Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Sistema insulina/fator de crescimento semelhante à insulina 1 (insulina/IGF-1) em hipocampo de roedores: efeitos da interação com exercício físico, dieta hiperpalatável e envelhecimento

Alexandre Pastoris Muller

Porto Alegre, maio de 2011.

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Doutorando

Alexandre Pastoris Muller

Orientador

Prof. Dr. Luis Valmor Portela

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Porto Alegre, maio de 2011.

APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As sessões Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas encontram-se nos próprios artigos.

Os itens Introdução, Discussão e Conclusões encontradas nesta tese apresentam interpretações e comentários gerais sobre os resultados contidos nos artigos científicos do presente trabalho. As referências bibliográficas referem-se somente às citações que aparecem nos itens supracitados.

As informações técnicas mais precisas sobre cada metodologia utilizada poderão ser encontradas nos artigos científicos correspondentes.

SUMÁRIO

RESUMO

Insulina e o fator de crescimento semelhante à insulina-1 (IGF-1) afetam positivamente as funções cerebrais. O exercício físico é um mediador de adaptações do SNC, enquanto o envelhecimento pode ser considerado um fator de risco para o desenvolvimento de doenças neurodegenerativas. Neste trabalho investigamos o efeito do exercício físico, dieta hiperpalatável e envelhecimento no sistema insulina/IGF-1 em hipocampo de roedores. No trabalho onde utilizamos uma dieta hiperpalatavel, para mimetizar o estilo de vida ocidental, causou uma diminuição na passagem de IGF-1 pela barreira sangue cérebro devido ao aumento nos níveis de triglicerídeos séricos. Os trabalhos onde utilizamos camundongos/ratos envelhecidos para verificar os efeitos do sistema insulina/IGF-1no SNC mostraram que, apesar de ocorrer uma compensação do cérebro em aumentar o transporte de IGF-1 pela barreira sangue cérebro e aumento dos receptores em hipocampo, ocorreu uma diminuição da sinalização pós-receptor, assim como perda da melhora cognitiva induzida pela insulina em animais jovens que induziu a produção de fatores tróficos. Nossos resultados apontam que exercício físico aumenta a sensibilidade à insulina no hipocampo por aumentar a translocação e ativação do receptor de insulina e ativação nas proteínas intracelulares. Os resultados apontam para um efeito antioxidante da insulina em preparações de sinaptossoma de ratos quando estimulamos a respiração mitocondrial com succinato. Estes resultados demonstram que fatores ambientais afetam profundamente a ação de hormônios responsáveis pela manutenção da homeostasia cerebral.

ABSTRACT

Insulin and insulin like growth factor-1 (IGF-1) affect the brain function. Physical exercise is proposed as a neurotrophic factor, while aging is considered a key factor to neurodisease. In our work we analyze the effect of physical exercise, high palatable diet and aging in insulin/IGF-1 system in hippocampus of the mice. High palatable diet decreased the transport of IGF-1 in blood brain barrier (BBB) by increased in the serum levels of triglycerides. Aged mice/rats had decrease in insulin/IGF-1 system in brain. Aged mice try to compensate this impair in IGF-1 signaling by increase the transport of hIGF-1 in BBB and increased the levels of IGF-1 receptor in hippocampus, however the intracellular signaling were decreased in aged mice. Aged rats showed resistance to benefits effect of insulin in cognition. Physical exercise increases the insulin sensitivity in hippocampus by increase the translocation and activation of intracellular proteins. Moreover insulin showed an antioxidant effect in synaptossome of the rats. Our results pointing to environmental factors modulated the insulin/IGF-1 system and affect the brain function.

Parte I

1. INTRODUÇÃO

1.1 Fatores ambientais e o sistema nervoso central (SNC)

Fatores ambientais como composição da dieta, atividade física e envelhecimento são componentes do estilo de vida da sociedade moderna que influenciam a funcionalidade dos tecidos periféricos, a produção de neuromoduladores e, em conseqüência, as suas ações no SNC (Stranahan and Mattson 2008). O cérebro é protegido de muitas substâncias presentes no sangue por uma barreira física composta de células especializadas, denominada barreira sangue-cérebro. Esta barreira transporta seletivamente tanto substâncias do sangue para o SNC quanto inversamente, além de estar envolvida em doenças neurológicas, quando a sua integridade está alterada (Neuwelt et al. 2011). Desta maneira, várias substâncias como hormônios, por exemplo, produzidos em tecidos periféricos, podem atravessar a barreira sangue-cérebro e atuar como neuromoduladores influenciando positivamente várias funções cerebrais.

Como resultado do alto teor calórico das dietas e do sedentarismo, a resistência à insulina e o Diabetes Mellitus (DM) estão rapidamente se tornando mais prevalente em todo o mundo (Marx 2005). Ambas as doenças envolvem uma disfunção da sinalização da insulina a qual parece estar envolvida na etiologia de comorbidades como renais, musculares, cardíacas assim como doenças do SNC como doença de Alzheimer (DA) e outras demências (Ott et al. 1999). Em indivíduos obesos, alterações como

intolerância à glicose, resistência periférica à insulina, hipertensão, dislipidemias e aterosclerose estão freqüentemente presentes como comorbidades. O conjunto destes fatores é denominado síndrome metabólica (SM) e a sua prevalência é de 30% na população adulta entre 20 e 60 anos (Antunes et al. 2010). Alguns estudos demonstram que o consumo exagerado de uma dieta rica em carboidratos simples (hiperpalatável) e/ou rica em gorduras saturadas (hiperlipídica) é capaz de provocar o aparecimento da SM, além de provocar alterações comportamentais associadas a doenças neuropsiquiátricas (Mattson 2010). Na maior parte dos casos, a SM evolui para o diabete mellitus tipo 2 e/ou para doença cardiovascular. Portanto, o tipo de dieta pode ser considerado um fator determinante para adiposidade corporal bem como para o aparecimento ou a prevenção das patologias associadas à obesidade (Schwartz and Porte 2005). Os efeitos deletérios de uma dieta rica em carboidratos simples e gordura saturada no SNC são conhecidos por diminuir a capacidade cognitiva (Molteni et al. 2004), alterar a funcionalidade de proteínas intracelulares (Muller et al. 2008), diminuir a plasticidade sináptica, alterar a permeabilidade de membrana e diminuir a sensibilidade à ação de hormônios (Gomez-Pinilla 2008).

O aumento do percentual de gordura corporal desencadeia uma série de desequilíbrios endócrinos, envolvendo principalmente o tecido adiposo, que também pode ser descrito como um órgão endócrino (McNay 2007). Ele secreta produtos solúveis (hormônios) como leptina, resistina e adiponectina, que exercem um importante papel no sistema cardiovascular, imunológico e na regulação do metabolismo. Além disso, estes produtos solúveis exercem uma influência direta no SNC, como demonstrado na figura 1 (Schwartz and Porte

2005). Outro aspecto importante é que o aumento de gordura corporal induz a resistência periférica à insulina (McGarry 2002), mas, além disso, também tem sido descrito que o cérebro pode aprsentar resistência a sinalização desse hormônio (Banks 2004). A barreira sangue-cérebro é um fator determinante para a passagem de hormônios e neuropeptídeos produzidos perifericamente para o SNC. O plexo coróide, que atua como uma barreira sangue líquido cerebroespinal, possui transportadores de hormônios e proteínas que participam nos mecanismos de neuroproteção e regulatórios além de estar envolvida na comunicação entre os tecidos periféricos e o SNC (Carro et al. 2002).
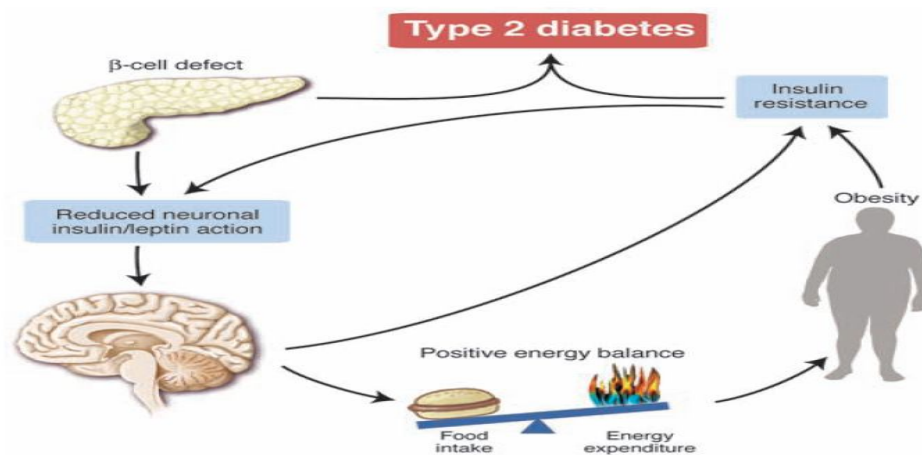
Figura 1. Inter-relações entre tecidos periféricos e a produção de hormônios com o sistema nervoso central na diabetes mellitus tipo 2. Aumento do consumo de alimentos e diminuição do gasto calórico gera alterações na produção de hormônios responsáveis por regular o consumo alimentar (adaptado de Schwartz and Portr Jr 2005).

Fisiologicamente, as ações da insulina e do fator de crescimento semelhante à insulina-1 (IGF-1) podem ser otimizadas pela prática de exercício físico regular (Cotman et al. 2007). Algumas estratégias poderiam ser usadas

com o objetivo de tentar minimizar os problemas da obesidade e as patologias associadas a ela. Assim intervenções voltadas para a o controle dos hábitos alimentares, e mudanças no estilo de vida, parecem ser alternativas apropriadas. O exercício físico regular tem sido proposto como umas das principais intervenções ambientais para melhorar a qualidade de vida das pessoas e para o tratamento da obesidade (Chakravarthy and Booth 2004), uma vez que além de aumentar o gasto calórico e também atua como protetor contra patologias associadas com a obesidade (Molteni et al. 2004). Atualmente, vários estudos demonstram que o exercício físico afeta diretamente o SNC, aumentando a neurogênese, angiogênese, melhorando os processos de sinalização celular e aumentando a síntese e secreção de fatores tróficos (Cotman and Berchtold 2002; Dietrich et al. 2005).

1.1.1 Efeitos da insulina no SNC

O cérebro foi considerado um órgão insensível à insulina, porém atualmente já se sabe que a insulina exerce importantes funções dentro do SNC. Essas funções vão desde a regulação do metabolismo energético, assim como efeitos tróficos importantes para o desenvolvimento e sobrevivência de células neurais (Park 2001). Alguns estudos demonstraram que o receptor de insulina (IR) está presente em todas as regiões do cérebro, principalmente nos neurônios de áreas relacionadas com aprendizado e memória como hipocampo, córtex cerebral e amígdala. Neste sentido, já foi observado em diferentes modelos animais que a interrupção ou alteração na sinalização cerebral da insulina causa prejuízo cognitivo e dano neuronal (Lannert and Hoyer 1998).

A insulina age ativando, tanto perifericamente como no SNC, vias de sinalização intracelular. Estudos apontam também que à insulina exerce um efeito neuroprotetor e neuromodulador (Frolich et al. 1998). Classicamente uma das mais conhecidas rotas modulada pela insulina após ligação ao seu receptor é a da proteína serina/treonina cinase B, conhecida como AKT. A insulina se liga a seus receptores e recruta a AKT para a membrana celular na proximidade dos seus ativadores, incluindo a proteína fosfatidil-dependente cinase 1 (PI3K). Uma vez fosforilada, a AKT atua como um regulador chave para a sobrevivência neuronal inibindo proteínas pro-apoptóticas como a glicogênio sintase cinase 3 beta (GSK3ß) e caspases (Miyawaki et al. 2009). Além disso, a AKT é capaz de ativar fatores de transcrição de genes envolvidos na sobrevivência neuronal como a proteína responsiva ao AMP cíclico (CREB) e a proteína alvo de rapamicina em mamíferos 2 (mTOR) e inativa fatores de transcrição de genes que promovem morte celular como o *forkhead box sub-group O* (FOXO1) (van der Heide et al. 2006).

A insulina atua no hipotálamo, ativando a liberação de neuropeptideos anorexigênicos como melacorticotropina e inibindo a liberação de neuropeptideo Y, um orexígeno (Niswender et al. 2003). Em animais obesos a ação da insulina e do fator de IGF-1 no SNC está diminuída devido a alterações na barreira sangue-cérebro (pré-receptor), e também nos receptores neurais ou na sua cascata de sinalização (pós-receptores) (Carro et al. 2002; Niswender et al. 2003). Alterações no metabolismo da glicose, resistência à insulina e síndrome metabólica estão sendo relacionadas por vários estudos epidemiológicos com demências, disfunções cognitivas, e doenças neurodegenerativas como Alzheimer e Parkinson (Craft and Watson 2004).

Estudos em animais apontam que a insulina, além de exercer um efeito sobre o metabolismo de carboidratos e lipídios em tecidos periféricos, é de fundamental importância para uma atividade cerebral normal (Moosavi et al. 2006) (figura 2).

Apesar de não se saber ao certo se a resistência periférica a insulina se reflete necessariamente em resistência cerebral, prevenir ou corrigir anormalidades periféricas tem demonstrado ser benéfico em reduzir os prejuízos na função cerebral. Curiosamente, quando a insulina é administrada perifericamente em doses que não alteram consideravelmente a glicemia, algumas perdas cognitivas em pacientes com a DA são revertidas (Neumann et al. 2008). Quando se administra insulina em animais, na maioria das vezes, este hormônio causa uma melhora da função cognitiva (Babri et al. 2007).

Os estudos realizados com animais para avaliar a disfunção na sinalização cerebral da insulina são baseados em modelos de resistência à insulina induzidos por obesidade associada ao consumo de dieta hiperlipídica e/ou hiperpalátével (Schwartz and Porte 2005) ou pela administração intracerebroventricular (icv) de estreptozotocina (STZ) (Hoyer and Lannert 2007). No primeiro modelo, há uma tentativa de mimetizar o consumo de alimentos com alto teor de gordura, hiperpalatáveis e de fácil acesso da sociedade ocidental. O segundo modelo mimetiza alguns achados bioquímicos em pacientes e modelos animais geneticamente modificados de DA como hiperfosforilação da proteína Tau e da GSK 3ß. A interrupção ou alteração na sinalização cerebral da insulina pela administração icv de STZ está relacionada com um aumento na produção e/ou diminuição da depuração de proteínas como a tau hiperfosforilada e a β-amilóide que são fortemente associadas à etiopatogenia da DA (Grunblatt et al. 2007).

Nestes modelos animais têm sido descritas alterações importantes como à diminuição da atividade mitocondrial e o aumento na produção de radicais livres, (Kilbride et al. 2008), bem como a diminuição do uso de glicose pelo cérebro (Hoyer 2004b; Morais and De Strooper). As alterações observadas nestes modelos parecem contribuir para o desenvolvimento de doenças neurodegenerativas como a DA. Assim, os mecanismos pelos quais estes hormônios atuam no SNC em condições fisiológicas e patológicas têm sido alvo de estudos. Em estudos *post mortem* de cérebros humanos, foi proposto que a resistência cerebral a insulina seria um "Diabetes tipo 3" (Steen et al. 2005).
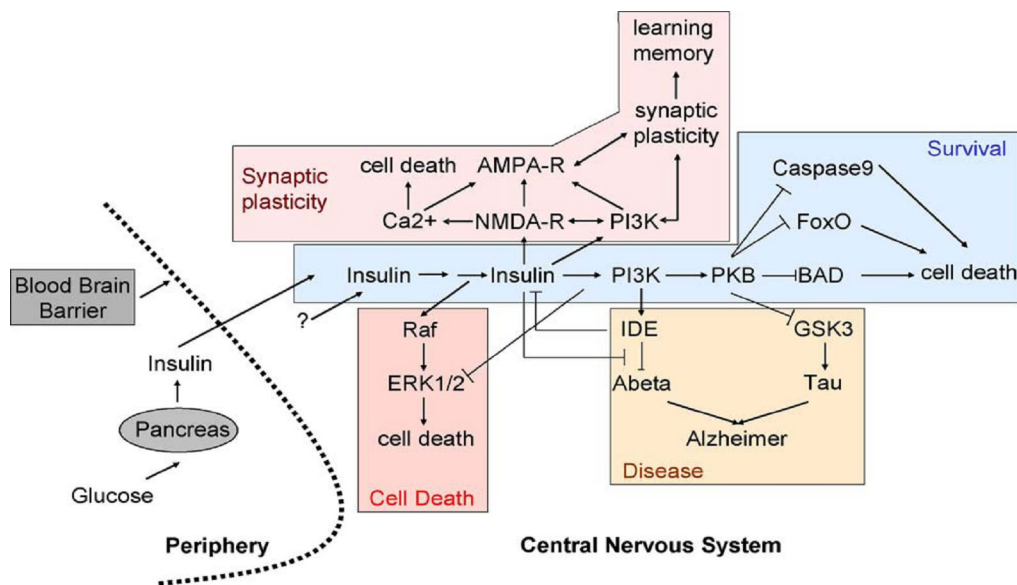

Figura 2. Efeitos da insulina no sistema nervoso central relacionados a aprendizado e memória, sobrevivência neuronal e doenças neurodegenerativas (adaptado de van der Reide 2006).

1.1.2 Efeitos do fator de crescimento semelhante à insulina (IGF-1) no SNC

Durante muitos anos o fator de crescimento semelhante à insulina-1 (IGF-1) foi considerado protetor para quase todos os tipos de célula, deste modo este hormônio foi considerado benéfico para a saúde. Entretanto, resultados de estudos epidemiológicos demonstraram que os níveis sanguíneos de IGF-1 se correlacionavam positivamente com risco de aparecimento alguns tipos de câncer (LeRoith and Roberts 2003). Além disso, o sistema insulina/IGF-1 está relacionado com a diminuição da expectativa de vida em modelos animais (Lin et al. 2001). Hoje em dia se propõe que as ações benéficas e/ou deletérias desse sistema dependem de uma fina regulação da sua concentração e/ou sinalização celular. Desta maneira, ações neuroprotetoras do IGF-1 ainda tem sido relatadas em pacientes idosos e em doenças neurológicas (Trejo et al. 2004b; Saute et al. 2011).

O papel do IGF-1 em regular neurodesenvolvimento foi estabelecido a partir de estudos com animais que não apresentavam a produção deste hormônio. A falta de IGF-1 logo após o nascimento é letal ou causa muitas anormalidades cerebrais (Vicario-Abejon et al. 2003). Cérebros adultos necessitam de IGF-1 para neurogênese, crescimento vascular e produção de mielina (Torres-Aleman 2010). No plexo coróide, uma das regiões da barreira sangue-cérebro, a passagem de IGF-1 do sangue para o cérebro é controlada por uma proteína denominada de megalina (Carro et al. 2002). Após sua entrada no SNC, o IGF-1 tem importante papel modulador na neurotransmissão, excitabilidade neuronal e plasticidade sináptica (Aleman and Torres-Aleman 2009), e, por isso, é muitas vezes considerado um fator neuroprotetor (figura 3) (Trejo et al. 2004a).

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Em humanos, os níveis de IGF-1 séricos diminuem com o envelhecimento, a partir da terceira década de vida, e a sua administração melhora a capacidade de aprendizado e memória (Markowska et al. 1998), plasticidade sináptica (Sonntag et al. 2000), crescimento vascular (Sonntag et al. 1997), e diminui o estresse oxidativo no cérebro (Puche et al. 2008) (Puche et al., 2008). Os níveis de IGF-1 se correlacionam positivamente com o desempenho cognitivo tanto em pessoas jovens (Aleman et al. 1999), como em camundongos (Trejo et al. 2008).
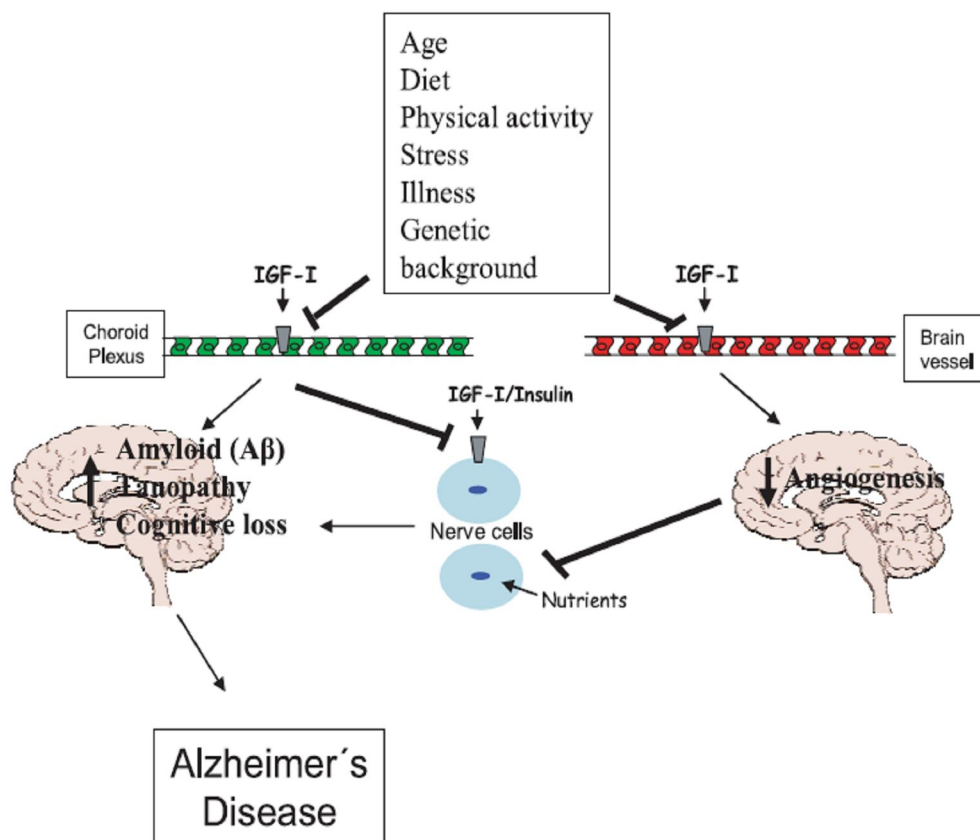
Figura 3. Efeitos neurotróficos do IGF-1 relacionados a fatores ambientais e doenças neurodegenerativas. Diminuição da ação deste hormônio causa aumento das (adaptado de Trejo et al. 2004).
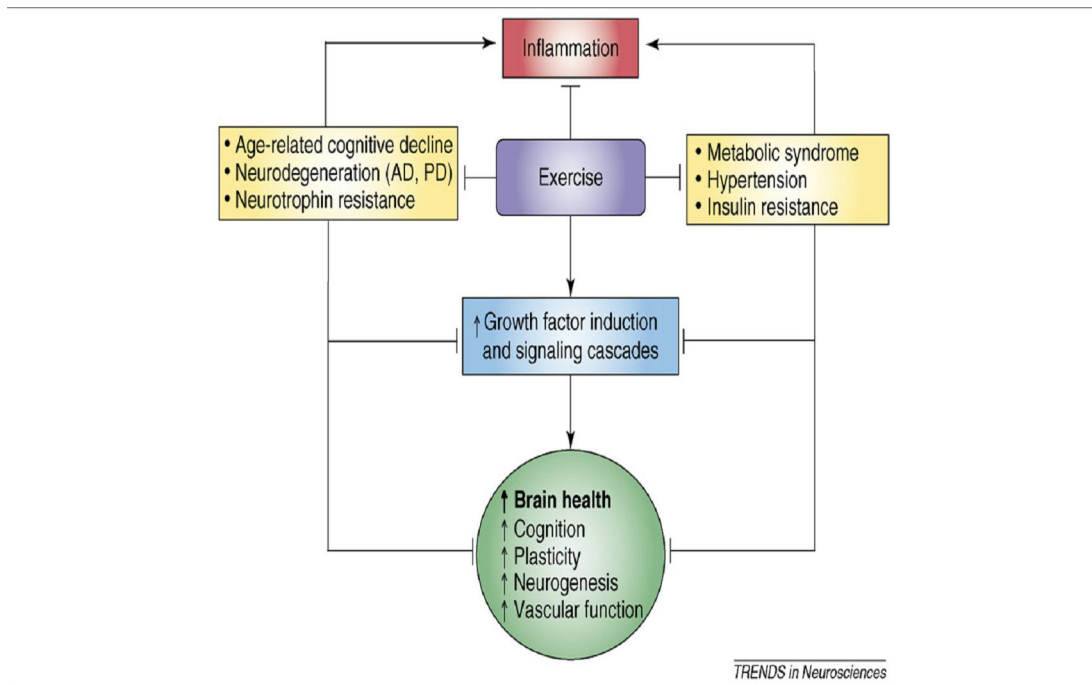
1.1.3 Efeitos do exercício físico no SNC

O exercício físico pode contribuir para prevenir e diminuir as complicações inerentes ao envelhecimento e tem sido proposto como um mediador de adaptações do SNC, podendo levar à estimulação fisiológica do sistema glutamatérgico, que é benéfica ao cérebro. Estudos em animais têm demonstrado que o exercício protege os neurônios de vários danos cerebrais (Adlard et al. 2005) e também promove a proliferação glial (Cotman and Berchtold 2002).

Fortes evidências apontam que o exercício físico é capaz de afetar positivamente o cérebro, induzindo adaptações celulares e moleculares que envolvem os fatores tróficos, proteínas de sinalização celular e sistemas de neurotransmissão (Parachikova et al. 2008). O aumento dos níveis cerebrais do fator neurotrófico derivado do cérebro (BDNF), e do IGF-1 têm sido relatados após a prática de exercício físico regular (Kanaley et al. 2005). Entre os possíveis alvos dos benefícios cerebrais do exercício físico está a modulação do sistema glutamatérgico, o principal sistema excitatório cerebral, por fatores neurotróficos e hormonais incluindo a insulina. A ativação dos receptores glutamatérgicos, principalmente de receptores ionotrópicos N-metil-D-aspartato (NMDAr), está envolvida em diversas funções cerebrais como síntese protéica, sinalização celular e aprendizado/memória. O NMDAr é um receptor glutamatérgico ionotrópico constituído de subunidades essenciais NR1, e por subunidades regulatórias NR2 (NR2A/B/C/D) e NR3 (A, B) (Collingridge 1987). Entretanto, a hiperativação do sistema glutamatergico via seus receptores inotrópicos, principalmente o NMDA, causa excitoxicidade, um mecanismo

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envolvido na fisiopatologia de diferentes doenças cerebrais, incluindo DA (Li et al. 2001).

Atividade glutamatérgica tem sido implicada em vários processos fisiológicos e patológicos associados ao envelhecimento e a doenças neurodegenerativas, e por esse motivo, receptores e proteínas que constituem a sinapse glutamatérgica estão sendo estudados como possíveis alvos dos efeitos do exercício no cérebro (Dietrich et al. 2005; Maragakis and Rothstein 2004). Assim a atividade física regular pode potencialmente diminuir os efeitos deletérios da obesidade e do envelhecimento no SNC, como demonstrado na figura 4 (Cotman et al. 2007).

Embora se saiba que o exercício físico melhora a resposta a testes cognitivos em animais e humanos, além de exercer efeitos neuroprotetores, os alvos moleculares onde esta ação ocorre ainda não estão bem elucidados. Neste sentido, a modulação da atividade da insulina no cérebro em áreas relacionadas com aprendizado e memória deve ser mais bem explorada. Flores et al. 2006 mostraram que o exercício físico agudo aumenta a sensibilidade do hipotálamo a hormônios como a insulina e leptina, que atuam em neurônios desta região regulando o gasto calórico e a fome.

Figura 4. Efeitos benéficos do exercício físico para tecidos periféricos como prevenção e tratamento para síndrome metabólica, hipertensão e resistência à insulina e no SNC como indução de fatores neurotróficos (adaptado Cotman 2007).

1.1.4 Efeitos do envelhecimento no SNC

O envelhecimento é o principal fator de risco para o desenvolvimento de doenças neurodegenerativas. A prevalência de DA está aumentando drasticamente em todo o mundo. Estima-se que 50 milhões de pessoas tenham DA nos Estados Unidos no ano de 2050 (Puglielli 2008). Durante o envelhecimento ocorre uma diminuição da ação de hormônios como insulina e IGF-1 (Bishop et al. 2010) o que contribui também para alterações estruturais e funcionais de várias regiões cerebrais. A perda de células neuronais em regiões específicas como córtex frontal e hipocampo, que ocorre durante o envelhecimento, além da perda da função de sistemas cerebrais, resulta em contínuo e progressivo declínio cognitivo, sensório e motor (Hofer et al. 2003;

Whitwell and Jack 2005). Durante envelhecimento ocorre uma série de alterações celulares e moleculares tais como dano ao DNA, aumento da resposta inflamatória e prejuízo das ações de hormônios tanto nos tecidos periféricos como no SNC (Cole and Frautschy 2007; Amor et al. 2010), o que pode estar diretamente relacionado às adaptações/alterações fisiológicas inerentes ao processo de envelhecimento.

Estudos epidemiológicos recentes demonstram que resistência cerebral à insulina é considerado um fator de risco para o desenvolvimento de doenças cerebrais e para o declínio cognitivo associado à idade (de la Monte et al. 2009; de la Monte and Wands 2005; Porte et al. 2005). Isto se relaciona com dados que demonstram que durante o envelhecimento há uma diminuição da ação da insulina no SNC (Cohen and Dillin 2008). Além disso, no envelhecimento ocorre uma ativação de células gliais, resultando no aumento da produção de fatores pró-inflamatórios como o fator de necrose tumoral alfa (TNF-α) e da proteína nuclear associada à cromatina polimerase 1 (PARP-1)(Block et al. 2007; Swardfager et al. 2010). O aumento da produção de fatores pró-inflamatórios e de estresse celular pelo envelhecimento pode levar a resistência cerebral à insulina (Altmeyer and Hottiger 2009; Beneke 2008; De Martinis et al. 2005). Proteínas envolvidas na regulação da sobrevivência/morte celular como a proteína alvo da rapamicina (mTOR) and FOXO1 também podem ser moduladas pelo envelhecimento (Hay and Sonenberg 2004; Cheng and White 2011).

2. OBJETIVOS

2.1 Objetivo geral

Investigar os efeitos do exercício físico, dieta hiperpalátavel e envelhecimento em parâmetros bioquímicos, morfológicos e comportamentais em relação ao sistema insulina/IGF-1 em cérebro de ratos e/ou camundongos.

2.2 *Objetivos específicos*

- Avaliar o efeito de uma dieta hiperpalatável sobre a passaagem de IGF-1 pela barreira sangue-cérebro, bem como sobre a sinalização intracelular deste hormônio em hipocampo de ratos;

- Avaliar o efeito do envelhecimento sobre transporte de IGF-1 pela barreira sangue-cérebro, bem como sobre a sinalização intracelular deste hormônio em hipocampo de camundongos;

- Estudar o efeito da administração icv de insulina em ratos jovens e envelhecidos sobre parâmetros metabólicos, comportamentais; morfológicos e inflamatórios no hipocampo.

- Investigar a resposta hipocampal da administração de insulina intracerebroventricular (icv) em camundongos submetidos exercício físico voluntário especialmente sobre a cascata de sinalização intracelular de insulina bem como parâmetros glutamatérgicos;

- Avaliar o efeito da insulina sobre a produção peróxido de hidrogênio e potencial de membrana mitocondrial em sinaptossomas e mitocôndrias isoladas de cérebro de ratos;

- Estudar o efeito do exercício físico voluntário após a administração icv de estreptozotocina em parâmetros comportamentais e de produção de peróxido de hidrogênio em sinaptossomas e mitocôndrias isoladas de cérebro de camundongos;

Parte II

Capitulo 1.

Artigo publicado no periódico Neuromolecular Medicine

ORIGINAL PAPER

Western Style Diet Impairs Entrance of Blood-Borne Insulin-like Growth Factor-1 into the Brain

**Marcelo O. Dietrich · Alexandre Muller · Marta Bolos · Eva Carro ·
Marcos L. Perry · Luis V. Portela · Diogo O. Souza · Ignacio Torres-Aleman**

Abstract It is increasingly recognized that life-style factors, such as physical exercise or diet influence brain health. In the present work we analyzed the effect of a western-style diet ("cafeteria diet") on the entrance to the brain of circulating IGF-1, a neuroprotective agent that has been related to different neurodegenerative diseases. Rats under a cafeteria diet showed reduced passage of systemic IGF-1 across the choroid plexus, a main site of IGF-1 entrance into the brain through the cerebrospinal fluid. Furthermore, the IGF-1 receptor at the choroid plexus of rats fed with a cafeteria diet showed enhanced sensitivity toward IGF-1 while receptor levels remained unchanged. Examination of possible mechanisms underlying reduced entrance of systemic IGF-1 to the brain showed that tri- glycerides that increased in blood after a cafeteria diet, diminished the passage of IGF-1 across choroid plexus epithelia. This effect of triglycerides was achieved by altering the interaction of IGF-1 with megalin, a choroid plexus transporter involved in transcytosis of IGF-1 from the circulation into the brain. Reduced brain entrance of circulating IGF-1 elicited by a western-style diet suggests that the higher incidence of brain diseases related to inadequate diets is due in part to diminished neurotrophic support.

Keywords Insulin-like growth factor 1 · Choroid plexus · Diet · Megalin · IGF-1 receptor · Neuroprotection · Life-style factors

Marcelo O. Dietrich and Alexandre Muller contributed equally to this work.

M. O. Dietrich · A. Muller · M. L. Perry ·
L. V. Portela · D. O. Souza
Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

M. O. Dietrich · A. Muller · M. Bolos · E. Carro ·
I. Torres-Aleman (✉)
Laboratory of Neuroendocrinology, Cajal Institute, CSIC, Madrid, Spain
e-mail: torres@cajal.csic.es

Present Address:
E. Carro
Hospital 12 de Octubre, Madrid, Spain

Introduction

Relatively poorly characterized interactions between life-style factors such as diet or physical activity and genetic makeup contribute to brain health. Since human genes were selected under intermittent food availability, among other environmental variables, those genes able to maintain homeostasis under that particular environmental conditions (fasting periods and intense physical activity) were cate- gorized as "thrifty genes" (Neel 1962). Recent changes in human life-style have disrupted the balance between a thrifty genotype and the environment (Booth et al. 2002). These changes have been driven mainly by the urban way of life, in which palatable high-caloric foods are associated with low levels of physical activity. This scenario is putatively associated to many epidemic chronic diseases that have emerged in relatively recent times.

Previous notions that the brain is isolated from sys- temic influences through the blood-brain barriers (BBB) have been refined by recent evidence that the periphery and the central nervous system are in constant crosstalk. Among the signals involved in a blood-brain interface, we recently proposed the wide-spectrum trophic factor insulin-like growth factor 1 (IGF-1) (Trejo et al. 2004).

This growth factor is a neuroprotective circulating peptide that enters into the brain in response to physical exercise (Carro et al. 2000) and is involved in important aspects of brain function (Trejo et al. 2004). Significantly, blood-borne IGF-1 signaling onto the choroid plexus BBB is pivotal for promoting Aß clearance (Carro et al. 2002), a process involved in amyloidosis (Carro et al. 2006), and therefore in Alzheimeŕs disease (Kosik 1992). Since the entrance of insulin into the brain was previously shown to be inhibited by diet (Kaiyala et al. 2000), we determined whether brain entrance of IGF-1, a close member of the insulin family of hormones, is also affected by diet. Transport of IGF-1 into the CSF through the choroid plexus is a key step in mediating its benefits in the brain (Carro et al. 2006).

Materials and Methods

Animals

Adult male (3-month-old) and infant rats from both sexes were kept in standard laboratory conditions (temperature and light/dark cycle) according to guidelines for Animal Care at both Porto Alegre and Madrid sites supervised by local Ethical Committees (European directive 86/609/EEC). Water was provided *ad libitum*.

In vivo Experiments

To mimic human fast food diets, we fed adult rats with a highly palatable diet enriched in simple sugars and saturated fat (high-fat, high-calorie "cafeteria diet", CD)—Standard diet: 62.5% carbohydrates from starch; 25.3% of protein; 4.5% fat (soybean oil); 5% salt; 2.7% fibers, and vitamins. Cafeteria diet: 57% carbohydrates (34% in condensed milk, 15% in starch, and 8% from sucrose), 25.3% of protein, 10% fat (soybean oil); 5% salt; 2.7% fibers, and vitamins. Saturated fats accounted for 16% of fats in both diets. Standard diet contains 3.9 Kcal/g and cafeteria diet 4.04 Kcal/g.

Rats were weighed weekly. After 1 or 3 months under this diet, a glucose tolerance test was performed. Briefly, rats were fasted overnight and injected with glucose (2 g/kg body weight). Blood was taken from the tail at 0, 30, 60, and 120 min. After completing the diet schedule, animals were anesthetized with ketamine/xylazine and the right carotid artery was cannulated as described previously (Carro et al. 2000). Recombinant human IGF-1 (hIGF-1, 5 µg) was injected in 100 µl of sterile saline during 1 min with a Hamilton's syringe. After 15 min, the cerebrospinal fluid

(CSF) was collected from the *cisterna magna* (100–150 µl) with a 31G needle attached to a 300 µl insulin syringe, and fast frozen. In case of accidental puncture (CSF mixed with blood), the CSF sample was discarded. Thereafter, rats were killed by decapitation and serum stored at –80°C. The carcase was given to a blinded investigator who dissected out the fat from retroperitoneal and epidydimal regions and weighed it for fat body composition analysis, as described (Parekh et al. 1998). The liver was weighed and fast frozen in small samples for further analysis. The brain was taken out of the skull, the choroid plexus dissected out, and fast frozen.

Biochemical Assays

Serum triglycerides (TGL), total cholesterol, and HDL cholesterol were analyzed using commercial kits (Labtest, MG, Brazil). The reactions were performed with Labmax equipment (Labtest). Blood glucose was measured with a commercial glucometer (Roche, Switzerland). Blood glucose levels along time were plotted and the "area under the curve" (AUC) used to compare glucose tolerance between groups (see Fig. 1). Liver was homogenized 1:10 with saline and 20 µl were used to determine liver TGL using a commercial kit (Labtest). Liver glycogen was determined by the colorimetric method described by Krisman (1962).

In vitro Experiments

A double-chamber choroid plexus epithelial cell-culture system mimicking the blood-CSF interface was used for in vitro studies, as described previously (Carro et al. 2002). Briefly, the choroid plexus was dissected from P7 rats, enzymatically digested with DNAase and Protease (both from Sigma, USA), and filtered. Plates were coated with laminin (10 µg/ml; Sigma), the cells plated ∼200,000 cells/well and grown to confluence for 7 days. In the day of the experiment, fresh medium was added to both chambers and 30 min later a TGL mix was added to the upper chamber (10 µg/µl; Supelco, Sigma). After another 30 min, digoxigenin-labeled IGF-1 (Dig-IGF-1, 100 nM) was added to the upper chamber. An hour later, the lower chamber medium was collected to measure Dig-IGF-1 levels. In experiments analyzing IGF-1 interactions with megalin or IGF-1 receptor (IGF-1R), choroid plexus cultures were exposed to Dig-IGF-1 and 5 min later cross-linking of the cells with dimethyl pimelydate was performed as described in detail elsewhere (Carro et al. 2005).

Fig. 1 Cafeteria diet and entrance of systemic IGF-1 into the brain. (**A**), Levels of triglycerides (TGLs) in blood increase after 1 and 3 month of cafeteria (CD) diet as compared to animals fed with an standard laboratory diet ($P < 0.05$ versus controls; $n = 10$–14 per group). (**B**), After a bolus injection of glucose (2 g/kg), blood levels were similarly increased in CD- and standard diet-fed rats after 1 month, but were significantly elevated over controls after 3 months of CD diet, as determined by AUC values, indicating glucose intolerance in CD rats (**$P < 0.01$ versus controls). (**C**), After 1 or 3 months of CD diet systemic injection of hIGF-1 (5 µg/rat) resulted in reduced CSF levels 15 min later, as compared to rats under standard diet (*$P < 0.05$)
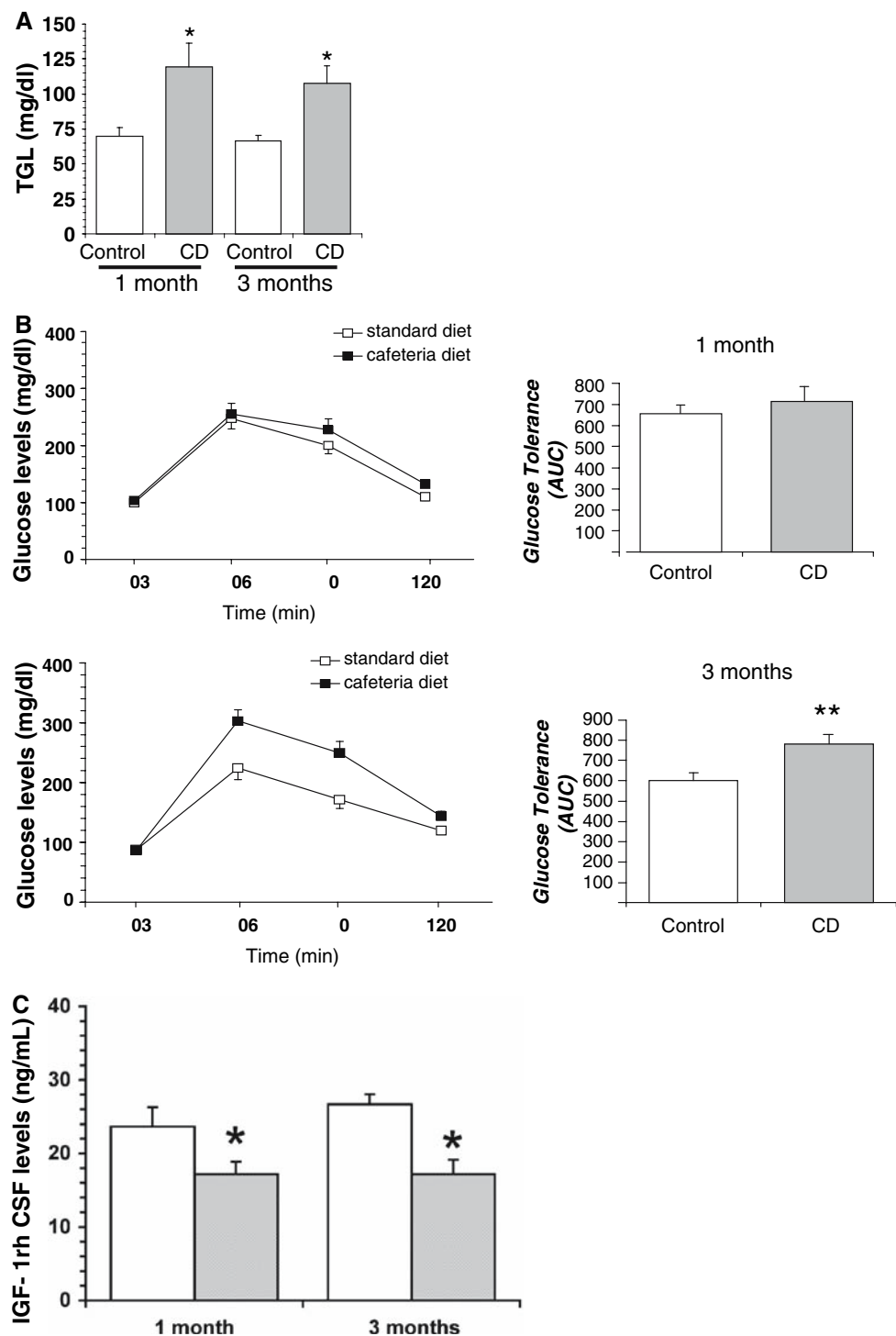
IGF-1 was labeled with digoxigenin-3-O-methylcarbonyl-aminocaproic-acid-N-hydroxy-succinimide ester (Boehringer Mannheim, Germany), as indicated by the manufacturer. IGF-1 was labeled to distinguish it from endogenous IGF-1 from the cultures. Experiments were run in triplicate and replicated at least four times.

Immunoassays

CSF samples (50 µl) were extracted using C_{18} affinity chromatography columns (Millipore) as described elsewhere (Maiter et al. 1989; Pons et al. 1991) to eliminate interference from CSF IGFBPs, and used in duplicate to

measure exogenously injected hIGF-1 with a commercially available kit that does not cross-react with rat IGF-1 (DSL, USA). Western blot (WB) and immunoprecipitation were performed as described previously (Carro et al. 2002). Levels of the protein under study were expressed relative to protein load in each lane. Densitometric analysis was performed using Quantity One software (Bio-Rad, USA). Antibodies used were against the IGF-1 receptor (1:1000), megalin (1:500), Akt1/2 (1:1000) from Santa Cruz (USA); phospho-IGF-1 receptor (1:1000), phospho-IRS-1 (1:1000) from Abcam (UK); phospho-Akt (1:1000) from Cell Signaling (USA) and digoxigenin (1:1000) from Boehringer (Germany). Secondary HRP-linked antibodies were from Santa Cruz or from BioRad.

Statistical Analysis

Results are shown as means ± SEM. One-way ANOVA was used to compare between groups followed by a post-hoc test. $P < 0.05$ was considered statistically significant.

Results

A Cafeteria Diet Impairs the Entrance of Systemic IGF-1 into the CSF

Rats submitted to a high-fat, high-calorie cafeteria diet (CD) showed a marked increase in adiposity, without

Table 1 Body composition and serum lipid profile of rats submitted to 3 months of cafeteria diet

|	Standard diet	Cafeteria diet	Statistics (P)
Body weight (g)			
Initial	234.00 ± 2.17	237.70 ± 2.55	>0.05
Final	317.60 ± 8.84	338.25 ± 6.19	>0.05
Fat tissue (g)			
Total	1.99 ± 0.21	2.83 ± 0.20	0.012
Visceral	1.02 ± 0.12	1.59 ± 0.12	0.007
Epididimal	0.97 ± 0.10	1.31 ± 0.08	0.013
Serum Lipid profile			
HDL[a]	28.20 ± 1.45	31.35 ± 0.99	>0.05
Total cholesterol[b]	43.60 ± 2.75	53.05 ± 1.14	<0.001
TGL[c]	66.40 ± 4.33	107.60 ± 12.79	0.034
Liver			
Weight (g)	10.01 ± 0.39	11.15 ± 0.39	>0.05
TGL[c]	2.83 ± 0.20	3.76 ± 0.28	0.016

[a] *HDL*, high-density lipoprotein expressed in mg/dl

[b] Total cholesterol expressed in mg/dl

[c] TGL, triglycerides in mg/dl

significant increases in body weight, as compared to normal diet: 42% increase after CD vs. 35% after SD (Table 1). The CD diet raised serum TGL levels within 1 month (Fig. 1A), while glucose intolerance, as assessed by significantly higher blood levels after a glucose bolus, was observed after 3 months of CD diet (Fig. 1B). In CD-fed rats, transport of serum IGF-1 into the brain across the blood-CSF barrier at the choroid plexus was diminished already after 1 month, and remained reduced thereafter. Thus, 15 min after injection of human IGF-1 into the carotid artery, levels of hIGF-1 in the CSF of 1- and 3-month CD-fed rats were ∼30% lower than in rats receiving a standard laboratory diet ($P < 0.05$ versus controls, Fig. 1C).

