UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

ALTERAÇÕES DO DIABETES MELLITUS NO SISTEMA NERVOSO CENTRAL: AVALIAÇÃO DE PARÂMETROS ASTROCITÁRIOS, COMPORTAMENTAIS, DA FUNCIONALIDADE DAS BARREIRAS HEMATOENCEFÁLICA E HEMATOLIQUÓRICA E O PAPEL DA EXENDINA-4 NA NEUROPROTEÇÃO

Caroline Zanotto de Boeckel

Orientador: Prof. Carlos Alberto Saraiva Gonçalves

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“Feliz aquele que transfere o que sabe e aprende o que ensina”

Cora Coralina

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SUMÁRIO

RESUMO

O Diabetes mellitus (DM) é uma desordem metabólica caracterizada principalmente por hiperglicemia crônica. Durante o DM, a atenção está voltada principalmente para doenças que afetam sistemas periféricos, contudo, as complicações do DM podem resultar em danos ao sistema nervoso central (SNC), podendo levar a prejuízos cognitivos.

Em vista disso, o objetivo desta tese foi investigar alterações no SNC, particularmente relacionadas às funções astrocitárias e do funcionamento das barreiras encefálicas em modelo animal de DM baseando-se nas alterações periféricas. Além disso, foi analisado o efeito da exendina-4 (EX-4), um agonista dos receptores do peptídeo semelhante ao glucagon (GLP-1), em reverter os parâmetros avaliados.

Os resultados observados mostraram prejuízos periféricos e no SNC decorrentes do DM. O DM foi responsável pela excessiva produção de AGEs e alterações em parâmetros periféricos como peso corporal, glicemia, hemoglobina glicada (HbA1c) e peptídeo C. Ao analisar o SNC, observamos prejuízo nas funções cognitivas, em parâmetros astrocitários, disfunções nas barreiras encefálicas e diminuição do conteúdo da subunidade GluN1 do receptor N-metil D-Aspartato (NMDA). O tratamento com EX-4 reverteu o prejuízo cognitivo, o dano nas barreiras encefálicas, a captação de glutamato e o conteúdo de GluN1. No entanto, a EX-4 não teve efeito sobre a glicemia e formação de AGEs.

O dano em funções astrocitárias e nas barreiras encefálicas observado no DM pode ser decorrente da ativação de vias de sinalização desencadeadas pelos elevados níveis de AGEs. Da mesma maneira, o prejuízo no comportamento cognitivo provavelmente possa ser atribuído aos danos astrocitários e nas barreiras encefálicas observados no DM.

O efeito da EX-4 na melhora cognitiva possivelmente seja devido aos seus efeitos na captação de glutamato, no conteúdo de GluN1 e na recuperação das barreiras do encéfalo. Ainda, os papéis já estabelecidos da EX-4 na ativação de vias de sinalização relacionadas com a aprendizagem e memória e na diminuição da expressão de mediadores inflamatórios, podem ter atenuado os danos causados pela hiperglicemia e pelo acúmulo de AGEs. No entanto, mais estudos são necessários para estabelecer o mecanismo de ação da EX-4 na recuperação dos danos causados pelo DM no SNC.

ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder characterized primarily by chronic hyperglycemia. During the DM, attention is mainly focused on diseases affecting peripheral systems. However, DM complications may result in damage to the central nervous system (CNS), leading to cognitive impairments.

In view of this, the aim of this thesis was to investigate changes in the CNS, particularly related to astrocytic functions and the functioning of the brain barriers in animal model of DM relying on the peripheral changes and to analyze the effect of exendin-4 (EX-4), an agonist of glucagon like peptide-1 (GLP-1) receptors, in reversing the parameters evaluated.

The observed results showed peripheral and CNS damage resulting from DM. The DM was responsible for the excessive production of AGEs and changes in peripheral parameters such as body weight, blood glucose, glycated hemoglobin (HbA1c) and C-peptide When assessing the CNS, we observed impairment in cognitive functions, astrocytic parameters, dysfunctions in the brain barriers and decrease in N-methyl-D-aspartate (NMDA) GluN1 subunit content. Treatment with EX-4 reversed the cognitive impairment, damage in brain barriers, glutamate uptake and GluN1 content. However, the EX-4 had no effect in the glycemia and ADEs formation.

The loss of astrocytic and brain barriers functions observed in DM may be due to the activation of signalling pathways triggered by high levels of AGEs. In addition, impairment in cognitive behavior can probably be attributed to astrocyte and brain barriers damage observed in DM.

The effect of EX-4 on the cognitive improvement possibly be due to its effects on glutamate uptake, in the GluN1 content and in the recovery of the brain barriers. In addition, the EX-4 established roles in the activation of signalling pathways associated with learning and memory and in the decrease expression of inflammatory mediators, can attenuate the damage caused by hyperglycemia and by accumulation of AGE. However, further studies are needed to establish the mechanism of EX-4 action in the recovery of damage caused by DM in CNS.

LISTA DE ABREVIATURAS

AGEs - Produtos finais de glicação avançada

Akt – Serina treonina cinase

AMPc - Adenosina 3',5'-monofosfato cíclico

AQP1- Aquaporina 1

AQP4- Aquaporina 4

AQP9- Aquaporina 9

AQPs - Aquaporinas

BDNF- Fator neurotrófico derivado do encéfalo

BHE - Barreira hematoencefálica

BHL - Barreira hematoliquórica

CD45 - Glicoproteína expressa na membrana dos linfócitos T

COX-1 – Ciclooxigenase 1

COX-2 – Ciclooxigenase 2

CREB – Proteína ligante de resposta ao AMPc

DM - Diabetes Mellitus

DPP-IV- Dipeptidil peptidase-4

EAAC1- Carreadores de aminoácidos excitatórios

EAAT-2 - Transportador de aminoácidos excitatórios do tipo 2

EAATs - Transportadores de aminoácidos excitatórios

ERK- Cinases reguladas por sinais extracelulares

EX-4 - Exendina-4

FDA - *Food and Drug Administration*

GFAP - Proteína fibrilar glial ácida

GIP - Polipeptídeo inibitório gástrico

GLAST - Transportador de glutamato e aspartato

GLO 1 - Glioxalase 1

GLO 2 - Glioxalase 2

GLP-1 - Peptídeo semelhante ao glucagon

GLT-1- Transportador glial de glutamato

GluN1 – Subunidade N1 do receptor N-metil-D-Aspartato

GLUT-1- Transportador de glicose independente de sódio do tipo 1

GLUT-3- Transportador de glicose independente de sódio do tipo 3

GLUT-4- Transportador de glicose independente de sódio do tipo 4

GLUTs - Transportadores de glicose independentes de sódio

HbA1c - Hemoglobina glicada

IFN-γ - Interferon gama

IL-1 - Interleucina 1

IL-1β - Interleucina 1 beta

IL-6 - Interleucina 6

iNOS - Óxido nítrico sintase induzível

LCR - Líquido cefalorraquidiano

MAPK - Proteína cinase ativada por mitógenos

NF-κB - Fator nuclear kappa B

NMDA - N-metil-D-Aspartato

PI3K - Fosfatidilinositol-3-cinase

PKA - Proteína cinase A

PKB - Proteína cinase B

PKC - Proteína cinase C

RAGE- Receptor para produtos finais de glicação avançada

SGLTs - Transportadores de glicose dependentes de sódio

SNC - Sistema nervoso central

TGF-β - Fator de transformação de crescimento beta

TLR4 - Receptor do tipo *toll* 4

TNF-α- Fator de necrose tumoral alfa

VCAM-1 - Molécula de adesão da célula vascular 1

ZO-1 - Proteína de zônula oclusiva 1

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PARTE I

INTRODUÇÃO

1. Diabetes Mellitus

O Diabetes Mellitus (DM) pode ser definido como uma desordem do metabolismo de carboidratos, lipídeos e proteínas, caracterizado principalmente por hiperglicemia crônica, frequentemente acompanhado por dislipidemia, hipertensão arterial e disfunção endotelial (Coleman et al., 2010). O DM representa um problema de saúde pública devido às sérias complicações desenvolvidas a longo prazo. Ademais, esta doença está assumindo proporções epidêmicas, atualmente, estima-se que em torno de 382 milhões de pessoas sofram de DM em todo o mundo, e este número pode chegar a 592 milhões até 2035 (Kade e Rocha, 2013; International Diabetes Federation, 2015).

O DM é caracterizado principalmente pela incapacidade das células beta do pâncreas em produzir insulina (Nejsum et al., 2008) e/ou o organismo não conseguir utilizar de maneira eficaz a insulina secretada (DM tipo 2) (Chu et al., 2014) e ambos os tipos de DM podem estar associados com prejuízos funcionais e estruturais do sistema nervoso central (SNC) (Li e Sima, 2004). Outro tipo de DM encontrado com frequência e cuja etiologia ainda não está completamente esclarecida é o diabetes gestacional, o qual é detectado no rastreamento pré-natal (Ministério da Saúde/ Secretaria de Atenção à Saúde, Departamento de Atenção Básica, 2013). Cabe ressaltar que são conhecidos outros tipos específicos de DM menos frequentes, os quais podem resultar de defeitos genéticos da função das células beta pancreáticas, defeitos genéticos da ação da insulina, doenças do pâncreas exócrino, endocrinopatias, efeitos colaterais de medicamentos, infecções e outras síndromes genéticas associadas ao DM (American Diabetes Association, 2015; Ministério da

Saúde/ Secretaria de Atenção à Saúde, Departamento de Atenção Básica, 2013).

O efeito comum do DM é a hiperglicemia, a qual pode induzir diversas alterações, tais como a auto-oxidação da glicose, a geração de produtos finais de glicação avançada (AGEs), o desequilíbrio entre a geração de radicais livres e defesas antioxidantes, distúrbios vasculares, inflamação, além de alterações na homeostase do cálcio e o aumento da peroxidação lipídica (Beauquis et al., 2010; Gispen e Biessels, 2000; Parvizi et al., 2014).

A hiperglicemia é a causa de complicações micro e macrovasculares (Rosiak et al., 2014). As complicações macrovasculares incluem obstruções dos vasos, como doenças das artérias coronárias, aterosclerose e doenças vasculares periféricas. As patologias microvasculares incluem retinopatia, nefropatia e neuropatia, que pode levar à cegueira, insuficiência renal e amputações de membros inferiores, respectivamente (Chilelli et al., 2013; Group, 1998). As complicações microvasculares decorrentes da hiperglicemia devem-se, pelo menos, a cinco mecanismos principais: o aumento do fluxo de glicose e outros açúcares através da via do poliol; o aumento da formação de AGEs; interação entre AGEs e os receptores para produtos finais de glicação avançada (RAGEs), levando à sinalização intracelular, a qual prejudica a função das células; a persistente ativação de isoformas da proteína cinase C (PKC); e o aumento da atividade da hexosamina (Chilelli et al., 2013).

Dentre os casos de DM, estima-se que 85-95% sejam de DM tipo 2 e 5-15% de DM gestacional ou tipo 1, no entanto as complicações micro e macrovasculares apresentam uma maior prevalência neste último grupo de pacientes (Rodrigues et al., 2010b).

2. Alterações Periféricas do Diabetes Mellitus

Os sintomas clássicos do DM são poliúria, polidipsia, polifagia e perda involuntária de peso. Outros sintomas que levantam a suspeita clínica são: fadiga, fraqueza, letargia, prurido cutâneo e vulvar e infecções de repetição. Algumas vezes o diagnóstico é feito a partir de complicações crônicas como neuropatia, retinopatia ou doença cardiovascular aterosclerótica, podendo ser também assintomático em proporção significativa dos casos (Nathan, 2015). Geralmente, o diagnóstico do DM é feito quando observados níveis de hemoglobina glicada (HbA1c) acima de 6,5%. Além disso, níveis glicêmicos maiores que 126 mg/dL em jejum, ou maiores que 200 mg/dL a qualquer momento do dia (glicemia casual) ou duas horas após a ingestão de 75 g de glicose dissolvida em água (teste de tolerância à glicose) podem ser utilizados como indicativo de DM (American Diabetes Association, 2015; Ministério da Saúde/ Secretaria de Atenção à Saúde, Departamento de Atenção Básica, 2013).

O monitoramento de marcadores metabólicos como a pressão arterial, peso corporal, perfil lipídico, glicemia e HbA1c são essenciais para o manejo clínico dos pacientes com DM (Yuan et al., 2014). Além disso, é muito importante a obtenção e a manutenção do controle glicêmico no tratamento do DM, especialmente em pacientes tratados com insulina (Braga et al., 2012).

2.1 Hemoglobina Glicada

A HbA1c é formada pela glicação não-enzimática da glicose com molécula de hemoglobina e reflete o número de moléculas de glicose ligadas à hemoglobina nos eritrócitos. Como o tempo médio de vida dos eritrócitos é de 120 dias e a formação da

HbA1c é uma reação contínua e lenta durante toda a vida eritrocitária, a medida de HbA1c reflete como esteve a concentração de glicose sanguínea nos últimos 120 dias (Kohnert et al., 2015). Dessa forma, a HbA1c é um fator importante utilizado para monitorar o equilíbrio da glicose no sangue a longo prazo e pode ser relacionado com os resultados obtidos no teste de tolerância à glicose (Hsieh et al., 2013) .

A HbA1c, além de ser um marcador amplamente aceito para verificar a glicemia em longos períodos, o aumento em seus índices pode prever riscos em consequência aos altos níveis de glicose (Nathan et al., 2008). Sabe-se que a redução nos níveis de HbA1c diminui os riscos de prejuízos relacionadas ao DM, como infarto do miocárdio e danos microvasculares (Stratton et al., 2000). O tratamento do DM, então, objetiva manter os níveis de HbA1c o mais próximo possível dos valores ideias (entre 4,5% e 5,7%) para reduzir ou prevenir estas complicações (Kohnert et al., 2015).

Não obstante, a avaliação da HbA1c apresenta algumas limitações: ela mostra a exposição total à glicose nos últimos 120 dias, mas não capta pequenas alterações na glicemia e, além disso, não tem a capacidade de prever a ocorrência de uma possível hipoglicemia durante o período (Kohnert et al., 2015).

2.2. Peptídeo C

Durante a biossíntese de insulina, a pró-insulina é clivada em insulina e peptídeo C. Ambos são estocados nas glândulas secretórias das células beta pancreáticas e, eventualmente, são liberados na circulação em níveis equimolares. Diversos testes foram realizados na tentativa de descobrir se o peptídeo C possui efeito semelhante à insulina, no entanto, os resultados foram negativos (Wahren e

Larsson, 2015).

O peptídeo C possui uma estabilidade muito maior que a insulina, por isso sua dosagem é frequentemente utilizada para verificar a função das células beta pancreáticas (Yosten e Kolar, 2015). Além disso, estudos mais recentes têm relatado efeitos anti-inflamatórios, antioxidantes e antiapoptóticos deste peptídeo (Wahren e Larsson, 2015). Pacientes com DM tipo 1 possuem uma deficiência de insulina e peptídeo C devido à destruição das células beta pancreáticas. A insulina é administrada de forma terapêutica, no entanto, estes pacientes continuam deficientes de peptídeo C, o que poderia contribuir para a inflamação e estresse oxidativo frequentemente presentes no DM tipo 1 (Yosten e Kolar, 2015).

2.3. Produtos Finais de Glicação Avançada

A hiperglicemia observada no DM promove a glicação não-enzimática de proteínas, lipídeos ou ácidos nucléicos através da chamada reação de Maillard, em um processo que engloba uma série de oxidações, desidratações e reações cíclicas que dão origem aos chamados AGEs endógenos. Dessa forma, os AGEs são compostos termodinamicamente instáveis que normalmente se acumulam em proteínas plasmáticas ou intracelulares e lipoproteínas (Chilelli et al., 2013).

O principal precursor dos AGEs é o metilglioxal e em situações normais as células estão protegidas da toxicidade deste composto por diferentes mecanismos, em particular pelo sistema glioxalase, o qual representa a rota mais importante de detoxificação do metilglioxal. A via da glioxalase é composta pelas enzimas glioxalase-1 (GLO 1) e glioxalase-2 (GLO 2) e seu funcionamento é essencial para a proteção celular contra a glicação e o estresse oxidativo (Allaman et al., 2015).

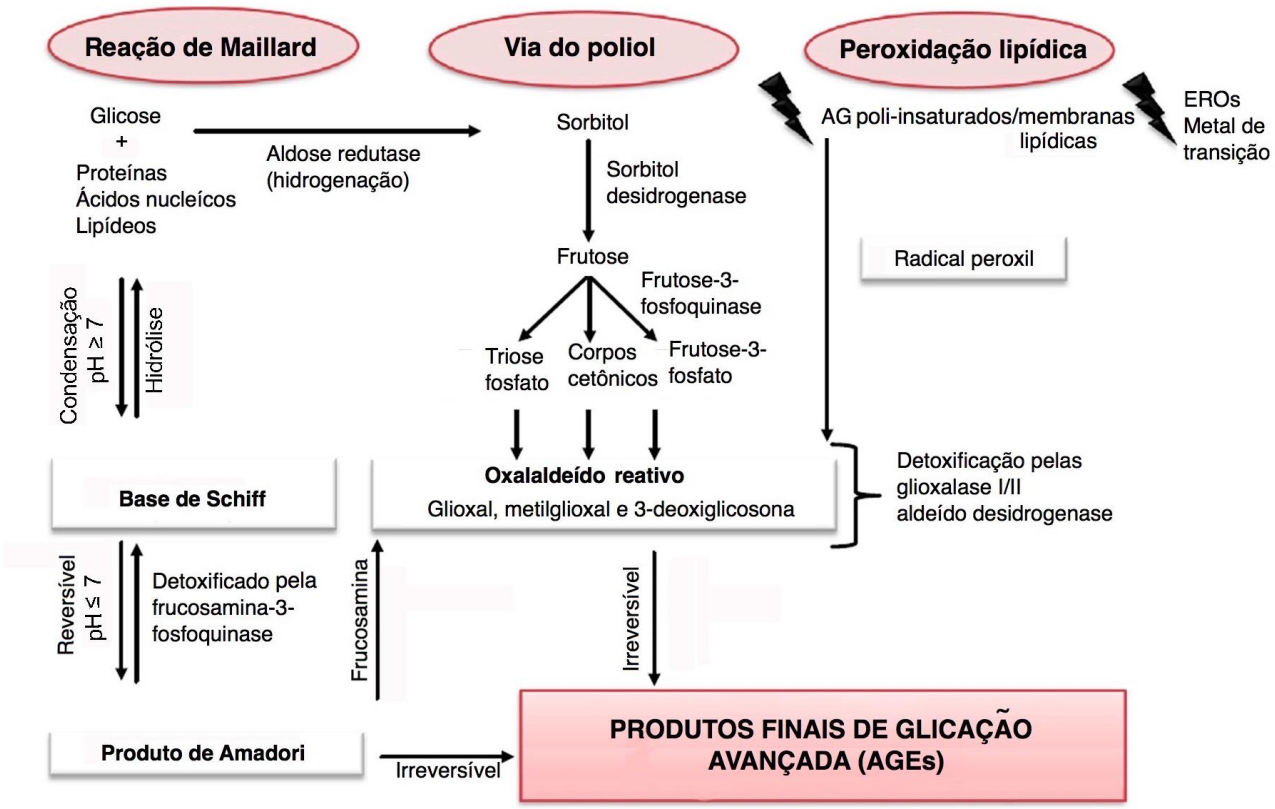

Figura 1. Representação esquemática da formação de AGEs. Adaptado de Ott et al., 2014

Os AGEs apresentam vários alvos intra e extracelulares, de modo que podem ser vistos como uma ponte entre o dano intracelular e extracelular. Além disso, independentemente do nível de hiperglicemia, os AGEs produzirão a glicação de proteínas da cadeia respiratória mitocondrial, o que formará espécies reativas de oxigênio que, por sua vez, levarão à formação de AGEs (Chilelli et al., 2013).

Diversos estudos têm identificado um receptor celular específico para AGEs, denominado RAGE (Nathan et al., 2008). RAGE, um membro da superfamília de imunoglobulinas codificado a partir de um gene no cromossomo 6, é uma proteína transmembrana e foi originalmente identificado em vários tipos celulares, tais como, monócitos/macrófagos, linfócitos T, fibroblastos, células musculares lisas, células

endoteliais, neurônios, astrócitos, eritrócitos e células mesangiais (Akirav et al., 2012; Li et al., 1998). O receptor RAGE tem diversos ligantes e não liga somente AGEs, mas também moléculas pró-inflamatórias e as proteínas ligantes de cálcio S100 (Yan et al., 2008).

3. Alterações Centrais no Diabetes Mellitus

Quando fala-se em DM, o maior foco está voltado para doenças que afetam rins, visão e sistema nervoso periférico. No entanto, as complicações do DM podem causar prejuízos estruturais e funcionais no SNC, levando a danos cognitivos transitórios ou permanentes (McCrimmon et al., 2012; Sima et al., 2004). No DM, principalmente no DM tipo 1, ocorrem flutuações nas concentrações da glicose plasmática, que associadas com alterações hormonais e metabólicas, podem contribuir para o desenvolvimento de alterações cognitivas (McCrimmon et al., 2012).

As concentrações de glicose no SNC desempenham um papel fundamental na regulação do metabolismo energético. A glicose é a principal fonte bioenergética do encéfalo, apesar da importância já reconhecida do lactato, dos corpos cetônicos e dos aminoácidos, como glutamina e glutamato (Amaral, 2013). Além disso, é armazenada como glicogênio nos astrócitos para evitar episódios de hiperglicemia (Garcia-Caceres et al., 2012).

O metabolismo energético cerebral consiste em um grupo complexo de vias e de mecanismos de trocas entre todos os componentes celulares encefálicos e fornece energia para manter a maioria das funções cerebrais (Amaral, 2013). O cérebro é muito sensível às alterações metabólicas, já que tanto neurônios como células gliais expressam uma variedade de receptores, transportadores e reguladores metabólicos

(Garcia-Caceres et al., 2012). Em particular, o metabolismo de astrócitos e de neurônios está intimamente interligado para suportar a atividade sináptica devido ao alto grau de compartimentalização celular e subcelular ao nível de enzimas, transportadores e conjunto de metabólitos (Amaral, 2013).

