de que a microglia tem papel importante na formação e suporte das sinapses bem como no funcionamento da memória, (Parkhurst et al., 2013) seria possível especular que o sinal microglial da insulina seja de extrema importância para que o efeito desta sobre a memória se concretize. Até o momento, foi assumido que a proliferação e ativação das células microgliais assim como a expressão de marcadores pró-inflamatórios, como IL-1β and IL-6, seriam apenas reações maléficas ao sistema e sempre teriam o objetivo de matar células (Biber et al., 2014). Esses conceitos, hoje, se mostram ultrapassados e, nos últimos anos, uma quebra de paradigmas vem sendo vista nesse sentindo. Assim muitos estudos estão mostrando que processos neuroinflamatório, inclusive os pró-inflamatórios, são primariamente benéficos ao cérebro e podem assumir papel tóxico a medida que se tornam processos crônicos (Biber et al., 2014).

Assim, seguindo a tendência e movimento mundial acerca desse tema, é possível interpretar que os desfechos pró-inflamatórios encontrados em animais jovens tratados com insulina i.c.v. estariam suportando a função sináptica e processamento da memória, uma vez que a microglia é uma célula altamente ramificada que vigia o nicho celular e fornece fatores inflamatórios e tróficos para exercer seu papel crítico no mantimento da homeostase (Wu et al., 2015). Os efeitos da insulina sobre a microglia visualizados neste segundo capítulo experimental levam a crer que a insulina estaria agindo diretamente na microglia, entretanto com os resultados encontrados não é possível afirmar isso com certeza, mesmo que dentre escassa literatura, outros grupos de pesquisa já tenham mostrado que a glia é sensível à sinalização direta de insulina (Garcia-Caceres et al., 2016; Garwood et al., 2015; Mamik et al., 2016). Assim, uma pergunta formulada a partir desses experimentos foi se a insulina estaria agindo diretamente na microglia ou se seria preciso uma atividade combinada das células cerebrais para que os efeitos da insulina sobre processos de aprendizado e memória possam acontecer.

Ainda no terceiro capítulo experimental, representado pelo manuscrito em preparação "Microglial responsiveness to insulin: different insulin effects in newborn and adult microglia", a ser submetido, foi visto que, usando o modelo experimental de cultura primária de microglia, obtida através do cérebro de camundongos neonatos, 30 minutos de insulina aumenta a fosforilação de Akt e que esse efeito acontece via PI3K, uma vez que foi inibido pela presença do inibidor da fosforilação de PI3K Wortmanina. Além disso, os efeitos dessa sinalização direta foram investigados e foi encontrado que a insulina, quando em um ambiente *in vitro,* contendo apenas células microgliais, diminui a produção de EROS e aumenta a função mitocondrial, mas não apresenta nenhum efeito sobre a microglia em seu estado basal na expressão das interleucinas avaliadas (IL-1β, IL-6 e TNF-α). Entretanto, quando a microglia foi exposta ao LPS após um tratamento prévio com insulina os níveis de IL-1β

foram aumentados ainda mais quando comparados ao desafio somente com LPS. Quando avaliado o efeitos da insulina e microglia isolada de animais adultos, foi encontrado, novamente, um efeito pró-inflamatório, sendo esse caracterizado pelo aumento da expressão genética de TNF-α. Os resultados deste capítulo trazem importantes achados conceituais acerca da sinalização microglial de insulina.

Nosso grupo de pesquisa já havia relatado as ações da insulina sobre a produção de EROS em preparações de sinaptossoma via PI3K (Muller et al., 2013). Da mesma forma que nos resultados previamente relatados nós encontramos uma inibição na produção de ROS em microglia primária. Por se tratar de um tipo de macrófago e que esses tem metade da sua produção de ROS provinda da NADPH oxidase (Nayernia et al., 2014), o inibidor desta, a Apocinina, foi utilizado com o intuito de investigar se a insulina estaria atuando também nessa enzima. Foi observado que provavelmente a cascata de sinalização de insulina também tenha como alvo a NADPH oxidase e que esse efeito seja dose-dependente. Mais experimentos seriam necessários para afirmar com certeza os mecanismos de ação da insulina sobre as fontes de EROS em microglia primária neonatal.