Because both the IGF-1 receptor and the choroid plexus multitransport protein megalin were previously shown to be involved in the transport of serum IGF-1
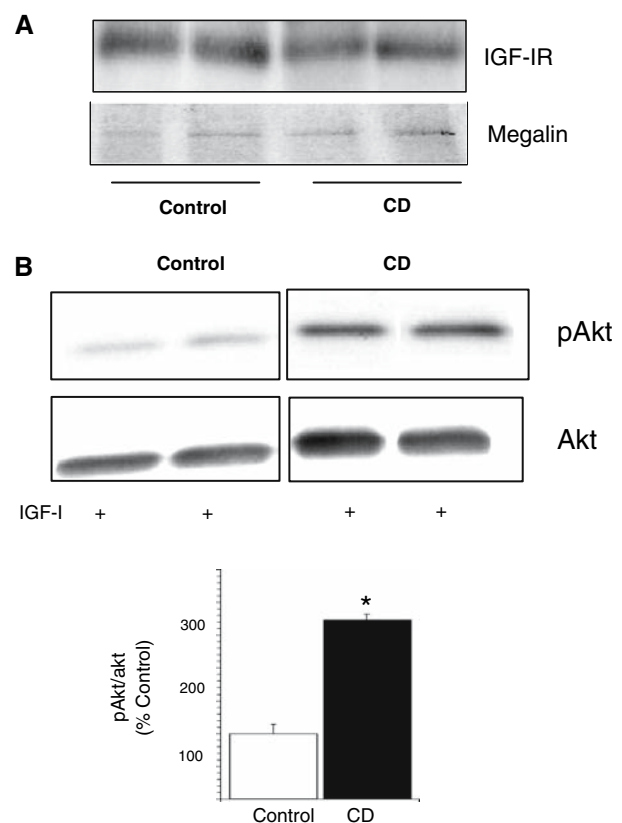
Fig. 2 IGF-1 signalling in the choroid plexus of CD-fed rats. (**A**), Levels of IGF-1 receptor and megalin, a protein involved in transport of IGF-1 across the choroid plexus (Carro et al 2005) were not changed after 3 months of CD diet. Two representative animals per group are shown. (**B**), The response of choroid plexus to a bolus injection of IGF-1 resulted in higher phosphoAkt (pAkt) levels in CD-fed rats. Representative levels of two animals/group are shown. Histograms show densitometric quantification of pAkt after normalization for total Akt (lower blots). (*$P < 0.05$ versus controls, $n = 10$–14 per group)
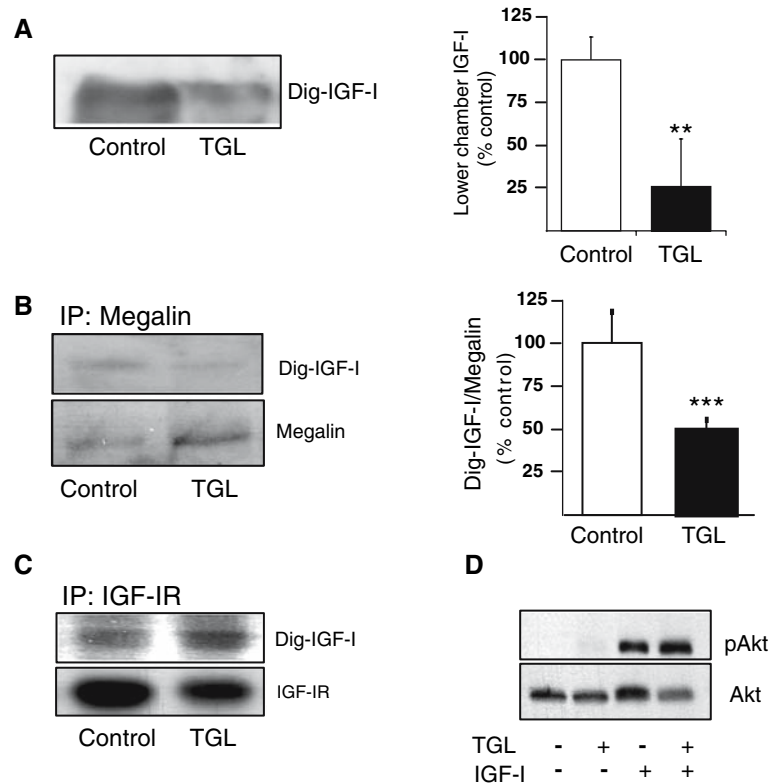
Fig. 3 Triglycerides reduce the passage of IGF-1 across the choroid plexus. (**A**), Passage of digoxigenin-labeled IGF-1 (Dig-IGF-1) across a monolayer of epithelial choroid plexus cells is significantly inhibited in the presence of triglycerides (TGLs). Dig-IGF-1 in the lower culture chamber was determined using anti-digoxigenin antibodies and western blot. Representative blot is shown. Histograms: densitometric quantification of the blots. $**P < 0.01$ versus control cultures ($n = 4$). (**B**), Co-immunoprecipitation of megalin with Dig-IGF-1 was significantly diminished in the presence of TGLs. Representative blots showing western blot with anti-digoxigenin of immunoprecipitates with anti-megalin. Western blot of megalin in lower blot corresponds to re-blotting of upper blot. Histograms: quantification of blots after correcting for megalin load ($***P < 0.001$ versus controls; $n = 4$). (**C**), Co-immunoprecipitation of the IGF-1 receptor with Dig-IGF-1 was not altered by TGLs. Representative blots showing western blot with anti-digoxigenin of immunoprecipitates with anti-IGF-1 receptor. Western blot of IGF-1 receptor in lower blot corresponds to re-blotting of upper blot ($n = 4$). (**D**), Representative western blot of pAkt in choroid plexus cultures in the presence or absence of TGLs. Increased pAkt levels in response to IGF-1 were not modified by TGLs

into the CSF (Carro et al. 2005), we examined their levels in choroid plexus and found them normal after CD diet (Fig. 2A). However, the response of CP cells to circulating IGF-1 was increased in CD-fed rats as determined by higher increases in CP levels of the active form of Akt (phospho-Akt), a kinase downstream of the IGF-1 receptor, 15 min after intracarotid administration of hIGF-1 (Fig. 2B).

Triglycerides Reduce Entrance of Serum IGF-1 into the Brain

An abnormal serum lipid profile in CD-fed rats made us consider that high TGLs associated to this diet could underlie the blockade of IGF-1 transport because TGLs inhibit passage of leptin through choroid plexus (Banks et al. 2004), a process that also involves megalin (Dietrich et al. 2007). In an in vitro double-chamber system mimicking the blood-CSF barrier we observed that a mix of TGLs produced a ~75% reduction in the passage of IGF-1 through a choroid plexus epithelial cell layer (Fig. 3A, $P < 0.001$ versus control cultures). The same inhibitory effect was found with whole milk, which is rich in TGLs ($23.2 \pm 14.0\%$ of control levels, $P < 0.001$). We then determined whether TGLs affected passage of IGF-1 through CP epithelial cells by altering the interactions of IGF-1 with megalin and/or the IGF-1 receptor. In the presence of TGLs, cultured CP epithelial cells showed a ~50% reduction in the interaction of IGF-1 with megalin but not with the IGF-1 receptor, as determined by co-immunoprecipitation of Dig-IGF-1 with either megalin (Fig. 3B, $P < 0.0001$ versus control) or the IGF-1 receptor (Fig. 3C). Normal activation of Akt by IGF-1 in the presence of TGLs corroborated that TGLs did not affect the interaction of IGF-1 with its receptor (Fig. 3D).

Discussion

The effects of diet and other life-style factors on brain function are currently a subject of great interest (Mattson 2003). In the present work we have found that a western style ("cafeteria") diet with a nutritional profile that resembles an average "fast food menu" in many western countries, diminished IGF-1 passage from blood into the CSF. Examination of the underlying processes indicated that increased circulating TGLs brought about by the cafeteria diet appeared to elicit this effect. TGLs disrupted the interaction of IGF-1 with megalin, a choroid plexus protein cargo involved in the transport of IGF-1, and many other blood-borne proteins across the CP epithelium (Barth et al. 2001), without affecting IGF-1 signaling through its receptor. Because blood-borne IGF-1 is an important determinant of brain health, reduced serum IGF-1 input to the brain may contribute to the deleterious actions of diet on brain function (Mattson 2003). A similar scenario was previously suggested by us to link the brain health risks of sedentary life with reduced serum IGF-1 input to the brain (Trejo et al. 2002). Altogether these data provide a mechanistic insight into the connection between life-style factors and brain health.

As discussed elsewhere, lower serum IGF-1 input to the brain is associated to many disturbances such as cognitive loss (Davila et al. 2007), reduced levels of the wide-spectrum neurotrophic factor BDNF (Ding et al. 2006), decreased neuronal resilience to insults (Carro et al. 2001), lowered angiogenesis (Lopez-Lopez et al. 2004), and with neuropathological changes reminiscent of Alzheimer′s disease (Carro et al. 2006). Importantly, at least part of these deleterious effects has also been related to inadequate diets (Molteni et al. 2004). For instance, a high-fat diet was previously shown to disturb brain BDNF and its downstream effectors (Vaynman et al. 2006), while reduced calorie intake was shown to produce the opposite effect (Lee et al. 2000). In turn, BDNF appears related to IGF-1 input to the brain (Ding et al. 2006); therefore, it is possible that reduced IGF-1 input to the brain induced by diet will result in perturbed BDNF action. An unhealthy diet is also associated to poorer cognitive performance and accelerates AD pathology in mouse models of the disease (Ho et al. 2004). Altogether these observations may contribute to explain the deleterious actions of high-fat, high-calorie diets, and/or obesity on cognitive performance and the increased risk of AD associated to obesity (Elias et al. 2003; Gustafson et al. 2003).

We also observed that a cafeteria diet enhanced the sensitivity of choroid plexus cells to IGF-1, as CP levels of phospho-Akt were enhanced after intracarotid injection of IGF-1 compared to rats receiving a standard diet. However, in vitro acute exposure to a TGL mix did not affect the response of Akt to IGF-1, which suggests that a prolonged in vivo exposure to high TGLs is required to modulate the effect of IGF-1 on its receptor. Alternatively, other unknown factors associated to the cafeteria diet may underlie IGF-1R sensitization of CP cells (Clodfelder-Miller et al. 2005). Conversely, acute exposure of choroid plexus cells to TGLs decreased the crossing of IGF-1 through the double-chamber culture system, pointing out to an acute effect of TGLs on the passage of IGF-1 across the choroid plexus.

In summary, because blood-borne IGF-1 is involved in many aspects of brain function (Torres-Aleman 2000), our results point to a potentially new mechanism by which diet can be regulating brain homeostasis. Reduced serum IGF-1 input to the brain very likely results in a reduced ability of brain tissue to respond to deleterious stimuli. The present results highlight the importance of a strict control of the serum lipid profile in maintaining an appropriate crosstalk between the periphery and the CNS.

References

Banks, W. A., Coon, A. B., Robinson, S. M., Moinuddin, A., Shultz, J. M., Nakaoke, R., & Morley, J. E. (2004). Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes*, *53*, 1253–1260.

Barth, J. L., & Argraves, W. S. (2001). Cubilin and megalin: Partners in lipoprotein and vitamin metabolism. *Trends in Cardiovascular Medicine*, *11*, 26–31.

Booth, F. W., Chakravarthy, M. V., & Spangenburg, E. E. (2002). Exercise and gene expression: Physiological regulation of the human genome through physical activity. *Journal of Physiology*, *543*, 399–411.

Carro, E., Nunez, A., Busiguina, S., & Torres-Aleman, I. (2000). Circulating insulin-like growth factor I mediates effects of exercise on the brain. *Journal of Neuroscience*, *20*, 2926–2933.

Carro, E., Spuch, C., Trejo, J. L., Antequera, D., & Torres-Aleman, I. (2005). Choroid plexus megalin is involved in neuroprotection by serum insulin-like growth factor I. *Journal of Neuroscience*, *25*, 10884–10893.

Carro, E., Trejo, J. L., Busiguina, S., & Torres-Aleman, I. (2001). Circulating insulin-like growth factor I mediates the protective effects of physical exercise against brain insults of different etiology and anatomy. *Journal of Neurosciences*, *21*, 5678–5684.

Carro, E., Trejo, J. L., Gomez-Isla, T., LeRoith, D., & Torres-Aleman, I. (2002). Serum insulin-like growth factor I regulates brain amyloid-beta levels. *Nature Medicine*, *8*, 1390–1397.

Carro, E., Trejo, J. L., Spuch, C., Bohl, D., Heard, J. M., & Torres-Aleman, I. (2006). Blockade of the insulin-like growth factor I receptor in the choroid plexus originates Alzheimer's-like neuropathology in rodents: New cues into the human disease? *Neurobiology of Aging*, *27*, 1618–1631.

Clodfelder-Miller B., De Sarno P., Zmijewska A. A., Song L., & Jope, R. S. (2005). Physiological and pathological changes in glucose

regulate brain Akt and glycogen synthase kinase-3. *The Journal of Biological Chemistry*, *280*, 39723–39731.

Davila, D., Piriz, J., Trejo, J. L., Nunez, A., & Torres-Aleman, I. (2007). Insulin and insulin-like growth factor I signalling in neurons. *Frontier in Biosciences*, *12*, 3194–3202.

Dietrich, M. O., Spuch, C., Antequera, D., Rodal, I., de Yebenes, J. G., Molina, J. A., Bermejo, F., & Carro, E. (2007). Megalin mediates the transport of leptin across the blood-CSF barrier. *Neurobiology of Aging*, doi:10.1016/j.neurobiolaging.2007.01.008.

Ding, Q., Vaynman, S., Akhavan, M., Ying, Z., & Gomez-Pinilla, F. (2006). Insulin-like growth factor i interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. *Neuroscience, 140*, 823–833.

Elias, M. F., Elias, P. K., Sullivan, L. M., Wolf, P. A., & D'Agostino, R. B. (2003). Lower cognitive function in the presence of obesity and hypertension: The Framingham heart study. *International Journal of Obesity and Related Metabolic Disorders*, *27*, 260–268.

Gustafson, D., Rothenberg, E., Blennow, K., Steen, B., & Skoog, I. (2003). An 18-year follow-up of overweight and risk of Alzheimer disease. *Archives of Internal Medicine*, *163*, 1524–1528.

Ho, L., Qin, W., Pompl, P. N., Xiang, Z., Wang, J., Zhao, Z., Peng, Y., Cambareri, G., Rocher, A., Mobbs, C. V., Hof, P. R., & Pasinetti, G. M. (2004). Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *Official Publication of the Federation of American Societies for Experimental Biology Journal*, *18*, 902–904.

Kaiyala, K. J., Prigeon, R. L., Kahn, S. E., Woods, S. C., & Schwartz, M. W. (2000). Obesity induced by a high-fat diet is associated with reduced brain insulin transport in dogs. *Diabetes*, *49*, 1525–1533.

Kosik, K. S. (1992). Alzheimer's disease: A cell biological perspective. *Science*, *256*, 780–783.

Krisman, C. R. (1962). A method for the colorimetric estimation of glycogen with iodine. *Analytical Biochemistry*, *4*, 17–23.

Lee, J., Duan, W., Long, J. M., Ingram, D. K., & Mattson, M. P. (2000). Dietary restriction increases the number of newly generated neural cells, and induces BDNF expression, in the dentate gyrus of rats. *Journal of Molecular Neuroscience*, *15*, 99–108.

Lopez-Lopez, C., LeRoith, D., & Torres-Aleman, I. (2004). Insulin-like growth factor I is required for vessel remodeling in the adult brain. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 9833–9838.

Maiter, D., Fliesen, T., Underwood, L. E., Maes, M., Gerard, G., Davenport, M. L., & Ketelslegers, J. M. (1989). Dietary protein restriction decreases insulin-like growth factor I independent of insulin and liver growth hormone binding. *Endocrinology*, *124*, 2604–2611.

Mattson, M. P. (2003). Gene-diet interactions in brain aging and neurodegenerative disorders. *Annals of Internal Medicine*, *139*, 441–444.

Molteni, R., Wu, A., Vaynman, S., Ying, Z., Barnard, R. J., & Gomez-Pinilla, F. (2004). Exercise reverses the harmful effects of consumption of a high-fat diet on synaptic and behavioral plasticity associated to the action of brain-derived neurotrophic factor. *Neuroscience*, *123*, 429–440.

NeeL, J. V. (1962). Diabetes mellitus: A "thrifty" genotype rendered detrimental by "progress"? *American Journal of Human Genetics*, *14*, 353–362.

Parekh, P. I., Petro, A. E., Tiller, J. M., Feinglos, M. N., & Surwit, R. S. (1998). Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism*, *47*, 1089–1096.

Pons, S., Rejas, M. T., & Torres-Aleman, I. (1991). Ontogeny of insulin-like growth factor I, its receptor, and its binding proteins in the rat hypothalamus. *Brain Research Developmental Brain Research*, *62*, 169–175.

Torres-Aleman, I. (2000). Serum growth factors and neuroprotective surveillance. *Molecular Neurobiology*, *21*, 153–160.

Trejo, J. L., Carro, E., Garcia-Galloway, E., & Torres-Aleman, I. (2004). Role of insulin-like growth factor I signaling in neurodegenerative diseases. *Journal of Molecular Medicine*, *82*, 156–162.

Trejo, J. L., Carro, E., Nunez, A., & Torres-Aleman, I. (2002). Sedentary life impairs self-reparative processes in the brain: the role of serum insulin-like growth factor-I. *Reviews in the Neurosciences*, *13*, 365–374.

Vaynman, S., & Gomez-Pinilla, F. (2006). Revenge of the "sit": How lifestyle impacts neuronal and cognitive health through molecular systems that interface energy metabolism with neuronal plasticity. *Journal of Neuroscience Research*, *84*, 699–715.

Capitulo 2:

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REDUCED BRAIN INSULIN-LIKE GROWTH FACTOR I FUNCTION DURING AGING

Alexandre Pastoris Muller1, Ana M. Fernandez2, Clarissa Haas 1, Eduardo Zimmer1, Luis Valmor Portela1, and Ignacio Torres-Aleman2

1Departamento de Bioquímica, ICBS, UFRGS. Programa de Pós Graduação em Ciências Biológicas-Bioquímica. Porto Alegre, RS, Brazil. 2Cajal Institute and CIBERNED, Madrid, Spain

Corresponding author: Ignacio Torres-Aleman. Cajal Institute, 28002 Madrid Spain. Phone: 34 91 5854723; email: torres@cajal.com

Abstract

Peripheral insulin-like growth factor I (IGF-I) function progressively deteriorates with aging. However, whether IGF-I function is impaired in the aged brain is not yet firmly established. As serum IGF-I can enter into the brain through the cerebrospinal fluid (CSF), we analyzed serum-to-CSF traffic of IGF-I in aged mice. To distinguish endogenous murine IGF-I from exogenously applied IGF-I we injected intra-peritoneously human IGF-I (hIGF-I). We found that CSF levels of hIGF-I were significantly higher in old (2 year-old) than in young (4-month-old) mice. In spite of enhanced uptake of human IGF-I in old mice, murine brain and plasma IGF-I levels were reduced in naïve old mice. Moreover, IGF-I signaling was deteriorated in the brain of aged animals. After systemic injection of IGF-I, activation of the brain IGF-I receptor/Akt/GSK3 pathway was markedly reduced even though old mice have higher levels of brain IGF-I receptors. These data suggest that increased brain IGF-I receptors and uptake of peripheral IGF-I during aging are ineffective because IGF-I function remains reduced and aged mice are cognitively impaired, a trait dependant on preserved serum IGF-I input to the brain.

Abbreviations: blood-brain-barrier: BBB; cerebrospinal fluid: CSF; insulin-like growth factor I: IGF-I;

1. Introduction

During aging, different neuroactive hormones decline in parallel with gradually impaired brain function (Conrad and Bimonte-Nelson, 2010). Among these, insulin-like growth factor I (IGF-I), a pleiotropic neurotrophic factor, has been proposed to be directly involved in age-associated brain deterioration due to its ample spectrum of neuroprotective activities, including pro-cognitive effects (Aleman and Torres-Aleman, 2009). Conceivably, decreased brain IGF-I function in aging could explain several deficits in learning tasks in old rodents and humans (Ron-Harel et al., 2008). Conversely, recent evidence points to a deleterious action of the insulin/IGF-I pathway in brain aging (Cohen et al., 2009).

Serum IGF-I is synthesized by the liver in response to growth hormone and can be transported through the blood brain barrier (BBB) into the brain (Pardridge, 1993). Circulating levels of IGF-I determine in part the rate of its transport into the brain as systemic administration of IGF-I increases brain IGF-I levels (Carro et al., 2000). However, while both serum and brain IGF-I levels decrease in aged individuals (Breese et al., 1991; Niblock et al., 1998), whether its rate of serum-to-brain transport is also reduced and in this way contributes to reduced brain IGF-I input is not known. In this work we have found that aged mice show increased uptake of peripheral IGF-I even though brain IGF-I function is reduced.

2. Material and methods

2.1 Materials and animals: Young (4 months-old) and old (>22 months) C57BL6 (Spanish site) and CF1 (Brazilian site) inbred male mice were kept under controlled environmental conditions (12 hour light cycle, 22±2ºC). Comparisons between young and old animals were done within the same strain. Animals had free access to food and water. All experiments were approved by the corresponding Animal Welfare committees. Primary antibodies against the IGF-I receptor (Santa Cruz, 1:1000), phosphoTyrosine (pTyr, Santa Cruz, 1: 1000), pSer473 Akt (Cell Signaling, 1:1000) Akt (Cell Signaling, 1:3000), pSer19GSK 3ß (Cell Signaling, 1:1000), GSK 3ß (Cell signaling, 1:3000), βactin (Sigma, 1:5000), and secondary antibodies (1:3000, anti-rabbit, Cell Signaling; 1:5000 anti-mouse, Santa Cruz) were used. Protein A-agarose (Invitrogen) was used in immunoprecipitations.

2.2 Experimental design: To test transport of systemic IGF-I into the cerebrospinal fluid (CSF) recombinant human IGF-I (hIGF-I, 1μg/gr) was injected intraperitoneously (ip). After 120 min, CSF was collected from the cisterna magna with a 31G needle attached to a 300 μl insulin syringe, and fast frozen at –80º C. In case the CSF mixed with blood the sample was discarded. This dose of hIGF-I resulted in serum hIGF-I levels around 500-fold higher than in CSF, confirming that a minimal fraction of circulating IGF-I enters this compartment (Armstrong et al. 2000). To test the effects of systemic IGF-I on brain IGF-I receptor function hIGF-I was injected ip and 2 hours later mice received 50 mg/kg pentobarbital and intracardially perfused with saline. Brains and sera were collected and frozen.

2.3 Immunoassays: Western blots and immunoprecipitations were performed as described before (Pons and Torres-Aleman 2000). Briefly, after electrophoresis of hippocampal proteins (30 ug/lane), gels were transferred and thereafter membranes incubated overnight at 4 oC with primary antibodies and then incubated with secondary antibodies during 2h at room temperature. The films were scanned and band intensity was analyzed using Image J software. Human- and murine-specific ELISAs were used as described (Trejo et al. 2007) to determine hIGF-I in CSF and brain and serum IGF-I, respectively. Briefly, serum samples were extracted using Sep-Pak C18 cartridges while tissue samples were acidified in 1 N acetic acid, boiled at 100ºC/20 min, lyophilized prior to analysis, and reconstituted in assay buffer (Trejo et al., 2007).

2.4 Behavioral tests: Spatial learning was evaluated using a standard water maze test (n=12 per group) as described (Trejo et al., 2007). In brief, mice were trained in a 4-trial water maze task for 4 consecutive days. The next day a probe trial without the platform was performed to evaluate preference for the platform quadrant (short-term retention). A cued version protocol was conducted to rule out possible sensorimotor and motivational differences between experimental groups. Swimming speed was found to be similar in all groups at all days (not shown). For object recognition, mice were randomly allocated to individual boxes (50 x 50 x 50 cm) placed in soundproof and diffusely illuminated room, for 3 min in day 1. On day 2, mice were familiarized with two identical plastic objects placed in the cage, one in each corner. Each session lasted 3 min. Following the novel-place

test trial, the mice were returned to their cages for 24h and then placed in the arena for a novel object recognition test, in which one of the objects was replaced with a novel object. All objects and the arena were thoroughly cleaned with 10% ethanol between trials to remove odors. The number of exploratory events (exploratory index) for each object during the training and testing trials was recorded. 'Exploration' was defined as approaching the object nose-first within 1 cm, sniffing, and touching the object with the tip of the nose and/or with the paws.

2.5 Statistics. A t-test was used when comparing two groups and post-hoc comparisons. Water maze results were analyzed with two methods. In the learning curves, escape latencies of all animals across days and groups were compared with a repeated measures ANOVA and post-hoc tests. In Figure 3A, representing escape latencies, significant differences between groups are indicated with asterisks. In the probe trials a Student´s t-test was used.

3. Results

We first confirmed that both serum and brain IGF-I levels were significantly reduced in old as compared to young mice (Figure 1A,B). However, transport of serum IGF-I into the brain was enhanced in old animals (Figure 1C). Thus, after intraperitoneal injection of hIGF-I, CSF hIGF-I levels were slightly but significantly higher in old than in young mice ($p<0.05$). This cannot be attributed to changes in the levels of IGF-I receptor

in the choroid plexus as these were similar in young and old animals (Figure 2A).

Because IGF-I function is reduced during aging (Arvat et al. 2000) we next determined whether brain IGF-I receptors (IGF-IR) respond to a bolus systemic injection of IGF-I (1 µg/gr) as previously shown in young animals (Carro et al. 2005). We measured brain levels of the active forms of the IGF-I receptor in hippocampus (pTyrIGF-IR), and its downstream kinase Akt (pSer473 Akt), as well as the Akt target, GSK3β (pSer9) that becomes phosphorylated in serine residues and in this way is inactivated. At baseline, activation of the brain IGF-I signaling pathway in hippocampus was similar between young and old animals, as illustrated by the same level of pAkt and pGSK3β (Figure 2 C and D respectively). However, while young animals responded to systemic IGF-I by increasing brain IGF-IR, Akt and GSK phosphorylation levels, old animals were significantly less responsive to IGF-I (Figure 2 C-D). Despite this reduced response to systemic IGF-I, hippocampal IGF-I receptor levels were significantly higher in old mice (Figure 2D).

As expected from previous observations, and in parallel with reduced brain IGF-I signaling, old mice had significantly impaired learning and memory in the water maze and object recognition tasks as compared to young animals (Figure 3A,B). Furthermore, hippocampal levels of the vesicular glutamate transporter 1 (VGlut1), a biomarker of glutamatergic synapses that is decreased in cognitively deteriorated young mice with reduced serum IGF-I levels (Trejo et al., 2007), were significantly decreased in old animals (Figure 3C). Conversely, levels of GAD, a marker of

GABAergic synapses, were unaltered (not shown), as previously shown also in young mice with reduced serum IGF-I levels (Trejo et al., 2007).

4. Discussion

An increased life-span in modern societies has brought a greater incidence of impaired cerebral function usually associated with aging (Bishop et al., 2010). Understanding the molecular mechanisms involved in age-associated brain impairments is thus required to propose novel interventions aiming to improve or maintain brain health. The present results indicate that aging is associated to increased transport of systemic IGF-I to the brain together with increased levels of brain IGF-I receptors. However, these increases do not result in enhanced brain IGF-I function as the canonical Akt/GSK3 pathway downstream of the IGF-I receptor is clearly impaired in the brain of old, cognitively deteriorated mice. Therefore, what may be interpreted as an attempt to compensate reduced IGF-I function during brain aging appears to be ineffective.

Putative compensatory increases in brain IGF-I receptors have already been described in age-associated neurological conditions such as Alzheimer´s disease (Moloney et al., 2010). Also, increased brain uptake of serum insulin has been documented in a mouse model of this condition (Poduslo et al., 2001). Intriguingly, transgenic mice with AD-like pathology have low serum IGF-I levels (Carro et al., 2002) and high brain IGF-I receptors (Lopez-Lopez et al., 2007), while mutant mice with deleted brain IGF-I receptors show higher serum IGF-I levels (Kappeler et al., 2008). Furthermore, treatment of AD mice with IGF-I at early stages of the disease

normalized brain IGF-I receptor levels (Lopez-Lopez et al., 2007). Collectively, these data suggest the existence of a bidirectional inverse relationship between serum IGF-I levels and brain IGF-I receptors.

In this regard, we previously hypothesized that higher levels of brain IGF-I receptors probably reflect a situation of resistance/deficiency to IGF-I/insulin in age-associated brain diseases (Carro and Torres-Aleman, 2004). Our present results suggest that this impaired response to IGF-I may also be present in normal brain aging. Indeed, resistance to IGF-I has already been documented in the normal aged muscle (Cao et al., 2007). Thus, it seems reasonable to assume that aging causes a defect in intracellular IGF-I signaling in target tissues. As increased levels of oxidative stress are associated to the aging process (Droge, 2003) and also impair neuronal IGF-I signaling (Davila and Torres-Aleman, 2008), oxidative stress may constitute a mechanism for IGF-I resistance in the aging brain. But this possibility remains speculative at present.

Reduced IGF-I signaling probably contributes to cognitive deterioration associated to aging because administration of IGF-I to aged rodents restores cognition (Markowska et al., 1998). In turn, reduced serum IGF-I levels during aging (Breese, Ingram, and Sonntag 1991) probably contributes to reduced brain IGF-I function.

Alternatively, reduced brain IGF-I signaling may be aimed to reduce its putative deleterious actions in the aging brain (Cohen et al., 2009) even though increased brain IGF-I receptors in aged animals suggests the opposite. Regardless of its ultimate beneficial or detrimental effects, we may conclude that IGF-I signaling is deteriorated in the aged brain and ensuing

homeostatic responses such as increased receptor levels and increased CSF uptake appear insufficient to compensate this loss. Whether increased CSF uptake reflects different pharmacokinetics between old and young animals of exogenously administered human IGF-I, differences in IGF-binding proteins composition, or underlies changes in the blood-CSF interface needs further studies. In this regard, normal levels of IGF-I receptors (present results), together with low levels of megalin (Carro et al., 2005) in the aged choroid plexus hints to an imbalance in the IGF-I transport system at this level.

Conclusions

Increased peripheral uptake of serum IGF-I, together with increased levels of IGF-I receptors in the aging brain may be interpreted as ineffective compensatory mechanisms in view of the reduced IGF-I function found in the aged brain. Disturbed brain IGF-I function during aging likely contributes to cognitive deterioration.

Acknowledgements

Reference List

Aleman,A., Torres-Aleman,I., 2009. Circulating insulin-like growth factor I and cognitive function: neuromodulation throughout the lifespan. Prog.Neurobiol. 89, 256-265.

Armstrong,C.S., Wuarin,L., Ishii,D.N., 2000. Uptake of circulating insulin-like growth factor-I into the cerebrospinal fluid of normal and diabetic rats and normalization of IGF-II mRNA content in diabetic rat brain. J.Neurosci.Res. 59, 649-660.

Arvat,E., Broglio,F., Ghigo,E., 2000. Insulin-Like growth factor I: implications in aging. Drugs Aging 16, 29-40.

Bishop,N.A., Lu,T., Yankner,B.A., 2010. Neural mechanisms of ageing and cognitive decline. Nature 464, 529-535.

Breese,C.R., Ingram,R.L., Sonntag,W.E., 1991. Influence of age and long-term dietary restriction on plasma insulin- like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. J.Gerontol. 46, B180-B187.

Cao,J.J., Kurimoto,P., Boudignon,B., Rosen,C., Lima,F., Halloran,B.P., 2007. Aging Impairs IGF-I Receptor Activation and Induces Skeletal Resistance to IGF-I. J Bone Miner.Res. 22, 1271.

Carro,E., Nunez,A., Busiguina,S., Torres-Aleman,I., 2000. Circulating insulin-like growth factor I mediates effects of exercise on the brain. Journal of Neuroscience 20, 2926-2933.

Carro,E., Torres-Aleman,I., 2004. The role of insulin and insulin-like growth factor I in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. Eur.J Pharmacol. 490, 127-133.

Carro,E., Trejo,J.L., Gomez-Isla,T., LeRoith,D., Torres-Aleman,I., 2002. Serum insulin-like growth factor I regulates brain amyloid-beta levels. Nat.Med. 8, 1390-1397.

Carro,E., Spuch,C., Trejo,J.L., Antequera,D., Torres-Aleman,I., 2005. Choroid Plexus Megalin Is Involved in Neuroprotection by Serum Insulin-Like Growth Factor I. Journal of Neuroscience 25, 10884-10893.

Cohen,E., Paulsson,J.F., Blinder,P., Burstyn-Cohen,T., Du,D., Estepa,G., Adame,A., Pham,H.M., Holzenberger,M., Kelly,J.W., Masliah,E., Dillin,A., 2009. Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. Cell 139, 1157-1169.

Conrad,C.D., Bimonte-Nelson,H.A., 2010. Impact of the hypothalamic-pituitary-adrenal/gonadal axes on trajectory of age-related cognitive decline. Prog.Brain Res. 182, 31-76.

Davila,D., Torres-Aleman,I., 2008. Neuronal Death by Oxidative Stress Involves Activation of FOXO3 through a Two-Arm Pathway That Activates Stress Kinases and Attenuates Insulin-like Growth Factor I Signaling. Molecular Biology of the Cell 19, 2014-2025.

Droge,W., 2003. Oxidative stress and aging. Adv.Exp.Med Biol 543, 191-200.

Kappeler,L., De Magalhaes Filho,C.M., Dupont,J., Leneuve,P., Cervera,P., Perin,L., Loudes,C., Blaise,A., Klein,R., Epelbaum,J., Le,B.Y., Holzenberger,M., 2008. Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism. PLoS.Biol 6, e254.

Lopez-Lopez,C., Dietrich,M.O., Metzger,F., Loetscher,H., Torres-Aleman,I., 2007. Disturbed cross talk between insulin-like growth factor I and AMP-

activated protein kinase as a possible cause of vascular dysfunction in the amyloid precursor protein/presenilin 2 mouse model of Alzheimer's disease. J Neurosci 27, 824-831.

Markowska,A.L., Mooney,M., Sonntag,W.E., 1998. Insulin-like growth factor-1 ameliorates age-related behavioral deficits. Neuroscience 87, 559-569.

Moloney,A.M., Griffin,R.J., Timmons,S., O'Connor,R., Ravid,R., O'Neill,C., 2010. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. Neurobiology of Aging 31, 224-243.

Niblock,M.M., Brunso-Bechtold,J.K., Lynch,C.D., Ingram,R.L., McShane,T., Sonntag,W.E., 1998. Distribution and levels of insulin-like growth factor I mRNA across the life span in the Brown Norway x Fischer 344 rat brain. Brain Res. 804, 79-86.

Pardridge,W.M., 1993. Transport of insulin-related peptides and glucose across the blood- brain barrier. Ann.N.Y.Acad.Sci. 692, 126-137.

Poduslo,J.F., Curran,G.L., Wengenack,T.M., Malester,B., Duff,K., 2001. Permeability of proteins at the blood-brain barrier in the normal adult mouse and double transgenic mouse model of Alzheimer's disease. Neurobiol.Dis. 8, 555-567.

Pons,S., Torres-Aleman,I., 2000. Insulin-like Growth Factor-I Stimulates Dephosphorylation of Ikappa B through the Serine Phosphatase Calcineurin (Protein Phosphatase 2B). Journal of Biological Chemistry 275, 38620-38625.

Ron-Harel,N., Segev,Y., Lewitus,G.M., Cardon,M., Ziv,Y., Netanely,D., Jacob-Hirsch,J., Amariglio,N., Rechavi,G., Domany,E., Schwartz,M., 2008.

Age-dependent spatial memory loss can be partially restored by immune activation. Rejuvenation.Res. 11, 903-913.

Trejo,J.L., Piriz,J., Llorens-Martin,M.V., Fernandez,A.M., Bolos,M., LeRoith,D., Nunez,A., Torres-Aleman,I., 2007. Central actions of liver-derived insulin-like growth factor I underlying its pro-cognitive effects. Mol Psychiatry 12, 1118-1128.

LEGENDS TO FIGURES

Figure 1: Changes in IGF-I input during aging. A, B, Serum and brain levels of IGF-I are significantly decreased in old mice (n=6 per group). C, In response to a systemic injection of hIGF-I, old mice transport significantly more hIGF-I into the CSF (n= 14). *$p<0.05$, ***$p<0.001$. Bars represent mean ± SEM.

Figure 2: IGF-I signaling is impaired in the aged hippocampus. (A) Levels of IGF-IR in choroids plexus did not change in both groups. (B) In response to a bolus systemic injection of hIGF-I, phosphorylation of the IGF-I receptors (IGF-IR) in the hippocampus is reduced in old mice as compared to younger animals (n=6). IGF-IR were immunoprecipitated from hippocampus and blotted against pTyr residues and thereafter against IGF-IR for load control. C, Similarly, Akt phosphorylation in response to systemic hIGF-I is reduced

in hippocampus of the old mice (n=14). However the basal levels of the Akt phosphorylation as well as total Akt protein did not change in old mice. D, Phosphorylation of GSK3 is also reduced in hippocampus of the aged mice after hIGF-I administration in comparison to young animals (n=14). However the basal levels of the GSK3 phosphorylation as well as total GSK3 protein did not change in old mice. E, Old mice have higher levels IGF-I receptors in hippocampus as compared to young mice (n=14). *p<0.05, ***p<0.001.

Figure 3: Cognitive deterioration and glutamatergic imbalance in old mice. A, Learning in the water maze was significantly impaired in old mice as shown by slower learning capacity (left) and poorer short-term recollection of the platform location (right bars). B, Old mice also showed lower ability to recognize novel objects. C, Brain levels of the glutamate transporter VGlut1, an indicator of glutamatergic synapses, were significantly reduced in old, cognitively impaired mice. n=14 per group in all experiments. *p<0.05.
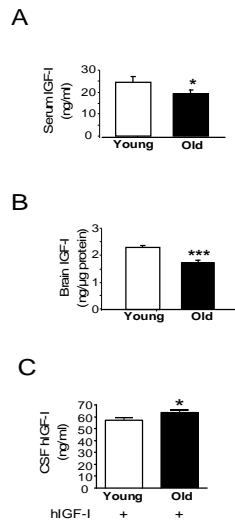
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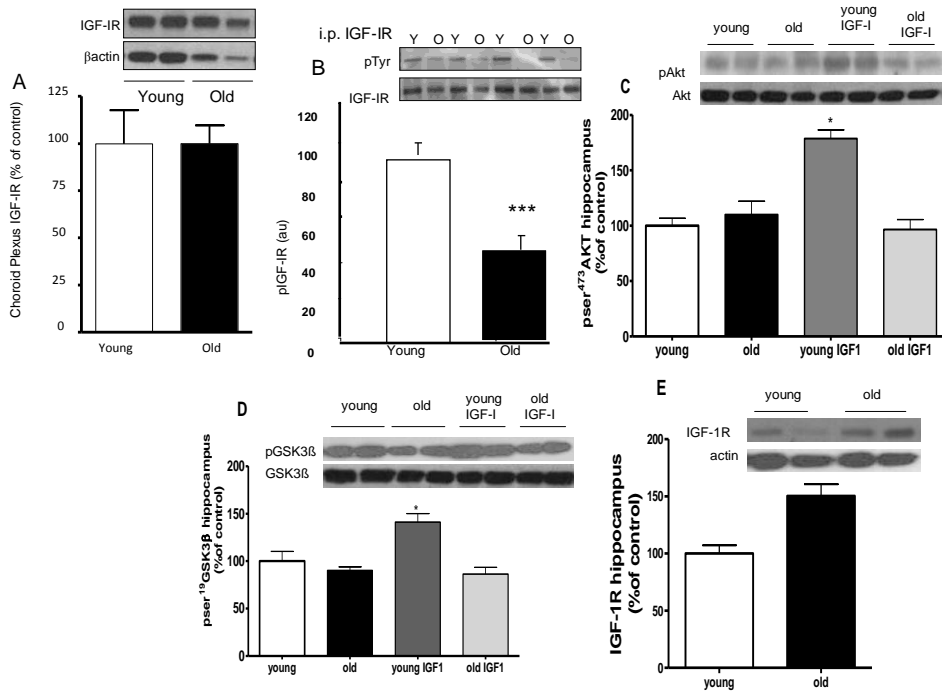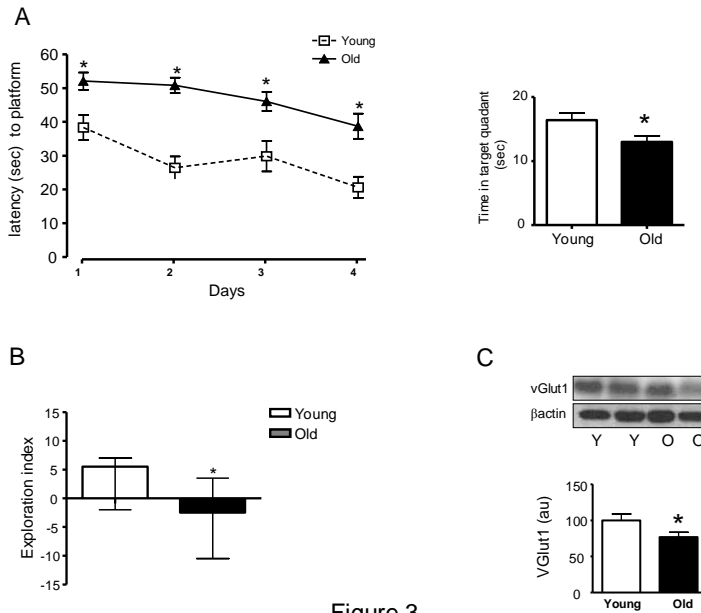
Capitulo 3:

Artigo submetido ao periódico Neurobiology of Aging

Insulin intracerebroventricular administration improves spatial memory and BDNF levels in young but not in aged rats: morphological and inflammatory responses in the hippocampus

Clarissa B. Haasa, Eduardo Kalininea, Eduardo Rigon Zimmera, Jean Pierre Osesa,b, Jussânia Gnoattoa, Diogo O. Souzaa, Luis V. Portelaa, Alexandre P. Mullera*

a. Departamento de Bioquímica, ICBS, UFRGS. Programa de Pós Graduação em Ciências Biológicas-Bioquímica. Rua Ramiro Barcelos, 2600 anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

b. Programa de Pós-Graduação em Saúde & Comportamento Centro de Ciências da Vida e da Saúde Universidade Católica de Pelotas. Rua Almirante Barroso,1202 sala G109 96010-280 Pelotas, Rio Grande do Sul Brasil.

 Corresponding author:

Prof. Alexandre Pastoris Muller, MSc

Departamento de Bioquímica, ICBS, UFRGS

Rua Ramiro Barcelos, 2600 anexo

CEP 90035-003

Porto Alegre, RS, Brazil

Email: alexandrep.muller@gmail.com

Telephone: 55 51 33085557

Fax: 55 51 33085544

 Abstract

Impaired hippocampal insulin signaling is associated with cognitive decline with aging. The modulation of brain-derived neurotrophic factor (BDNF), mammalian target of rapamycin (mTor), forkhead transcription factor 1 (FOXO1), tumor necrosis factor-α (TNFα) and chromatin-associated nuclear protein poly (ADP-ribose) polymerase 1 (PARP-1) by insulin participate in the mechanisms of neuronal survival/degeneration during aging. We investigated the effects of insulin on spatial memory performance (water maze) and hippocampal morphology. Hippocampal immunocontent of BDNF, mTor, FOXO1, PARP-1, and TNF-α were also evaluated. Wistar rats aged (26 months,n=22) and young (4 months,n= 24) received icv insulin 20 mU/vehicle once a day for five consecutive days. In young rats, insulin improved spatial memory, increased neuronal fluorescence density (CA1) and BDNF levels compared to aged rats. Moreover, insulin diminished PARP-1 levels in aged rats, while decreased FOXO1, TNF-α, phosphorylated mTORser4881 and ser4888 levels in young and aged rats. In conclusion, insulin improved spatial memory performance in young rats by increasing BDNF and CA1 density. Furthermore, insulin decreased proinflammatory factors in aged and young rats.

Key words: Aging, insulin resistance, inflammation, learning and memory, brain.

Introduction

Aging is a major risk factor for the development of brain structural and functional alterations associated with neurodegenerative disorders. Glial and neuronal cells in the central nervous system (CNS) are affected by aging, which results in a continuous and progressive decline in sensory, motor and cognitive function (Hofer et al., 2003). The loss of neuronal cells along with the disruption of neural systems that participate in different aspects of cognitive function, particularly in the frontal cortex and hippocampus, has been implicated in the cognitive decline associated with aging (Whitwell and Jack, 2005). Although the majority of brain regions are affected, the hippocampus has been shown to have an increased vulnerability to cell death associated with aging. Even the hippocampal neurons from the CA1, CA3 and dentate gyrus present differences in relation to their physiology and their vulnerability to cell death (Jackson and Foster, 2009). The cellular and molecular mechanisms of the brain that participate in aging include DNA damage, increased inflammatory response and impaired hormonal signaling (Amor et al.; Mattson). Furthermore, peripheral hormones modulate many aspects of structure, function and neural plasticity (Dietrich et al., 2008; McNay, 2007; McNay et al.; Muller et al.); however, these effects can be disrupted by aging (Cole and Frautschy, 2007; Gispen and Biessels, 2000). Insulin receptors (IR) are primarily localized in neurons and have neuromodulatory and neurotrophic roles (van der Heide et al., 2006). Insulin/IR mediate brain responses through a variety of signaling proteins and transcription factors, including the mammalian target of rapamycin

(mTor) (Hoeffer and Klann) and the forkhead transcription factor 1 (FOXO1) (Cheng and White), which have been implicated in the control of neuronal survival/degeneration. Moreover, depending on the brain region, insulin has distinct functional roles. Insulin/IR signaling was shown to improve learning, memory formation and synaptic plasticity in the hippocampus and cerebral cortex (Plum et al., 2005), whereas in the hypothalamus insulin suppressed feeding behavior (Bruning et al., 2000). Interestingly, the beneficial effects of insulin on cognitive function and neural plasticity are proposed to be mediated by brain-derived neurotrophic factor (BDNF) (Mattson et al., 2004). Recently, it has been proposed that the decreased brain insulin/IR signaling that occurs through central insulin resistance is involved in the pathophysiology of neurodegenerative disorders and impaired cognitive function (de la Monte et al., 2009; de la Monte and Wands, 2005; Porte et al., 2005). Thus, disturbances in insulin signaling have been linked to neurodegeneration during the aging process (Cohen and Dillin, 2008). Although the precise mechanisms of action are unclear, aging has been shown to influence glial activation, resulting in the increased production of the pro-inflammatory cytokine, tumor necrosis factor-α (TNFα) (Block et al., 2007; Swardfager et al.). Furthermore, the chromatin-associated nuclear protein, poly (ADP-ribose) polymerase 1 (PARP-1), is also activated by age-related metabolic and inflammatory stress signals (Altmeyer and Hottiger, 2009; Beneke, 2008; De Martinis et al., 2005).

