UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: NEUROCIÊNCIAS

AVALIAÇÃO DOS EFEITOS DO EXERCÍCIO FÍSICO SOBRE AS CÉLULAS ASTROCITÁRIAS E A BARREIRA HEMATOENCEFÁLICA NO HIPOCAMPO E NO ESTRIADO, E SOBRE O COMPORTAMENTO COGNITIVO E MOTOR EM RATOS DIABÉTICOS.

Tese de Doutorado

Priscylla Nunes de Senna

Porto Alegre
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Priscylla Nunes de Senna

Orientadora Profa. Dra. Matilde Achaval

Tese apresentada ao Programa de Pós-Graduação em Neurociências, da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Doutor em Neurociências.

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RESUMO

O diabetes mellitus tipo 1 (DM1) é associado ao comprometimento cognitivo, sendo capaz de induzir morte neuronal, de alterar o número e a morfologia das células astrocitárias, de aumentar a permeabilidade da barreira hematoencefálica (BBB) e de promover dano neurovascular. O exercício físico, por outro lado, possui efeitos benéficos sobre o sistema nervoso central (SNC), induzindo plasticidade e melhorando o processo de aprendizagem, de memória e a atividade locomotora.

Em nosso primeiro estudo, investigamos os efeitos de cinco semanas de exercício aeróbico sobre a prevenção ou reversão do déficit de memória espacial produzido pelo diabetes, e sobre alterações bioquímicas e imuno-histoquímicas de astrócitos no hipocampo, em ratos machos *Wistar* com DM1 induzido por estreptozotocina. Foram utilizados quatro grupos: controle não treinado (NTC); controle treinado (TC); diabético não treinado (NTD); e diabético treinado (TD). Neste estudo demonstramos que o exercício reverteu o dano na memória espacial causado pelo DM1, aumentou os níveis de glutationa (GSH) e de glutamina sintetase (GS) nos animais TC, mas não no grupo TD; aumentou a densidade de astrócitos GFAP positivos nos grupos TC e TD, e promoveu ramificação astrocitária no grupo TD. Não observamos nenhuma alteração na captação de glicose e de glutamato entre todos os grupos.

No segundo estudo, com os mesmos grupos experimentais, avaliamos os efeitos do exercício sobre a memória de reconhecimento do novo objeto, sobre o comportamento motor no rotarod, e sobre a expressão das proteínas claudina-5 e aquaporina-4 (AQP4), proteínas associadas à integridade da BBB, no hipocampo e no estriado de animais diabéticos. Observamos que o exercício melhorou a memória não espacial e a habilidade motora nos animas TC e TD. O diabetes produziu uma diminuição da expressão da claudina-5 no hipocampo e no estriado, e reduziu os níveis de AQP4 hipocampal. O exercício preservou os níveis de claudina-5 no estriado dos animais TD, mas não no hipocampo, e não alterou os níveis reduzidos de AQP4 hipocampal produzidos pelo diabetes.

Nossos resultados indicam que o exercício físico reverte o déficit de memória espacial induzido pelo DM1, melhora a memória de reconhecimento do novo objeto, e a atividade motora, induz importantes alterações morfológicas nas células astrocitárias, e afeta importantes componentes da BBB no estriado. Nossos dados contribuem para a compreensão dos benefícios comportamentais, motores, histofisiológicos e neuroquímicos do exercício físico no DM1.

ABSTRACT

Type 1 diabetes mellitus (T1DM) affects cognitive domains, induces neuronal death, changes in the number and in morphology of astrocytes, increases blood brain barrier (BBB) permeability and promotes neurovascular impairment. Physical exercise, on the other hand, has beneficial effects on brain functions, inducing brain plasticity and improving learning, memory and locomotor activity.

In our first study, we investigated the effects of five weeks of aerobic exercise to prevent or reverse spatial memory deficits produced by diabetes and some biochemical and immunohistochemical changes in hippocampal astrocytes of streptozotocin-induced T1DM in male Wistar rats. The rats were divided in four groups: trained control (TC), non-trained control (NTC), trained diabetic (TD) and non-trained diabetic (NTD). In this study, we demonstrated that exercise reversed spatial memory impairments generated by T1DM, increased glutathione (GSH) and glutamine synthetase (GS) levels in TC animals, but not in TD rats; increased the density of GFAP immunoreactive astrocyte in TC and TD groups, and increased astrocyte ramification in TD group. Glucose and glutamate uptake were not affected.

In the second study, using the same experimental groups, we evaluated the effects of exercise on novel object recognition task, on motor behavior in rotarod test, and on the expression of proteins related to BBB integrity, such as claudin-5 and aquaporin-4 (AQP4) in the hippocampus and striatum in diabetic rats. We showed that exercise enhanced the non-spatial memory performance and rotarod ability in the TC and TD animals. Diabetes produced a decrease in claudin-5 expression in the hippocampus and striatum and reduced AQP4 in the hippocampus. Physical exercise preserved the claudin-5 content in the striatum of TD rats, but not in the hippocampus, and the reduction of AQP4 levels produced by diabetes was not reversed by exercise.

Our findings indicate that physical exercise reverses the cognitive deficits present in T1DM, improves novel object recognition task retention, enhances motor performance, induces important morphological astrocytic changes, and affects important structural components of the striatal BBB. Our date could enhance the knowledge regarding the behavioral, locomotor, histophysiological and neurochemical benefits of exercise in diabetes.

LISTA DE ABREVIATURAS

AGE´s.....do inglês *Advanced glycation end-product*; produtos finais de glicação avançada

AOIs……do inglês *Areas of interest;* áreas de interesse

AQPs.....Aquaporinas

AQP4.....Aquaporina-4

BBB.....do inglês *Blood brain barrier*; barreira hematoencefálica

BDNF…….do inglês *Brain-derived neurotrophic factor;* fator neurotrófico derivado do encéfalo

BHR.....Barreira hemato-retiniana

CA.....Corno de Ammon

DM1.....Diabetes Mellitus Tipo 1

EAAT1/GLAST.....Transportador de aminoácido excitatório 1

EAAT2/GLT-1.....Transportador de aminoácido excitatório 2

EGF……do inglês *Epidermal growth factor*; fator crescimento epidérmico

FGF……do inglês *Fibroblast growth factor*; fator de crescimento fibroblástico

GABA.....Ácido gama-amino butírico

GFAP.....do inglês *Glial fibrillary acidic protein;* proteína glial fibrilar ácida

GLU.....Glutamato

GLUT2.....do inglês *Glucose transporter type 2*; transportador de glicose do tipo 2

GS.....do inglês *Glutamine synthetase*; glutamina sintetase

GSH.....do inglês *Gluthatione*; glutationa

HLA.....Antígeno leucocitário humano

IL-1.....Interleucina-1

LTP.....do inglês *Long term potention*; potencial de longa duração

MET….....do inglês *Maximal exercise test*; teste de esforço máximo

MMP .....do inglês *Matrix metalloproteinases*; metaloproteinases de matriz

NMDA……...N-metil-D-aspartato

NOPR.....do inglês *Novel object-placement recognition task*; tarefa de reconhecimento do objeto reposicionado

NOR……do inglês *Novel object recognition task*; tarefa de reconhecimento do novo objeto

NTC…….……....do inglês *Non-trained control*; controle não treinado

NTD……do inglês *Non-trained diabetic*; diabético não treinado

PKC.....Proteína quinase C

ROS.....do inglês *Reactive oxygen species*; espécies reativas de oxigênio

SNC.....Sistema nervoso central

STZ.....do inglês *Streptozotocin*; estreptozotocina

T1DM……...….....do inglês Type 1 diabetes mellitus; diabetes mellitus tipo 1

TC…….…….....do inglês *Trained control*; controle treinado

TD……...…….....do inglês *Trained diabetic*; diabético treinado

TJ.....do inglês *Tight junction*; junções de oclusão

TNF.....do inglês *Tumoral necrosis factor*; fator de necrose tumoral

VEGF.....do inglês *Vascular endothelial growth fator*; fator de crescimento do endotélio vascular

ZO.....Zônulas de oclusão

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INTRODUÇÃO

1. Diabetes Mellitus Tipo 1

O diabetes mellitus tipo 1 (DM1) é definido como uma síndrome metabólica caracterizada pelo comprometimento do metabolismo da glicose e de outros substratos produtores de energia, decorrente da produção deficiente do hormônio insulina, mediante a destruição das células β pancreáticas. É considerado um problema de saúde pública prevalente, em ascendência e oneroso do ponto de vista social e econômico (CRAIG et al., 2009).

A sua prevalência mundial não é exata, mas somente nos Estados Unidos estima-se um total de 3 milhões de indivíduos com DM1. Anualmente, 78 mil jovens são diagnosticados com DM1 no mundo, com a maior incidência na Finlândia (superior a 64/100.000/ano), seguida por populações caucasianas na Europa e na América do Norte (cerca de 10-20/100.000/ano; CHIANG et al., 2014). No Brasil, a incidência é de 8/100.000/ano (KARVONEM et al., 2000).

O custo anual direto (medicamentos, insulina, hemoglicoteste) com a doença soma no Brasil mais de 4 milhões de dólares, com um orçamento de US$ 1.319,15 por indivíduo. Os custos aumentam com o tempo de duração do diabetes, duplicando após 15 anos de diagnóstico que, integrados ao custo indireto (absenteísmo, presenteísmo), exercem importante impacto econômico à sociedade (COBAS et al., 2013; TAO; TAYLOR, 2010).

O diagnóstico clínico do DM1 baseia-se na apresentação de polidipsia, polifagia, poliúria e perda de massa corporal. A sua confirmação ocorre laboratorialmente pela realização da dosagem de hemoglobina glicada (valores ≥ 6,5%), ou pela combinação dos exames "glicemia de jejum" e "teste oral de tolerância à glicose, cujos níveis maiores que 126 mg/dL e 200 mg/dL, respectivamente, indicam a presença do diabetes (*American Diabetes Association*, 2014).

Múltiplos genes estão envolvidos na etiopatogênese do DM1, sendo os genes do sistema antígeno leucocitário humano (HLA) de classe II, no braço curto do cromossomo 6, os principais determinantes à suscetibilidade à doença. Os lócus DR e DQ são responsáveis por 40% a 50% do risco genético de desenvolver o DM1, com os alelos DR*03 ou DR*04 os mais frequentes nos pacientes diabéticos (Figura 1; CONCANNON et al., 2009).

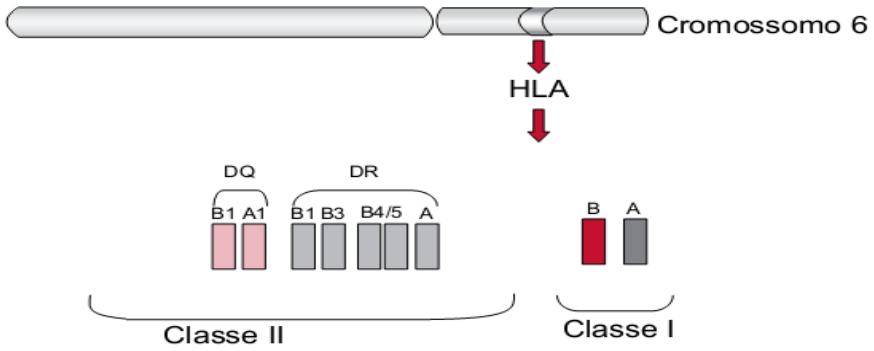

Figura 1. Representação dos principais haplótipos do sistema HLA de classe II e classe I no braço curto do cromossomo 6 associados à suscetibilidade genética do DM1 (modificado de SILVA et al., 2008).

A suscetibilidade genética, contudo, não é suficiente para desencadear o processo autoimune de destruição das células β. Um fator ambiental parece ser necessário para desengatilhar o processo, e as evidências mostram que infecções virais por enterovírus, rotavirus, citomegalovírus, paramyxovirus e rubivirus são os principais candidatos virais que determinariam o início do evento autoimune. O enterovírus, particularmente, possui tropismo pelo pâncreas, e acredita-se que ative linfócitos T a partir da liberação de antígenos por células β lisadas ou pela expressão de antígenos virais na superfície das células β. Outros fatores, como elementos da dieta alimentar – vitamina D e leite de vaca – são apontados como possíveis determinantes para o evento autoimune; contudo, os estudos ainda são limitados (VAN DER WERF, 2007; VIRTANEN, KNIP, 2003).

No que se refere aos mecanismos de destruição das células β pancreáticas, incluem-se: reações de hipersensibilidade do tipo tardio, mediada por linfócitos T "helper" $CD4^+$ (T_H1 $CD4^+$) reativos a antígenos das ilhotas pancreáticas; lise das células β mediada por linfócitos T citolítico; produção local de citocinas, como fator de necrose tumoral (TNF) e interleucina-1 (IL-1); e autoanticorpos contra autoantígenos das células β. Ao início dos sintomas clínicos no DM1, 70 a 90% das células β já se encontram destruídas (CABRERA et al., 2015).

Cronicamente, o DM1 causa uma série de complicações neuropáticas e vasculares, como neuropatias motora e autonômica, retinopatia, nefropatia, doença cardiovascular, doença cerebrovascular, com prejuízo cognitivo e risco à demência (PIROLA et al., 2010; GRZEDA; WISNIEWSKA, 2008; MCCARTHY et al., 2002). O

controle glicêmico, deste modo, a insulinoterapia, dieta controlada e realização de exercícios físicos são essenciais para a prevenção e o tratamento das comorbidades (*American Diabetes Association*, 2014).

1.1 Encefalopatia Diabética

As primeiras notificações da presença de déficits cognitivos em pacientes DM1 datam de 1922 (MILES, ROOT, 1922). O conjunto destes déficits, mesmo que modestos, juntamente com alterações estruturas do sistema nervoso central (SNC), tem sido denominado de encefalopatia diabética (SIMA, 2010).

O início precoce do DM1 é forte preditor do grau dos prejuízos cognitivos na infância, na fase jovem e adulta dos indivíduos com a doença (BIESSELS et al., 2013; FERGUSON et al., 2005). Crianças com diagnóstico precoce tendem a apresentar menor desempenho nas habilidades escolares e verbais (ler, soletrar, contar) quando comparadas a crianças saudáveis (HANNONEN et al., 2010; NAGUIB et al., 2009). A menor capacidade de atenção, a menor velocidade de processamento das informações e a pior função executiva na infância (BIESSELS et al., 2008; SIMA et al., 2010), resultam em notas escolares mais baixas e menores quocientes de inteligência (QI) - ainda que discretos em alguns casos - em relação a crianças sem o diagnóstico (GAUDIERI et al., 2008; HANNONEN et al., 2012; NORTHAM et al., 2009). Outros domínios cognitivos, como inteligência, atenção, percepção visual, são afetados na idade jovem e adulta, podendo comprometer o desempenho acadêmico e o sucesso profissional (JONER, 2013; MEO et al., 2013; MCCRIMMON et al., 2012; PERSSON et al.,2013).

Exames de imagem mostram alterações neuroanatômicas nos pacientes com DM1 correlacionadas a alterações cognitivas. São relatados em adultos diabéticos quando comparado a não diabéticos: aumento do tamanho dos ventrículos; atrofia cortical; diminuição da densidade da substância cinzenta no tálamo, no hipocampo, no córtex insular, e nos giros temporal superior esquerdo e parahipocampal direito; diminuição do volume da substância branca na área pré-frontal (BIESSELS, REIJMER, 2014; MUSEN et al., 2006; PERANTIE et al., 2007).

A suscetibilidade aos danos estruturais e ao prejuízo cognitivo é atribuída, sobretudo, a dois períodos cruciais da vida humana: (1) a infância, na qual ocorre o

desenvolvimento do SNC, e (2) a velhice, com a presença de alterações neurodegenerativas (BIESSELS et al., 2008). Além da hiperglicemia, os picos de hipoglicemia decorrentes à insulinoterapia, somados a comorbidades como hipertensão arterial sistêmica, dislipidemias, contribuem para o risco da encefalopatia diabética (Figura 2). Deste modo, devido aos diferentes fatores que influenciam a neurofisiopatologia do DM1, o déficit cognitivo pode estar presente em apenas parte dos indivíduos, e em alguns destes os déficits não progridem acentuadamente ao longo dos anos (BRANDS et al., 2005; BIESSELS et al., 2013).

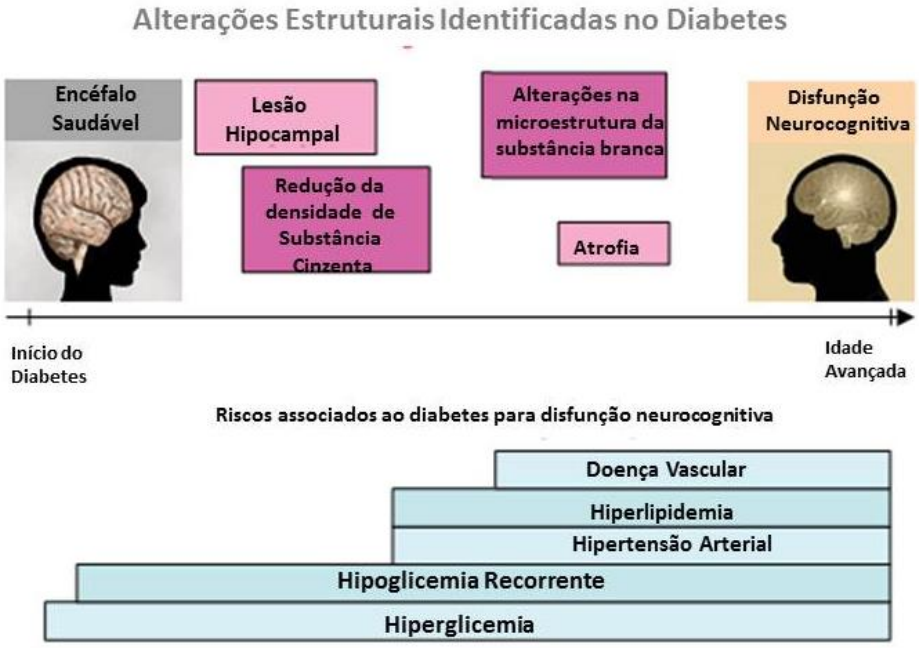

Figura 2: Representação esquemática dos dois períodos mais críticos (infância e envelhecimento) para a suscetibilidade dos danos estruturais e cognitivos no DM1 (modificado de SEAQUIST, 2010).