Quando um tratamento intranasal de insulina foi utilizado e a microglia de camundongos adultos foi isolada, a expressão genética de TNF-α foi encontrada aumentada. Essa observação corrobora com o aumento da expressão de IL-1β encontrada após tratamento i.c.v. com insulina, relatado no capítulo experimental número 2 desta tese e citado acima (Haas et al., 2017 – manuscrito em preparação). Embora com os resultados dos experimentos apresentados no capítulo experimental 2 e 3 ainda não seja possível afirmar que existe uma relação do aumento da expressão do gene do TNF-α na microglia com o aumento da expressão proteica de IL-1β no hipocampo total, e, ainda, que esses dois fatores estariam contribuindo para a sinalização neuroinflamatória da insulina, e, consequentemente, os efeitos

benéficos da mesma sobre a memória, eles se mostram evidências sólidas para embasar perspectivas para o presente trabalho. Isso seria reforçado pelo fato de que TNF-α pode apresentar sinal neuroprotetor já relatado por (Vinet et al., 2012).

Na presente tese foram apresentados resultados intrigantes e ao mesmo tempo desafiadores que ganharam embasamento científico ao longo dos anos de pesquisa e de realização da mesma. Além de resultados importantes, já relatados na literatura ou ainda em preparação, relacionados à sinalização cerebral de insulina e às alterações desencadeadas pela resistência da mesma no envelhecimento, consideramos que esse trabalho proporcionou contribuições importantes para entendimento dos mecanismos de envelhecimento cerebral. Em conjunto com a literatura, foi demostrado por esta tese que existe uma ruptura de paradigmas na interpretação dos processos neuroinflamatórios, que deixam de ser vistos somente como um fator tóxico ao cérebro, mas também como um artifício elementar de adaptação do SNC aos diversos estímulos que as células nervosas recebem durante o curso da vida, desde o nascimento até o envelhecimento.

7. CONCLUSÃO

Esta tese demonstra que a sinalização de insulina causa diferentes respostas funcionais, celulares e moleculares em cérebro de ratos velhos e jovens, o que poderia significar resistência cerebral a sinalização de insulina. Mais ainda, a microglia de roedores jovens e velhos responde a desafios com insulina e expressa proteínas inflamatórias de forma diferente. Estes resultados proporcionam novos componentes para o entendimento do envelhecimento cerebral em humanos e a sua relação com a sinalização neuroendócrina.

8. PERSPECTIVAS

Algumas perguntas que não foram respondidas com os experimentos apresentados nessa tese são propostas como perspectivas para futuros experimentos complementares ou não aos manuscritos em preparação apresentados.

8.1. Avaliação da resposta neuroinflamatória específica da microglia à sinalização cerebral de insulina em animais jovem e velhos;

8.2. Avaliação da expressão de fatores tróficos pela microglia isolada de animais jovens e velhos em resposta à sinalização cerebral de insulina;

8.3. Investigação do mecanismo de sinalização da insulina que diminui a produção de EROS na microglia primária;

8.4. Investigação do metabolismo energético e de oxigênio da microglia e sua relação com os efeitos da insulina previamente encontrados.

8.5. Investigação dos mecanismos bioquímicos de sinalização de insulina sobre a modulação da expressão microglial de IL-1β.

8.6. Investigação da possível relação do aumento da expressão genética de TNF-α em microglia isolada com o aumento dos níveis de IL-1β no hipocampo e melhora da memória espacial causada pela insulina.

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ANEXOS

ANEXO 1

Experimental Neurology 247 (2013) 66–72

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Experimental Neurology

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Insulin prevents mitochondrial generation of H_2O_2 in rat brain

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ABSTRACT

The mitochondrial electron transport system (ETS) is a main source of cellular ROS, including hydrogen peroxide (H_2O_2). The production of H_2O_2 also involves the mitochondrial membrane potential ($\Delta\Psi_m$) and oxygen consumption. Impaired insulin signaling causes oxidative neuronal damage and places the brain at risk of neurodegeneration. We evaluated whether insulin signaling cross-talks with ETS components (complexes I and F_oF_1ATP synthase) and $\Delta\Psi_m$ to regulate mitochondrial H_2O_2 production, in tissue preparations from rat brain. Insulin (50 to 100 ng/mL) decreased H_2O_2 production in synaptosomal preparations in high Na^+ buffer (polarized state), stimulated by glucose and pyruvate, without affecting the oxygen consumption. In addition, insulin (10 to 100 ng/mL) decreased H_2O_2 production induced by succinate in synaptosomes in high K^+ (depolarized state), whereas wortmannin and LY290042, inhibitors of the PI3K pathway, reversed this effect; heated insulin had no effect. Insulin decreased H_2O_2 production when complexes I and F_oF_1ATP synthase were inhibited by rotenone and oligomycin respectively suggesting a target effect on complex III. Also, insulin prevented the generation of maximum level of $\Delta\Psi_m$ induced by succinate. The PI3K inhibitors and heated insulin maintained the maximum level of $\Delta\Psi_m$ induced by succinate in synaptosomes in a depolarized state. Similarly, insulin decreased ROS production in neuronal cultures. In mitochondrial preparations, insulin neither modulated H_2O_2 production or oxygen consumption. In conclusion, the normal downstream insulin receptor signaling is necessary to regulate complex III of ETS avoiding the generation of maximal $\Delta\Psi_m$ and increased mitochondrial H_2O_2 production.