The purpose of this study was to investigate the effects of intracerebroventricular insulin administration on spatial memory performance and hippocampal morphology in aged rats. In addition, we examined

whether insulin modulated hippocampal proteins (BDNF, mTor, FOXO1, PARP-1, TNF-α) participate in the mechanisms involved in neural cell survival or degeneration.

Material and methods

Animals

Male Wistar rats (n=24) were housed in plastic cages in a temperature-controlled room under a 12 hr light/12 hr dark cycle with free access to food and water. The rats (4-5 per cage) were assigned to one of following groups: young (4 months age) (n=12), young insulin (n=12), aged (24 months age) (n=12) and aged insulin (24-26 months age) (n=10). 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 of the Federal University of Rio Grande do Sul, Brazil.

Surgical procedure and insulin icv treatment

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 at 0.9 mm posterior to the bregma, 1.5 mm right of the midline and 1.0 mm above the right lateral brain ventricle. The cannula was implanted 2.6 mm ventral to the superior surface of the skull through a 2-mm hole made in the cranial bone (Torres et al.). On the third day post-surgery, the rats already displayed normal food intake and water consumption as well as spontaneous locomotion; therefore, they were considered ready for in vivo experiments. Animals received 5μL of insulin

(20 mU) (Lilly, Humulin) or vehicle for 5 min (1 µL/min) via intracerebroventricular (icv) once a day for 5 consecutive days.

Body weight and Food intake

Body weight and food intake were concomitantly monitored for five days of insulin or saline icv administration. Each day, food was weighed and added to the cages. The ratio between these days was divided by the number of the animals per cage and was used to estimate food intake.

Glucose tolerance test - GTT

To assess the potential influence of icv insulin treatment on peripheral glucose sensitivity, we measured glucose levels (n=8 per group) 1 day after the last icv injection (day 6). The rats were fasted for 12 h, and blood was then collected via a small puncture in the tail; blood was collected immediately before (0 min) and 30, 60, and 120 min after an i.p. injection of glucose (2 mg/g body weight). Glucose was measured using a glucosimeter (AccuChek Active, Roche Diagnostics®, USA).

Spontaneous locomotor activity – open field task

All experiments were conducted between 09:00 a.m. and 04:00 p.m. Two days after the last insulin icv administration (day 7), rats were randomly allocated into four individual wooden boxes (50 x 50 x 50 cm) placed on the floor of a soundproof and diffusely illuminated room. Videos of the spontaneous locomotion were obtained for 5 min and analyzed using the N-Maze program.

Spatial memory - Morris water maze task

Three days after the last insulin icv administration, the rats performed the spatial memory task (days 8 to 13). The apparatus is a black circular pool

(200 cm in diameter) with a water temperature of 21 ± 1 °C. During training, the rats learned to escape from the water by finding a hidden rigid black platform submerged about 1 cm below the water surface in a fixed location. The rats were trained over 4 trials in a water maze task for five consecutive days, with each trial lasting up to 60 s, and the rats were allowed to rest on a hidden black platform for 20 s. Each trial was separated by a minimum of 12 min. If the rat failed to find the platform in 60 s, it was placed on the platform and allowed to rest for 20 s. The maze was located in a well-lit white room with several visual stimuli hanging on the walls to provide spatial cues. The escape latency during each trial was measured as an indicator of learning. A probe test was performed without the platform twenty-four hours after the five day of acquisition, and the time spent in the target quadrant was measured as an indicator of memory retention. Videos were obtained and analyzed using the N-Maze program.Twenty-four hours after the Morris water maze task, the animals were sacrificed by decapitation (day 14) for collecting biological samples.

Hippocampal BDNF and TNF-α levels

After decapitation, the hippocampus was dissected and homogenized in PIK buffer (1 % NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM CaCl2, 1 mM MgCl2, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 0.1 % phosphatase inhibitor cocktails I and II; Sigma-Aldrich, USA). The homogenate was centrifuged, and the supernatant was collected. The total protein content was measured as described by Peterson (Peterson, 1977). The homogenates were stored at -70 oC until analysis. The BDNF and TNF-α assays were performed using

commercially available ELISA kits (refs. DY248 and DY510). The protocol was conducted according to the instructions of the manufacturer (R&D systems, USA).

Western blot

For western blot analysis, 30 μg of protein from hippocampal tissue homogenates were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to nitrocellulose membranes. Non-specific binding sites were blocked with Tween–Tris buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5) containing 5% albumin for 2 h and then incubated overnight at 4 °C with polyclonal antibodies against PARP1 (Cell Signaling, 1:500), pmTor ser4881 (Cell Signaling, 1:1000), pmTor ser4888 (Cell Signaling, 1:1000), mTor (Cell Signaling, 1:1000) and actin (Sigma, 1:5000). The membranes were rinsed 3x for 10 min with TTBS and incubated with secondary antibodies (1:3000 dilution, anti-rabbit, Cell Signaling Technology; 1:5000 dilution anti-mouse, Santa Cruz Technology) for 2 h at room temperature. Next, the membranes were rinsed 4x for 10 min with TTBS and incubated with peroxidase-conjugated for 5 min at room temperature. The resulting reaction was displayed on autoradiographic film by chemiluminescence. The films were scanned, and band intensity was analyzed using Image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Histochemistry and Immunohistochemistry

For the histochemical and immunohistochemical studies, the animals (4-6 rats per group) were sacrificed, and the left hemispheres were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 24 h and then

placed in a 30% sucrose solution in PB at 4 °C. Coronal sections (50 µm) were obtained using a Vibratome (Leica, Germany) and stored in PBS 0.2 M buffer. One out of 8 random series were collected for analyses. First, we analyzed the cellular density. Briefly, the sections were hydrated in 95% and 70% ethanol and distilled water, stained with filtered cresyl violet solution (5 mg/mL cresyl violet acetate) for 5 minutes, rinsed in water, and then dehydrated with 70%, 95%, and 100% ethanol. The slides were mounted using Canada balsam. The histochemistry images were acquired on an Olympus microscope (Olympus, Japan). Double immunohistochemical staining was used to identify neurons and glial cells. The sections were incubated for 48 h at 4 °C with polyclonal rabbit GFAP antiserum (Dako, UK, 1:500) and NeuN (Chemicon, 1:500) in PBS-Tx containing 2% BSA. After several PBS washes, the sections were incubated with 594 nm Alexa-conjugated donkey anti-rabbit and 488 nm Alexa-conjugated donkey anti-mouse antibodies for 24 hr at 4 °C. Next, the slices were stained with DAPI (0.0001%; Milipore) to stain the nuclei. After 24 h, the sections were mounted with FluorSave Reagent (Calbiochem). To determine the density of fluorescence, sections were photographed using a confocal fluorescence microscopy system (Olympus FV1000) and analyzed using Image J software. All images were analyzed using the ROI manager Image J toll. Three ROIs were used from the same coordinate and in the same image area. After each ROI analyses, the mean of the ROI fluorescence was estimated.

Statistical analysis

The results were calculated and expressed as the means ± S.E.M. The data from the water maze task was analyzed using a repeated-measures analysis of variance (ANOVA) followed by a post-hoc Tukey test. To evaluate the effect of insulin between groups, we performed a Student's t-test between groups. For analyzing differences between ages, we used a two-way ANOVA. To analyze the differences between groups, we used one-way ANOVA followed by a post-hoc Tukey test. The differences were considered statistically significant at $p < 0.05$.

Results

Food intake, body weight and glucose metabolism monitoring

Insulin injected icv decreased food intake during the five days of administration in both young and aged rats (Table 1; young > young insulin; aged > aged insulin $p < 0.05$). Furthermore, insulin decreased the food intake 70% in young and 35% in aged rats. Consistent with these results, the body weight also decreased after five days of icv insulin administration (body weight young > young insulin; aged > aged insulin $p < 0.05$). The body weight was decreased 34% in young and 23% in aged rats (Table 1). Although both groups were affected, insulin decreased the food intake and body weight significantly more in young than in old rats. Thus, we showed that young rats are more sensitive to icv insulin administration than aged rats in their feeding behavior and body mass control. Next, we analyzed the effect of insulin on peripheral glucose homeostasis 24 h after the five days of icv administration. Insulin slightly improved GTT in young rats. The area

under the curve decreased in young insulin rats compared to the other groups (young insulin < young, aged and aged insulin; $p < 0.05$) (Table 1). Two hours after icv administration of insulin, the serum glucose levels were not significantly different between the groups (data not shown).

Aging decreased spontaneous locomotion and insulin had no effect

To evaluate the effects of insulin on locomotor activity, we performed an open field task two days after the five days of insulin icv administration (day 7). Decreased locomotor activity was found in the aged rats compared to the young groups, and insulin did not affect locomotor activity in the young and aged rats (young and young insulin>aged and aged insulin; $p < 0.05$). Moreover, the aged rats showed reduced speed during exploration compared to the young rats, and insulin did not affect the speed of both young and aged rats (Figure 1B-C).

Insulin improves spatial memory performance in young rats and increases BDNF hippocampal levels

On the third day after insulin icv administration, the spatial memory performance was evaluated using the Morris water maze task (MWM) (from days 8 to 13). The hippocampus plays a key role in the formation of memory in this paradigm. From the third to fifth days of the acquisition phase, the young insulin animals showed an improvement in learning (decreased time to find hidden platform) compared to the other groups (latency of young insulin<young, aged and aged insulin; $p<0.05$). In addition, the time the aged rats spent finding the platform was not different on days 3-5, whereas the young vehicle group trended toward a decrease in time spent finding the platform until the last day of the acquisition phase. The memory retention

phase was evaluated in a probe trial performed without the platform on day eight after icv insulin injection (day 13). The young insulin icv animals spent more time in the target quadrant when compared to the other groups (young insulin > other groups; $p < 0.05$) (Figure 2A-B), and the swim speed was not different between the groups (data not shown).

We also investigated whether insulin icv administration could modulate the level of BDNF in hippocampal tissue, which might positively impact neural plasticity and would reflect an improvement in the performance of the spatial memory task. Indeed, nine days after the last injection of insulin (day 14), hippocampal BDNF levels increased in young animals as compared to the young vehicle (young insulin > young vehicle, $p < 0.05$). The young vehicle, aged vehicle and aged insulin showed similar hippocampal BDNF levels (Figure 2C).

Insulin icv administration attenuates the hippocampal inflammatory response and the phosphorylation status of signaling proteins involved in cell death/survival

The aged rats showed increased levels of PARP-1 in the hippocampus compared to the young rats, but icv insulin administration reduced PARP-1 to the levels of the young animals (Figure 3A, aged vehicle > other groups; $p < 0.05$). In addition, insulin decreased the hippocampal TNF-α levels in both the young and aged rats when compared to their respective vehicle counterparts (Figure 3F; young vehicle > young insulin and aged vehicle > aged insulin, $p < 0.05$).

Regarding signaling proteins, there were increased hippocampal levels of mTor associated with aging (Figure 3D, aging vehicle > other groups; $p <$

0.05). Insulin administration decreased the phosphorylation levels of pmTor ser4881 and pmTor ser4888 (Figure 3B-C, young vehicle > young insulin and age vehicle > aged insulin, $p < 0.05$). In addition, the total level of FOXO1 was increased in aged rats and was significantly decreased in the young insulin animals compared to the young vehicle (Figure 3E; young vehicle > young insulin, $p < 0.05$), whereas in aged animals insulin had no effect.

Insulin treatment increases neuronal and glial immunoreactivity in hippocampal subfields

We analyzed the cellular density and the fluorescence density of neurons, glial cells, and nuclei in hippocampal CA1, CA3 and dentate gyrus subfields.

Effect on CA1 subfield

The cresyl violet stained images (Figure 4 A-D) showed that aging decreased the cellular density in the CA1 (4C). However, insulin icv administration increased the cellular density in the CA1 in both the young and aged rats (Figure 4B and D).

When examining the neuronal fluorescence density (NeuN), the aged rats showed a decreased fluorescence density compared to the young rats (Figure 5A young > aged; *$p < 0.05$). Insulin icv treatment increased the fluorescence density of NeuN in the young and aged rats when compared with the vehicle groups (Figure 5A young insulin > young; aged insulin > aged; *$p < 0.05$). The fluorescence density of GFAP positive cells, a glial marker, was not significantly different between the groups or treatment. Furthermore, there was a decreased nuclear fluorescence density of DAPI in the aged rats compared to the young rats (young > aged; *$p < 0.05$). Insulin

increased the nuclear fluorescence density in the young and aged rats (young insulin>young; aged insulin > aged; *p < 0.05). Overall, the neurons in the CA1 showed an increased fluorescence density after insulin icv administration (Figure 5A-C).

Effect on CA3 subfield

The cresyl violet stained images (figure 4 E-H). Aging decreased the cellular density in the CA3 subfield (Figure 4G), and insulin had no effect (Figure 4H). Moreover, aging decreased the NeuN fluorescence density compared to the young rats, and insulin did not change the fluorescence density (Figure 6A young and young insulin > aged and aged insulin; $p<0.05$). Neither the GFAP nor DAPI fluorescence density was affected by aging or insulin treatment (Figure 6B-C).

Effect on dentate gyrus subfield

The cresyl violet stained images (Figure 4I-L). Insulin had no effect in young animals (Figure 4 I-J). Aging decreased (Figure 4K), and insulin increased (Figure 4L) the cellular density in the dentate gyrus of aged animals. Insulin treatment did not affect the fluorescence density of neurons (NeuN) in the young compared to the young vehicle rats. The aged rats showed a decreased NeuN fluorescence density when compared to the young rats (Figure 7A young > aged; *p < 0.05); however, insulin icv treatment raised the neuronal fluorescence of aged rats to a similar level to that of the young rats (aged insulin = young groups). Astrocytic and nuclear (GFAP and DAPI, respectively) fluorescence were not affected by aging or insulin treatment (Figure 7B-C).

Discussion

Our study provides further support that insulin has important functional roles in the brain and suggests that aging decreases central insulin sensitivity. This study showed that insulin acts as a neurotrophic factor in the hippocampus by increasing BDNF levels in young animals and improves learning and memory performance in the MWM; however, in aged rats, the action of insulin was less effective when compared to young animals.

The hippocampus has an essential role in rodent spatial memory and navigation (Moosavi et al., 2006). Subfields from the hippocampus have many different functions in learning and memory. It has been demonstrated that the CA3 lesioned rats showed impaired performance in the water maze task; however; the CA1 lesioned rats were not impaired. However, the CA3 subfield is also important for the acquisition and encoding of spatial information in short-term memory with a duration of seconds to minutes (Kesner, 2007). Our results showed an increase in the fluorescence density of neurons in the CA1 subfield by insulin in both the young and aged rats; however, in the CA3 subfield of aged rats, there was a decrease in the fluorescence density, whereas insulin had no effect. Aged rats are consistently impaired in learning the location of the escape platform (Rosenzweig and Barnes, 2003). However, Rosenzweig et al. (Rosenzweig and Barnes, 2003) reported that more than 30% of aged rats performed as well as the adult rats. Therefore, in our work, the aged animals did not show any impairment in spatial cognition. However, the aged animals stabilized the time to found the platform on the third day (decreased the latency to find the platform by 40%; first to last day), whereas the young animals trended

towards a decrease in the time to find the platform until the last day of acquisition (decreased the latency to find the platform by 55%, first to last day); however, these results did not reach statistical significance.

Insulin icv treatment facilitated learning and memory at optimal doses in young rats (Moosavi et al., 2007; Park et al., 2000), and the insulin receptor has been shown to be selectively enriched in the CA subfields of the hippocampus (Chiu et al., 2008). However, the effect of insulin on memory performance in aged rats has yet to be elucidated. We demonstrated that insulin icv administration improved spatial learning in young rats and increased BDNF levels in the hippocampus. The increase in the neuronal fluorescence density in the CA1, the normal neuronal CA3 subfield and the increase in BDNF levels correlated with the improvement in memory performance induced by insulin in young rats, but these changes were not correlated in the aged animals. We propose that a decrease in intracellular insulin signaling, as shown by Solas et al. 2010, which was associated with changes in the connections between the cells of the hippocampal network, could reflect the lack of an effect of insulin in the spatial memory performance of aged animals. This will need to be studied further.

Inflammatory mediators are increased with aging and participate in the etiology of brain disorders and events that culminate in apoptotic cell death (Uranga et al.). In this study, we showed that insulin icv administration decreased the hippocampal levels of PARP-1, which contributes to 'inflammaging', an increased brain inflammation associated with advanced aging (Altmeyer and Hottiger, 2009; Beneke, 2008; De Martinis et al., 2005). Moreover, insulin treatment decreased the levels of TNF-α in the

hippocampus in both the young and aged animals. Thus, insulin administration showed a neuroprotective effect in the hippocampus of both the young and aged rats by mediating the production of proinflammatory factors. In contrast, insulin resistance in the aged brain is associated with an increased vulnerability of neurons to cell death and impaired cognition function (de la Monte et al., 2009). Moreover, the total mTOR levels and mTOR phosphorylated at Ser 2441 and Ser 2448 increased significantly in Alzheimer's disease, which positively correlated with an aberrant phosphorylation of Tau protein (Li et al., 2005). Similarly, we demonstrated that aged rats have an increased total mTOR content as compared to the young rats, and icv administration of insulin decreased the phosphorylation state at mTOR ser2441 and ser2448 in both the young and aged rats. In addition, FOXO1, a transcriptional factor that activates genes involved in cell death, was significantly elevated in the aged rats, and insulin administration decreased the content in only the young rats. During the aging process, both mTOR and FOXO1 could mediate neuronal death by apoptosis and autophagy (Cheng and White; Hay and Sonenberg, 2004). Thus, decreasing the hippocampal neurotrophic and anti-inflammatory properties impaired the insulin signaling with aging and made the brain more vulnerable, which increased the risk of massive neuronal death and cognitive decline.

Insulin is an anorexic hormone, and impairments in the insulin signaling pathway have been associated with obesity and neurodegenerative diseases (Schwartz and Porte, 2005). We showed that aged rats are resistant to the anorexic effect of insulin compared to the young rats. This was reflected by an increased body mass and visceral adiposity, which is a

common feature of aging process (Uranga et al.). We demonstrated that the insulin icv administration was able to regulate peripheral glucose metabolism in the young rats, and the aged rats were resistant to the icv insulin effects. As previously proposed, the brain resistance to insulin in the aged rats has been implicated in cognitive decline and metabolic disorders (Schubert et al., 2004).

In summary, our data showed that insulin icv administration increased neuronal fluorescence in the CA1 and dentate gyrus in both the young and aged rats. Insulin also improved the spatial memory performance in the young rats, which correlated with increased hippocampal BDNF levels and normal CA3 cells. Furthermore, insulin mediated the production of trophic and proinflammatory factors involved in neuronal survival or degeneration in the hippocampus of aged and young rats.

Disclosure of Potential Conflict of Interest: The authors have nothing to disclose

References

Altmeyer, M., Hottiger, M,O., 2009. Poly(ADP-ribose) polymerase 1 at the crossroad of metabolic stress and inflammation in aging. Aging (Albany NY) 1(5),458-69.

Amor, S., Puentes, F., Baker, D., van der Valk, P. 2010. Inflammation in neurodegenerative diseases. Immunology 129(2),154-69.

Beneke, S., 2008. Poly(ADP-ribose) polymerase activity in different pathologies--the link to inflammation and infarction. Exp Gerontol 43(7),605-14.

Block, M.L., Zecca, L., Hong JS., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8(1),57-69.

Bruning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Muller-Wieland, D., Kahn, C.R., 2000. Role of brain insulin receptor in control of body weight and reproduction. Science 289(5487),2122-5.

Cheng, Z., White, M.F., Targeting Forkhead box O1 from the concept to metabolic diseases: lessons from mouse models. Antioxid Redox Signal 14(4),649-61.

Chiu, S.L., Chen, C.M., Cline, H.T., 2008. Insulin receptor signaling regulates synapse number, dendritic plasticity, and circuit function in vivo. Neuron 58(5),708-19.

Cohen, E., Dillin, A., 2008. The insulin paradox: aging, proteotoxicity and neurodegeneration. Nat Rev Neurosci 9(10),759-67.

Cole, G.M,, Frautschy, S.A., 2007. The role of insulin and neurotrophic factor signaling in brain aging and Alzheimer's Disease. Exp Gerontol 42(1-2),10-21.

de la Monte, S.M., Longato, L., Tong, M., Wands, J.R., 2009. Insulin resistance and neurodegeneration: roles of obesity, type 2 diabetes mellitus and non-alcoholic steatohepatitis. Curr Opin Investig Drugs 10(10),1049-60.

de la Monte, S.M., Wands, JR., 2005. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J Alzheimers Dis 7(1),45-61.

De Martinis, M., Franceschi, C., Monti, D., Ginaldi, L., 2005. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. FEBS Lett 579(10),2035-9.

Dietrich, M.O., Spuch, C., Antequera, D., Rodal, I., de Yebenes, J.G., Molina, J.A., Bermejo F, Carro E. 2008. Megalin mediates the transport of leptin across the blood-CSF barrier. Neurobiol Aging 29(6),902-12.

Gispen, W.H., Biessels, G.J., 2000. Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 23(11),542-9.

Hay, N., Sonenberg, N., 2004. Upstream and downstream of mTOR. Genes Dev 18(16),1926-45.

Hoeffer, C.A., Klann, E. mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci 33(2):67-75.

Hofer, S.M., Berg, S., Era, P., 2003. Evaluating the interdependence of aging-related changes in visual and auditory acuity, balance, and cognitive functioning. Psychol Aging 18(2),285-305.

Jackson, T.C., Foster, T.C., 2009. Regional Health and Function in the hippocampus: Evolutionary compromises for a critical brain region. Biosci Hypotheses 2(4),245-251.

Kesner, R.P., 2007. Behavioral functions of the CA3 subregion of the hippocampus. Learn Mem 14(11),771-81.

Li, X., Alafuzoff, I., Soininen, H., Winblad, B., Pei, J.J., 2005. Levels of mTOR and its downstream targets 4E-BP1, eEF2, and eEF2 kinase in relationships with tau in Alzheimer's disease brain. FEBS J 272(16),4211-20.

Mattson, M.P., The impact of dietary energy intake on cognitive aging. Front Aging Neurosci 2,5.

Mattson, M.P., Maudsley, S., Martin, B., 2004. A neural signaling triumvirate that influences ageing and age-related disease: insulin/IGF-1, BDNF and serotonin. Ageing Res Rev 3(4),445-64.

McNay, E.C., 2007. Insulin and ghrelin: peripheral hormones modulating memory and hippocampal function. Curr Opin Pharmacol 7(6),628-32.

McNay, E.C., Ong, C.T., McCrimmon, R.J., Cresswell, J., Bogan, J.S., Sherwin, R.S., 2010 Hippocampal memory processes are modulated by insulin and high-fat-induced insulin resistance. Neurobiol Learn Mem. 93(4),546-53.

Moosavi, M., Naghdi, N., Choopani, S., 2007. Intra CA1 insulin microinjection improves memory consolidation and retrieval. Peptides 28(5),1029-34.

Moosavi, M., Naghdi, N., Maghsoudi, N., Zahedi, Asl, S., 2006. The effect of intrahippocampal insulin microinjection on spatial learning and memory. Horm Behav 50(5):748-52.

Muller, A.P., Gnoatto, J., Moreira, J.D., Zimmer, E.R., Haas, C.B., Lulhier, F., Perry, M.L., Souza, D.O., Torres-Aleman, I., Portela, L.V., Exercise increases insulin signaling in the hippocampus: Physiological effects and pharmacological impact of intracerebroventricular insulin administration in mice. Hippocampus. in press

Park, C.R., Seeley, R.J., Craft, S., Woods, S.C., 2000. Intracerebroventricular insulin enhances memory in a passive-avoidance task. Physiol Behav 68(4),509-14.

Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83(2),346-56.

Plum, L., Schubert, M., Bruning, J.C., 2005. The role of insulin receptor signaling in the brain. Trends Endocrinol Metab 16(2),59-65.

Porte ,D, Jr., Baskin, D.G., Schwartz, M.W., 2005. Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. Diabetes 54(5),1264-76.

Rosenzweig, E.S., Barnes, C.A., 2003. Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. Prog Neurobiol 69(3),143-79.

Schubert, M., Gautam, D., Surjo, D., Ueki, K., Baudler, S., Schubert, D., Kondo, T., Alber, J., Galldiks, N., Kustermann, E. and others. 2004. Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci U S A 101(9),3100-5.

Schwartz, M.W., Porte, D, Jr., 2005. Diabetes, obesity, and the brain. Science 307(5708),375-9.

Solas, M., Aisa, B., Mugueta, M.C., Del Río, J., Tordera, R.M., Ramírez, M.J., 2010. Interactions between age, stress and insulin on cognition: implications for Alzheimer's disease. Neuropsychopharmacology 35(8),1664-73.

Swardfager, W., Lanctot, K., Rothenburg, L., Wong, A., Cappell, J., Herrmann, N., A meta-analysis of cytokines in Alzheimer's disease. Biol Psychiatry 68(10),930-41.

Torres, F.V., da Silva Filho, M., Antunes, C., Kalinine, E., Antoniolli, E., Portela, L.V., Souza, D.O., Tort, A.B., Electrophysiological effects of guanosine and MK-801 in a quinolinic acid-induced seizure model. Exp Neurol 221(2),296-306.

Uranga, R.M., Bruce-Keller, A.J., Morrison, C.D., Fernandez-Kim, S.O., Ebenezer, P.J., Zhang, L., Dasuri, K., Keller, J.N., Intersection between metabolic dysfunction, high fat diet consumption, and brain aging. J Neurochem 114(2),344-61.

van der Heide, L.P., Ramakers, G.M., Smidt, M.P., 2006. Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol 79(4),205-21.

Whitwell, J.L., Jack, C.R. Jr., 2005. Comparisons between Alzheimer disease, frontotemporal lobar degeneration, and normal aging with brain mapping. Top Magn Reson Imaging 16(6),409-25.

Legends

1) Timeline of the experimental protocol and locomotor activity behavior

A) Time (days) of the experimental protocols. B) The distance traveled (m) during the 10 min in the open field task. The aged animals had reduced locomotor activity compared to the young animals (*aged vehicle and insulin< young vehicle and insulin; two-way ANOVA analysis $p<0.05$). Insulin had no effect. C) The mean speed (m/s) in the open field task. The aged animals had a decreased mean speed compared to the young animals (*aged vehicle and insulin< young vehicle and insulin; two-way ANOVA analysis $p<0.05$), and insulin had no effect (n= 10-12).

2) Spatial learning memory performance and BDNF levels in the hippocampus.

A) The latency to find the platform for five days in the Morris water maze task (MWM). The young insulin animals decreased the time spent finding the platform by the third day in the acquisition phase compared to the other groups (*young insulin< other groups; repeated-measures analysis of variance $p<0.05$). B) The young insulin animals increased the time spent in the target quadrant during the probe day compared to the other groups (*young insulin< other groups; one-way ANOVA followed by a post-hoc Tukey test $p<0.05$). C) The young insulin animals showed increased hippocampal BDNF levels compared to the young insulin animals (*young insulin> young vehicle; one-way ANOVA followed by a post-hoc Tukey test $p<0.05$) (n= 10-12).

3) Inflammatory parameters and proteins involved in cell death/survival in hippocampus by western blotting analysis

A) Aging increased the level of PARP-1 in the hippocampus, and insulin icv treatment decreased the levels in aged animals (*aged vehicle< other groups; PARP-1 relative to actin; one-way ANOVA followed by a post-hoc Tukey test $p<0.05$). B) and C) Insulin icv treatment decreased the phosphorylation state of mTor in Ser2441 and Ser2448 in young and aged animals (pSer2441 and pSer2448 relative to mTor; *young vehicle< young insulin, *aged vehicle< aged insulin; one-way ANOVA followed by a post-hoc Tukey test $p<0.05$). D) Aging increased the level of total mTor in the hippocampus, and insulin had no effect (mTor relative to actin; *aged young and insulin> young and vehicle, two-way ANOVA analysis $p<0.05$). E) Aging increased the level of FOXO 1 in the hippocampus, but insulin had no effect (FOXO1 relative to actin; *aged young and insulin> young and vehicle, Two-way ANOVA analysis $p<0.05$). F) Insulin icv treatment decreased the TNF-α level in the hippocampus in both the young insulin and aged insulin groups compared to the vehicle groups (*young insulin>young vehicle and aged insulin>aged vehicle; t-test $p<0.05$). G) Representative images of PARP-1, pmTor ser 2441, pmTor ser 2448, mTor, FOXO 1, actin and ponceau of membranes from western blots (n= 5-8).

4) Cellular density in CA1, CA3 and the dentate gyrus of the hippocampus

A-D) Representative images of cresyl violet staining in the CA1 subfield. Aging decreased (C) and insulin increased the cellular density in the young and aged animals (B and D). E-H) Representative images of cresyl violet staining in the CA3 subfield. Aging decreased (G) and insulin had no effect on the cellular density in the young and aged animals (F-H). I-L) Representative images of cresyl violet staining in the dentate gyrus subfield.

Aging decreased (K) and insulin increased the cellular density in the aged animals (L) (n= 4-6).

5) Morphological effects of aging and insulin icv administration in the CA1 of the hippocampus

A) Aging decreased the fluorescence density of the neuronal marker, NeuN. Insulin icv treatment increased the fluorescence density in the young and aged animals (*young insulin>young vehicle and aged insulin>aged vehicle; t-test $p<0.05$). B) The astrocytic marker, GFAP, was not altered by aging or insulin icv treatment. C) The nuclei marker, DAPI, was increased by insulin in the young and aged animals. (*young insulin>young vehicle and aged insulin>aged vehicle; one-way ANOVA followed by a post-hoc Tukey test $p<0.05$) (n= 4-6).

6) Morphological effects of aging and insulin icv administration in the CA3 of the hippocampus

A) Aging decreased the fluorescence density of the neuronal marker, NeuN. Insulin icv treatment had no effect on the fluorescence density in the young and aged animals (*young vehicle and young insulin> aged vehicle and aged insulin; t-test $p<0.05$). B) The astrocytic marker, GFAP, was not altered by aging or insulin icv treatment. C) The nuclei marker, DAPI, was not altered by insulin in the young and aged animals (*young vehicle and young insulin> aged vehicle and aged insulin; two-way ANOVA $p<0.05$) (n= 4-6).

7) Morphological effects of aging and insulin icv administration in the dentate gyrus of the hippocampus

A) Aging decreased the fluorescence density of the neuronal marker, NeuN. Insulin icv treatment increased the fluorescence density in the aged animals

(*aged insulin>aged vehicle; t-test p<0.05). B) The astrocytic marker, GFAP, was not altered by aging or insulin icv treatment. C) The nuclei marker, DAPI, was not altered by insulin in the young and aged animals (*young vehicle and young insulin> aged vehicle and aged insulin; two-way ANOVA p<0.05) (n= 4-6).

Figure 1

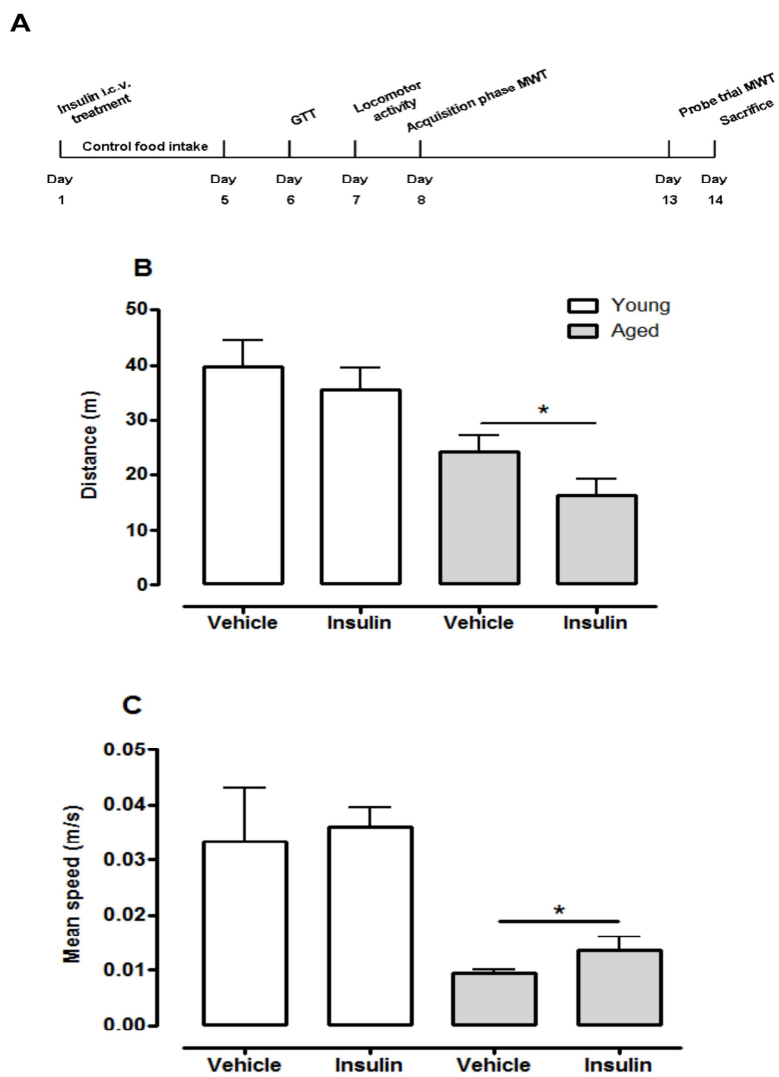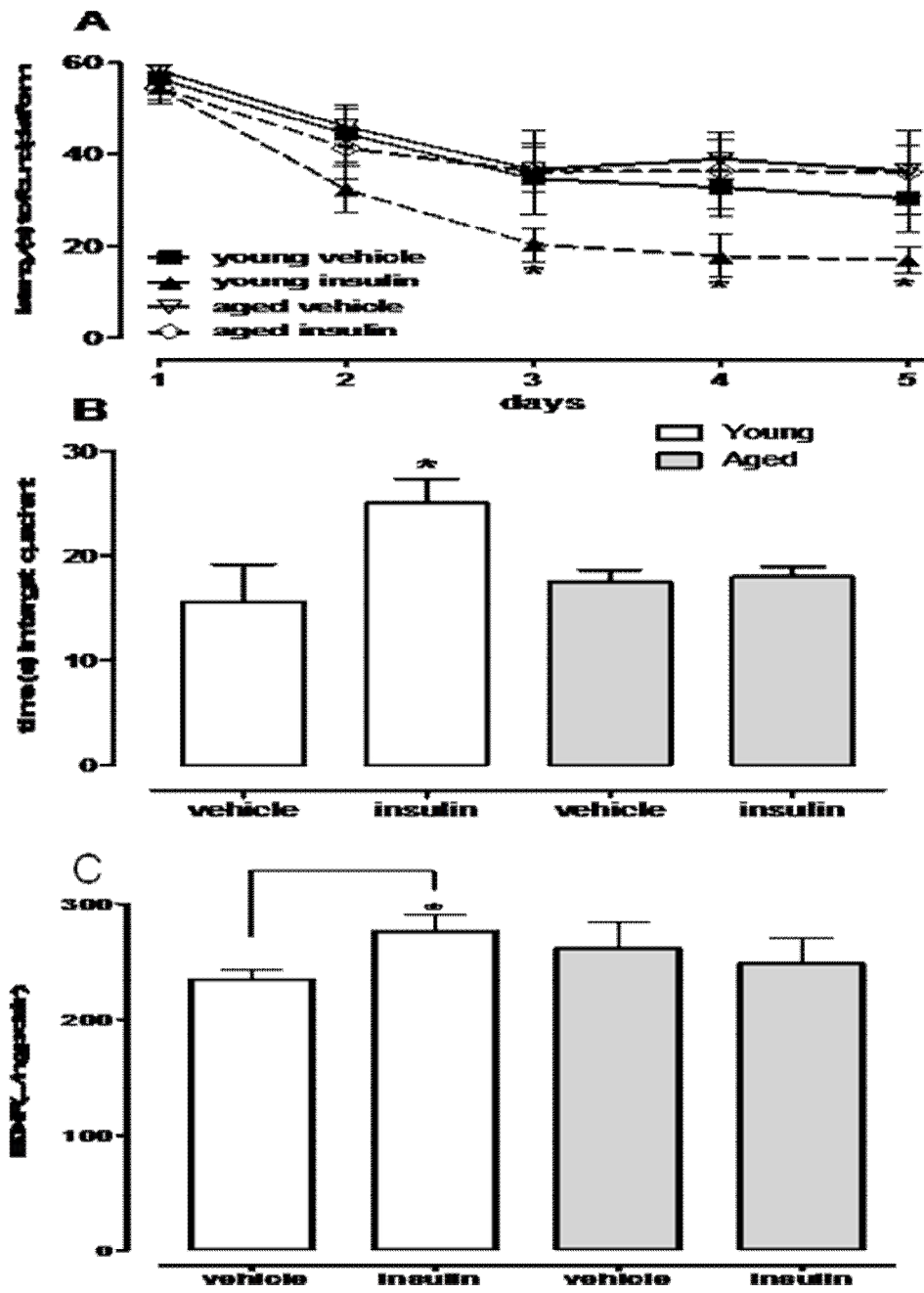Figure 2

Figure 3

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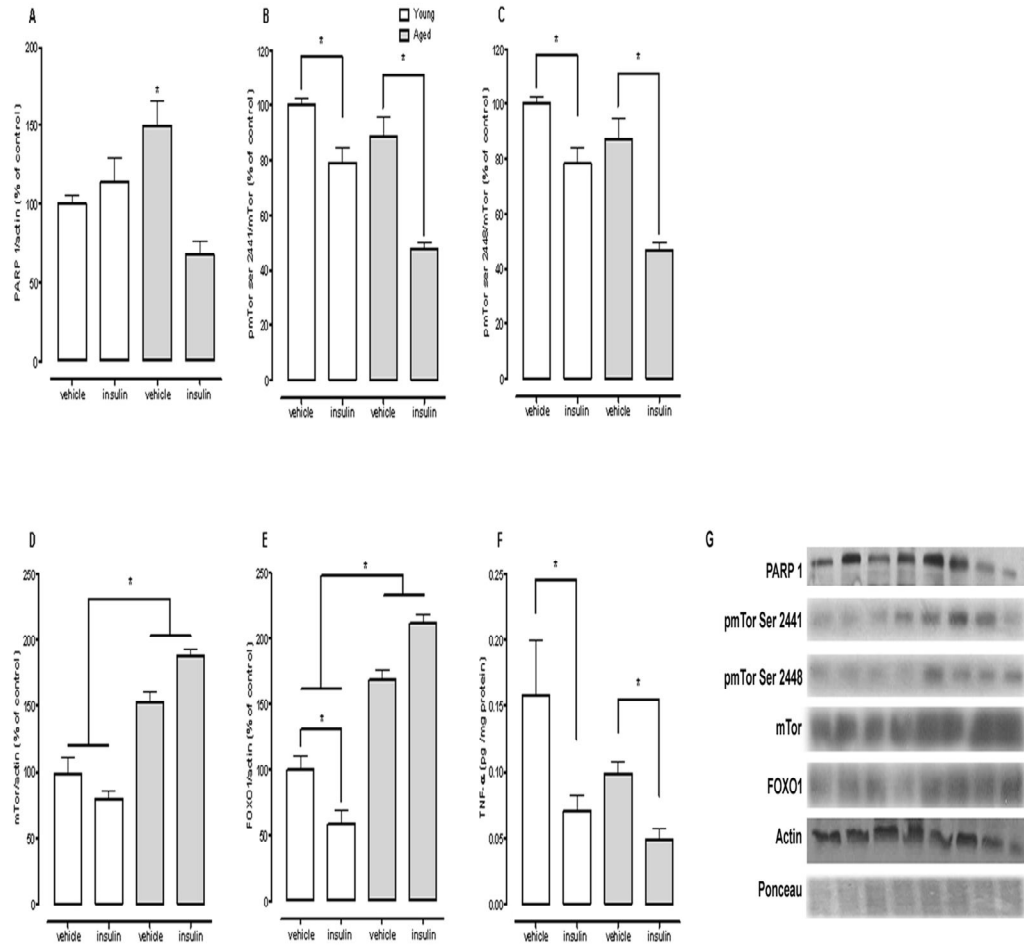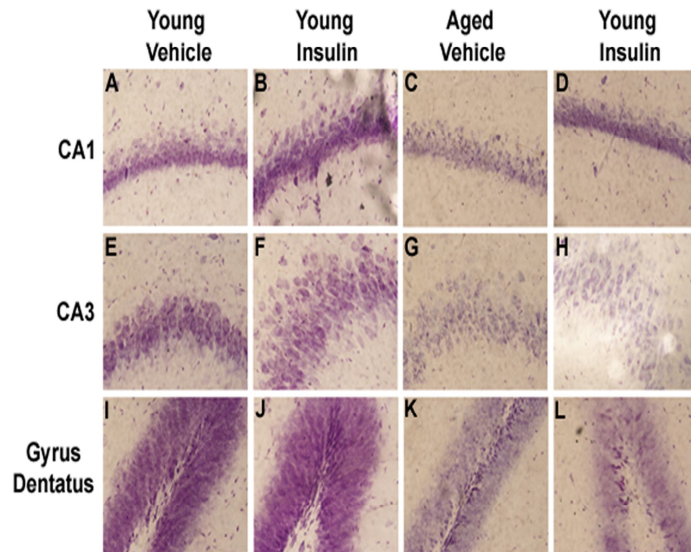
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Figure 5

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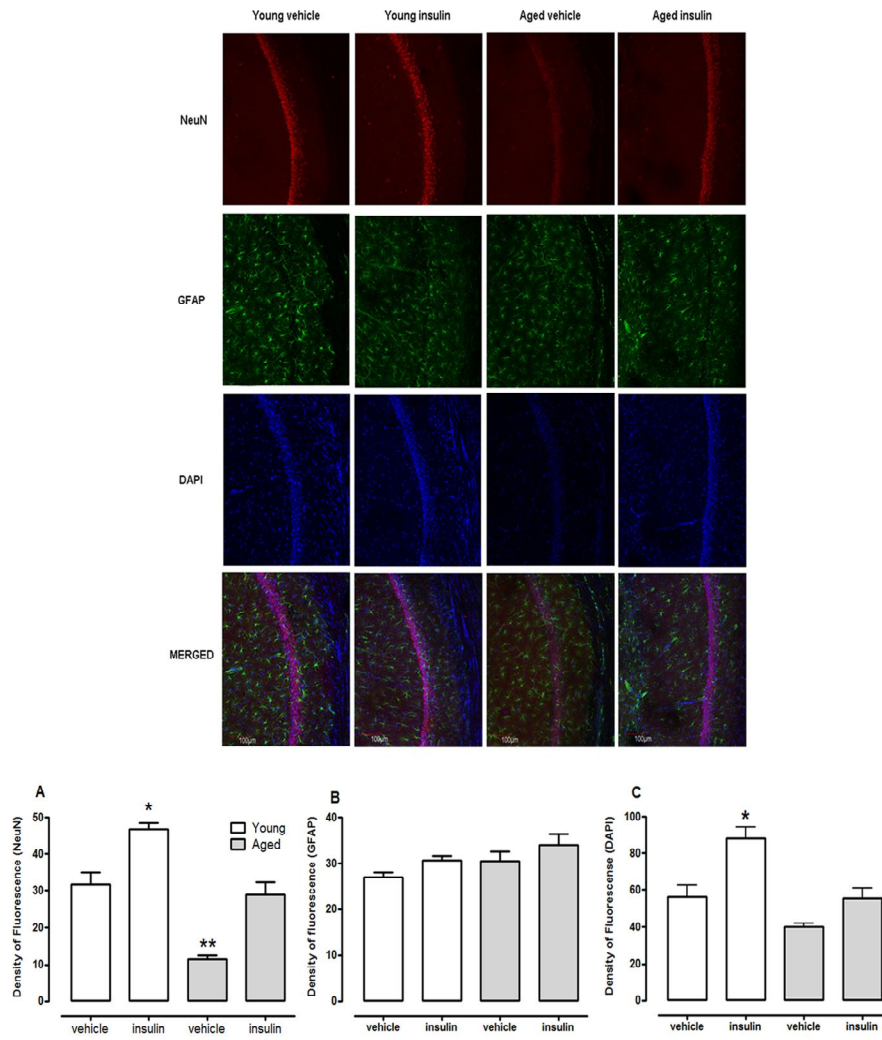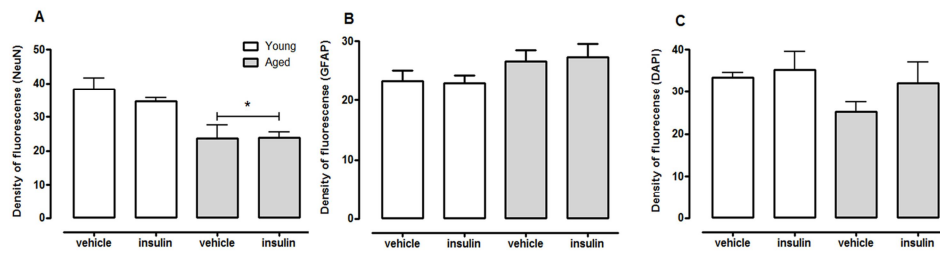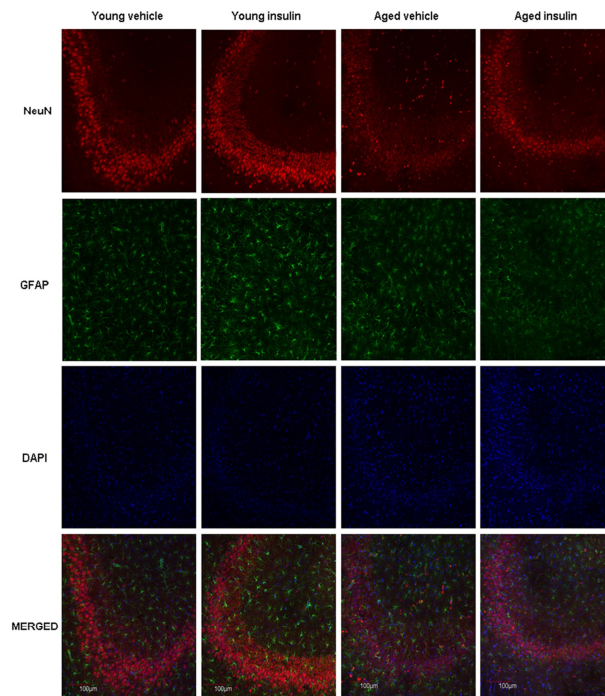
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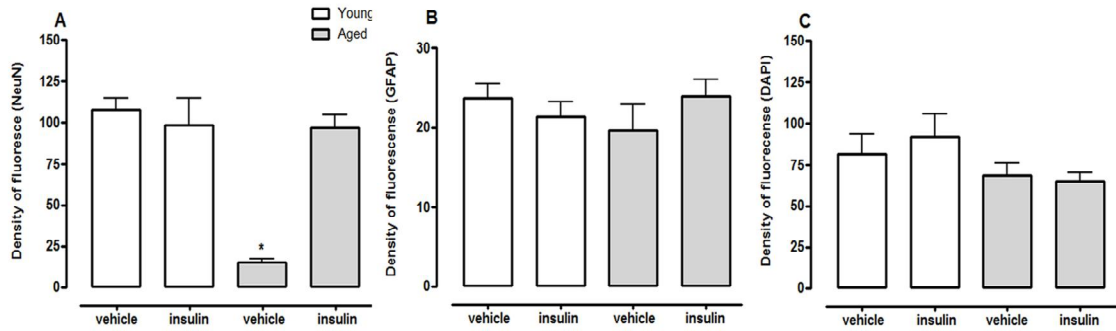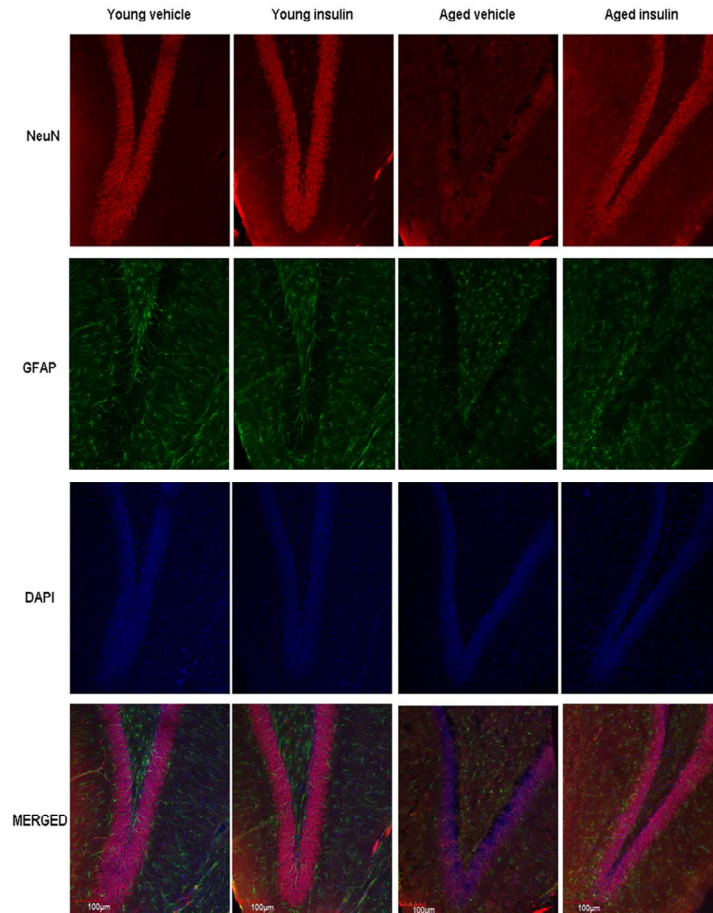
Table 1: Body weight, area under curve and food intake after insulin icv administration (Mean±SEM)

	Body weight (g) after 5 days insulin icv treatment	*AUC*	*Food intake*
Young vehicle	*294±40*	*502±30*	*37±1.8*
Young insulin	*224±29*	*421±43***	*12±1.0#*
Old vehicle	*409±60**	*553±39*	*28±1.1*
Old insulin	*317±21*	*584±47*	*18±0.6&*

*>other groups (p<0.05)

**<other groups (p<0.05)

\# young insulin < young vehicle (p<0.05)

& aged insulin < aged vehicle (p<0.05)

Capitulo 4

Artigo publicado no periódico Hippocampus

Exercise Increases Insulin Signaling in the Hippocampus:
Physiological Effects and Pharmacological Impact of Intracerebroventricular Insulin Administration in Mice

Alexandre P. Muller,[1] Jussânia Gnoatto,[1] Julia D. Moreira,[1] Eduardo R. Zimmer,[1] Clarissa B. Haas,[1] Francisco Lulhier,[1] Marcos L.S. Perry,[1] Diogo O. Souza,[1] Ignácio Torres-Aleman,[2] and Luis V. Portela[1]*

ABSTRACT: Increasing evidence indicates that physical exercise induces adaptations at the cellular, molecular, and systemic levels that positively affect the brain. Insulin plays important functional roles within the brain that are mediated by insulin-receptor (IR) signaling. In the hippocampus, insulin improves synaptic plasticity, memory formation, and learning via direct modulation of GABAergic and glutamatergic receptors. Separately, physical exercise and central insulin administration exert relevant roles in cognitive function. We here use CF1 mice to investigate (i) the effects of voluntary exercise on hippocampal insulin signaling and memory performance and (ii) whether central insulin administration alters the effects of exercise on hippocampal insulin signaling and memory performance. Adult mice performed 30 days of voluntary exercise on running wheel and afterward both, sedentary and exercised groups, received intracerebroventricular (icv) injection of saline or insulin (0.5–5 mU). Memory performance was assessed using the inhibitory avoidance and water maze tasks. Hippocampal tissue was measured for [U-^{14}C] glucose oxidation and the immunocontent of insulin receptor/signaling (IR, pTyr, pAktser473). Additionally, the phosphorylation of the glutamate NMDA receptor NR2B subunit and the capacity of glutamate uptake were measured, and immunohistochemistry was used to determine glial reactivity. Exercise significantly increased insulin peripheral sensitivity, spatial learning, and hippocampal IR/pTyrIR/pAktser473 immunocontent. Glucose oxidation, glutamate uptake, and astrocyte number also increased relative to the sedentary group. In both memory tasks, 5 mU icv insulin produced amnesia but only in exercised animals. This amnesia was associated a rapid (15 min) and persistent (24 h) increase in hippocampal pNR2B immunocontent that paralleled the increase in glial reactivity. In conclusion, physical exercise thus increased hippocampal insulin signaling and improved water maze performance. Overstimulation of insulin signaling in exercised animals, however, via icv administration impaired behavioral performance. This effect was likely the result of aberrant phosphorylation of the NR2B subunit. © 2010 Wiley-Liss, Inc.