Nos modelos experimentais de DM1, o déficit de memória e aprendizado é observado nas tarefas do labirinto aquático de Morris – *Water Maze* –, e de Reconhecimento de Objeto Reposicionado (NOPR), por exemplo. Os animais diabéticos apresentam maior latência e maior deslocamento para alcançar a plataforma alvo no teste de *Water Maze* (GRZĘDA; WIŚNIEWSKA, 2008; TUZCU; BAYDAS, 2006), enquanto no teste do NOPR despendem um menor tempo na exploração do

objeto reposicionado (DE SENNA et al., 2011; PIAZZA et al., 2010; REVSIN et al., 2009).

No SNC destes animais são encontradas alterações morfológicas induzidas pelo diabetes. No hipocampo ocorre uma diminuição de espinhos dendritos dos neurônios piramidais, diminuição da neurogênese, da proliferação celular e apoptose neural, diminuição da expressão da proteína glial fibrilar ácida ([GFAP]; (ALVAREZ et al., 2009; BEAUQUIS et al., 2006; LI et al., 2002; MALONE et al., 2008; DE SENNA et al., 2011).

Apesar de os mecanismos responsáveis pela encefalopatia diabética serem complexos e ainda não totalmente compreendidos, estes são, em sua maior parte, mediados pela hiperglicemia. A hiperglicemia aumenta o fluxo da (1) via do poliol, (2) da via da hexosamina, induz a (3) formação de produtos finais de glicação avançada (AGE´s) e (4) o excesso ou ativação inadequada de isoformas da proteína quinase C (PKC), resultando em dano celular e vascular (BROWNLEE, 2005; MOORE et al., 2009). O dano microvascular causa perda da integridade da barreira hematoencefálica (BBB), reduz o metabolismo cerebral, contribuindo para a disfunção glial e neuronal (Figura 3). Além disso, com o aumento da produção de espécies reativas de oxigênio (ROS), ocorre uma redução da produção de óxido nítrico e prostaciclina, acometendo a regulação do tônus vascular e, consequentemente, prejudicando a regulação do fluxo sanguíneo encefálico (BRANDS et al., 2004; ERGUL et al., 2012; MANSCHOT et al., 2003; PRASAD et al., 2014).

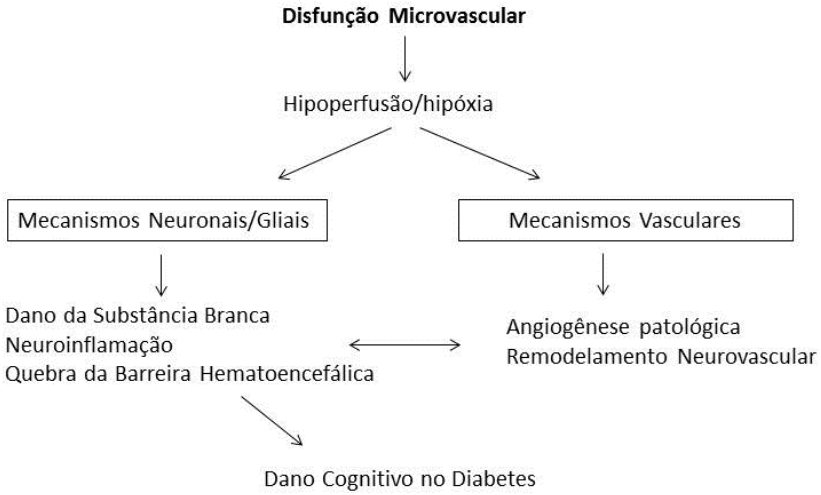

Figura 3: Representação esquemática do dano cognitivo induzido pela hiperglicemia no DM1 (adaptado de ERGUL et al, 2012).

1.2 Modelo Experimental de Diabetes Mellitus Tipo 1

O modelo animal de DM1 induzido pela estreptozotocina (STZ) tem sido amplamente utilizado para os estudos experimentais. A STZ é um antibiótico derivado da *Streptomycetes achromogene.* Possuindo afinidade pelos transportadores de glicose do tipo 2 (GLUT2), a STZ é transportada pela membrana plasmática das células β pancreáticas, agindo toxicamente sobre elas por meio da metilação de DNA, e pela formação de espécies reativas de oxigênio (LENZEN, 2008).

A administração intravenosa da droga via veia caudal é a forma mais utilizada pelo menor risco de mortalidade dos animais e pela maior confiabilidade de seu efeito diabetogênico, embora a mesma severidade do diabetes possa ser atingida via veia sublingual ou injeção intraperitoneal de STZ (DELFINO et al., 2002). Doses únicas de STZ, entre 40 a 60 mg/kg da massa corporal, são o suficientes para indução de hiperglicemia, hiperosmolaridade plasmática, hipoinsulinemia, hiperfagia, polidipsia e perda de massa corporal que caracterizam o estado diabético (SERINO et al., 1998).

2. Astrócitos

Os astrócitos constituem o tipo de células da glia mais abundante do SNC (ARAQUE; NAVARRETE, 2010). Possuem um corpo celular de formato estrelado, de onde partem prolongamentos astrocíticos curtos e ramificados, ou longos e pouco ramificados, conforme o subtipo protoplasmático ou fibroso, respectivamente (SOFRONIEW; VINTERS, 2010).

As células astrocitárias são essenciais para homeostase, plasticidade cerebral, transmissão sináptica, estando em contato com as células neuronais e com os vasos sanguíneos cerebrais. Dentre as suas funções, destacam-se o papel destas células sobre: metabolismo de neurotransmissores; metabolismo energético; facilitação do potencial de longa duração (LTP); manutenção da BBB; tamponamento do meio extracelular, seja pela remoção direta de íons e de neurotransmissores da fenda sináptica, seja por comporem a unidade neurovascular; produção e modulação de fatores tróficos, regulando proliferação, diferenciação e sobrevivência das células neuronais e gliais; orientação do processo de migração dos neurônios durante o desenvolvimento do SNC e

controle da microcirculação cerebral (GIBBS et al., 2008; HAMM et al., 2004; HENNEBERGER et al., 2010; PELLERIN, 2005).

2.1 Proteína Glial Fibrilar Ácida

A proteína glial fibrilar ácida (GFAP) é uma proteína de filamentos intermediários do tipo III do citoesqueleto glial, marcadora específica de astrócitos. É considerada como um dos principais antígenos para a identificação e estudo destas células (ENG et al., 2000).

Na presença de um evento neuropatológico ou mediante o processo de neuroplasticidade, os astrócitos tornam-se reativos, uma resposta fisiológica na tentativa de proteger o SNC de agentes infecciosos, de células inflamatórias, e de regular o microambiente sináptico em resposta à atividade neural (PEKNY et al., 2014a; SOFRONIEW, VINTERS, 2010; VIOLA et al., 2009).

A reatividade astroglial envolve alterações no conteúdo de GFAP. O aumento da expressão de GFAP pode indicar proliferação de astrócitos, hipertrofia do corpo celular, aumento do número e/ou do comprimento dos prolongamentos astrocitários. A diminuição de sua expressão, por outro lado, pode indicar morte astrocitária, redução de proliferação ou alteração morfológica com redução do número e/ou do comprimento de sua arborização (SAUR et al., 2013 ; PEKNY, PEKNA, 2004).

Estudos prévios demonstraram que uma diminuição da expressão de GFAP no cerebelo e no hipotálamo de animas diabéticos é causada por uma redução do número de astrócitos e do comprimento dos seus prolongamentos em resposta à hiperglicemia crônica (LECHUGA- SANCHO et al., 2006a, 2006b).

O ambiente enriquecido e o exercício físico, por outro lado, podem induzir o aumento de células GFAP positivas no hipocampo (SAMPEDRO-PIQUERO et al., 2014; SAUR et al., 2013; DE SENNA et al., 2011; VIOLA et al., 2009). Para animais diabéticos submetidos ao exercício físico, ainda não está descrito na literatura se o aumento na expressão de células GFAP positivas está associado ao aumento da densidade ou a alterações no fenótipo destas células.

2.2 Astrócitos e Metabolismo do Glutamato

No metabolismo de neurotransmissores, destaca-se o papel dos astrócitos sobre o metabolismo do glutamato (GLU), principal neurotransmissor excitatório do SNC.

Os astrócitos expressam transportadores de GLU do tipo EAAT-1 (ou GLAST; (transportador excitatório de glutamato 1) e do tipo EAAT-2 (ou GLT-1; transportador de aminoácido excitatório 2), sendo responsáveis por 90% da captação do GLU extracelular, crucial contra a excitotoxidade glutamatérgica. Intracelularmente, o GLU, através da enzima glutamina sintetase (GS), pode ser (1) convertido a glutamina, a qual é liberada pelos astrócitos e captada por neurônios para sua conversão novamente à GLU – ou ainda ao ácido gama-amino butírico (GABA) –, processo denominado de "ciclo glutamina-glutamato" (Figura 4), ou pode ter outros destinos, como (2) desaminado a α-cetoglutarato, produto intermediário do ciclo do ácido carboxílico, e utilizado para síntese de glutationa ([GSH]; SCHOUSBOE et al., 2013)

A GSH, existente em nosso organismo na forma reduzida e oxidada, atua em diferentes processos biológicos (POPE et al., 2008). Na forma reduzida, ela é maior antioxidante do SNC, agindo na detoxicação de espécies reativas de oxigênio de uma forma não enzimática (DRINGEN, 2000).

No DM1, são escassos os estudos sobre o metabolismo do glutamato, com alguns resultados controversos entre estes (AHMED, ZAHRA, 2011; BAYDAS et al., 2005; COLEMAN et al., 2010; DUARTE et al., 2009).

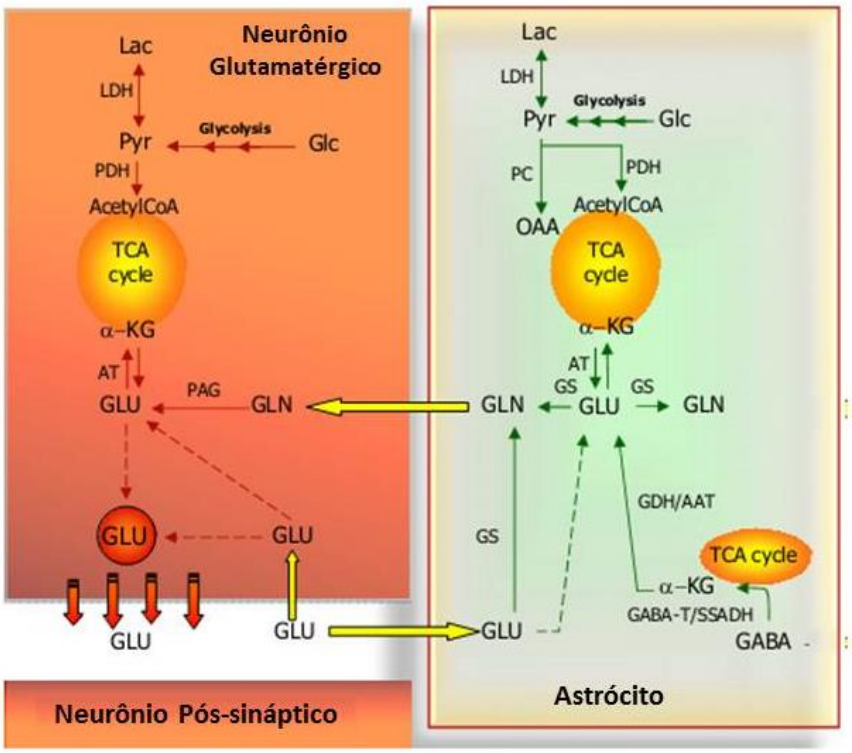

Figura 4: Representação esquemática mostrando o papel dos astrócitos no ciclo glutamina-glutamato. Lac: lactato; Pyr: piruvato; Glc: glicose; Glu: glutamato; α-cetoglutarato; GS: glutamina sintetase; GLN: glutamina (modificado de SCHOUSBOE et al., 2013).

2.3 Astrócitos e Metabolismo Energético

No que se refere ao metabolismo energético, os astrócitos captam glicose, metabolizam-na diretamente a piruvato ou estocam-na sobre a forma de glicogênio. O glicogênio estocado pode ser rapidamente metabolizado e convertido a lactato, importante fonte energética neuronal, ou direcionado para a biossíntese de glutamato. (Figura 5). Os produtos intermediários da via glicolítica podem ser utilizados para biossíntese de aminoácidos importantes como D-serina, co-ativador de receptores N-metil-D-aspartato ([NMDA]; GIBBS et al., 2008; PELLERIN, MAGISTRETTI, 2004).

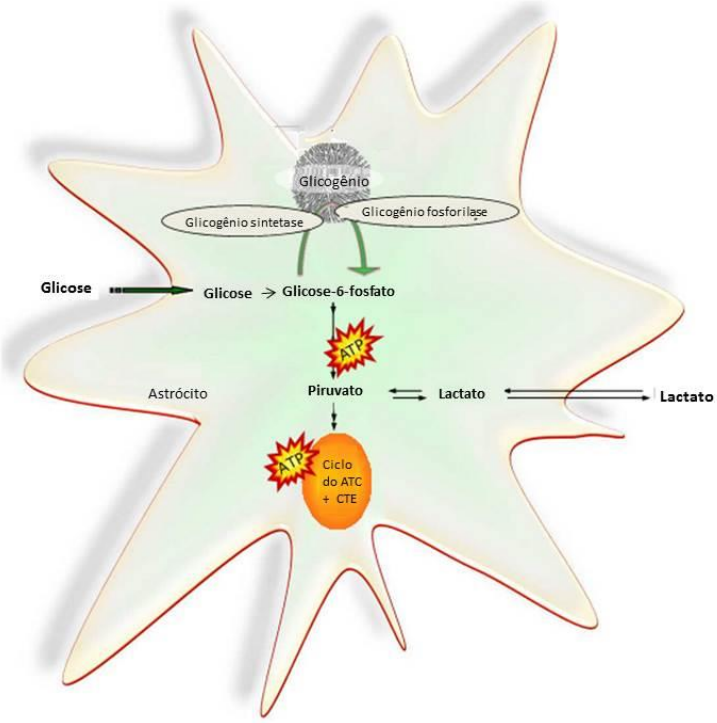

Figura 5: Esquema simplificado do metabolismo energético nos astrócitos mostrando: estoque da glicose na forma de glicogênio; conversão da glicose à piruvato; síntese de lactato. ATC: ácido tricarboxílico; CTE: cadeia transportadora de elétrons (modificado de SCHOUSBOE et al., 2013).

3. Barreira Hematoencefálica

A barreira hematoencefálica (BBB), anatomicamente, é formada pelas células endoteliais dos capilares em associação a componentes perivasculares, como células musculares lisas, pericitos, lâmina basal, que juntamente com astrócitos, neurônios e microglia, constituem a unidade neurovascular (Figura 6; BONKOWSKI et al., 2011).

Funcionalmente, é uma barreira de difusão e proteção do SNC, responsável por isolar o espaço extracelular encefálico de possíveis flutuações plasmáticas provenientes do compartimento extracelular corporal (REDZIC, 2011). Para isto, entre as células endoteliais adjacentes, são encontradas junções de oclusão (TJ), que limitam o fluxo paracelular de solutos, de íons e de água (SANDOVAL, WITT, 2008).

Este isolamento do SNC em relação ao meio plasmático protege as estruturas encefálicas de eventos como excitotoxidade glutamatérgica e apoptose celular. A

restrição entre os sistemas garante que não haja a troca, entre os meios, de neurotransmissor glutamato - cuja concentração plasmática é elevada -, e garante a impermeabilidade a proteínas de alto peso molecular, como albumina, protrombina, plasminogênio, as quais podem induzir dano celular, apoptose, ativação glial e convulsão (ABBOT et al., 2010; GINGRICH, TRAYNELIS, 2000).

A BBB, contudo, não inibe a troca de nutrientes, metabólicos e íons indispensáveis ao SNC entre o sistema vascular e o fluido intersticial cerebral. Através de uma combinação de transportadores e canais iônicos, a barreira permite uma pequena permeabilidade dos elementos essenciais aos tecidos do SNC, como glicose, e mantém uma composição iônica ótima para a sinalização sináptica (ABBOTT et al., 2010). A concentração do fluido intersticial cerebral e do líquido cérebro-espinhal é mantida, assim, em torno de 2,5 a 2,9 mM, divergindo da concentração plasmática (4,5 mM). O pH também é regulado pela BBB (NISCHWITZ et al., 2008; SOMJEN, 2004).

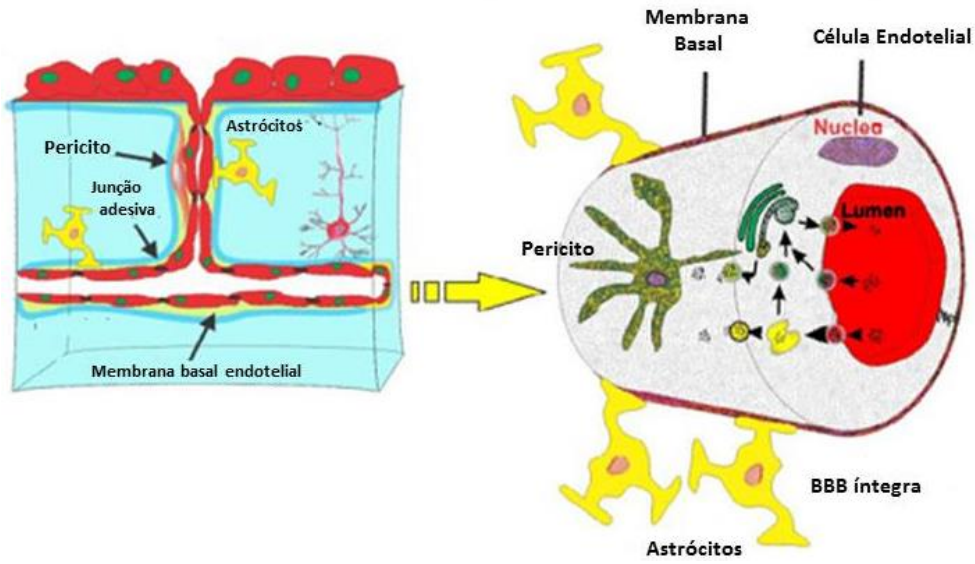

Figura 6: Representação esquemática da barreira hematoencefálica (BBB) e de componentes da unidade neurovascular (modificado de PRASAD et al., 2014).