Introduction

The brain consumes large amounts of oxygen to generate the ATP and phosphocreatine required for the maintenance of cellular functional homeostasis (Halliwell, 2006). However, part of the oxygen is physiologically diverted to the generation of reactive oxygen species (ROS) (Floyd and Hensley, 2002). Given their potentially harmful effects, the balance between ROS production and antioxidant defenses determines the degree of oxidative stress on cellular membranes, proteins and DNA; therefore a tight regulation is required to maintain a normal cell functioning (Nicholls and Budd, 2000).

Evidence indicates the complexes I and III, and F_oF_1ATP synthase of the mitochondrial electron transport system (ETS) as main sources of cellular ROS production, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Meyer et al., 2006). Although all organs can potentially be modified by oxidative stress damage, brain comprises one especially sensitive tissue as consequence of its reduced antioxidant defenses and high metabolic rate. This is particularly relevant during brain aging, metabolic disorders and in the pathogenesis of neurodegenerative diseases including Alzheimer's disease (Reger et al., 2006). In this scenario, the mitochondrial membrane potential ($\Delta\Psi_m$) is at the center of the cell's interactions, controlling ATP synthesis, mitochondrial Ca^{2++} accumulation, superoxide generation and redox activity (Aldinucci et al., 2007). Thus, the mechanisms involved in the regulation of mitochondrial function could link ROS production and neurodegenerative process (Dumont and Beal, 2011).

Insulin was recognized as a neurotrophic factor that acts on insulin receptors (IRs) distributed in neurons from different brain regions (Zhao and Alkon, 2001). Activation of insulin receptor downstream signaling proteins (PI3K and AKT) modulates neuronal survival (Valenciano et al., 2006), synaptic plasticity, and inhibitory/excitatory neurotransmission (Dou et al., 2005; Lee et al., 2005; Stranahan et al., 2008; Zhao et al., 1999). Moreover, insulin signaling modulates the activity of AKT, GSK3, and cytochrome c proteins localized in mitochondrial compartment (Cheng et al., 2010; Mookherjee et al., 2007; Petit-Paitel et al., 2009). Consistent data of the literature have supported the concept that impaired peripheral and central IR

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ANEXO 2

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Regular article

Nandrolone-induced aggressive behavior is associated with alterations in extracellular glutamate homeostasis in mice

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ABSTRACT

Nandrolone decanoate (ND), an anabolic androgenic steroid (AAS), induces an aggressive phenotype by mechanisms involving glutamate-induced N-methyl-D-aspartate receptor (NMDAr) hyperexcitability. The astrocytic glutamate transporters remove excessive glutamate surrounding the synapse. However, the impact of supraphysiological doses of ND on glutamate transporters activity remains elusive. We investigated whether ND-induced aggressive behavior is interconnected with GLT-1 activity, glutamate levels and abnormal NMDAr responses. Two-month-old untreated male mice (CF1, n = 20) were tested for baseline aggressive behavior in the resident–intruder test. Another group of mice (n = 188) was injected with ND (15 mg/kg) or vehicle for 4, 11 and 19 days (short-, mid- and long-term endpoints, respectively) and was evaluated in the resident–intruder test. Each endpoint was assessed for GLT-1 expression and glutamate uptake activity in the frontoparietal cortex and hippocampal tissues. Only the long-term ND endpoint significantly decreased the latency to first attack and increased the number of attacks, which was associated with decreased GLT-1 expression and glutamate uptake activity in both brain areas. These alterations may affect extracellular glutamate levels and receptor excitability. Resident males were assessed for hippocampal glutamate levels via microdialysis both prior to, and following, the introduction of intruders. Long-term ND mice displayed significant increases in the microdialysate glutamate levels only after exposure to intruders. A single intraperitoneal dose of the NMDAr antagonists, memantine or MK-801, shortly before the intruder test decreased aggressive behavior. In summary, long-term ND-induced aggressive behavior is associated with decreased extracellular glutamate clearance and NMDAr hyperexcitability, emphasizing the role of this receptor in mediating aggression mechanisms.