[1] Departamento de Bioquímica, ICBS, UFRGS, Programa de Pós Graduação em Ciências Biológicas-Bioquímica, Rua Ramiro Barcelos, 2600 anexo, CEP 90035-003, Porto Alegre, RS, Brazil; [2] Cajal Institute, Consejo Superior de Investigaciones Científicas and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), 28002 Madrid, Spain

INTRODUCTION

Increasing evidence indicates that physical exercise induces adaptations at the cellular, molecular, and systemic levels that positively affect the health of the brain (Dietrich et al., 2005; Droste et al., 2006; Stranahan et al., 2009). These effects include an increase in synaptic plasticity and peripheral sensitivity to insulin as well as a slowing of age-related cognitive decline and a delay in the onset of neurodegenerative diseases. Among the possible mechanisms underlying these central benefits of physical exercise, it is the modulation of neurotransmitter systems by trophic factors and hormones, including insulin (Laurin et al., 2001; Cotman and Berchtold, 2002; Duarte et al., 2003; Park et al., 2005; Flores et al., 2006; Balkau et al., 2008).

The brain was long considered an insulin-insensitive organ; however, this assumption is now challenged by recent studies showing that insulin has important functional roles within the brain that are mediated by insulin-receptor signaling (Gispen and Biessels, 2000; Dou et al., 2005; van der Heide et al., 2006). Moreover, insulin and its receptors (IR) have been associated with distinct, region-specific functional roles (Plum et al., 2005). For instance, insulin/IR signaling has been shown to improve learning, memory formation, and synaptic plasticity in the hippocampus and cerebral cortex. In contrast, a resistance to the effects of insulin in the brain is closely related to the etiology of neurodegenerative diseases and impaired cognitive function, suggesting that brain IR may be potential pharmacological targets in the treatment of cognitive disorders (Park, 2001). Some human and animal studies have proposed that intranasal or systemic insulin administration might exert memory-enhancing action (Park, 2001; Hallschmid and Schultes, 2009). Studies delivering insulin directly into the brains of rodents, however, have produced conflicting results regarding

memory performance. In general, low to intermediate doses have produced no memory changes while high doses tended to improve memory performance (Schwarzberg et al., 1989; Park et al., 2000; Moosavi et al., 2006, 2007a,b; Babri et al., 2007; McNay et al., 2010). These differences could be related to insulin's modulation of GABAergic and glutamatergic receptors and, in consequence, its modulation of synaptic transmission during learning and memory processes (Wan et al., 1997; Ott et al., 1999; Zhao et al., 2004; Watson and Craft, 2006).

Given the important roles attributed to N-methyl-D-aspartate receptors (NMDARs) in synaptic plasticity and cognitive function (Collingridge, 1987), NMDARs may be a target of insulin/IR signaling and may be involved in changes to learning and memory processes. It has been suggested that insulin transiently modulates the phosphorylation of the NR2A and NR2B subunits and potentiates NMDA responses (Christie et al., 1999). In addition, alterations in the expression and function of NMDARs have been implicated in the behavioral and electrophysiological abnormalities observed in insulin-deficient rats (Di Luca et al., 1999). Neuronal transmission requires that glutamate stimulation of NMDARs be kept at physiological levels because of the delicate threshold between normal and excitotoxic responses caused by this receptor (Danbolt, 2001). Thus, it is plausible that central insulin signaling may participate in fine-tuning the modulation of NMDAR responses via its interaction with functional regulatory subunits.

Drugs that improve insulin sensitivity are currently being tested in human trials for use in treating functional deficits associated with Alzheimer's disease (Cole and Frautschy, 2007; McNay et al., 2010). A number of studies have demonstrated that physical exercise improves cognitive function and peripheral insulin sensitivity, but little is known regarding its possible role in enhancing brain insulin sensitivity. We speculate that physical exercise may increase the brain's sensitivity and response to insulin, thereby resulting in a positive influence on memory performance. Taking these factors into considerations we use mice to investigate: (i) the effects of voluntary exercise on memory performance and hippocampal insulin signaling and (ii) whether insulin delivery into the brain alters the effects of voluntary exercise on memory performance and hippocampal insulin signaling.

METHODS

Animals and Exercise Protocol

Two-month-old CF1 mice were housed in standard cages (48 × 26 cm^2) with four animals per cage (van Praag et al., 1999, 2005; Dietrich et al., 2005). To avoid social isolation, the animals were not confined in individual housing (Leasure and Decker, 2009). Animals were kept in a room with controlled temperature (22°C) under a 12 h light/12 h dark cycle and had free access to food and water. Mice were divided into a sedentary group and a voluntary exercise group, which had

free access to a running wheel. After 4 weeks of access to the running wheel, each mouse ran an average of about 3.500 m. All experiments followed the guidelines of the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

Surgical Procedure

Sedentary and exercised animals were anesthetized by an intraperitoneal (i.p.) injection of ketamine (100 mg kg^{-1} body weight) and xylazine (10 mg kg^{-1} body weight). A 27-gauge, 7-mm guide cannula was placed 1 mm posterior to the bregma, 1 mm right from the midline and 1 mm above the lateral brain ventricle. Through a 2-mm hole made at the cranial bone, the cannula was implanted 1.5 mm ventral to the superior surface of the skull and fixed with jeweler's acrylic cement (Schmidt et al., 2005). On the third day postsurgery, the mice already displayed normal food intake and water consumption as well as spontaneous locomotion; they were thus considered ready for in vivo experiments.

MEMORY TASKS

Inhibitory Avoidance Task-Aversive Memory

The behavioral apparatus consisted of a 50 × 25 × 25 cm^3 acrylic box with a floor composed of parallel-caliber stainless steel bars (1 mm diameter) spaced 1 cm apart (Insight Equipments, SP, Brazil). A platform 2 cm wide and 2.5 cm high was placed against the left wall of the box. To evaluate aversive memory performance, we used a footshock aversive task. Sedentary and exercised mice (n = 12–18 per group) were placed on the platform and their latency to step down on the grid floor with four paws was measured with an automatic device. The latencies in the training session were similar in all groups (data not shown). In training sessions, animals received a 1 s, 0.4 mA footshock when they stepped onto the grid. After the shock, they were immediately injected icv for 5 min (1 μl min^{-1}) with vehicle or insulin (0.5–5 mU) and returned to their home cages. Test sessions were performed 24 h after training to evaluate long-term memory. For test sessions, mice were returned to the platform and the latency to step down was used as a measure of retention (a 180 s cut-off time was used). The footshock was omitted during the testing session.

Morris Water Maze Task-Spatial Memory

The apparatus was a black, circular pool (110 cm diameter) with a water temperature of 21°C ± 1°C. Sedentary and exercised mice (n = 10–12 per group) were trained daily in a four-trial water maze task for three consecutive days; each trial lasted up to 60 s and was followed by 20 s of rest on a hidden black platform. During training, mice learned to escape from the water by finding a hidden, rigid, black platform submerged about 1 cm below the water surface in a fixed location. If the

animal failed to find the platform in 60 s, it was manually placed on the platform and allowed to rest for 20 s. Each trial was separated by at least 12 min to avoid hypothermia (the variations of rectal temperature were equal for all groups) and facilitate memory acquisition. Immediately after each daily training session, mice were injected icv for 5 min (1 µl min^{-1}) with vehicle or insulin in a single, 5 mU dose and returned to their home cages. The maze was located in a well-lit, white room with several visual stimuli hanging on the walls to provide spatial cues. Latency to find the platform during each trial was measured as an indicator of learning. A probe test without the platform was performed on the fourth day. The time spent in the target quadrant was measured as an indicator of memory retention.

Glucose and Insulin Serum Levels

To assess peripheral insulin sensitivity, serum glucose and insulin levels ($n = 8$ per group) were measured after 30 days of treatment. The mice fasted for 12 h and blood was then collected via a small puncture in the tail; blood was collected immediately before (0 min) and 30 min after an i.p. injection of glucose (2 mg/g body weight). Glucose was measured with a glucosimeter (AccuChek Active, Roche Diagnostics$^{®}$, USA) and serum insulin levels were measured with a commercially available radioimmunoassay kit (BioChem ImmunoSystems$^{®}$, Italy). The Homeostasis Model Assessment (HOMA2) was used to estimate insulin resistance based on fasting insulin and glucose serum levels [glucose serum (mg dl^{-1}) × insulin serum (µIU ml^{-1})/405].

Liver Glycogen and Epididymal Fat Pad

Fed mice were sacrificed and liver glycogen was determined using the colorimetric method described by Krisman, (1962). Fat tissue from epididymal regions was dissected and weighted as previously described (Muller et al., 2008).

Hippocampal [U-^{14}C] Glucose Oxidation

Considering the key role of the hippocampus in memory processes and the importance of glucose as an energy substrate in normally functioning neural cells, we evaluated the potential influence of exercise and insulin on glucose oxidation following the protocol by Gilbert and Bergold (2005).

After 30 days, the voluntary exercise group and the sedentary group ($n = 8$ per group) were decapitated. Hippocampal slices between 20 and 25 mg were incubated in 0.5 ml buffer (KRb + 5.0 mM D-glucose, pH 7.4) containing 0.1 µCi [U-^{14}C] glucose and vehicle or insulin (5 mU). Incubation was performed in flasks after KRb medium was gasified with 95% O_2:5% CO_2, and flasks were then sealed with rubber caps. Hippocampal slices were incubated at 37°C for 1 h in a Dubnoff metabolic shaker (60 cycles/min) following the method of Dunlop et al. (1975). Incubation was stopped by adding 0.2 ml of 50% TCA through the rubber cap, and 0.1 ml of 1M sodium hydroxide was then injected into the central wells. The

flasks were shaken an additional 30 min at 37°C to trap the CO_2, and the contents of the central well were then transferred to vials and assayed for CO_2 radioactivity in a liquid-scintillation counter (Wallac 1,409).

### Synaptic Membrane and Homogenate Preparations of the Hippocampus

In the third day post surgery, mice were injected icv for 5 min (1 µl min^{-1}) with vehicle or insulin in a single, 5 mU dose. After 15 min or 24 h, mice ($n = 8$ per group) were decapitated and the right hippocampus was immediately dissected and stored at −70°C for synaptic membrane and homogenate preparations.

Synaptic membrane preparations were obtained from the hippocampus as previously described by Dietrich et al. (2005) and stored at −70°C. Membranes were thawed at 37°C for 30 min and washed three times with 5 mM Tris-HCl, pH 7.4 at 27,000g for 15 min. The final pellet was resuspended in PIK buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} aprotinin, and 0.1% phosphatase inhibitor cocktails I and II. Sigma-Aldrich, USA). Total protein content was measured using the method described by Peterson (1977).

Hippocampal homogenates were prepared in PIK buffer and centrifuged. Supernatants were collected and total protein was measured using the method described by Peterson (1977).

Western Blotting

For Western blot analysis, 30 µg of protein from hippocampal homogenates and synaptic membrane preparations were loaded into each well, and IR immunoprecipitates were separated by electrophoresis on a 10% polyacrylamide gel and electrotransfered to PVDF membranes. Nonspecific binding sites were blocked with Tween-Tris buffered saline (TTBS, 100 mM Tris-HCl, pH 7.5) containing 5% albumin for 2 h and then incubated overnight at 4°C with polyclonal antibodies against insulin receptors (IR, Santa Cruz Technology, 1:1,000), pTyrosine (pTyr, Santa Cruz Technology, 1:1,000), pAktser-473 (Cell Signaling Technology, 1:1,000), Akt (Cell Signaling Technology, 1:3,000), pNR2B (Upstate, 1:250) and actin (Sigma, 1:5,000). Membranes were rinsed 3× 10 min with TTBS and incubated with secondary antibodies (1:3,000 dilution, antirabbit, Cell Signaling Technology; 1:5,000 dilution antimouse, Santa Cruz Technology) for 2 h at room temperature. Membranes were then rinsed 4× 10 min with TTBS and incubated with peroxidase-conjugated for 5 min at room temperature. The resulting reaction was displayed on autoradiographic film by chemiluminescence. The films were scanned and band intensity was analyzed using Image J software (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). The pTyr levels in the hippocampus were measured via IR immunoprecipitation. Fifty micrograms of hippocampal protein was incubated with 5 µg
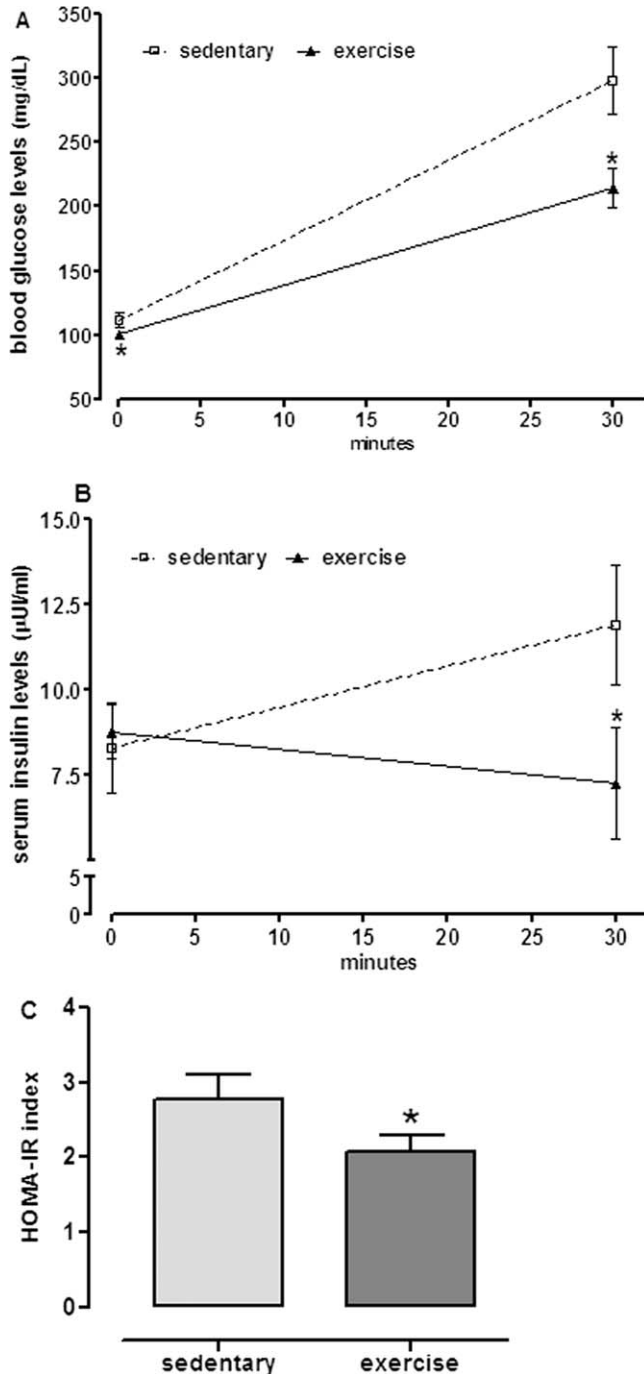
FIGURE 1.   Exercise increases peripheral insulin sensitivity. (A) After 30 days of exercise, mice presented decreased fasting blood glucose levels (0 min) and less increment at 30 min after glucose input (2 mg glucose/g mice) when compared to sedentary animals. (B) Fasting insulin serum levels were not altered by exercise, and a glucose input did not increase insulin serum levels in exercised mice. (C) There was a decrease in the HOMA-IR index as a result of exercise. □ = sedentary; ▲ = exercised (*$P < 0.05$ exercised vs. sedentary). Values are mean ± SE of eight mice per group.

primary IR antibody overnight. Protein A-agarose (Invitrogen) was added to the antigen–antibody mixture and incubated while gently stirring for 2 h. The immunoprecipitate was

washed three times with the same lysis buffer then resuspended, electrophoresed, and transferred to a nitrocellulose membrane; it was then analyzed by Western blot as previously described.

Total and Na$^+$-Independent [^{3}H] Glutamate Uptake

Sedentary and exercised mice were injected icv with saline or insulin (5 mU) and after 24 h were sacrificed by decapitation. Hippocampal slices were preincubated with HBSS at 37°C for 15 min, followed by the addition of 100 μM [^{3}H] glutamate. Incubation was stopped after 5 min with two ice-cold washes of l mL HBSS. After washing, 0.5N NaOH was immediately added to the slices and they were stored overnight. Na$^+$-independent uptake was measured using the above-described protocol with alterations in the temperature (4°C) and the composition of the medium (N-methyl-D-glucamine instead of sodium chloride). Results (Na$^+$-dependent uptake) were measured as the difference between the total uptake and the Na$^+$-independent uptake. Each incubation was performed in triplicate (Thomazi et al., 2004). Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1,409).

Immunohistochemistry

For the immunohistochemical study, animals (four mice per group) were killed and left hemispheres were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 and then cryoprotected in a 30% sucrose solution in PB at 4°C. Coronal sections (50 μm) were obtained using a Vibratome (Leica, Germany). One-in-8 random series were collected for immunohistochemistry. The sections were incubated for 48 h at 4°C with polyclonal rabbit GFAP antiserum (Dako, UK, 1:500) and NeuN (Chemicon, 1:250) in phosphate buffered saline (PBS)-Tx containing 2% bovine serum albumin (BSA). After being washed several times with PBS, the sections were incubated with 594 alexa-conjugated donkey antirabbit and 488 alexa-conjugated donkey antimouse antibodies for 2 h at room temperature. To determine fluorescence insensitivity, sections were photographed with a confocal microscope (Olympus, Japan) and analyzed using Image J software. Fluorescence insensitivity was expressed as the ratio of GFAP-/NeuN-positive cells. Astrocyte

TABLE 1.

Body Weight, Fat Pad, and Liver Glycogen

	Body weight (g)	Epididymal fat pad	Liver glycogen (mg%)
Sedentary $n = 10$	49.7 ± 2.6	2.5 ± 0.4	1.9 ± 0.2
Exercise $n = 10$	45.8 ± 1.6	1.7 ± 0.3*	2.6 ± 0.2*

Epididymal fat pat pad: exercise < sedentary *$P < 0.05$.
Liver glycogen: exercise > sedentary *$P < 0.05$.

Statistical Analysis

Results were presented as means ±standard error of mean (SEM) with the exception of the step-down latencies in the inhibitory avoidance task, which were expressed as median and interquartile ranges. The data from the water maze task were analyzed using repeated-measures analysis of variance (ANOVA) followed by Tukey's post hoc test. Likewise, the differences between all groups were analyzed using ANOVA and Tukey's post hoc test. The differences between the sedentary and exercised groups were analyzed using Student's t test. The data from the inhibitory avoidance task were analyzed using the Mann-Whitney U test. The differences between groups were considered statistically significant if $P < 0.05$.

RESULTS

Exercise Improved Spatial Learning: Amnesic Actions of Central Insulin Administration

We confirmed that exercise increases peripheral sensitivity to insulin. At fasting, glucose levels were higher in sedentary animals when compared to exercised ones (Fig. 1A; $P < 0.05$) while serum insulin levels were unchanged (Fig. 1B). Moreover, the exercise protocol caused an increase in the liver glycogen and decrease in epididymal fat pad (Table 1). In the inhibitory avoidance task, the sedentary and exercised animals presented a similar long-term memory. Furthermore, the icv administration of vehicle or insulin at 0.5 and 2 mU doses had no effect in the sedentary and exercised groups. However, 5 mU icv insulin had an amnesic effect only in the exercised group (Fig. 2A; $P < 0.05$). Because of this dose response, we decided to evaluate spatial memory using only 5 mU of insulin in subsequent test. In the Morris water maze task, the exercised animals had better learning and retention relative to the

FIGURE 2. Exercise improves performance in spatial memory and intracerebroventricular (icv) insulin administration had an amnesic effect on both aversive and spatial memory in exercised mice. (A) Sedentary and exercised mice treated with vehicle or insulin at 0.5 or 2 mU icv showed an increased latency to step down onto the platform in the inhibitory avoidance task 24 h after training, demonstrating successful long-term learning. However, 5 mU icv insulin had an amnesic effect on the exercised group (*$P < 0.05$ training vs. long-term memory). Values are median, interquartile range of 12–18 mice per group. (B and C) Exercise improved spatial learning and memory in the Morris water maze task. Insulin (5 mU, icv) did not alter the performance of the sedentary group; however, this dose also had an amnesic effect on the exercised group during memory acquisition and retention. ■ = sedentary/vehicle; △ = sedentary/insulin 5mU; ○ = exercised/vehicle; ● = exercised/insulin 5mU (*$P < 0.05$ exercised vehicle vs. other groups). Values are mean ± SE of 10–12 mice per group.

number was estimated by counting the number of GFAP-positive cells using a Nikon Eclipse E-600 microscope (500×, Japan) coupled to a Nikon DXM 1200C CCD camera.

FIGURE 3. Exercise increases hippocampal glucose oxidation. Exercise increased the [U-^{14}C] glucose oxidation by hippocampal slices. Insulin did not affect [U-^{14}C] glucose oxidation in both groups (*$P < 0.05$ exercised vs. sedentary groups). Values are mean ± SE of eight mice per group.
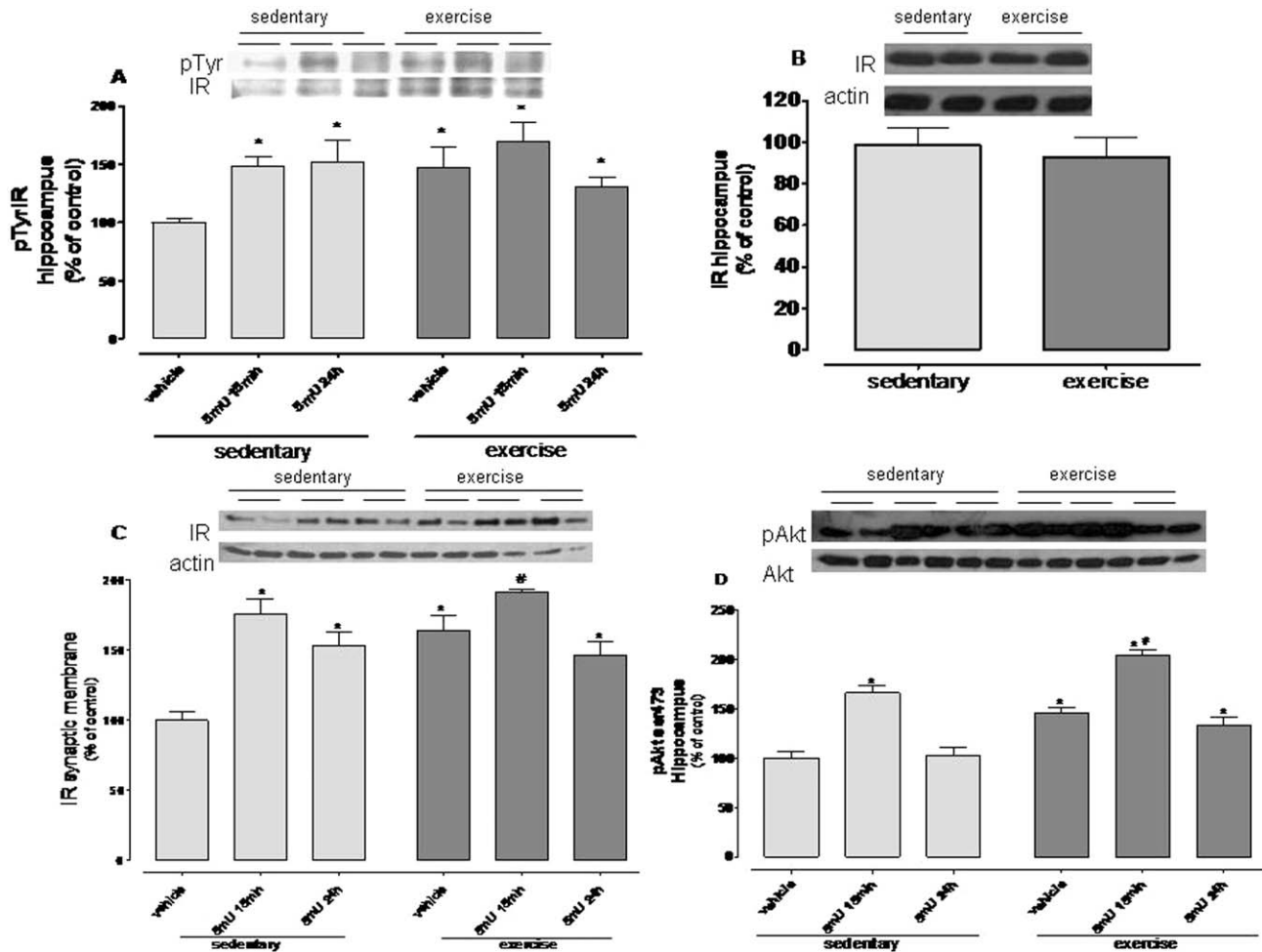
FIGURE 4.   Exercise increases insulin signaling in the hippocampus. (A) Exercise increased the phosphorylation status of Tyr in the immunoprecipitate of insulin receptors (pTyr-IR) in the hippocampus. Insulin increased pTyr-IR only in sedentary groups. (B) Exercise did not increased total IR immunocontent in the hippocampus. (C) Both exercised and sedentary groups showed an increased IR immunocontent in synaptic membranes. (D) Exercise increased the immunocontent of pAkt ser-473 in the hippocampus relative to sedentary mice. Insulin (5 mU, icv) increased pAkt-ser473 at 15 min after administration in both sedentary and exercised groups (*$P < 0.05$ sedentary vehicle vs. other groups). (#$P < 0.05$ exercised vehicle vs. exercised insulin at 15 min). Values are mean ± SE of six to eight mice per group.

sedentary groups. Again, central insulin administration had an amnesic effect only in the exercised group (Figs. 2B,C; $P < 0.05$). After these initial findings, we then investigated the possible mechanisms underlying the effects of exercise and central insulin administration on memory performance and insulin signaling.

We first performed ex vivo experiments to determine whether insulin could affect the ability of the hippocampus to oxidize glucose. There was a significant increase of [U-^{14}C] glucose oxidation in hippocampal slices from exercised mice relative to sedentary mice (Fig. 3; $P < 0.05$). Insulin did not have an additional affect on glucose oxidation in exercised mice (Fig. 3).

Exercise Increased Hippocampal Insulin Receptors and Signaling

After 30 days of exercise, the IR immunoprecipitate from hippocampal homogenates showed an increased phosphoryla-tion in tyrosine residues (pTyr-IR) compared to the sedentary group (Fig. 4A, $P < 0.05$); however, there was no change in total IR immunocontent in the hippocampal homogenate (Fig. 4B). The IR immunocontent in hippocampal synaptic membranes was significantly increased (64%) by exercise when compared to sedentary mice (Fig. 4C; $P < 0.05$), suggesting that physical exercise may activate molecular machinery that is involved in IR translocation to the synaptic membrane. The phosphorylation of AKT at ser473 (pAKTser473), a down-stream protein activated by insulin/IR signaling, was significantly increased by exercise relative to sedentary mice (Fig. 4D, $P < 0.05$). Overall, these results suggest that insulin signaling in the hippocampus is increased by exercise. As previously demonstrated by Li et al. (2005) and Trejo et al. (2008), we also found an increased number of GFAP-positive cells in the hippocampus of exercised animals when compared to sedentary mice (Fig. 6A, $P < 0.05$).

Implications of icv Insulin Administration on Hippocampal Signaling in Exercised and Sedentary Mice

After 15 min and 24 h of insulin (5 mU) administration to sedentary mice, there was increased phosphorylation of pTyr-IR in hippocampal homogenates as well as increased IR immuno-content in the synaptic membranes of the hippocampus (sedentary vs. sedentary insulin; Fig. 4A, $P < 0.05$). Insulin administration did not further improve the effects of exercise on pTyr-IR phosphorylation and IR immunocontent in the hippocampus. Exercise significantly increased hippocampal pAKTser473 immunocontent relative to the sedentary group and icv insulin administration further increased the immunocontent of this protein after 15 min in both the sedentary and exercised groups (Fig. 4D $P < 0.05$).

Central Insulin Administration in Exercised Mice Increases NR2B Phosphorylation and Causes Glial Reactivity

We postulated that alterations in NMDA receptor (NMDAR) activity may be involved in the aversive and spatial memory deficits observed in exercised mice receiving icv insulin; this hypothesis was based on previous evidence showing that these receptors are involved in learning and memory processes (Collingridge, 1987). We thus measured the phosphorylation of NR2B (pNR2B), a functional subunit of the NMDAR. Physical exercise did not affect NR2B phosphorylation in hippocampal homogenates; however, when insulin was combined with physical exercise, there was a rapid (15 min; 73%) and persistent (24 h; 39%) increase in the immunocontent of pNR2B (Figs. 5A,B, $P < 0.05$).

Impaired control of glutamate levels in the synaptic cleft is closely associated with excitotoxic events via NMDAR. We thus investigated glutamate uptake capacity as a potential factor influencing NMDAR NR2B activation. Hippocampal slices obtained from exercised groups displayed an increased glutamate uptake capacity (30%) relative to sedentary mice (Fig. 5C; $P < 0.05$). However, insulin had no effect on uptake capacity. Thirty days of exercise increased the number of GFAP-positive cells (astrocyte proliferation) with no apparent activation of glial cells (Figs. 6A,B, $P < 0.05$). In the exercised group, however, insulin administration (5 mU) caused a marked activation of glial cells at 24 h with no change in the number of GFAP-positive cells (Figs. 6A,B, $P < 0.05$).

DISCUSSION

Although physical exercise is known to increase peripheral insulin sensitivity, little is known regarding its effects on brain insulin sensitivity. In this study, we showed that exercise enhanced performance in spatial learning memory and increased insulin signaling in the mouse hippocampus. These
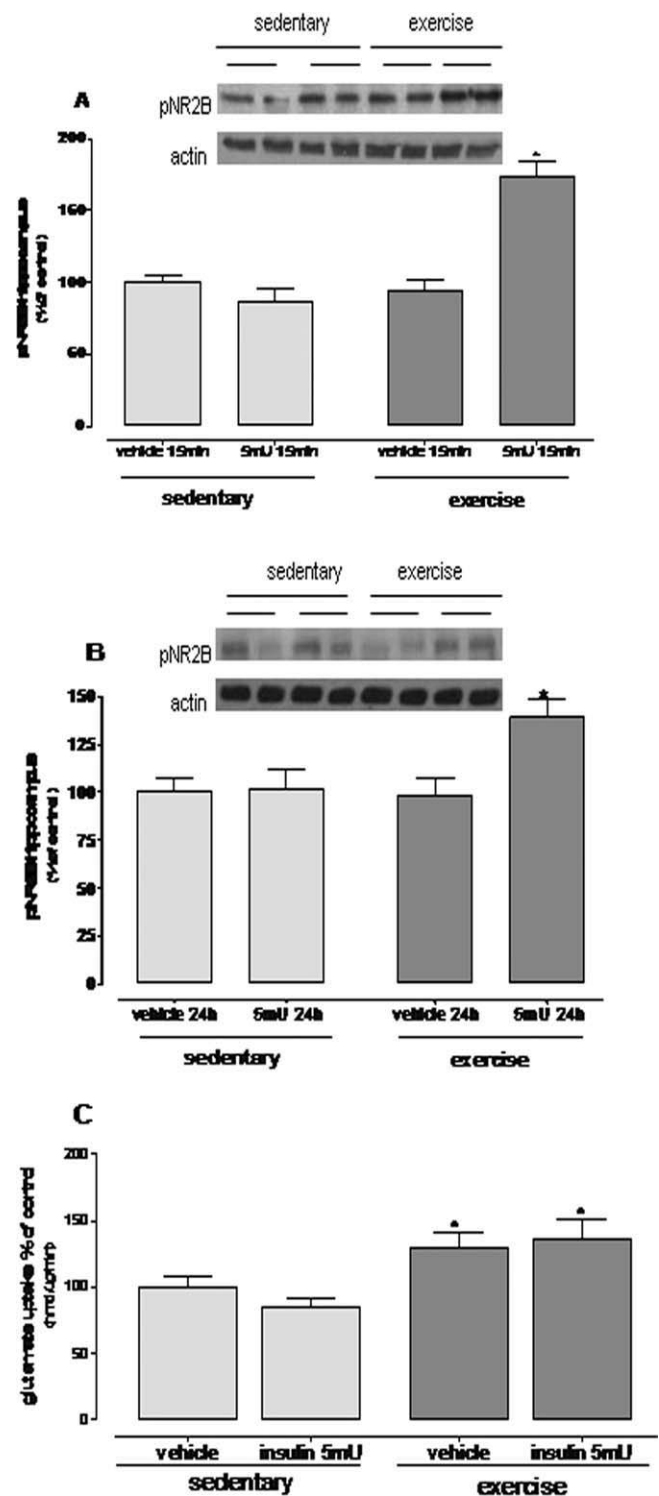
FIGURE 5. Exercise and/or 5mU icv insulin increases NMDAR activation and increases glutamate uptake. Insulin (5 mU, icv) increased the phosphorylation state of the NR2B subunit in the exercised group after 15 min (A) and 24 h (B) of administration. Neither insulin nor exercise alone altered the phosphorylation state. (C) Exercise increased glutamate uptake in hippocampal slices independently of insulin (*$P < 0.05$ exercised vs. sedentary groups). Values are mean ± SE of 8–10 mice per group.
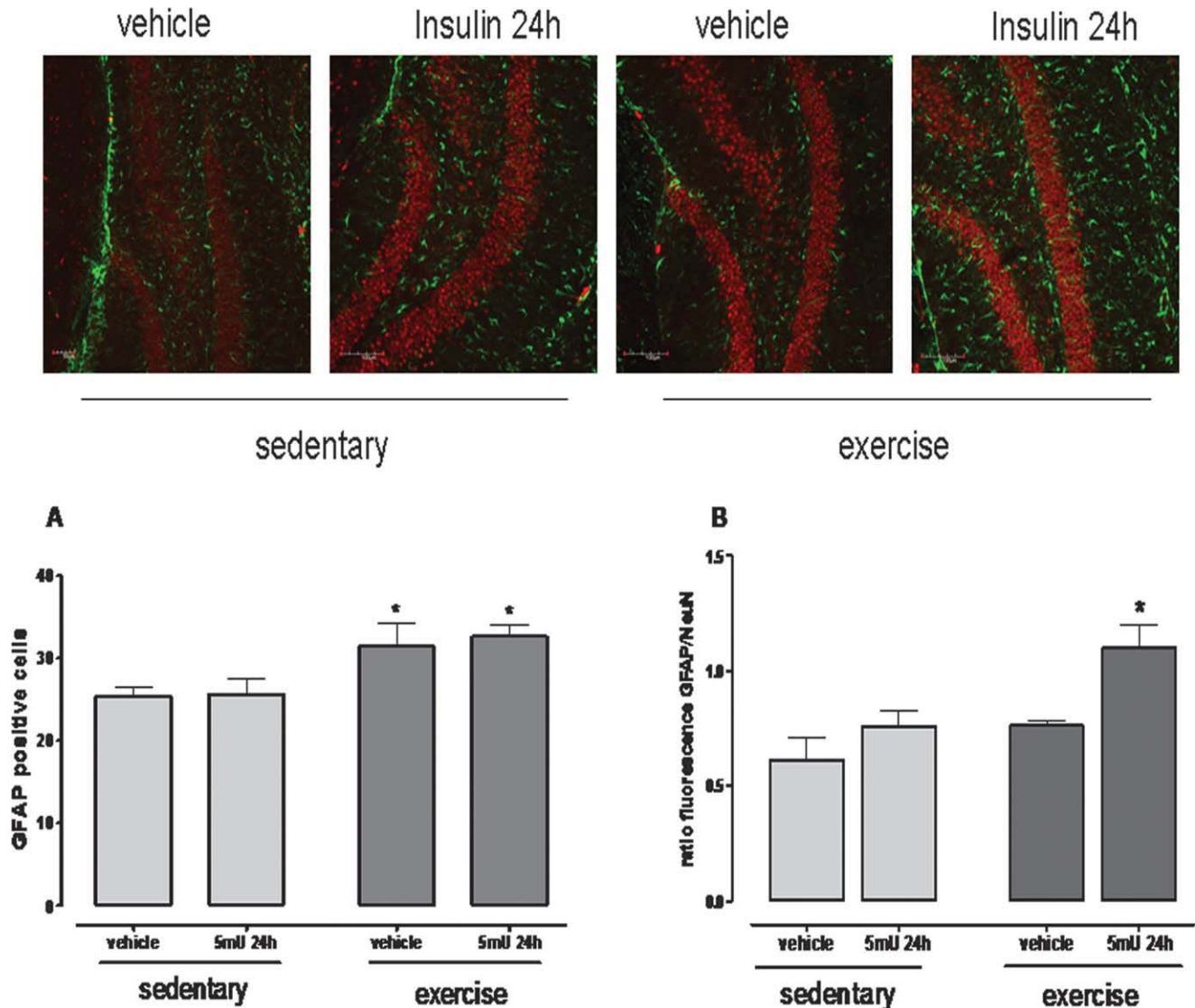
FIGURE 6.   Exercise and 5 mU icv insulin increase glial reactivity in the hippocampus. (A) Exercise increased the number of GFAP-positive cells in the hippocampus independently of insulin (*$P < 0.05$ exercised vs. sedentary groups). (B) Exercise and 5 mU icv insulin increased the fluorescence intensity of GFAP in the hippocampus at 24 h (*$P < 0.05$ exercised insulin 24 h vs. other groups). Values are mean ± SE of four mice per group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

results may be related to the observed increase in IR translocation/activation (pTyr-IR) and the increased responsiveness of downstream proteins to insulin signaling (e.g., pAKTser473). The presence of IR in the hippocampus and cerebral cortex suggests its functional involvement in learning and memory processes (Zhao et al., 1999). Indeed, insulin has been shown to enhance memory in both humans and experimental animals (Zhao et al., 2004). Similarly, physical exercise positively affects hippocampal plasticity and memory function (Cotman et al., 2007).

It is now recognized that brain insulin administration controls peripheral glucose homeostasis in a dose-dependent manner. In addition, peripheral or central glucose injections improve cognitive processes in both rodents and humans.

These behavioral findings support the hypothesis that glucose acts directly on the brain to facilitate memory processing. However, there is still intense debate regarding insulin's ability to regulate brain glucose metabolism (Messier, 2004). In our study, icv insulin (5 mU) administration did not alter serum glucose levels measured at 30 min, 2 h, and 24 h in either sedentary or exercised mice (data not shown), ruling out the possibility that insulin-induced changes in glucose availability could account for the differences in memory performance. Dietrich et al. (2008) showed that spontaneous physical exercise increased mitochondrial activity in synaptic terminals in mouse hippocampus. Here, the increase in glucose oxidation might reflect the high energy demands of synaptic terminals, demands that parallel the increased mitochondrial activity induced by

exercise. Insulin did not affect glucose oxidation in the ex vivo hippocampus of sedentary and exercised mice, supporting the previous theory that insulin plays a neuromodulatory role in the brain rather than controlling glucose metabolism.

The fact that IR is mainly detected in neuronal cells may indicate a cell-type specific modulation. Indeed, emerging data suggest that the brain is an insulin target and that insulin receptor signaling within the brain is involved in diverse functions including neuronal survival (Valenciano et al., 2006), synaptic plasticity (Lee et al., 2005; Stranahan et al., 2008) and learning and memory (Zhao et al., 1999; Dou et al., 2005). Here, we showed that exercise increased IR signaling (as indicated by increased levels of pTyr-IR and pAktser473 immunocontent) and increased IR levels in hippocampal synaptic membrane preparations without affecting the total levels of IR. However, insulin administration did not further increase IR immunocontent in exercised animals, suggesting that physical exercise may be a nonpharmacological, therapeutic intervention for neurological disorders associated with brain insulin resistance. The increased translocation and activation of IR was thus achieved independently of insulin administration. It has been suggested that insulin/IR signaling is activated in the early stages of memory formation and may play a role in sorting memories for long-term storage (Zhao et al., 2004).

Although several studies have suggested that insulin may improve memory performance in rats, there are few studies employing mice protocols. In the present study, we combined a lifestyle intervention known to positively affect peripheral insulin sensitivity and brain function with icv insulin intervention, trying to improve learning and memory performance. While we found that exercise improved performance in the water maze as previously described (Molteni et al., 2004; Parachikova et al., 2008), neither exercise nor icv insulin alone were able to modulate aversive memory in the inhibitory avoidance task. Surprisingly, we found that 5 mU of insulin had amnesic effects only in exercised animals. This effect was time-dependent, once after 15 days of exercise; insulin did not impaired performance on inhibitory avoidance task (data not shown).