Muito diferente da disfunção da barreira hemato-retiniana (BHR), bem estabelecida no DM1, a fisiopatologia da disfunção da BBB ainda não é totalmente clara nesta patologia (CUI et al., 2012; CURTIS et al., 2011; IANDIEV et al., 2007).

No diabetes, a permeabilidade microvascular no SNC parece ser mais regional que global e ainda tempo dependente. Inicialmente, em animais com 14 dias de DM1, é encontrado aumento da permeabilidade à sacarose, com as diferenças regionais de permeabilidade surgindo em torno de 28 dias após a indução do diabetes no mesencéfalo e após 56 dias no córtex cerebral e nos núcleos da base (HAWKINS et al., 2007). Para moléculas maiores, como a inulina e a albumina, a permeabilidade parece ocorrer mais tardiamente, sendo observada cerca de duas a três semanas após a indução do modelo também nas regiões de córtex cerebral, mesencéfalo e núcleos da base. No hipocampo, o aumento de permeabilidade é observado somente para pequenas moléculas dentro de um tempo de 56 a 90 dias após a indução do modelo de DM1 (HUBER et al., 2006). O acometimento das junções oclusivas (*tight junction* – TJ), desta forma, tem sido evidenciado como fator importante para a disfunção da BBB no diabetes (CHEHADE et al., 2002).

3.1 Junções Oclusivas

As junções oclusivas (*tight junction* – TJ) constituem a estrutura mais apical da fenda intercelular da BBB, formando uma espécie de vedamento entre as células endoteliais, o que limita o fluxo paracelular de moléculas hidrofílicas (SANDOVAL, WITT, 2008). Em condições fisiológicas, substâncias com peso molecular de 180 Da não ultrapassam as TJ (KIPTOO et al., 2011).

Três proteínas transmembrana compõem as TJ, sendo encontradas em diferentes isoformas e estados de fosforilação conforme o tecido e a atividade regulatória. São elas: proteínas claudina, ocludina e as moléculas de junções adesivas (SANDOVAL, WITT, 2008). Suas regiões citoplasmáticas ancoram-se ao citoesqueleto através de ligações com proteínas intracelulares. Uma redução na expressão destas proteínas juncionais compromete a integridade das TJ, aumentando a permeabilidade paracelular (CORREALE, VILLA, 2009).

3.2 Proteína Claudina-5

As proteínas claudinas constituem um grupo de pelo menos 24 membros, das quais os tipos claudina-3, 5 e 12 contribuem para formação das TJ da BBB (ABBOTT et al. 2010; PIONTEK et al. 2008). Estruturalmente, cada uma destas proteínas cruza a bicamada lipídica da célula endotelial e se adere fortemente à outra proteína claudina da célula adjacente. Internamente, para que se fixem ao citoesqueleto, ligam-se as ZO (Z0-1, ZO-2 e ZO-3, proteínas de ancoragem (Figura 7; REDZIC, 2011).

A claudina-5, especificamente, regula a permeabilidade paracelular de partículas de pequeno tamanho molecular na BBB (PIONTEK et al., 2008). Algumas drogas podem aumentar a sua expressão na barreira, diminuindo a permeabilidade paracelular (HONDA et al., 2006), enquanto eventos neuropatológicos, como a reperfusão cerebral pós evento isquêmico, podem reduzir a sua expressão, contribuindo para uma disfunção da BBB (JIAO et al., 2011)

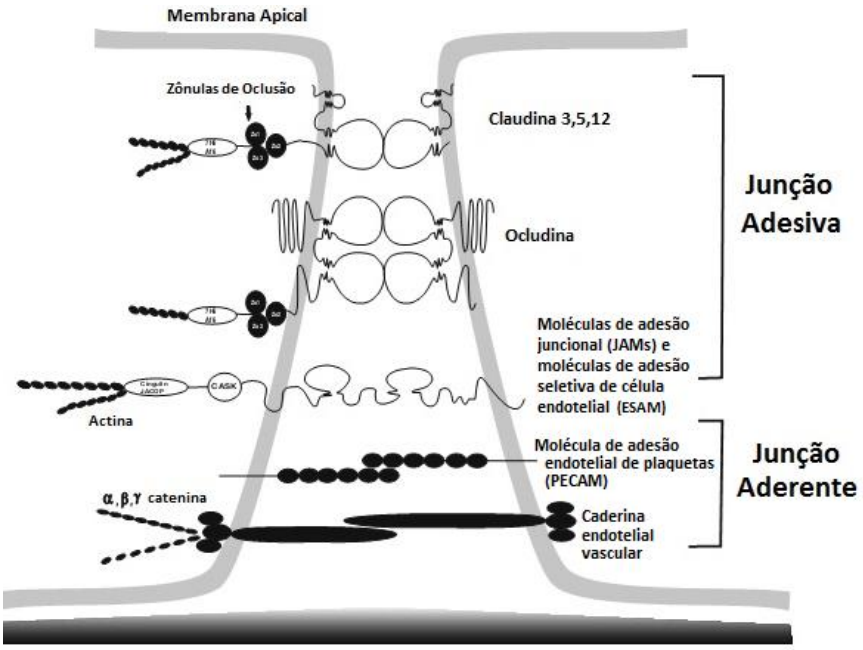

Figura 7. Representação da organização molecular da junção oclusiva, mostrando as proteínas claudinas ligadas ao citoesqueleto através das zônulas de oclusão (adaptado de CORREALE, VILLA, 2009).

3.3. Proteínas Aquaporinas

As aquaporinas (AQPs) representam uma família de proteínas de membrana as quais funcionam como canais de água. Além do controle crítico no controle do conteúdo hídrico celular, algumas destas proteínas também permitem o transporte de ânios, solutos voláteis (CO_2) e amônia (ZHENG et al., 2010) Com um peso molecular em torno de 30 kDa, distribuem-se amplamente pelos tecidos corporais. Dos 13 tipos de AQPs identificadas até o momento (AQP0-AQP12), seis encontram-se no SNC (AQP 1, 3, 4, 5, 8 e 9), distribuindo-se pelo plexo coroide (AQP1), pelos astrócitos (AQP 1, 3, 4, 5, 8 e 9), pelos oligodendrócitos (AQP8), pelas células ependimárias (AQP 1, 4 e 9) e neuronais (AQP 1, 5 e 8; BARBARA, 2012; LIU et al., 2008).

3.3.1 Proteína Aquaporina-4

A proteína APQ4 é a principal AQP do SNC de mamíferos, fortemente expressa nos astrócitos e nas células ependimárias do encéfalo e da medula espinal. Localiza-se nos sítios de contato entre o parênquima cerebral com os espaços subaracnóide e o sistema ventricular, e nos astrócitos – preferencialmente nos pés astrocitários –, recobrindo 95% da superfície dos capilares da BBB (BARBARA, 2010; PARDRIDGE, 1999).

No hipocampo, a co-localização de proteínas APQ4 com a proteína S100β e GFAP confirma que os canais de AQP4 são quase exclusivamente encontrados nos pés astrocitários perivasculares do tecido hipocampal. Apesar de estarem amplamente distribuídos pelo parênquima, os estratos lacunoso e molecular da região 1 de Corno de Ammon (CA1) são as regiões de maior expressão dos canais de AQP4, certamente por constituírem o local de penetração dos vasos sanguíneos no hipocampo (HSU et al., 2011).

As AQP4 permitem o fluxo bidirecional de água entre os tecidos encefálicos e os vasos sanguíneos. Os canais de AQP4 exercem importante função no edema vasogênico – evento vinculado à perda da integridade da BBB –, facilitando a remoção de água do parênquima cerebral (FRANCESCA, REZZANI, 2010).

Estudos na retina de modelo animal de DM1 mostram uma redução na expressão da proteína AQP4, associada ao agravamento da retinopatia, com aumento do edema e

do processo inflamatório (CUI et al., 2012; CURTI et al., 2011; IANDIEV et al., 2007). Para as demais estruturas do SNC acometidas pela hiperglicemia, não há dados na literatura sobre o comportamento da proteína AQP4.

4. Hipocampo e Memória

O hipocampo, localizado no lobo temporal de ambos os hemisférios cerebrais, é uma das estruturas mais plásticas e mais estudadas no DM1. Anatomicamente, é dividido em Corno de Ammon (CA1 a CA4, ou hipocampo propriamente dito) e em giro denteado.

O Corno de Ammon é estratificado em seis camadas microscopicamente distintas: *alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunoso* e *stratum moleculare*. No *stratum piramidale* são encontrados os somas dos neurônios piramidais, estando os seus axônios no *stratum alveus* e os seus dendritos no *stratum radiatum* (FALOUGY et al., 2008). No *stratum radiatum*, particularmente da região de CA1, são encontrados astrócitos de formato em sua maioria fusiforme, orientados paralelamente aos dendritos apicais dos neurônios piramidais (BUSHONG et al., 2002).

A área de CA1 é a região com a menor densidade de capilares, o que a torna suscetível aos diferentes insultos encefálicos e, portanto, uma das regiões mais estudadas do hipocampo (CAVAGLIA et al., 2001). Desta, partem fibras excitatórias ao subículo, e deste ao córtex entorrinal, o qual faz conexões aferentes e eferentes com o córtex pré-frontal, córtices associativos parietal, occipital, cingulado anterior e outras áreas do córtex temporal, integrando os distintos e específicos mecanismos de formação de memórias (IZQUIERDO et al., 1997).

Funcionalmente, o hipocampo está envolvido com o processo de formação de memórias declarativas, as quais se referem à retenção de experiências sobre fatos e eventos passados, divergindo das memórias procedurais relacionadas a habilidades motoras ou sensoriais (IZQUIERDO et al., 2002).

As memórias declarativas podem ser divididas conforme a sua duração em "memórias de curta e de longa duração", e conforme o seu conteúdo em "episódica e semântica". Em modelos animais, os testes comportamentais cuja memória é evocada

em até três horas após a exposição a um evento, avaliam a memória de curta duração (IZQUIERDO et al., 2002; SHARMA et al., 2010).

Duas formas de memória episódica – tipo de memória cujas lembranças dependem de um contexto temporal ou espacial – são a "memória espacial", envolvida com localização e movimentação dentro de um ambiente, e a "memória de reconhecimento de objetos". Ambas são amplamente utilizadas para estudar a efetividade de tratamentos envolvidos para o processo de aprendizado e de memória em modelos experimentais (COHEN; STACKMAN, 2014; GRAYSON et al., 2014; SHARMA et al., 2010).

5. Estriado

Em roedores, o estriado dorsomedial recebe aferências de áreas corticais associativas, estando envolvido com as fases iniciais de aprendizado motor. O estriado dorsolateral recebe aferências sensoriomotoras, sendo crítico para a aquisição gradual do comportamento motor habitual e automático (PAN et al., 2010; YIN et al., 2009) .

No estriado de modelos animais de DM1, foram mostrados comprometimento na neurotransmissão colinérgica, dopaminérgica e glutamatérgica, com redução da habilitada motora (ROBINSON et al., 2009; SHERIN et al., 2012). Aumento da permeabilidade da BBB também foi encontrado no estriado de animais diabéticos (KAROLCZAK et al., 2012).

Para avaliação do aprendizado motor e da integridade do estriado dorsal, o teste de rotarod tem sido amplamente utilizado nos estudos com modelos animais (DO NASCIMENTO et al., 2011; YIN et al., 2009), sendo demonstrado que o exercício físico melhora o desempenho no teste do rotarod em animais diabéticos (DO NASCIMENTO et al., 2011).

6. Exercício Físico

A prática de pelo menos 150 min por semana de exercício físico aeróbico em intensidade moderada é recomendada aos indivíduos com DM1 no intuito de auxiliar o controle dos níveis glicêmico, de reduzir os riscos cardiovasculares e a incidência de

lesões microvasculares associada à hiperglicemia (AMERICAN DIABETES ASSOCIATION, 2014; BALDUCCI et al., 2006).

Os benéficos do exercício físico aplicam-se também aos aspectos neurocognitivos. A sua prática regular está associada a menores riscos de desenvolvimento da doença de Alzheimer e outras demências, e, deste modo, possivelmente eficaz em prevenir ou diminuir déficits cognitivos (ALAE et al., 2007; ALBECK et al., 2006; LAURIN et al., 2001; QIANG et al., 2006). No DM1 experimental, o exercício físico melhora a função cognitiva dos animais treinados, não estando ainda claro se seus efeitos revertem ou previnem o dano cognitivo (DIEGUES et al., 2014; REISE et al., 2009; DE SENNA et al., 2011).

Dentre os achados que contribuem para um melhor desempenho cognitivo associado ao treinamento físico, destaca-se à capacidade deste de: induzir neurogênese, angiogênese, sinaptogênese; diminuir a expressão de genes associados com o estresse oxidativo; aumentar a expressão de genes associados à plasticidade sináptica, à função mitocondrial, aos espinhos dendríticos; melhorar a função da BBB; de induzir proliferação e alterações na morfologia dos astrócitos (GUO et al., 2008; LISTA, SORRENTINO, 2010; MUNEHIRO et al., 2006; SAUR et al., 2013; STRANAHAN et al., 2010).

O exercício físico, conforme a intensidade de treinamento, melhora a atividade locomotora e o desempenho motor, induzindo o aumento da imunorreatividade da tirosina hidroxilase na substância nigra *pars compacta*, promovendo a liberação de fatores neurotróficos, protegendo os neurônios dopaminérgicos no estriado, e aumentando a expressão de células GFAP positivas associadas à angiogênese e ao fortalecendo da unidade neurovascular (ALAEI et al., 2008; DE SENNA et al., 2011; DO NASCIMENTO et al., 2011; LI et al., 2005; MALYSZ et al., 2010; SMITH, ZIGMOND, 2003).

OBJETIVOS

Objetivo Geral

Avaliar o efeito do exercício físico sobre o comportamento cognitivo, motor, alterações astrocitárias e sobre a barreira hematoencefálica, no hipocampo e no estriado, de ratos machos com diabetes mellitus tipo 1 induzido por estreptozotocina (STZ).

Objetivos Específicos

i. Avaliar se o exercício físico previne ou reverte o dano na memória espacial presente em ratos diabéticos induzidos por STZ, através da tarefa "Reconhecimento do Objeto Reposicionado";

ii. Avaliar a influência do exercício físico sobre a memória não espacial, através da tarefa "Reconhecimento do Novo Objeto", em ratos diabéticos por STZ;

iii. Avaliar o efeito do exercício físico sobre o comportamento motor, em ratos diabéticos por STZ, através do teste rotarod;

iv. Estimar a densidade de astrócitos GFAP positivos no *stratum radiatum* de CA1 hipocampal e avaliar a morfologia astrocitária pela técnica dos círculos concêntricos de Sholl;

v. Avaliar a captação de glicose e glutamato no hipocampo de ratos diabéticos por STZ, e a influência do exercício físico sobre estas variáveis;

vi. Mensurar os níveis de glutationa reduzida e a atividade da enzima glutamina sintetase no hipocampo de ratos diabéticos por STZ, e a influência do exercício físico sobre estas variáveis;

vii. Avaliar o conteúdo da proteína claudina-5 e aquaporina-4 no hipocampo e no estriado de ratos diabéticos por STZ, e a influência do exercício físico sobre seus níveis.

MODO DE APRESENTAÇÃO DOS MÉTODOS, RESULTADOS E DISCUSSÃO

As seções de “material e métodos”, “resultados” e “discussão” serão apresentadas na forma de dois artigos científicos.

O primeiro artigo foi submetido ao periódico *Metabolic Brain Disease* (FI= 2.398). O segundo artigo está publicado no periódico *Brain Research* (1618: 75–82; 2015; FI= 2.828).

Artigo 1 - Artigo submetido na revista *Metabolic Brain Disease.*

Priscylla Nunes de Senna, Léder Leal Xavier, Pamela Brambilla Bagatini, Lisiani Saur, Fabiana Galland, Larissa Bobermin, Caren Bernardi, Patrícia Severo do Nascimento, Cristiane Batassini, Lucas Tortorelli, Ana Carolina Tramontina, Patrícia Nardin, Jocemar Ilha, Carlos Alberto Gonçalves, Matilde Achaval. **Physical exercise improves spatial memory and astroglial alterations in the hippocampus of diabetic rats**.

Metabolic Brain Disease

Physical exercise reverses spatial memory deficit and induces hippocampal astrocyte plasticity in diabetic rats.

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Corresponding Author:	matilde achaval, Ph.D., M.D. Universidade Federal do Rio Grande do Sul Porto Alegre, BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidade Federal do Rio Grande do Sul
Corresponding Author's Secondary Institution:	
First Author:	Priscylla Nunes de Senna, MSc.
First Author Secondary Information:	
Order of Authors:	Priscylla Nunes de Senna, MSc.
	Leder Leal Xavier, Ph.D.
	Pamela Brambilla Bagatini, MSc.
	Lisiani Saur, MSc.
	Fabiana Galland, MSc.
	Larissa Bombermin, Ph.D.
	Caren Bernardi, Ph.D.
	Patrícia Severo do Nascimento, Ph.D.
	Cristiane Batassini, Ph.D.
	Lucas Tortorelli, MSc.
	Ana Carolina Tramontina, Ph.D.
	Patrícia Nardin, Ph.D.
	Jocemar Ilha, Ph.D.
	Carlos Alberto Gonçalves
	matilde achaval, Ph.D., M.D.
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Physical exercise can induce brain plasticity and reduce the cognitive decline observed in type 1 diabetes mellitus (T1DM). We investigated the effects of physical exercise to prevent or reverse spatial memory deficits produced by diabetes and some biochemical and immunohistochemical changes in hippocampal astrocytes of T1DM model. In this study, 56 Wistar rats were divided in four groups: trained control (TC), non-trained control (NTC), trained diabetic (TD) and non-trained diabetic (NTD). 27 days after streptozotocin-induced (STZ) diabetes, the exercise groups were submitted to 5 weeks of aerobic exercise. All groups were assessed in a novel object-placement

	recognition task (NOPR) before and after training. The glial fibrillary acidic protein (GFAP) positive astrocytes were evaluated using planar morphology, optical densitometry and Sholl's concentric circles method. Glucose and glutamate uptake, reduced glutathione (GSH) and glutamine synthetase (GS) levels were measured using biochemical assays. Our main results are: 1-Exercise reversed spatial memory impairments generated by T1DM; 2-Exercise increased GSH and GS in TC but not in TD rats; 3-Exercise increased astrocytic density in the TC and TD groups and increased astrocyte ramification in TD animals. Our findings indicate that physical exercise reverses the cognitive deficits present in T1DM and induces important morphological astrocytic changes.
Suggested Reviewers:	Vivaldo Moura Neto, PhD Professor and Research, Universidade Federal do Rio de Janeiro vivaldo@anato.ufrj.br
	Andre Ricardo Massensini Universidade Federal de Minas Gerais massen@icb.ufmg.br
	Francesco Amenta Università di Camerino, 62032 Camerino, Italy francesco.amenta@unicam.it
	Jaderson Costa Dacosta, M.D., Ph.D. Pontifícia Universidade Católica do Rio Grande do Sul – PUCRS jcc@pucrs.br
	Giyasettin Baydas Firat University, Turkey baydas@hotmail.com
	Helle S. Waagepetersen University of Copenhagen, Universitetsparken helle.waagepetersen@sund.ku.dk

Physical exercise reverses spatial memory deficit and induces hippocampal astrocyte plasticity in diabetic rats.