Embora a relação entre a glicose encefálica intersticial e a glicose plasmática seja 1:15 e estes níveis permaneçam constantes em situações de controle glicêmico, o cérebro utiliza aproximadamente 25% da glicose circulante para obter energia. Em decorrência disso, em condições de alterações da glicose plasmática, o encéfalo pode sofrer sérios prejuízos (McCrimmon et al., 2012; Prasad et al., 2014).

A glicose entra no encéfalo via duas classes de transportadores presentes principalmente na barreira hematoencefálica (BHE): transportadores de glicose independentes de sódio (GLUTs) e transportadores de glicose dependentes de sódio (SGLTs) (Shah et al., 2012). Os transportadores GLUTs exercem o transporte bidirecional de glicose a favor do gradiente de concentração, já os transportadores SGLTs transportam glicose e galactose contra o gradiente de concentração com simultâneo transporte de íons sódio (Shah et al., 2012).

A família de transportadores GLUTs possui 14 membros, sendo o transportador de glicose independente de sódio do tipo 1 (GLUT-1) o melhor estudado e caracterizado. Enquanto o GLUT-1 das células endoteliais da microvasculatura encefálica e dos astrócitos carrega glicose através da BHE, o transportador de glicose independente de sódio do tipo 3 (GLUT-3) é considerado o principal, mas não exclusivo, transportador de glicose neuronal. O transportador de glicose independente de sódio do tipo 4 (GLUT-4), o qual é o transportador de glicose sensível à insulina, e os receptores de insulina nas células endoteliais da BHE, também desempenham um papel central na regulação do transporte de glicose para o

SNC (Prasad et al., 2014).

Devido à permeabilidade restrita da BHE e à falta de estoques de carboidratos encefálicos, a expressão e a função desses transportadores é essencial para suprir com nutrientes e solutos o encéfalo. Assim, quaisquer alterações na sua função ou expressão pode afetar seriamente o metabolismo energético e a homeostase da glicose no SNC (Shah et al., 2012).

As complicações degenerativas e funcionais no SNC de diabéticos geralmente são consequências da hiperglicemia crônica (Amin et al., 2013). Essas desordens acarretam prejuízo das funções encefálicas, como alterações morfológicas, diminuição da cognição e alterações no metabolismo da glicose no SNC (Northam et al., 2009; Strachan et al., 1997).

É importante mencionarmos que a hipoglicemia, frequentemente presente em pacientes diabéticos insulinodependentes, também compromete seriamente as funções encefálicas. Os níveis normais da glicose sanguínea variam entre 3,9 a 7,1 mM (70 mg/dL a 127 mg/dL) e da glicose encefálica entre 0,8 a 2,3 mM (14 mg/dL a 41 mg/dL). Quando os níveis da glicose sanguínea estão abaixo de 2 mM (36 mg/dL), a concentração da glicose encefálica pode chegar a zero, já que o consumo sanguíneo torna-se superior ao transporte para o encéfalo, podendo acarretar coma e até morte (Amaral, 2013).

Um dos principais mecanismos que precede o DM é o aumento da glicose intracelular. Devido a isso é crucial compreender a função, a expressão e a regulação dos transportadores de glicose no encéfalo, pois alterações nesses parâmetros podem ser passos limitantes na patogênese de complicações associadas ao SNC de diabéticos (Shah et al., 2012).

3.1. Prejuízo cognitivo

Já foi comprovado que o DM, tanto o tipo 1 como o tipo 2, aumenta em pelo menos duas vezes o risco de demência e disfunções cognitivas (Umegaki, 2014; Xu et al., 2009; Ott et al., 1999). Prejuízos na aprendizagem, memória, atenção, capacidade de resolução de problemas, processamento de informações e alterações na velocidade para desempenhar tarefas motoras e mentais foram demonstradas em humanos e roedores com DM (Gispen e Biessels, 2000; Beauquis et al., 2008; Biessels et al., 1996).

Pelo menos 3 alterações bioquímicas têm sido estudadas e associadas com os déficits cognitivos no DM: alterações vasculares, resistência à insulina e falha na regulação da glicose (Nardin, 2014). Além disso, o hipocampo, estrutura encefálica responsável por diversas formas de aprendizagem e memória, é particularmente sensível a alterações na homeostase da glicose, sendo que tanto a hipo como a hiperglicemia podem afetar seu funcionamento (Amin et al., 2013).

A duração do DM é um fator de risco para o aumento do declínio cognitivo. Foi observado que os decréscimos no funcionamento cognitivo em pacientes com DM tipo 1 é mais evidente naqueles com início na infância. Então, isso pode estar relacionado com o tempo de exposição a níveis elevados de insulina combinado com a gravidade da doença (Williamson et al., 2012). No entanto, pacientes com DM tipo 1 com severas complicações microvasculares apresentam um declínio cognitivo muito maior do que pacientes com DM tipo 1 sem essas complicações (Biessels e Reijmer, 2014).

3.2. Astrócitos

Os astrócitos são um dos principais tipos celulares e as células mais abundantes do SNC, estima-se que 99% da superfície cerebrovascular seja coberta por processos astrocitários (Mathiisen et al., 2010). Os astrócitos podem proteger os neurônios de diferentes insultos estressores, como da produção de espécies reativas de oxigênio devido a sua alta capacidade antioxidante, e podem liberar fatores neurotróficos que aumentam a sobrevivência neuronal, como o fator neurotrófico derivado do encéfalo (BDNF) e fatores de crescimento neuronal. Além disso, os astrócitos regulam a homeostase do cálcio e os níveis basais de íons e neurotransmissores (Chen et al., 2005; Finsterwald et al., 2015; Pirttimaki e Parri, 2013).

Os astrócitos também desempenham papeis importantes em várias outras atividades encefálicas, como na manutenção da BHE, no controle do fluxo sanguíneo cerebral (Alvarez et al., 2013), na remoção do glutamato da fenda sináptica e no tamponamento do potássio (Cheung et al., 2015). Além disso, podem desempenhar papeis centrais na progressão de doenças neurodegenerativas (Pekna e Pekny, 2012).

As células em questão desempenham importantes funções no metabolismo energético cerebral, como na modulação dos níveis periféricos e centrais de glicose e no fornecimento de glicose para o espaço extracelular, onde será captada pelos neurônios. Ademais, também captam e estocam a glicose sanguínea como glicogênio, a partir do qual será produzido lactato, que será transferido para os neurônios como fonte de energia (Garcia-Caceres et al., 2012). A comunicação entre astrócitos e neurônios é necessária para que a glicose seja utilizada como fonte de combustível, assim, astrócitos, neurônios e vasos sanguíneos trabalham juntos como uma unidade

funcional (Wang et al., 2015a). Dessa forma, o fornecimento de glicose e seus metabólitos para os neurônios, assim como quaisquer alterações nos níveis de glicose encefálica poderiam afetar principalmente os astrócitos (Nagayach et al., 2014). Além da glicose, os astrócitos transportam para o SNC ácidos graxos, cetonas e lactato, e as necessidades energéticas do cérebro estão ligadas aos tipos de nutrientes disponíveis (Jakovcevic e Harder, 2007). Todas essas propriedades dos astrócitos sugerem que eles podem reagir precocemente às alterações no metabolismo da glicose relacionadas ao DM (Lebed et al., 2008).

Em particular, os astrócitos podem ser ativados e mudar a sua aparência em resposta a condições fisiopatológicas, fenômeno este chamado de gliose ou astrogliose, onde ocorrem alterações morfológicas, proliferação celular, expressão de marcadores específicos de ativação e aumento da geração de citocinas e interleucinas pró-inflamatórias (Anderson et al., 2014). A ativação glial atua geralmente de duas maneiras: auxilia na eficácia da restauração das células encefálicas danificadas ou provoca um ambiente ameaçador que acarreta posterior disfunção cerebral dependendo do estímulo e da progressão da doença (Pekny e Pekna, 2014).

A ativação de células gliais altera a expressão gênica de marcadores bastante utilizados na astrogliose em diversas situações envolvendo doença encefálica: a proteína glial fibrilar ácida (GFAP) e a proteína S100B (Goncalves et al., 2008). Além disso, a astrogliose provoca alterações no metabolismo do glutamato e em defesas antioxidantes (Eng et al., 2000; Donato et al., 2009).

3.2.1. Proteína Glial Fibrilar Ácida

A GFAP é uma proteína de filamento intermediário de astrócitos, com peso molecular de aproximadamente 50 kDa, regulada por fosforilação, que além do seu papel como integrador estrutural, pode fornecer sítios de ligações para diversas enzimas envolvidas na geração de diferentes respostas astrocitárias (Lebed et al., 2008). Esta proteína é comumente utilizada como marcador de astrogliose e ativação de astrócitos em distintas situações envolvendo dano encefálico e é comumente utilizada para analisar a distribuição de células gliais em resposta a danos neuronais (Hamby e Sofroniew, 2010).

O aumento da GFAP em consequência da astrogliose protege as células do SNC através da captação do glutamato excitotóxico, promove o aumento da produção do antioxidante glutationa e do neuroprotetor adenosina (Dringen et al., 2000), provoca degradação do peptídeo beta amilóide (Koistinaho et al., 2004) e limita a propagação de células inflamatórias (Voskuhl et al., 2009).

As alterações na GFAP em modelos animais de DM ocorrem geralmente em períodos precoces da doença, o que poderia contribuir para o entendimento da patofisiologia de doenças encefálicas provocadas pelo DM (Coleman et al., 2010).

3.2.2. S100B

A S100B pertence à família de 25 membros de proteínas S100 que receberam esta denominação por serem solúveis em solução 100% saturada de sulfato de amônio (Moore, 1965). A S100B apresenta estrutura homodimérica, onde cada monômero beta possui peso molecular aproximado de 10,5 kDa, exibindo dois sítios de ligações do tipo *EF-hand* para o cálcio e sítios de ligações independentes para o zinco

(Donato, 2001). A S100B é expressa principalmente em astrócitos no SNC de vertebrados, no entanto outros tipos celulares como linfócitos, adipócitos, algumas linhagens tumorais, células progenitoras neurais, dentre outros tipos celulares também expressam esta proteína (Donato et al., 2009).

Intracelularmente no SNC, a S100B interage com diferentes proteínas envolvidas em uma variedade de rotas biológicas, como na regulação do citoesqueleto, proliferação, sobrevivência e diferenciação celular, além da modulação do metabolismo energético astrocitário (Van Eldik e Wainwright, 2003; Donato, 2001).

A proteína S100B executa efeitos autócrinos e parácrinos em astrócitos, neurônios e microglia, está associada com a proliferação astrocitária, mas sabe-se que também estimula a ativação de células microgliais (Nagayach et al., 2014).

Em cultura de neurônios a S100B demonstrou ter efeitos tróficos quando presente em concentrações picomolares e nanomolares, enquanto concentrações micromolares desta proteína apresentaram efeitos tóxicos, promovendo apoptose (Van Eldik e Wainwright, 2003).

Além disso, sabe-se que o papel extracelular da S100B também é mediado pelo receptor RAGE e envolve vias de sinalização como de proteínas cinases reguladas por sinais extracelulares (ERK) e do fator nuclear kappa B (NF-κB) (Donato et al., 2009; Goncalves et al., 2008).

3.2.3. Glutamato

O glutamato é o principal neurotransmissor excitatório do SNC de mamíferos e 40% dentre todas as sinapses são glutamatérgicas. Visando manter a homeostase encefálica, o glutamato deve ser removido rapidamente da fenda sináptica pelos

astrócitos (Kim et al., 2011). No interior do astrócito, por sua vez, o glutamato é convertido em glutamina pela enzima glutamina sintetase, e a glutamina é captada pelos neurônios glutamatérgicos e reconvertida em glutamato, para então agir em novas sinapses (Magistretti e Pellerin, 1996). Quando ocorrem disfunções astrocitárias, pode ocorrer uma diminuição da captação do glutamato e consequente acúmulo extracelular, podendo provocar excitotoxicidade e morte neuronal. Desta maneira, para avaliação das funções astrocitárias no tecido encefálico, a medida da captação de glutamato astrocitária é bastante utilizada (Stobart e Anderson, 2013).

O glutamato transportado para dentro do astrócito ativa a glicólise intracelular, aumenta a produção de lactato e sua distribuição para os neurônios, controlando assim a disponibilidade de nutrientes (Garcia-Caceres et al., 2012). Além disso, o glutamato também pode ser utilizado para a síntese *per se* de glutationa (Dringen et al., 1999) e parece estar envolvido no controle da migração, diferenciação e sobrevivência neuronal, crescimento de neuritos e sinaptogênese (Suzuki et al., 2006).

Já foram identificados cinco tipos de transportadores de glutamato em humanos, os chamados transportadores de aminoácidos excitatórios (EAATs). Estes transportadores são bombas iônicas e desempenham importante papel na regulação das concentrações de glutamato no espaço extracelular e manutenção de baixos níveis fisiológicos que promovem as funções biológicas sem causar toxicidade. Eles estão altamente expressos em astrócitos, onde desempenham importante papel na comunicação entre células gliais e neurônios (Wang e Qin, 2010). No encéfalo de ratos, três homólogos destes transportadores estão presentes: transportador de glutamato e aspartato (GLAST), transportador glial de glutamato (GLT-1) e carreador de aminoácidos excitatórios (EAAC1). O transportador de aminoácidos excitatórios

do tipo 2 (EAAT-2) ou GLT-1 é responsável por, pelo menos, 80% da captação de glutamato no encéfalo de adultos (Soni et al., 2014).

A captação de glutamato é regulada de diversas maneiras. A expressão dos transportadores é regulada pela adenosina 3',5'-monofosfato cíclico (AMPc), fatores neuronais e em resposta a diferentes doenças encefálicas. Já a atividade dos transportadores pode ser regulada por fosforilação, oxidação da sulfidrila, ácido araquidônico, dentre outros fatores (Kim et al., 2011).

Sabe-se que o aumento nos níveis de glutamato extracelular tem diversos efeitos danosos, como a disfunção na homeostase do cálcio, aumento da produção de óxido nítrico, ativação de proteases, aumento de fatores de transcrição citotóxicos e aumento da formação de radicais livres (Wang e Qin, 2010).

4. Barreiras no Sistema Nervoso Central

O SNC é parcialmente impermeável às moléculas presentes no sangue, principalmente pela BHE e pela barreira hematoliquórica (BHL) (Engelhardt e Sorokin, 2009).

A BHE consiste em um sistema complexo formado por diversos tipos celulares. A unidade estrutural e funcional básica da BHE é a chamada unidade neurovascular, a qual é composta por pelo menos quatro tipos celulares: as células endoteliais, que compreendem os capilares da vasculatura encefálica; os pericitos que situam-se em cima das células endoteliais e partilham a mesma membrana basal; os astrócitos que cercam os capilares com os pés astrocitários; e os neurônios que inervam diretamente a microcirculação, juntas essas células da unidade neurovascular controlam a função da barreira (Obermeier et al., 2013).

A BHE possui características distintas, como a falta de fenestrações e atividade pinocitótica baixa, o que torna limitada a disponibilidade de glicose e outros nutrientes aos tecidos neurais em comparação com os órgãos periféricos (Shah et al., 2012). Além disso, a funcionalidade da BHE é manifestada por mecanismos de transporte permanentemente ativos, expressos especificamente por células endoteliais dos capilares cerebrais que garantem o transporte de nutrientes para o SNC e, ao mesmo tempo, o impedimento da passagem de moléculas que podem prejudicar o ambiente necessário para a transmissão neuronal (Engelhardt e Sorokin, 2009).

A perda da integridade da BHE expõe o encéfalo a substâncias potencialmente danosas como, por exemplo, componentes plasmáticos, moléculas imunológicas, células, íons, aminoácidos, proteínas e outras macromoléculas que podem perturbar a homeostase encefálica e levar à disfunção e degeneração neuronal (Obermeier et al., 2013; Daneman, 2012). Além disso, a BHE é uma estrutura heterogênea e em certas regiões encefálicas está mais vulnerável ao dano oxidativo e desacoplamento neurovascular (VanGilder et al., 2009).

Os plexos coróides são estruturas que consistem de uma extensiva rede de capilares fenestrados revestidos por uma única camada do epitélio cuboidal e sua principal função é produzir e secretar o líquido cefalorraquidiano (LCR). A BHL é encontrada na superfície das junções oclusivas apicais entre as células epiteliais dos plexos coróides, inibindo a difusão paracelular de moléculas solúveis em água. A barreira e a função secretória destas células epiteliais são mantidas pela expressão de sistemas de transporte, permitindo a passagem direta de íons e nutrientes para dentro do LCR. Em patologias do SNC, características da BHL são alteradas, levando à formação de edema e recrutamento de células inflamatórias para dentro do SNC (Engelhardt e Sorokin, 2009).

Em ambas, tanto na BHE como na BHL, a difusão paracelular simples de metabólitos e íons é impedida pela presença de junções oclusivas nas células endoteliais e epiteliais, respectivamente (De Bock et al., 2014).

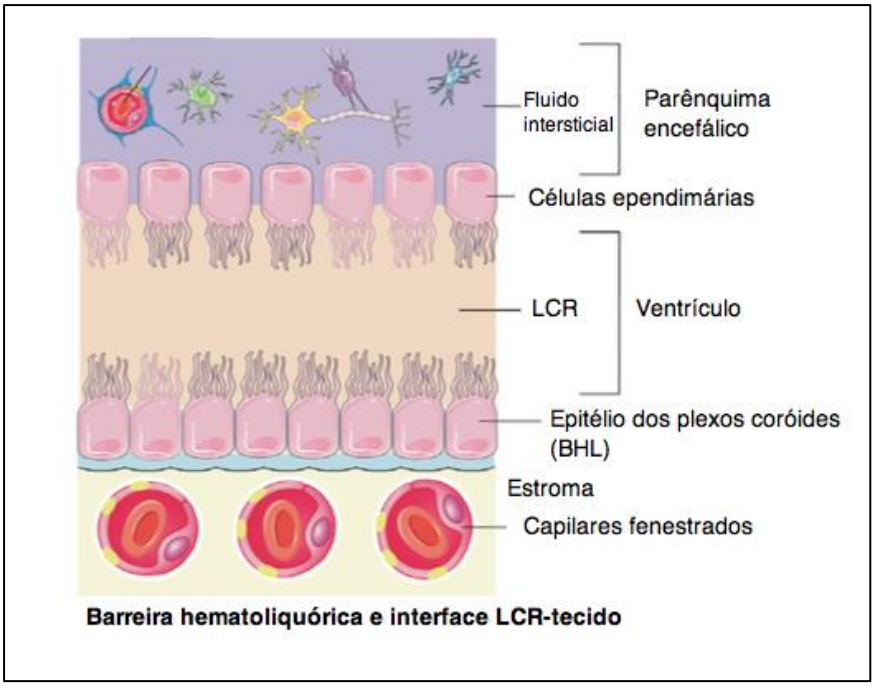

Figura 2. Representação gráfica da formação da BHL e da interface entre LCR e tecido encefálico. Adaptado de De Bock et al., 2014

4.1. Junções Oclusivas

O complexo juncional entre as células endoteliais dos microvasos do SNC incluem as junções aderentes e as junções oclusivas, já nas células epiteliais há somente as junções oclusivas. Esses dois tipos de junções são componentes estruturais destas barreiras que impedem o movimento paracelular de íons, solutos e proteínas, além do transporte de água que medeia o influxo de substâncias essenciais e o efluxo de moléculas deletérias através da BHE e BHL (De Bock et al., 2014).

As junções oclusivas, tanto da BHE como da BHL, são formadas por um complexo proteico transmembrana e citosólico que vedam os espaços entre as células endoteliais e epiteliais vizinhas, respectivamente e, assim, estabelecem uma barreira passiva e física que regulam a passagem de solutos e íons do sangue para o encéfalo (Coisne e Engelhardt, 2011). As junções oclusivas exercem pelo menos duas funções nos tecidos, como barreira e como cerca limitante, as quais são essenciais para o desenvolvimento e homeostase cerebral, tanto quanto para a manutenção da polaridade das células como um limite para os sítios da membrana plasmática apical e basolateral (Goncalves et al., 2013). Dessa forma, a BHE e a BHL equilibram o influxo de nutrientes e o efluxo de toxinas, resíduos e drogas para manter a homeostase encefálica (Liu e Liu, 2014).

Dentre as várias proteínas deste complexo estão as primeiras proteínas integrais de membrana descritas, as ocludinas, localizadas exclusivamente nas junções oclusivas da BHE. Alguns estudos demonstraram que elas não são essenciais para o desenvolvimento normal das junções oclusivas desta barreira (Engelhardt e Sorokin, 2009). Já a família de 27 membros das claudinas é essencial e suficiente para a formação das ligações das junções oclusivas.

As claudinas possuem entre 20 e 34 kDa, possuem quatro hélices transmembrana e não estão uniformemente distribuídas nos tecidos. Está bem estabelecido que as claudinas 1, 3, 5 e 12 regulam a permeabilidade paracelular de pequenas moléculas através da BHE, sendo a claudina 5 a mais abundante e um regulador crítico da permeabilidade através da BHE. As claudinas 1, 2, 3 e 11 são encontradas principalmente nas junções oclusivas das células epiteliais dos plexos coroides. A BHL possui menor resistência física e permeabilidade menos restrita que a BHE e isto pode ser explicado por possuir diferentes tipos de claudinas, já que estas

proteínas desempenham um importante papel na função de barreira e no controle do movimento paracelular de íons e moléculas (Coisne e Engelhardt, 2011; Goncalves et al., 2013).