Introduction

Anabolic androgenic steroids (AAS), such as nandrolone decanoate (ND), are synthetic derivatives of testosterone that were developed to improve anabolic functions with fewer androgenic effects (Jones and Lopez, 2006; Shahidi, 2001). However, humans and rodents submitted to high AAS dose regimens may display exaggerated emotional reactivity and aggressive behavior, which ultimately is associated with glutamatergic hyperexcitability in brain areas such as the hypothalamus, cortex, and hippocampus (Breuer et al., 2001; Carrillo et al., 2009, 2011a; Diano et al., 1997; Kanayama et al., 2010; Le Greves et al., 1997; McGinnis, 2004; Ricci et al., 2007; Robinson et al., 2012; Talih et al., 2007).

The mechanism underlying the AAS-induced aggressive phenotype is dynamic and not restricted to proteins of the synaptic milieu. For instance, select hypothalamic neurons express dramatic increases in phosphate-activated glutaminase, the rate-limiting enzyme in the synthesis of glutamate, in aggressive, adolescent, AAS-treated, male Syrian hamsters (Fischer et al., 2007). Steroids also increase the rate of glutamate and aspartate release, thus increasing the binding probability of glutamate to NMDA or AMPA receptors (Brann and Mahesh, 1995; Ventriglia and Di Maio, 2013). Actually, AAS-induced aggression is mechanistically associated with glutamatergic hyperexcitability. This concept has been supported by studies on genetically modified animals and pharmacological studies addressing glutamate fast-acting ionotropic receptors, i.e. kainate (KAr), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAr), and N-methyl-D-aspartate (NMDAr) (Fischer et al., 2007). To date, studies on knockout mice lacking the AMPAr 1 subunit (GluR1) show reduced aggressive behavior (Vekovischeva et al., 2004), whereas AAS-treated aggressive hamsters

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ANEXO 3

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ORIGINAL PAPER

Intracerebroventricular Metformin Decreases Body Weight But Has Pro-oxidant Effects and Decreases Survival

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Abstract Metformin (Met), which is an insulin-sensitizer, decreases insulin resistance and fasting insulin levels. The precise molecular target of Met is unknown; however, several reports have shown an inhibitory effect on mitochondrial complex I of the electron transport chain (ETC), which is a related site for reactive oxygen species production. In addition to peripheral effects, Met is capable of crossing the blood–brain barrier, thus regulating the central mechanism involved in appetite control. The present study explores the effects of intracerebroventricular (i.c.v.) infusion of Met on ROS production on brain, insulin sensitivity and metabolic and oxidative stress outcomes in CF1 mice. Metformin (Met 50 and 100 μg) was injected i.c.v. in mice daily for 7 days; the brain mitochondrial H_2O_2 production, food intake, body weight and fat pads were evaluated. The basal production of H_2O_2 of isolated mitochondria from the hippocampus and hypothalamus was significantly increased by Met (100 μg). There was increased peripheral sensitivity to insulin (Met 100 μg) and glucose tolerance tests (Met 50 and 100 μg). Moreover, Met decreased food intake, body weight, body temperature, fat pads and survival rates. Additionally, Met (1, 4 or 10 mM) decreased mitochondrial viability and increased the production of H_2O_2 in neuronal cell cultures. In summary, our data indicate that a high dose of Met injected directly into the brain has remarkable neurotoxic effects, as evidenced by hypothermia, hypoglycemia, disrupted mitochondrial ETC flux and decreased survival rate.

Keywords Brain metabolism · Food intake · Body weight · Body temperature · Insulin resistance · Fat pads · Mitochondrial function

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Introduction

Type 2 diabetes mellitus (T2D), which is a progressive and complex disorder, has a considerable impact on individuals and society. The majority of patients are unable to sustain normal glycemia without pharmacological interventions targeted at improving insulin receptor responsiveness [1]. Metformin (1,1-dimethylbiguanide) (Met), which is an oral insulin sensitizer, is widely prescribed for treating hyperglycemia in patients with T2D because it improves insulin sensitivity and peripheral glucose homeostasis [2, 3]. Although the precise molecular targets associated with these benefits have not been established, the increasing expression of insulin receptor and activity of tyrosine kinase present as likely candidates [4]. The inhibition of complex I of the mitochondrial electron transport chain