Priscylla Nunes de Senna[a,b], Léder Leal Xavier[b], Pamela Brambilla Bagatini[a], Lisiani Saur[b], Fabiana Galland[c], Larissa Bobermin[c], Caren Bernardi[d], Patrícia Severo do Nascimento[e], Cristiane Batassini[c], Lucas Tortorelli[c], Ana Carolina Tramontina[f], Patrícia Nardin[c], Jocemar Ilha[a], Carlos Alberto Gonçalves[a,c], Matilde Achaval[a*]

[a]Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[b]Laboratório de Biologia Celular e Tecidual, Departamento de Ciências Morfofisiológicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

[c]Departamento de Bioquímica, Instituto de Ciências Básica da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[d]Universidade Federal de Ciências da Saúde de Porto Alegre, RS, Brazil

[e]Universidade Federal de Santa Maria, RS, Brazil

[f]Universidade Estadual do Rio Grande do Sul, Porto Alegre, Brazil

*Corresponding author:

Matilde Achaval (MD,PhD)

Laboratório de Histofisiologia Comparada, Departamento de Ciências Morfológicas, ICBS, Universidade Federal do Rio Grande do Sul (UFRGS). Rua Sarmento Leite 500, Porto Alegre, RS, Brazil, CEP: 90050-170.

E-mail address: machaval@pq.cnpq.br

Keywords: Type 1 Diabetes Mellitus; Physical Exercise; Spatial Memory; Astrocytes

Abstract

Physical exercise can induce brain plasticity and reduce the cognitive decline observed in type 1 diabetes mellitus (T1DM). We investigated the effects of physical exercise to prevent or reverse spatial memory deficits produced by diabetes and some biochemical and immunohistochemical changes in hippocampal astrocytes of T1DM model. In this study, 56 Wistar rats were divided in four groups: trained control (TC), non-trained control (NTC), trained diabetic (TD) and non-trained diabetic (NTD). 27 days after streptozotocin-induced (STZ) diabetes, the exercise groups were submitted to 5 weeks of aerobic exercise. All groups were assessed in a novel object-placement recognition task (NOPR) before and after training. The glial fibrillary acidic protein (GFAP) positive astrocytes were evaluated using planar morphology, optical densitometry and Sholl's concentric circles method. Glucose and glutamate uptake, reduced glutathione (GSH) and glutamine synthetase (GS) levels were measured using biochemical assays. Our main results are: 1-Exercise reversed spatial memory impairments generated by T1DM; 2-Exercise increased GSH and GS in TC but not in TD rats; 3-Exercise increased astrocytic density in the TC and TD groups and increased astrocyte ramification in TD animals. Our findings indicate that physical exercise reverses the cognitive deficits present in T1DM and induces important morphological astrocytic changes.

INTRODUCTION

Central nervous system damage is increasingly recognized as a microvascular complication of chronic diabetes mellitus (Gispen et al. 2000). The hippocampus is one of the most affected brain structures in type 1 diabetes (T1DM) and different neuropathological mechanisms such as neuronal death, impaired long-term potentiation (LTP) have been demonstrated in this region (Gardoni et al. 2002; Gispen et al. 2000; Li et al. 2002).

Astrocytes play many physiologically roles in the brain, performing diverse functions such as glycogen synthesis, glutamate uptake and glutathione (GSH) synthesis (Costa et al. 2012; Hansen et al. 2012). In glutamate metabolism, astrocytes are responsible for removing 90% of total glutamate from the synaptic cleft, and its conversion, through glutamine synthetase (GS), into glutamine for replacement in the neurons (Santin et al. 2011; Tanaka et al. 1997). Astrocytic plasticity is important to maintain and reorganize brain areas post-injury and it may reflect a substantial increase in astroglial metabolism and protein synthesis in response to increased physiologic demands (Eddleston et al. 1993).

In this way, GFAP is an intra-cellular intermediate filament protein, essential for the formation of stable astrocytic processes in response to neuronal damage or physiologic demands, and is used as an astrocytic marker (Baydas et al. 2005).

Beneficial effects of physical exercise have been widely reported in T1DM cognitive impairment, although it is not so clear if physical activity prevents or reverts it (de Senna et al. 2011; Tuzco and Baydas 2006). Exercise can increase the release of neurotrophic factors, induce LTP, neurogenesis, and increase hippocampal synaptic and structural plasticity markers, such as synapsin I, neurofilaments, microtubule-associated

protein 2, as well as augment the glial fibrillary acidic protein (GFAP) expression and GFAP positive cells density, which may be involved in enhancing cognitive function (de Senna et al. 2011; Lista and Sorrentino 2010; Saur et al. 2013; Uda et al. 2006; van Praag et al. 1999).

Therefore, this study aimed to evaluate the effects of physical exercise on astrocytic biochemical parameters, such as glucose and glutamate uptake, glutathione content and glutamine synthetase activity in the hippocampus, and astrocytic density and morphology in the hippocampal CA1 area, as well as investigate whether it prevents or reverses the spatial memory decline observed in STZ- induced T1DM in Wistar rats.

RESEARCH DESIGN AND METHODS

Animal Model

In this study were used three-month old male Wistar rats, weighing 270 g to 400 g, obtained from a local breeding colony (ICBS, *Universidade Federal do Rio Grande do Sul*, Brazil). The animals were housed in standard plexiglass boxes (three per cage) and kept in a temperature-controlled colony room with food and water available *ad libitum* and maintained under a 12:12 light/dark cycle (lights on at 8:00 h). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the Brazilian Laws (11794/2008) for animal care and ethical use of animals.

Experimental design

Fifty-six animals were randomly divided into four groups as follows: non-trained control (NTC) (n=15), trained control (TC) (n=15), non-trained diabetic (NTD) (n=13) and trained diabetic (TD) (n=13). For the biochemical assays, each group was subdivided, and 8 animals were used from both the NTC and TC groups and 7 animals from both the NTD and TD groups. The remaining animals (n= 7 in NTC and TC; n=6 in NTD and TD) were used for the GFAP immunohistochemical evaluation.

A timeline with our experimental design can be seen in figure1.

Diabetic Model

After a 6 h fasting period, T1DM was induced with a single intravenous injection into the tail vein of streptozotocin (STZ) (Sigma Chemicals, Co., USA) dissolved in 0.01 M citrate buffer, pH 4.5 (50 mg/kg of corporal weight). Control rats received only vehicle (citrate buffer). Diabetes was verified by glycaemia levels greater than 300 mg/dl, 48 h post-injection. Blood glucose concentrations were evaluated in blood collected from rat-tail using a glucose test strip (Acon Laboratorie, INC. San Diego, CA 92121, USA), following a 5 h morning fasting.

On the 20^{th} day after diabetes induction, before the animals underwent adaptation to a treadmill apparatus, a spatial memory task was employed to assess cognitive function in all the animals.

Novel object-placement recognition task (NOPR)

To clarify if physical training prevents or reverses the spatial memory impairment induced by diabetes, we evaluated spatial memory before and after the animals were submitted to the physical exercise protocol.

The novel object-placement recognition task (NOPR) was used to assess spatial memory and two identical objects (0.5 L plastic bottles filled with water) were used to evaluate object exploration. One day before the memory task, animals were habituated to an open field apparatus (50 × 60 × 40 cm^3). The rats were gently placed in the center of the box and released to explore the environment for 3 min. All the tests were recorded using a mounted digital video camera.

Each animal received one sample trial and a test trial. In the sample trial, animals were placed into the center of the open field containing two identical plastic bottles placed one in the northwest zone and another in the southwest zone and then allowed 5 min for exploration. The rats were returned to their home cages and 50 min after the end of sample trial, they were submitted to the test trial, which consisted of identical conditions except that the object previously in the southwest zone was moved to the east zone in order to analyze the exploration of the relocated object.

The percentage of preference for exploring the relocated object was calculated as follows: Exploration time of relocated object/Sum of exploration of both objects x 100 (Revsin et al. 2009).

Six weeks after the first memory task and 1 day after the end of the physical exercise protocol, the animals were again submitted to the NOPR.

Treadmill adaptation and maximal exercise test (MET)

Rats were habituated with the treadmill apparatus to minimize novelty stress by walking for 10 min at 5 m/min for 4 days. On the fifth day, the rats were submitted to the MET, which consisted of a graded treadmill exercise with speed increments of 5m/min every 3min, starting at 5m/min and continuing up to the maximal intensity attained by each rat. The values obtained in the MET were used to plan the treadmill training program as a moderate intensity exercise protocol, which started in the 5th week after diabetes induction (de Senna et al. 2011).

Training Protocol

The physical exercise consisted of running on the treadmill for 20 min on the first day. This training period was progressively increased everyday up to 60 min on the sixth day, which was maintained for the next 4 weeks. Each training session included a warm up period of 5 min running at 30% of the maximal speed reached in the MET (4.5 m/min for TC and TD group), 10 – 50 min running at 50 – 60% (~ 8.25 m/min for TC and TD group) and 5 min recovery at 30%, 5 sessions per week, once a day during 5 weeks, at 0 º of inclination. The training program was considered moderate-intensity endurance (de Senna et al. 2011).

Biochemical analysis

One day after the animals were newly submitted to the NOPR, 30 animals (n=8 from NTC and TC; n=7 from NTD and TD) were anesthetized with an intramuscular injection of ketamine and xylazine (75 and 10 $mg.Kg^{-1}$, respectively) and then killed by decapitation. The brains removed and the hippocampi were quickly dissected on ice.

Hippocampal samples were then used for biochemical measurements, described as follows:

Glucose uptake assay

Glucose uptake was performed in hippocampal slices using the procedures as previously described with modification (Hansen et al. 2012). Briefly, slices were transferred to 24-well plates and incubated for 30 min at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4.7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by addition of 0.1 µCi/mL [2,3-^{3}H]deoxi-D-glucose. Incubation was stopped after 30 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The slices were then lysed in a solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Non-specific uptake was determined by using 25 µM cytochalasin B. Final glucose uptake was obtained by subtracting the non-specific uptake of the total one to obtain the specific uptake.

Glutamate uptake assay

Glutamate uptake was measured as previously described (Tramontina et al. 2012). Hippocampal slices were transferred immediately to 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. Medium was replaced by HBSS. The assay started by the addition of 0.1 mM L-glutamate and 0.66 Ci.mL−1 L-[2,3-^{3}H] glutamate. Incubation was stopped after 5 min by removal of the medium and rinsing the slices twice with ice-cold HBSS. Slices were then lysed in a

solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of NaCl. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the total to obtain the specific uptake. Results are expressed as nmol/mg protein/min.

Glutathione Content

Reduced glutathione (GSH) concentration was determined as previously described (Nardin et al. 2009). Briefly, slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdehyde (1 mg/mL of methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard glutathione solutions (0–500 μM). Glutathione concentrations were expressed as nmol/mg protein.

Glutamine synthetase (GS) activity

The enzymatic activity of glutamine synthetase was determined using the procedures described previously with modifications (Minet et al. 1997). Briefly, homogenized tissue samples were added to a reaction mixture containing (in mM): 50 imidazole, 50 hydroxylamine, 100 L-glutamine, 25 sodium arsenate dibasic heptahydrate, 0.2 ADP, 0.5 manganese chloride, pH 6.2; and incubated for 15 min at 37°C. The reactions were terminated with the addition of 0.2 mL of 0.37 M $FeCl_3$, 0.3

M trichloroacetic acid, and 0.6 M HCl. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate acid (Sigma) treated with ferric chloride reagent. Glutamine synthetase activity was expressed as μmol/h/mg protein.

GFAP immunohistochemistry

Two days after the animals were newly submited to the NOPR memory task, twenty six animals (n=7 from NTC and TC; n=6 from NTD and TD) were anesthetized using ketamine (90 mg/kg) and xylazine (15 mg/kg) (i.p.). All animals were perfused through the left cardiac ventricle, using a peristaltic pump (Milan, Brazil, 50 mL/min), with 200 mL of saline solution followed by 200 ml of fixative solution containing 4% paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB), pH 7.4. Brains were extracted from the skull, post-fixed for 4 h in the same fixative solution at room temperature, cryoprotected in a 30% sucrose (Synth, Brazil) solution in PB at 4°C until they sank, and then frozen in isopentane previously cooled in liquid nitrogen (Nitrovet, Brazil). After these procedures the brains kept in a freezer (−70 °C) for further analyses. Using a cryostat (Leica, Germany), coronal brain sections (50 μm) were obtained from between the coordinates interaural 6.7 mm, bregma 2.3 mm and interaural 4.8 mm, bregma −4.16 mm, according to Paxinos and Watson's Atlas. Brain sections were processed for GFAP immunohistochemistry as previously described (de Senna et al. 2011). The brains in both experimental groups were fixed and post-fixed for the same time in identical solutions, processed at the same time, and incubated in the same immunostaining medium for the same period of time, in order to minimize differences in the staining of astrocytes and in background levels.

Estimation of astrocytic density

The number of GFAP-immunoreactive astrocytes per mm^2 in the *stratum radiatum* of CA1 was estimated using an Olympus BX 50 microscope, coupled to a Motic Images Plus 2.0 camera and Image Pro Plus software (Image Pro Plus 6.1, Media Cybernetics, Silver Spring, EUA). 15 images (20x) from the *stratum radiatum* were analyzed per animal (5 sections, 3 digitized images from each section). Three randomized squares measuring 7389 μm^2, named areas of interest (AOIs), were overlaid on each image, and GFAP-reactive the astrocytes located inside each square or intersected by the upper and/or right edges of the squares were counted. Astrocytes intersected by the lower and/or left edges of the squares were not counted. 45 AOIs were analyzed in each animal.

Morphological analysis of astrocytes

Morphological analysis was performed on 15 astrocytes per animal, using the same images employed to measure cellular optical density. Astrocytes in which the soma and processes were clearly visible were selected. For a general analysis of astrocytic ramification, an adaptation of Sholl's concentric circles technique was used. Briefly, seven virtual circles with 4.4 μm intervals were drawn around each astrocyte. The degree of ramification of the astrocytes was measured by the number of times the astrocytic processes intersected with each virtual circle in both the lateral (i.e. right/left) and central (i.e. superior/inferior) quadrants surrounding the astrocytes. Primary process quantification was performed by counting the processes extending directly from the soma in both the lateral and central quadrants. Finally, the longest primary process in

each quadrant was delineated and measured using Image Pro Plus software (Image Pro Plus 6.1, Media Cybernetics, Silver Spring, EUA; [Saur et al. 2013]).

Statistical analysis

Data for all variables are expressed as mean ± standard error. Repeated measures analysis of variance (ANOVA) was used for blood glucose and weight comparisons between groups. Two-way ANOVA followed by Newman-Keuls *post-hoc* were used to analyze all other data. STATISTICA 9 software was used in the statistical analyses were ($P<0.05$).

RESULTS

Blood Glucose Levels and Body Weight

Blood glucose concentrations were significantly higher in diabetic groups compared to control groups ($P<0.001$) 48 hours after diabetes induction. Diabetic rats also showed significant weight loss when compared to the NTC and TC groups ($P<0.001$). No differences between the NTD and TD groups were observed in relation to glycemia or body weight during the experiment ($P>0.05$; Table 1).

Spatial memory analysis

Analyzing the behavioral effects as shown by the use of the novel object-placement recognition task, it can be seen that, before the animals were submitted to the

training protocol, both diabetic groups explored the relocated object for significantly less time than the control groups ($P<0.05$), which explored the relocated object for similar amounts of time (Fig.2a). After physical training, the analysis of the exploration behavior showed that NTD rats spent less time exploring the relocated object than all the other groups ($P<0.05$), this reduction was reverted by physical training in the TD group (Fig.2b).

Biochemical Assays

Glucose uptake, glutamate uptake, GSH content and GS activity

Glucose and glutamate uptake were not significantly affected in the hippocampus in all groups ($P>0.05$; Fig.3a and 3b).

Physical exercise induced a significant increase in GSH and GS content in the hippocampus of the TC group, when compared to the others groups ($P<0.05$; Fig.3c and 3d).

Density of GFAP immunoreactive astrocytes

A significantly increased number of GFAP positive astrocytes/mm² was seen in the *stratum radiatum* of CA1 area in TC and TD groups as compared to NTC and NTD animals ($P<0.01$), showing that exercise was able to increase the density of GFAP immunoreactive astrocytes (Fig.4ab).

Analysis of astrocytic ramification

A significant increase in the number of total ramifications was observed in TD rats ($P<0.05$; Fig.5Aa). This increase was seen in both the central and lateral orientations in TD animals, indicating that exercise in diabetic rats was able to induce astrocytic ramification in all directions ($P<0.05$; Fig.5Abc).

Analysis of primary processes

Primary process counting revealed that neither the total number nor the number of centrally located primary processes were affected in the diabetic or training groups ($P>0.05$; Fig.5Bab). However, there was a significant decrease in the number of primary process found in the lateral quadrant in NTD rats when compared to all other groups ($P<0.05$; Fig.5Bc).

Length of the primary processes

Analysis of the processes length showed there was no significant difference between the groups ($P>0.05$; Fig.5Ca,b,c).

DISCUSSION

Diabetic rats showed hyperglycemia and weight loss, and as previous demonstrated, the physical exercise protocol used in this study did not affect blood glucose levels or body weight (de Senna et al. 2011).

Physical exercise can improve memory in diabetic animals, although few studies have attempted to elucidate whether exercise is able to reverse diabetes-induced memory impairment (Alae et al. 2006; de Senna et al. 2011). Thus, our study provides evidence that T1DM-induced spatial memory impairments are reversed by physical training (Fig.2). This finding are probably related to improvements in parameters such as hippocampal neural plasticity, LTP, astrocytic proliferation, neurogenesis and oxidative stress prevention (Gomes da Silva et al. 2010; Li et al. 2005; Reisi et al. 2009; Stranahan et al. 2008; Uda et al. 2006).