Além das ocludinas e claudinas, as proteínas de zônula oclusiva também fazem parte das junções oclusivas da BHE e BHL, sendo a proteína de zônula oclusiva 1 (ZO-1) a mais descrita na literatura. A ZO-1 é uma proteína acessória que está ligada às proteínas das junções oclusivas permitindo seu ancoramento à actina (proteínas do citoesqueleto celular). Alterações na expressão e conteúdo de ZO-1 também podem prejudicar as funções da BHE e BHL (Gunzel e Yu, 2013).

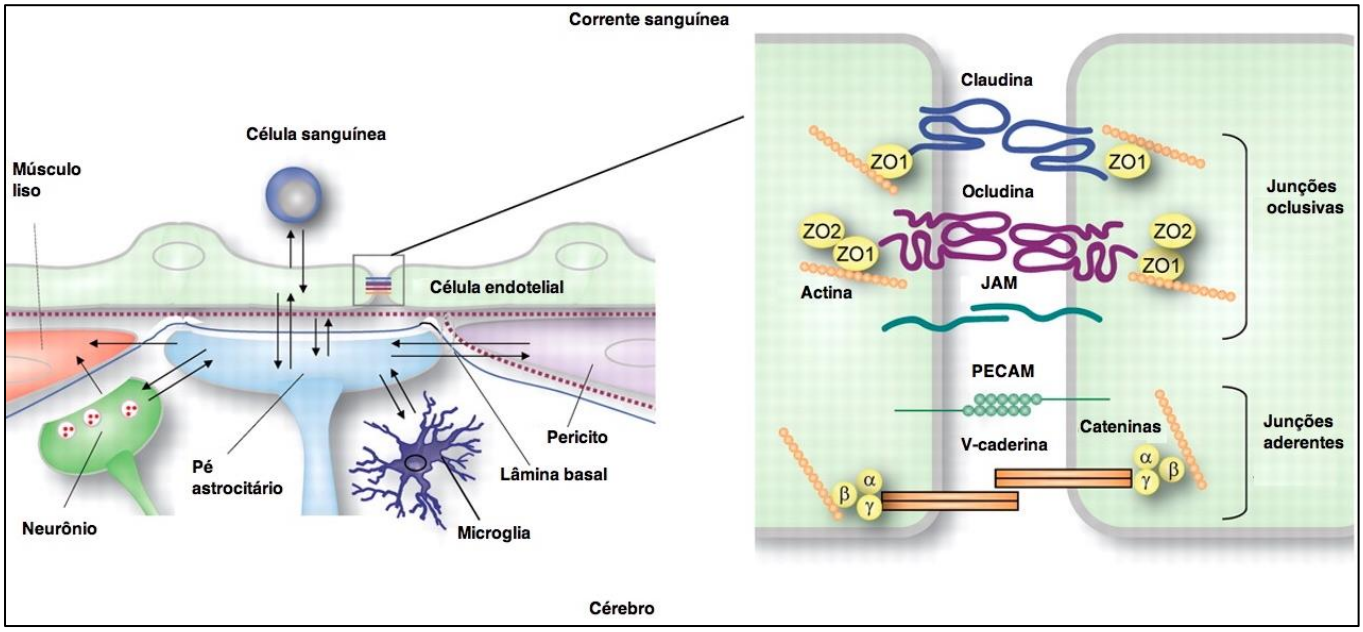

Figura 3. Representação esquemática das células que formam a BHE e das junções oclusivas presentes nesta barreira. Adaptado de http://www.neurology.org/content/78/16/1268/F1.expansion.html-

4.2. Aquaporinas

As aquaporinas (AQPs) representam uma família de proteínas integrais de membrana que funcionam como canais de água e já foram identificados 13 tipos de AQPs em mamíferos (Vella et al., 2015). As AQPs possuem peso molecular em torno de 30 kDa e desempenham um papel crítico no controle do conteúdo de água das células e, em alguns casos, facilitam o transporte de outros pequenos solutos através das membranas. Esses canais são amplamente distribuídos em todos os organismos e estão presentes, principalmente, onde uma rápida ou alta passagem de moléculas de água é necessária, permitindo que a função celular seja desempenhada (Zheng et al., 2010). Essas proteínas contribuem significativamente para a homeostase de água no organismo e tem importantes papéis tanto em condições fisiológicas como patológicas. No entanto, as AQPs têm sido associadas com outras funções celulares, como migração celular, difusão de gases e alterações em sua expressão em tecidos tumorais (Papadopoulos et al., 2008; Badaut et al., 2014).

No SNC de mamíferos já foi demonstrado a expressão das AQPs 1, 4 e 9. A aquaporina 1 (AQP1) localiza-se principalmente no epêndima e na membrana apical do epitélio dos plexos coroides, onde está relacionada com a produção do LCR. Além disso, trabalhos mais recentes relataram a presença de AQP1 no septo neuronal, com aumento após danos encefálicos, o que sugere um papel na plasticidade e reparo dos neurônios (Badaut et al., 2014; Fukuda et al., 2012). No entanto, mais estudos são necessários para esclarecer seu papel nestas células.

A aquaporina-4 (AQP4) é a AQP mais abundante no encéfalo e está localizada no epêndima, na glia limitante e, principalmente, é encontrada em grande quantidade nos pés astrocitários perto dos vasos encefálicos, onde desempenha

importante função no transporte de água para fora do parênquima cerebral por aumentar o fluxo de água transmembrana nos astrócitos e no tamponamento do potássio (Bonomini e Rezzani, 2010; Filippidis et al., 2011). Além disso, a AQP4 parece estar envolvida em diversas outras funções, como na adesão celular, na remoção de substâncias potencialmente danosas ao SNC, como o peptídeo beta amilóide, na remoção de gases e solutos e na migração astrocitária durante a astrogliose (Badaut et al., 2014).

A aquaporina 9 (AQP9) facilita tanto a difusão de água como de glicerol, ureia e monocarboxilatos. A AQP9 está presente nos astrócitos, células endoteliais e neurônios catecolaminérgicos, e parece ser alterada por mudanças no metabolismo energético cerebral (Zelenina, 2010; Badaut et al., 2014). Além disso, já foi demonstrado um aumento desta proteína em neurônios de animais diabéticos (Badaut et al., 2011; Badaut, 2010).

Como as funções encefálicas normais estão ligadas à homeostase de água, não é de estranhar que desde a sua descoberta, as AQPs tomaram um papel central no estudo de diversas condições encefálicas e a determinação da sua expressão durante diferentes doenças pode contribuir para um melhor entendimento das funções das barreiras encefálicas (Vella et al., 2015).

5. Agonistas dos Receptores de GLP-1

O Peptídeo Semelhante ao Glucagon (GLP-1) é um peptídeo produzido pelas células L enteroendócrinas do intestino e, juntamente com o polipeptídeo inibitório gástrico (GIP), formam a família de hormônios incretinas, que coletivamente estimulam 50-70% do total da secreção de insulina pós-prandial (Unger, 2013; Pettus

et al., 2013). O GLP-1 possui 30 aminoácidos, sendo em torno de 50% da sequência homóloga ao glucagon, e exerce uma variedade de efeitos pancreáticos, incluindo o aumento da biossíntese e liberação da insulina pós-prandial, diminuição da secreção de glucagon, aumento da massa e da sensibilidade das células beta à glicose e diminuição da apoptose (Seufert e Gallwitz, 2014).

O efeito do hormônio GLP-1 nativo tem limitado valor farmacológico devido a sua meia-vida curta de 1-2 minutos, atribuída à degradação pela enzima dipeptidil peptidase-4 (DPP-IV) (Mentlein et al., 1993). Devido a isto, têm sido desenvolvido análogos mais estáveis, com efeito mais longo ao do GLP-1 e resistentes à degradação pela enzima DPP-IV, os agonistas dos receptores de GLP-1 (Green et al., 2004). Estes compostos são drogas bem estabelecidas para tratar o DM tipo 2 e sabe-se que elas estimulam a produção e secreção de insulina pelas células beta pancreáticas de uma maneira dependente de glicose (Drucker e Nauck, 2006). Atualmente, dois agonistas dos receptores de GLP-1 estão aprovados para o tratamento do DM tipo 2: a exendina-4 (EX-4; Exenatide®, Byetta®) e a liraglutida (Victoza®). Estes análogos são injetados de forma subcutânea e não afetam os níveis de glicose sanguínea em pacientes normoglicêmicos, por isso podem ser administrados em pacientes não-diabéticos (Vella et al., 2002). A EX-4 foi o primeiro análogo GLP-1 de ação longa descrito que foi aprovado pela *Food and Drug Administration* (FDA) em 2005 para ser utilizado no tratamento do DM tipo 2. Esta substância apresenta uma meia-vida muito mais longa (120 minutos) que o GLP-1, o que possibilita sua utilidade clínica (Hamilton et al., 2011).

Os receptores para GLP-1 pertencem à família que abrange sete receptores transmembrana acoplados à proteína G e estão expressos em vários tecidos como no SNC e periférico, coração, endotélio, rins, pâncreas, pulmões e trato gastrointestinal,

o que demonstra que sua ação não está restrita ao pâncreas (Unger, 2013). Após a ligação ao receptor, a principal via de sinalização envolve a ativação da adenilil ciclase, levando a um aumento dos níveis intracelulares de AMPc e uma série de eventos de sinalização subsequente que regulam diversas funções celulares (Salcedo et al., 2012). No pâncreas, o GLP-1 pode ativar a proteína cinase B/serina treonina cinase (PKB/Akt) através da fosforilação dependente de AMPc de proteínas ligantes responsivas ao AMPc e resultar na ativação da via da insulina (Wang e Brubaker, 2002).

No SNC, os receptores GLP-1 estão amplamente expressos principalmente pelos neurônios, em especial em neurônios piramidais no hipocampo e neocórtex e em células de Purkinje no cerebelo. Em células gliais não foram encontrados esses receptores, no entanto, elas induzem sua expressão quando ativadas em resposta inflamatória (Holscher, 2014). Os análogos do GLP-1 atravessam a BHE, propriedade que é de vital importância para serem utilizados no tratamento de desordens degenerativas do SNC (Kastin e Akerstrom, 2003; Hamilton e Holscher, 2009).

Estudos recentes têm investigado o papel de agonistas dos receptores de GLP-1 em pacientes com DM tipo 1 (Traina et al., 2014). Na maioria destes pacientes, a resposta regulatória do glucagon à hipoglicemia induzida pela insulina é deficiente, e os agonistas dos receptores de GLP-1 poderiam melhorar esta resposta (Kramer et al., 2014; Christensen et al., 2015). Como os pacientes com DM tipo 1 tem uma função residual das células beta de 20 a 30%, os agonistas dos receptores de GLP-1 poderiam atuar aumentando a massa dessas células e consequentemente a biossíntese de insulina (Unger, 2013) ou, ainda, diminuindo a apoptose e, dessa forma, aumentando a sobrevivência celular (Drucker, 2003). Estas características aumentam

a possibilidade de utilizar esses compostos no início da doença como agentes imunomodulatórios, para auxiliar a compensar o insulto autoimune e a perda da massa das células beta pancreáticas (Pettus et al., 2013). Além disso, esses agentes podem aumentar o controle glicêmico independente dos efeitos nas células beta, inibindo o glucagon, promovendo a saciedade e atrasando o esvaziamento gástrico (Nauck et al., 1996).

Como mencionado anteriormente, o DM é uma desordem metabólica caracterizado por mudanças funcionais e estruturais no SNC, aumentando em pelo menos duas vezes o risco de demência e disfunções cognitivas. Neste sentido, a hipótese deste trabalho foi confirmar alterações periféricas e o prejuízo cognitivo em um modelo de DM tipo 1 induzido por estreptozotocina (STZ), bem como verificar possíveis alterações bioquímicas e astrocitárias associadas com o prejuízo cognitivo no DM e sua (s) provável (is) origem (ns). Ainda, buscou-se investigar a eventual função neuroprotetora de um agonista dos receptores de GLP-1.

OBJETIVOS

OBJETIVO GERAL

O objetivo geral desta tese foi investigar alterações no SNC, particularmente às funções astrocitárias e das barreiras encefálicas em modelo animal de DM induzido por STZ tendo como base as alterações periféricas, bem como analisar o efeito da EX-4, um agonista dos receptores de GLP-1, em reverter os parâmetros alterados.

PARTE II

CAPÍTULO I

Peripheral Levels of AGEs and Astrocyte Alterations in the Hippocampus of STZ-Diabetic Rats

Patrícia Nardin[1x*], Caroline Zanotto[1x], Fernanda Hansen[1], Cristiane Batassini[1], Manuela Sangalli Gasparin[1], Patrícia Sesterheim[2], Carlos-Alberto Gonçalves[1]

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Artigo publicado no periódico *Neurochemical Research*

OBJETIVOS ESPECÍFICOS

1. Confirmar alterações periféricas no modelo de DM induzido por STZ;
2. Analisar alterações bioquímicas relacionadas à captação de glicose encefálica;
3. Investigar funções astrocitárias através da dosagem das proteínas GFAP, S100B e da captação de glutamato.

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ORIGINAL PAPER

Peripheral Levels of AGEs and Astrocyte Alterations in the Hippocampus of STZ-Diabetic Rats

Patrícia Nardin[1] · Caroline Zanotto[1] · Fernanda Hansen[1] · Cristiane Batassini[1] · Manuela Sangalli Gasparin[1] · Patrícia Sesterheim[2] · Carlos-Alberto Gonçalves[1]

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Abstract Diabetic patients and streptozotocin (STZ)-induced diabetes mellitus (DM) models exhibit signals of brain dysfunction, evidenced by neuronal damage and memory impairment. Astrocytes surrounding capillaries and synapses modulate many brain activities that are connected to neuronal function, such as nutrient flux and glutamatergic neurotransmission. As such, cognitive changes observed in diabetic patients and experimental models could be related to astroglial alterations. Herein, we investigate specific astrocyte changes in the rat hippocampus in a model of DM induced by STZ, particularly looking at glial fibrillary acidic protein (GFAP), S100B protein and glutamate uptake, as well as the content of advanced glycated end products (AGEs) in serum and cerebrospinal fluid (CSF), as a consequence of elevated hyperglycemia and the content of receptor for AGEs in the hippocampus. We found clear peripheral alterations, including hyperglycemia, low levels of proinsulin C-peptide, elevated levels of AGEs in serum and CSF, as well as an increase in RAGE in hippocampal tissue. We found specific astroglial abnormalities in this brain region, such as reduced S100B content, reduced glutamate uptake and increased S100B secretion, which were not accompanied by changes in GFAP. We also observed an increase in the glucose transporter, GLUT-1. All these changes may result from RAGE-induced inflammation; these astroglial alterations together with the reduced content of GluN1, a subunit of the NMDA receptor, in the hippocampus may be associated with the impairment of glutamatergic communication in diabetic rats. These findings contribute to understanding the cognitive deficits in diabetic patients and experimental models.

Keywords Advanced glycation end products (AGEs) · Diabetes mellitus · GFAP · Glutamate neurotransmission · S100B secretion

Introduction

Diabetes mellitus (DM) is a metabolic and multifactorial disease, associated with systemic and central abnormalities [1], whose prevalence has reached alarming proportions [2]. DM is characterized by high concentrations of glucose in the tissues, including the blood and brain [3], and the resulting glucotoxicity is among the causes of DM complications [4]. One group of toxic glucose derivatives is the advanced glycation end products (AGEs), which are produced from non-enzymatic glycation and glycoxidation processes and are formed on proteins, lipids or nucleic acids in a pro-oxidant environment [5]. DM is associated with several adverse effects in the brain, including changes in brain energy metabolism, increased free calcium levels, as well as glucose-mediated increases in oxidative stress and inflammation, possibly mediated by AGEs [6, 7].

Animal models have greatly contributed to the study of the DM pathophysiology, clinical features and potential therapy. In chemically induced-DM models, insulin

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producing β-cells are destroyed, leading to hyperglycemia [8]. Streptozotocin (STZ), a nitrosourea derivative isolated from *Streptomyces achromogenes*, is an alkylating antineoplastic drug often used to induce DM in experimental animals [9]. The cytotoxicity of STZ in the β-cells appears to depend on the GLUT-2 glucose transporter, but its full mechanism is still unclear [10].

The experimental model of STZ-induced DM has demonstrated evidence of brain dysfunction, such as astrocyte activation [11], β-amyloid deposition [12], neuronal damage and memory impairment verified by behavioral tests [13]. Interestingly, DM is associated with an increased risk of Alzheimer's disease (AD) and other dementias [14, 15].

Astrocytes, the most abundant cells of the central nervous system (CNS), provide nutrients, maintain brain homeostasis and regulate neuronal metabolic function [16]. As such, astrocytes participate in glucose metabolism and respond to alterations in glucose levels by providing lactate to the extracellular space for uptake by neurons [17]. Astrocytes that surround capillaries capture and transport glucose into the brain, principally through the glucose transporter 1 (GLUT-1). There are two isoforms of GLUT-1, one more glycosylated form (GLUT-1 55 kDa), located on the vascular endothelial cells, which ensures transport of glucose across the blood–brain barrier (BBB), and another less glycosylated form (GLUT-1 45 kDa), which is located in astrocytes [18].

The astrocytes become reactive in response to numerous injuries to the CNS, modifying the cellular morphology and expression of specific markers [19]. The glial fibrillary acidic protein (GFAP) and S100B protein are astrocyte markers for activity and brain injury in several neuropsychiatric disorders [20]. Intracellular S100B interacts with several target proteins (e.g. GFAP [21] and calcineurin [22]), modulating astroglial activity and extracellular S100B, acting on receptors for AGE [23], and is considered to be a neurotrophic or inflammatory cytokine [24, 25].

Glutamate is the major excitatory neurotransmitter and changes in glutamate receptors are related to synaptic plasticity in cognitive tasks such as memory and learning [26]. However, extracellular glutamate accumulation leads to neurotoxicity, mediated by the same receptors, and calcium overload [27]. The glutamate uptake by specific astrocytic transporters is a defense system against excitotoxicity that contributes to the efficient clearance and recycling of glutamate [28]. Alterations in glutamate transport have been associated with DM and this mechanism could be involved in the pathogenesis of brain disorders related to this disease [14, 29].

Cognitive changes observed in diabetic patients and experimental models could be related to alterations in glutamate neurotransmission and S100B secretion, parameters intimately related to astrocyte function. Herein, we investigate specific astrocyte changes in the rat hippocampus in a model of DM induced by STZ, looking particularly at GFAP, S100B and glutamate uptake, as well as the content of AGE and RAGE in this brain tissue.

Results

Changes in Peripheral Metabolic Parameters Validate the Diabetic Model

To validate our model of DM in WKY rats, we measured peripheral parameters – body weight, glycemia, glycated hemoglobin and C-peptide, after 8 weeks of STZ administration. We observed that the diabetic rats exhibited significantly lower body weight than age-matched control rats (Fig. 1a; $p < 0.0001$). The diabetic rat group presented an increase in serum glucose levels (Fig. 1b; $p < 0.0001$), urine glucose levels (data not shown) and in the glycated hemoglobin concentration in total blood (Fig. 1c; $p < 0.0001$), when compared with the control group. Confirming insulin deficiency, the diabetic rats presented a lower concentration of C-peptide in the serum (Fig. 1d; $p = 0.0022$).

AGEs and RAGE are Altered in Diabetic Rats

To evaluate glucotoxicity, we determined AGE content in the serum and CSF of diabetic rats. An increase in AGE levels was observed in the serum (Fig. 2a; $p = 0.0022$) and in the CSF (Fig. 2b; $p = 0.0057$) of diabetic rats compared with control rats. Moreover, a potential receptor for AGE [23] was measured in hippocampal tissue and, RAGE level was augmented in STZ-diabetic rats, compared with control rats (Fig. 2c; $p = 0.0369$).

Brain Glucose Transport is Modified in Diabetic Rats

We measured the glucose uptake activity and the GLUT-1 content in hippocampal slices. The [^{3}H] glucose uptake in diabetic rats is not different from control (Fig. 3a; $p = 0.5821$). However, in diabetic rats, the amount of glucose transporter GLUT-1 is increased (Fig. 3b; $p = 0.0328$).

The Astroglial Marker, S100B, is Altered in the Hippocampus of Diabetic Rats

Two astroglial markers, GFAP and S100B, were analyzed in hippocampal tissue. We did not find changes in the content of GFAP in diabetic rats compared with age-

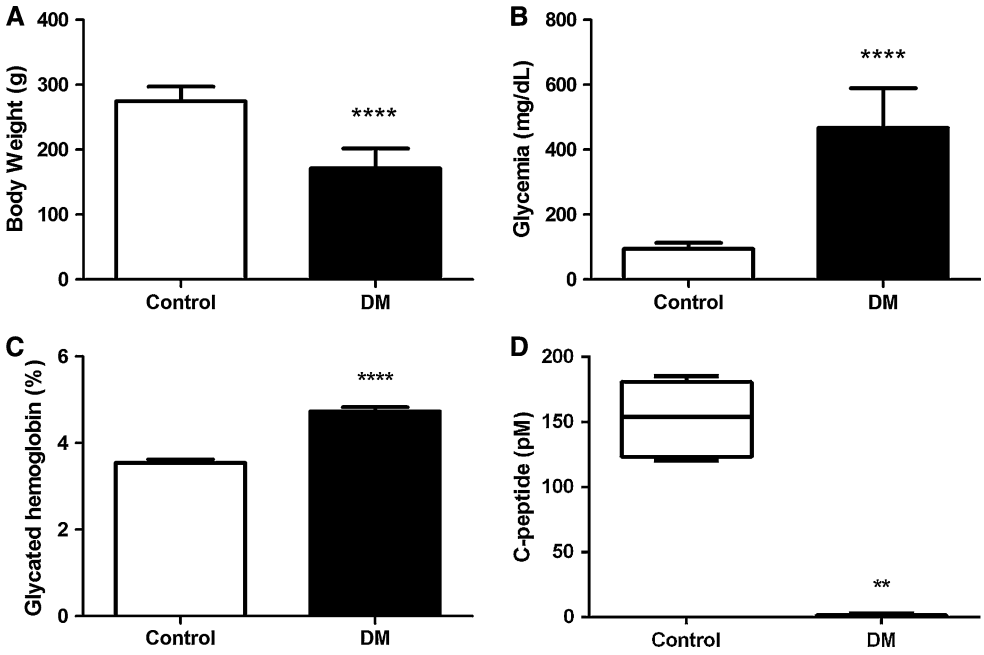

Fig. 1 Changes in peripheral metabolic parameters validate the diabetic model. Body weight (**a**), glycemia (**b**), glycated hemoglobin (**c**) and C-peptide (**d**) were evaluated in control and DM (60 days after STZ-induction) groups. Results represent the mean ± S.E.M (**a**–**c**) or the median and interquartile range (**d**). N = 12, in **a** and **b**; N = 6, in **c**, **d**. $^{**}p < 0.01$ or $^{****}p < 0.0001$ significantly different from control, as determined by unpaired Student's *t* test or Mann–Whitney test (**d**)

matched control rats (Fig. 4a; $p = 0.3736$). However, the content of the S100B protein was reduced in the hippocampus of diabetic rats (Fig. 4b; $p = 0.0461$).