Impairments in memory performance after insulin treatment have previously been described. Schwarzberg et al. (1989) reported that icv insulin administration prior to a retention trial impaired performance in a passive-avoidance task, while Kopf and Baratti (1999) showed that an intraperitoneal injection of insulin delivered to mice immediately after training impaired retention memory in an inhibitory avoidance task. In contrast to these findings, Park et al. (2000) showed that delivery of icv insulin to rats at 4 mU improved memory consolidation in a passive avoidance task. Even considering these aspects, we believe that some methodological differences (for example, route of administration, dose, memory task employed and rodent strain) could account for discrepancies among these studies [for a review see (Park, 2001)]. As previously proposed by Park (2001), the conflicting results observed in memory tasks after insulin administration may be caused by specific aspects of memory modulation such as acquisition, retrieval, or consolidation. In addition, different concentrations of insulin may modulate memory or other memory-influencing processes

in opposite directions, resulting in a range of physiological effects. Here, we showed that icv insulin administration (5 mU) in exercised animals impaired long-term memory. Because the effects of insulin were examined after training, we propose that insulin affects the consolidation and retrieval of memory.

Insulin can potentiate NMDA activity and synaptic transmission in the hippocampal CA1 region (Liu et al., 1995). Because the activity of the NMDAR is dependent on its phosphorylation status, it has been proposed that insulin may directly alter NMDAR phosphorylation through multiple signal transduction pathways. The modulation of NMDA activity by insulin involves the phosphorylation of the functional NR2A and NR2B subunits (Christie et al., 1999). In the present study, the administration of icv insulin to exercised mice resulted in a rapid and persistent activation of the NMDAR NR2B subunit. We propose that this hyperactivation may be related to excitotoxic events as previously shown by Schumann et al. (2008), who found that NR2B hyperactivation is associated with brain damage. Our conjecture is based on the increased glial activation observed in insulin exercised mice. Physiologically, glial reactivity may have either neurotoxic or neuroprotective effects (Ren and Dubner, 2008). Therefore, the administration of insulin when insulin signaling is already increased, as it is after exercise, may be detrimental to brain functioning. Our results thus highlight the fine balance in brain insulin signaling and shed light into the neuromodulatory effects of insulin on the glutamatergic system. In accordance with this conjecture (Ramsden et al., 2003) showed that exercise animals have increased neuronal vulnerability to excitotoxicity induced by kainate when compared to sedentary ones. Thus, exercised animals seem to be more sensitive to a neurotoxic insult associated with overexcitation of glutamatergic system.

CONCLUSION

In conclusion, our main findings were that physical exercise increased hippocampal insulin receptor/signaling (IR/pTyrIR/pAktser473), glucose utilization and glutamate uptake. These mechanisms might underlie the enhanced spatial memory performance in exercised mice. In contrast, icv insulin administration (5 mU) had an amnesic effect in exercised mice likely caused by hyperactivation of NMDAR subunit NR2B; this effect paralleled increased glial activation.

REFERENCES

Babri S, Badie HG, Khamenei S, Seyedlar MO. 2007. Intrahippocampal insulin improves memory in a passive-avoidance task in male wistar rats. Brain Cogn 64:86–91.

Balkau B, Mhamdi L, Oppert JM, Nolan J, Golay A, Porcellati F, Laakso M, Ferrannini E. 2008. Physical activity and insulin sensitivity: The RISC study. Diabetes 57:2613–2618.

Christie JM, Wenthold RJ, Monaghan DT. 1999. Insulin causes a transient tyrosine phosphorylation of NR2A and NR2B NMDA

receptor subunits in rat hippocampus. J Neurochem 72:1523–1528.

Cole GM, Frautschy SA. 2007. The role of insulin and neurotrophic factor signaling in brain aging and Alzheimer's Disease. Exp Gerontol 42:10–21.

Collingridge G. 1987. Synaptic plasticity. The role of NMDA receptors in learning and memory. Nature 330:604–605.

Cotman CW, Berchtold NC. 2002. Exercise: A behavioral intervention to enhance brain health and plasticity. Trends Neurosci 25:295–301.

Cotman CW, Berchtold NC, Christie LA. 2007. Exercise builds brain health: Key roles of growth factor cascades and inflammation. Trends Neurosci 30:464–472.

Danbolt NC. 2001. Glutamate uptake. Prog Neurobiol 65:1–105.

Di Luca M, Ruts L, Gardoni F, Cattabeni F, Biessels GJ, Gispen WH. 1999. NMDA receptor subunits are modified transcriptionally and post-translationally in the brain of streptozotocin-diabetic rats. Diabetologia 42:693–701.

Dietrich MO, Mantese CE, Porciuncula LO, Ghisleni G, Vinade L, Souza DO, Portela LV. 2005. Exercise affects glutamate receptors in postsynaptic densities from cortical mice brain. Brain Res 1065:20–25.

Dietrich MO, Andrews ZB, Horvath TL. 2008. Exercise-induced synaptogenesis in the hippocampus is dependent on UCP2-regulated mitochondrial adaptation. J Neurosci 28:10766–10771.

Dou JT, Chen M, Dufour F, Alkon DL, Zhao WQ. 2005. Insulin receptor signaling in long-term memory consolidation following spatial learning. Learn Mem 12:646–655.

Droste SK, Schweizer MC, Ulbricht S, Reul JM. 2006. Long-term voluntary exercise and the mouse hypothalamic-pituitary-adrenocortical axis: Impact of concurrent treatment with the antidepressant drug tianeptine. J Neuroendocrinol 18:915–925.

Duarte AI, Santos MS, Seica R, de Oliveira CR. 2003. Insulin affects synaptosomal GABA and glutamate transport under oxidative stress conditions. Brain Res 977:23–30.

Dunlop DS, van Elden W, Lajtha A. 1975. Optimal conditions for protein synthesis in incubated slices of rat brain. Brain Res 99:303–318.

Flores MB, Fernandes MF, Ropelle ER, Faria MC, Ueno M, Velloso LA, Saad MJ, Carvalheira JB. 2006. Exercise improves insulin and leptin sensitivity in hypothalamus of Wistar rats. Diabetes 55:2554–2561.

Gilbert E, Bergold PJ. 2005. Oxidation of 14C-labeled substrates by hippocampal slice cultures. Brain Res Brain Res Protoc 15:135–141.

Gispen WH, Biessels GJ. 2000. Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 23:542–549.

Hallschmid M, Schultes B. 2009. Central nervous insulin resistance: A promising target in the treatment of metabolic and cognitive disorders? Diabetologia 52:2264–2269.

Kopf SR, Baratti CM. 1999. Effects of posttraining administration of insulin on retention of a habituation response in mice: Participation of a central cholinergic mechanism. Neurobiol Learn Mem 71:50–61.

Krisman CR. 1962. A method for the colorimetric estimation of glycogen with iodine. Anal Biochem 4:17–23.

Laurin D, Verreault R, Lindsay J, MacPherson K, Rockwood K. 2001. Physical activity and risk of cognitive impairment and dementia in elderly persons. Arch Neurol 58:498–504.

Leasure JL, Decker L. 2009. Social isolation prevents exercise-induced proliferation of hippocampal progenitor cells in female rats. Hippocampus 19:907–912.

Lee CC, Huang CC, Wu MY, Hsu KS. 2005. Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway. J Biol Chem 280:18543–18550.

Li J, Ding YH, Rafols JA, Lai Q, McAllister JP II, Ding Y. 2005. Increased astrocyte proliferation in rats after running exercise. Neurosci Lett 386:160–164.

Liu L, Brown JC III, Webster WW, Morrisett RA, Monaghan DT. 1995. Insulin potentiates N-methyl-D-aspartate receptor activity in Xenopus oocytes and rat hippocampus. Neurosci Lett 192:5–8.

McNay EC, Ong CT, McCrimmon RJ, Cresswell J, Bogan JS, Sherwin RS. 2010. Hippocampal memory processes are modulated by insulin and high-fat-induced insulin resistance. Neurobiol Learn Mem 93:546–553.

Messier C. 2004. Glucose improvement of memory: A review. Eur J Pharmacol 490:33–57.

Molteni R, Wu A, Vaynman S, Ying Z, Barnard RJ, Gomez-Pinilla F. 2004. Exercise reverses the harmful effects of consumption of a high-fat diet on synaptic and behavioral plasticity associated to the action of brain-derived neurotrophic factor. Neuroscience 123:429–440.

Moosavi M, Naghdi N, Maghsoudi N, Zahedi Asl S. 2006. The effect of intrahippocampal insulin microinjection on spatial learning and memory. Horm Behav 50:748–752.

Moosavi M, Naghdi N, Choopani S. 2007a. Intra CA1 insulin microinjection improves memory consolidation and retrieval. Peptides 28:1029–1034.

Moosavi M, Naghdi N, Maghsoudi N, Zahedi Asl S. 2007b. Insulin protects against stress-induced impairments in water maze performance. Behav Brain Res 176:230–236.

Muller AP, Cammarota M, Dietrich MO, Rotta LN, Portela LV, Souza DO, Izquierdo I, Bevilaqua LR, Perry ML. 2008. Different effect of high fat diet and physical exercise in the hippocampal signaling. Neurochem Res 33:880–885.

Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. 1999. Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology 53:1937–1942.

Parachikova A, Nichol KE, Cotman CW. 2008. Short-term exercise in aged Tg2576 mice alters neuroinflammation and improves cognition. Neurobiol Dis 30:121–129.

Park CR. 2001. Cognitive effects of insulin in the central nervous system. Neurosci Biobehav Rev 25:311–323.

Park CR, Seeley RJ, Craft S, Woods SC. 2000. Intracerebroventricular insulin enhances memory in a passive-avoidance task. Physiol Behav 68:509–514.

Park S, Jang JS, Jun DW, Hong SM. 2005. Exercise enhances insulin and leptin signaling in the cerebral cortex and hypothalamus during dexamethasone-induced stress in diabetic rats. Neuroendocrinology 82:282–293.

Peterson GL. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356.

Plum L, Schubert M, Bruning JC. 2005. The role of insulin receptor signaling in the brain. Trends Endocrinol Metab 16:59–65.

Ramsden M, Berchtold NC, Patrick Kesslak J, Cotman CW, Pike CJ. 2003. Exercise increases the vulnerability of rat hippocampal neurons to kainate lesion. Brain Res 971:239–244.

Ren K, Dubner R. 2008. Neuron-glia crosstalk gets serious: Role in pain hypersensitivity. Curr Opin Anaesthesiol 21:570–579.

Schmidt AP, Avila TT, Souza DO. 2005. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem Res 30:69–73.

Schumann J, Alexandrovich GA, Biegon A, Yaka R. 2008. Inhibition of NR2B phosphorylation restores alterations in NMDA receptor expression and improves functional recovery following traumatic brain injury in mice. J Neurotrauma 25:945–957.

Schwarzberg H, Bernstein HG, Reiser M, Gunther O. 1989. Intracerebroventricular administration of insulin attenuates retrieval of a passive avoidance response in rats. Neuropeptides 13:79–81.

Stranahan AM, Norman ED, Lee K, Cutler RG, Telljohann RS, Egan JM, Mattson MP. 2008. Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. Hippocampus 18:1085–1088.

Stranahan AM, Lee K, Martin B, Maudsley S, Golden E, Cutler RG, Mattson MP. 2009. Voluntary exercise and caloric restriction enhance hippocampal dendritic spine density and BDNF levels in diabetic mice. Hippocampus 19:951–961.

Thomazi AP, Godinho GF, Rodrigues JM, Schwalm FD, Frizzo ME, Moriguchi E, Souza DO, Wofchuk ST. 2004. Ontogenetic profile of glutamate uptake in brain structures slices from rats: Sensitivity to guanosine. Mech Ageing Dev 125: 475–481.

Trejo JL, Llorens-Martin MV, Torres-Aleman I. 2008. The effects of exercise on spatial learning and anxiety-like behavior are mediated by an IGF-I-dependent mechanism related to hippocampal neurogenesis. Mol Cell Neurosci 37:402–411.

Valenciano AI, Corrochano S, de Pablo F, de la Villa P, de la Rosa EJ. 2006. Proinsulin/insulin is synthesized locally and prevents caspase- and cathepsin-mediated cell death in the embryonic mouse retina. J Neurochem 99:524–536.

van der Heide LP, Ramakers GM, Smidt MP. 2006. Insulin signaling in the central nervous system: Learning to survive. Prog Neurobiol 79:205–221.

van Praag H, Kempermann G, Gage FH. 1999. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.

van Praag H, Shubert T, Zhao C, Gage FH. 2005. Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25:8680–8685.

Wan Q, Xiong ZG, Man HY, Ackerley CA, Braunton J, Lu WY, Becker LE, MacDonald JF, Wang YT. 1997. Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. Nature 388:686–690.

Watson GS, Craft S. 2006. Insulin resistance, inflammation, and cognition in Alzheimer's Disease: Lessons for multiple sclerosis. J Neurol Sci 245:21–33.

Zhao WQ, Chen H, Xu H, Moore E, Meiri N, Quon MJ, Alkon DL. 1999. Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. J Biol Chem 274:34893–34902.

Zhao WQ, Chen H, Quon MJ, Alkon DL. 2004. Insulin and the insulin receptor in experimental models of learning and memory. Eur J Pharmacol 490:71–81.

Capitulo 5.

Insulin prevents the mitochondrial generation of H_2O_2 in synaptosomes by

intracellular signaling

Alexandre Pastoris Muller[1], Clarissa Branco Haas[1], Juliana Camamacho-Pereira[2], Diogo Onofre Souza[1], Antonio Galina[2], Luis Valmor Portela[1]

1. Departamento de Bioquímica, ICBS, UFRGS. Programa de Pós Graduação em Ciências Biológicas-Bioquímica. Rua Ramiro Barcelos, 2600 anexo, CEP 90035-003, Porto Alegre, RS, Brazil.
2. Instituto de Bioquímica Médica, Programa de Biofísica e Bioquímica Celular e Programa de Biologia Molecular e Biotecnologia, Universidade Federal do Rio de Janeiro, Laboratório de Bioenergética e Fisiologia Mitocôndria, Cidade Universitária, Rio de Janeiro 21941–590, Brazil.

Corresponding author:

Prof. Dr. Luis Valmor Portela
Departamento de Bioquímica, ICBS, UFRGS
Rua Ramiro Barcelos, 2600 anexo
CEP 90035-003
Porto Alegre, RS, Brazil
Email: roskaportela@gmail.com
Telephone: 55 51 33085558
Fax: 55 51 33085544

Abstract

In humans 20% of oxygen is used by central nervous system some of them may be deviated to generate reactive oxygen species (ROS). Antioxidant enzyme activities in brain are lower, which put neural cells under increased risk of oxidative stress damage. Mitochondrial electron transport system (ETS) is the main source of cellular ROS including peroxide (H_2O_2). The H_2O_2 production is regulated by many mechanisms as mitochondrial potential of membrane ($\Delta\Psi_m$). Insulin activates variety of physiological effects including neuronal survival, synaptic plasticity and modulation of inhibitory and excitatory neurotransmission. Moreover insulin could regulate mitochondrial proteins in brain. Impaired insulin signaling on brain is currently proposed as a risk factor to development of Alzheimer disease (AD). The main objective of this work was to evaluate the effect of insulin in the H_2O_2 production and $\Delta\Psi m$ in synaptossome and mitochondria of rat brain. Insulin (from 0.1 to 20 mU) with or without pre-incubation in synaptosomes, decreases synaptosomal H_2O_2 production induced by succinate. Wortmannin at 15 min and more prominent at 30 min reversed the neuroprotective effect of lower doses of insulin (from 0.1 to10 mU). Wortmannin keep almost normal the $\Delta\Psi_m$ induced by succinate in sinaptosomes with or without 30min pre-incubation with insulin. In isolated mitochondrial fraction, insulin had few effects on $\Delta\Psi_m$ modulation. Considering the main findings of this work, we propose that insulin may exert antioxidant properties in brain. The results showed that insulin more robustly affect synaptosomes than mitochondria, thus we intend to propose that a complete activation of signaling

pathway is necessary to achieve such effect. These findings strongly suggest that insulin signaling is required for normal mitochondrial function in brain.

Introduction

In humans, almost 20% of oxygen is used by central nervous system and, from this amount, 2 to 5% may be partially reduced deviated to generate reactive oxygen species (ROS) (Floyd and Hensley 2002). In addition, antioxidant enzyme activities in brain are lower, which put neural cells under increased risk of oxidative stress damage (Meyer et al. 2006). A major reason for the high O_2 uptake by brain is the vast amounts of ATP needed to maintain neuronal homeostasis in the face of all the openings and closings of ion channels associated with propagation of action potentials and neurosecretion (Halliwell 2006). In this context, a normal mitochondrial function is essential to support the flux of energy as well as for many cellular functions, including intermediary metabolism, calcium buffering, regulation of signaling cascades, and apoptosis regulation (Morais and De Strooper).

Along with the involvement in the cellular energy homeostasis, mitochondrial electron transport system (ETS) has been suggested as the main source of cellular ROS including superoxide ($O2^{.-}$) and peroxide (H_2O_2). Thus, the continuous generation of ROS without an efficient scavenger activity leads to an imbalance between oxidative/antioxidant status and play important role in ROS induced-cell damage in neurodegenerative diseases and in normal aging processes (Brewer ; Patten et al.). It has been postulated that the neurotoxic effects caused by H_2O_2 is associated to its capacity in induce apoptosis, carbonilation of proteins, damage to cell membranes and to DNA (Nicholls and

Budd 2000). The majority of H_2O_2 production occurs in states 2 and 4 of the mitochondrial respiration in complexes I and III (Chen et al. 2003) and, this production is regulated by many mechanisms as mitochondrial potential of membrane ($\Delta\Psi_m$) and oxygen consumption. High $\Delta\Psi_m$ values causes increased H_2O_2 production (Starkov and Fiskum 2003). In addition, high cellular oxygen consumption may negatively impact $\Delta\Psi_m$ thus increasing the H_2O_2 production (Korshunov et al. 1997; Starkov and Fiskum 2003).

In the last years insulin was recognized as a neurotrophic factor acting on insulin receptors (IR) distributed in neurons from different brain regions (Zhao and Alkon 2001). After binding to IR insulin activates downstream signaling proteins (PI3K, AKT, GSK) resulting a variety of physiological effect including neuronal survival (Valenciano et al. 2006), synaptic plasticity and modulation of inhibitory and excitatory neurotransmission (Dou et al. 2005; Lee et al. 2005; Stranahan et al. 2008; Zhao et al. 1999). Impaired insulin signaling on brain is currently proposed as a risk factor to development of Alzheimer disease (AD) (Neumann et al. 2008; Ott et al. 1999). Insulin deficient signaling on brain decreases clearance of β-amyloids, impairs learning and memory processes and disrupt glucose metabolism (van der Heide et al. 2006). Some recent works have shown that insulin is able to modulate mitochondrial signaling/related proteins such as AKT avoiding neuronal death (Cheng et al. ; Mookherjee et al. 2007; Petit-Paitel et al. 2009) as well as to block cytochrome c release by brain mitochondria through the PI3K activation (Sanderson et al. 2008).

The main objective of this work was to evaluate the effect of insulin in the H_2O_2 production and $\Delta\Psi m$ in synaptossome and mitochondria of rat brain. The

data suggest that insulin binding to insulin receptors in synaptossomal membranes preparations consuming oxygen sustained by succinate starts signaling cascade events that culminate in a small depolarization of $\Delta\Psi_m$ and a blockage of mitochondrial electron leaks from ETS.

Material and methods

Animals: Adult (3-5months) Wistar male rats were kept in a room with controlled temperature (22^oC) under a 12 h light/12 h dark cycle and had free access to food and water. All experiments followed the guidelines of the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

Chemicals reagents

Insulin was purchased form Lilly, Humulin; fatty acid-free bovine serum albumin, succinate, rotenone, antimycin A, safranine O, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), horseradish peroxidase, catalase and wortmannin, were all purchased from Sigma. Amplex Red was purchased from Invitrogen; Percoll was from Amersham Biosciences. Hydrogen peroxide was from Merck.

Isolation of synaptosomes and mitochondria.

Synaptosomes and mitochondria were isolated by method of Sims 1990 (Sims 1990). Briefly, the brains were rapidly removed to ice-cold "isolation buffer" containing 0.32 M sucrose, 1 mM EDTA (K' salt), and 10 mM Tris-HC1 (pH 7.4). The cerebellum and underlying structures were removed and the remaining material used as the forebrain. Forebrain was homogenate in isolation buffer.

The homogenate was centrifuged at 4,000 rpm (1,330 g) for 3 min. The supernatant was carefully decanted and the pellet resuspended in isolation buffer. This homogenate was recentrifuged as above, the supernatant retained, and the pellet discarded. The pooled supernatant was centrifuged at 16,000 rpm (21,200 g) for 10 min. The decanted supernatant was discarded and the pellet resuspended in 3ml 15%. The discontinuous density gradient was prepared (3.5 ml of 23% Percoll above 3.5 ml of 40% Percoll). Tubes were centrifuged for 5 rnin at 19,000 rpm (30,700 g). The fraction 2 was used as fraction enriched in synaptosomes and fraction 3 was used as fraction highly enriched mitochondria as described by Sims et al (Sims 1990; Sims and Anderson 2008).

ROS production

Mitochondrial release of H_2O_2 was assessed by the Amplex Red oxidation method. Synaptossome and mitochondria (0.1 mg protein/mL) were incubated in the standard respiration buffer supplemented with 10 µM Amplex Red and 2 units/mL horseradish peroxidase. Fluorescence was monitored at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit5 nm), a multi-mode microplate reader spectrofluoremeter Spectra Max M5 (Molecular Devices). Each experiment was repeated at least five times with different sinaptosomes and mitochondrial preparations. In all experiments, we detected small changes in the rate of H_2O_2 formation among different preparations, but the overall pattern of response to different modulators was not affected. The maximal rate (100%) of mitochondrial H_2O_2 formation was assumed to be the difference between the rate of H_2O_2 formation in the

absence of oxidable substrate and that measured after the addition of succinate up to 30 min.

Mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by using the fluorescence signal of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria. Synaptossome and mitochondria (0.1 mg protein/mL) were incubated in the standard respiration buffer supplemented with 10 µM safranine. FCCP (5 µM) was used to collapse $\Delta\Psi_m$ as a control. Fluorescence was detected with an excitation wavelength of 495 nm (slit 5 nm) and an emission wavelength of 586 nm (slit 5 nm) using a multi-mode microplate reader spectrofluoremeter Spectra Max M5 (Molecular Devices). Data are reported as percentage of maximal depolarization induced by succinate during 30min (no substate-succinate). Each experiment was repeated at least three times with different mitochondrial preparations.

Western blotting

For Western blot analysis, 30 µg of protein (total protein content was measured using the method described by Peterson 1977 (26); from sinaptossome and mitochondria (homogenate in PIK buffer, 1 % NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 0.1 % phosphatase inhibitor cocktails I and II. Sigma-Aldrich, USA), were loaded into each well; separated by electrophoresis on a 10% polyacrylamide gel and electrotransfered to PVDF membranes. Non-specific binding sites were blocked with Tween–Tris buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5) containing 5% albumin for 2 h and then incubated overnight at 4 ^{o}C with polyclonal

antibodies against insulin receptors pAktser-473 (Cell Signaling Technology, 1:1000) and Akt (Cell Signaling Technology, 1:3000). Membranes were rinsed 3 x 10 min with TTBS and incubated with secondary antibodies (1:3000 dilution, anti-rabbit, GE healthcare life sciences) for 2 h at room temperature. Membranes were then rinsed 4 x 10 min with TTBS and incubated with peroxidase-conjugated for 5 min at room temperature.

Results

Insulin decreases synaptossomal and mitochondrial H_2O_2 production.

Firstly, as showed in figure 1A and 1B, brain tissue preparations enriched in synaptosomes and highly enriched in mitochondria were responsive to succinate, ADP and oligomycin. Moreover as showed in figure 1C we demonstrate that insulin signaling was present in our preparation. In synaptossome insulin increased the phosphorylation state of AKT (ser473) in a dose dependent manner at 15min of incubation. The mitochondria enriched preparation showed total and phosphorylated AKT but with no effect of insulin (figure 1C). These were performed to confirm the functional viability of preparations.

Afterward we showed that insulin (from 1 to 20 mU) without pre-incubation in synaptosomes, decreases synaptosomal H_2O_2 production induced by succinate (up to 30 min) (Figure 2A). Pre-incubation (without succinate) at 37^oC with insulin (from 0.1 to 20 mU) in synaptosomes during 15 min and 30 min decreases synaptosomal H_2O_2 production induced by succinate (up to 30min) (Figures 2B, 2C respectively). Comparing without pre-incubation with pre-incubation protocols, we observed that the concentration of insulin necessary to achieve the protective effects on H_2O_2 production is lower to the

later protocol. To investigate if the insulin signaling pathway is necessary to decrease H_2O_2 production we used an insulin signaling phosphatidylinositol 3-kinase (PI3-K) inhibitor, wortmannin at 200nM. The results showed that wortmannin at 15 min (2B) and more prominent at 30 min (2C) completely reversed the neuroprotective effect of lower doses of insulin (from 0.1 to10 mU).

In isolated forebrain mitochondria, insulin at doses of 10 and 20 mU decreased the H_2O_2 production (2E), however this effect was lower than in synaptosomes preparations. Moreover, pre-incubation with insulin had no effect on H_2O_2 production in isolated mitochondria (data not show). Likewise, wortmannin had no effect on isolated mitochondria. This observation suggests that the insulin effect's observed for in isolated mitochondria preparation could be related to sinaptosomal cross-contamination. Taking into consideration these findings, we assumed that insulin signaling in mitochondrial enriched preparations is absent or restricted.

Insulin prevents alterations in the mitochondrial potential of membrane ($\Delta\Psi_m$)

We further postulated that decreased H_2O_2 production by insulin treatment could be implicated in the modulation of $\Delta\Psi_m$. Thus, we induced $\Delta\Psi_m$ alterations by succinate on both, synaptosomes and mitochondria preparations. Insulin efficiently prevents the decreased in $\Delta\Psi_m$ induced by succinate in synaptosomes. Wortmannin keep almost normal the $\Delta\Psi_m$ induced by succinate in sinaptosomes with or without 30min pre-incubation with insulin (Figure 3A, B).

In isolated mitochondrial fraction, insulin had few effects on $\Delta\Psi_m$ modulation. Moreover wortmanin had no affect on $\Delta\Psi_m$ of isolated mitochondria as observed in the H_2O_2 production (Figure 3C).

Discussion

Our results suggest that insulin prevents the oxidative stress in forebrain rats. This proposed functional role may be closely associated with the modulation exerted by insulin on $\Delta\Psi_m$ in state 2 of mitochondria. In the last few years, brain insulin receptors mainly found in neurons have been implicated in neuromodulatory, neurotrophic and metabolic functions (Cole and Frautschy 2007). Moreover, insulin was proposed to have antioxidant role in brain (Duarte et al. 2004) and to enhances $\Delta\Psi_m$ of culture of neurons (Huang et al. 2005). We observed H_2O_2 production is more prominent decreased with pre-incubation with insulin and the $\Delta\Psi_m$ modulation do not require these pre-treatment. In this way we proposed that insulin signaling could affect neurons in different ways.

We do not assume that decreased the H_2O_2 production induced by insulin is only due to $\Delta\Psi_m$ modulation, but it was described that very small decreases in $\Delta\Psi_m$ values could impair the semi-ubiquinone radical formation leading to an drastic decrease in the electron leaks from ETS to form superoxide anion (Korshunov et al. 1997). In agree with these, kinases modulated the $\Delta\Psi_m$ (Meyer et al. 2006), moreover oxygen consumption and scavengers enzymes modulated the H_2O_2 production (Santiago et al. 2008) therefore we can't ruling out the effect of insulin in regulated these pathways. However insulin did not affect the catalase activity neither the oxygen consumption in our preparations (data not showed). A normal intracellular

insulin signaling is putatively involved in several aspects of brain physiology including neuronal survival (Plum et al. 2005), however, alterations in this signaling pathway may causes cellular damage (Hoyer and Lannert 2007). Here, we showed that insulin decreased H_2O_2 production and $\Delta\Psi_m$ in synaptosomes whereas wortmanin, a PI3K insulin signaling inhibitor, abolished such effects.

Insulin modulated GABAergic and glutamatergic neurotransmitter systems (Christie et al. 1999; Duarte et al. 2003; van der Heide et al. 2006). It was reported that insulin transiently activates the NR2A and NR2B NMDA glutamatergic receptor subunits (Christie et al. 1999). Once this receptor is activated, it promotes the influx of Ca^{2+} inside the cell, which can acts as a modulator of $\Delta\Psi_m$ (Komary et al.). Our results showed that insulin more robustly affect synaptosomes than mitochondria, thus we intend to proposed that a complete activation of IR and downstream signaling pathway is necessary to achieve such effect.

The brain has a high capacity to generate ROS associated due to high metabolic demands and to a lower antioxidant capacity. Considering the main findings of this work, we can propose that insulin may exert antioxidant properties in brain. Interestingly, in conditions where brain insulin signaling are deficient or altered including aging, Alzheimer disease and diabetes mellitus there are increased H_2O_2 production (Kilbride et al. 2008). Overall, strategies maintenance/improvement of brain insulin signaling (Muller et al.), or improve mitochondrial function (Dietrich et al. 2008) could be helpful to keep normal the balance of redox state in brain. These findings strongly suggest that insulin

signaling is required for normal mitochondrial function in metabolism as described by Cheng et al (Cheng et al. 2009).

Impair insulin signaling in brain are related to neurodegenerative disease (Steen et al. 2005). In summary our results showed that insulin decreased mitochondrial H_2O_2 generation, which was closely related in avoiding depolarization induced by succinate on $\Delta\Psi_m$ but not with oxygen consumption and catalase. Thus insulin may exert potential neuroprotective roles against neurodegenerative diseases through antioxidant properties and modulation of mitochondrial function.

References

1. **Floyd RA, Hensley K** 2002 Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. Neurobiol Aging 23:795-807

2. **Meyer LE, Machado LB, Santiago AP, da-Silva WS, De Felice FG, Holub O, Oliveira MF, Galina A** 2006 Mitochondrial creatine kinase activity prevents reactive oxygen species generation: antioxidant role of mitochondrial kinase-dependent ADP re-cycling activity. J Biol Chem 281:37361-37371

3. **Halliwell B** 2006 Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634-1658

4. **Morais VA, De Strooper B** Mitochondria dysfunction and neurodegenerative disorders: cause or consequence. J Alzheimers Dis 20 Suppl 2:S255-263

5. **Brewer GJ** Epigenetic oxidative redox shift (EORS) theory of aging unifies the free radical and insulin signaling theories. Exp Gerontol 45:173-179

6. **Patten DA, Germain M, Kelly MA, Slack RS** Reactive oxygen species: stuck in the middle of neurodegeneration. J Alzheimers Dis 20 Suppl 2:S357-367

7. **Nicholls DG, Budd SL** 2000 Mitochondria and neuronal survival. Physiol Rev 80:315-360

8. **Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ** 2003 Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 278:36027-36031

9. **Starkov AA, Fiskum G** 2003 Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. J Neurochem 86:1101-1107

10. **Korshunov SS, Skulachev VP, Starkov AA** 1997 High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 416:15-18

11. **Zhao WQ, Alkon DL** 2001 Role of insulin and insulin receptor in learning and memory. Mol Cell Endocrinol 177:125-134

12. **Valenciano AI, Corrochano S, de Pablo F, de la Villa P, de la Rosa EJ** 2006 Proinsulin/insulin is synthesized locally and prevents caspase- and cathepsin-mediated cell death in the embryonic mouse retina. J Neurochem 99:524-536

13. **Lee CC, Huang CC, Wu MY, Hsu KS** 2005 Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway. J Biol Chem 280:18543-18550

14. **Stranahan AM, Norman ED, Lee K, Cutler RG, Telljohann RS, Egan JM, Mattson MP** 2008 Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. Hippocampus 18:1085-1088

15. **Dou JT, Chen M, Dufour F, Alkon DL, Zhao WQ** 2005 Insulin receptor signaling in long-term memory consolidation following spatial learning. Learn Mem 12:646-655

16. **Zhao W, Chen H, Xu H, Moore E, Meiri N, Quon MJ, Alkon DL** 1999 Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. J Biol Chem 274:34893-34902

17. **Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM** 1999 Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology 53:1937-1942

18. **Neumann KF, Rojo L, Navarrete LP, Farias G, Reyes P, Maccioni RB** 2008 Insulin resistance and Alzheimer's disease: molecular links & clinical implications. Curr Alzheimer Res 5:438-447

19. **van der Heide LP, Ramakers GM, Smidt MP** 2006 Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol 79:205-221

20. **Petit-Paitel A, Brau F, Cazareth J, Chabry J** 2009 Involvment of cytosolic and mitochondrial GSK-3beta in mitochondrial dysfunction and neuronal cell death of MPTP/MPP-treated neurons. PLoS One 4:e5491

21. **Mookherjee P, Quintanilla R, Roh MS, Zmijewska AA, Jope RS, Johnson GV** 2007 Mitochondrial-targeted active Akt protects SH-SY5Y neuroblastoma cells from staurosporine-induced apoptotic cell death. J Cell Biochem 102:196-210

22. **Cheng Z, Tseng Y, White MF** Insulin signaling meets mitochondria in metabolism. Trends Endocrinol Metab

23. **Sanderson TH, Kumar R, Sullivan JM, Krause GS** 2008 Insulin blocks cytochrome c release in the reperfused brain through PI3-K signaling and by promoting Bax/Bcl-XL binding. J Neurochem 106:1248-1258

24. **Sims NR** 1990 Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. J Neurochem 55:698-707

25. **Sims NR, Anderson MF** 2008 Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. Nat Protoc 3:1228-1239

26. **Peterson GL** 1977 A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346-356

27. **Cole GM, Frautschy SA** 2007 The role of insulin and neurotrophic factor signaling in brain aging and Alzheimer's Disease. Exp Gerontol 42:10-21

28. **Duarte AI, Santos MS, Seica R, Oliveira CR** 2004 Oxidative stress affects synaptosomal gamma-aminobutyric acid and glutamate transport in diabetic rats: the role of insulin. Diabetes 53:2110-2116

29. **Huang TJ, Verkhratsky A, Fernyhough P** 2005 Insulin enhances mitochondrial inner membrane potential and increases ATP levels through phosphoinositide 3-kinase in adult sensory neurons. Mol Cell Neurosci 28:42-54

30. **Santiago AP, Chaves EA, Oliveira MF, Galina A** 2008 Reactive oxygen species generation is modulated by mitochondrial kinases: correlation with mitochondrial antioxidant peroxidases in rat tissues. Biochimie 90:1566-1577

31. **Plum L, Schubert M, Bruning JC** 2005 The role of insulin receptor signaling in the brain. Trends Endocrinol Metab 16:59-65

32. **Hoyer S, Lannert H** 2007 Long-term abnormalities in brain glucose/energy metabolism after inhibition of the neuronal insulin receptor: implication of tau-protein. J Neural Transm Suppl:195-202

33. **Christie JM, Wenthold RJ, Monaghan DT** 1999 Insulin causes a transient tyrosine phosphorylation of NR2A and NR2B NMDA receptor subunits in rat hippocampus. J Neurochem 72:1523-1528

34. **Duarte AI, Santos MS, Seica R, de Oliveira CR** 2003 Insulin affects synaptosomal GABA and glutamate transport under oxidative stress conditions. Brain Res 977:23-30

35. **Komary Z, Tretter L, Adam-Vizi V** Membrane potential-related effect of calcium on reactive oxygen species generation in isolated brain mitochondria. Biochim Biophys Acta

36. **Kilbride SM, Telford JE, Davey GP** 2008 Age-related changes in H2O2 production and bioenergetics in rat brain synaptosomes. Biochim Biophys Acta 1777:783-788

37. **Muller AP, Gnoatto J, Moreira JD, Zimmer ER, Haas CB, Lulhier F, Perry ML, Souza DO, Torres-Aleman I, Portela LV** Exercise increases insulin signaling in the hippocampus: Physiological effects and pharmacological impact of intracerebroventricular insulin administration in mice. Hippocampus

38. **Dietrich MO, Andrews ZB, Horvath TL** 2008 Exercise-induced synaptogenesis in the hippocampus is dependent on UCP2-regulated mitochondrial adaptation. J Neurosci 28:10766-10771

39. **Cheng Z, Guo S, Copps K, Dong X, Kollipara R, Rodgers JT, Depinho RA, Puigserver P, White MF** 2009 Foxo1 integrates insulin signaling with mitochondrial function in the liver. Nat Med 15:1307-1311

40. **Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM** 2005 Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis 7:63-80

Legends to figure

Figure 1. Viability of synaptossome and mitochondria enriched fraction respectively. A) and B) Succinate increase the H_2O_2 production when compared to basal levels (Succinate>Basal; $p<0.05$). The additions of ADP decrease the H_2O_2 production when compared to succinate (Succinate+ADP< succinate; $p<0.005$). The addition of oligomicin decrease the H_2O_2 production when compared to succinate (Oligomicin< succinate; $p<0.05$). C) Insulin increased the phosphorylation state of Akt[ser 473] at dose 0f 0.5-20mU in synaptossome but not in mitochondrial fraction.

Figure 2. Insulin decreases the H_2O_2 production induced by succinate in synaptossome by insulin intracellular signaling. A) Insulin decrease the H_2O_2 production at dose 1-20mU without pre-incubation (vehicle>insulin 1-20mU; $p<0.05$). The PI3K inhibitors, wortmannin, abolish this effect in 1mU but not in other doses (vehicle= wortmannin+insulin 1mU). B) The pre-incubation with insulin for 15min increase the insulin antioxidant effect (vehicle>insulin 0.5-20mU; $p<0.05$). The pre incubation with insulin+ wortmannin abolish the effect of insulin in lower doses (vehicle= wortmannin+insulin 0.1-1mU; $p<0.05$).C) The pre-incubation with insulin for 30min increase the insulin antioxidant effect (vehicle>insulin 0.1-20mU; $p<0.05$). The pre incubation with insulin+ wortmannin abolish the effect of insulin (vehicle= wortmannin+insulin 0.1-10mU; $p<0.05$). D) Neither insulin nor insulin+wortmannin had no effect in mitochondrial enriched fraction.

Figure 3. Insulin efficiently prevents the decreased in $\Delta\Psi_m$ induced by succinate in synaptosomes. A) The incubation with insulin decreased the $\Delta\Psi_m$ at dose of 0.1mU and other doses had the same pattern (basal>insulin 0.1-20mU; $p<0.05$). The incubation with wortmannin abolish this effect in synaptossome. B) The pre-incubation for 30min with insulin decreased the $\Delta\Psi_m$ at dose of 0.1mU and other doses had the same pattern (basal>insulin 0.1-20mU; $p<0.05$). The incubation with wortmannin abolishes this effect in synaptossome. C) Neither insulin nor insulin+wortmannin had no effect in mitochondrial enriched fraction.

Figure 1
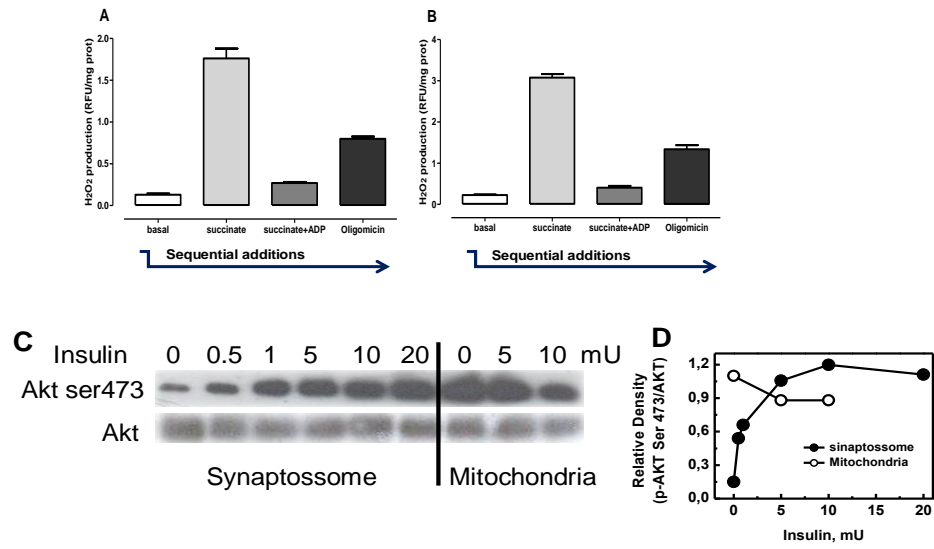
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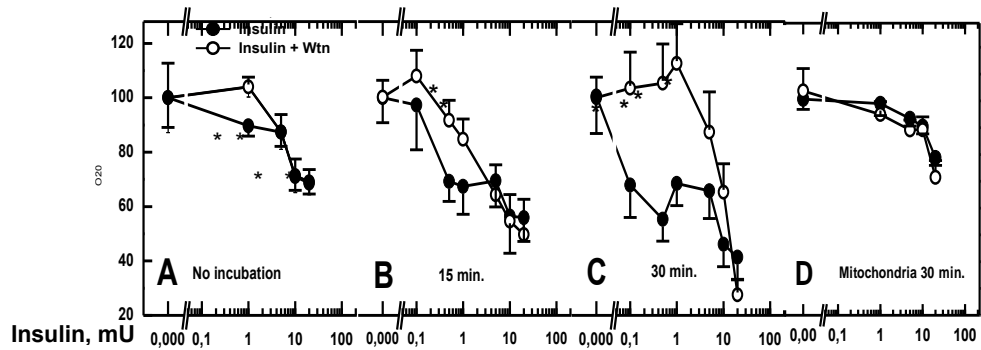
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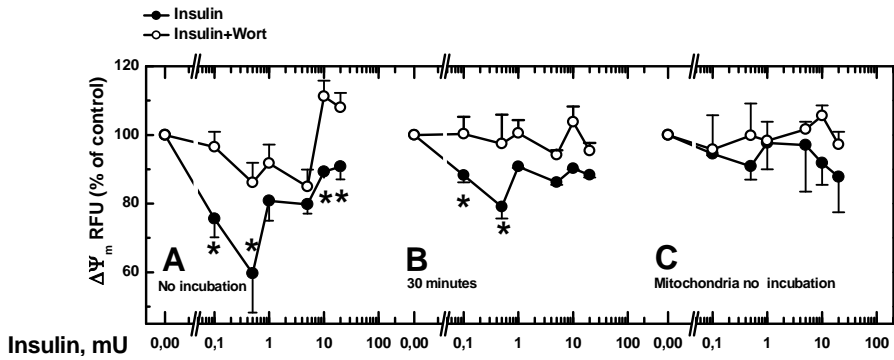
Capitulo 6.

Artigo submetido ao periódico Journal of Alzheimer Disease.

Physical exercise worsens insulin resistant brain state induced by

streptozotocin but improves insulin antioxidant effect in mice

Running title: Exercise increases insulin resistance brain induced by STZ

Alexandre P. Muller[a*]; Eduardo Zimmer[a]; Eduardo Kalinine[a]; Clarissa B.

Haas[a],   Jean Pierre Oses[a,b]; Antonio Galina[c], Diogo O. Souza[a]; Luis

Valmor Portela[a]

a. Departamento de Bioquímica, ICBS, UFRGS. Programa de Pós Graduação em Ciências Biológicas-Bioquímica. Rua Ramiro Barcelos, 2600 anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

b. Programa de Pós-Graduação em Saúde & Comportamento Centro de Ciências da Vida e da Saúde Universidade Católica de Pelotas. Rua Almirante Barroso,1202 sala G109 96010-280 Pelotas, Rio Grande do Sul Brasil.

c. Instituto de Bioquímica Médica, Programa de Biofísica e Bioquímica Celular e Programa de Biologia Molecular e Biotecnologia, Universidade Federal do Rio de Janeiro, Laboratório de Bioenergética e Fisiologia Mitocôndria, Cidade Universitária, Rio de Janeiro 21941–590, Brazil.

Corresponding author: Alexandre Pastoris Muller, MSc

Departamento de Bioquímica, ICBS, UFRGS

Rua Ramiro Barcelos, 2600 anexo

 CEP 90035-003

 Porto Alegre, RS, Brazil

Email: alexandrep.muller@gmail.com

Telephone: 55 51 33085557

Fax: 55 51 33085544

Abstract

Insulin resistant brain state is related to cognitive deficits and Alzheimer's disease. Oxidative stress and damage to mitochondria may participate in the pathogenesis of these alterations. Conversely, physical exercise improves cognitive function and brain insulin signaling. Intracerebroventricular (i.c.v.) administration of streptozotocin (STZ) in rodents has been used as a model of brain insulin resistance. The aim of this work was to evaluate in mice the effects of voluntary physical exercise on memory performance of STZ i.c.v (1 and 3 mg/kg) treated mice and whether insulin (0.5 and 1mU) modulates H_2O_2 generation by mitochondria in synaptossomes. S100B level was assessed as marker of glial activation in hippocampus. Sedentary and exercised vehicle mice had similar performance in object recognition memory. In water maze task exercised vehicle mice showed improvement performance in both acquisition and retrieval phases. STZ 1mg/kg impaired recognition and spatial memory only in exercised mice whereas 3mg/kg impaired the performance of sedentary and exercised groups. Moreover, STZ 3mg/kg increased S100B levels in hippocampus in both groups. Insulin more prominently decreased the mitochondrial H_2O_2 production in exercised compared to sedentary mice. Both STZ doses abolished this antioxidant effect. In summary, a normal brain insulin signaling participates in the mechanism necessary to improve cognition function induced by exercise. This result could be associated with increase mitochondrial activity induced by exercise. However, blockade of brain insulin signaling by STZ induced insulin resistant brain state abolish the benefits of exercise on memory performance and in mitochondrial H_2O_2 regulation.

Key words: Physical exercise, insulin, Insulin-resistant brain state, Alzheimer disease, mitochondria, cognition performance.

Introduction

There is a growing interest in determining the importance of insulin signaling for a normal brain function and whether a state of central insulin resistance could be related to neurodegenerative diseases and cognitive deficits (de la Monte and Wands 2005; Fulop et al. 2003; Hoyer 2004a; Salkovic-Petrisic and Hoyer 2007). In accordance with these conjectures, studies have demonstrated that impaired brain insulin/IGF1 pathways may cause mitochondrial dysfunction along with increased oxidative stress damage to neural cells. The insulin resistance brain state is believed to play a pivotal role in the pathogenesis of neurodegenerative disorders including Alzheimer's disease (AD) (Dietrich et al. 2007; Lin and Beal 2006; Muller et al. 2008).