Hyperglycemia is the initiating cause of the diabetic tissue damage that is clinically seen. However, only some cell types are involved in diabetic complications, such as capillary endothelial cells in the retina, mesangial cells in the renal glomerulus and Schwann cells in peripheral nerves, because, when exposed to hyperglycemia. most cells are able to reduce the internal transport of glucose (Brownlee et al. 2005). Downregulation of glucose transporter has been reported in brain under chronic hyperglycemia and this may be able to maintain the internal glucose concentration cell constant (Sickman and Waagepetersen 2014). Previous studies showed that glucose uptake was not altered in the hippocampus of STZ-treated rats and glucose concentration gradient inside the hippocampus was sustained (Duarte et al. 2009; Simpson et al. 1999). Together with these observations, our results suggest glucose uptake in the hippocampus is not affected by experimental diabetes (Fig.3a).

Furthermore, in our study, physical exercise did not affect glucose uptake in hippocampus (Fig.3a). This is probably associated to the intensity of exercise, as it has been reported that high intensity of running exercise decreases brain glucose uptake (Kemppainen et al. 2005), while moderated intensity did not affect the glucose uptake

(Bernardi et al. 2013). We used a moderated intensity protocol in our study, similarly to Bernardi et al. (2013).

Our data show that glutamate uptake in hippocampus slices was not significantly different between the groups (Fig.3b). Coleman et al (2010) suggests that glutamate uptake is increased in diabetic rats. However, their study did not include an analysis of isolated regions of the brain. In the hippocampus, proton magnetic resonance (MR) spectroscopy showed no difference in glutamate levels after 30 days of STZ-induced hyperglycemia (Duarte et al. 2009). Furthermore, astrocytic glutamate uptake is positively modulated by extracellular S100B protein (Tramontina et al. 2006, 2012), and we demonstrated in another study that hippocampal S100B protein levels are unaltered in diabetic animals (de Senna et al. 2011), which is in agreement with the results for glutamate uptake in the present study. Similarly, an animal model of dementia with spatial cognitive deficit did not alter hippocampal glutamate uptake activity or S100B protein levels (Costa et al. 2012). Furthermore, astrocyte glutamate transporter expression in the hippocampus was unaffected in diabetic rats (Coleman et al. 2010). Other possibility, to be investigated, is that regional differences in glutamate uptake exist in diabetes.

Glutathione levels (GSH) in the hippocampal slices were not affected in diabetic groups (Fig.3c), which is in agreement with Baydas et al. (2005). Nevertheless, decreases in GSH levels have been reported in STZ-induced diabetes (Duarte et al. 2009). The discrepancies between these studies and our results may be related to the different protocols used to assay GSH peptide, as they used MR spectroscopy, which provides neurochemical detection *in vivo*. In addition, in the present study, the GSH content is in accordance with the glucose uptake observed in the diabetic groups (Fig.3c). Decreased GSH is associated with an increase in the polyol pathway under

hyperglycemic conditions (Brownlee et al. 2005). It is likely the absence of increased glucose uptake observed in our study did not affect the cellular glucose concentration, thus ensuring the necessary NADPH for the regeneration of oxidized GSH (Tramontina et al. 2012). Acute and chronic high-glucose environments activate the pentose phosphate pathway in astroglia, which is associated with regeneration of reduced GSH from oxidized glutathione (GSSG) and may thus preserve GSH concentrations (Darmaun et al. 2012).

Physical exercise increased hippocampal GSH levels in the TC rats (Fig.3c). This result is in accordance with a previous study that used the same protocol applied in the present study (Rodrigues et al. 2010), demonstrating that this aerobic training protocol can induce an important mechanism against oxidative stress (Dringen et al. 2000). On the other hand, physical exercise did not affect GSH content in the TD animals (Fig.3c). In contrast to the present study, Alipour et al (Alipour et al. 2012) reported that treadmill exercise increases GSH content in training-diabetic rats, which may be explained by the different intensities and duration of exercise protocols employed. The physical exercise protocol used in our study may not have been sufficiently demanding to induce hippocampal GSH changes in the TD rats, but it is important to consider that it may have protected against oxidative damage because it reduces nitrosative stress (Dringen et al. 2000).

Our findings regarding the hippocampal glutamine synthetase (GS) content show there is no difference between the sedentary control and diabetic groups (Fig.3d). A decline in GS activity in the cerebral cortex and medulla oblongata has been reported in T1DM animals (Ahmed and Zahra 2011), but prior to the present study, no experimental approach had shown changes in hippocampal GS levels in experimental T1DM using a biochemical assay. Duarte et al (2009) and Wang et al (2012) found no

difference in glutamine content in the hippocampus measured using MR spectroscopy. Glutamine is synthesized from glutamate in astrocytes so as to return the glutamate that is removed from the synaptic cleft after release from the presynaptic neuron (Ahmed and Zahra 2011). Thus, our result regarding glutamate uptake (Fig.3b) seems be in accordance with the GS response.

The exercise protocol proved effective at increasing GS activity in the TC group, but it was unable to influence the TD animals (Fig.3d). Glutamine released from astrocytes is used by neurons as a precursor for the glutamate necessary for GSH synthesis (Dringen et al. 2000). The increment in GS activity observed in the TC group may explain the increased GSH observed in the hippocampus in our study, as suggested in a previous study (Dringen et al. 2000). GSH synthetase activity in neurons should not be overlooked, and it is a matter to be further investigated.

It is important to mention that glutamate–glutamine cycle and *de novo* synthesis of glutamate from glucose in astrocytes is also essential for learning and memory consolidation (Gibbs et al. 2008). To our surprise, in our study, the biochemical parameters did not influence the memory result in the diabetic animals.

Exercise has been shown to increase the density of GFAP immunoreactive astrocytes in the frontoparietal cortex, striatum and hippocampus (Li et al. 2005; Saur et al. 2013), and stimulate astrocyte proliferation in the subgranular zone of the dentate gyrus in the hippocampus of healthy animals (Uda et al. 2006). Our data show an increase in GFAP positive astrocyte density, confirming that exercise induces similar astrocytic plasticity in the *stratum radiatum* of CA1 in healthy and diabetic rats (Fig.4b). Astrogliosis has been previously related to the reinforcement of the blood brain barrier and the angiogenesis caused by exercise, and so is not exclusively

associated with a harmful response to injury, but also represents a protective reaction of the CNS (Liberto et al. 2004).

The main change in astrocytic morphology observed in our study was an increase in astrocytic ramification observed in the TD group (Fig.5Aa), which was due to increased ramification in the central and lateral quadrants (Fig.5Abc). It is important to clarify that an increased number of intersections in Sholl´s method might represent not only an increase in the ramification of each primary process, but also an increase in the number of primary processes or an increase in process length (Viola et al. 2009). Thus, since no differences were seen in the total number of primary processes per astrocyte (Fig.5Ba) or in the length of the processes between all groups (Fig.5C), we conclude that the increased ramification observed in diabetic animals submitted to exercise is mainly generated by primary process ramification. This change in astrocytic ramification could be related to post-exercise changes in factors such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF-2) and/or epidermal growth factor ([EGF]; Ferreira et al. 2011; Lista and Sorrentino 2010).

Some studies have reported changes in astrocytic polarization in healthy animals submitted to exercise and enriched environment, but this was not observed in our study (Saur et al. 2013; Viola et al. 2009). The remodeling of astrocytic processes found in our study is probably closely linked to the increased neuronal damage induced by diabetes, and may represent astrocytic plasticity to compensate the reduction in neuronal synapses that occurs in diabetes (Li et al. 2012). Furthermore, in contrast with Saur et al (2013), exercise did not affect the TC animals in our study. This discrepancy between the studies may be explained because the animals used in former study were younger than ours and consequently in a different stage of astrocytic maturation, thus indicating age could be a key factor in determining the effects of exercise in healthy animals

(Catalani et al. 2002). The fact morphological change was only observed in the astrocytes of the TD group suggests these cells in diabetic animals become more susceptible to exercise.

In summary, our findings show that treadmill running reduces diabetes-induced spatial memory deficits, since the cognition impairment present in both diabetic groups before physical exercise was reverted in the TD rats. We also show that glutamate metabolism was positively modulated by exercise in the healthy animals. Additionally, an increase in astrocytic density and morphological changes in astrocytic processes were observed in the TD animals, supporting the idea that physical training also induces relevant astroglial plasticity in diabetes.

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Legends

Table 1 – Glycemia and body weight values obtained pre, post and following 9 weeks after STZ induction. ***P<0.001 comparing diabetic groups (NTD,TD) with controls groups (NTC,TC; mean ± SE). Legends: NTC = non-trained control; TC = trained control; NTD = non-trained diabetic; TD = trained diabetic.

Fig. 1 – Time line of the experimental design. D = day; NOPR = novel object-placement recognition task; MET = maximal exercise test.

Fig. 2 – Exploration measurements of the relocated object in the novel object-placement recognition task. Graphs **a:** Before the physical exercise training; *P<0.05 when compared with non-trained control and trained control group; **b:** After the physical exercise training. *P<0.05 comparing non-training diabetic to all groups (mean ± SE). Legends: NTC = non-trained control; TC = trained control; NTD = non-trained diabetic; TD = trained diabetic.

Fig. 3 – Biochemical analyses showing the effects of physical exercise on (**a)** glucose uptake, (**b)** glutamate uptake, (**c**) glutathione levels and (**d**) glutamine synthetase activity in hippocampal slices. *P<0.05 comparing all groups (mean ± SE). Legends: NTC = non-trained control; TC = trained control; NTD = non-trained diabetic; TD = trained diabetic.

Fig. 4 – GFAP immunoreactivity in the stratum radiatum (SR) of hippocampal CA1 region. Graphs: (**a**) digitized images of a coronal section in SR area from the NTC, TC, NTD, TD groups, showing astrocytes counted in the area of interest (AOI); (**b**) astrocytic density per mm^2 in the stratum radiatum of CA1. **P<0.01 compared with the NTC and NTD groups (mean±SE). ■ = areas of interest at 20x. The orientation and location of the images presented are shown on a schematic drawing of the brain slice (adapted from Paxinos and Watson, 1998). Legends: P = stratum pyramidale; R = stratum radiatum; LM = stratum lacunosum moleculare and M = stratum moleculare; NTC = non-trained control; TC = trained control; NTD = non-trained diabetic; TD = trained diabetic. D = dorsal; M = medial; L= lateral; V = ventral. Calibration bar: 60 µm.

Fig. 5 – Quantitative analyses of GFAP positive astrocytes in the stratum radiatum of CA1, using Sholl´s concentric circles method. Graphs: (**A**) ramification of the astrocytes as (**a**) total number of intersections with concentric circles, number of intersections in the (**b**) central and (**c**) lateral quadrants, and (**d**) a schematic representation of process intersections; (**B**) number of primary processes of astrocytes as (**a**) total number of primary processes per astrocyte, number

of primary processes per astrocyte in (**b**) central and (**c**) lateral quadrants, and (**d**) a schematic representation of primary processes; (**C**) length of the longest astrocytic processes as (**a**) mean length of longest process per astrocyte, length of the longest processes in (**b**) central and (**c**) lateral quadrants, and (**d**) a schematic representation of the longest processes. *P<0.05 comparing all groups (mean±SE). Legends: NTC = non-trained control; TC = trained control; NTD = non-trained diabetic; TD = trained diabetic.

Table 1

Experimental Groups	Pre-STZ		Post- STZ		After Training Protocol	
	Glycemia (mg/dL)	Body Weight (g)	Glycemia (mg/dL)	Body Weight (g)	Glycemia (mg/dL)	Body Wheigt (g)
NTC	97±2.1	346±9.5	96±9.4	388±10	106±10.8	415±11
TC	98±2.0	326±8.8	92±10.8	370±9.6	113±12.3	389±10
NTD	99±2.0	362±8.8	375±10,8***	281±9.6***	534±12.3***	270±10***
TD	100±2.0	338±9.1	388±10.2***	272±9.9***	556±12.3***	288±11***

Figure 1

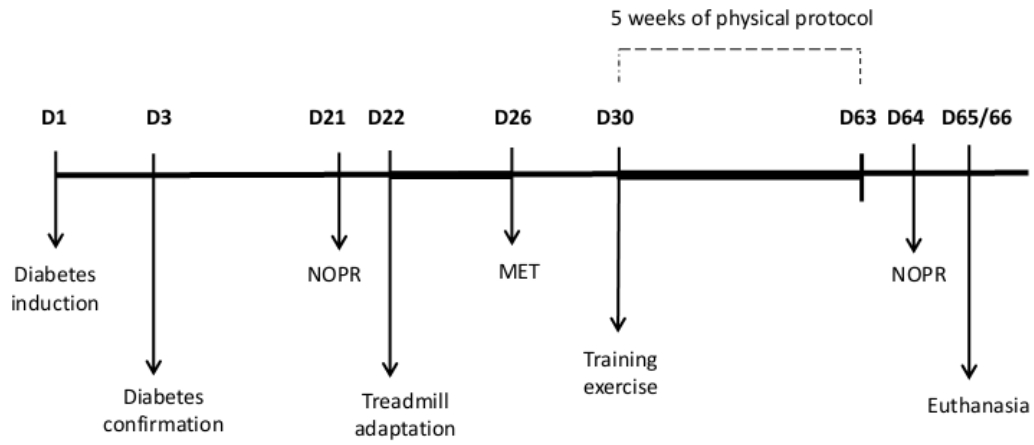

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Figure 2.

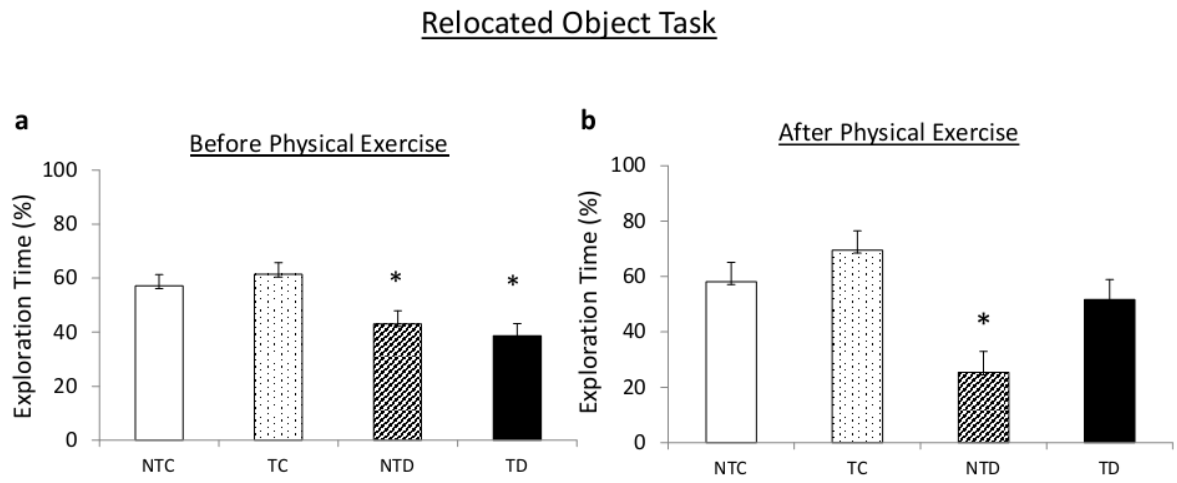
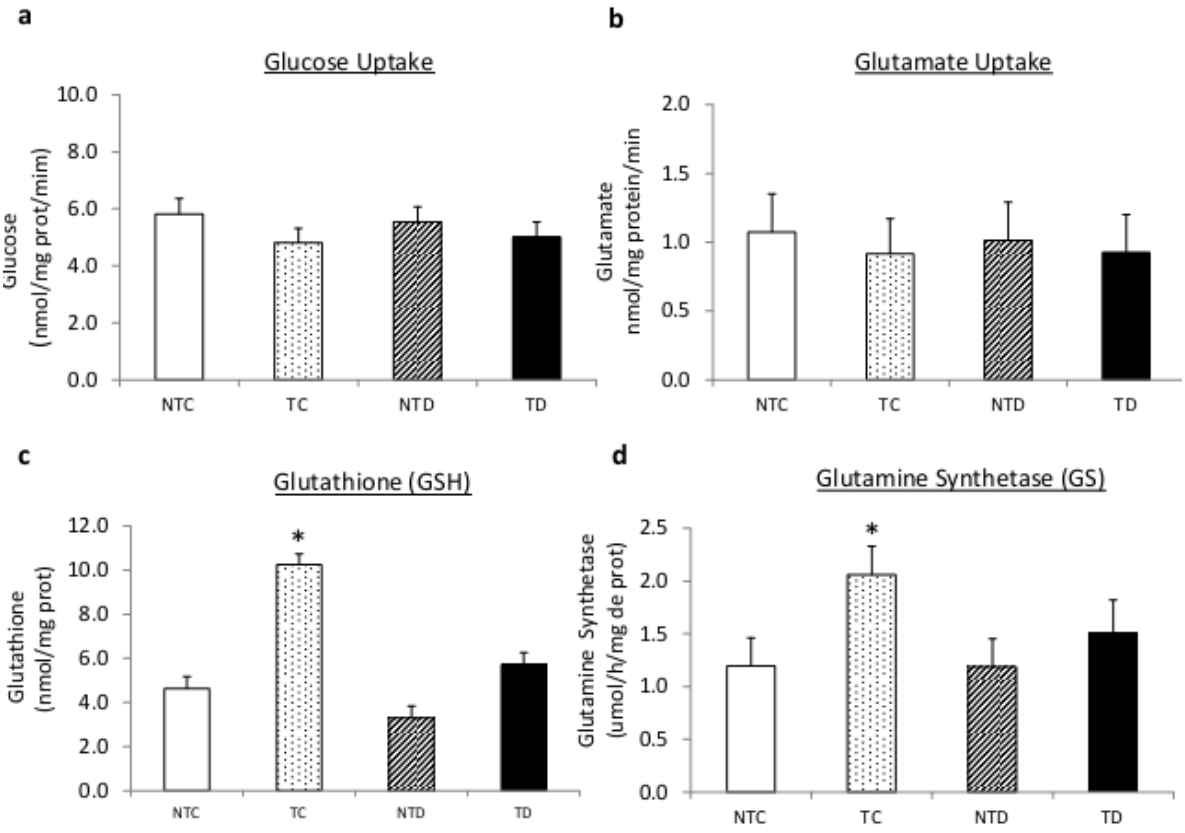


Figure 3.