Extracellular Levels of S100B are Altered in Diabetic Rats

In the CNS, S100B protein is mainly produced and secreted by astrocytes and variations of extracellular levels of this protein have been reported in diabetic patients and models. We measured serum and CSF levels of S100B and found a dramatic decrease in serum S100B (Fig. 5a; $p < 0.0001$), but without changes in CSF levels (Fig. 5b; $p = 0.1125$). Moreover, we measured the S100B content in the extracellular medium of acute hippocampal slices. In this ex vivo assay we observed an increase in S100B secretion (Fig. 5c; $p = 0.0083$).

Glutamatergic Neurotransmission is Modified in Diabetic Rats

Assuming the key role of astrocytes in connecting energy metabolism and glutamatergic neurotransmission, we investigated glutamate transport in hippocampal slices. We found a decrease in glutamate uptake in the diabetic rats (Fig. 6a; $p = 0.0054$). Conversely, the protein levels of the main astrocytic glutamate transporters, GLT-1 and GLAST, did not differ between control and diabetic rats (Fig. 6b; $p = 0.9986$ and Fig. 6c; $p = 0.3467$, respectively). Moreover, the GluN1 subunit of the receptor NMDA for glutamate was found to be reduced in the hippocampus of diabetic rats. (Figure 6d; $p = 0.0308$).

Discussion

Animal models have contributed immensely to our understanding of the biochemical modifications of DM. The peripheral alterations in our STZ-induced model of DM are in accordance with those of the literature [30–32]. More recently, such models have been used to address questions of central neurochemical modifications in DM, particularly in relation to cognitive impairment and even dementia [33, 34].

Chronic hyperglycemia is associated with the formation and accumulation of AGEs in DM [35]. Accordingly, we found an augment in the AGE levels in the serum and CSF of diabetic rats. AGEs are cleared by scavenger receptors (see [36] for a review). However, AGE activate the multiligand receptor associated with the inflammatory response, RAGE (receptor for AGE) [37].

RAGE activation plays a key role in the pathogenesis of diabetic vascular complications [38] and neurodegenerative disorders, including AD [39]. The AGE-RAGE pathway investigated in epidemiological studies has reinforced the association between DM and AD [40, 41]. We, herein, demonstrate an increase in the levels of RAGE in the hippocampus of diabetic rats. Activation of AGE-RAGE signaling involves an inflammatory response that is mediated by NF-kB and cytokines [42].

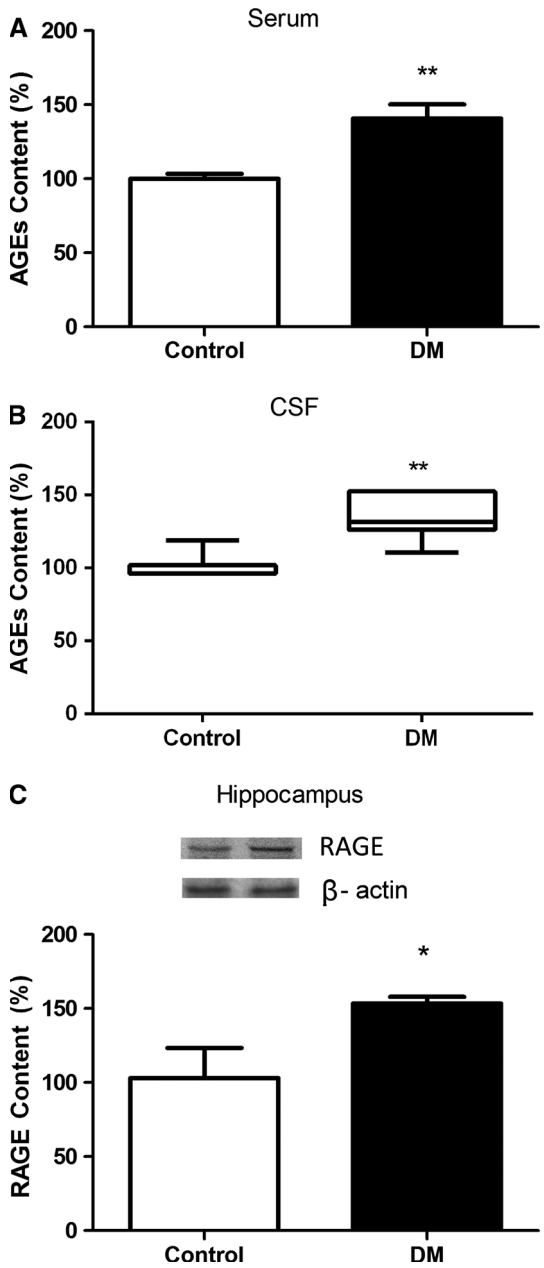

Fig. 2 AGEs and RAGE are altered in diabetic rats. We found augmented AGE levels in the serum (**a**) and in CSF (**b**), as evaluated by ELISA. Western blotting showed an augment in RAGE protein in the hippocampus of diabetic rats compared with controls (**c**). Results represent the mean ± S.E.M (**a**) or the median and interquartile range (**b**). Representative Western blots are presented above each respective graph. The results are normalized to β-actin and represent the mean ± S.E.M., expressed as arbitrary units (**c**). N = 6 in all panels. $^{*}p < 0.05$ or $^{**}p < 0.01$ significantly different from control, as determined by unpaired Student's *t* test (**a**, **c**) or Mann–Whitney test (**b**)

Two astroglial markers have been widely investigated in DM [30, 43]; however, there are conflicting results in the literature. We did not find any alteration in hippocampal GFAP content, but we observed a decrease in S100B in STZ-diabetic rats. Some authors have reported a significant increase in GFAP protein [44, 45], while others have related a decrease of this protein in the hippocampus of diabetic animals [6, 31, 46, 47]. In part, these discrepancies might be due to methodological differences in the animal models used, the heterogeneity of glial cells, time analysis and assays employed for GFAP measurement. It is also important to mention that, although GFAP is the most common marker of astrogliosis, astroglial activation does not necessarily involve GFAP increment [48]. With regard to the S100B protein, it has been reported that no change in the number of S100B-containing cells occurs in diabetic rats [31, 47, 49], despite other glial abnormalities. In another study, a transitory increase of S100B-positive cells in the hippocampus was observed in the first week after STZ administration [7].

The serum S100B has been proposed as a marker of glial activation or brain damage [50], but many extracerebral sources, such as adipose tissue, can contribute to serum S100B levels [51]. Steiner et al. observed a correlation between serum S100B and body mass index in humans [52]. We found a reduction in the serum S100B levels, which was in accordance with some clinical reports [53, 54]. To our knowledge, this is the first time that serum S100B has been measured in STZ-induced DM. The decreased serum S00B was not accompanied by changes in CSF levels. Moreover, it is important to mention that in other study where STZ was administered in the intracerebroventricular space (to induce dementia), we observed a decrease in CSF S100B, without changes in serum S100B. Together these data reinforce the idea that S100B changes in CSF and serum are not necessarily related [20].

The AGE-RAGE signaling pathway leads to NF-kB activation, which in turn, induces the expression of pro-inflammatory cytokines, such as IL-1β and TNF-α [55]. Such inflammatory mediators could cause the decrease in glutamate uptake [56] that we observed. A correlation between RAGE content and glutamate uptake was not found in the hippocampus ($r^2 = 0.11$; $p = 0.52$). However, RAGE content does not necessarily express RAGE activation and the small "N" in this study limits this analysis. Moreover, another mechanism of impairment of glutamate uptake in diabetic rats involving AGEs, but independent of RAGE, cannot be ruled out [57]. Despite the decrease in glutamate uptake, we did not find any changes in the levels of glutamate transporters (GLT-1 and GLAST). Regardless of the mechanism, impairment of glutamate uptake could result in elevated extracellular levels of glutamate and, therefore, excitotoxicity. Accordingly, the GluN1 subunit of the NMDA receptor, which is downregulated by chronic excitotoxicity [58], was found to be reduced in our STZ-diabetic animals.

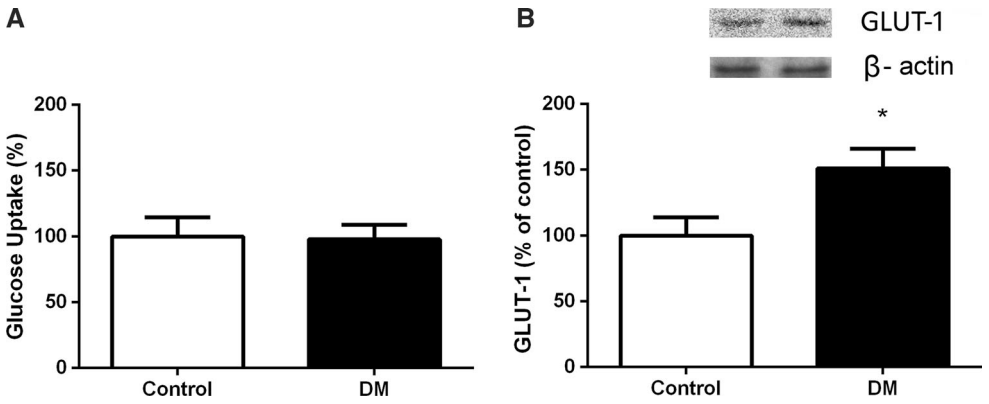

Fig. 3 Brain glucose transport is modified in diabetic rats. The DM rats did not present any alteration in glucose uptake activity, when compared with the controls (**a**). Western blotting showed an augment in GLUT-1 protein levels in diabetic rats, compared with the controls (**b**). Representative Western blots are presented above each respective graph. The results are normalized to β-actin and represent the mean ± S.E.M., expressed as arbitrary units, N = 6, $*p < 0.05$ significantly different from control, as determined by unpaired Student's *t* test

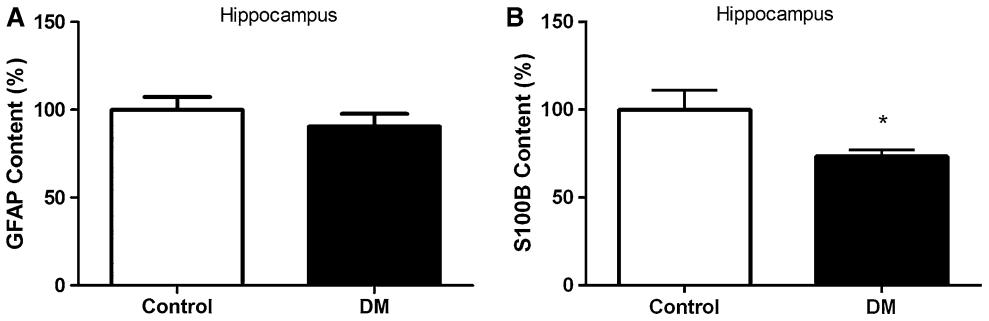

Fig. 4 Astroglial marker S100B is altered in the hippocampus of diabetic rats. ELISA showed no alterations in hippocampal GFAP (**a**) and a decrease in S100B content (**b**) in DM rats, compared with controls. Results represent the mean ± S.E.M., N = 6, $*p < 0.05$ by unpaired Student's *t* test

Pro-inflammatory cytokines induced by RAGE activation can trigger S100B secretion in hippocampal slices [59]. Therefore, although we did not find elevated CSF levels of S100B, the putative inflammatory state could explain the higher basal S100B secretion observed in the acute hippocampal slices of diabetic rats. On the other hand, we know that high extracellular levels of glutamate reduce S100B secretion in hippocampal slices [60]. We searched for a possible correlation between RAGE content and S100B secretion ($r^2 = 0.29$ and $p = 0.26$), as well as between glutamate uptake and S100B secretion ($r^2 = 0.09$ and $p = 0.54$). The lack of correlations found in this preliminary approach is limited by the small experimental "N" and we suggest that this issue deserves further investigation. It is important to emphasize that extracellular S100B binds to RAGE and, in this condition, it could work as a positive feedback mediator, reinforcing the inflammatory signaling.

We found an increase in the hippocampal amount of GLUT-1, but we did not find any modification in glucose uptake activity in the hippocampal slices of diabetic animals. Accordingly, an increase in GLUT-1 mRNA in brain cortical microvessels and no alterations in glucose uptake in the hippocampus and cerebral cortex of STZ-induced diabetic rats has been described in the literature [61]. An increase in GLUT-1 also was observed in a human BBB endothelial cell line exposed to a medium with high levels of glucose for 24 h [62]. However, Wang et al. found that the glucose transport across BBB was not altered in uncontrolled DM for up to 10 weeks in STZ-rats [63].

The mechanism of GLUT-1 increase in diabetic models and its significance is unclear at the moment. Expression of this transporter is increased by hypoglycemia [61] and in vitro results suggest that it can be regulated by inflammatory processes (see [18] for a review). Therefore, RAGE activation could help us explain the increase in GLUT-1 in diabetic rats. However, at this moment, we cannot estimate how much of the glucose uptake is due to astrocyte activity or how much of the GLUT-1 content is from astrocytes in hippocampal slices.

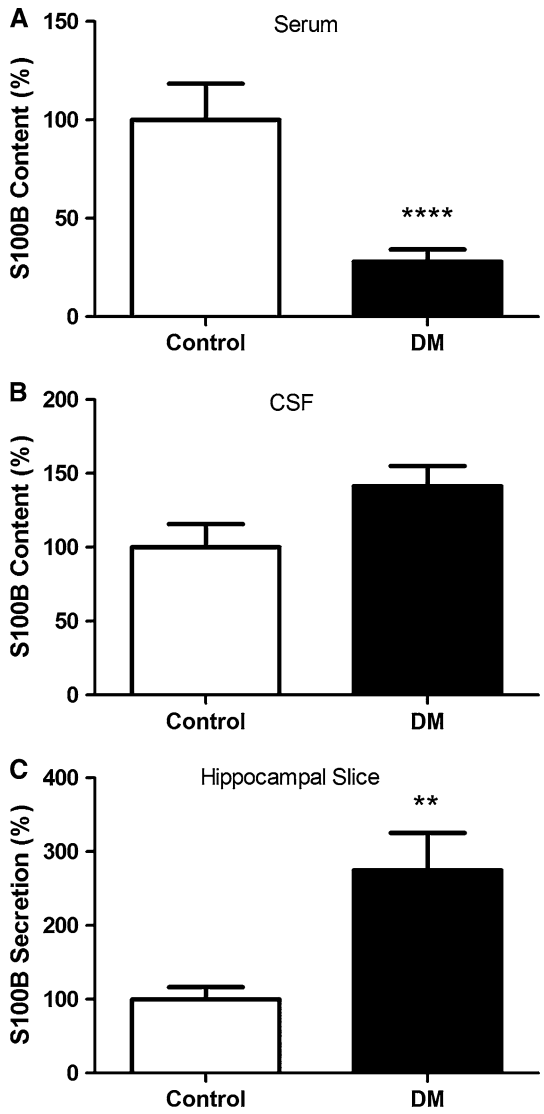

Fig. 5 Extracellular levels of S100B are altered in diabetic rats. ELISA showed a decrease in S100B levels in the serum (**a**) and no alterations in the CSF (**b**). The S100B secretion in hippocampal slices (**c**) was augmented in DM rats, compared with controls. Results represent the mean ± S.E.M., N = 6, $^{**}p < 0.01$ or $^{****}p < 0.0001$ by unpaired Student's *t* test

Several studies have demonstrated an association between DM and learning and memory impairments. The cognitive deficit observed in DM is associated, among other factors, with changes in hippocampal synaptic plasticity [64]. In this study, we observed a decrease in glutamate uptake and in the content of the GluN1 subunit of the NMDA glutamate receptor. Our findings support the hypothesis that glutamatergic transmission is altered in DM. Consistent with this idea, posttranslational alterations in the GluN1 of STZ-diabetic rats have been reported [65]. On the other hand, decreased GluN2B subunit (but not GluN1 or GluN2A) protein level was observed in post-synaptic density fractions of hippocampi from of STZ-diabetic rats [66].

In summary, in this STZ-induced model of DM, we found significantly elevated levels of AGEs in the serum and CSF of animals as well as an increase of RAGE in hippocampal tissue. We found specific astroglial abnormalities in this brain region, such as reduced S100B content, reduced glutamate uptake and increased S100B secretion, that were not accompanied by changes in GFAP. We also observed an increase in the glucose transporter, GLUT-1. All these changes are may be due to RAGE-induced inflammation. These astroglial alterations, together with the reduced content of the GluN1 receptors in the hippocampus, may be associated with the impairment of glutamatergic communication in diabetic rats and possibly contribute to the cognitive deficits that are observed in diabetic patients and experimental models.

Experimental Procedure

Experimental Animals

A total of twenty-six isogenic male Wistar-Kyoto (WKY) rats (8-weeks-old, weighing 160–270 g) were obtained from our breeding colony. Rats were maintained under a 12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C and had with free access to a 20 % (w/w) protein commercial chow and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project approval number 20375). All efforts were made to minimize animal suffering and reduce the number of animals used.

STZ-Induced Diabetic Model and Experimental Design

DM was induced in 14 WKY rats by intraperitoneal injection of streptozotocin (STZ) (75 mg/kg in citrate buffer with an injection volume of 300 µL/kg body weight, pH 4.5); 12 age-matched animals that received only vehicle (citrate buffer) were used as control. The animals were then housed (3–4 per cage) for a period of 60 days. Body weight, blood and urine glucose levels were recorded every 2 weeks. Glycemia was measured with an Accu-Chek® Active Kit (Roche Diagnostics, Basilea, Switzerland), and the animals were considered diabetic if their blood glucose levels were more than 250 mg/dL. Twelve (12 of 14) rats became diabetic (as indicated by glycemic parameters measured 48 h after STZ administration). Glycosuria was

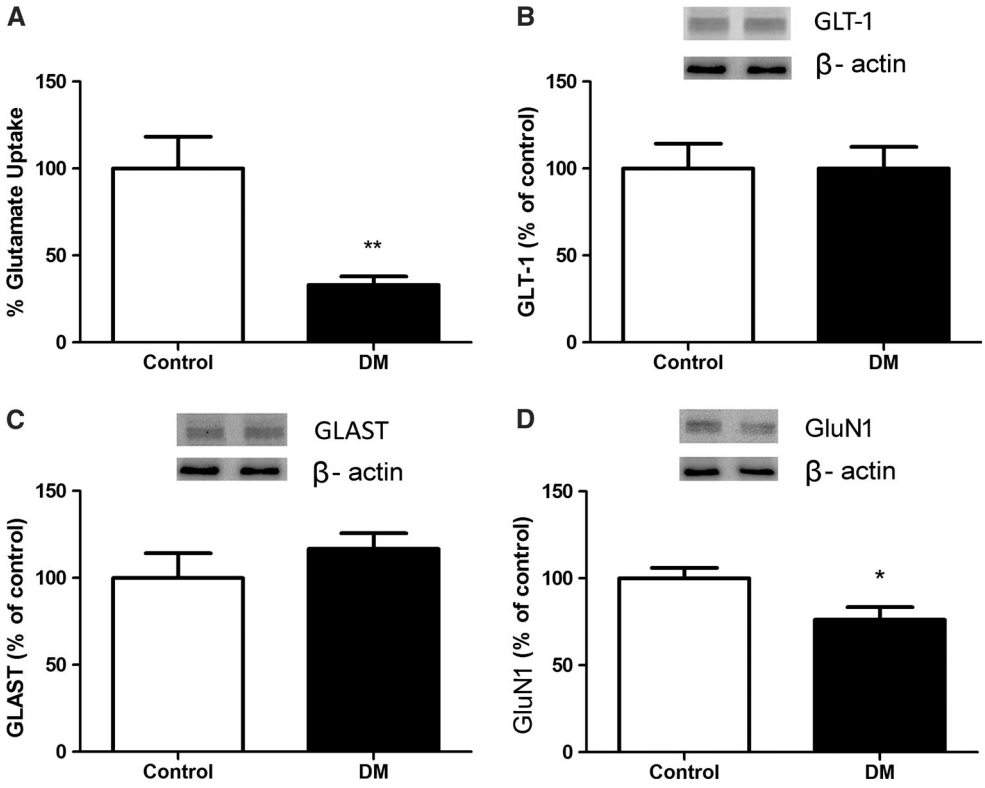

Fig. 6 Glutamatergic neurotransmission is modified in diabetic rats. Glutamate uptake in hippocampal slices revealed a decrease in DM rats, compared to control rats (**a**). Western blotting showed no alterations in protein levels of GLT-1 (**b**) and GLAST (**c**), but showed a decrease in GluN1 protein levels (**d**) in diabetic rats, compared with the controls. Representative Western blots for each protein are presented above each respective graph. The results are normalized to β-actin and represent the mean ± S.E.M., expressed as arbitrary units, N = 6, $*p < 0.05$ or $**p < 0.01$ significantly different from control, as determined by unpaired Student's *t* test

confirmed with urinalysis strips (Sensi 10, Guangxl, China). Biochemical analyses were performed 60 days after DM induction and in the respective controls. Diabetic (N = 12) and control (N = 12) rats were randomly divided into two groups for biochemical analysis. One group (N = 6) was anesthetized (see item Cerebrospinal Fluid and Serum Samples) and used for CSF and blood sampling. The other group (N = 6) was euthanized by decapitation and used for obtaining hippocampal samples.