Moreover, there is increased number of evidences that mitochondria *per se* can be a site of damage in neurodegenerative diseases. Indeed, Aβ amyloid protein is taken up and interacts with various mitochondrial proteins causing reductions in respiratory chain complex activities and increasing generation of reactive oxygen species (ROS) (Readnower et al.). Hydrogen peroxide (H_2O_2) is a reactive oxygen species important for inducing oxidative damage and regulating redox-sensitive signaling (Murphy et al.). Under normal conditions, the majority of cellular H_2O_2 is produced by mitochondria and is promptly metabolized, however in a scenario of decreased mitochondrial antioxidant capacity, H_2O_2 raises causing oxidative damage impaired metabolism and cellular degeneration (Du et al. 2009; Manczak et al. 2006). Thus, mitochondrial dysfunction and ROS generation emerge as one important factor associated to AD pathogenesis. Conversely, it has been shown that regular physical exercise positively modulate brain insulin signaling (Muller et al.)], protects against

neurodegenerative disorders (Cotman and Berchtold 2002)] and increases antioxidant defenses (Radak et al. 2008)]. Moreover, physical exercise increases the number and activity of neuronal mitochondria supporting energy enough for exercise-induced neuroplasticity phenomenon (Radak et al. 2008) .

In the last years brain oxidative damage to mitochondria has been a field of interest in AD research (Davey et al. 1998). Indeed, alterations in the respiratory chain complexes impair brain energy supply leading to neural cells to degenerate (Bowling et al. 1993). Streptozotocin (STZ) is commonly used at high doses (> 65 mg/kg, i.p.) to induce experimental diabetes mellitus in rats following peripheral administration (Szkudelski 2001). However, intracerebroventricular (i.c.v.) STZ administration at very low doses (1–3 mg/kg) in rodents does not alter peripheral glucose and is used as a reliable experimental model for late-onset Sporadic Alzheimer's disease (SAD) (Lannert and Hoyer 1998; Plaschke and Hoyer 1993; Prickaerts et al. 1999; Sharma and Gupta 2001). The proposed mechanisms for STZ induced AD are multifactorial and include: i) impair in glucose metabolism particularly in brain structures with high glucose demands and high insulin sensitivity (Henneberg and Hoyer 1995); ii) decreased intracellular insulin/IGF1 signaling pathways (Blokland and Jolles 1993; Mayer et al. 1990); and iii) increase oxidative stress (Sharma and Gupta 2001). These alterations are associated with impaired learning and memory performance reported in this AD model.

The aim of this work was to evaluate the effects of physical exercise on memory performance of STZ i.c.v treated mice as well as whether insulin modulates H_2O_2 generation by mitochondria. With this in mind we used spatial and recognition memory tasks and forebrain synaptossomal preparations. Our

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results indicate that STZ i.c.v causes cognitive impairment by decreasing insulin signaling and increasing mitochondrial H_2O_2 generation. Physical exercise did not prevent the deleterious STZ effects.

Material and methods

Animals, exercise protocol and surgical procedure

Two-month-old CF1 mice were housed in standard cages (48 x 26 cm) with four animals per cage. To avoid social isolation, the animals were not confined in individual housing (Leasure and Decker 2009). Animals were kept in a room with controlled temperature (22^oC) under a 12 h light/12 h dark cycle and had free access to food and water. Animals were anesthetized by an intraperitoneal (i.p.) injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). STZ (1mg/kg or 3mg/kg) or saline was i.c.v injected in both hemispheres. After 3 days of surgery mice were divided into sedentary group and exercise groups. Exercise animals had free access to a running wheel. After 4 weeks of access to the running wheel, each mouse ran an average of about 3500 m. All experiments followed the guidelines of the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil. The experiments were carried out after four weeks of voluntary exercise and during behavioral protocols animals still have free access to running wheel.

Object recognition memory task

Mice were randomly allocated to individual wooden boxes (50 x 50 x 50 cm) placed on the floor of a soundproof and diffusely illuminated room, for 3 min in day 1. On day 2, mice were familiarized with two identical plastic objects placed in the cage, one in each corner, 8.5 cm from the walls. Each session lasted 3 min. Following the novel-place test trial, the mice were returned to their

cages for 24h and then placed in the arena for a novel object recognition test, in which one of the objects was replaced with a novel object that differed in shape, color, and texture. All objects and the arena were thoroughly cleaned with 10% ethanol between trials to remove odors. The numbers of exploring each object during the familiarization training and the testing trials was recorded. Exploration was defined as approaching the object nose-first within 1 cm, sniffing, and touching the object with the tip of the nose and/or with the paws. Standing next to the object or on top of it was not considered as explorative activity. Videos were obtained and analyzed by N-Maze program.

Morris water maze–spatial memory task

The apparatus was a black, circular pool (110cm diameter) with a water temperature of 21 ± 1°C. Mice (n=10-12 per group) were trained daily in a 4-trial water maze task for 3 consecutive days; each trial lasted up to 60 s and was followed by 20 s of rest on a hidden black platform. During training, mice learned to escape from the water by finding a hidden, rigid, black platform submerged about 1 cm below the water surface in a fixed location. If the animal failed to find the platform in 60 s, it was manually placed on the platform and allowed to rest for 20 s. Each trial was separated by at least 12 min to avoid hypothermia (the variations of rectal temperature were equal for all groups) and facilitate memory acquisition. The maze was located in a well-lit, white room with several visual stimuli hanging on the walls to provide spatial cues. Latency to find the platform during each trial was measured as an indicator of learning. A probe test without the platform was performed on the fourth day. The time spent in the target quadrant was measured as an indicator of memory retention. Videos were obtained and analyzed by N-Maze program.

Preparation of synaptosomes

Synaptosomes were isolated by method of Sims 1990. Briefly, the brains were rapidly removed to ice-cold "isolation buffer" containing 0.32 M sucrose, 1 mM EDTA (K' salt), and 10 mM Tris-HC1 (pH 7.4). The cerebellum and underlying structures were removed and the remaining material used as the forebrain. Forebrain was homogenate in isolation buffer.

The homogenate was centrifuged at 4,000 rpm (1,330g) for 3 min. The supernatant was carefully decanted and the pellet resuspended in isolation buffer. This homogenate was recentrifuged as above, the supernatant retained, and the pellet discarded. The pooled supernatant was centrifuged at 16,000 rpm (21,200 g) for 10 min. The decanted supernatant was discarded and the pellet resuspended in 3ml 15% Percoll. The discontinuous density gradient was prepared (3.5 ml of 23% Percoll above 3.5 ml of 40% Percoll. Tubes were centrifuged for 5 min at 19,000 rpm (30,700 g). The fraction 2 was used as fraction enriched in synaptosomes and fraction 3 was used as fraction highly enriched mitochondria as described by Sims et al (Sims 1990; Sims and Anderson 2008).

Mitochondrial activity - H_2O_2 production

Mitochondrial H_2O_2 production was assessed by the Amplex Red oxidation method. Synaptossomes (0.1 mg protein/mL) were incubated in the standard respiration buffer supplemented with 10 mM Amplex Red and 2 units/mL horseradish peroxidase with vehicle or insulin (0.5 and 1mU) in each well. Fluorescence was monitored at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit5 nm), a multi-mode microplate reader Spectra Max M5 (Molecular Devices). Each experiment was repeated at least five times with different synaptosomes. In all experiments, we detected small

changes in the rate of H_2O_2 formation among different preparations, but the overall pattern of response to different modulators was not affected. The maximal rate (100%) of mitochondrial H_2O_2 formation was assumed to be the difference between the rate of H_2O_2 formation in the absence of oxidable substrate and that measured after the addition of succinate up to 30min.

S100 beta ELISA assay

After decapitation the hippocampus was dissected and homogenized in PIK buffer (1 % NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 0.1 % phosphatase inhibitor cocktails I and II. Sigma-Aldrich, USA). The homogenate was centrifuged and the supernatant was collected. Total protein content was measured using the method described by Peterson. Homogenates were stored at -70 ^{o}C until analysis. S100B protocol was conducted according to the instructions of the manufacturer (Diasorin, Italy).

Results

Recognition and spatial memory performance

There was no statistical difference between sedentary vehicle and exercise vehicle groups in object recognition task. However, 1mg/kg STZ i.c.v. impaired recognition memory performance in exercised compared to exercise vehicle group. Nevertheless, 3mg/kg STZ i.c.v. caused impair in recognition memory in both groups compared to vehicle groups (Fig 1A; $p<0.05$).

In the water maze task exercise vehicle animals showed improvement memory performance on both, acquisition and retrieval phases, as revealed by

decreasing latency to found platform and more time spent in the target quadrant. STZ 1mg/kg impaired the spatial memory performance in exercised animals whereas sedentary animals were not affected. The administration of 3mg/kg STZ impaired memory performance in sedentary and exercise animals (Fig 1B-C; $p<0.05$). The mean swim speed were not statistically different among groups (Fig 1D;).

Mitochondrial activity

We estimate mitochondrial activity through the production of H_2O_2 in isolated forebrain synaptossomes. Exercise increased the H_2O_2 production induced by succinate in synaptossomes. Incubation of insulin at 0.5 and 1 mU in synaptossomal preparations decreased the H_2O_2 production induced by succinate in both groups. The ability of insulin in decrease H_2O_2 production was higher in exercise than in sedentary animals (38% *vs* 26%) (Fig 2A; $p<0.05$). STZ i.c.v at doses of the 1 and 3 mg/Kg abolished the effect of insulin in decreasing H_2O_2 production in both groups (Fig 2B-C). Moreover, exercised STZ i.c.v groups (1 and 3mg/kg) showed decreased H_2O_2 production at basal levels compared to exercise vehicle (Fig 2B-C; $p<0.05$).

Glial reactivity

We analyzed the content of hipocampal S100B as an indicator of glial response to exercise and STZ. Exercise did not increase hippocampal S100B compared to sedentary group but after thirty days of STZ i.c.v 3mg/kg administration there was an increased S100 beta content in sedentary and exercise groups compared to their respective control and to 1 mg/Kg groups (Fig 3; $p<0.05$).

Discussion

In this work we demonstrated that voluntary physical exercise increased the sensitivity to cognitive deficits induced by STZ i.c.v insulin resistance brain state. The administration of STZ i.c.v. 3mg/kg caused impair in the recognition and spatial memory in both sedentary and exercised animals, while 1mg/kg caused memory deficits only in exercised animals. In addition, the ability of insulin in decreasing mitochondrial H_2O_2 production induced by succinate in synaptossomal preparations was more prominent in exercised animals. Furthermore, both STZ doses abolished the ability of insulin in decreasing mitochondrial H2O2 generation suggesting that the antioxidant properties of insulin require normal brain insulin signaling from membrane to mitochondria.

Brain insulin receptors are mainly localized in regions associated with learning and memory processes such as cortex and hippocampus. Deficits in brain insulin signaling have been consistently linked with cognitive deficits in experimental model and in Alzheimer's disease patients (Craft 2006). Moreover, insulin administration at optimal doses improves cognitive performance in both rodents and humans (Park 2001). It is well accepted that voluntary exercise improves hippocampal plasticity and spatial memory performance of rodents. The results presented here corroborate this assumption. We previous showed that voluntary exercise increased insulin signaling in hippocampus (Muller et al.), a region closely associated with spatial memory. The administration of STZ i.c.v. causes a broad range of neural changes including impaired metabolism, decrease in insulin signaling and increase oxidative stress damage, associated to undesired consequences to the processes of learning and memory formation (Sharma and Gupta 2001). Interestingly, cognitive deficits are long-term and

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progressive, observed as early as 2 weeks after STZ-icv administration and are maintained up to 12 weeks post treatment (Grunblatt et al. 2007; Lannert and Hoyer 1998; Salkovic-Petrisic and Hoyer 2007). As expected, after four weeks of STZ icv administration animals still presented memory deficits, which were not reduced by exercise. We showed that even the low dose of the STZ i.c.v. (1mg/kg) affected the memory performance of exercised animals in both tasks (recognition and spatial). It seems that exercise increases sensitization of IR receptors to be damaged by STZ. Alternatively, we intend to suggest that more receptors were translocated to membrane induced by exercise and thus, are more susceptible to damage by STZ than sedentary animals. Thus, an intact hippocampal insulin signaling is required to achieve benefits of exercise on recognition and spatial memory processes (Muller et al.).

Searching for possible mechanisms involved in cognitive impairment associated to STZ administration we evaluated mitochondrial H_2O_2 generation. H_2O_2 is a major ROS produced by mitochondria that is important for oxidative damage (Stone and Yang 2006). Increased mitochondrial H_2O_2 is reported to participate of the aging processes (Schriner et al. 2005) and was also shown to be associated with AD pathogenesis (Manczak et al. 2006). We showed that exercise increased the mitochondrial H_2O_2 production induced by succinate in sinaptossomes. This could represent one aspect of the mitochondrial activation induced by exercise. Moreover, insulin decreased H_2O_2 production in synatossomes as showed by our group (Muller et al, unpublished data) and that effect was reached more efficiently in exercised (38% exercise and 26% sedentary) compared to sedentary animals reinforcing our previous postulation that exercise increase brain insulin sensitive (Muller et al.). On the other hand,

STZ i.c.v. did not affect basal levels production of H_2O_2 in sedentary group. However, the basal levels production of H_2O_2 was decreased by both STZ icv doses (1 and 3 mg/kg) in exercise animals. As insulin was not able to reduce H_2O_2 production in synaptossomes of STZ mice but did it in vehicle mice we can suggests that a normal insulin signaling system has neuroprotective relevance through the modulation of brain mitochondrial H_2O_2 generation. Indeed, disturbing insulin signaling by STZ 3mg/kg elicited S100B protein to increases in hippocampus, which may reflect an activation of glia cells in responses to toxic effects of STZ. Further, it have been demonstrated by Chen et al. 2011 (Chen et al.) that a transgenic mice over expressing mitochondrial peroxiredoxin 3 had significantly decreased cognitive deficits induced by paraquat model of AD along with decreased brain mitochondrial H_2O_2 generation (Chen et al. 2008). Taking together, these results reinforce the major role of mitochondrial H_2O_2 generation in neurodegenerative processes and impaired cognitive function as well as the importance of insulin and exercise on the modulation mitochondrial function.

In summary our results point to normal brain insulin signaling as a mechanism necessary to improve cognition function induced by exercise. This result could be associated with increase mitochondrial activity induced by exercise. However, blockade of brain insulin signaling by STZ induced insulin resistant brain state abolish the benefits of exercise on memory performance and mitochondrial regulation.

References

1. Hoyer S, (2004) Causes and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: therapeutic implications. *Adv Exp Med Biol* 541:135-152

2. de la Monte SM, Wands JR, (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *J Alzheimers Dis* 7:45-61

3. Salkovic-Petrisic M, Hoyer S(2007) Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach *J Neural Transm Suppl*:217-233, 4. Fulop T, Larbi A, Douziech N: Insulin receptor and ageing. *Pathol Biol (Paris)* 51:574-580, 2003

5. Muller AP, Cammarota M, Dietrich MO, Rotta LN, Portela LV, Souza DO, Izquierdo I, Bevilaqua LR, Perry ML, (2008) Different effect of high fat diet and physical exercise in the hippocampal signaling. *Neurochem Res* 33:880-885

6. Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, Souza DO, Torres-Aleman I, (2007) Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. *Neuromolecular Med* 9:324-330

7. Lin MT, Beal MF: Alzheimer's APP mangles mitochondria (2006) *Nat Med* 12:1241-1243

8. Readnower RD, Sauerbeck AD, Sullivan PG, (2011) Mitochondria, Amyloid beta, and Alzheimer's Disease. *Int J Alzheimers Dis* 104545

9. Murphy MP, Holmgren A, Larsson NG, Halliwell B, Chang CJ, Kalyanaraman B, Rhee SG, Thornalley PJ, Partridge L, Gems D, Nystrom T, Belousov V, Schumacker PT, Winterbourn CC: Unraveling the biological roles of reactive oxygen species. *Cell Metab* 13:361-366

10. Du Y, Wooten MC, Wooten MW, (2009) Oxidative damage to the promoter region of SQSTM1/p62 is common to neurodegenerative disease. *Neurobiol Dis* 35:302-310,

11. Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH, (2006) Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. *Hum Mol Genet* 15:1437-1449

12. Muller AP, Gnoatto J, Moreira JD, Zimmer ER, Haas CB, Lulhier F, Perry ML, Souza DO, Torres-Aleman I, Portela LV: Exercise increases insulin signaling in the hippocampus: Physiological effects and pharmacological impact of intracerebroventricular insulin administration in mice. *Hippocampus*

13. Cotman CW, Berchtold NC, (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends Neurosci* 25:295-301

14. Radak Z, Chung HY, Goto S, (2008) Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic Biol Med* 44:153-159

15. Davey GP, Peuchen S, Clark JB, (1998) Energy thresholds in brain mitochondria. Potential involvement in neurodegeneration. *J Biol Chem* 273:12753-12757

16. Bowling AC, Mutisya EM, Walker LC, Price DL, Cork LC, Beal MF, (1993) Age-dependent impairment of mitochondrial function in primate brain. *J Neurochem* 60:1964-1967

17. Szkudelski T, (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol Res* 50:537-546

18. Lannert H, Hoyer S, (1998) Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. *Behav Neurosci* 112:1199-1208

19. Prickaerts J, Fahrig T, Blokland A, (1999) Cognitive performance and biochemical markers in septum, hippocampus and striatum of rats after an i.c.v. injection of streptozotocin: a correlation analysis. *Behav Brain Res* 102:73-88

20. Sharma M, Gupta YK, (2001) Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. *Life Sci* 68:1021-1029

21. Plaschke K, Hoyer S, (1993) Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus. *Int J Dev Neurosci* 11:477-483

22. Henneberg N, Hoyer S, (1995) Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? *Arch Gerontol Geriatr* 21:63-74

23. Mayer G, Nitsch R, Hoyer S, (1990) Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats. *Brain Res* 532:95-100

24. Blokland A, Jolles J, (1993) Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. *Pharmacol Biochem Behav* 44:491-494

25. Leasure JL, Decker L: Social isolation prevents exercise-induced proliferation of hippocampal progenitor cells in female rats. *Hippocampus* 19:907-912, 2009

26. Sims NR, (1990) Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. *J Neurochem* 55:698-707

27. Sims NR, Anderson MF, (2008) Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. *Nat Protoc* 3:1228-1239

28. Craft S, (2006) Insulin resistance syndrome and Alzheimer disease: pathophysiologic mechanisms and therapeutic implications. *Alzheimer Dis Assoc Disord* 20:298-301

29. Park CR, (2001) Cognitive effects of insulin in the central nervous system. *Neurosci Biobehav Rev* 25:311-323

30. Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S, (2007) Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. *J Neurochem* 101:757-770

31. Stone JR, Yang S, (2006) Hydrogen peroxide: a signaling messenger. *Antioxid Redox Signal* 8:243-270

32. Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS, (2005) Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* 308:1909-1911

33. Chen L, Yoo SE, Na R, Liu Y, Ran Q: Cognitive impairment and increased Abeta levels induced by paraquat exposure are attenuated by enhanced removal of mitochondrial H(2)O(2). *Neurobiol Aging*

34. Chen L, Na R, Gu M, Salmon AB, Liu Y, Liang H, Qi W, Van Remmen H, Richardson A, Ran Q, (2008) Reduction of mitochondrial H2O2 by

overexpressing peroxiredoxin 3 improves glucose tolerance in mice. *Aging Cell* 7:866-878

Legends to figure

Figure 1. STZ i.c.v 1mg/kg cause cognitive deficits in exercised but not in sedentary animals. A) Exercise and sedentary vehicles animals showed similar performance in object recognition task; however 1m/kg of STZ caused impair in recognition performance only in exercised mice(# exercise vehicle > exercise STZ 1mg/kg; p<0.005). At dose of 3mg/kg of STZ i.c.v both groups showed impair in recognition memory (*sedentary vehicle and exercise vehicle > sedentary STZ 3mg/kg and exercise STZ 3mg/kg; ; p<0.05). B) Exercised mice showed improve spatial memory performance during acquisition phase in the water maze task (*latency to found platform exercise vehicle < other groups; p<0.05). STZ i.c.v 1mg/kg impairs exercise effects on memory (a; latency to found platform= exercise 1mg/kg>exercise vehicle; p<0.05). STZ i.c.v. 3mg/kg impaired the performance of both groups when compared to respective vehicles (b, c; latency to found platform= sedentary vehicle and exercise vehicle < sedentary STZ 3mg/kg and exercise STZ 3mg/kg; p<0.05). C) In retrieval phase exercise improve the spatial memory (*exercise vehicle > other groups; p<0.05). STZ i.c.v at 1mg/kg decrease the time spent in target quadrant in exercise group when compared to exercise vehicle (# exercise vehicle>exercise STZ 1mg/kg and; p<0.05). STZ 3mg/kg decrease the time spent in target quadrant in both groups (#sedentary STZ 3mg/kg and exercise STZ 3mg/kg < other groups). D) The mean speed did not change with the treatments.

Figure 2. Exercise induced increase ROS production and insulin antioxidant effect in synaptossome. A) Exercised animals showed more H_2O_2 production induced by succinate in synaptossome when compared to sedentary mice (**exercise>sedentary, $p<0.05$). Insulin at dose of 0.5 and 1mU decrease the H_2O_2 production induced by succinate in synaptossome in both groups, however the effects in exercised mice were more prominent (exercise vehicle > exercise 0.5mU > exercise 1mU; and sedentary > sedentary 0.5 and sedentary 1mU, $p<0.05$). B) and, C) STZ i.c.v at 1 and 3mg/kg abolish the effect of exercise increase the basal H_2O_2 production and antioxidant effects of insulin.

Figure 3. STZ i.c.v. (3mg/kg) increased the hippocampal S100B levels in sedentary and exercised mice (sedentary 3mg/kg and exercise 3mg/kg> other groups, $p<0.05$).

Figure 1

A — numbers of exploration

sedentary vehicle, sedentary STZ 1mg, sedentary STZ 3mg, exercise vehicle, exercise STZ 1mg, exercise STZ 3mg

B — latency (s) to found platform vs days of training

sedentary vehicle, sedentary STZ 1mg, sedentary STZ 3mg, exercise vehicle, exercise STZ 1mg, exercise STZ 3mg

C — time (s) in target quadrant

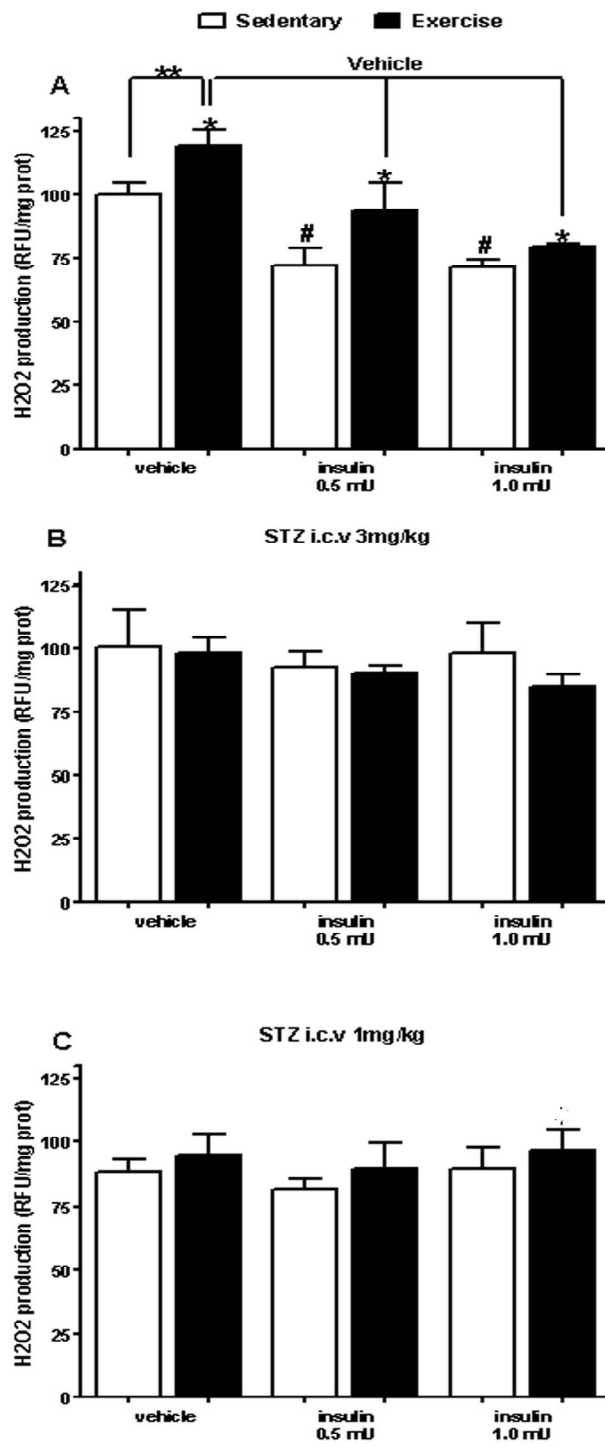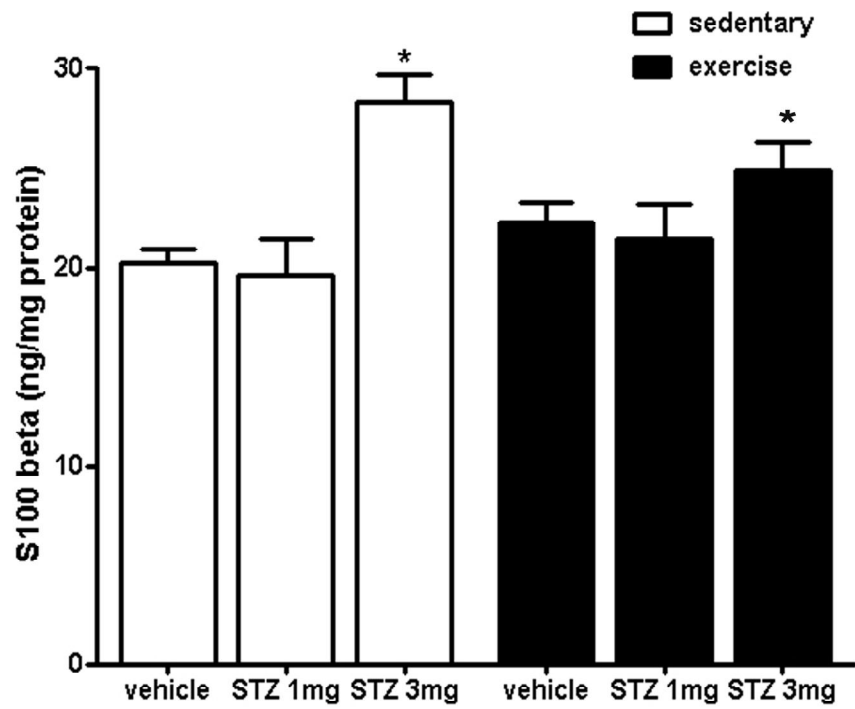D — mean speed (m/s)

Sedentary, exercise
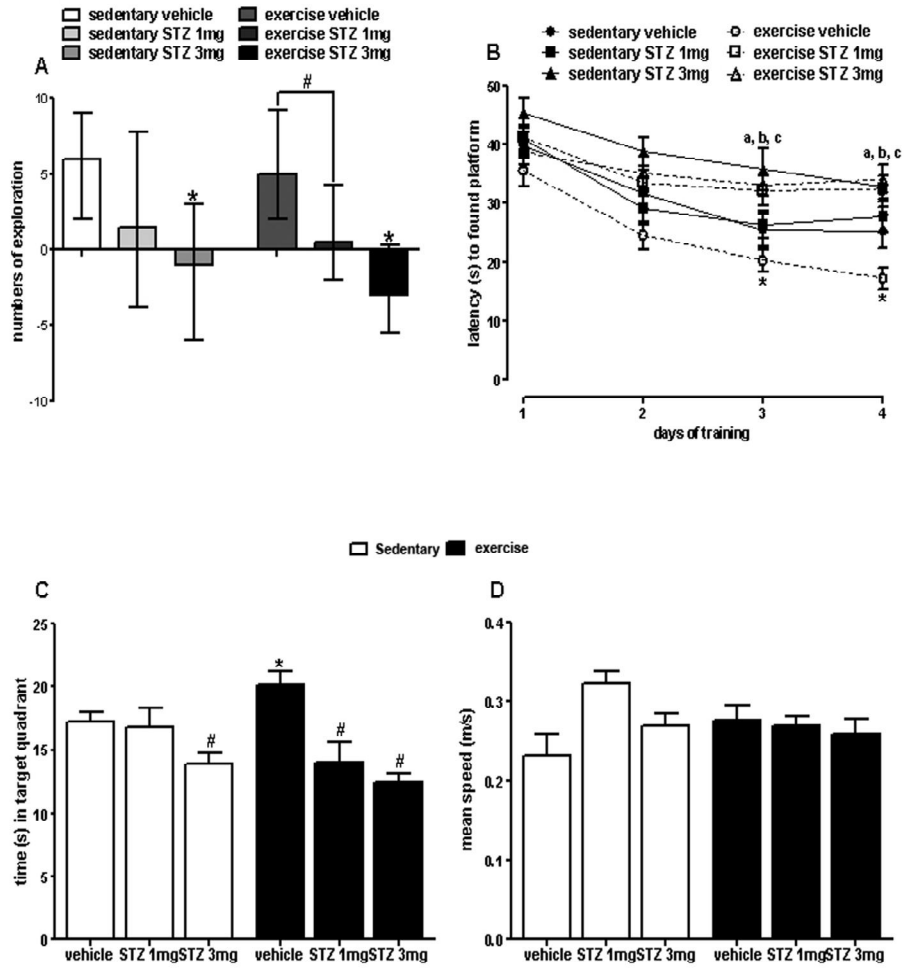
Figure 2

Figure 3

Parte III

4. DISCUSSÃO

Os efeitos dos fatores ambientais como composição da dieta, atividade física e envelhecimento são atualmente de grande interesse no meio científico principalmente em relação as suas ações no SNC (Mattson 2003). No primeiro capítulo nós mimetizamos um modelo dieta de estilo ocidental, rica em carboidratos simples e gordura saturada, altamente palatável aos roedores, o que causou aumento de massa corporal e aumento dos níveis séricos de triglicerídeos. Ocorreu também uma diminuição na passagem de IGF-1 pela barreira sangue cérebro. Estes altos níveis de TGL séricos interagem com muitas proteínas sanguíneas e podem alterar sua ligação aos seus transportadores na barreira sangue-cérebro (Banks et al. 1999), como foi no caso da ligação do IGF-1 ao seu principal transportador no plexo coróide, a proteína megalina, sem afetar sua ligação ao seu recepto.

A barreira sangue-cérebro tem um importante papel na homeostasia cerebral e, um reduzido aporte de um importante fator neurotrófico como o IGF-1, pode ser uma das causas dos efeitos indesejáveis provocados por uma dieta hiperpalátavel (Mattson) 2011. Realmente tem sido demonstrado que a diminuição da passagem de IGF-1 do sangue para o cérebro pode causar distúrbios cognitivos (Davila et al. 2007), redução dos níveis de BDNF (Ding et al. 2006), diminuição da angiogênese (Lopez-Lopez et al. 2004) e alterações neuropatológicas semelhantes à doença de Alzheimer (Carro and Torres-Aleman 2004). Dieta rica em carboidratos e lipídeos também causa alterações semelhantes (Molteni et al. 2004) além de diminuição dos níveis de BDNF cerebrais (Vaynman et al. 2006).

Entretanto nós observamos que a dieta ocidental aumenta a sensibilidade do plexo coróide ao IGF-1, evidenciado pelo aumento dos níveis de fosforilação da proteína AKT em plexo coroide em resposta a administração de IGF-1 humano. Contudo quando tentamos mimetizar um efeito agudo em cultura de células epiteliais do plexo coróide não obtivemos o mesmo resultado, demonstrando que o aumento e manutenção de altos níveis por longos períodos de TGL como o ocorrido no experimento *in vivo* é necessário para causar essas alterações no SNC. Estes resultados apontam para a necessidade de manutenção dos níveis de lipídeos séricos em níveis normais para a manutenção da homeostasia cerebral associada a sinalização do IGF-1.

O aumento na expectativa de vida é outro aspecto da sociedade atual que afeta o SNC. Usualmente o envelhecimento está associado a perda massiva de células neurais, aumento da neuroinflamação e do dano oxidativo cerebral e a prejuízos da função cognitva (Bishop et al. 2010). Entender os mecanismos moleculares que causam as alterações cérebrais durante o envelhecimento é de fundamental importância para buscar intervenções que possam prevenir ou aumentar a saúde cerebral.

No segundo capítulo onde utilizamos camundongos envelhecidos para verificar os efeitos do IGF-1 no SNC nossos resultados mostraram que, apesar de tentar haver uma compensação do cérebro em aumentar o transporte de IGF-1 pela barreira sangue cérebro e aumento dos receptores em hipocampo, ocorreu uma diminuição da sinalização pós-receptor. As proteínas de sinalização intracelular são fundamentais para as ações neurotróficos do IGF-1 (Torres-Aleman 2010). Deste modo a tentativa de compensar a alteração da sinalização intracelular do IGF-1 por aumento de transporte pela barreira

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sangue cérebro e aumento de receptor foi ineficiente, uma vez que estes animais apresentaram prejuízos cognitivos. Mecanismos compensatórios relacionados com IGF-1 já foram relatados em na DA (Carro et al. 2002; Moloney et al. 2008). Entretanto modelos experimentais de DA demonstram baixos níveis séricos de IGF-1 (Carro et al. 2002) e altas concentrações de receptores de IGF-1 no cérebro (Lopez-Lopez et al. 2007). Estes dados apontam uma relação inversa entre os níveis séricos de IGF-1 e receptores de IGF-1 cerebral tanto em modelos animais da DA como em animais envelhecidos (Poduslo et al. 2001). O prejuizo cognitivo observado em animais envelehcidos pode estar relacionado com a diminuição da ação de IGF-1 no hipocampo. (Aleman and Torres-Aleman 2009; Trejo et al. 2007).

No terceiro capítulo estudamos o efeito do envelhecimento sobre à ação da insulina no SNC. Os resultados demonstram, assim como quando estudamos os efeitos do IGF-1, uma diminuição da ação da insulina em alguns parâmetros em ratos envelhecidos quando comparados com animais jovens. Nossos dados apontaram para um efeito positivo da administração de insulina 20mU icv na memória espacial e no conteúdo de BDNF no hipocampo.

O trabalho analisou o efeito da insulina na morfologia de subáreas do hipocampal, uma região cerebral fundamental para processos de memória e aprendizado espacial (Moosavi et al. 2006). Ratos que sofreram lesão na região CA3 do hipocampo apresentam déficit cognitivo no teste de labirinto aquático de Morris, enquanto que quando lesionados na região CA1 não apresentam este déficit (Kesner 2007). Nossos resultados mostraram um aumento na densidade de fluorescência do marcador neuronal (NeuN) na região CA1 do hipocampo em animais jovens e envelhecidos induzida por

insulina. Os animais envelhecidos não tratados com insulina apresentavam uma diminuição deste marcador. Na região CA3 a administração de insulina não reverteu o déficit na densidade de fluorescência neuronal em animais velhos.

Ratos envelhecidos têm prejuízo na memória espacial no teste do labirinto aquático de Morris (Rosenzweig and Barnes 2003). Contudo Rosenzweig et al. 2003 relatou que 30% dos animais velhos tem desempenho igual a jovens neste teste. Nossos resultados não apontaram nenhum déficit cognitivo aparente nos animais envelhecidos tanto na fase de aquisição quanto na fase evocação da memória no labirinto aquático de Morris. No entanto, os animais velhos demonstraram uma estabilização da aprendizagem no terceiro dia de treino enquanto animais jovens apresentaram uma diminuição na latência para encontrar a plataforma até o último dia de treinamento. Entretanto, como demonstrado em outros trabalhos, insulina causou uma melhora no desempenho cognitivo (Park et al. 2000; Moosavi et al. 2007) somente em animais jovens associado a um aumento nos níveis de BDNF no hipocampo. (Solas et al. 2010) demonstraram que o envelhecimento causa resistência cerebral à insulina. Nós propomos aqui que esta resistência cerebral associada a mudanças morfológicas no hipocampo e falta de efeito da insulina em aumentar as concentrações de BDNF em hipocampo se refletem no prejuízo cognitivo observado nos animais velhos.

Outro enfoque deste estudo foi investigar o efeito do envelhecimento e da administração cerebral de insulina em parâmetros relacionados com neuroinflamação, uma vez que um estado pró-inflamatório persistente, como ocorre no envelhecimento, pode aumentar a expressão de proteínas envolvidas

em neurodegeneração (Uranga et al. 2010). Neste sentido, nós observamos que a administração de insulina icv diminuiu os níveis de PARP-1 no hipocampo de ratos envelhecidos (De Martinis et al. 2005; Altmeyer and Hottiger 2009). Além disso, o tratamento com insulina diminuiu os níveis de TNF-α no hipocampo de animais jovens e envelhecidos. Os níveis hipocampais de proteínas totais mTor e FOXO1 foram aumentadas pelo envelhecimento e não foram influenciados pela administração de insulina icv. O tratamento com insulina reduziu o estado de fosforilação da mToR Ser 2441 e 2448. Estas duas proteínas participam dos mecanismos de morte celular no envelhecimento por apoptose e autofagia (Hay and Sonenberg 2004; Li et al. 2005; Cheng and White).

No quarto capítulo estudamos os efeitos da atividade física regular como fator neuroprotetor. A possível modulação do exercício físico na resposta cerebral a insulina tem sido pouco explorada. O exercício físico é reconhecido por aumentar a sensibilidade à insulina em tecidos periféricos, entretanto os efeitos desta intervenção na sensibilidade cerebral à insulina ainda não foram investigados. Nossos resultados apontam para um aumento na translocação e ativação do receptor de insulina e ativação nas proteínas intracelulares responsivas à insulina no hipocampo. A presença de uma alta densidade de receptores de insulina em regiões como hipocampo e córtex cerebral sugerem que ela participa de processos de aprendizagem e memória (Zhao et al. 1999). A diminuição da sinalização hipocampal da insulina em roedores tratados com STZ i.c.v ou a redução dos receptores de insulina em no hipocampo de pacientes com DA se correlaciona com prejuízos cognitivos (Steen et al. 2005).

Um grande número de evidencias tem demonstrado que separadamente tanto a insulina (Zhao et al. 2004) como o exercício físico (Cotman et al. 2007) aumentam o desempenho cognitivo em humanos e roedores, embora poucos estudos tem sido endereçados ao entendimento do papel da mitocôndria nestes processos (Dietrich et al. 2008). Já está bem descrito que o aumento da atividade cerebral aumenta a demanda metabólica dos neurônios, embora a capacidade da insulina em regular o metabolismo de glicose ou a função mitocondrial cerebral ainda é controverso (Messier 2004). Nossos resultados sugerem um aumento do consumo de glicose provocado pelo exercício físico, mas a insulina na dose de 5mU não alterou o consumo de glicose em fatias de hipocampo. Estes dados suportam a hipótes de que insulina tem um papel neuromodulatório ao invés de regulador do metabolismo no SNC. Este papel envolve sobrevivência neuronal (Valenciano et al. 2006), plasticidade sináptica (Lee et al. 2005; Stranahan et al. 2008) e aprendizado e memória (Dou et al. 2005; Zhao et al. 1999). Em nosso trabalho, quando usamos insulina 5mU icv, ocorreu um efeito amnésico em animais que se exercitaram durante 30 dias. A dose de 5mU icv de insulina, usada no nosso trabalho, não demonstrou nenhum efeito no aprendizado e memória no grupo sedentário. Um dos possíveis mecanismos nos quais a insulina modula o aprendizado e memória é por regular a fosforilação das subunidades dos receptores NMDA de glutamato e, em conseqüência a atividade do receptor (Christie et al. 1999; Liu et al. 1995). Além de modulações em proteínas intracelulares, a insulina causou uma ativação da subunidade NR2B do receptor NMDA de glutamato 15 min após administração icv que se manteve por 24 h no hipocampo dos animais exercitados. Morfologicamente foi observado um aumento da reatividade

densidade de fluorescência de GFAP, um marcador astrocitário, demonstrando que a dose de 5mU poderia causar astrogliose via hiperativação do sistema glutamatérgico no hipocampo que exacerbaria um estado de estimulação causada pelo exercício físico.

Recentemente o principal compartimento celular envolvido na produção de energia na célula, a mitocôndria, tem sido alvo de grande interesse dos pesquisadores, uma vez que tem destacado efeito em processos fisiológicos e também em doenças neurodegenerativas. O cérebro utiliza uma grande quantidade de oxigênio, em torno de 20% do consumo corporal e com isso gera uma grande quantidade de espécies reativas de oxigênio (ROS), entretanto apresenta uma baixa capacidade antioxidante (Kilbride et al. 2008).

No quinto capítulo do nosso trabalho os resultados apontam para um efeito antioxidante da insulina em preparações de sinaptossoma de ratos quando estimulamos a respiração mitocondrial com succinato. Quando incubamos insulina em doses crescentes (0.1-20mU) ocorreu um aumento deste efeito. Isto também se relacionou com o tempo de incubação, uma vez que pré-incubação com insulina (15 e 30 min) potencializou este efeito. Nós postulamos que este efeito é dependente da sinalização de insulina uma vez em preparações enriquecidas de mitocôndria e tratadas com inibidor da proteína PI3K, wortmanina, ocorreu uma completa inibição do efeito da insulina em baixas doses e uma parcial inibição em altas doses de insulina. Já foi demonstrado que insulina pode exercer efeitos antioxidantes (Duarte et al. 2004) por modular a resposta de proteínas da mitocôndria como a   por alterar proteínas como AKT.

Os resultados do nosso trabalho apontam para um efeito da sinalização intracelular, e além disso sugerem que insulina modula o potencial de membrana mitocondrial. Pequenas alterações no potencial de membrana da mitocôndria modulam grandemente a produção de H_2O_2 (Korshunov et al. 1997). Insulina foi capaz de reduzir o potencial de membrana mitocondrial induzido por succinato em sinaptossoma e isto poderia ser um dos mecanismos pelos quais insulina pode exercer efeitos neuroprotetores antioxidantes. Entretanto outros fatores que podem modular a atividade mitocondrial como cinases (Meyer et al. 2006) e consumo de O2 e enzimas antioxidantes podem alterar a produção de H_2O_2 (Santiago et al. 2008). Com estes dados sugerimos que a insulina exerce efeito antioxidante no cérebro necessariamente via ativação de sinalização da membrana em direção a mitocôndria, mas não diretamente na mitocôndria, uma vez que a insulina não afetou a respostas em mitocôndrias isoladas.

O uso de modelos que possam mimetizar os efeitos do envelhecimento e/ou de doenças neurodegenerativas é de fundamental importância para entendermos e criarmos abordagens que possam ser efetivas para prevenção e tratamento. Um modelo utilizado é o uso de STZ icv, proposto como modelo de doença de Alzheimer esporádica. Este modelo se caracteriza por diminuir a atividade mitocondrial (Sharma and Gupta 2001).

No capítulo 6 utilizamos um modelo de resistência cerebral á insulina e nossos resultados mostram que exercício físico potencializa o efeito negativo na cognição induzida por STZ i.c.v., uma vez que uma baixa dose de STZ (1mg/Kg) causou um efeito amnésico tanto na memória de reconhecimento como na memória espacial o que não foi observado nos animais sedentários.

Na dose de 3mg/Kg ambos o grupos apresentaram o efeito amnésico. Este efeito parece ser relacionado a mecanismos de aprendizado e memória uma vez que a capacidade locomotora e velocidades de natação não foram alteradas por STZ.

Devido aos efeitos moduladores da insulina na cognição, do exercício físico aumentar sensibilidade à insulina no hipocampo e por este hormônio ter efeito antioxidante cerebral, nós avaliamos se essas variáveis poderiam estar associadas a produção de H_2O_2 em sinaptossomas em um modelo de camundongos com resistência cerebral à insulina. Como esperado, a insulina não afetou a produção de H_2O_2 em sinaptossoma de ratos que receberam STZ icv, sugerindo mais uma vez a necessidade de haver uma íntegra cascata de sinalização da insulina, para ativar os mecanismos antioxidantes. Outro resultado relevante foi o efeito antioxidante da insulina foi maior em animais exercitados. Isso pode estar relacionado ao aumento de receptores de insulina induzido pelo exercício.

5. CONCLUSÕES

- O consumo de uma dieta hiperpalatavel causa uma diminuição do transporte de IGF-1 pela barreira sangue cérebro pelo seu principal transportador no plexo coróide, a megalina, devido ao aumento da concentração de triglicerídeos no sangue;

- O envelhecimento causa uma diminuição da ação intracelular de IGF-1 em hipocampo, associado a um mecanismo compensatório representado pelo aumento do transporte de IGF-1 pela barreira sangue cérebro e do imunoconteúdo do seu receptor (IGF-1R) em hipocampo;

- O envelhecimento causa resistência à ação da insulina icv no que se refere em promover a melhora do desempenho em tarefas de memória de reconhecimento e espacial. A administração de insulina icv aumenta os níveis hipocampais de BDNF em ratos jovens, mas não em ratos envelhecidos. Além disso, a insulina diminui a expressão de fatores pro inflamatórios e pró-apoptóticos em hipocampo de ratos jovens e envelhecidos. Nos ratos jovens e envelhecidos, a insulina icv causa um aumento da densidade de fluorescência neuronal (NeuN) na região CA1 do hipocampo em animais jovens e envelhecidos;

- O exercício físico voluntário causa um aumento da sensibilidade à insulina em hipocampo de camundongos, por aumentar a translocação do receptor de insulina para a membrana celular e causar a ativação de proteínas pósreceptor. Entretanto, a administração de insulina icv na dose de 5mU causa déficit cognitivo nas tarefas de memória aversiva e espacial associadas a hiperativação da subunidade NR2B do NMDA e aumento da reatividade glial;

- A insulina diminui a produção de H_2O_2 induzida por succinato em sinaptossomas sugerindo que no cérebro ela pode atuar como propriedade. Além disso, este efeito antioxidante é dependente de sinalização intracelular, considerando a inibição da sinalização intracelular por wortmanina extingue a ação antioxidante;

- O exercício físico voluntário exacerba o prejuízo cognitivo causado pela estreptozotocina considerando que a dose de 1mg/kg não causou prejuízo em animais sedentários, mas prejudicou o aprendizado na memória espacial e de reconhecimento dos animais exercitados. O mecanismo envolvido nesta alteração parece estar relacionado com o aumento da atividade mitocondrial observado em animais exercitados o que é perdido quando utilizado este composto que diminui a ação da insulina no SNC.

6. PERSPECTIVAS

- Avaliar o efeito de fármacos, reconhecidamente efetivos em aumentar a sensibilidade à insulina em tecidos periféricos, no SNC de roedores submetidos a protocolos de dietas hiperpalatáveis e exercício físico;

- Investigar o efeito do envelhecimento sobre a atividade mitocondrial cerebral em animais envelhecidos bem como sua resposta as ações da insulina e de fármacos que potencializam as ações deste hormônio;

- Estudar os efeitos do exercício físico em outros modelos de doenças neurodegenerativas para avaliar o possível papel neuroprotetor da insulina;

- Avaliar o efeito do exercício físico em modular a sinalização da insulina no SNC em animais que já tenham desenvolvido resistência periférica à insulina.