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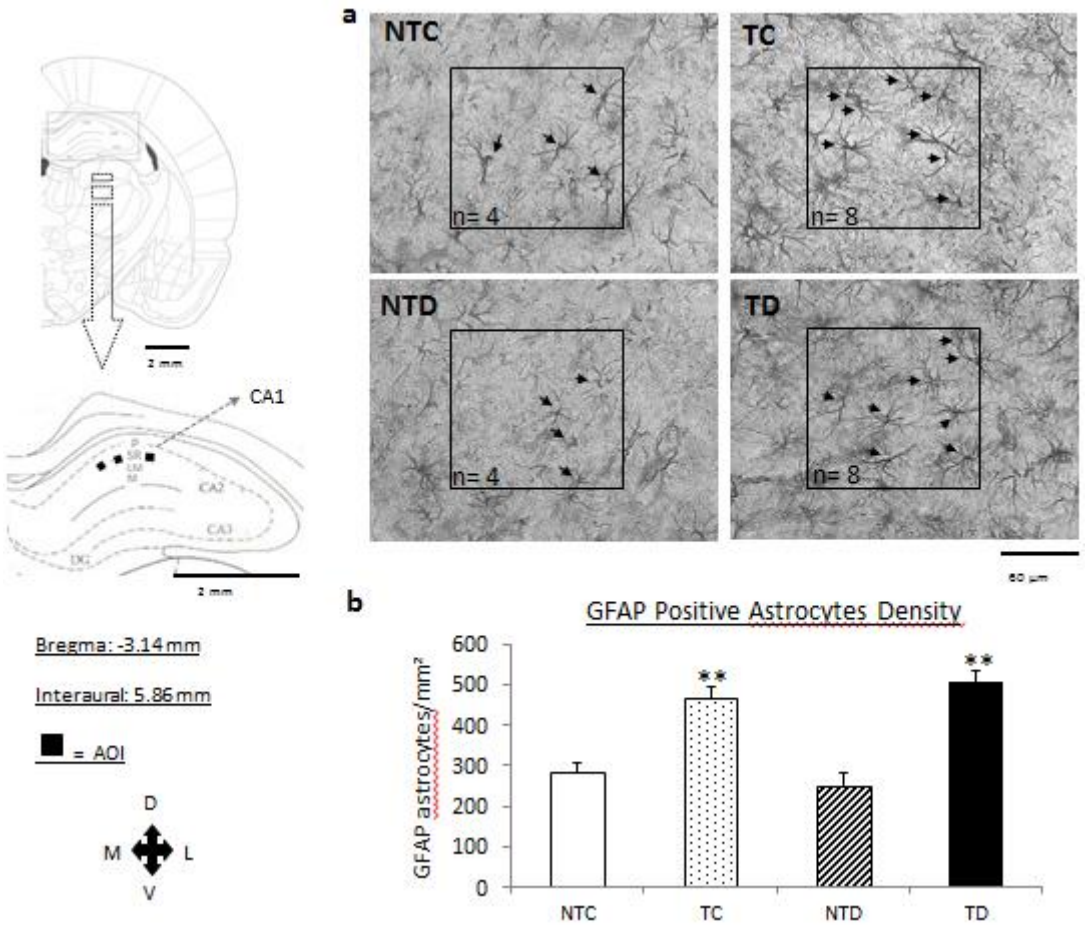
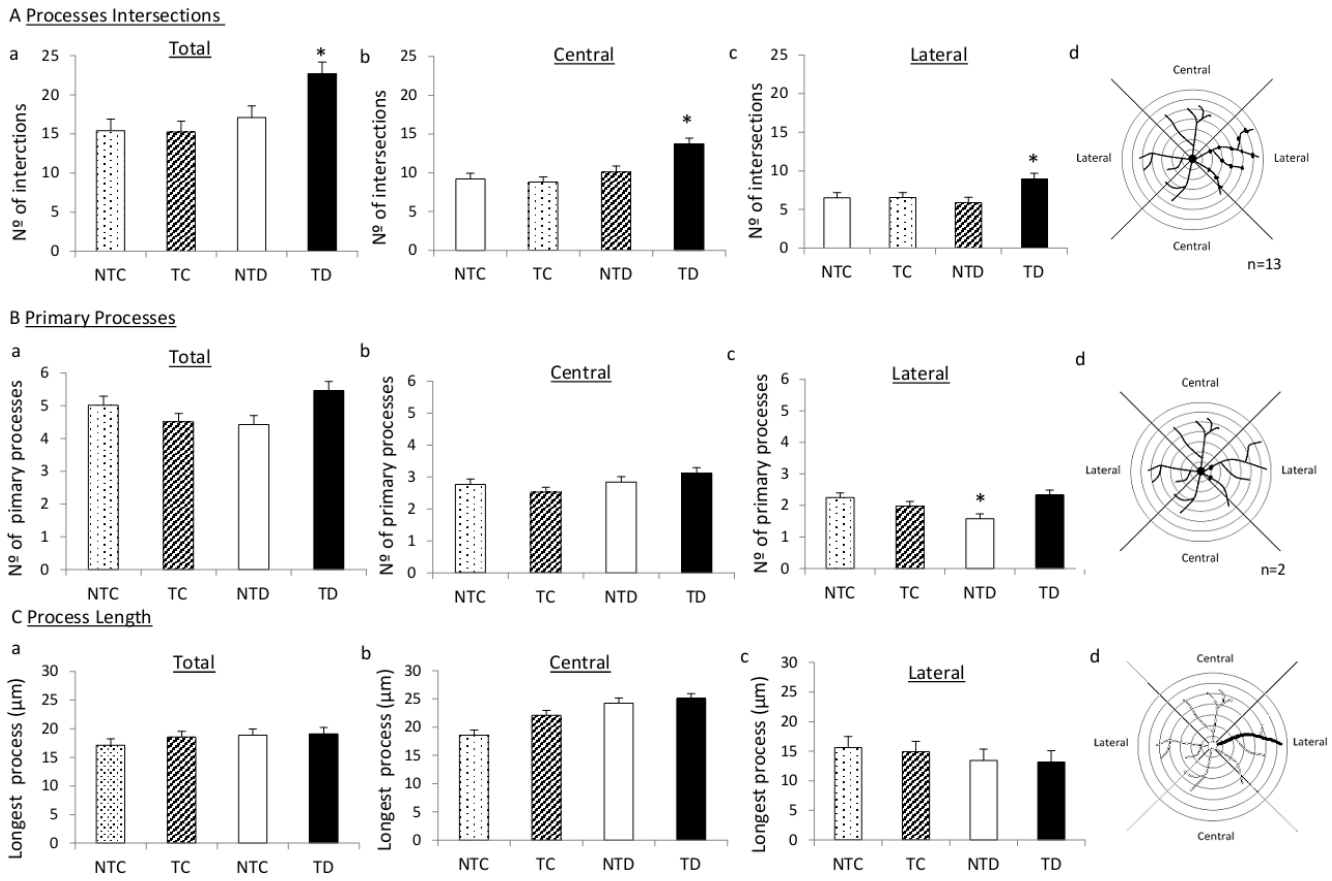

a
NTC
TC
NTD
TD
n= 4
n= 8
n= 4
n= 8
60 µm
CA1
CA2
CA3
2 mm
2 mm
Bregma: -3.14mm
Interaural: 5.86mm
= AOI
D
M
L
V
b
GFAP Positive Astrocytes Density
GFAP astrocytes/mm²
600
500
400
300
200
100
0
**
**
NTC
TC
NTD
TD

Figure 5.


A Processes Intersections
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Total
Nº of interctions
b
Central
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Nº of intersections
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Central
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n=13
NTC
TC
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TD
B Primary Processes
a
Total
Nº of pimary processes
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c
Lateral
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d
Central
Lateral
Lateral
Central
n=2
C Process Length
a
Total
Longest process (µm)
b
Central
Longest process (µm)
c
Lateral
Longest process (µm)
d
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Lateral
Lateral
Central

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Brain Research

Research Report

Physical training improves non-spatial memory, locomotor skills and the blood brain barrier in diabetic rats

Priscylla Nunes de Senna[a,b], *Léder Leal Xavier*[b], *Pamela Brambilla Bagatini*[a], *Lisiani Saur*[b], *Fabiana Galland*[c], *Caroline Zanotto*[c], *Caren Bernardi*[d], *Patrícia Nardin*[c], *Carlos Alberto Gonçalves*[a,c], *Matilde Achaval*[a,*]

[a]*Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[b]*Laboratório de Biologia Celular e Tecidual, Departamento de Ciências Morfofisiológicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[c]*Departamento de Bioquímica, Instituto de Ciências Básica da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[d]*Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil*

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ABSTRACT

Type 1 diabetes mellitus (T1DM) progressively affects cognitive domains, increases blood–brain barrier (BBB) permeability and promotes neurovascular impairment in specific brain areas. Physical exercise, on the other hand, has beneficial effects on brain functions, improving learning and memory. This study investigated the effects of treadmill training on cognitive and motor behavior, and on the expression of proteins related to BBB integrity, such as claudin-5 and aquaporin-4 (AQP4) in the hippocampus and striatum in diabetic rats. For this study, 60 Wistar rats were divided into four groups ($n=15$ per group): non-trained control (NTC), trained control (TC), non-trained diabetic (NTD), trained diabetic (TD). After diabetic induction of 30 days by streptozotocin injection, the exercise groups were submitted to 5 weeks of running training. After that, all groups were assessed in a novel object-recognition task (NOR) and the rotarod test. Additionally, claudin-5 and AQP4 levels were measured using biochemical assays. The results showed that exercise enhanced NOR task performance and rotarod ability in the TC and TD animals. Diabetes produced a decrease in claudin-5 expression in the hippocampus and striatum and reduced AQP4 in the hippocampus. Exercise preserved the claudin-5 content in the striatum of TD rats, but not in the hippocampus. The reduction of AQP4 levels produced by diabetes was not reversed by exercise. We conclude that exercise improves short-term memory retention, enhances motor performance in diabetic rats and affects important

*Correspondence to: Laboratório de Histofisiologia Comparada, Departamento de Ciências Morfológicas, ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Sarmento Leite 500, CEP 90050-170 Porto Alegre, RS, Brazil.
E-mail address: machaval@pq.cnpq.br (M. Achaval).

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structural components of the striatal BBB. The results obtained could enhance the knowledge regarding the neurochemical benefits of exercise in diabetes.

1. Introduction

Experimental evidence from human patients and animal models of diabetes has demonstrated that hyperglycemia increases blood–brain barrier (BBB) permeability, altering the levels of tight junction proteins, such as claudin-5 (Hawkins et al., 2007; Huber et al., 2006; Liu et al., 2012; VanGilder et al., 2009). In the same way, diabetes induces downregulation and mislocalization of aquaporin-4 (AQP4) in glial cells of diabetes rats (Curtis et al., 2011; Iandiev et al., 2007). However, in the literature there is little information about the effects of diabetes on the expression of claudin-5 and AQP4 in important brain regions such as the hippocampus and striatum, and how these expressions are related to cognitive and motor skills performances.

On the other hand, it is well documented that physical exercise has beneficial neurological effects in diabetes, including improvement in learning, memory and locomotor activity (Reisi et al., 2009; de Senna et al., 2011). Thus, our goals were to evaluate the effects of diabetes induced by streptozotocin (STZ) and physical exercise on cognitive and locomotor skills and claudin-5 and AQP4 expression in the hippocampus and the striatum.

2. Results

2.1. Blood glucose levels and body weight

There were no differences in the blood glucose and body weight between the NTC (97±2.1 mg/dl; 346±9.5 g), TC (98±2.0 mg/dl; 326±8.8 g), NTD (99±2.0 mg/dl; 362±8.8 g) and TD (100±2.0 mg/dl; 338±9.1 g) groups before diabetes induction ($P>0.05$). Forty eight hours after diabetic induction, and throughout the experiment, diabetic rats showed significantly higher blood glucose concentrations and weight loss when compared to control groups ($P>0.05$; data not show). At the time of sacrifice, the blood glucose levels and body weight were as follows: NTC: 106±10.8 mg/dl, 415±11 g; TC: 113±12,3 mg/dl, 389±10 g; NTD: 534±12.3 mg/dl, 270±10 g; TD: 556±12.3 mg/dl, 288±11 g ($P<0.001$ comparing control animals to diabetic animals). No significant differences were found between the diabetic groups in any of these variables ($P>0.05$).

2.2. Novel object-recognition task analysis

No differences were observed between the groups during the T1, that is, all animals spent a comparable percentage of time exploring both objects, expressed as percentage of preference for the object that was changed in the test trial. As revealed by Student's *t*-test, no differences were found for the object exploration time between trial 1 and 2 in the NTC and NTD animals ($P>0.05$), while there was an increase in the exploration index in trial 2 compared to trial 1 in the TC and TD groups ($P<0.001$; Fig. 1). In the T2, exploration time to the novel object was significantly higher in the TC and TD groups in relation to the NTC and NTD groups ($P<0.01$), and no differences were found between the NTC and NTD groups ($P>0.05$; Fig. 1).

2.3. Rotarod

Animals from group TC and TD presented a higher latency to fall when compared to those from the NTC and NTD groups ($P<0.05$; Fig. 2a). However, no differences were seen between the groups in the number of falls ($P>0.05$; Fig. 2b).

2.4. Claudin-5 and AQP4

A decrease in claudin-5 was seen in the hippocampus of NTD and TD rats when compared to the NTC and TC animals ($P<0.05$; Fig. 3a). In the striatum, claudin-5 content was significantly lower in the NTD group when compared to all groups ($P<0.05$; Fig. 3b). This decrease was prevented/reverted by exercise in the TD animals, which presented similar claudin-5 values to those found in the NTC and TC groups.

STZ-diabetes induction decreases AQP4 expression in the hippocampus and exercise failed to revert this reduction, as observed in the NTD and TD groups ($P<0.05$; Fig. 3c). AQP4

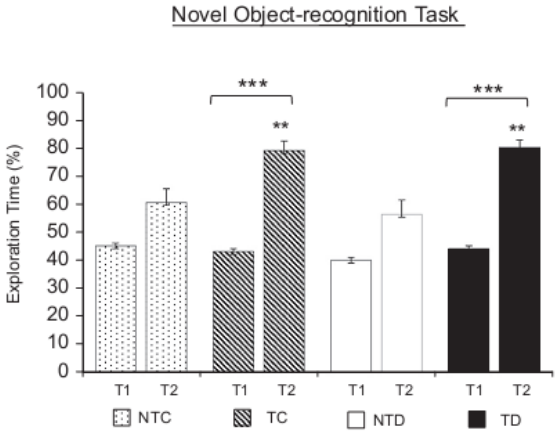

Fig. 1 – Exploration time measurements in trial 1(T1) and trial 2 (T2) of the novel object-recognition task. (*$P<0.001$), comparing T1 to T2. (**$P<0.01$) in relation to the NTC and NTD groups. Data are expressed as mean±SE. Legends: NTC=non-trained control; TC=trained control; NTD=non-trained diabetic; TD=trained diabetic.**

levels in the striatum were not affect by diabetes or exercise ($P > 0.05$; Fig. 3d).

3. Discussion

Every rat injected with STZ exhibited hyperglycemia and body weight loss throughout the experiment, and exercise did not affect these parameters. Some inconsistency exists regarding blood glucose results after exercise, as some studies have shown that exercise can reduce the blood glucose level in trained animals (Delbin et al., 2012; Hwang et al., 2011; Reisi et al., 2008), while others do not (de Senna et al., 2011; do Nascimento et al., 2011; Keeley et al., 2014; Kim et al., 2003). Different intensities and duration of exercise protocols employed may explain these findings, as the studies investigating the effect of moderate training have failed to demonstrate a benefit in glycemia, in contrast to the studies involving vigorous exercise which have reported a blood glucose benefit.

The novel object-recognition task (NOR) was performed to judge short-term memory retention. Our findings showed that diabetic rats did not exhibit damage to the short-term 60 min retention interval, in agreement with the observations made by Ceretta et al. (2012), which used a 90 min retention interval. However, it is important to note that some other studies revealed a significant effect of diabetes induction using the NOR task, applying a shorter interval between trial 1 and trial 2 and/or a longer object exploration time in each trial (Bhutada et al. 2012; King et al., 2013). Hence, studies should be conducted to clarify which NOR task protocol is the most sensitive regarding short-term memory in diabetic animals.

Physical training enhances object-recognition memory performance in TC and TD animals. Exercise can improve object recognition memory (Griffin et al., 2009; Hopkins, Bucci, 2010), but also impair it (Drumond et al., 2012; Mello et al., 2008), depending on the duration and type of activity, and on the integrity of the perirhinal cortex and hippocampus (García-Capdevila et al., 2009). Our results demonstrated that both exercise groups had stronger preference for the novel object, indicating that treadmill running, at moderate intensity, is able to enhance the performance in a non-spatial memory task in control and diabetic rats.

In the rotarod test, the training protocol positively influenced the retention time on the rotating rod, as the TC and TD rats showed a greater latency to fall than the NTC and NTD groups. It has been shown that treadmill training prevented abnormal mechanical sensitivity and recuperates tyrosine hydroxylase in the substantia nigra pars compacta in trained diabetic animals, which may contribute to greater motor coordination and balance in the rotarod rod test (do Nascimento et al., 2011, 2013). Thus, our results support the idea that exercise contributes to motor learning.

Claudins are the principal barrier-forming proteins, and claudin-5 is especially important in actively regulating small molecule permeability (Redzic, 2011). It has been reported that in diabetes progression, the extent of microvascular leakage to small molecules increases (Hawkins et al., 2007; Huber et al., 2006; Karolczak et al., 2012; Sajja et al., 2014). This increase in neurovascular permeability is probably related to claudin-5 reduction, as found in the NTD and TD groups. To support this idea, it has been reported that high glucose levels reduce the expression of tight-junction proteins, such as claudin-5, in cell culture (Liu et al., 2012).