Material

Streptozotocin; mouse monoclonal anti-actin antibody (1A4 clone); Ponceau S; cytochalasin B; mouse monoclonal anti-S100B antibody (SH-B1 clone); *L*-glutamate; 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and *o*-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-GFAP and anti-S100 antibodies were obtained from DAKO (Glostrup, Denmark). Goat polyclonal anti-RAGE and anti-GLUT-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-GluN1 (R1JHL clone) was obtained from Millipore (Darmstadt, Germany). The rabbit polyclonal anti-GLAST and anti-GLT-1 antibodies were purchased from ABCAM (Cambrigde, UK). The peroxidase-conjugated immunoglobulin (IgG) antibody and *L*-[2,3-^{3}H] glutamate were obtained from Amersham (Buckingamshire, UK). *D*-[3-^{3}H] glucose (20 Ci/mmol) was obtained from Perkin-Elmer (Boston, MA, USA). Anti-AGE antibody (6D12) was purchased from Cosmo Bio (Tokyo, Japan). All other chemicals were purchased from local commercial suppliers.

Cerebrospinal Fluid and Serum Samples

Animals were anesthetized using ketamine and xylazine (Syntec, São Paulo, Brazil) administered at doses of 75 and 10 mg/kg, respectively, and then positioned in a stereotaxic holder for collection of 100 μL (approximately) of CSF from the cisterna magna. The puncture was performed using an insulin 1 mL syringe and 31 G needle (0.25 mm diameter, 6 mm length). Rats were then removed from the stereotaxic apparatus and placed on a flat surface, where approximately 3 mL of whole blood was obtained through an intracardiac puncture using a 5 mL syringe and 21 G needle (0.80 mm diameter, 25 mm length) inserted into the intercostal space above the sternum. Serum was separated by centrifugation at 3000 g for 10 min. CSF and serum samples were frozen (−80 °C) until further analysis [67].

Glycated Hemoglobin Assay

Glycated hemoglobin was measured in whole blood using a Glycated Hemoglobin Doles Kit Assay (Goiânia, Goias, Brazil) according to the manufacturer's manual.

C-Peptide Assay

Proinsulin C-peptide was assessed using a Rat/Mouse C-Peptide 2 ELISA kit (Millipore, Darmstadt, Germany) according to the manufacturer's recommendations. Briefly, serum was added to a 96-well flat-bottom plate and incubated with a mixture of capture and detection antibodies, at 1:1, for 2 h at room temperature. Subsequently, the plate was incubated with streptavidin-horseradish peroxidase for 30 min at room temperature. The color reaction produced with tetramethylbenzidine (TMB) was then quantified in a plate reader at 450 and 590 nm. The standard C-peptide curve ranged from 0 to 1600 pM.

AGE Measurement

AGEs were measured in serum and CSF by an enzyme-linked immunosorbent assay (ELISA), as previously described [68] with some modifications. The wells of a microtiter plate were coated overnight with 0.1 μg protein in 0.1 mL of 50 mM carbonate bicarbonate buffer (pH 9.6). The wells were washed three times with washing buffer (PBS containing 0.05 % Tween 20) and then incubated for 3 h with 2 % albumin from chicken egg white to block nonspecific binding. Subsequently, wells were washed again with washing buffer and incubated with 100 μL of anti-AGE (6D12) for 1 h. After three washes, wells were incubated with 100 μL of peroxidase-conjugated secondary antibody for 1 h. The reactivity of peroxidase was determined by incubation with OPD for 30 min. The reaction was stopped by the addition of 50 μL sulfuric acid (3 M). Absorbance measurements were taken at 492 nm. Results were calculated and expressed as a percentage of the control.

Preparation and Incubation of Hippocampal Slices

After obtaining whole blood by intracardiac pucture, the animals were decapitated with the aid of a guillotine, the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4, and 10 glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately into 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. The medium was changed every 15 min with fresh saline medium at room temperature (maintained at 25 °C). Following a 120-min equilibration period, the medium was removed and replaced with basal media for 60 min at 30 °C in a warm plate. Thirty microliters of media were collected for S100B secretion measurement and the slices were collected for GFAP and S100B content measurement [69].

Glucose Uptake Assay

Glucose uptake was performed as previously described [70], with some modifications. Briefly, hippocampal slices were incubated at 35° 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 $Na_2HPO_4{\cdot}7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, and 5.6 glucose, adjusted to pH 7.2. The assay was started by the addition of 0.1 μCi deoxy-*D*-[3-^{3}H] glucose/well. The incubation was stopped after 30 min by removing the medium and rinsing the slices twice with ice-cold HBSS. The slices were then lysed in a solution containing 0.5 M NaOH. Glucose uptake was calculated by subtracting the non-specific uptake, obtained by the glucose transporter inhibitor, cytochalasin B (10 μM), from the total uptake in order to obtain the specific uptake. Radioactivity was measured in a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentage of the control.

Glutamate Uptake Assay

Glutamate uptake was performed as previously described [69]. The hippocampal slices were incubated in HBSS containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$, and 5.6 glucose, in pH 7.2. The assay was started by the addition of 0.1 mM *L*-glutamate and 0.66 μCi/ml 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 0.1 M NaOH and 0.01 % SDS. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the specific uptake. Radioactivity was measured with a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentage of the control.

Western Blotting

Equal amounts (30 μg) of proteins from each sample were boiled in sample buffer [0.0625 M Tris–HCl pH 6.8, 2 % (w/v) SDS, 5 % (w/v) β-mercaptoethanol, 10 % (v/v) glycerol, 0.002 % (w/v) bromophenol blue] and electrophoresed in 10 % (w/v) SDS–polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane and transference was confirmed with Ponceau S staining [71]. After incubating for 1 h at room temperature

with the primary antibody (anti-GluN1, anti-GLUT-1, anti-GLAST, anti-GLT-1 or anti-RAGE at 1:5000 dilution), the filters were washed and incubated with anti-rabbit, anti-mouse or anti-goat peroxidase-conjugated IgG (at dilution of 1:10000), for 1 h. Protein loading in each lane was normalized by the actin content (measured using antibody anti-actin, at 1:2000 dilution). The chemiluminescence signal was detected using an ECL Western Blotting Detection Kit from Amersham and captured using an ImageQuant LAS400 (GE).

S100B Measurement

S100B was determined by ELISA, as previously described [72]. Briefly, 50 µL of sample plus 50 µL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B antibody. Polyclonal anti-S100 was incubated for 30 min and then peroxidase-conjugated anti-rabbit IgG antibody was added for an additional 30 min. The color reaction with OPD was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL.

GFAP Measurement

ELISA for GFAP was performed as previously described [73]. Briefly, microtiter plates were coated with 100 µL of samples for 24 h at 4° C and then incubated with a polyclonal anti-GFAP from rabbit for 1 h, followed by incubation with a secondary antibody conjugated with peroxidase for 1 h at room temperature. A colorimetric reaction with OPD was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng/mL.

Protein Determination

The protein content was measured by Lowry's method using bovine serum albumin (BSA) as a standard [74].

Statistical Analysis

Data normality (by Kolmogorov–Smirnov test) and statistical analyses were performed using PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA), assuming $p < 0.05$ as significant. Parametric data from the experiments are presented as mean ± standard error (S.E.M.) and were statistically evaluated by Student's t test. Non–parametric data (CSF AGE and C-peptide) are presented as medians and interquartile range and were statistically evaluated by Mann–Whitney test.

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Compliance with ethical standards

Conflict of interest No conflict of interest, financial or otherwise, are declared by the authors.

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CAPÍTULO II

Exendin-4 Reverses Biochemical and Functional Alterations in the Blood-brain and Blood-CSF Barriers in Diabetic Rats

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OBJETIVOS ESPECÍFICOS

1. Avaliar o prejuízo cognitivo no modelo de DM;
2. Avaliar possíveis alterações na BHE e BHL;
3. Investigar o efeito da EX-4 em reverter as alterações cognitivas e bioquímicas observadas no modelo de DM.

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Exendin-4 Reverses Biochemical and Functional Alterations in the Blood–Brain and Blood–CSF Barriers in Diabetic Rats

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Abstract Diabetes mellitus (DM) is a metabolic disorder associated with micro- and macrovascular alterations that contribute to the cognitive impairment observed in diabetic patients. Signs of breakdown of the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB) have been found in patients and animal models of DM. Breakdown of the BBB and BCSFB can lead to disruptions in cerebral homeostasis and eventually neural dysfunction and degeneration. However, our understanding of the biochemistry underlying barrier protein modifications is incomplete. Herein, we evaluated changes in the levels of specific proteins in the BBB (occludin, claudin-5, ZO-1, and aquaporin-4) and BCSFB (claudin-2 and aquaporin-1) in the hippocampus of diabetic rats, and we also investigated the functional alterations in these barriers. In addition, we evaluated the ability of exendin-4 (EX-4), a glucagon-like peptide-1 agonist that can cross the BBB to reverse the functional and biochemical modifications observed in these animals. We observed a decrease in BBB proteins (except ZO-1) in diabetic rats, whereas the EX-4 treatment recovered the occludin and aquaporin-4 levels. Similarly, we observed a decrease in BCSFB proteins in diabetic rats, whereas EX-4 reversed such changes. EX-4 also reversed alterations in the permeability of the BBB and BCSFB in diabetic rats. Additionally, altered cognitive parameters in diabetic rats were improved by EX-4. These data further our understanding of the alterations in the central nervous system caused by DM, particularly changes in the proteins and permeability of the brain barriers, as well as cognitive dysfunction. Furthermore, these data suggest a role for EX-4 in therapeutic strategies for cognitive dysfunction in DM.

Keywords Blood–brain barrier · Blood–cerebrospinal fluid barrier · Cognitive impairment · Diabetes mellitus · GLP-1 agonist

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Introduction

Diabetes mellitus (DM) is a systemic metabolic disorder that leads to the development of micro- and macrovascular complications and is associated with an increased risk of mild cognitive impairment, dementia, and stroke [1]. Hyperglycemia is one of the main causes of endothelial dysfunction and a precursor of microvascular complications in DM [2]. Disturbances in cerebral microvessels may contribute to cognitive and functional deficits in specific brain regions, such as the hippocampus [3]. It has been demonstrated that the

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development of cognitive deficits in adults with type 1 diabetes mellitus (T1DM) is usually slow compared with healthy individuals, except in subgroups of patients with microvascular complications who may present a more marked decline [4]. Supporting these data, T1DM has been associated with reduced cognitive functioning, particularly in the presence of microangiopathy [5, 6]. However, despite evidence of vascular pathology in diabetic patients and experimental models, the possibility that ruptures may occur in the blood–brain barrier (BBB), and their association with cognitive impairment, is still in debate [7, 8].

The central nervous system (CNS) is selectively impermeable to molecules circulating in the blood through the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB), providing a stable microenvironment that is critical for brain function [9]. The tight junctions (TJ) of the brain barriers are formed by a transmembrane protein complex that constitute physical barriers by regulating the passage of ions and solutes through the paracellular slot [10]. Endothelial TJ of the BBB include proteins such as zonula occludens protein-1 (ZO-1), occludin, and claudin-5 [9]. Breakdown of the BBB that accompanies several disorders, such as ischemia [11] and epilepsy [12], exposes the brain to neurotoxins, plasma components, immune molecules and cells that may cause dysfunction and neural degeneration [13]. Epithelial TJ of the BCSFB contain the protein claudin-2. Moreover, several types of aquaporins (AQPs), which are water channel proteins that play a critical role in controlling the water content of cells, have been studied in pathological brain conditions, including edema, trauma, and dementia, and may be altered during neuroinflammation, which is frequently associated with changes in BBB and BCSFB permeability [14, 15]. The primary AQPs related to BBB and BCSFB disorders are aquaporin-4 (AQP4) and aquaporin-1 (AQP1). AQP4 is specifically found in astrocytes, particularly in the endfeet surrounding the BBB endothelium, whereas AQP1 is found in the choroid epithelium of the BCSFB and plays an important role in cerebrospinal fluid (CSF) formation [15].

Glucagon-like peptide-1 (GLP-1) receptor agonists stimulate insulin secretion from pancreatic β-cells in a glucose-dependent manner and are well-established drugs for treating type 2 diabetes mellitus (T2DM) [16]. Moreover, some studies have investigated the role of GLP-1 agonists in patients [17] with T1DM and rodents [18] and have reported benefits including increased proliferation of beta cells with a reduction in apoptosis and improved glycemic control in patients [17, 19].

Exendin-4 (EX-4) is a long-acting analogue of GLP-1 that can cross the BBB [20]. When it is administered peripherally, it appears to have beneficial effects on cognitive functioning in mice with chronic hyperglycemia associated-inflammation [21]. GLP-1 mimetics show an impressive range of protective effects in the CNS, and studies have shown positive effects on neurogenesis, synaptogenesis, and cell repair, as well as reductions in the chronic inflammatory response and levels of amyloid plaques [20]. These findings demonstrate that GLP-1 agonists have effects beyond insulin homeostasis.

Our working hypothesis is that vascular alterations in DM elapse with changes in BBB, as well as in BCSFB, and that such alterations contribute to the cognitive impairment observed in patients and experimental models. Because cognitive decline in diabetes may be associated with alterations in the hippocampus, a region related to learning and memory, we evaluated specific BBB (occludin, claudin-5, ZO-1, and AQP4) and BCSFB (claudin-2 and AQP1) proteins in the hippocampus of STZ-diabetic rats, and we investigated the functional changes in these barriers 30 and 60 days after DM induction. In addition, we evaluated the ability of EX-4 to reverse the functional and biochemical changes observed in these animals, as well as its possible protective role against cognitive deficit.

Materials and Methods

Experimental Design and Animals

A total of 75 male Wistar Kyoto (WKY) rats (8 weeks old, weighing 160–270 g) were obtained from our breeding colony. Rats were maintained under a 12-h light/12-h dark cycle at a constant temperature of 22 ± 1 °C and had free access to a commercial chow and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23), and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project number 24076). All efforts were made to minimize animal suffering and reduce the number of animals used.

DM was induced in 45 WKY rats by intraperitoneal injection of streptozotocin (STZ) (75 mg/kg in citrate buffer with an injection volume of 300 μL/kg body weight, pH 4.5). Forty-one (41 of 45) rats became diabetic (as demonstrated by glycemic parameters measured 48 h after STZ administration); 30 age-matched animals that received only vehicle (citrate buffer) were used as controls. The animals were then housed (3–4 per cage) for a period of 30 or 60 days. Body weight and blood glucose levels were recorded every 2 weeks. Glycemia was measured with an Accu-Chek® Active Kit (Roche Diagnostics, Basilea, Switzerland), and the animals were considered diabetic if their blood glucose levels were more than 250 mg/dL.

Behavioral and other biochemical analyses were performed 30 (DM 30) and 60 (DM 60) days after DM induction and in their respective controls. At 30 days, 10 DM rats and 10 control rats were submitted to cognitive evaluation, followed by euthanasia and biochemical analysis. At 60 days, 30 DM rats

and 20 controls were submitted to cognitive evaluation. After performing the cognitive task, rats in the DM 60 group were randomly divided in three subgroups. One was euthanized for biochemical analysis ($n = 10$) and two other were assigned: DM-vehicle ($n = 10$) and DM-EX-4 ($n = 10$). The DM-EX-4 animals received EX-4 10 μg/kg intraperitoneally once a day for 28 days, whereas the DM-vehicle animals received an equal volume of saline solution. Note that, at 30 days after STZ, there were 10 DM and 10 control rats; at 60 days there were 10 DM and 10 control rats for biochemical analysis, and a total of 30 DM and 20 control rats for behavioral analysis; finally, at 28 days after EX-4 treatment, 10 DM-EX-4 and 10 DM-vehicle rats were used for cognitive evaluation and biochemical analysis. For biochemical analysis, half of the animals from each subgroup was used for Evan blue assay and measurements of acid ascorbic and albumin. The other half was used for Western blotting assays. The numbers of animals used in each evaluated parameter are detailed in the figure legends.

Material

STZ, Evans blue, mouse monoclonal anti-actin antibody, EX-4, and Ponceau S were obtained from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-AQP4 and anti-AQP1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and Millipore (Darmstadt, Germany), respectively. Peroxidase-conjugated IgG antibodies and ECL Western Blotting Detection Kit were obtained from Amersham (Buckingamshire, UK). The antibodies mouse monoclonal anti-claudin-5 and anti-occludin, mouse polyclonal anti-claudin-2 and rabbit polyclonal anti-ZO-1 were purchased from Invitrogen (Carlsbad, USA). The Ascorbate Assay Kit was purchased from BioVision Research Products (Mountain View, CA, USA). All other chemicals were purchased from local commercial suppliers.

Glycated Hemoglobin Assay

Glycated hemoglobin was measured in whole blood using a Glycated Hemoglobin Doles Kit Assay (Goiânia, Goias, Brazil) according to the manufacturer's manual.

Cognitive Evaluation

The DM 30 and DM 60 groups underwent training in the Morris water maze [22]. Following the task, the DM 60 group was randomly divided into DM-vehicle or DM-EX-4 subgroups. After 28 days, rats in both subgroups repeated the water maze task. The task apparatus consisted of a circular pool filled with water that was placed in a room with spatial cues. An escape platform was placed 1.5 cm below the water surface in the middle of one of the quadrants and was equidistant from the maze walls and the middle of the pool. The escape platform was located in the same quadrant in every trial. Four different starting positions were equally spaced around the perimeter of the pool, and the positions were used in a random sequence each training day. A trial began by placing the animal in the water at one of the starting points. If the animal failed to escape within 60 s, then it was gently conducted to the platform, where it was allowed to stay for 20 s. The rats were trained for 5 days and were submitted to a test session 24 h after the last training session. The submerged platform was removed before the test session. The retention test consisted of placing the animals in the water for 1 min. The number of crossings over the original platform position and the time spent in the target quadrant compared with the opposite quadrant were measured.

Evans Blue Extravasation

BBB permeability was evaluated according to the distribution in the rat brain of Evans blue dye administered at a dose of 2 mg/kg by an intracarotid injection [23]. Rats were anesthetized using ketamine and xylazine (Syntec, São Paulo, Brazil) administered at doses of 75 and 10 mg/kg, respectively. The dye was injected through a P10 catheter via a small incision in the right common carotid artery. After 5–10 min, the rats were decapitated and the brain was dissected.

For quantification, the hemispheres were separated along the sagittal suture. Both hemispheres were weighed and immersed in formamide (1 mL/100 mg) at 60 °C for 24 h. The content of the dye extracted from each brain was determined with a spectrophotometer (at 620 nm). The quantitative calculation of the dye content was based on the external standards dissolved in the same solvent [24].

Cerebrospinal Fluid and Serum Samples

Animals were anesthetized as described above and then positioned in a stereotaxic holder for collection of 100 μL (approximately) of CSF from the cisterna magna. The puncture was performed using an insulin 1 mL syringe and 31 G needle (0.25 mm diameter, 6 mm length). Rats were then removed from the stereotaxic apparatus and placed on a flat surface, where approximately 3 mL of whole blood was obtained through an intracardiac puncture using a 5-mL syringe and 21 G needle (0.80 mm diameter, 25 mm length) inserted into the intercostal space above the sternum. Serum was separated by centrifugation at 3000 g for 10 min. CSF and serum samples were frozen (−80 °C) until further analysis [25].

Protein Determination

Protein levels were measured by Lowry's method using bovine serum albumin as a standard [26].

Determination of CSF/Serum Albumin Ratio

Serum and CSF were collected as described above. The CSF/serum albumin ratio was analyzed using the bromocresol green assay kit from Doles (Goiânia, Brazil). The modified method included the mixture of 10 μL of serum or 50 μL of CSF sample with 2 mL or 500 μL of color reagent, respectively. The samples were incubated for 10 min at 37 °C, and the absorbance of each sample was measured at 546 nm against a reagent blank using a spectrophotometer [27].

Determination of CSF/Serum Ascorbic Acid Ratio

Serum and CSF were collected as described above. Ascorbic acid in the blood or CSF was measured with the Ascorbic Acid Colorimetric Assay Kit II, BioVision (Mountain View, CA, USA) according to the manufacturer's instructions.

Western Blotting

Equal amounts (30 μg) of proteins from each sample were boiled in sample buffer [0.0625 M Tris–HCl pH 6.8, 2 % (*w*/*v*) SDS, 5 % (*w*/*v*) β-mercaptoethanol, 10 % (*v*/*v*) glycerol, 0.002 % (*w*/*v*) bromophenol blue] and electrophoresed on a 10 % (*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 [28]. After incubating for 1 h at room temperature with the primary antibodies (anti-AQP4, anti-AQP1, anti-claudin-5, anti-claudin-2, anti-occludin, or anti-ZO-1 at 1:5000 dilution), the filters were washed and incubated with anti-rabbit or anti-mouse peroxidase-conjugated immunoglobulin (IgG) (at dilution of 1:10,000). The chemiluminescence signal was detected using an ECL Western Blotting Detection Kit from Amersham.

Statistical Analysis

The escape latency parameter in the Morris water maze task was evaluated by two-way ANOVA with a repeated measures analysis of variance followed by Tukey's post hoc test using PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA), assuming $p < 0.05$ as significant. Biochemical data from the experiments are presented as the mean ± standard error and were statistically evaluated by *Student's t test*, assuming $p < 0.05$ as significant. The peripheral parameters (in Table 1) were analyzed by one-way ANOVA followed by Tukey's post hoc test.