- Avaliar os efeitos do exercício físico e de dietas em relação ao mecanismo da lançadeira de lactato em cérebro de roedores.

Referências

Adlard PA, Perreau VM, Pop V, Cotman CW (2005) Voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease. J Neurosci 25: 4217-21

Aleman A, Torres-Aleman I (2009) Circulating insulin-like growth factor I and cognitive function: neuromodulation throughout the lifespan. Prog Neurobiol 89: 256-65

Aleman A, Verhaar HJ, De Haan EH, De Vries WR, Samson MM, Drent ML, Van der Veen EA, Koppeschaar HP (1999) Insulin-like growth factor-I and cognitive function in healthy older men. J Clin Endocrinol Metab 84: 471-5

Altmeyer M, Hottiger MO (2009) Poly(ADP-ribose) polymerase 1 at the crossroad of metabolic stress and inflammation in aging. Aging (Albany NY) 1: 458-69

Amor S, Puentes F, Baker D, van der Valk P Inflammation in neurodegenerative diseases. Immunology 129: 154-69

Antunes LC, Levandovski R, Dantas G, Caumo W, Hidalgo MP Obesity and shift work: chronobiological aspects. Nutr Res Rev 23: 155-68

Babri S, Badie HG, Khamenei S, Seyedlar MO (2007) Intrahippocampal insulin improves memory in a passive-avoidance task in male wistar rats. Brain Cogn 64: 86-91

Banks WA (2004) The source of cerebral insulin. Eur J Pharmacol 490: 5-12

Banks WA, DiPalma CR, Farrell CL (1999) Impaired transport of leptin across the blood-brain barrier in obesity. Peptides 20: 1341-5

Beneke S (2008) Poly(ADP-ribose) polymerase activity in different pathologies--the link to inflammation and infarction. Exp Gerontol 43: 605-14

Bishop NA, Lu T, Yankner BA Neural mechanisms of ageing and cognitive decline. Nature 464: 529-35

Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8: 57-69

Blokland A, Jolles J (1993) Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. Pharmacol Biochem Behav 44: 491-4

Bowling AC, Mutisya EM, Walker LC, Price DL, Cork LC, Beal MF (1993) Age-dependent impairment of mitochondrial function in primate brain. J Neurochem 60: 1964-7

Brewer GJ Epigenetic oxidative redox shift (EORS) theory of aging unifies the free radical and insulin signaling theories. Exp Gerontol 45: 173-9

Carro E, Torres-Aleman I (2004) The role of insulin and insulin-like growth factor I in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. Eur J Pharmacol 490: 127-33

Carro E, Trejo JL, Gomez-Isla T, LeRoith D, Torres-Aleman I (2002) Serum insulin-like growth factor I regulates brain amyloid-beta levels. Nat Med 8: 1390-7

Chakravarthy MV, Booth FW (2004) Eating, exercise, and "thrifty" genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases. J Appl Physiol 96: 3-10

Chen L, Na R, Gu M, Salmon AB, Liu Y, Liang H, Qi W, Van Remmen H, Richardson A, Ran Q (2008) Reduction of mitochondrial H2O2 by overexpressing peroxiredoxin 3 improves glucose tolerance in mice. Aging Cell 7: 866-78

Chen L, Yoo SE, Na R, Liu Y, Ran Q Cognitive impairment and increased Abeta levels induced by paraquat exposure are attenuated by enhanced removal of mitochondrial H(2)O(2). Neurobiol Aging

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ (2003) Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 278: 36027-31

Cheng Z, Guo S, Copps K, Dong X, Kollipara R, Rodgers JT, Depinho RA, Puigserver P, White MF (2009) Foxo1 integrates insulin signaling with mitochondrial function in the liver. Nat Med 15: 1307-11

Cheng Z, Tseng Y, White MF Insulin signaling meets mitochondria in metabolism. Trends Endocrinol Metab

Cheng Z, White MF Targeting Forkhead box O1 from the concept to metabolic diseases: lessons from mouse models. Antioxid Redox Signal 14: 649-61

Christie JM, Wenthold RJ, Monaghan DT (1999) Insulin causes a transient tyrosine phosphorylation of NR2A and NR2B NMDA receptor subunits in rat hippocampus. J Neurochem 72: 1523-8

Cohen E, Dillin A (2008) The insulin paradox: aging, proteotoxicity and neurodegeneration. Nat Rev Neurosci 9: 759-67

Cole GM, Frautschy SA (2007) The role of insulin and neurotrophic factor signaling in brain aging and Alzheimer's Disease. Exp Gerontol 42: 10-21

Collingridge G (1987) Synaptic plasticity. The role of NMDA receptors in learning and memory. Nature 330: 604-5

Cotman CW, Berchtold NC (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. Trends Neurosci 25: 295-301

Cotman CW, Berchtold NC, Christie LA (2007) Exercise builds brain health: key roles of growth factor cascades and inflammation. Trends Neurosci 30: 464-72

Craft S (2006) Insulin resistance syndrome and Alzheimer disease: pathophysiologic mechanisms and therapeutic implications. Alzheimer Dis Assoc Disord 20: 298-301

Craft S, Watson GS (2004) Insulin and neurodegenerative disease: shared and specific mechanisms. Lancet Neurol 3: 169-78

Davey GP, Peuchen S, Clark JB (1998) Energy thresholds in brain mitochondria. Potential involvement in neurodegeneration. J Biol Chem 273: 12753-7

Davila D, Piriz J, Trejo JL, Nunez A, Torres-Aleman I (2007) Insulin and insulin-like growth factor I signalling in neurons. Front Biosci 12: 3194-202

de la Monte SM, Longato L, Tong M, Wands JR (2009) Insulin resistance and neurodegeneration: roles of obesity, type 2 diabetes mellitus and non-alcoholic steatohepatitis. Curr Opin Investig Drugs 10: 1049-60

de la Monte SM, Wands JR (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J Alzheimers Dis 7: 45-61

De Martinis M, Franceschi C, Monti D, Ginaldi L (2005) Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. FEBS Lett 579: 2035-9

Dietrich MO, Andrews ZB, Horvath TL (2008) Exercise-induced synaptogenesis in the hippocampus is dependent on UCP2-regulated mitochondrial adaptation. J Neurosci 28: 10766-71

Dietrich MO, Mantese CE, Porciuncula LO, Ghisleni G, Vinade L, Souza DO, Portela LV (2005) Exercise affects glutamate receptors in postsynaptic densities from cortical mice brain. Brain Res 1065: 20-5

Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, Souza DO, Torres-Aleman I (2007) Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. Neuromolecular Med 9: 324-30

Ding Q, Vaynman S, Akhavan M, Ying Z, Gomez-Pinilla F (2006) Insulin-like growth factor I interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. Neuroscience 140: 823-33

Dou JT, Chen M, Dufour F, Alkon DL, Zhao WQ (2005) Insulin receptor signaling in long-term memory consolidation following spatial learning. Learn Mem 12: 646-55

Du Y, Wooten MC, Wooten MW (2009) Oxidative damage to the promoter region of SQSTM1/p62 is common to neurodegenerative disease. Neurobiol Dis 35: 302-10

Duarte AI, Santos MS, Seica R, de Oliveira CR (2003) Insulin affects synaptosomal GABA and glutamate transport under oxidative stress conditions. Brain Res 977: 23-30

Duarte AI, Santos MS, Seica R, Oliveira CR (2004) Oxidative stress affects synaptosomal gamma-aminobutyric acid and glutamate transport in diabetic rats: the role of insulin. Diabetes 53: 2110-6

Flores MB, Fernandes MF, Ropelle ER, Faria MC, Ueno M, Velloso LA, Saad MJ, Carvalheira JB (2006) Exercise improves insulin and leptin sensitivity in hypothalamus of Wistar rats. Diabetes 55: 2554-61

Floyd RA, Hensley K (2002) Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. Neurobiol Aging 23: 795-807

Frolich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Turk A, Hoyer S, Zochling R, Boissl KW, Jellinger K, Riederer P (1998) Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. J Neural Transm 105: 423-38

Fulop T, Larbi A, Douziech N (2003) Insulin receptor and ageing. Pathol Biol (Paris) 51: 574-80

Gomez-Pinilla F (2008) The influences of diet and exercise on mental health through hormesis. Ageing Res Rev 7: 49-62

Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S (2007) Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem 101: 757-70

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97: 1634-58

Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18: 1926-45

Henneberg N, Hoyer S (1995) Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? Arch Gerontol Geriatr 21: 63-74

Hofer SM, Berg S, Era P (2003) Evaluating the interdependence of aging-related changes in visual and auditory acuity, balance, and cognitive functioning. Psychol Aging 18: 285-305

Hoyer S (2004a) Causes and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: therapeutic implications. Adv Exp Med Biol 541: 135-52

Hoyer S (2004b) Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. Eur J Pharmacol 490: 115-25

Hoyer S, Lannert H (2007) Long-term abnormalities in brain glucose/energy metabolism after inhibition of the neuronal insulin receptor: implication of tau-protein. J Neural Transm Suppl: 195-202

Huang TJ, Verkhratsky A, Fernyhough P (2005) Insulin enhances mitochondrial inner membrane potential and increases ATP levels through phosphoinositide 3-kinase in adult sensory neurons. Mol Cell Neurosci 28: 42-54

Kanaley JA, Frystyk J, Moller N, Dall R, Chen JW, Nielsen SC, Christiansen JS, Jorgensen JO, Flyvbjerg A (2005) The effect of submaximal exercise on immuno- and bioassayable IGF-I activity in patients with GH-deficiency and healthy subjects. Growth Horm IGF Res 15: 283-90

Kesner RP (2007) Behavioral functions of the CA3 subregion of the hippocampus. Learn Mem 14: 771-81

Kilbride SM, Telford JE, Davey GP (2008) Age-related changes in H2O2 production and bioenergetics in rat brain synaptosomes. Biochim Biophys Acta 1777: 783-8

Komary Z, Tretter L, Adam-Vizi V Membrane potential-related effect of calcium on reactive oxygen species generation in isolated brain mitochondria. Biochim Biophys Acta

Korshunov SS, Skulachev VP, Starkov AA (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 416: 15-8

Lannert H, Hoyer S (1998) Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav Neurosci 112: 1199-208

Leasure JL, Decker L (2009) Social isolation prevents exercise-induced proliferation of hippocampal progenitor cells in female rats. Hippocampus 19: 907-12

Lee CC, Huang CC, Wu MY, Hsu KS (2005) Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway. J Biol Chem 280: 18543-50

LeRoith D, Roberts CT, Jr. (2003) The insulin-like growth factor system and cancer. Cancer Lett 195: 127-37

Li BS, Sun MK, Zhang L, Takahashi S, Ma W, Vinade L, Kulkarni AB, Brady RO, Pant HC (2001) Regulation of NMDA receptors by cyclin-dependent kinase-5. Proc Natl Acad Sci U S A 98: 12742-7

Li X, Alafuzoff I, Soininen H, Winblad B, Pei JJ (2005) Levels of mTOR and its downstream targets 4E-BP1, eEF2, and eEF2 kinase in relationships with tau in Alzheimer's disease brain. FEBS J 272: 4211-20

Lin K, Hsin H, Libina N, Kenyon C (2001) Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet 28: 139-45

Lin MT, Beal MF (2006) Alzheimer's APP mangles mitochondria. Nat Med 12: 1241-3

Liu L, Brown JC, 3rd, Webster WW, Morrisett RA, Monaghan DT (1995) Insulin potentiates N-methyl-D-aspartate receptor activity in Xenopus oocytes and rat hippocampus. Neurosci Lett 192: 5-8

Lopez-Lopez C, Dietrich MO, Metzger F, Loetscher H, Torres-Aleman I (2007) Disturbed cross talk between insulin-like growth factor I and AMP-activated protein kinase as a possible cause of vascular dysfunction in the amyloid precursor protein/presenilin 2 mouse model of Alzheimer's disease. J Neurosci 27: 824-31

Lopez-Lopez C, LeRoith D, Torres-Aleman I (2004) Insulin-like growth factor I is required for vessel remodeling in the adult brain. Proc Natl Acad Sci U S A 101: 9833-8

Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH (2006) Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. Hum Mol Genet 15: 1437-49

Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. Neurobiol Dis 15: 461-73

Markowska AL, Mooney M, Sonntag WE (1998) Insulin-like growth factor-1 ameliorates age-related behavioral deficits. Neuroscience 87: 559-69

Marx J (2005) Alzheimer's disease. Play and exercise protect mouse brain from amyloid buildup. Science 307: 1547

Mattson MP The impact of dietary energy intake on cognitive aging. Front Aging Neurosci 2: 5

Mattson MP (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromolecular Med 3: 65-94

Mayer G, Nitsch R, Hoyer S (1990) Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats. Brain Res 532: 95-100

McGarry JD (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes 51: 7-18

McNay EC (2007) Insulin and ghrelin: peripheral hormones modulating memory and hippocampal function. Curr Opin Pharmacol 7: 628-32

Messier C (2004) Glucose improvement of memory: a review. Eur J Pharmacol 490: 33-57

Meyer LE, Machado LB, Santiago AP, da-Silva WS, De Felice FG, Holub O, Oliveira MF, Galina A (2006) Mitochondrial creatine kinase activity prevents reactive oxygen species generation: antioxidant role of mitochondrial kinase-dependent ADP re-cycling activity. J Biol Chem 281: 37361-71

Miyawaki T, Ofengeim D, Noh KM, Latuszek-Barrantes A, Hemmings BA, Follenzi A, Zukin RS (2009) The endogenous inhibitor of Akt, CTMP, is critical to ischemia-induced neuronal death. Nat Neurosci 12: 618-26

Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C (2008) Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. Neurobiol Aging

Molteni R, Wu A, Vaynman S, Ying Z, Barnard RJ, Gomez-Pinilla F (2004) Exercise reverses the harmful effects of consumption of a high-fat diet on synaptic and behavioral plasticity associated to the action of brain-derived neurotrophic factor. Neuroscience 123: 429-40

Mookherjee P, Quintanilla R, Roh MS, Zmijewska AA, Jope RS, Johnson GV (2007) Mitochondrial-targeted active Akt protects SH-SY5Y neuroblastoma cells from staurosporine-induced apoptotic cell death. J Cell Biochem 102: 196-210

Moosavi M, Naghdi N, Choopani S (2007) Intra CA1 insulin microinjection improves memory consolidation and retrieval. Peptides 28: 1029-34

Moosavi M, Naghdi N, Maghsoudi N, Zahedi Asl S (2006) The effect of intrahippocampal insulin microinjection on spatial learning and memory. Horm Behav 50: 748-52

Morais VA, De Strooper B Mitochondria dysfunction and neurodegenerative disorders: cause or consequence. J Alzheimers Dis 20 Suppl 2: S255-63

Muller AP, Cammarota M, Dietrich MO, Rotta LN, Portela LV, Souza DO, Izquierdo I, Bevilaqua LR, Perry ML (2008) Different effect of high fat diet and physical exercise in the hippocampal signaling. Neurochem Res 33: 880-5

Muller AP, Gnoatto J, Moreira JD, Zimmer ER, Haas CB, Lulhier F, Perry ML, Souza DO, Torres-Aleman I, Portela LV Exercise increases insulin signaling in the hippocampus: Physiological effects and pharmacological impact of intracerebroventricular insulin administration in mice. Hippocampus

Murphy MP, Holmgren A, Larsson NG, Halliwell B, Chang CJ, Kalyanaraman B, Rhee SG, Thornalley PJ, Partridge L, Gems D, Nystrom T, Belousov V, Schumacker PT, Winterbourn CC Unraveling the biological roles of reactive oxygen species. Cell Metab 13: 361-6

Neumann KF, Rojo L, Navarrete LP, Farias G, Reyes P, Maccioni RB (2008) Insulin resistance and Alzheimer's disease: molecular links & clinical implications. Curr Alzheimer Res 5: 438-47

Neuwelt EA, Bauer B, Fahlke C, Fricker G, Iadecola C, Janigro D, Leybaert L, Molnar Z, O'Donnell ME, Povlishock JT, Saunders NR, Sharp F, Stanimirovic D, Watts RJ, Drewes LR (2011) Engaging neuroscience to advance translational research in brain barrier biology. Nat Rev Neurosci 12: 169-82

Nicholls DG, Budd SL (2000) Mitochondria and neuronal survival. Physiol Rev 80: 315-60

Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG, Jr., Seeley RJ, Schwartz MW (2003) Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes 52: 227-31

Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM (1999) Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology 53: 1937-42

Parachikova A, Nichol KE, Cotman CW (2008) Short-term exercise in aged Tg2576 mice alters neuroinflammation and improves cognition. Neurobiol Dis 30: 121-9

Park CR (2001) Cognitive effects of insulin in the central nervous system. Neurosci Biobehav Rev 25: 311-23

Park CR, Seeley RJ, Craft S, Woods SC (2000) Intracerebroventricular insulin enhances memory in a passive-avoidance task. Physiol Behav 68: 509-14

Patten DA, Germain M, Kelly MA, Slack RS Reactive oxygen species: stuck in the middle of neurodegeneration. J Alzheimers Dis 20 Suppl 2: S357-67

Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83: 346-56

Petit-Paitel A, Brau F, Cazareth J, Chabry J (2009) Involvment of cytosolic and mitochondrial GSK-3beta in mitochondrial dysfunction and neuronal cell death of MPTP/MPP-treated neurons. PLoS One 4: e5491

Plaschke K, Hoyer S (1993) Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus. Int J Dev Neurosci 11: 477-83

Plum L, Schubert M, Bruning JC (2005) The role of insulin receptor signaling in the brain. Trends Endocrinol Metab 16: 59-65

Poduslo JF, Curran GL, Wengenack TM, Malester B, Duff K (2001) Permeability of proteins at the blood-brain barrier in the normal adult mouse and double transgenic mouse model of Alzheimer's disease. Neurobiol Dis 8: 555-67

Porte D, Jr., Baskin DG, Schwartz MW (2005) Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. Diabetes 54: 1264-76

Prickaerts J, Fahrig T, Blokland A (1999) Cognitive performance and biochemical markers in septum, hippocampus and striatum of rats after an i.c.v. injection of streptozotocin: a correlation analysis. Behav Brain Res 102: 73-88

Puche JE, Garcia-Fernandez M, Muntane J, Rioja J, Gonzalez-Baron S, Castilla Cortazar I (2008) Low doses of insulin-like growth factor-I induce mitochondrial protection in aging rats. Endocrinology 149: 2620-7

Puglielli L (2008) Aging of the brain, neurotrophin signaling, and Alzheimer's disease: is IGF1-R the common culprit? Neurobiol Aging 29: 795-811

Radak Z, Chung HY, Goto S (2008) Systemic adaptation to oxidative challenge induced by regular exercise. Free Radic Biol Med 44: 153-9

Readnower RD, Sauerbeck AD, Sullivan PG Mitochondria, Amyloid beta, and Alzheimer's Disease. Int J Alzheimers Dis 2011: 104545

Rosenzweig ES, Barnes CA (2003) Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. Prog Neurobiol 69: 143-79

Salkovic-Petrisic M, Hoyer S (2007) Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach. J Neural Transm Suppl: 217-33

Sanderson TH, Kumar R, Sullivan JM, Krause GS (2008) Insulin blocks cytochrome c release in the reperfused brain through PI3-K signaling and by promoting Bax/Bcl-XL binding. J Neurochem 106: 1248-58

Santiago AP, Chaves EA, Oliveira MF, Galina A (2008) Reactive oxygen species generation is modulated by mitochondrial kinases: correlation with mitochondrial antioxidant peroxidases in rat tissues. Biochimie 90: 1566-77

Saute JA, da Silva AC, Muller AP, Hansel G, de Mello AS, Maeda F, Vedolin L, Saraiva-Pereira ML, Souza DO, Arpa J, Torres-Aleman I, Portela LV, Jardim LB Serum insulin-like system alterations in patients with spinocerebellar ataxia type 3. Mov Disord 26: 731-5

Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS (2005) Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 308: 1909-11

Schwartz MW, Porte D, Jr. (2005) Diabetes, obesity, and the brain. Science 307: 375-9

Sharma M, Gupta YK (2001) Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. Life Sci 68: 1021-9

Sims NR (1990) Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. J Neurochem 55: 698-707

Sims NR, Anderson MF (2008) Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. Nat Protoc 3: 1228-39

Solas M, Aisa B, Mugueta MC, Del Rio J, Tordera RM, Ramirez MJ Interactions between age, stress and insulin on cognition: implications for Alzheimer's disease. Neuropsychopharmacology 35: 1664-73

Sonntag WE, Lynch C, Thornton P, Khan A, Bennett S, Ingram R (2000) The effects of growth hormone and IGF-1 deficiency on cerebrovascular and brain ageing. J Anat 197 Pt 4: 575-85

Sonntag WE, Lynch CD, Cooney PT, Hutchins PM (1997) Decreases in cerebral microvasculature with age are associated with the decline in growth hormone and insulin-like growth factor 1. Endocrinology 138: 3515-20

Starkov AA, Fiskum G (2003) Regulation of brain mitochondrial H2O2 production by membrane potential and NAD(P)H redox state. J Neurochem 86: 1101-7

Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis 7: 63-80

Stone JR, Yang S (2006) Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal 8: 243-70

Stranahan AM, Mattson MP (2008) Impact of energy intake and expenditure on neuronal plasticity. Neuromolecular Med 10: 209-18

Stranahan AM, Norman ED, Lee K, Cutler RG, Telljohann RS, Egan JM, Mattson MP (2008) Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. Hippocampus 18: 1085-8

Swardfager W, Lanctot K, Rothenburg L, Wong A, Cappell J, Herrmann N A meta-analysis of cytokines in Alzheimer's disease. Biol Psychiatry 68: 930-41

Szkudelski T (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50: 537-46

Torres-Aleman I Toward a comprehensive neurobiology of IGF-I. Dev Neurobiol 70: 384-96

Trejo JL, Carro E, Garcia-Galloway E, Torres-Aleman I (2004a) Role of insulin-like growth factor I signaling in neurodegenerative diseases. J Mol Med 82: 156-62

Trejo JL, Carro E, Lopez-Lopez C, Torres-Aleman I (2004b) Role of serum insulin-like growth factor I in mammalian brain aging. Growth Horm IGF Res 14 Suppl A: S39-43

Trejo JL, Llorens-Martin MV, Torres-Aleman I (2008) The effects of exercise on spatial learning and anxiety-like behavior are mediated by an IGF-I-dependent mechanism related to hippocampal neurogenesis. Mol Cell Neurosci 37: 402-11

Trejo JL, Piriz J, Llorens-Martin MV, Fernandez AM, Bolos M, LeRoith D, Nunez A, Torres-Aleman I (2007) Central actions of liver-derived insulin-like growth factor I underlying its pro-cognitive effects. Mol Psychiatry 12: 1118-28

Uranga RM, Bruce-Keller AJ, Morrison CD, Fernandez-Kim SO, Ebenezer PJ, Zhang L, Dasuri K, Keller JN Intersection between metabolic dysfunction, high fat diet consumption, and brain aging. J Neurochem 114: 344-61

Valenciano AI, Corrochano S, de Pablo F, de la Villa P, de la Rosa EJ (2006) Proinsulin/insulin is synthesized locally and prevents caspase- and cathepsin-mediated cell death in the embryonic mouse retina. J Neurochem 99: 524-36

van der Heide LP, Ramakers GM, Smidt MP (2006) Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol 79: 205-21

Vaynman SS, Ying Z, Yin D, Gomez-Pinilla F (2006) Exercise differentially regulates synaptic proteins associated to the function of BDNF. Brain Res 1070: 124-30

Vicario-Abejon C, Yusta-Boyo MJ, Fernandez-Moreno C, de Pablo F (2003) Locally born olfactory bulb stem cells proliferate in response to insulin-related factors and require endogenous insulin-like growth factor-I for differentiation into neurons and glia. J Neurosci 23: 895-906

Whitwell JL, Jack CR, Jr. (2005) Comparisons between Alzheimer disease, frontotemporal lobar degeneration, and normal aging with brain mapping. Top Magn Reson Imaging 16: 409-25

Zhao W, Chen H, Xu H, Moore E, Meiri N, Quon MJ, Alkon DL (1999) Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. J Biol Chem 274: 34893-902

Zhao WQ, Alkon DL (2001) Role of insulin and insulin receptor in learning and memory. Mol Cell Endocrinol 177: 125-34

Zhao WQ, Chen H, Quon MJ, Alkon DL (2004) Insulin and the insulin receptor in experimental models of learning and memory. Eur J Pharmacol 490: 71-81

ANEXOS

Anexo 1. Artigo publicado no periódico Behavioural Pharmacology

Metabolic and behavioral effects of chronic olanzapine treatment and cafeteria diet in rats

Alexandre P. Muller[a], Ana H. Tort[a], Jussânia Gnoatto[a], Julia D. Moreira[a], Elsa R. Vinadé[a], Marcos L. Perry[a], Diogo O. Souza[a], Diogo R. Lara[b] and Luis V. Portela[a]

Olanzapine and highly palatable diets can alter metabolism and brain function. We investigated the interaction of chronic treatment (4 months) with olanzapine and a cafeteria diet on metabolic parameters, memory tasks (spatial and aversive), the elevated plus maze and locomotor activity induced by d-amphetamine. Male Wistar rats were separated into the following groups: standard diet vehicle, standard diet and olanzapine, cafeteria diet vehicle and cafeteria diet and olanzapine. Olanzapine was administered in the drinking water (approximately 1.5 mg/kg/day), and after 3 days of treatment, the rats exhibited an expected anxiolytic effect and reduced amphetamine-induced hyperlocomotion. After 4 months of treatment, cafeteria diet vehicle and cafeteria diet olanzapine rats exhibited an increased body weight and heavier fat pads compared with the standard diet groups. Olanzapine increased only the epididymal and mesenteric fat pads. The cafeteria diet and olanzapine group showed greater glucose intolerance compared with all other groups. The cafeteria diet altered the effects of chronic olanzapine on the performance in the water maze and inhibitory avoidance tasks. Chronic olanzapine treatment failed to affect amphetamine-induced locomotion and to produce anxiolytic effects in the elevated plus maze task, regardless of the diet. Our results suggest that chronic olanzapine caused an increase in fat pads, which is putatively involved in the etiology of many metabolic diseases. Rats on the cafeteria diet were overweight and exhibited glucose intolerance. We did not observe these effects with olanzapine treatment with the standard diet. Moreover, the chronic treatment regimen caused tolerance to the antipsychotic and anxiolytic effects of olanzapine and seemed to potentiate some of the metabolic effects of the cafeteria diet. The cafeteria diet also modified the effects of chronic treatment with olanzapine on cognitive tasks, which may represent an undesirable effect of poor diets in psychiatric patients. *Behavioural Pharmacology* 21:668–675 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

[a]Departamento de Bioquímica, ICBS, UFRGS, Programa de Pós Graduação em Ciências Biológicas-Bioquímica, Rua Ramiro Barcelos and [b]Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, CEP, Porto Alegre, RS, Brazil

Correspondence to Luis V. Portela, PhD, Departamento de Bioquímica, ICBS, UFRGS, Rua Ramiro Barcelos, 2600 Anexo, Porto Alegre CEP 90035-003, RS, Brazil
E-mail: roskaportela@gmail.com

Introduction

The atypical antipsychotic olanzapine is associated with a robust therapeutic response in schizophrenia and mania (Lieberman *et al.*, 2005), and with insulin resistance, weight gain and enhanced abdominal obesity and adiposity (Allison *et al.*, 1999; Newcomer, 2005). In rodents, olanzapine can disrupt the performance of animals in spatial memory tasks designed to assess specific components of cognition that are affected in schizophrenia (Terry *et al.*, 2002, 2008; Didriksen *et al.*, 2006); however, olanzapine is able to reverse drug-induced cognitive dysfunction (Egashira *et al.*, 2008). In addition, acute treatment with olanzapine reverses amphetamine (AMPH)-induced locomotion and induces an anxiolytic effect in rodents (Frye and Seliga, 2003; Samaha *et al.*, 2007; Mead *et al.*, 2008).

Weight gain, especially when manifested by intra-abdominal obesity, is a significant long-term health issue associated with impaired metabolism (Newcomer, 2005,

2007). Data from human studies indicate that olanzapine is able to induce weight gain and cause metabolic alterations, such as changes in glucose metabolism, insulin resistance and metabolic syndrome (Casey, 2005; Lieberman *et al.*, 2005; Haupt, 2006; Minet-Ringuet *et al.*, 2006). Many authors have attempted to model such effects induced by olanzapine in a range of animal species, including rats (Minet-Ringuet *et al.*, 2006; Cooper *et al.*, 2007). These studies have been controversial, because animal models differ in the atypical antipsychotic used, strain, diet composition and duration of treatment. Moreover, major discrepancies regarding weight gain are related to sex. Atypical antipsychotics, such as olanzapine, induced weight gain and metabolic changes much more readily in female rats with a standard diet (Cooper *et al.*, 2005; Kirk *et al.*, 2009). However, male rats treated with olanzapine (6 weeks, 0.5 and 2 mg/kg) and fed with a standard laboratory diet resembling the western diet presented an obese phenotype (Minet-Ringuet *et al.*,

2006). More recent studies performed without changes in diet composition have shown no weight gain, but have shown increased visceral adiposity in males (Cooper *et al.*, 2007, 2008; Chintoh *et al.*, 2008).

Diet composition is an essential component of a healthy life. Psychiatric patients are more prone to eating poorer diets that are rich in fat and low in fiber (Brown *et al.*, 1999; McCreadie, 2003). It has been established that some atypical antipsychotics, especially olanzapine and clozapine, may increase appetite and food intake in patients (Casey and Zorn, 2001); however, the impact of the western style diet with a 'cafeteria' or 'fast food' nutritional profile requires additional investigation. An elevated consumption of highly palatable foods, rich in fat and/or sugar (cafeteria diet), has a clear association with metabolic derangements and impaired brain function (Molteni *et al.*, 2002). In rodent models, a high-fat diet worsened hippocampal signaling and performance in a variety of tasks (Molteni *et al.*, 2004; Muller *et al.*, 2008). Moreover, the consumption of cafeteria diet was harmful to neural cells (Molteni *et al.*, 2002; Souza *et al.*, 2007), impaired insulin-like growth factor 1 entrance to the brain through the choroid plexus (Dietrich *et al.* 2007) and induced anxiety-like behavior (Souza *et al.*, 2007).

Separately, both olanzapine therapy and cafeteria diet consumption may cause weight gain and metabolic derangements, and they may modify behavior and brain function. The main goal of this study was to evaluate the interactions of chronic treatment with olanzapine and cafeteria diet on metabolic parameters, memory, anxiety and locomotor activity in male rats.

Methods
Subjects
Male Wistar rats were maintained in plastic cages (five per cage) under a 12-h light/dark cycle at 22–24°C. Rats were assigned to one of following groups starting at 3 months of age: standard diet vehicle (SD, $n = 10$), standard diet and olanzapine (SDO, $n = 10$), cafeteria diet vehicle (CD, $n = 10$), and cafeteria diet and olanzapine (CDO, $n = 10$). 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 of the Federal University of Rio Grande do Sul, Brazil.

Olanzapine treatment
We had earlier measured volume of water consumed in 24 h and observed that subjects drank about 20 ml of drinking water per day. We diluted olanzapine in drinking water to obtain an estimated dose of 1.5 mg/kg per rat/day (20 mg olanzapine/liter of water). This dose was chosen because olanzapine at 1 and 3 mg/kg (intraperotoneal) has been shown to block approximately 70% of dopamine D2 receptors in rats, to exert antipsychotic-like activity (Zhang and Bymaster, 1999) and to induce weight gain

(Minet-Ringuet *et al.*, 2006). The dose remained constant throughout the study. Olanzapine (Zyprexa, Eli Lilly of Brasil, São Paulo, Brazil) was dissolved in 0.1 mol/l HCl and then diluted with tap water and given to rats in drinking water. Olanzapine solution was changed every 2 days and did not affect the volume consumed compared with the water groups. The pH of the water and olanzapine solution was similar. Importantly, olanzapine diluted in water has been shown to be stable for at least 96 h at room temperature (Terry *et al.*, 2008).

Subacute treatment
Three-month-old adult male Wistar rats ($n = 20$), on a standard diet, were treated with olanzapine (SDO, $n = 10$) or vehicle (SD, $n = 10$) for 3 days evaluated for locomotion and in the elevated plus maze (EPM) (see below).

Chronic treatment
Male Wistar rats ($n = 40$) were assigned to one of the four groups starting at 3 months of age until 7 months of age: standard diet vehicle (SD, $n = 10$), standard diet and olanzapine (SDO, $n = 10$), cafeteria diet vehicle (CD, $n = 10$) and cafeteria diet and olanzapine (CDO, $n = 10$).

Cafeteria diet
Diet effects were only evaluated during chronic treatment, with or without olanzapine. To mimic human fast food diets, we fed adult male rats with a highly palatable diet enriched with simple sugars and saturated fat (high fat, high-calorie 'cafeteria diet'). This diet was chosen because it produced consistent weight gain and metabolic changes in our earlier studies (Dietrich *et al.*, 2007). The cafeteria diet contained the following: 57.0% carbohydrates (34.0% condensed milk, 15.0% starch and 8.0% sucrose), 25.3% protein, 10.0% fat, 5.0% salt and 2.7% fibers and vitamins. The standard diet contained the following: 62.5% carbohydrates from starch, 25.3% protein, 4.5% fat, 5% salt and 2.7% fibers and vitamins. Saturated fats accounted for 16% of fats in both the diets. The standard diet contained 3.9 kCal/g and the cafeteria diet contained 4.04 kCal/g (Dietrich *et al.*, 2007). All subjects had free access to food and water or olanzapine solution during the treatment.

Evaluation of antipsychotic-like and anxiolytic effects of subacute olanzapine treatment
To evaluate whether the doses of olanzapine used in drinking water had antipsychotic-like and/or anxiolytic effects, we performed a subacute treatment (3 days) to confirm the effects of olanzapine, evidenced by decreased locomotor activity induced by AMPH (Samaha *et al.*, 2007) and reduced anxiety-related behavior (Frye and Seliga, 2003; Mead *et al.*, 2008).

Locomotor activity
All experiments were conducted between 09:00 and 16:00 h. Rats were randomly allocated to four individual wooden boxes (50 × 50 × 50 cm) placed on the floor of

a soundproof and diffusely illuminated room. The locomotor activity of four rats was recorded simultaneously by a video-computerized system, with image analysis at four frames per second. The software (Tort *et al.*, 2006) tracked the animals by distinguishing their white color from the black background of the floor, registering x and y horizontal coordinates. The method was set to examine horizontal locomotor activity, ignoring small movements, such as breathing, head and tail actions, and tremors. Rats were individually placed in the locomotor activity cages and left undisturbed for 60 min to measure baseline levels of locomotor activity (habituation period). After that, the subjects were injected with AMPH (0.5 mg/kg), and locomotor activity was recorded for another 60 min. Hyperlocomotion induced by AMPH is a well-recognized pharmacological model with predictive value for antipsychotics, that is, subacute administration of antipsychotics reduces AMPH-induced locomotor activity (Dall'Igna *et al.*, 2005).

Elevated plus maze test
Subjects were tested in the EPM to assess the anxiolytic effects of olanzapine. The EPM consisted of two open (50 cm long × 10 cm wide) and two-walled arms (50 cm long × 10 cm wide × 38.5 cm high) with an open roof, arranged around a central platform (10 × 10 cm), and with the two arms of each type placed opposite to each other. Illumination (0.3 and 48 lx on the closed and open arms, respectively) was provided by diffuse ceiling lighting. Subjects were placed onto the central platform, facing one of the open arms. The subject was allowed to explore the maze for 5 min. The parameters used to evaluate the anxiety were time (s) spent in the open and closed arms and entries into the open and closed arms.

Evaluation of the metabolic, cognitive effect, and locomotor activity of chronic treatment with olanzapine and the cafeteria diet
Morris water maze task
The apparatus was a black circular pool (200 cm in diameter) with a water temperature of 21 ± 1 °C. During training, rats learned to escape from the water by finding a hidden rigid black platform submerged about 1 cm below the water surface in a fixed location. Rats were trained in a 4-trial water maze task for 5 consecutive days, with each trial lasting up to 60 s, with 20 s of rest on the hidden platform. If the rat failed to find the platform in 60 s, it was placed gently on the platform and allowed to rest for 20 s. The maze was located in a well-lit white room with several visual stimuli hanging on the walls to provide spatial cues. The escape latency during each trial was measured as an indicator of learning. A probe test was performed without the platform, and the time spent in the target quadrant was measured as an indicator of memory retention.

Inhibitory avoidance task
The apparatus was a 50 × 25 × 25 cm acrylic box with a floor of parallel caliber stainless steel bars (1 mm in diameter) spaced 1 cm apart (Insight Equipments, São Paulo, Brazil). A 7-cm wide, 2.5-cm high platform was placed against the left wall of the box. Subjects were placed on the platform, and their latencies to step down on the floor with four paws were measured with an automatic device. In the training sessions, when the animals stepped down onto the grid they received a 0.7 mA foot shock for 1 s, and were immediately returned to their home cages. In the test sessions, performed 24 h after training to evaluate long-term memory, the rats were returned to the platform, and the latency to step down (180-s maximum) was used as a measure of retention. The foot shock was omitted during testing. Data for the inhibitory avoidance task are shown as the median (interquartile ranges) of the latencies to step down on the grid in both the sessions.

Locomotor activity and elevated plus maze task
The same protocols described above were used following chronic treatment.

Glucose tolerance test
Glucose (2 mg/g body weight) was administered intraperotoneally to 8-h fasted rats (at 14:00 h) after 4 months of treatment. Blood was collected by a small puncture in the tail immediately before (0 min), 30, 60, and 120 min after the injection. At each time point, glucose was measured using a glucosimeter (AccuChek Active, Roche Diagnostics, Indianapolis, Indiana USA).

Blood biochemical evaluation and fat pad weights
Rats were killed by decapitation after 4 months of treatment. Blood was collected and centrifuged at 5000g/10 min to obtain serum samples, which were stored at $-80°C$ until the day of analysis. Serum triacylglycerol (TAG), total cholesterol, and HDL cholesterol were measured using commercial kits (Labtest, Lagoa Santa, Brazil) and the Labmax equipment (Labtest). Fat tissues from retroperitoneal and epididymal regions were dissected and weighed as described earlier (Parekh *et al.*, 1998).

Statistical analysis
The results were calculated and expressed as means ± SEM, except for the inhibitory avoidance and EPM results, which are expressed as median ± interquartile ranges. The data from the water maze task and glucose tolerance test were analyzed using repeated-measures analysis of variance (ANOVA) followed by a post-hoc Duncan test. Data from the blood biochemical evaluation and fat pad weights were analyzed by a one-way ANOVA followed by a post-hoc Duncan test. The Kruskal–Wallis one-way ANOVA was used for the inhibitory avoidance data because of a cutoff time. The Kruskal–Wallis one-way ANOVA was used for the EPM data, as the distribution was asymmetrical in the olanzapine group according to the Shapiro–Wilk analysis. To evaluate the effect of olanzapine

or cafeteria diet alone, we performed a Student's *t*-test between groups. Differences between groups were considered statistically significant at *P* value less than 0.05.

Results
Subacute effects of olanzapine
Locomotor effects of amphetamine and anxiolytic activity

As shown in Fig. 1a, olanzapine at a dose of 1.5 mg/kg/day in drinking water over 3 days was able to prevent the increased locomotor activity induced by AMPH ($P < 0.05$). Locomotor activity at the time of AMPH injection (60-min habituation) did not differ between the groups. Subacute olanzapine also produced an anxiolytic effect, as shown by increased time spent in the open arms of the EPM task (Fig. 1b, $P < 0.05$). The other EPM parameters did not differ between the groups (data not shown). These results indicated that the dose and administration procedures of olanzapine produced the expected antipsychotic-like and anxiolytic effects.

Chronic effects of olanzapine and cafeteria diet
Morris water maze and inhibitory avoidance task

Spatial and aversive memories were evaluated by the Morris water maze and inhibitory avoidance tasks, respectively. In the water maze task (Fig. 2a), all groups showed a similar latency to find the platform until the third day of the acquisition phase. However, from the fourth day, the SDO group displayed a higher latency to find the platform compared with all the other groups (SDO > CD, CDO and SD, $P < 0.05$). There were no statistically significant differences between groups in retention memory in the probe test (sixth day, data not shown).

In the inhibitory avoidance task (Fig. 2b), the SD, SDO and CD groups showed an increased step-down latency in the test session compared with the training session ($P < 0.05$), whereas in the CDO group, test time latencies were not significantly different from the training session.

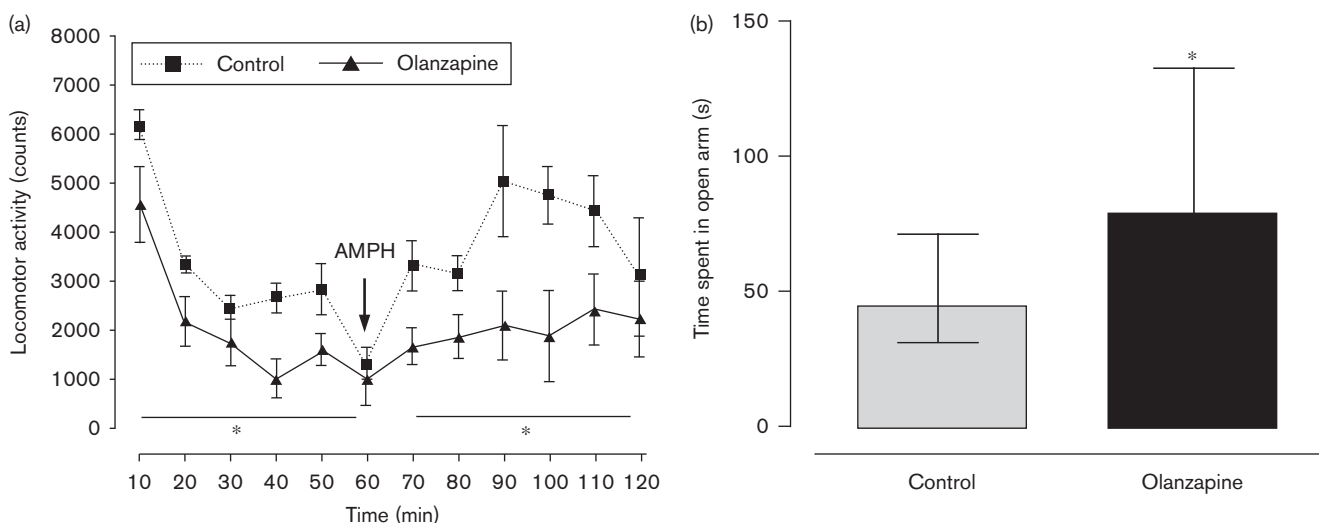#### Locomotor effects of amphetamine and anxiolytic activity

After 4 months of treatment, olanzapine or CD alone, or their combination, was not able to decrease the locomotor activity induced by AMPH (Fig. 2c). As expected, locomotion during the habituation period (60 min) was lower in the chronic treatment group than in the subacute treatment group (Fig. 1a). However, at the time of AMPH administration, locomotion was similar and the effect of AMPH also reached comparable levels even in the olanzapine groups, suggesting a true tolerance effect. Similarly, the time spent in the open arms of the EPM task did not differ between the groups (Fig. 2d).

Glucose tolerance test, body weight, and fat pads

As shown in Table 1, the fasting CDO group showed higher blood glucose levels when compared with the other groups ($P < 0.05$). At 30 and 60 min after glucose administration, the cafeteria groups presented higher glucose levels compared with the standard diet groups (Fig. 3a, SD and SDO < CD and CDO, $P < 0.05$). At 120 min, blood glucose levels in the CDO group remained significantly different when compared with all other groups (CDO > CD, SD and SDO, $P < 0.05$).

Total body weight (Fig. 3b), and the mesenteric (Fig. 3c) and epididymal (Fig. 3d) fat pads were heavier in the cafeteria diet groups ($P < 0.05$) compared with the

Fig. 1

Amphetamine (AMPH)-induced locomotor and anxiolytic activity in rats after acute treatment with olanzapine. (a) Acute olanzapine decreased locomotor activity during the habituation period and after AMPH administration (*$P<0.05$). (b) Acute olanzapine increased the time spent in the open arms of the elevated plus maze (*$P<0.05$).