Exercise was unable to reverse the hippocampal decrease in claudin-5 expression produced by diabetes. On the other hand, claudin-5 was also reduced in the striatum of NTD animals, but this decrease was not observed in the TD rats. These differences between striatum and hippocampus could be related to levels of matrix metalloproteinases (MMP) in these regions. Claudin-5 degradation and BBB breakdown have been associated to enhanced MMP-2 and MMP-9 activity and expression (Aggarwal et al., 2015; Chiu and Lai, 2014; Feng et al., 2011). High levels of MMP have been documented in the brain of diabetic animals and in the plasma of diabetic patients (Chen et al., 2011; Derosa et al., 2007; Lee et al., 2005; Zhao et al., 2013). Exercise decreased MMP-9 activity in the striatal area (Chaudhry et al., 2010; Guo et al., 2008), while in the hippocampus physical training increased MMP-9 activity (Nishijima et al., 2015), suggesting that exercise could promote hippocampal claudin-5 degradation, or, at least, does not induce increases in the levels of this protein, as presented in our findings.

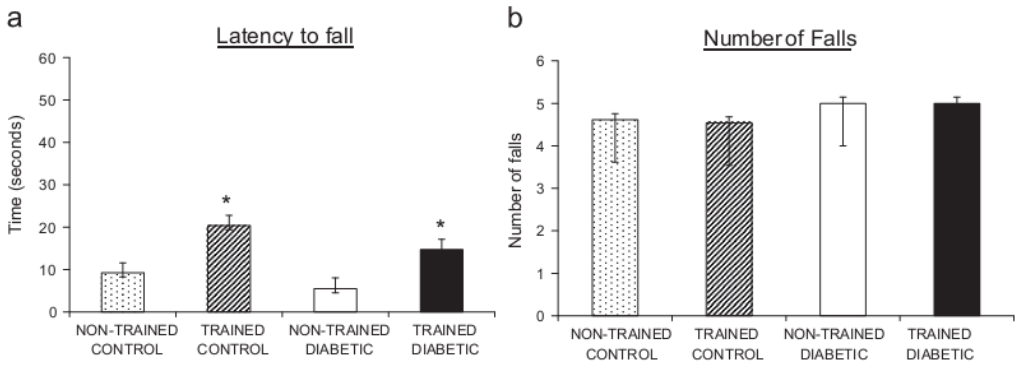

Fig. 2 – Motor skills measurements (mean ± SEM) in rotarod test after the physical exercise. Graphs (a) latency to fall; (b) number of falls. *$P < 0.05$ when compared with non-trained control and non-trained diabetic groups. Data are expressed as mean ± SE.

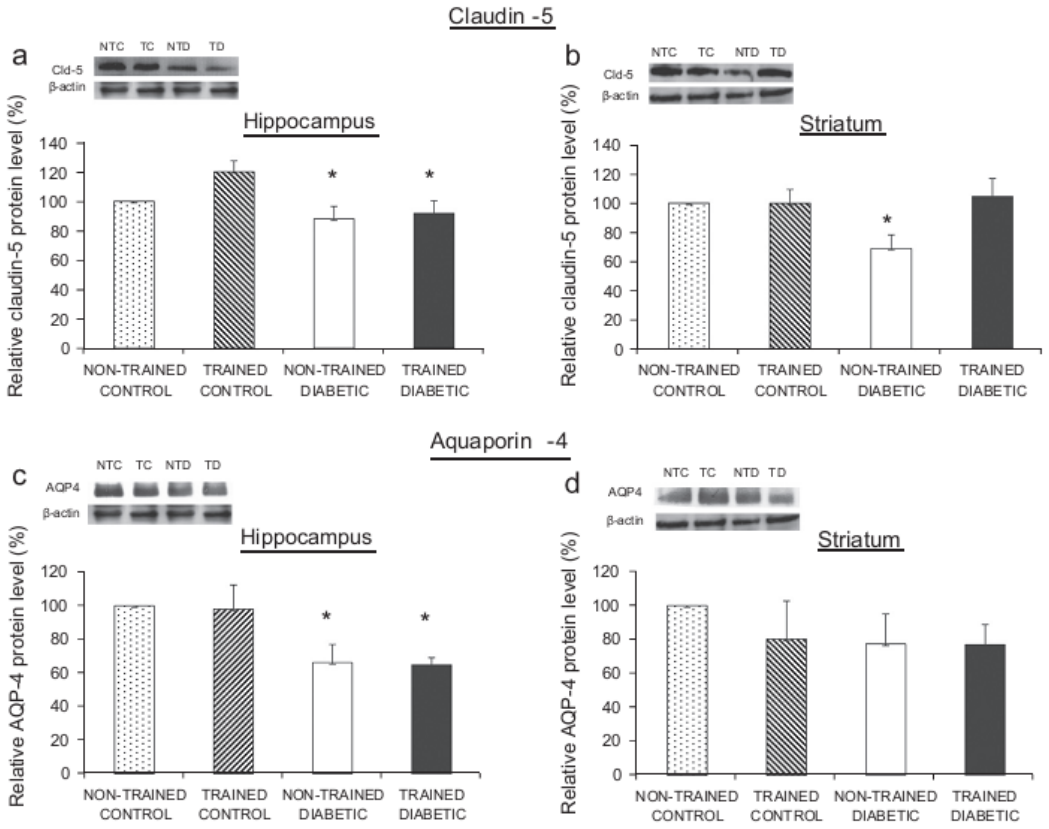

Fig. 3 – Graphs: claudin-5 in hippocampus (a) with *P<0.05 when compared with non-trained control and trained control groups, and in striatum (b) with *P<0.05 comparing all groups. AQP4 protein in hippocampus (c) (*P<0.05 when compared with non-trained control and trained control groups), and AQP4 in striatal slices (d). Western blots images are expression in all graphs. Legends: Cld5=claudin-5; AQP4=aquaporin-4. Data are expressed as mean±SE.

Previous studies have demonstrated that AQP4 proteins are downregulated and mislocalized in retinal glial cells in diabetic rats (Curtis et al., 2011; Iandiev et al., 2007), but no one has attempted to elucidate AQP4 expression in other brain structures in diabetes. Our study showed that AQP4 expression decreases in the hippocampus of diabetic animals and is unchanged in the striatum. Given that diabetic animals presented a decrease in claudin-5 expression in the hippocampus and the striatum, we would have expected there to be increases in AQP4 protein in these brain areas, presumably due to BBB impairment and in order to clear the brain parenchyma of water (Wang et al., 2014). It was reported that AQP4 is upregulated in the diabetic retina as a compensatory response to retinal edema (Cui et al., 2012). On the other hand, AQP4 downregulation might be a consequence of astrocytic changes in hippocampus (de Senna et al., 2011). Thus, our results provide evidence that the AQP4 system is impaired in the diabetic hippocampus, which in association with BBB permeability, may induce imbalanced water homeostasis (Chiu et al., 2013), and since no differences were found in the striatum it may be that regional differences exist in the AQP4 system in diabetes.

Furthermore, the unchanged results for hippocampal and striatal AQP4 in the TC group were surprising because AQP4 is strongly expressed in astrocytic endfeet abutting the abluminal membrane area of cerebral capillaries, and since running exercise induces astrocyte proliferation coupled to angiogenesis, we expected AQP4 expression would be enhanced by exercise (Borght et al., 2009; Li et al., 2005; Nagelhus and Ottersen, 2013; Saur et al., 2013).

All results showed that exercise was able to enhance memory performance in the TD rats, despite the decreased hippocampal levels of claudin-5 and AQP4. Previous studies showed that AQP4 is important in memory consolidation and synaptic plasticity (Fan et al., 2013; Skucas et al., 2011), while an increase in claudin-5 expression was unable to improve cognition (Zhao et al., 2012). Since each element within the neurovascular unit is important to the integrity of the BBB and given that different brain circuitry and biochemical events are required in the NOR task (Francis et al., 2010; Intlekofer et al., 2013), other brain mechanisms, such as astrocytic alterations and increased Brain-derived Neurotrophic Factor (BDNF), may have been induced by the physical exercise and thus compensated the breakdown of the BBB and contributed to cognitive performance (de Senna et al., 2011; Hamm et al., 2004; Intlekofer et al., 2013; Li et al., 2005; Saur et al., 2013; Stranahan et al., 2009). Furthermore, BBB disruption has been associated to functional deficit in the rotarod test (Ding et al., 2014), while an improvement in locomotor behavior was seen when BBB damage was

attenuated (Amenta et al., 2012; Jang et al., 2014; Yu et al., 2012). Thus, we believe that the striatal levels of claudin-5 and other positive neurochemical effects promoted by exercise are related to the satisfactory locomotor behavior found in the TD animals.

In conclusion, the present study provides data to show exercise enhances performance in a non-spatial memory task in normal and, for the first time, in diabetic rats, and that our running protocol is also able to improve motor ability and thus support the idea that running exercise improves motor ability. We also showed that claudin-5 expression is decreased in the hippocampus and striatum of diabetic rats, which may reflect BBB impairment in these brain regions, and that exercise can protect the striatal BBB in training diabetic rats. Finally, our study provides the first reported evidence of decreased hippocampal AQP4 protein in diabetes, suggesting that brain osmotic equilibrium is probably disrupted in T1DM.

4. Experimental procedures

4.1. Animal model

The experiment used three-month old male Wistar rats, weighing about 270–400 g, obtained from a local breeding colony (ICBS, *Universidade Federal do Rio Grande do Sul*, Brazil). The animals were housed in standard plexiglass boxes (three per cage) and kept in a temperature-controlled colony room with food and water available ad libitum and maintained under a 12:12 light/dark cycle (lights on at 8:00 h). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and the Brazilian Laws (11794/2008) for animal care and ethical use of animals.

4.2. Experimental design

Sixty animals were randomly divided equally into four groups as follows: non-trained control (NTC), trained control (TC), non-trained diabetic (NTD) and trained diabetic (TD).

4.3. Diabetic model

After a 6 h fasting period, T1DM was induced with a single intravenous injection into the tail vein of streptozotocin (STZ) (Sigma Chemicals, Co., USA) dissolved in 0.01 M citrate buffer, pH 4.5 (50 mg/kg of corporal weight). Control rats received only vehicle (citrate buffer). Diabetes was verified by glycemia levels greater than 300 mg/dl, 48 h post-injection (de Senna et al., 2011). Blood glucose concentrations were evaluated in blood collected from rat-tail using a glucose test strip (Acon Laboratorie, INC. San Diego, CA 92121, USA), following a 5 h morning fasting. On the 20th day after diabetes induction, all animals underwent adaptation to a treadmill apparatus.

4.4. Treadmill adaptation and maximal exercise test (MET)

Rats were habituated with the treadmill apparatus to minimize novelty stress by walking for 10 min at 5 m/min for 4 days. On the fifth day, the rats were submitted to the MET, which consisted of a graded treadmill exercise with speed increments of 5 m/min every 3 min, starting at 5 m/min and continuing up to the maximal intensity attained by each rat. The values obtained in the MET were used to plan the treadmill training program as a moderate intensity exercise protocol, which started in the 5th week after diabetes induction (de Senna et al., 2011).

4.5. Training protocol

The physical exercise consisted of running on the treadmill for 20 min on the first day. This training period was progressively increased everyday up to 60 min on the sixth day, which was maintained for the next 4 weeks. Each training session included a warm up period of 5 min running at 30% of the maximal speed reached in the MET (4.5 m/min for TC and TD group), 10–50 min running at 50–60% (~8.25 m/min for TC and TD group) and 5 min recovery at 30%, 5 sessions per week, once a day during 5 weeks, at 0° of inclination. The training program was considered moderate-intensity endurance (de Senna et al., 2011). One day after the last training session, all animals were submitted to a short-term memory test.

4.6. Novel object-recognition task (NOR)

All animals were habituated to the open field apparatus ($50 \times 60 \times 40$ cm^3) one day before the memory task. The rats were gently placed in the center of the box and released to explore the environment for 3 min. Testing consisted of two trials, each lasting 5 min. In the first trial (T1), two different objects were placed in the testing box. Trial two (T2) was conducted in the same manner as T1, but with a new object in place of one object from T1. The inter-trial interval was 1 h. The objects differed in shape, surface, color, contrast and texture. All the tests were recorded using a mounted digital video camera. The times spent exploring the sample and the novel objects were recorded separately. The exploration time of both objects in T1 was calculated as "exploration time of the new object/sum of exploration of both objects" to check any possible object preference (Das et al., 2005).

4.7. Rotarod test

On the day after the memory task, the rats were trained to remain on the rotarod apparatus (Insight, Brazil) with the speed adjusted to 12 rpm for 60 s. The following day, the rats were tested in the apparatus with the speed adjusted to 16 rpm for five trials with 60 s of duration. The latency to fall (data presented as the mean of the five trials) and the number of falls were evaluated (do Nascimento et al., 2011).

4.8. Biochemical analysis

Five days after the end of the training session, 28 animals ($n=7$ per group) were anesthetized with an intramuscular injection of ketamine and xylazine (75 and 10 mg Kg^{-1}, respectively) and then euthanized by decapitation. The brains were removed and the hippocampi and striata were quickly dissected on ice. Hippocampal and striata samples were then used for biochemical measurements, described as follows.

4.8.1. Claudin-5 protein and aquaporin protein (AQP4)

The samples were homogenized in sample buffer containing 0.0625 M Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and after it was boiled for 5 min. Proteins were measured and then added in the samples 5% (w/v) β-mercaptoethanol and 0.002% (w/v) bromophenol blue. After, electrophoresis was performed in 12% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). Anti-claudin-5 (Millipore) or anti-AQP4 (Invitrogen) was used at a dilution of 1:2000. After incubating with the primary antibody for 1 h at room temperature, filters were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:4000. The chemiluminescence signal was detected using an ECL kit from Amersham (Zanotto et al., 2013). The protein content was measured by Lowry's method using bovine serum albumin as standard (Lowry et al., 1951).

4.9. Statistical analysis

Data for all variables are expressed as mean ± standard error. Repeated measures analysis of variance (ANOVA) was used for blood glucose and weight comparisons between groups. Differences between exploration times in trial 1 and trial 2 in the NOR task were analyzed using a paired (Student's) t test. Two-way ANOVA followed by Newman–Keuls post-hoc were used to analyze all other data. STATISTICA 9 software was used in the statistical analyses were ($P<0.05$).

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DISCUSSÃO

Este trabalho teve como objetivo geral avaliar os efeitos do exercício físico sobre o comportamento cognitivo e motor, parâmetros astrogliais e BBB, em ratos adultos diabéticos induzidos por STZ.

No primeiro artigo apresentado nesta tese, demonstramos que o déficit de memória espacial observado após 30 dias da indução do DM1 por STZ é revertido com o treinamento físico. Este dado foi associado ao aumento da densidade de células GFAP positivas e ao aumento da ramificação dos prolongamentos astrocíticos, sendo estes, portanto, possíveis mecanismos de proteção aos danos no SNC induzidos pelo diabetes e participantes na melhora da memória espacial encontrada nos ratos treinados (SAMPEDRO-PIQUERO et al., 2014).

As possíveis bases neuroquímicas do processo de aprendizagem e de memória – o potencial de longa duração (LTP) e a depressão de longa duração (LTD) – mostram-se alteradas no DM1. Estudos têm demostrado que o DM1 inibe a indução do LTP e facilita a depressão de longa duração no hipocampo (KAMAL et al., 1999, 2006; STEVENS, 2008; STRANAHAN et al, 2010). Alterações moleculares como redução da expressão da subunidade NR2B dos receptores NMDA e da subunidade GluR1 dos receptores AMPA, redução da fosforilação da proteína quinase dependente de cálcio/calmodulina (CaMKII) foram demonstradas em animais com DM1 (GARDONI et al., 2002; KLEIN; WAXMAN, 2003; VISWAPRAKASH et al., 2015). Estas e outras alterações neuroquímicas possivelmente estão relacionadas aos déficits de memória espacial encontrados nos animais diabéticos, em nosso estudo.

O exercício, por outro lado, induz neurogênese e plasticidade sináptica. O treinamento físico eleva a expressão de sinaptofisina, da CaMKII, de BDNF, de subunidades dos receptores de glutamato, facilitando o LTP (REISE et al., 2008; VAN PRAAG et al., 1999; VOSS et al., 2013).

Os astrócitos, ao liberarem gliotransmissores como o aminoácido D-serina, estão envolvidos na indução do LTP através da regulação da ativação de receptores de glutamato do tipo NMDA. Em nosso estudo demonstramos que o exercício físico aumenta as ramificações astrocitárias, e possivelmente este aumento faz com que os astrócitos tenham uma maior influência sobre a sinapse neuronal, reforçando o processo de aprendizado e de consolidação da memória (HENNEBERGER et al., 2010; SAMPEDRO-PIQUERO et al., 2014; SAUR et al., 2013; WENZE et al., 1991).

O grau da expressão de GFAP nos astrócitos, juntamente com a análise da densidade, da forma e do tamanho dos astrócitos está intimamente relacionada com o tipo de resposta que será apresentada por estas células (BRIONES et al., 2009). O aumento da expressão de GFAP nos astrócitos pode indicar a existência de astrócitos hipertrofiados, apresentando maior imunorreatividade para GFAP no soma, com incapacidade de manter o equilíbrio osmótico, causando dano neuronal. Estes astrócitos hipertróficos possuem prolongamentos mais grossos, muitas vezes mais longos – astrogliose anisomórfica –, exacerbam o dano no tecido nervoso pela liberação de citocinas inflamatórias e ROS, e são frequentemente associados à cicatriz glial. Por outro lado, astrócitos que adquirem um formato estrelado – astrogliose isomórfica – aumentam sua produção de enzimas citosólicas, de antioxidantes, de proteínas estruturais, de fatores de crescimento, favorecendo a remodelação tecidual e a restauração da homeostase (LIBERTO et al., 2004; ROCHA et al., 1998).

Na literatura, apesar de existirem estudos sobre a expressão de GFAP no SNC de animais diabéticos, são poucos os que associaram esta mensuração à densidade celular e à morfologia destas células. Aparentemente, ocorre uma resposta astroglial tempo-dependente ao diabetes mellitus, indicando diferentes respostas e possíveis adaptações dessas células frente ao dano hiperglicêmico. Os dados da literatura, porém, ainda são controversos quanto ao que representam estas adaptações astrogliais (LEBED et al., 2008; NAGAYACH et al., 2014).

No estudo de Lebed et al (2008) foi demonstrado, no hipocampo de animais com diabetes induzido por STZ, uma variação dos níveis de GFAP juntamente com alterações fenotípicas dos astrócitos nos primeiros três, sete e 14 dias após a indução do DM1. Inicialmente, foi identificado na região CA1 do hipocampo dos animas diabéticos uma redução de 20% do número de células GFAP positivas, acompanhado de uma redução em 44% da área de secção transversa destas células em relação aos animais controle. Aos sete dias, houve uma recuperação destes parâmetros morfológicos nos animais diabéticos, os quais apresentaram um aumento em densidade e em área astrocitária. Este aumento de área mostrou-se significamente maior após 14 dias da indução do DM1 por STZ. Diferentemente, a densidade de astrócitos mostrou-se similar entre animais diabéticos e controles aos 14 dias da indução do DM1.