Results

Exendin-4 Did Not Alter the Peripheral Parameters in Diabetic Rats

To validate our DM animal model in WKY rats, we measured some peripheral parameters (Table 1) and observed that the DM 60 group exhibited significantly lower body weight than age-matched control rats ($p < 0.0001$). We then analyzed the glycemia and glycated hemoglobin concentrations in total blood, and the DM 60 group showed an augment in these factors compared with the control group ($p < 0.0001$). After 28 days of EX-4 administration to the subgroup randomly derived from DM 60 group, we still observed a body weight reduction and an increase in glycemia and glycated hemoglobin concentrations in the DM-EX-4 subgroup when compared with the control group. Interestingly, the glycated hemoglobin concentration increased in the DM-EX-4 subgroup compared with the DM 60 group ($p < 0.0001$) (Table 1).

Exendin-4 Improves Cognitive Deficits in Diabetic Rats

The Morris water maze was used to evaluate reference memory in the DM 30 and DM 60 groups. When the DM 30 group and their respective control group were analyzed, both showed significantly reduced escape latencies during the acquisition phase; however, there were no differences between the groups [treatment × days, $F(4.76) = 4.079$, $p = 0.0048$; days, $F(4.76) = 24.56$, $p < 0.0001$; treatment, $F(1.19) = 0.3745$, $p = 0.5478$]. In the test trial, controls and DM 30 did not show any changes in the parameters analyzed, demonstrating that these animals did not show cognitive deficits ($p > 0.05$) (data not shown).

The DM 60 group and its respective control group demonstrated significant improvements in task performance in the training sessions. Main effects of days [$F(4.232) = 93.18$, $p < 0.0001$] and treatment [$F(1,58) = 15.12$, $p = 0.0003$] were observed. No interactions between these factors were observed [treatment × days, $F(4,232) = 1,393$, $p = 0.2373$; Fig. 1a]. These effects can be attributed to the latencies during the days of the training session (Fig. 1a), which decreased for both groups as the animals showed improved times for finding the platform. However, the entire control group showed a better performance compared with DM 60. In the probe trial, the DM 60 group showed a longer latency for finding the original platform location compared with its control group (Fig. 1c $p < 0.0001$). Moreover, the number of crossings over the platform location was significantly lower in the DM 60 compared with controls (Fig. 1e; $p < 0.0001$).

On the test day, both the control and the DM 60 groups demonstrated an increase in the time spent in the target quadrant compared with the opposite quadrant [Fig. 1g; treatment × quadrant, $F(1.116) = 7.566$, $p = 0.0069$; quadrants,

Table 1 Exendin-4 did not alter the peripheral parameters observed in diabetic rats. Body weight, glycemia, and glycated hemoglobin were evaluated in the DM 60 ($n=30$) and control ($n=20$) groups 60 days after STZ induction. A randomly derived subgroup from DM 60 ($n=7$) is showed at right column, that was treated for 28 days with EX-4

	Control	DM 60	DM-EX-4
Body weight (g)	273.9 ± 9.9	180.9 ± 11.0****	208.9 ± 8.1****
Glycemia (mg/dL)	92.4 ± 10.8	531.7 ± 37.6****	546.6 ± 18.0****
Glycated hemoglobin (%)	3.5 ± 0.1	4.6 ± 0.1****	5.5 ± 0.2 $^{****}_{\#\#\#\#}$

All values are expressed as the mean ± S.E.M

*Significantly different from the control group, **** $p<0.0001$ # Significantly different from the DM 60 group, #### $p<0.0001$

F (1.116) = 43.59, $p<0.0001$; treatment, F (1.116) = 1.269, $p=0.2623$)]. Moreover, the control group spent more time in the target quadrant compared with the DM 60 group. The time spent in the opposite quadrant did not differ between the groups.

The DM 60 group was then randomly divided into two subgroups, DM-EX-4 and DM-vehicle, which received EX-4 or vehicle, respectively, for 28 days. After 28 days, the two subgroups exhibited in the training sessions similar performances and improved latencies for finding the platform during the acquisition phase [Fig. 1b; treatment × days, F (4, 184) = 3.530, $p=0.0084$; days, F (4.184) = 27.82, $p<0.0001$; treatment, F (1,46) = 14.93, $p=0.0003$].

In the test trial, the DM-EX-4 subgroup demonstrated improvement in both latency and crossings (Fig. 1d $p<0.0001$; Fig. 1f; $p<0.0001$). When the time spent in each quadrant was analyzed, both DM-vehicle and DM-EX-4 subgroups spent more time in the target quadrant than in the opposite quadrant. Additionally, the DM-EX-4 remained in the target quadrant for a longer duration than the DM-vehicle [Fig. 1h; treatment × quadrant, F (1,91) = 1416, $p=0.2371$; quadrants, F (1,91) = 14.48, $p=0.0003$; treatment, F (1,91) = 3.443, $p=0.0668$)]. Taken together, these results demonstrate a clear effect of EX-4 treatment in recuperating cognitive decline.

Diabetic Rats Exhibited Disruption of the BBB That Was Recovered by Exendin-4 Administration

The functional permeability of the BBB was evaluated in the DM 30 and DM 60 groups and their respective controls using Evans blue staining assay and the CSF/serum albumin ratio. A significant increase in Evans blue staining was observed (Fig. 2a, b) and quantified (Fig. 2e, $p=0.0198$) in the DM 60 group. Accordingly, an increase in the CSF/serum albumin ratio was observed in the same group (Fig. 2g, $p=0.0014$). In contrast with the DM-EX-4 subgroup, the DM-vehicle subgroup exhibited a strong staining with Evans blue (Fig. 2c), (Fig. 2d). The difference in Evans blue staining was quantified (Fig. 2f, $p=0.0213$) to characterize the recovery attributed to EX-4. In addition, the CSF/serum ratio of albumin decreased in DM-EX-4 rats (Fig. 2h, $p=0.0015$). Importantly, no change was observed in the Evans blue staining assay ($p=0.89$) or in the CSF/serum albumin ratio ($p=0.59$) in the DM 30 group (data not shown).

Diabetic Rats Exhibited Disruption of the BCSFB That Was Recovered by Exendin-4 Administration

We measured the CSF/serum ratio of ascorbate to evaluate the function of the BCSFB. No difference was observed in the DM 30 group (data not shown). However, we observed a decrease in CSF/serum ascorbate levels in the DM 60 group (Fig. 3a; $p=0.0016$). BCSFB function was reevaluated after EX-4 administration (or vehicle), which revealed the CSF/serum ratio of ascorbate was augmented in the DM-EX-4 subgroup compared with the DM-vehicle subgroup (Fig. 3b; $p=0.0279$).

Levels of Specific BBB Proteins Are Altered in Diabetic Rats

To identify protein alterations related to the BBB in diabetic rats, we measured the levels of occludin, claudin-5, ZO-1, and AQP4 by Western blotting, using actin as a standard. Decreases in the levels of occludin (Fig. 4a, $p=0.0012$), claudin-5 (Fig. 4c, $p=0.0185$), and AQP4 (Fig. 4g, $p=0.0396$) were observed in the DM 60 group. No change was observed in the levels of ZO-1 protein (Fig. 4e, $p=0.0599$). After treatment with EX-4 (or vehicle), the levels of these proteins were reevaluated. Interestingly, occludin (Fig. 4b, $p=0.0427$) and AQP4 (Fig. 4h, $p=0.0458$) increased in the DM-EX-4 subgroup, suggesting a recovery. This effect was not observed for the claudin-5 (Fig. 4d, $p=0.0620$). In addition, EX-4 failed to induce any change in ZO-1 protein levels (Fig. 4f, $p=0.4361$).

Levels of BCSFB Proteins Were Altered in Diabetic Rats

To identify alterations in BCSFB proteins in diabetic rats, we measured the levels of claudin-2 and AQP1 with Western blotting, using actin as a standard. We observed a decrease in the levels of claudin-2 (Fig. 5a, $p=0.0054$) and AQP1

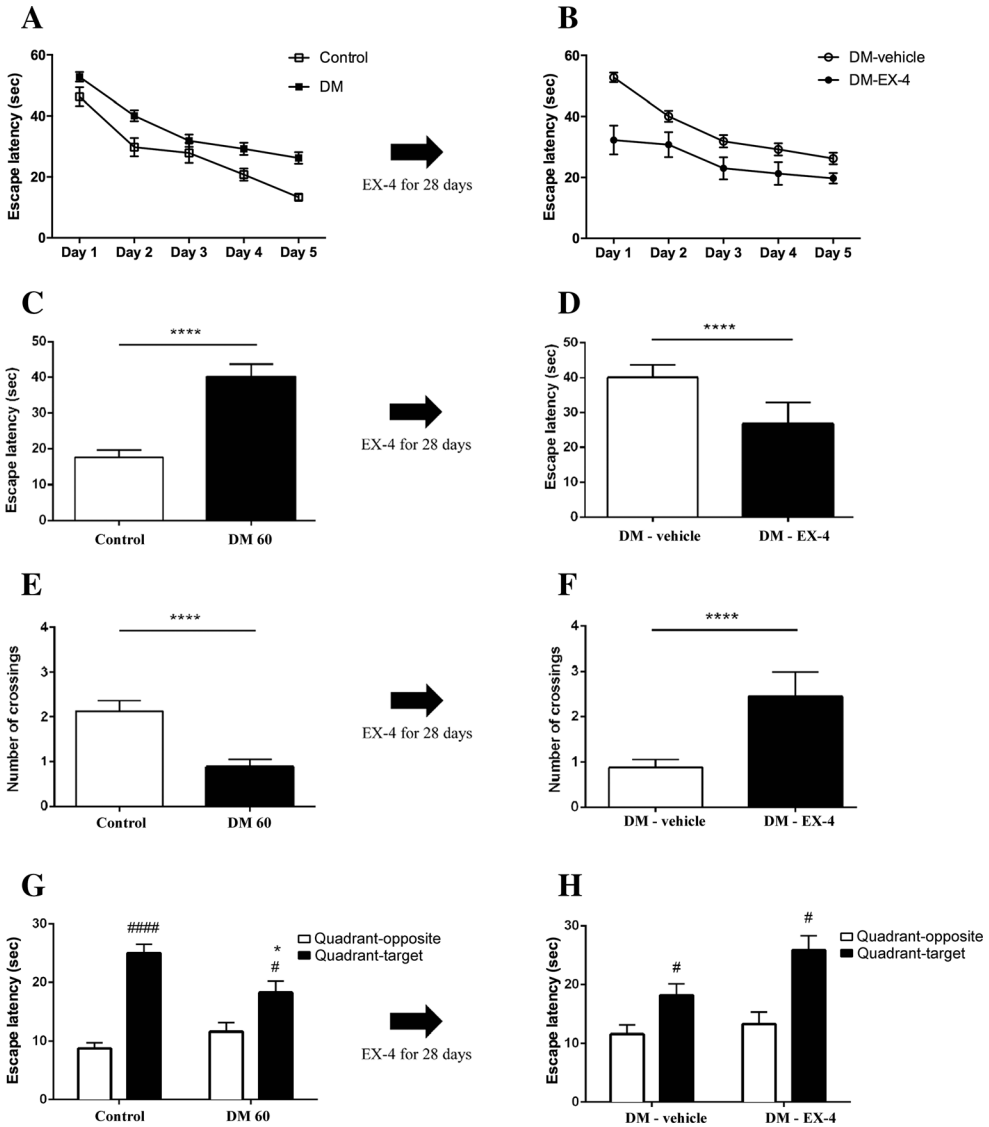

Fig. 1 Exendin-4 improved cognitive deficits in diabetic rats. Rats were tested in the Morris water maze at 60 days after induction of DM. The DM 60 and control groups demonstrated improvements in task performance in the training sessions (**a**). In the probe trials, the DM 60 group demonstrated increased latencies for finding the platform location (**c**) and a decreased number of crossings over the platform location in relation to the control group (**e**). The DM 60 group was randomly divided into two subgroups, DM-vehicle or DM-EX-4, which received vehicle or EX-4, respectively, for 28 days. In the training session, the two subgroups demonstrated improved latencies for finding the platform location in the acquisition phase (**b**). The DM-EX-4 subgroup showed improved escape latencies (**d**) and a higher number of crossings over the platform location (**f**) when compared with the DM-vehicle subgroup. Both groups (**g**) and subgroups (**h**) showed an increase in the time spent in the target quadrant compared with the opposite quadrant. However, the control group (**g**) and the subgroup DM-EX-4 (**h**) spent more time in the target quadrant compared with the DM 60 and DM-vehicle, respectively. Data are expressed as the mean ± S.E.M. Differences in escape latencies during the training sessions, and the time spent in quadrants were analyzed by two-way ANOVA with a repeated-measures analysis of variance followed by Tukey's post hoc test, assuming $p < 0.05$ as significant. Differences in escape latencies and the number of crossings on the test day were analyzed using an unpaired *Student's t test.* *Significantly different between the treatments. #Significantly different between the quadrants. *or #$p < 0.05$; **or ##$p < 0.01$; ***or ###$p < 0.001$ ****or ####$p < 0.0001$. Control ($n = 20$), DM 60 ($n = 30$), DM-vehicle ($n = 10$) and DM-EX-4 ($n = 10$)

(Fig. 5c, $p < 0.0001$) in the DM 60 group. After treatment with EX-4 (or vehicle), the levels of these proteins were reevaluated. The levels of both claudin-2 (Fig. 5b, $p = 0.0153$) and AQP1 (Fig. 5d, $p = 0.0055$) increased in the DM-EX-4 subgroup, possibly due to the functional recovery of the BCSFB.

Discussion

Accumulating data suggest that cognitive impairment occurs in diabetic patients and animal models, but the brain pathophysiology of DM is poorly understood. Advanced imaging techniques have shown

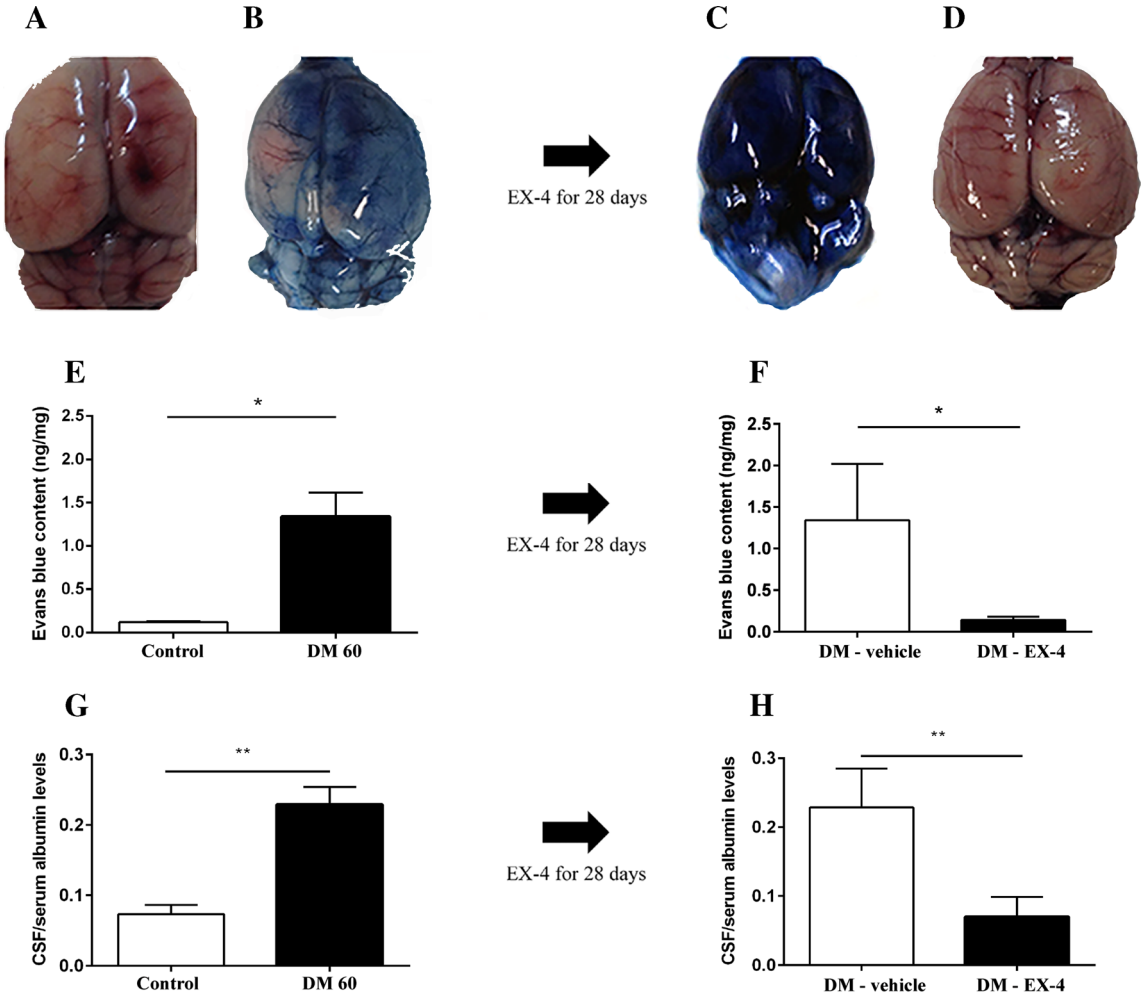

Fig. 2 Diabetic rats demonstrated BBB disruption that was reversed by EX-4 administration. Evans blue staining assay and the CSF/serum albumin ratio were used to evaluate BBB permeability at 60 days after DM induction. The DM 60 group (**b**) showed extravasation of Evans blue dye, which was not observed in the control group (**a**). The DM-vehicle subgroup (**c**) exhibited strong staining with Evans blue, but the DM-EX-4 subgroup (**d**) demonstrated less permeation. The quantification of the Evans blue dye confirmed the above results (**e** and **f**). The DM 60 group demonstrated an increased CSF/serum albumin ratio (**g**); a decline in this ratio was observed in the DM-EX-4 subgroup (**h**) when compared with the DM-vehicle subgroup. The results represent the mean ± S.E.M., $n = 4$, $*p < 0.05$; $**p < 0.01$ by unpaired *Student's t test*

microstructural abnormalities in the small vessels of diabetic patients, and these alterations might underlie DM-associated cognitive dysfunction [4]. Moreover, chronic exposure to hyperglycemia [29], vascular

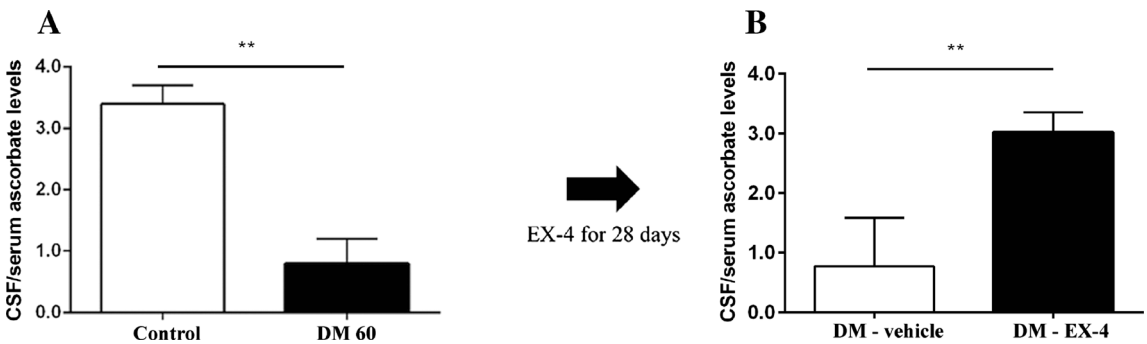

Fig. 3 Diabetic rats showed BCSFB disruption that was reversed by EX-4 administration. To evaluate the function of the BCSFB, we measured the CSF/serum ascorbate ratio in diabetic rats at 60 days after DM induction. A decrease in CSF/serum ascorbate level was observed in the DM 60 group when compared with the control groups (**a**). The DM-EX-4 subgroup demonstrated increased ascorbate levels when compared with the DM-vehicle subgroup (**b**). The results represent the mean ± S.E.M., $n = 5$, $**p < 0.01$ by unpaired *Student's t test*

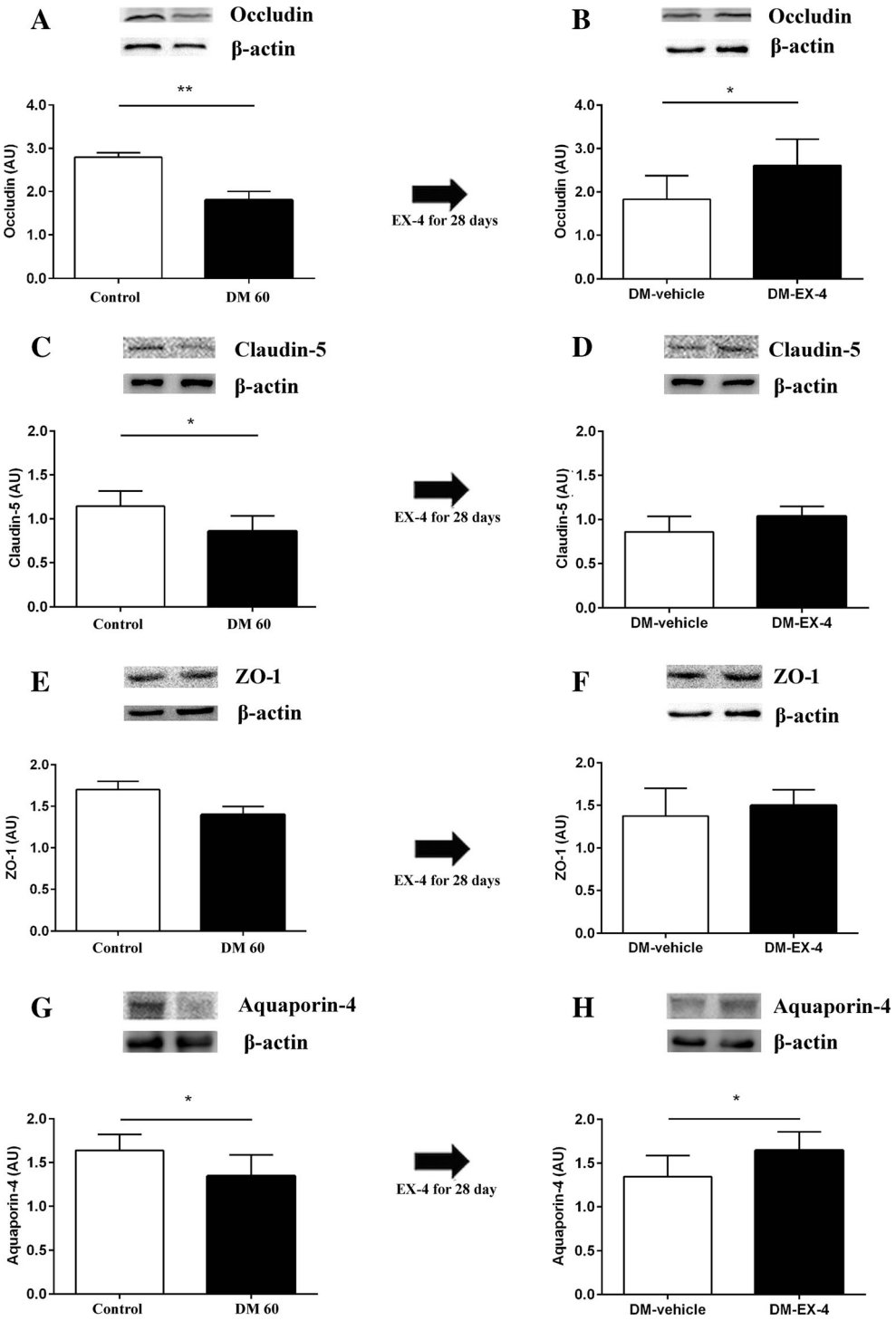

Fig. 4 Levels of specific BBB proteins were reduced in diabetic rats. Western blotting showed a decrease in occludin (**a**), claudin-5 (**c**), and AQP4 (**g**) protein levels in the DM 60 group compared with the control group. ZO-1 protein level was not altered (**e**). The EX-4 treatment recovered the occludin (**b**) and AQP4 (**h**) protein levels. The claudin-5 (**d**) and ZO-1 (**f**) proteins were not affected by EX-4 treatment. Representative Western blots for each protein are presented above each respective graph. The results are normalized to beta-actin, $n = 5$, and represent the mean ± S.E.M., expressed as arbitrary units. $*p < 0.05$, $**p < 0.01$ significantly different from control, as determined by unpaired *Student's t test*

disorder [30], dysregulation of the insulin-signaling pathway [31], and inflammatory mechanisms [32] are associated with both DM cognitive impairment and Alzheimer's disease, suggesting that these pathologies share some pathogenic aspects [29, 33, 34]. Vascular pathology has been characterized in diabetic patients and experimental models, but whether this leads to BBB rupture is unclear [8]. Moreover, it is also unclear as to whether such ruptures cause brain functional changes resulting in the type of cognitive or psychiatric symptoms observed in diabetic patients [35]. Our working hypothesis was that DM vascular pathology commits the BBB and other brain barriers (such as the BCSFB) structurally and functionally and that these changes contribute to the cognitive impairment observed in patients and experimental models.