Fig. 2
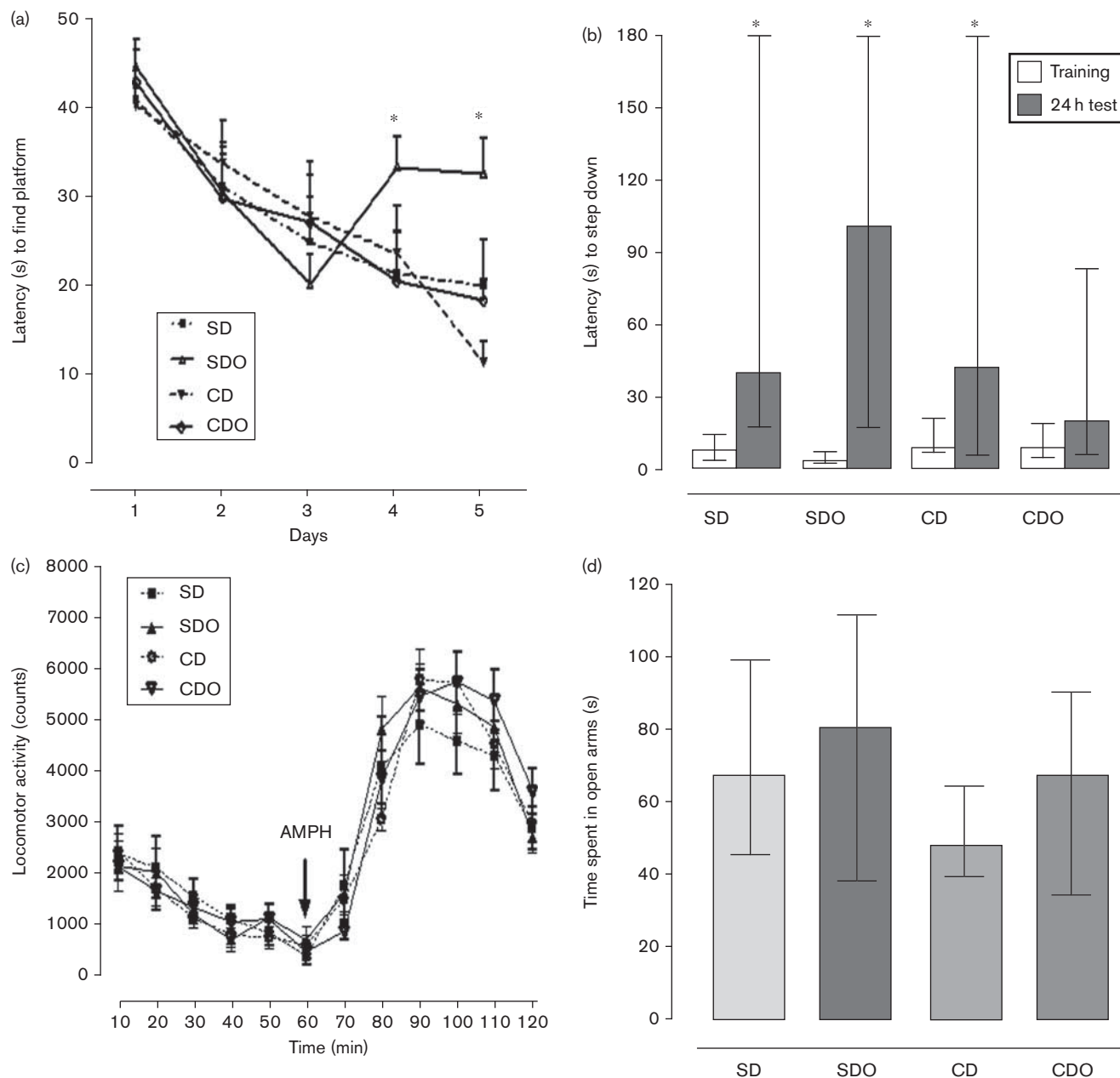
Memory tasks, amphetamine (AMPH)-induced locomotor activity, and anxiolytic effects in rats after 4 months of treatment with one of the following: standard diet vehicle (SD), standard diet and olanzapine (SDO), cafeteria diet vehicle (CD), or cafeteria diet and olanzapine (CDO). $n = 10$ rats per group. (a) Water maze: after the third day of acquisition, olanzapine increased the latency to find the platform only in the SDO group (SDO > SD, CD and CDO, *$P < 0.05$). (b) Inhibitory avoidance task: compared with the training trials, the SD, SDO, and CD, but not the CDO, group presented increased latency to step down on the test day (test > training, $P < 0.05$). (c) Locomotor activity induced by administration of AMPH in chronically treated rats: animals were habituated to an open field for 60 min, injected intraperitoneally with AMPH (0.5 mg/kg) and locomotor activity was evaluated for an additional 60 min. There were no significant differences among the groups in spontaneous and AMPH-induced locomotor activity. (d) Anxiolytic activity. There were no significant differences among the groups in the time spent in the open arms of the elevated plus maze task.

standard diet groups (CD and CDO > SD and SDO, $P < 0.05$). Olanzapine also caused an increase in the weight of fat pads in the standard diet groups (Fig. 3c and d; SDO > SD, $P < 0.05$).

Serum biochemical evaluation

Serum TAG, total cholesterol, and HDL cholesterol levels are shown in Table 1. The cafeteria diet groups presented increased TAG levels compared with the standard diet

Table 1 Serum fasting glucose levels and lipid profiles of rats treated during 4 months with olanzapine and cafeteria diet

Groups	Glucose (mg/dl)	Triacylglycerol (mg/dl)	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)
SD	78±4	92±12	56±3	39±1
SDO	85±2	88±10	58±4	41±3
CD	86±2	138±24**	56±6	37±4
CDO	103±3*	162±24**	78±5***	51±2***

CD, cafeteria diet and vehicle; CDO, cafeteria diet and olanzapine; HDL, high-density lipoprotein; SD, standard diet and vehicle; SDO, standard diet and olanzapine.
*CDO > SD, SDO, and CD, $P<0.05$.
**CD and CDO > SD and SDO, $P<0.05$.
***CDO > CD, SD and SDO, $P<0.05$.

Fig. 3
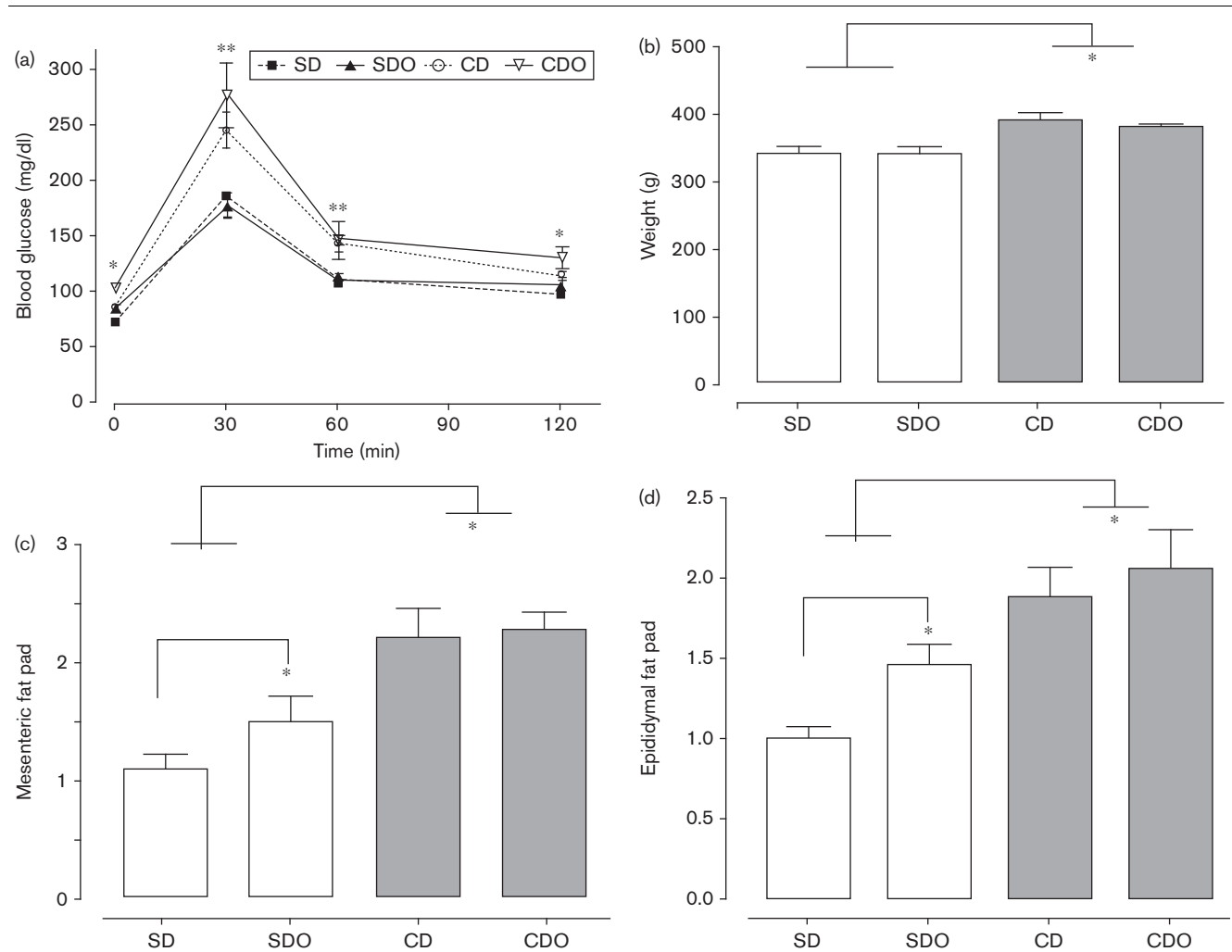
Glucose tolerance test (GTT), body weight, mesenteric, and epydidimal fat pads in rats after 4 months of treatment with one of the following: standard diet vehicle (SD), standard diet and olanzapine (SDO), cafeteria diet vehicle (CD), or cafeteria diet and olanzapine (CDO). $n=10$ rats per group. (a) GTT: blood glucose levels of the CDO group were significantly elevated at 0 min (baseline) and 120 min compared with the other groups (CDO>other groups, *$P<0.05$). At 30 and 60 min, the cafeteria diet groups showed increased blood glucose levels compared with the standard diet groups (CD and CDO>SD and SDO, **$P<0.05$). (b) Body weight: the cafeteria diet groups showed significantly increased body weights when compared with the standard diet groups (CD and CDO > SD and SDO, *$P<0.05$). (c) Mesenteric fat pad: olanzapine increased the weight of the mesenteric fat pad when compared with the control group (SDO>SD, *$P<0.05$). The cafeteria diet groups showed heavier mesenteric fat pads when compared with the other groups (CD and CDO>SD and SDO, *$P<0.05$). (d) Epididymal fat pad: olanzapine increased the epididymal fat pad when compared with the control group (SDO>SD, *$P<0.05$). The cafeteria diet groups had heavier epididymal fat pads when compared with the other groups (CD and CDO>SD and SDO, *$P<0.05$).

groups (CD and CDO > SD and SDO, $P < 0.05$). Total cholesterol and HDL cholesterol levels were higher in the CDO group when compared with all other groups (CDO > CD, SD and SDO, $P < 0.05$).

Discussion

In this study, we searched for interactions between olanzapine and the cafeteria diet treatments on metabolic and behavioral parameters in rats. In relation to metabolic parameters, the cafeteria diet caused harmful effects that were worsened by olanzapine treatment on some specific parameters. Regarding performance in memory tests, the cafeteria diet affected the behavioral response to olanzapine treatment in spatial memory and in aversive memory tasks.

Increased risk of insulin resistance, type-2 diabetes mellitus, and hyperglycemia have been associated with antipsychotic therapy; however, some patients with mental disorders present abnormalities in glucose regulation before medication (Haupt, 2006). In our study, olanzapine treatment failed to increase body weight, but caused an increase in the weight of the mesenteric and epididymal fat pads when compared with the standard diet. This finding is in agreement with the findings by Cooper et al. (2007), who showed that olanzapine (2–8 mg/kg/day, intraperotoneal, over 21 days) induced a significant increase in visceral adiposity without weight gain in male Wistar rats. In contrast, two doses of olanzapine (0.5 and 2.5 mg/kg/day in food for 6 weeks) produced obesity and heavier fat pads in male Sprague–Dawley rats fed on what resembled the typical human diet (Minet-Ringuet et al., 2006). In addition, our results showed that rats fed with the standard diet plus olanzapine presented normal glucose levels from fasting to 120 min and a normal lipid profile, despite heavier fat pads. This result is consistent with earlier findings showing no changes in glucose serum levels when olanzapine was associated with high-fat (Minet-Ringuet et al., 2005) or standard diets (Cooper et al., 2007).

A sedentary lifestyle and poor diet may contribute to metabolic alterations in psychiatric patients (Waitzkin, 1966; Newcomer, 2004). Here, we found that after 4 months of treatment, animals receiving the cafeteria diet presented increased body weight, adiposity, serum TAG, and glucose intolerance on glucose tolerance tests (at 30 and 60 min). Similar effects regarding body fat mass and TAG levels were reported earlier using a cafeteria diet (Chaves et al., 2006). Although in this study olanzapine alone was mostly devoid of metabolic effects when the standard diet was used, olanzapine combined with the cafeteria diet produced higher fasting and long-lasting high glucose levels compared with all the other groups. Although insulin resistance, elevated plasma glucose, and lipid levels are associated with increased fat mass (Tracy, 2001), the effects of certain second-generation antipsychotics on glucose metabolism were quite independent of adiposity (Newcomer et al., 2002). This finding may derive from

alterations in insulin signaling proteins and/or glucose transporters (Newcomer, 2004).

The effects of psychopharmacologic treatments on cognitive functions, such as attention, memory, and executive functions (Green et al., 2000) have received special attention in recent years. For example, chronic treatments with olanzapine have been shown to affect the cognitive performance of rats in a spatial water maze task (Terry et al., 2002, 2008; Didriksen et al., 2006). Here, we were able to replicate this finding in the SDO group. However, we found that this effect in the water maze was absent when olanzapine treatment was combined with the cafeteria diet (Fig. 3a). A possible mechanism for the effect of olanzapine is its ability to block dopamine D1 receptors (Zhang and Bymaster, 1999), which has been shown to impair water maze performance (Stuchlik et al., 2007). In contrast, palatable food increases D1-mediated signaling (Rauggi et al., 2005), which may counteract the effects of olanzapine. Moreover, in the SDO group, olanzapine had no effect on the inhibitory avoidance task, but the CDO group displayed impaired performance (Fig. 3b). The mechanism to explain this result is unclear, but overall these results indicate that the palatable diet seems to have independent influences on the effects of olanzapine on different cognitive functions.

Locomotor activity stimulated by AMPH is a classical parameter that is sensitive to acute treatment with antipsychotic drugs. Accordingly, treatment with olanzapine (1.5 mg/kg) for 3 days decreased spontaneous and AMPH-induced locomotion (Fig. 1a). In addition, treatment with olanzapine produced an anxiolytic effect in the EPM task, as reported earlier (Locchi et al., 2008). However, after 4 months of treatment, olanzapine (1.5 mg/Kg) failed to affect spontaneous or AMPH-induced locomotion and failed to produce anxiolytic effects in the EPM task, regardless of the diet (Fig. 3c). Accordingly, Samaha et al. (2007) reported that chronic treatment with haloperidol and olanzapine leads to a progressive loss of efficacy against AMPH-induced locomotion and conditioned avoidance response, and this effect was linked to an increase in the number and sensitivity of D2 receptors. Our results confirmed that the rats developed a tolerance to chronic olanzapine administration and did not show the typical decrease in AMPH -induced locomotion or the normal anxiolytic effects in the EPM. Thus, antipsychotic tolerance and cross tolerance can readily be studied in subjects and is a clinically important topic that may underlie the symptomatic relapse (Goudie et al., 2007; Goudie and Cole, 2008).

In conclusion, our results show that chronic olanzapine caused an increase in fat pad weight, which is a change that is putatively involved in the etiology of many metabolic diseases. The cafeteria diet was associated with overweight and glucose intolerance, but not with olanzapine treatment, compared with the standard diet. In addition,

we found that chronic treatment with olanzapine induced a tolerance to its antipsychotic and anxiolytic actions. The cafeteria diet also modified the effects of chronic treatment with olanzapine on cognitive tasks, which may represent an undesirable effect of poor diets in psychiatric patient treatment.

Acknowledgements

References

Allison DB, Mentore JL, Heo M, Chandler LP, Cappelleri JC, Infante MC, Weiden PJ (1999). Antipsychotic-induced weight gain: a comprehensive research synthesis. *Am J Psychiatry* **156**:1686–1696.

Brown S, Birtwistle J, Roe L, Thompson C (1999). The unhealthy lifestyle of people with schizophrenia. *Psychol Med* **29**:697–701.

Casey DE (2005). Metabolic issues and cardiovascular disease in patients with psychiatric disorders. *Am J Med* **118** (**Suppl 2**):15S–22S.

Casey DE, Zorn SH (2001). The pharmacology of weight gain with antipsychotics. *J Clin Psychiatry* **62** (**Suppl 7**):4–10.

Chaves VE, Frasson D, Martins-Santos ME, Boschini RP, Garofalo MA, Festuccia WT, *et al.* (2006). Glyceroneogenesis is reduced and glucose uptake is increased in adipose tissue from cafeteria diet-fed rats independently of tissue sympathetic innervation. *J Nutr* **136**:2475–2480.

Chintoh AF, Mann SW, Lam TK, Giacca A, Remington G (2008). Insulin resistance following continuous, chronic olanzapine treatment: an animal model. *Schizophr Res* **104**:23–30.

Cooper GD, Pickavance LC, Wilding JP, Halford JC, Goudie AJ (2005). A parametric analysis of olanzapine-induced weight gain in female rats. *Psychopharmacology (Berl)* **181**:80–89.

Cooper GD, Pickavance LC, Wilding JP, Harrold JA, Halford JC, Goudie AJ (2007). Effects of olanzapine in male rats: enhanced adiposity in the absence of hyperphagia, weight gain or metabolic abnormalities. *J Psychopharmacol* **21**:405–413.

Cooper GD, Harrold JA, Halford JC, Goudie AJ (2008). Chronic clozapine treatment in female rats does not induce weight gain or metabolic abnormalities but enhances adiposity: implications for animal models of antipsychotic-induced weight gain. *Prog Neuropsychopharmacol Biol Psychiatry* **32**:428–436.

Dall'Igna OP, Tort AB, Souza DO, Lara DR (2005). Cinnarizine has an atypical antipsychotic profile in animal models of psychosis. *J Psychopharmacol* **19**:342–346.

Didriksen M, Kreilgaard M, Arnt J (2006). Sertindole, in contrast to clozapine and olanzapine, does not disrupt water maze performance after acute or chronic treatment. *Eur J Pharmacol* **542**:108–115.

Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, *et al.* (2007). Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. *Neuromolecular Med* **9**:324–330.

Egashira N, Ishigami N, Mishima K, Iwasaki K, Oishi R, Fujiwara M (2008). Delta9-Tetrahydrocannabinol-induced cognitive deficits are reversed by olanzapine but not haloperidol in rats. *Prog Neuropsychopharmacol Biol Psychiatry* **32**:499–506.

Frye CA, Seliga AM (2003). Olanzapine's effects to reduce fear and anxiety and enhance social interactions coincide with increased progestin concentrations of ovariectomized rats. *Psychoneuroendocrinology* **28**:657–673.

Goudie AJ, Cole JC (2008). Switching antipsychotics. Antipsychotic tolerance, withdrawal and relapse: unresolved issues and research implications. *J Psychopharmacol* **22**:815–817.

Goudie AJ, Cole JC, Sumnall HR (2007). Olanzapine and JL13 induce cross-tolerance to the clozapine discriminative stimulus in rats. *Behav Pharmacol* **18**:9–17.

Green MF, Kern RS, Braff DL, Mintz J (2000). Neurocognitive deficits and functional outcome in schizophrenia: are we measuring the 'right stuff'? *Schizophr Bull* **26**:119–136.

Haupt DW (2006). Differential metabolic effects of antipsychotic treatments. *Eur Neuropsychopharmacol* **16** (**Suppl 3**):S149–S155.

Kirk SL, Glazebrook J, Grayson B, Neill JC, Reynolds GP (2009). Olanzapine-induced weight gain in the rat: role of 5-HT2C and histamine H1 receptors. *Psychopharmacology (Berl)* **207**:119–125.

Lieberman JA, Stroup TS, McEvoy JP, Swartz MS, Rosenheck RA, Perkins DO, *et al.* (2005). Effectiveness of antipsychotic drugs in patients with chronic schizophrenia. *N Engl J Med* **353**:1209–1223.

Locchi F, Dall'olio R, Gandolfi O, Rimondini R (2008). Olanzapine counteracts stress-induced anxiety-like behavior in rats. *Neurosci Lett* **438**:146–149.

McCreadie RG (2003). Diet, smoking and cardiovascular risk in people with schizophrenia: descriptive study. *Br J Psychiatry* **183**:534–539.

Mead A, Li M, Kapur S (2008). Clozapine and olanzapine exhibit an intrinsic anxiolytic property in two conditioned fear paradigms: contrast with haloperidol and chlordiazepoxide. *Pharmacol Biochem Behav* **90**:551–562.

Minet-Ringuet J, Even PC, Guesdon B, Tome D, de Beaurepaire R (2005). Effects of chronic neuroleptic treatments on nutrient selection, body weight, and body composition in the male rat under dietary self-selection. *Behav Brain Res* **163**:204–211.

Minet-Ringuet J, Even PC, Lacroix M, Tome D, de Beaurepaire R (2006). A model for antipsychotic-induced obesity in the male rat. *Psychopharmacology (Berl)* **187**:447–454.

Molteni R, Barnard RJ, Ying Z, Roberts CK, Gomez-Pinilla F (2002). A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning. *Neuroscience* **112**:803–814.

Molteni R, Wu A, Vaynman S, Ying Z, Barnard RJ, Gomez-Pinilla F (2004). Exercise reverses the harmful effects of consumption of a high-fat diet on synaptic and behavioral plasticity associated to the action of brain-derived neurotrophic factor. *Neuroscience* **123**:429–440.

Muller AP, Cammarota M, de Oliveira Dietrich M, Rotta LN, Portela LV, Souza DO, *et al.* (2008). Different effect of high fat diet and physical exercise in the hippocampal signaling. *Neurochem Res* **33**:880–885.

Newcomer JW (2004). Abnormalities of glucose metabolism associated with atypical antipsychotic drugs. *J Clin Psychiatry* **65** (**Suppl 18**):36–46.

Newcomer JW (2005). Second-generation (atypical) antipsychotics and metabolic effects: a comprehensive literature review. *CNS Drugs* **19** (**Suppl 1**):1–93.

Newcomer JW (2007). Metabolic syndrome and mental illness. *Am J Manag Care* **13**:S170–S177.

Newcomer JW, Haupt DW, Fucetola R, Melson AK, Schweiger JA, Cooper BP, Selke G (2002). Abnormalities in glucose regulation during antipsychotic treatment of schizophrenia. *Arch Gen Psychiatry* **59**:337–345.

Parekh PI, Petro AE, Tiller JM, Feinglos MN, Surwit RS (1998). Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism* **47**:1089–1096.

Rauggi R, Scheggi S, Cassanelli A, De Montis MG, Tagliamonte A, Gambarana C (2005). The mesolimbic dopaminergic response to novel palatable food consumption increases dopamine-D1 receptor-mediated signalling with complex modifications of the DARPP-32 phosphorylation pattern. *J Neurochem* **92**:867–877.

Samaha AN, Seeman P, Stewart J, Rajabi H, Kapur S (2007). 'Breakthrough' dopamine supersensitivity during ongoing antipsychotic treatment leads to treatment failure over time. *J Neurosci* **27**:2979–2986.

Souza CG, Moreira JD, Siqueira IR, Pereira AG, Rieger DK, Souza DO, *et al.* (2007). Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like behavior. *Life Sci* **81**:198–203.

Stuchlik A, Rehakova L, Telensky P, Vales K (2007). Morris water maze learning in Long-Evans rats is differentially affected by blockade of D1-like and D2-like dopamine receptors. *Neurosci Lett* **422**:169–174.

Terry AV Jr, Hill WD, Parikh V, Evans DR, Waller JL, Mahadik SP (2002). Differential effects of chronic haloperidol and olanzapine exposure on brain cholinergic markers and spatial learning in rats. *Psychopharmacology (Berl)* **164**:360–368.

Terry AV Jr, Warner SE, Vandenhuerk L, Pillai A, Mahadik SP, Zhang G, Bartlett MG (2008). Negative effects of chronic oral chlorpromazine and olanzapine treatment on the performance of tasks designed to assess spatial learning and working memory in rats. *Neuroscience* **156**:1005–1016.

Tort AB, Neto WP, Amaral OB, Kazlauckas V, Souza DO, Lara DR (2006). A simple webcam-based approach for the measurement of rodent locomotion and other behavioural parameters. *J Neurosci Methods* **157**:91–97.

Tracy RP (2001). Is visceral adiposity the 'enemy within'? *Arterioscler Thromb Vasc Biol* **21**:881–883.

Waitzkin L (1966). A survey for unknown diabetics in a mental hospital. II. Men from age fifty. *Diabetes* **15**:164–172.

Zhang W, Bymaster FP (1999). The in vivo effects of olanzapine and other antipsychotic agents on receptor occupancy and antagonism of dopamine D1, D2, D3, 5HT2A and muscarinic receptors. *Psychopharmacology (Berl)* **141**:267–278.

Anexo 2. Artigo de publicado no periódico Experimental Gerontology
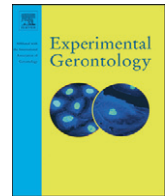
IGF-I and the aging mammalian brain

J. Piriz, A. Muller, J.L. Trejo, I. Torres-Aleman *

Cajal Institute and Ciberned, Madrid, Spain

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ABSTRACT

Insulin-like growth factors (IGFs) are important modulators of organismal life-span all along phylogeny. These growth factors are widely viewed as detrimental for long life by reducing tissue resistance to oxidative stress. However, IGF-I has been consistently shown to be a potent neuroprotective factor in mammals, and as such, a deterrent of brain aging. Conversely, recent data suggest that IGF-I may contribute to amyloid neurodegeneration underlying Alzheimer's disease. These opposing observations underline an incomplete understanding of the significance of this ancestral hormone pathway in relation to brain aging. It is possible that these opposite results are the consequence of using different experimental approaches. Thus, brain amyloid injury is reduced in mutant mice partially defective in IGF-I receptor function, whereas IGF-I is neuroprotective when administered to animal models of neurodegenerative disease or normal brain aging. This approach-dependent effect of IGF-I highlights a fundamental gap in our knowledge of the relationship between peripheral and brain IGF-I function and the actual biological impact of experimental modulation of brain IGF-I function. We suggest to directly address brain IGF-I function in the varying experimental approaches used to confirm that changes have taken place in the desired way.

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1. Introduction

For many years IGF-I was considered a cyto-protective factor for all type of cells in almost every tissue. Therefore, this hormone was broadly considered as beneficial for health. After epidemiological data indicated that blood levels of IGF-I were positively related to cancer risk (LeRoith and Roberts, 2003), this purported beneficial role turned the opposite way. New therapies in cancer are now relying on developing IGF-I receptor antagonists (Tao et al., 2007). Along this detrimental role of IGF-I another line of evidence from genetic studies in aging indicated that the insulin/IGF-I pathway shortens life-span (Kenyon, 2001). Hence, the idea that the general physiological action of IGF-I is detrimental for health is gaining acceptance. Nowadays it is undisputed that reduced insulin/IGF-I effects will result in prolonged life not only in primitive organisms such as worms (Kenyon, 2001) but also in mammals (Holzenberger et al., 2003), including humans (Suh et al., 2008). This is reinforced by the fact that in invertebrate species with a divergent evolutionary history, such as flies, this pathway is similarly detrimental (Clancy et al., 2001). It is probably in the specific case of the aging brain where this detrimental role of IGF-I may be challenged. Indeed, the neuroprotective actions of IGF-I encompass the aging brain and related diseases (Trejo et al., 2004a).

Based only in the amount of information, evidence portraying IGF-I as a wide spectrum neuroprotective agent greatly outweighs observations documenting the opposite.

2. IGF-I as a neuroprotective factor

The role of IGF-I in regulating brain growth during development was firmly established through classical genetic manipulation using overexpression and deletion of this growth factor in transgenic mice. The absence of IGF-I is perinatally lethal but the few surviving animals show pronounced microcephaly and many brain abnormalities (Vicario-Abejon et al., 2003; Baker et al., 1993). Conversely, overproduction of IGF-I leads to organomegaly affecting specific organs such as the brain (D'Ercole et al., 1996). In the latter, a general overgrowth effect was observed, although the myelin content was specifically increased.

In the adult brain, IGF-I is an important signal in brain homeostasis. The number of beneficial actions exerted by IGF-I is constantly growing. IGF-I promotes tissue remodelling by controlling the formation of new neurons, vessels and myelin in the adult brain (Torres-Aleman, 2010). IGF-I participates in brain metabolism in ways that are not fully understood but that appear essential to metabolic homeostasis (Bondy and Cheng, 2002). This growth factor directly affects higher brain functions through an array of actions on neurons, glia and the rest of cells that constitute brain tissue. In this way, IGF-I

* Corresponding author.
  E-mail address: torres@cajal.csic.es (I. Torres-Aleman).

has shown prominent effects on cognition by its underlying actions at the level of neurotransmission, neuronal excitability, and synaptic plasticity (reviewed in (Aleman and Torres-Aleman, 2009). For these reasons, IGF-I is currently considered a prototype neuroprotective factor in the adult brain (Trejo et al., 2004a).

3. IGF-I neuroprotection in the aging brain

In mammals, levels of IGF-I decrease with age. In humans this decrease starts relatively early, at the third decade of life. Whether this decrease is adaptive or merely reflects a general tissue decay is not known. At any rate, administration of IGF-I has shown widely neuroprotective actions in the aging brain. Namely, IGF-I improves learning and memory (Markowska et al., 1998), synaptic plasticity (Sonntag et al., 2000) or vascular growth (Sonntag et al., 1997), while diminishes oxidative stress in brain tissue (Puche et al., 2008). In elderly humans, serum IGF-I levels positively correlate with cognitive status (Aleman et al., 1999), and a similar correlation in mice unveiled a trophic action of circulating IGF-I on glutamate neurotransmission affecting synaptic plasticity and cognition (Trejo et al., 2007). Indeed, all major derangements commonly found in the aging brain, including cell atrophy/dysfunction, metabolic impairment, altered higher brain functions and accumulation of deleterious substances can be ascribed to reduced brain IGF-I input, a condition that is inherently linked to the aging process (Trejo et al., 2004b). Thus, lower IGF-I levels as individuals age are coupled to reduced sensitivity to this growth factor.

In this vein, as age-associated brain pathologies may be related to an exacerbated loss of IGF-I input (Trejo et al., 2004a), replacement therapy has shown promising effects in a number of neurodegenerative diseases (Torres-Aleman, 2007), most prominently in animal models of Alzheimer's disease (Carro et al., 2002, 2006), the most common type of neurodegeneration associated with aging. Clinical data have also reinforced a possible role of IGF-I in this disease. Thus, increased levels of serum IGF-I have been reported in early stages of AD, while at later stages serum IGF-I levels are low (Vardy et al., 2007; Watanabe et al., 2005). This agrees with the notion that circulating IGF-I levels will be increased in the initial stages of AD as a result of an ongoing resistance process while at later stages the levels of this serum growth factor would be decreased due to depletion of tissue resources (Carro and Torres-Aleman, 2004). Moreover, diverse drugs currently on the market, such as donepezil and glatiramer acetate (Butovsky et al., 2006; Tei et al.,
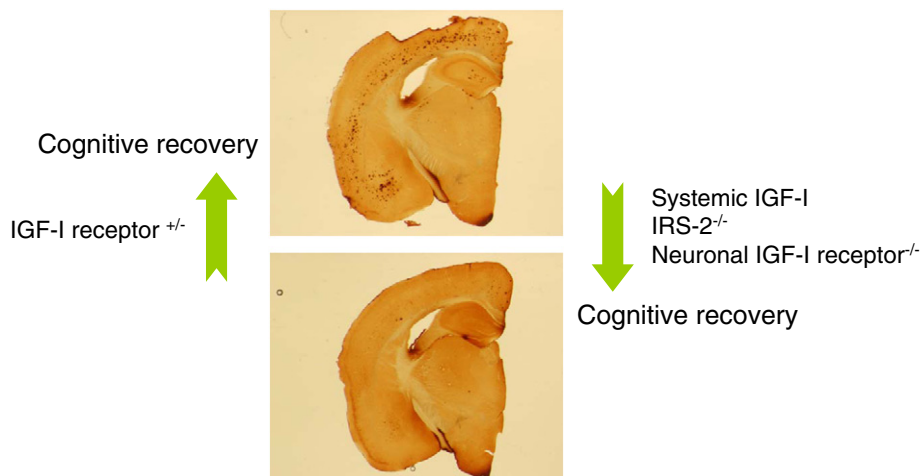
2008), together with novel compounds still in clinical trials (Bolos et al., 2010), display IGF-I-increasing effects. Recently, IGF-I levels have been proposed as a biomarker to predict therapeutic responses to donepezil in AD (Yamagata et al., 2010). In addition, a biomarker protein array recently described for AD includes insulin-like binding protein 6, a carrier protein for IGF-I (Ray et al., 2007).

Conversely, clinical trials with ghrelin agonists that stimulate growth hormone and in this way raise serum IGF-I levels have shown no efficacy in AD patients (Sevigny et al., 2008). Moreover, other authors failed to replicate the beneficial actions of IGF-I in animal models of AD-related amyloidosis (Lanz et al., 2008). Finally, recent publications concluded that genetic reduction of IGF-I/insulin signalling protects against AD-like pathology in animal models of the disease (Cohen et al., 2009; Freude et al., 2009; Killick et al., 2009).

4. The controversy on the role of IGF-I in the aging brain: the case of Alzheimer's dementia

Alzheimer's disease (AD) is the most common type of disturbance in the human aging brain. That previous evidence from animal models suggested that IGF-I could be of therapeutic use in AD (Carro et al., 2002, 2006), while more recent observations indicate that inhibition of IGF-I could equally be beneficial in AD (Cohen et al., 2009; Freude et al., 2009; Killick et al., 2009) exemplifies the confusing situation of the field (Fig. 1). Presently, these opposing views are difficult to reconcile. Thus, administration of either IGF-I receptor antagonists, currently in clinical trials for various cancers (Tao et al., 2007), or administration of IGF-I or its mimetics, would both seem a logical pursuit for treatment of AD based on these disparate observations. However, fueled by these apparently irreconcilable views, new research will hopefully lead to a better understanding of the significance of this growth factor in AD and in brain aging in general. Until new data becomes available several issues merit further discussion.

The first intriguing fact is that the controversy arises from data gathered using differing experimental approaches. Detrimental actions of IGF-I in the brain have been found after genetic ablation of either the IGF-I receptor or its downstream docking protein IRS-2. Conversely, all the beneficial actions of IGF-I have been reported after administration of the peptide to various animal models. Maybe the different approaches used explain the opposite results. While the underlying assumption of reducing IGF-I receptor is that the biological action of this pathway will be consequently reduced in the brain, and that administration of IGF-I

Cognitive recovery

IGF-I receptor $^{+/-}$

Systemic IGF-I
IRS-2$^{-/-}$
Neuronal IGF-I receptor$^{-/-}$

Cognitive recovery

Fig. 1. Cognitive deficits in animal models of Alzheimer's amyloidosis are corrected by manipulation of IIS signalling in differing and even opposite ways. Subcutaneous administration of IGF-I to transgenic mice overexpressing Aβ peptides reduces plaque load, gliosis and restores cognition (Carro et al., 2006). Similar results are obtained after crossing IRS-2 knockouts with Aβ-overproducing mice, although in this case animals displayed aberrant tau phosphorylation (Killick et al., 2009; Freude et al., 2009). Cross-breeding of Aβ-overproducing mice with neuronal IGF-I receptor knockouts also reduces plaque load and improves cognition (Freude et al., 2009). When IGF-I receptor heterozygous knockouts are bred with Aβ-overproducing mice, plaque size is increased but cognition is restored and neuronal loss mitigated (Cohen et al., 2009).

will lead to the opposite effect, the authors of the different studies did not provide direct evidence that the intended changes were taking place. More importantly, other unaccounted changes may occur, particularly when using knockout mice where the targeted deletion may produce pleiotropic effects along development. Another intriguing point is that both IRS-2 and neuronal IGF-I receptor knockouts present cognitive disturbances (unpublished observations) while after crossing them with Aβ-producing mice, cognitive disturbances present in both strains appear corrected.

An attempt to reconcile both observations was to suggest that increasing IGF-I levels will result in downregulation of brain IGF-I signaling (Cohen et al., 2009). This idea has not yet been tested, but the absence of neuronal IGF-I receptors is accompanied by increased serum IGF-I levels in mutant mice (Kappeler et al., 2008), suggesting that an opposite relationship between brain IGF-I receptors and serum IGF-I may exist. If this is the case, then low serum IGF-I levels, as seen in aged individuals, would result in higher IGF-I receptor function in the aging brain. However, IGF-I receptor function is reduced in the aging brain even though serum IGF-I levels are also reduced (Muller et al., submitted). This is not surprising as both IGF-I deficiency and resistance to IGF-I are associated to aging in other tissues (Trejo et al., 2004b). Thus, available evidence suggest that this apparent opposite relationship between serum IGF-I levels and brain IGF-I receptors is not always present. Furthermore, reduced serum IGF-I levels do not result in reduced brain IGF-I levels (Adams et al., 2009), suggesting that both compartments are regulated independently. It is clear then that the relationship between systemic and brain IGF-I function is complex, forbidding any preconceptions. Hence, experimental approaches aimed to either increase or decrease brain IGF-I function need to firmly establish that the intended changed is obtained. Namely, when either reducing IGF-I receptors/downstream molecules or administering IGF-I, brain IGF-I function needs to be assessed from a functional perspective, such as measuring Akt activation, a canonical pathway downstream of the IGF-I receptor.

Other non-exclusive possibilities may be envisaged. Thus, it is possible, as recently documented for BDNF (Ji et al., 2010), that distinct cellular responses to IGF-I may be triggered depending on the way this pathway is activated. That is, chronic reduction of IGF-I signalling in IGF-I receptor mutants or IRS-2 knockouts may not necessarily lead to reduced IGF-I activity as we currently know from acute exposure of cells to this growth factor. Rather, a distinct, an as yet unknown type of cellular response to prolonged activation of this pathway may be disrupted. Furthermore, administration of IGF-I may elicit cell/tissue responses that are not necessarily the opposite of those seen in heterozygous IGF-I receptor KOs, as recently suggested (Cohen et al., 2009). Thus, reduction/deletion of brain IGF-I receptors may elicit compensatory responses in related systems (i.e.: brain insulin receptors) or address other functions of this receptor that are currently poorly characterized, such as its role in APP metabolism.

5. Conclusions

In the face of declining IGF-I function with age, administration of IGF-I has proven widely beneficial to the aging brain. However, a wealth of evidence indicates that IGF-I is deleterious for aging. In addition, the neuroprotective effects of IGF-I have also been challenged in relation to Alzheimer's disease. The current controversy witnesses our incomplete understanding of the role of insulin and insulin-like peptides in the aging brain and emphasizes the urgent need to undertake new directions in the field.

Acknowledgements

References

Adams, M.M., Elizabeth, F.M., Constance, L.M., Riddle, D.R., Sonntag, W.E., Brunso-Bechtold, J.K., 2009. Stability of local brain levels of insulin-like growth factor-I in two well-characterized models of decreased plasma IGF-I. Growth Factors 1.

Aleman, A., Torres-Aleman, I., 2009. Circulating insulin-like growth factor I and cognitive function: neuromodulation throughout the lifespan. Prog. Neurobiol. 89, 256–265.

Aleman, A., Verhaar, H.J., de Haan, E.H., de Vries, W.R., Samson, M.M., Drent, M.L., van der Veen, E.A., Koppeschaar, H.P., 1999. Insulin-like growth factor-I and cognitive function in healthy older men. J. Clin. Endocrinol. Metab. 84, 471–475.

Baker, J., Liu, J.P., Robertson, E.J., Efstratiadis, A., 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75, 73–82.

Bolos, M., Fernandez, S., Torres-Aleman, I., 2010. Oral administration of a GSK3 inhibitor increases brain insulin-like growth factor-I levels. J. Biol. Chem. 285, 17693–17700.

Bondy, C.A., Cheng, C.M., 2002. Insulin-like growth factor-1 promotes neuronal glucose utilization during brain development and repair processes. Int. Rev. Neurobiol. 51, 189–217.

Butovsky, O., Koronyo-Hamaoui, M., Kunis, G., Ophir, E., Landa, G., Cohen, H., Schwartz, M., 2006. Glatiramer acetate fights against Alzheimer's disease by inducing dendritic-like microglia expressing insulin-like growth factor 1. Proc. Natl Acad. Sci. USA 103, 11784–11789.

Carro, E., Torres-Aleman, I., 2004. The role of insulin and insulin-like growth factor I in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. Eur. J. Pharmacol. 490, 127–133.

Carro, E., Trejo, J.L., Gomez-Isla, T., LeRoith, D., Torres-Aleman, I., 2002. Serum insulin-like growth factor I regulates brain amyloid-beta levels. Nat. Med. 8, 1390–1397.

Carro, E., Trejo, J.L., Gerber, A., Loetscher, H., Torrado, J., Metzger, F., Torres-Aleman, I., 2006. Therapeutic actions of insulin-like growth factor I on APP/PS2 mice with severe brain amyloidosis. Neurobiol. Aging 27, 1250–1257.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., Partridge, L., 2001. Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science 292, 104–106.

Cohen, E., Paulsson, J.F., Blinder, P., Burstyn-Cohen, T., Du, D., Estepa, G., Adame, A., Pham, H.M., Holzenberger, M., Kelly, J.W., Masliah, E., Dillin, A., 2009. Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. Cell 139, 1157–1169.

D'Ercole, A.J., Ye, P., Calikoglu, A.S., Gutierrez-Ospina, G., 1996. The role of the insulin-like growth factors in the central nervous system. Mol. Neurobiol. 13, 227–255.

Freude, S., Hettich, M.M., Schumann, C., Stohr, O., Koch, L., Kohler, C., Udelhoven, M., Leeser, U., Muller, M., Kubota, N., Kadowaki, T., Krone, W., Schroder, H., Bruning, J.C., Schubert, M., 2009. Neuronal IGF-1 resistance reduces A{beta} accumulation and protects against premature death in a model of Alzheimer's disease. FASEB J. 23, 3315.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P.C., Cervera, P., Le Bouc, Y., 2003. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421, 182–187.

Ji, Y., Lu, Y., Yang, F., Shen, W., Tang, T.T.-T., Feng, L., Duan, S., Lu, B., 2010. Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons. Nat. Neurosci. 13, 302–309.

Kappeler, L., De Magalhaes Filho, C.M., Dupont, J., Leneuve, P., Cervera, P., Perin, L., Loudes, C., Blaise, A., Klein, R., Epelbaum, J., Le, B.Y., Holzenberger, M., 2008. Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism. PLoS Biol. 6, e254.

Kenyon, C., 2001. A conserved regulatory system for aging. Cell 105, 165–168.

Killick, R., Scales, G., Leroy, K., Causevic, M., Hooper, C., Irvine, E.E., Choudhury, A.I., Drinkwater, L., Kerr, F., Al-Qassab, H., Stephenson, J., Yilmaz, Z., Giese, K.P., Brion, J.P., Withers, D.J., Lovestone, S., 2009. Deletion of Irs2 reduces amyloid deposition and rescues behavioural deficits in APP transgenic mice. Biochem. Biophys. Res. Commun. 386, 257–262.

Lanz, T.A., Salatto, C.T., Semproni, A.R., Marconi, M., Brown, T.M., Richter, K.E., Schmidt, K., Nelson, F.R., Schachter, J.B., 2008. Peripheral elevation of IGF-1 fails to alter Abeta clearance in multiple in vivo models. Biochem. Pharmacol. 75, 1093–1103.

LeRoith, D., Roberts Jr., C.T., 2003. The insulin-like growth factor system and cancer. Cancer Lett. 195, 127–137.

Markowska, A.L., Mooney, M., Sonntag, W.E., 1998. Insulin-like growth factor-1 ameliorates age-related behavioral deficits. Neuroscience 87, 559–569.

Puche, J.E., Garcia-Fernandez, M., Muntane, J., Rioja, J., Gonzalez-Baron, S., Castilla-Cortazar, I., 2008. Low doses of Insulin-like growth factor I induce mitochondrial protection in aging rats. Endocrinology 149, 2620–2627.

Ray, S., Britschgi, M., Herbert, C., Takeda-Uchimura, Y., Boxer, A., Blennow, K., Friedman, L.F., Galasko, D.R., Jutel, M., Karydas, A., Kaye, J.A., Leszek, J., Miller, B.L., Minthon, L., Quinn, J.F., Rabinovici, G.D., Robinson, W.H., Sabbagh, M.N., So, Y.T., Sparks, D.L., Tabaton, M., Tinklenberg, J., Yesavage, J.A., Tibshirani, R., Wyss-Coray, T., 2007. Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. Nat. Med. 13, 1359–1362.

Sevigny, J.J., Ryan, J.M., van Dyck, C.H., Peng, Y., Lines, C.R., Nessly, M.L., 2008. Growth hormone secretagogue MK-677: no clinical effect on AD progression in a randomized trial. Neurology 71, 1702–1708.

Sonntag, W.E., Lynch, C.D., Cooney, P.T., Hutchins, P.M., 1997. Decreases in cerebral microvasculature with age are associated with the decline in growth hormone and insulin-like growth factor 1. Endocrinology 138, 3515–3520.

Sonntag, W.E., Bennett, S.A., Khan, A.S., Thornton, P.L., Xu, X., Ingram, R.L., Brunso-Bechtold, J.K., 2000. Age and insulin-like growth factor-1 modulate N-methyl-D-aspartate receptor subtype expression in rats. Brain Res. Bull. 51, 331–338.

Suh, Y., Atzmon, G., Cho, M.O., Hwang, D., Liu, B., Leahy, D.J., Barzilai, N., Cohen, P., 2008. Functionally significant insulin-like growth factor I receptor mutations in centenarians. PNAS 105, 3438–3442.

Tao, Y., Pinzi, V., Bourhis, J., Deutsch, E., 2007. Mechanisms of disease: signaling of the insulin-like growth factor 1 receptor pathway—therapeutic perspectives in cancer. Nat. Clin. Pract. Oncol. 4, 591–602.

Tei, E., Yamamoto, H., Watanabe, T., Miyazaki, A., Nakadate, T., Kato, N., Mimura, M., 2008. Use of serum insulin-like growth factor-I levels to predict psychiatric non-response to donepezil in patients with Alzheimer's disease. Growth Horm. IGF Res. 18, 47–54.

Torres-Aleman, I., 2007. Targeting insulin-like growth factor-1 to treat Alzheimer's disease. Expert Opin. Ther. Targets 11, 1535–1542.

Torres-Aleman, I., 2010. Toward a comprehensive neurobiology of IGF-I. Dev. Neurobiol. 70, 384–396.

Trejo, J.L., Carro, E., Garcia-Galloway, E., Torres-Aleman, I., 2004a. Role of insulin-like growth factor I signaling in neurodegenerative diseases. J. Mol. Med. 82, 156–162.

Trejo, J.L., Carro, E., Lopez-Lopez, C., Torres-Aleman, I., 2004b. Role of serum insulin-like growth factor I in mammalian brain aging. Growth Horm. IGF Res. 14, 39–43 Suppl A.

Trejo, J., Piriz, J., Llorens-Martin, M.V., Fernandez, A.M., Bolos, M., LeRoith, D., Nunez, A., Torres-Aleman, I., 2007. Central actions of liver-derived insulin-like growth factor I underlying its pro-cognitive effects. Mol. Psychiatry 12, 1118–1128.

Vardy, E.R., Rice, P.J., Bowie, P.C., Holmes, J.D., Grant, P.J., Hooper, N.M., 2007. Increased circulating insulin-like growth factor-1 in late-onset Alzheimer's disease. J. Alzheimers Dis. 12, 285–290.

Vicario-Abejon, C., Yusta-Boyo, M.J., Fernandez-Moreno, C., de Pablo, F., 2003. Locally born olfactory bulb stem cells proliferate in response to insulin-related factors and require endogenous insulin-like growth factor-I for differentiation into neurons and glia. J. Neurosci. 23, 895–906.

Watanabe, T., Miyazaki, A., Katagiri, T., Yamamoto, H., Idei, T., Iguchi, T., 2005. Relationship between serum insulin-like growth factor-1 levels and Alzheimer's disease and vascular dementia. J. Am. Geriatr. Soc. 53, 1748–1753.

Yamagata, B., Watanabe, T., Tomioka, H., Kobayashi, H., Nakano, Y., Mimura, M., 2010. Preliminary use of insulin-like growth factor-I as a biomarker for sorting high-dose donepezil responders among Japanese patients with Alzheimer's disease. Regul. Pept. 163, 137–142.