A análise do conteúdo de GFAP no citosol e no citoesqueleto foi realizada no estudo de Lebed et al. (2008). A redução inicial da área dos astrócitos nos animais diabéticos foi associada à menor arborização destas células, uma vez que houve redução

da expressão de GFAP no citoesqueleto, sem diferença na expressão de GFAP citosólica. Aos 14 dias, por outro lado, demonstrou-se o dobro da marcação de GFAP no citosol, característica de hipertrofia, e mais que o quádruplo no citoesqueleto dos animais diabéticos em relação aos animais controle. Desafortunadamente, os pesquisadores analisaram apenas o perímetro e a área dos astrócitos, e não o comprimento e a ramificação dos prolongamentos astrocitários de forma direta, o que poderia estimar a arborização destas células. Uma vez que demonstraram um aumento do perímetro destas células em associação à hipertrofia celular, é provável que aos 14 dias da indução do diabetes STZ haja a presença de uma astrogliose anisomórfica.

De forma semelhante, após 11 dias da indução do diabetes, o estudo de Revsin et al. (2009) também demonstrou aumento da expressão de astrócitos GFAP positivos no *stratum radiatum* de CA1 hipocampal, associado ao déficit cognitivo. Ao tratar os animais com mifepristona, um antagonista de receptores glicocorticoide, estas alterações foram revertidas. Com 20 dias de DM1, também foi encontrado aumento da expressão de GFAP na região de CA1 (SARAIVA et al., 2006).

Após quatro semanas da indução de STZ, os dados na literatura são controversos. Tanto uma diminuição quanto um aumento dos níveis de células GFAP positivas foram relatados, o que ressalta a importância da avaliação do número e da morfologia dos astrócitos para uma melhor compreensão do papel adaptativo destas células (PEKNY; PEKNA, 2014b).

No estudo de Baydas et al. (2003a,b, 2008), seis semanas após a indução do diabetes por STZ foi demonstrado um aumento da expressão de GFAP hipocampal, concomitante ao aumento da peroxidação lipídica, e cujas alterações foram revertidas com administração de vitamina E e melatonina, ambas antioxidantes. Diferentemente, no estudo de Coleman et al. (2004), o conteúdo de astrócitos GFAP imunorreativos no hipocampo mostrou-se reduzido após quatro e oito semanas da indução do diabetes, sem alteração no número destas células.

A redução na expressão de astrócitos GFAP positivos, além de poder designar morte celular, também pode ser atribuída aos níveis alterados de secreção de insulina e aos níveis aumentados da proteína S100β no DM1. A insulina, quando presente, aumenta a imunorreatividade de células GFAP positivas ao induzir a transcrição gênica desta proteína (COLEMAN et al., 2010; TORAN-ALLERAND et al., 1991). A proteína S100β, produzida e secretada pelos astrócitos, pode inibir a fosforilação de GFAP (FRIZZO et al., 2004; LEBED et al., 2008).

Deste modo, em nosso estudo, o aumento da densidade astrocitária concomitante à ramificação dos prolongamentos astrocíticos nos animais diabéticos treinados deve indicar astrogliose isomórfica, possivelmente facilitando a comunicação neurônio-glia, remodelando a sinapse neuronal afetada com a hiperglicemia, e, assim, contribuindo para o desempenho cognitivo (HENNEBERGER et al., 2010; REISI et al., 2008; SASAKI-HAMADA et al., 2012; WENZEL et al., 1991).

Em nosso estudo, não encontramos alterações no metabolismo do glutamato e na captação de glicose nos animais diabéticos. Alguns trabalhos descrevem que o metabolismo glial está afetado no diabetes, contribuindo para a encefalopatia diabética (DUARTE et al., 2009; GARCIA-ESPINOSA et al., 2003; SICKMANN, WAAGEPETERSEN, 2014). Em pacientes com a doença de Alzheimer, por exemplo, a captação de glicose está prejudicada, sendo em parte atribuída a menor captação de glicose glial (ALEXANDER et al., 2002).

A literatura científica mostra resultados antagônicos para o metabolismo da glicose no diabetes mellitus. No estado hipoglicêmico, foi demonstrado aumento da concentração de glicose encefálica e aumento de seus transportadores na BBB, enquanto no estado hiperglicêmico os transportadores estão reduzidos (CRIEGO et al., 2005; KORANYI et al., 1991). Outros estudos, porém, tanto em modelo animal quanto em humanos com DM1, não encontraram diferença na concentração ou no transporte de glicose no SNC no estado hiperglicêmico (DUARTE et al., 2009; FANELLI et al., 1998; SEAQUIST et al., 2005 SIMPSON et al., 1999; WANG et al., 2010).

É possível que estas discrepâncias de dados estejam relacionadas com as diferentes metodologias de análise, intensidade da hiperglicemia, e com o tempo de evolução do diabetes (SICKMANN, WAAGEPETERSEN, 2014). Entretanto, é sugerido que o SNC seja mais eficaz em promover ajustes protetores e compensatórios ao estado hipoglicêmico que ao hiperglicêmico (BINGHAM et al., 2002; SEAQUIST et al., 2005; SIMPSON et al., 1999).

Apesar de os resultados para o metabolismo do glutamato (GLU) também parecerem dúbios no diabetes, existem evidências de que este não se encontre alterado na hiperglicemia (COLEMAN et al., 2010; KADE et al., 2009). Utilizando análise por espectroscopia de ressonância magnética nuclear, o estudos de DUARTE et al. (2009) e Wang et al. (2012) não demonstraram diferença na captação de GLU hipocampal e cortical no diabetes. Na retina de animais diabéticos, também não foi encontrado

aumento da captação pelos transportadores de GLU do tipo EAA-T1 (MYSONA et al., 2009).

Os astrócitos são elementos chave no metabolismo do GLU, uma vez que são responsáveis por 90% da sua captação na fenda sináptica e pela sua conversão à glutamina. Além do mais, são células essenciais na defesa antioxidante pela produção de GSH (SCHOUSBOE et al., 2013). Um estudo em modelo animal de demência, embora não tenha demonstrado alteração na captação de glutamato, relatou disfunção astrocitária ao observar uma redução da atividade da GS e dos níveis de GSH, o que o diverge de nossos resultados (COSTA et al., 2012). Assim, com base em nossos resultados para captação de GLU, níveis de GS e GSH, e de acordo com os demais dados da literatura, é possível que as funções astrocitárias associadas ao metabolismo do GLU e à defesa antioxidante não estejam alteradas, pelo menos, após nove semanas da indução do DM1 por STZ.

O protocolo de exercício utilizado em nosso trabalho foi capaz de influenciar o metabolismo do GLU nos animais TC, mas não nos animais DT. O exercício, afortunadamente, também protege do estresse nitrosativo, o que pode ter ocorrido em nosso estudo, influenciando o comportamento cognitivo (RODRIGUES et al., 2010; SANTIN et al., 2010).

Em nosso segundo artigo, demostramos que o exercício físico de intensidade moderada atua positivamente sobre a memória não espacial em animais diabéticos, e corroboramos com os dados encontrados na literatura para animais saudáveis (GRIFFIN et al., 2009; HOPKINS et al., 2010).

Estudos em humanos e em animais trazem evidências do benefício do exercício físico sobre o aprendizado e a memória (ALAEI et al., 2008; ALBECK et al., 2006; BUCK et al., 2008; QIANG et al., 2006). Para estes benefícios, a intensidade do exercício físico tem sido reconhecida como fator crítico, uma vez que exercício de alta intensidade induz resposta inflamatória, estresse oxidativo, apoptose neural (BLUSTEIN et al., 2006; DE ALMEIDA et al., 2013; GARCÍA-CAPDEVILA et al., 2009; LI et al., 2013; SHIH et al., 2013). Nosso estudo confirma que uma intensidade moderada de treino deve ser priorizada.

Com nossos resultados para o teste de rotarod, renovamos a importância da prática de atividade física para melhorar o desempenho motor (DO NASCIMENTO et al., 2011). O exercício físico nos pacientes com diabetes mellitus pode ser uma importante estratégia para prevenir ou reduzir o risco de quedas, uma vez que

instabilidade postural, alteração da marcha, fraqueza muscular, diminuição da propriocepção, estão frequentemente presentes nestes indivíduos (LIM et al., 2014; STRECKMANN et al., 2014).

Os efeitos benéficos do exercício sobre a habilidade motora podem ser atribuídos aos seus efeitos tanto sobre o sistema nervoso periférico quanto sobre o SNC. O treinamento físico melhora parâmetros morfométricos do nervo sural e ciático, aumenta o volume de neurônios do tipo A do gânglio da raiz dorsal, protege contra o déficit da velocidade da condução nervosa e mantém a sensibilidade aos estímulos mecânicos e sensitivos (DO NASCIMENTO et al., 2010; MALYSZ et al., 2010; SHANKARAPPA et al., 2011). Em estruturas como córtex motor, cerebelo, estriado, o treino motor eleva os níveis de neurotrofinas, como BDNF, aumenta a densidade da substância cinzenta, induz angiogênese e plasticidade sináptica, promove proliferação neural e glial, e reverte a diminuição da imunorreatividade à tirosina hidroxilase na substância nigra observada em animais diabéticos (DO NASCIMENTO et al., 2011; LAN et al., 2014; LEE et al., 2014; LI et al., 2005; SUMIYOSH et al., 2014; WANG et al., 2015).

Além disso, é possível que um melhor desempenho motor possa ser atribuído ao aumento dos níveis de claudina-5 no estriado, como demonstrado em nosso segundo estudo, uma vez que a integridade da BBB está associada ao desempenho motor (AMENTA et al., 2012; DING et al., 2014). Ratos com lesão encefálica por trauma quando tratados com lítio, droga capaz de inibir a atividade de metaloproteinases de matriz (MMP), e logo, de inibir a degradação das junções oclusivas (*tight junction* – TJ), apresentam maior latência na tarefa do rotarod (YU et al., 2012). Estudos têm mostrado que o exercício físico reduz a atividade das MMP (CHAUDHRY et al., 2010; GUO et al., 2008).

Outros achados importantes de nosso segundo estudo estão relacionados com a BBB. Demonstramos que o diabetes mellitus diminui a expressão de claudina-5 no hipocampo e no estriado, reduz a expressão de AQP4 no hipocampo, e que o exercício é capaz de preservar o conteúdo de claudina-5 no estriado.

A BBB é uma estrutura protetora do SNC e a perda de sua integridade expõe o tecido encefálico a concentrações lesivas de substâncias da circulação periférica, como proteínas, aminoácidos, neurotransmissores, afetando a homeostase e a sinalização neural (ABBOTT et al., 2010). O diabetes mellitus, além de estar diretamente associado ao déficit cognitivo, é fator de risco para demências como doença de Alzheimer, e a

perda da integridade da BBB possivelmente é correlacionada a esta patologia (LIU et al., 2014).

Um dos principais determinantes para disfunção da BBB no diabetes mellitus é a disfunção das TJ. Em modelo de diabetes por STZ, ocorre uma redução da expressão das proteínas ocludina, claudina-5 e das zônulas de oclusão (ZO) no tecido encefálico (CHEHADE et al., 2002; HAWKINS et al., 2007; LIU et al., 2012). Sugere-se que uma maior atividade de metaloproteinases de matriz (MMP), em especial MMP-2 e MMP-9, induzida pelo estresse oxidativo, seja responsável pela alteração das TJ (KADOGLOU et al., 2005; HAWKINS et al., 2007). Além do mais, a ativação da PKCβ no estado hiperglicêmico induz a fosforilação das proteínas das TJ, aumentando a expressão de fator de crescimento do endotélio vascular (VEGF), comprometendo a BBB (ARGAW et al., 2009; HAORAH et al., 2007; PRASAD et al., 2014; SAJJA et al., 2014; SHIMIZU et al., 2013).

O papel benéfico do exercício físico sobre a BBB tem sido bem demonstrado em estudos com animais submetidos à evento isquêmico. O treinamento físico, como citado anteriormente, protege a lâmina basal endotelial e reduz a atividade de MMP no córtex cerebral e no estriado (GUO et al., 2008; HE et al., 2014). Em contrapartida, o exercício aumenta a atividade MMP no hipocampo, o que pode justificar a diferença entre os nossos achados para claudina-5 no hipocampo e no estriado (NISHIJIMA et al., 2015).

Apesar de não termos encontrados um aumento de claudina-5 no hipocampo dos animais do grupo TD, o exercício físico melhorou o desempenho cognitivo nestes animais. Há evidências de que a disfunção da BBB não é unicamente causada por alteração nas TJ e que claudina-5 não é essencial para a consolidação da memória e para a plasticidade sináptica (FAN et al., 2013; SKUCAS et al., 2011. ZHAO et al., 2012).

Em pacientes com a doença de Alzheimer, por exemplo, foi demonstrado aumento da permeabilidade da BBB sem dano na TJ (VIGGARS et al., 2011), e corroborando com estes achados, foi observado, em meio de cultura, aumento da permeabilidade paracelular com a remoção dos astrócitos e não com a perda da TJ (HAMM et al., 2013). Estes dados fortalecem a hipótese de que em nosso trabalho outros mecanismos induzidos pelo treinamento físico, como o aumento e a adaptação morfológica das células astrocitárias – componente importante da unidade neurovascular, e apontado em nosso primeiro artigo –, devem compensar o dano da BBB, contribuindo para a melhora cognitiva (DE SENNA et al., 2011; SAUR et al., 2013).

Em relação à proteína AQP4, nossos achados nos animais diabéticos salientam a necessidade de uma maior atenção sobre o desequilíbrio hídrico na encefalopatia diabética. As células astrocitárias, através da expressão de AQP4, participam do sistema glinfático. A deleção de AQP4 glial facilita edema vasogênico. Além do mais, o sistema glinfático participa na remoção de solutos intersticiais como β-amiloide, e o seu comprometimento pode estar associado à suscetibilidade à doença de Alzheimer nos indivíduos com diabetes mellitus (ILIFF; NEDERGAARD, 2013; SIMS-ROBINSON et al., 2010; THRANE et al., 2014).

Alterações da expressão de AQP4 são frequentemente observadas em associação com astrogliose. Em estudos que realizam lesão encefálica isquêmica e traumática de intensidade moderada e severa é relatado aumento da expressão de AQP4 associado à cicatrização glial. No dano encefálico por microinfarto foi demonstrado expressão elevada de AQP4 nas regiões com gliose após sete dias da lesão, com normalização dos seus níveis após 14 dias do microinfarto. Foi identificado que também ocorre uma redistribuição da AQP4 glial, com uma redução da sua expressão perivascular e um aumento no soma glial (ILIFF; NEDERGAARD, 2013; KIMBLER et al., 2012; NEAL et al., 2007).

Na verdade, se o aumento ou a diminuição de AQP4 é benéfico ou prejudicial ao SNC, dependerá do tipo de lesão, estrutura acometida e da sua fase aguda ou resolutiva (VERKMAN et al., 2006). Maior expressão desta proteína auxilia na resolutividade do edema vasogênico, mas também contribui com os processos neuroinflamatórios da astrogliose e da inativação microglial (KONG et al., 2008). A sua diminuição, por outro lado, é associada com menor astrogliose e menor edema citotóxico. O treinamento físico prévio em animais submetidos à isquemia encefálica reduz os níveis de AQP4 no estriado e no córtex cerebral ipslateral à lesão isquêmica, reduzindo o edema (HE et al., 2014).

Com base em nossos resultados e em estudos prévios analisando a retinopatia diabética, é possível que no DM1 um aumento da expressão de AQP4 ocorra associado à reatividade glial da fase aguda, auxiliando a redução do edema vasogênico, e que, posteriormente, esta expressão se reduza pela persistência da disfunção da BBB, o que poderia contribuir para limitar o influxo de água ao tecido nervoso (CUI et al., 2012; HAJ-YASEIN et al., 2011) .

Nossos dados, portanto, incitam para que novos estudos avaliem outros mecanismos pelas quais o exercício possa atuar para a integridade da BBB, bem como

quais os protocolos de treino podem ser mais eficazes para o tratamento preventivo ou terapêutico da encefalopatia diabética.

CONCLUSÕES E PERSPECTIVAS

Os resultados apresentados nesta tese permitem-nos concluir que:

- O diabetes mellitus induzido pela estreptozotocina provocou um déficit de memória espacial, avaliado no teste de reconhecimento do objeto reposicionado;
- O exercício físico foi capaz de reverter o comprometimento da memória espacial nos animais diabéticos treinados;
- O diabetes mellitus induzido pela estreptozotocina não alterou, no hipocampo, a captação de glicose, a captação de glutamato, os níveis de glutamina sintetase e de glutationa;
- O exercício físico induziu um aumento dos níveis de GSH e GS no hipocampo dos animais controle treinados;
- O exercício físico aumentou a densidade de astrócitos GFAP positivos no hipocampo de animais controle treinados e diabéticos treinados;
- O exercício físico promoveu ramificação dos processos astrocitários no hipocampo dos animais diabéticos treinados;
- O exercício físico foi capaz de melhorar o desempenho da memória de curta duração nos animais dos grupos controle treinado e diabético treinado;
- O exercício físico favoreceu o desempenho motor no teste de rotarod nos animais controle treinados e diabéticos treinados;
- O diabetes mellitus induzido pela estreptozotocina diminuiu os níveis das proteínas claudina-5 e AQP4 hipocampal, e de claudina-5 no estriado;
- O exercício físico preveniu/reverteu a queda dos níveis de claudina-5 no estriado dos animais diabéticos treinados.

Como perspectivas, sugerimos que sejam investigados:

- O metabolismo da glicose encefálica com a técnica de tomografia por emissão de pósitrons, Micro PET-CT, utilizando o marcador radiológico [^{18}F]FDG, nos animais diabéticos e a influência do exercício sobre esta variável;
- O efeito do protocolo de treino sobre o estresse nitrosativo no hipocampo e no estiado dos animais diabéticos;
- A expressão de AQP4 no hipocampo e no estriado, nas fases aguda e crônica do diabetes, com a avaliação da distribuição desta proteína no soma e nos prolongamentos astrocitários, e o efeito do exercício sobre estas variáveis;
- O efeito do diabetes e do exercício sobre o sistema glinfático.

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