ANEXO 4

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ORIGINAL PAPER

Cytokines in Machado Joseph Disease/Spinocerebellar Ataxia 3

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Abstract The aim of the present study is to describe the serum concentrations of a broad spectrum of cytokines in symptomatic and asymptomatic carriers of Machado Joseph disease (SCA3/MJD) CAG expansions. Molecularly confirmed carriers and controls were studied. Age at onset, disease duration, and clinical scales Scale for the Assessment and Rating of Ataxia (SARA), Neurological Examination Score for Spinocerebellar Ataxias (NESSCA), SCA Functional Index (SCAFI), and Composite Cerebellar Functional Score (CCFS) were obtained from the symptomatic carriers. Serum was obtained from all individuals and a cytokine panel "consisted of" eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, IFN-γ, interleukin (IL)-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-a, MIP-b, regulated on activation, normal T cell expressed and secreted (RANTES) and tumor necrosis factor (TNF)-α was analyzed. In a subgroup of symptomatic carriers, the cytokine panel was repeated after 360 days. Cytokine distribution among groups was studied by discriminant analysis; changes in serum levels after 360 days were studied by generalized estimation equation. Sixty-six symptomatic carriers, 13 asymptomatic carriers, and 43 controls were studied. No differences in cytokine patterns were found between controls and carriers of the CAG expansions or between controls and symptomatic carriers only. In contrast, eotaxin concentrations were significantly higher in asymptomatic than in symptomatic carriers or in controls (p=0.001, ANCOVA). Eotaxin did not correlate with age, disease duration, CAG expansion, NESSCA score, and SARA score. Among symptomatic carriers, eotaxin dropped after 360 days (p=0.039, GEE). SCA3/MJD patients presented a benign pattern of serum cytokines. In contrast, levels of eotaxin, a peptide secreted by astrocytes, were elevated in the asymptomatic carriers, suggesting that a specific response of these cells can be related to symptom progression, in SCA3/MJD.

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ANEXO 5

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Hyperpalatable Diet and Physical Exercise Modulate the Expression of the Glial Monocarboxylate Transporters MCT1 and 4

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Abstract Hyperpalatable diets (HP) impair brain metabolism, and regular physical exercise has an apparent opposite effect. Here, we combined a prior long-term exposure to HP diet followed by physical exercise and evaluated the impact on some neuroenergetic components and on cognitive performance. We assessed the extracellular lactate concentration, expression of monocarboxylate transporters (MCTs), pyruvate dehydrogenase (PDH), and mitochondrial function in the hippocampus. Male C57BL/6J mice were fed 4 months with HP or a control diet. Subsequently, they were divided in the following groups: control diet sedentary (CDS), control diet exercise (CDE), HP diet sedentary (HPS), and HP diet exercise (HPE) (n = 15 per group) and were engaged for an additional 30-day period of voluntary exercise and HP diet. Relative to the control situation, exercise increased MCT1, MCT4, and PDH protein levels, while the HP diet increased MCT1 and MCT4 protein levels. The production of hydrogen peroxide (H_2O_2) and the mitochondrial membrane potential ($\Delta\Psi_m$) stimulated by succinate in hippocampal homogenates were not significantly different between groups. ADP phosphorylation and the maximal respiratory rate induced by FCCP showed similar responses between groups, implying a normal mitochondrial function. Also, extracellular brain lactate levels were increased in the HPE group compared to other groups soon after performing the Y-maze task. However, such enhanced lactate levels were not associated with improved memory performance. In summary, hippocampal protein expression levels of MCT1 and 4 were increased by physical exercise and HP diet, whereas PDH was only increased by exercise. These observations indicate that a hippocampal metabolic reprogramming takes place in response to these environmental factors.

Keywords Hyperpalatable diet · Physical exercise · Lactate · Monocarboxylate transporters · Mitochondrial function

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Introduction

Beyond genetic components, sedentary habits and high intake of diets enriched in fat and sugar are significant contributors to the overweight and obesity epidemics in western societies. Such undesirable conditions increase the risk for the development of insulin resistance, diabetes, and neurodegenerative diseases [1–3]. This is paralleled by metabolic disturbances, which affect the capacity of peripheral tissues and brain to generate or use the energy necessary to sustain their function.

The high energetic supply needed to support brain function is mostly satisfied by glucose [4]. However, in conditions of high metabolic demands, other energetic substrates like lactate and ketone bodies can serve as additional energy substrates for neurons [5]. For instance, lactate is essential for long-term memory [6], and during moderate or intense exercise, the