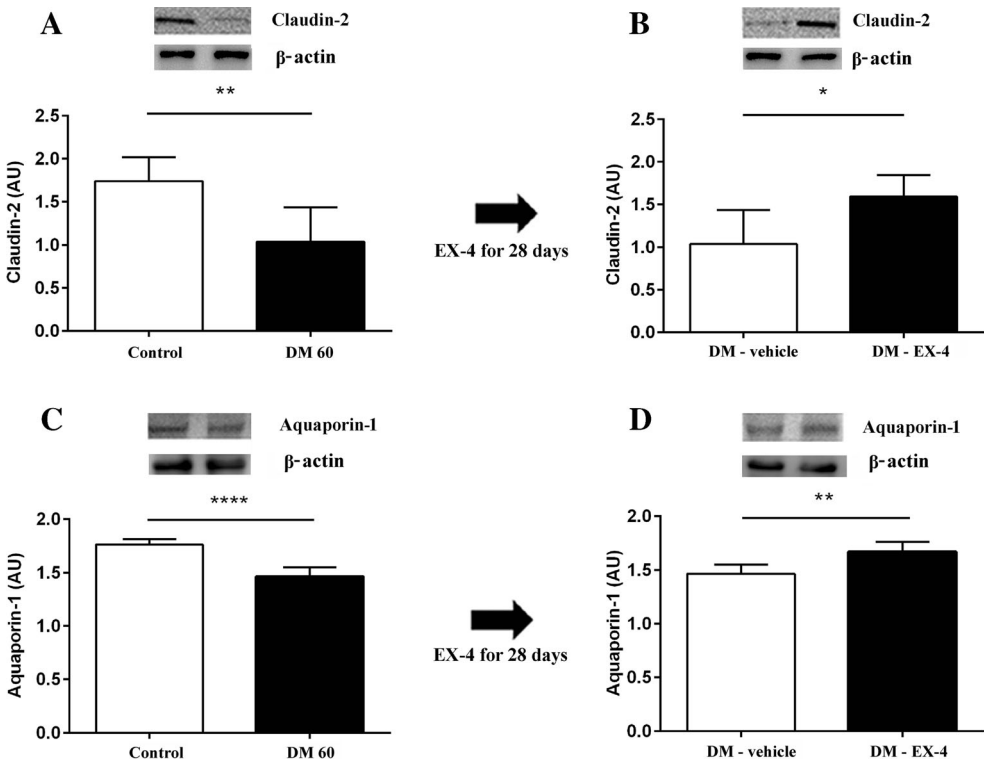

Fig. 5 Levels of specific BCSFB proteins were reduced in diabetic rats. Western blotting showed a decrease in claudin-2 (**a**) and AQP1 (**c**) protein levels in the DM 60 group compared with the controls. The EX-4 treatment reversed the alterations in claudin-2 (**b**) and AQP1 (**d**) protein levels. Representative Western blots for each protein are presented above each respective graph. The results are normalized to beta-actin, $n = 5$, and represent the mean ± S.E.M. expressed as arbitrary units. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ significantly different from control, as determined by unpaired *Student's t test*

It is important to emphasize that the peripheral alterations (hyperglycemia, body weight, and glycated hemoglobin) found in our diabetic rats are in accordance with the literature [36, 37] and validate our STZ-induced model of DM.

DM-induced CNS complications are multifactorial, and the impairment in BBB functioning constitutes one of these alterations. In fact, breakdown of the BBB is considered one of the key steps in diabetic encephalopathy [37, 38]. The BBB is a heterogeneous structure in which the vasculature in certain brain regions is more vulnerable to neurovascular uncoupling [3]; however, there is still controversy as to whether T1DM directly impairs the BBB. Bradburry et al. [39] and Dai et al. [40] found BBB permeability was unaffected in the cerebral cortex of STZ-induced diabetic rats (13–14 weeks of duration) and in diabetic patients, respectively. On the other hand, Hawkins et al. [38] and Mooradian et al. [41] found increased barrier permeability in animal models of STZ-induced DM (of different durations). We found an increase in the CSF/serum albumin ratio and a strong Evans blue staining in diabetic rats 60 days after STZ administration, indicating BBB disruption. Additionally, the decrease in the CSF/serum ascorbic acid ratio suggests a disruption of the BCSFB.

We evaluated the levels of specific TJ proteins of the BBB in the hippocampus and observed decreased levels of occludin and claudin-5 in diabetic rats; however, the ZO-1 level was not altered. A decrease in occludin has been described in two other reports in diabetic rats [38, 42], as well as in cultures of human brain microvascular endothelial cells (HBMEC) exposed to a high-glucose medium [43]. A decrease in claudin-5 has also been described in diabetic rats [37, 44] and in cultured HBMEC exposed to advanced glycation end products (AGE) [45]. However, the ZO-1 level in diabetic rats has been reported either as unaltered [42], as we found, or reduced [37, 38].

Epithelial cells in the choroid plexus contain TJ, produce CSF and are surrounded by fenestrated endothelial cells. Ascorbic acid levels are 3–5 times higher in CSF than in serum and are maintained by an active transport mechanism in the BCSFB [46]. As such, we measured the CSF/serum ascorbic acid ratio and found a decrease in the diabetic group. In addition, a decrease in the claudin-2 level, a BCSFB-related protein, was shown. Thus, to the best of our knowledge, we demonstrated herein for the first time that the biochemical integrity of the BCSFB is impaired in diabetic rats. The results of the present study corroborate those of Egleton et al., who observed an increase in ^{86}Rb efflux in the choroid plexus of STZ-induced diabetic rats [47].

The determination of the expression of AQPs is important in several neural disorders and could contribute to an improved understanding of the pathophysiological functions of AQPs associated with brain barriers. AQP1 is highly expressed in the choroid plexus and is involved in CSF production, whereas AQP4 is highly expressed in the perivascular processes of BBB astrocytes [15].

We observed a decrease in the AQP4 and AQP1 levels in the diabetic rat hippocampus. Our data demonstrating a decrease in

hippocampal AQP4 are in accordance with another independent and recent study that used the STZ model of DM in Wistar rats [48]. In that study, no alteration in AQP4 was found in the striatum, suggesting that DM-induced CNS insult is dependent on the brain region. In agreement with our results, Koves and collaborators [49] showed a decrease in AQP4 mRNA in neuroblastoma cell lines exposed to a hyperosmolar medium. In contrast, Deng et al. [50] observed an up-regulation of AQP4 protein after hypoglycemia-induced brain edema in rat cortical tissue. This is relevant because hypoglycemic episodes are common in diabetic patients.

The renal levels of AQP1 and AQP4 (but not other AQPs) appear to be unchanged in diabetic rats [51]. To the best of our knowledge, brain AQP1 has not yet been investigated in diabetic rats. The decrease in AQP1 that we observed could reflect alterations in the BCSFB. However, this protein is possibly involved in other brain functions, including cell plasticity (e.g., [52]), even as AQP4 [53, 54]). Although the precise contributions of brain AQP1 and AQP4 are not yet fully understood, they likely play roles in neuroprotection, and a reduction in their levels could be related to DM-induced CNS complications.

The standard therapeutic strategies available for DM may be insufficient to prevent cognitive decline and psychiatric comorbidities. Therefore, further research should attempt to explore novel therapeutic strategies to prevent or reduce the development of cognitive impairment or dementia in diabetic individuals [55]. Some proposed neuroprotective agents such as angiotensin receptor inhibitors [56], statins [41], and resveratrol [57] appear to improve BBB integrity. Our results show that EX-4, a GLP-1 agonist, could reverse cognitive impairment in diabetic rats, as well as reverse functional changes in the permeability of the brain barriers and restore levels of BBB and BCSFB proteins. The mechanisms mediating the effects of chronic hyperglycemia and elevated AGE levels on the brain barriers and their structural–functional correlations remain unknown. These mechanisms could be associated with the inflammatory response induced by high glucose [21] and AGE [58] levels, regulating the turnover of TJ proteins and AQPs, although other regulatory mechanisms (e.g., protein phosphorylation) could be involved [59, 60].

The BBB and BCSFB alterations induced in the STZ model of DM were reversed by EX-4. GLP-1 agonists have a variety of pancreatic effects and can improve glycemic control independently of their effects on the beta cells [19]. Additionally, extra-pancreatic effects have been reported in the literature [61]. Recently, GLP-1 mimetics have been associated with neurotrophic [62] and neuroprotective actions [20]. Considering that EX-4 treatment decreased inflammatory factors, such as interleukin-1 beta [21], this protein could modify the protein kinase A pathway, which regulates TJ protein phosphorylation, and consequently, the BBB and BCSFB. However, further studies are needed to determine the mechanism by which EX-4 reverses the breakdown in TJ and cognitive impairment.

Our results clearly show that biochemical and functional alterations in the BBB and BCSFB in the hippocampus, as well as cognitive impairment, are dependent on time. At 30 days after STZ exposure, it was possible to observe metabolic signals of DM, such as body weight reduction and an increase in glycemia (data not shown), but no functional alterations in the BBB or BCSFB, or decreased cognitive functioning, were observed. Alterations in both brain barriers and cognitive functioning were observed only at 60 days after STZ administration. Moreover, EX-4 treatment of diabetic animals reversed the functional rupture of these barriers and cognitive decline. These results contribute to the current debate about diabetic encephalopathy, increasing our understanding of the role of brain barriers in cognitive decline in DM and indicating the importance of these barriers and barrier proteins as biomarkers and therapeutic targets in the clinical practice.

Despite of these advances, the present study has some limitations. Firstly, although our study only focused on changes in BBB, BCSFB, and TJ proteins during STZ-induced DM, this disorder induces other brain abnormalities that can compromise cognitive functions [4, 63]. Therefore, it is currently not possible to affirm an exclusive and direct correlation between alterations in brain barriers and cognitive impairment. Secondly, we quantified TJ proteins only in the hippocampus because of the relationship between this brain region and cognitive tasks, as evaluated by the Morris water maze in the present study. However, TJ proteins should be quantified in the whole brain, and especially in the choroid plexus, in further studies to obtain a better understanding of BCSFB alterations in diabetic rats.

In summary, this study demonstrates that STZ-induced DM leads to increased BBB and BCSFB permeability, and these alterations are associated with decreases in TJ proteins. Our results corroborate previous data regarding BBB alterations in DM and also extend our understanding of both the functional and biochemical alterations in the BCSFB (claudin-2 and AQP1). A clear effect of DM on AQP4 and AQP1 was observed in this model. The functions of these proteins go beyond their roles in water flux (see [54] for a review), reinforcing the impact of this metabolic disorder on the CNS. In addition, this study demonstrated a neuroprotective effect of EX-4 on reversing functional and structural alterations in brain barriers, as well as cognitive impairments. These data further our knowledge regarding CNS alterations in DM, particularly changes in the proteins (TJ and aquaporins) and permeability of brain barriers, as well as cognitive dysfunction, and they suggest a role for EX-4 in therapeutic strategies in the clinical practice for treating cognitive dysfunction associated with DM.

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Compliance with Ethical Standards All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23), and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project number 24076). All efforts were made to minimize animal suffering and reduce the number of animals used.

Conflict of Interest The authors declare that they have no conflict of interest.

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CAPÍTULO III

Exendin-4 Reverses the Glutamatergic Transmission in Diabetic Rats

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Manuscrito em preparação

OBJETIVOS ESPECÍFICOS

1. Investigar o efeito da EX-4 em reverter as alterações relacionadas à transmissão glutamatérgica, além da hiperglicemia e formação de AGEs no modelo de DM.

Exendin-4 Reverses the Glutamatergic Transmission in Diabetic Rats

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Abstract

Diabetes mellitus (DM) is a metabolic disorder that results in glucotoxicity and cognitive impairment both, associated with several adverse effects in the brain. Exendin-4 (EX-4), an analogue of glucagon-like peptide-1 (GLP-1), appears to have beneficial effects on cognitive functioning in mice with chronic hyperglycemia. Herein, we investigate the ability of EX-4 to reverse changes in the glutamatergic transmission observed in a model of DM STZ-induced, looking particularly at glutamate uptake and GluN1 subunit content of N-methyl-D-aspartate (NMDA) receptor. Additionally, we evaluated peripheral metabolic parameters, as well as the content of AGEs in cerebrospinal fluid (CSF) and serum, and RAGE in the brain tissue. We found a decrease in glutamate uptake and GluN1 content in hippocampus of diabetic rats and EX-4 was able to reverse these parameters, but had no effect in the others evaluated parameters (glycemia, C-peptide, AGEs levels, RAGE and glyoxalase 1). The reversal in the glutamatergic neurotransmission may result from an improvement in brain inflammatory condition in hyperglycemic situations or a direct effect on the functioning of neurons and astrocytes. Both situations could reverse the glutamate uptake by astrocytes, avoiding the excitotoxicity and restore the GluN1 content. However, more studies are necessary to clarify the EX-4 mechanism in our diabetic rats.

Keywords: Exendin-4; Glutamate uptake; Glutamatergic neurotransmission

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized primarily by chronic hyperglycemia that results in glucotoxicity, including the AGEs formation, which is among the causes of DM complications. AGEs are a diverse group of compounds that are generated by non-enzymatic glycation or glycoxidation of proteins, lipids or nucleic acids through of a series of reactions (Vlassara and Uribarri, 2014). The main precursor of AGEs is the methylglyoxal and, in normal situations, the cells are protected from the toxicity of this compound by the glyoxalase system, a detoxification pathway formed by the enzymes glyoxalase 1 (GLO 1) and glyoxalase 2 (GLO 2) (Allaman et al., 2015), which catalyzes the conversion of methylglyoxal to *D*-lactate (Rabbani and Thornalley, 2011). DM is associated with several adverse effects in the brain, including increases in oxidative stress and inflammation, possibly mediated by AGEs and their receptor, RAGE (Amin et al., 2013).

Glutamate is the major excitatory neurotransmitter and in order to maintain the brain homeostasis and avoid the excitotoxicity, glutamate should be removed quickly from the synaptic cleft by specific astrocytes transporters (Kim et al., 2011). Extracellular glutamate accumulation leads to neurotoxicity, mediated by the glutamate receptors, such as N-methyl-D-aspartate (NMDA). The glutamate receptors play a key role in neuroplasticity, neural development and differentiation. Moreover, changes in NMDA receptors are related to impairment in cognitive tasks such as memory and learning (Lin et al., 2014).

Exendin-4 (EX-4) is an analogue of glucagon-like peptide-1 (GLP-1), which are well-established drugs for treating type 2 diabetes mellitus (T2DM). Some studies have investigated the role of GLP-1 agonists in type 1 diabetes mellitus (T1DM).

They reported benefits including improved glycemic control, increased in proliferation and reduction in apoptosis of beta cells (Pettus et al., 2013). Additionally, when administered peripherally, EX-4 appears to have beneficial effects on cognitive functioning in mice with chronic hyperglycemia associated-inflammation (Huang et al., 2012).

Herein, we investigated the ability of EX-4 to reverse changes in the glutamatergic transmission observed in a model of DM STZ-induced, looking particularly at glutamate uptake and NMDA content. Additionally, we evaluated peripheral metabolic parameters, as well as the content of AGEs in cerebrospinal fluid (CSF) and serum, and RAGE in the brain tissue.

2. Materials and Methods

2.1 Experimental Animals

A total of forty-five male Wistar Kyoto (WKY) rats (8 weeks old, weighing 160–270 g) were obtained from our breeding colony. Rats were maintained under a 12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C and had free access to a commercial chow and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23), and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project number 24076). All efforts were made to minimize animal suffering and reduce the number of animals used.

DM was induced in thirty-three WKY rats by intraperitoneal injection of streptozotocin (STZ) (75 mg/kg in citrate buffer with an injection volume of 300 μL/kg body weight, pH 4.5). Thirty (30 of 33) rats became diabetic (as demonstrated

by glycemic parameters measured 48 h after STZ administration); ten age-matched animals that received only vehicle (citrate buffer) were used as controls. The animals were then housed (3–4 per cage) for a period of 60 days. Body weight and blood glucose levels were recorded every two weeks. Glycemia was measured with an Accu-Chek® Active Kit (Roche Diagnostics, Basilea, Switzerland), and the animals were considered diabetic if their blood glucose levels were more than 250 mg/dL.

Biochemical analyses were performed 60 days after DM induction and in their respective controls. After 60 days of DM induction, the DM rats were randomly divided in three subgroups. One was euthanized for biochemical analysis (n = 10) together with controls (n=10), and two other were assigned: DM-vehicle (n = 10) and DM-EX-4 (n = 10). The DM-EX-4 animals received EX-4 10 μg/kg intraperitoneally once a day for 28 days, whereas the DM-vehicle animals received an equal volume of saline solution.

2.2. Material

STZ; mouse monoclonal anti-actin antibody (1A4 clone); Ponceau S; *L*-glutamate and *o*-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO, USA). Goat polyclonal anti-RAGE was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-GluN1 (R1JHL clone) was obtained from Millipore (Darmstadt, Germany). The peroxidase-conjugated immunoglobulin (IgG) antibodies and *L*-[2,3-^{3}H] glutamate were obtained from Amersham (Buckingamshire, UK). Anti-AGE antibody (6D12) was purchased from Cosmo Bio (Esposito et al., 2014). All other chemicals were purchased from local commercial suppliers

2.3. Cerebrospinal fluid and serum samples

Animals were anesthetized as described above and then positioned in a stereotaxic holder for collection of 100 μL (approximately) of CSF from the cisterna magna. The puncture was performed using an insulin 1 mL syringe and 31 G needle (0.25 mm diameter, 6 mm length). Rats were then removed from the stereotaxic apparatus and placed on a flat surface, where approximately 3 mL of whole blood was obtained through an intracardiac puncture using a 5 mL syringe and 21 G needle (0.80 mm diameter, 25 mm length) inserted into the intercostal space above the sternum. Serum was separated by centrifugation at 3000 g for 10 min. CSF and serum samples were frozen (-80 ° C) until further analysis (Netto et al., 2006).

2.4. C-peptide Assay

Proinsulin C-peptide was assessed using a Rat/Mouse C-Peptide 2 ELISA kit (Millipore, Darmstadt, Germany) according to the manufacturer's recommendations. Briefly, serum was added to a 96-well flat-bottom plate and incubated with a mixture of capture and detection antibodies, at 1:1, for 2 hours at room temperature. Subsequently, the plate was incubated with streptavidin-horseradish peroxidase for 30 min at room temperature. The color reaction produced with tetramethylbenzidine (TMB) was then quantified in a plate reader at 450 and 590 nm. The standard C-peptide curve ranged from 0 to 1600 pM.

2.5. AGE measurement

AGEs were measured in serum and CSF by ELISA, as previously described by (Ikeda et al., 1996) with some modifications. Briefly, the wells of a microtiter

plate were coated overnight with 0.1 µg protein in 0.1 mL of 50 mM carbonate bicarbonate buffer (pH 9.6). The wells were washed with washing buffer (PBS containing 0.05 % Tween 20) and then incubated for 3 h with 2 % albumin. Subsequently, wells were washed again and incubated with 100 µL of anti-AGE (6D12) for 1 h. After washes, wells were incubated with 100 µL of peroxidase-conjugated secondary antibody for 1 h. The reactivity of peroxidase was determined by incubation with OPD for 30 min. The reaction was stopped by the addition of 50 µL sulfuric acid (3M). Absorbance measurements were taken at 492 nm. Results were calculated and expressed as a percentage of the control.

2.6. Preparation for Glyoxalase Activity Assays

Hippocampal slices were lysed and homogenized in sodium phosphate buffer, pH 7.4. Subsequently, slices were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was used for enzymatic activity and protein content measurements (Hansen et al., 2012).

2.7. Glyoxalase 1 Activity Assay

GLO 1 activity was assayed according to (Mannervik et al., 1981) with some modifications. The assay was carried out in 96-well microplates using a microplate spectrophotometer (UV Star - Greiner). The reaction mixture (200 µL/well) contained 50 mM sodium-phosphate buffer pH 7.2, 2 mM methylglyoxal (MG) and 1 mM GSH (pre-incubated for 30 min at room temp). Protein from the sample (10– 20 µg per well) was added to the buffer. The formation of S-(D)-lactoylglutathione was linear and monitored at 240 nm for 15 min at 30°C. A unit of GLO 1 activity is defined as

the amount of enzyme that catalyzes the formation of 1 μmol of S-(D)-lactoylglutathione per minute. Specific activity was calculated in milliunits per milligram of protein (mU/mg protein).

2.8. Glutamate Uptake Assay

Glutamate uptake was performed as previously described (Nardin et al., 2009). The hippocampal slices were incubated in a Hank's balanced salt solution (HBSS), in pH 7.4. The assay was started by the addition of 0.1 mM *L*-glutamate and 0.66 μCi/ml 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 0.1 M NaOH and 0.01% SDS. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the specific uptake. Radioactivity was measured with a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentage of the control.

2.9. Western Blotting

Equal amounts (30 μg) of proteins from each sample were boiled in sample buffer [0.0625 M Tris–HCl pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and electrophoresed on a 10% (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 (Zanotto et al., 2013). After incubating for 1 h at room temperature with the primary antibodies (anti-GluN1 or anti-RAGE at 1:5000 dilution), the filters were washed and incubated with anti-mouse or anti-goat peroxidase-conjugated

immunoglobulin (IgG) (at dilution of 1:10000). The chemiluminescence signal was detected using an ECL Western Blotting Detection Kit from Amersham.

2.10. Protein Determination

Protein levels were measured by Lowry's method using bovine serum albumin as a standard (Lowry et al., 1951).

2.11. Statistical Analysis

Data normality (by Kolmogorov-Smirnov test) and statistical analysis were performed using PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA), assuming $p < 0.05$ as significant. Parametric data from the experiments are presented as mean ± standard error (S.E.M.) and were statistically evaluated by Student's t test. Non–parametric data (CSF AGE and C-peptide) are presented as median and interquartile range and were statistically evaluates by Mann-Whitney test.

3. Results

3.1. Glutamatergic neurotransmission is reverses by EX-4 in diabetic rats

Assuming the key role of astrocytes in connecting the energy metabolism to glutamatergic transmission, we investigated the glutamate transport in hippocampal slices in diabetic rats. We found a reduction in glutamate uptake ($p=0.0054$) and in the GluN1 subunit of the receptor NMDA ($p=0.0308$) in hippocampus of diabetic rats compared with controls rats. Glutamatergic metabolism was reevaluated after EX-4

administration (or vehicle), and revealed a reversal in glutamate uptake (p=0.0005) and also in the GluN1 (p=0.008) impaired by DM.

Conversely, the protein levels of the main astrocytes glutamate transporters, GLT-1 and GLAST, did not differ between control and DM group, and between DM-vehicle and DM-EX-4 subgroups (data not shown).

3.2. AGEs and RAGE are altered in diabetic rats

To assess glucotoxicity, we determined AGEs content in the serum and CSF of diabetic rats. An increase in AGE levels was observed in serum (p = 0.0022) and CSF (p = 0.0057) of the DM group compared with control group. Moreover, a potential receptor for AGEs was measured in hippocampal tissue, and RAGE levels were augmented in DM rats, compared with control rats (p = 0.0369). We measured the levels of the enzyme GLO 1 in hippocampus to evaluate the function of the glyoxalase system. Increase in GLO 1 levels were observed in DM group compared with control group (p=0.03).

After 28 days of EX-4 administration to the arbitrarily assigned DM group, intriguingly, we observed an increase in AGE serum concentrations in the DM-EX-4 subgroup compared with the DM-vehicle subgroup (p=0.003). The AGE concentrations in CSF (p=0.48) and RAGE levels in hippocampus (p=0.31) did not differ between DM-vehicle and DM-EX-4 subgroups. Accordingly, the GLO 1 levels did not differ between the subgroups (p=0.66) (Table 1).

Surprisingly, the glucose uptake activity did not differ in groups and subgroups analyzed, but the amount of glucose transporter GLUT-1 was increased in DM group compared with age-matched controls. The GLUT-1 content did not differ between DM-vehicle and DM-EX-4 subgroups (data not shown).

3.3. EX-4 do not recovered the peripheral metabolic parameters observed in diabetes model

We measured some peripheral parameters and observed that the rats from DM group exhibited higher glycemia than age-matched control rats ($p < 0.0001$). The C-peptide was significantly lower in DM group than control group ($p = 0.002$). After 28 days of EX-4 administration to the randomly assigned DM group, the glycemia did not differ between DM-vehicle and DM-EX-4 subgroups ($p=0.13$). Confirming insulin deficiency, the DM-vehicle and DM-EX-4 subgroups presented very low levels of serum C-peptide (Table 1).

4. Discussion

Assuming that the reversal of the cognitive dysfunction by EX-4 observed in STZ-induced diabetic rats in previous work of our group (Zanotto et al, *in press*) could not be attributed entirely to biochemical and functional alterations in brain barriers, we evaluated the neuroprotective role of EX-4 on changes in glutamatergic transmission.

When assessing the glutamatergic transmission, we found a decrease in glutamate uptake and GluN1 content in hippocampus of diabetic rats. The EX-4 was able to reverse the glutamatergic system, but had no effect in the others evaluated parameters (see table 1). The high glucose levels found in DM lead to glucotoxicity by glycation of biomolecules and formation of different AGEs. The exposure to these compounds could cause changes in glutamatergic activity of both ways, RAGE dependent manner in which the AGE/RAGE pathway activation (Barlovic et al., 2011) cause an

inflammatory response with the release of pro-inflammatory cytokines (IL-1β, TNF-α) (Barlovic et al., 2011) and thus, a reduction of the glutamate uptake (Tilleux and Hermans, 2007) and/or a RAGE independent manner (Hansen et al., 2015). Despite this decrease in glutamate uptake, we found no changes in the glutamate transporters levels present in astrocytes, GLT-1 and GLAST. Regardless of the mechanism, decreased glutamate uptake result in extracellular high levels of glutamate and subsequently excitotoxicity. Accordingly, the GluN1 subunit of the NMDA receptor, which is negatively regulated by chronic excitotoxicity (Gascon et al., 2005) was decreased in our diabetic rats. Togheter, excitotoxicity and alterations in the glutamate receptors expression could cause glutamatergic transmission dysfunction and, finally, cognitive deficit in DM (Gardoni et al., 2002).

The peripheral parameters evaluated in the present study, blood glucose and C-peptide, are according to the literature (Burvin et al., 1994; Goyal et al., 2015) and validate the model of STZ-induced DM. It is well established that the chronic hyperglycemia found in DM is directly related to the formation and accumulation of AGEs in both, serum and tissues (Ott et al., 2014). Accordingly, we found an augment of AGEs levels in the serum and in CSF of diabetic rats as well as, elevated hippocampal levels of the RAGE receptor and GLO-1, the key enzyme that detoxifies the methylglyoxal. Probably, the elevated levels of the enzyme GLO-1 observed in ours diabetic rats is a compensatory mechanism in an attempt to detoxify the CNS from the AGEs accumulation (Allaman et al., 2015).

Therapeutic standards strategies used in DM, such as the administration of insulin in insulin-dependent patients, appear to be insufficient to prevent or reduce CNS damage observed in long-term diabetic patients (McCall and Farhy, 2013). We observed a reversal in glutamatergic transmission in EX-4-treated rats, however, EX-

4 was not able to recover the others altered parameters observed in our DM animal model.

The reports in literature indicate a recovery of blood glucose in diabetic type 1 patients with detectable levels of C-peptide, which enable us to claim that these patients have a residual function of pancreatic beta cells (Ghazi et al., 2014). Our diabetic animals had no detectable C-peptide levels, so probably this was the reason for the EX-4 not has been capable to recover blood glucose and consequently AGEs formation.

It has been shown that EX-4 is able to improve inflammatory mediators such as IL-1β and NF-κB in hyperglycemic situations (Huang et al., 2012). In addition, GLP-1 agonists may be acting directly on neurons and astrocytes, improving their functioning (Long-Smith et al., 2013). Both situations could reverse the glutamate uptake by astrocytes, avoiding the excitotoxicity and restore the NMDA receptor content. However, more studies are necessary to clarify the EX-4 mechanism in our diabetic rats.

5. Conclusion

To conclude, our results indicate the potential role of EX-4, a GLP-1 agonist, to reverse the glutamatergic transmission, particularly glutamate uptake and GluN1 content, in diabetic rats. However, the EX-4 was not able to recover the glycemia and AGEs formation in STZ-induced diabetes. These results suggest that EX-4 could be an adjuvant treatment in brain damage related to excitotoxicity in type 1 DM. Future investigations are needed to clarify by which the EX-4 is acting to improve the glutamatergic transmission in DM.

Conflict of interest statement

The authors have no conflict of interest.

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Figure Caption

Figure 1. Glutamatergic neurotransmission is recovered by EX-4 in diabetic rats Glutamate uptake in hippocampal slices were evaluated in DM and control groups (A). After EX-4 or vehicle administration the measure were reevaluated (B). Western blotting were assessed to verified alterations in GluN1 protein levels in DM group and their age-matched controls (C).). After EX-4 or vehicle administration the measure were reevaluated (D). Representative Western blots for each protein are presented above each respective graph. The results are normalized to actin and represent the means ± S.E.M., expressed as arbitrary units, n = 8-10, * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$ significantly different from control, as determined by unpaired *Student's t test.*

Table 1. EX-4 do not recovered the peripheral metabolic parameters and AGE/RAGE altered in diabetes model Glycemia, C-peptide, AGEs in serum and CSF, RAGE content in hippocampus, and GLO 1 were evaluated in the DM and age-matched control rats. Rats of DM group that were not used for biochemical analyzes were randomly assigned to the DM-vehicle or DM-EX-4 subgroup and the measures were reevaluated. All values are expressed as the mean ± S.E.M. n=8-10. * Significantly different from the control group, * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$, **** $p < 0.0001$

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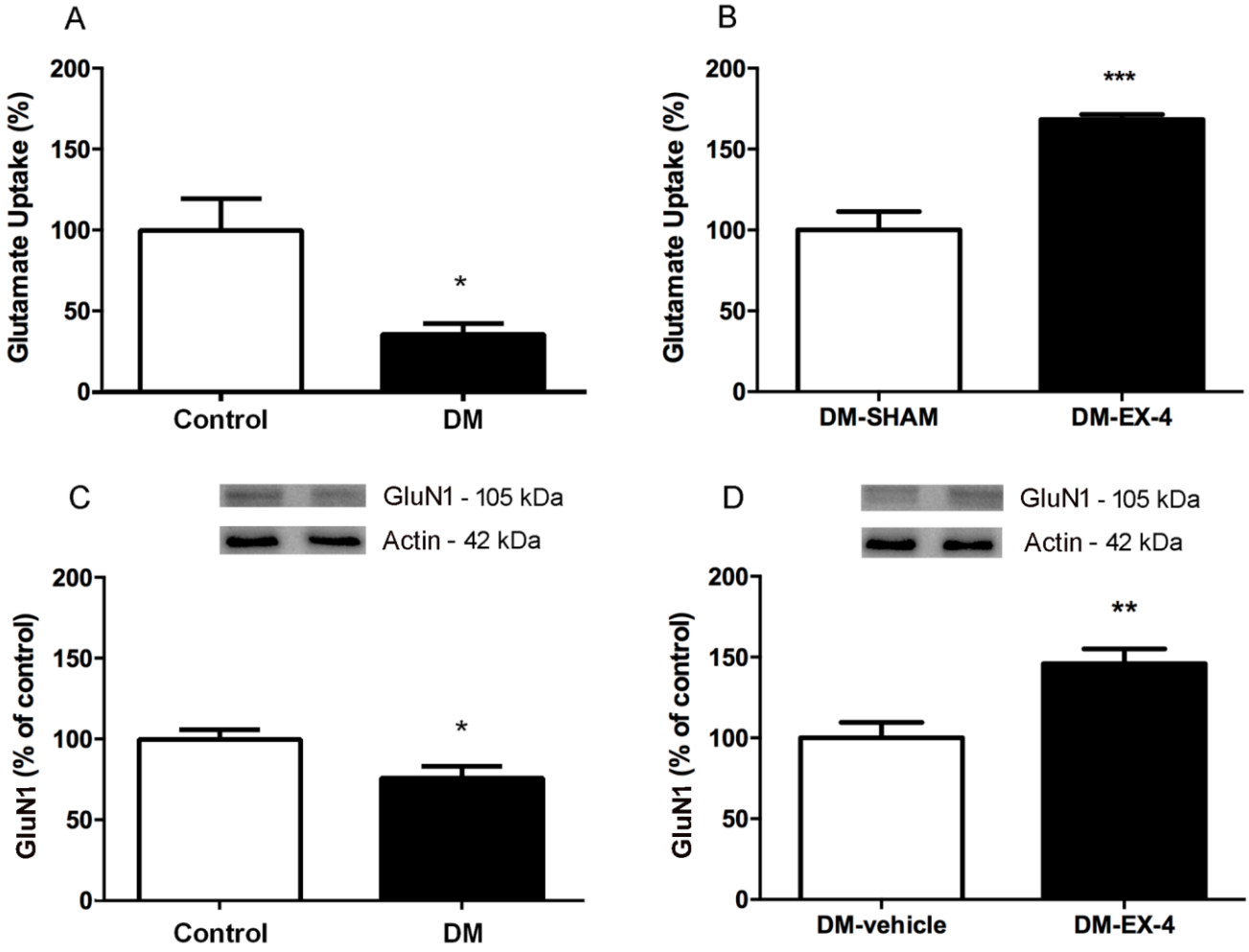
Figure 1:


A
Glutamate Uptake (%)
200
150
100
50
0
*
Control
DM
B
Glutamate Uptake (%)
200
150
100
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0
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DM-SHAM
DM-EX-4
C
GluN1 - 105 kDa
Actin - 42 kDa
GluN1 (% of control)
200
150
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*
Control
DM
D
GluN1 - 105 kDa
Actin - 42 kDa
GluN1 (% of control)
200
150
100
50
0
**
DM-vehicle
DM-EX-4

Table 1:

		Control	DM	DM-vehicle	DM-EX-4
Peripheric Parameters	Glycemia (mg/dL)	94.3 ± 4.9	477.4 ± 34.0****	439.5 ± 24.5	497.4 ± 26.71
	C-peptide (pM)	154 (123-180)	0 (0-1.5)***	0 (0-1.5)	0 (0-1.5)
	AGEs serum (%)	100.0 ± 3.3	140.8 ± 9.3*	100.0 ± 4.0	120.0 ± 4.2**
Central Parameters	AGEs CSF (%)	96.2 (96-101)	131.6 (126-152)**	100.0 ± 7.6	89.48 ± 11.22
	RAGE (%)	100.0 ± 18.0	151.8 ± 8.5*	100.0 ± 13.5	124.3 ± 18.87
	GLO 1(%)	100.0 ± 10.1	131.8 ± 9.9*	100.0 ± 7.6	95.2 ± 2.9

PARTE III

DISCUSSÃO

O DM é uma doença crônica caracterizada principalmente por hiperglicemia e alterações na homeostase celular, o que pode provocar danos vasculares e disfunções em diversos tecidos biológicos. A longo prazo, pacientes diabéticos frequentemente apresentam alterações macro e microvasculares (Chilelli et al., 2013).

O DM é responsável por danos no SNC e, devido a isso, modelos animais têm contribuído imensamente para a compreensão das alterações bioquímicas e funcionais, incluindo alterações neuroquímicas particularmente relacionadas com o comprometimento cognitivo e, até mesmo, demência (Flood et al., 1990; Duarte et al.; 2009; Duarte et al., 2012; Wang et al., 2015b). Corroborando com esses achados, esta tese teve por objetivo investigar alterações no SNC, particularmente relacionadas às funções astrocitárias e, posteriormente, das barreiras encefálicas em modelo animal de DM induzido por STZ tendo como base as alterações periféricas, bem como analisar o efeito da EX-4, um agonista dos receptores de GLP-1, em reverter os parâmetros avaliados.

Assim, os resultados desta tese foram divididos em três capítulos. No primeiro deles avaliamos determinados parâmetros periféricos confirmando a indução de DM no modelo induzido por STZ. Investigamos também o acúmulo de AGEs e alterações nas funções astrocitárias. No segundo capítulo verificamos o prejuízo cognitivo nos animais diabéticos e alterações nas barreiras encefálicas. Neste capítulo também verificamos a capacidade da EX-4 em reverter os danos encontrados. No terceiro e último capítulo avaliamos se a EX-4 foi capaz de reverter o prejuízo no metabolismo de glutamato e os parâmetros periféricos incluindo a formação de AGEs observado nos animais diabéticos.

Em nosso estudo utilizamos modelo animal induzido por injeção intraperitoneal de STZ, uma droga alquilante antineoplásica que destrói as células beta pancreáticas, mimetizando o DM tipo 1 (Aggarwal, et al., 2014). Este modelo é bem estabelecido na literatura para estudar o DM. Alterações em parâmetros periféricos, como diminuição do peso corporal, aumento da glicemia e da Hb1Ac, e uma redução bastante acentuada do peptídeo C, sugerindo uma destruição quase total das células beta pancreáticas que obtivemos em nosso trabalho, confirmam que o modelo animal estabelecido mimetiza as principais características do DM e torna-se adequado para avaliarmos as alterações comportamentais e bioquímicas decorrentes do DM (VanGilder et al., 2009; Aggarwal et al., 2014).

A hiperglicemia está associada com o aumento da formação e acúmulo de AGEs (Singh et al., 2014; Ott et al., 2014). Em conformidade com esta afirmação, observamos elevados níveis de AGEs no soro e no LCR de animais diabéticos. Além disso, quando avaliamos o sistema glioxalase, um aumento na atividade da enzima GLO 1 no hipocampo foi determinado, o que poderia ser um mecanismo compensatório do sistema de detoxificação contra o acúmulo de AGEs (Allaman et al., 2015). No entanto, este aumento na GLO 1 não foi suficiente para combater os elevados índices de AGEs nos animais diabéticos.

Os AGEs ativam receptores multi-ligantes associados à resposta inflamatória, os RAGEs (Chen et al., 2015). Estudos sugerem que a formação de AGEs e a ativação de RAGE poderia ser um dos mecanismos patogênicos interconectados mais importantes envolvido nas complicações microvasculares do DM (Chilelli et al., 2013). Além disso, sabe-se que os principais mecanismos envolvidos no dano tecidual causado por AGEs são a glicação intracelular, a formação de ligações cruzadas e a interação com RAGE (Chilelli et al., 2013; Chawla et al., 2014).

De acordo, em nosso trabalho verificamos um aumento no conteúdo de RAGE no hipocampo de animais diabéticos. Sabe-se que a ativação da via de sinalização AGE-RAGE está envolvida na resposta inflamatória mediada pelo NF-κB e produção de citocinas pró-inflamatória, e esta reação poderia estar mediando danos no SNC de animais diabéticos (Ahmed, 2005). Nesse sentido, já foi demonstrado o aumento de mediadores inflamatórios no DM, como o aumento do receptor do tipo *toll* 4 (TLR4), das ciclooxigenases 1 e 2 (COX-1 e COX-2, respectivamente), da glicoproteína expressa na membrana dos linfócitos T (CD45) e da enzima óxido nítrico sintase induzível (iNOS), além do NF-κB e interleucina 1 beta (IL-1β) (Huang et al., 2012). A interação de AGE com o receptor RAGE também pode induzir inflamação vascular, aumentando a regulação de moléculas de adesão endoteliais, como a molécula de adesão da célula vascular 1 (VCAM-1) (Piga et al., 2007).

O acúmulo intracelular de AGEs altera fatores citoplasmáticos e nucleares, incluindo proteínas envolvidas na transcrição gênica e altera vias envolvidas na sinalização celular em tecidos encefálicos, o que acarreta prejuízos cognitivos (Pugliese, 2008). Em vista disso, os AGEs e a ativação de RAGE desempenham um papel importante tanto na patogênese de complicações vasculares relacionadas ao DM, como em desordens neurodegenerativas, incluindo a doença de Parkinson e Alzheimer (Takeuchi e Yamagishi, 2008; Ramasamy et al., 2005; Yamagishi, 2011). Ainda, a via AGE-RAGE investigada em estudos epidemiológicos tem reforçado a associação entre DM e a doença de Alzheimer, e estudos com pacientes diabéticos sugerem que um dos possíveis mecanismos que conecta a disfunção astrocitária e a neurodegeneração relacionada ao DM é a formação de AGEs (Huang et al., 2014; Ouwens et al., 2014; Chu et al., 2014). De fato, nós observamos um aumento na formação de AGEs e alterações em parâmetros astrocitários (secreção de S100B e

captação de glutamato) em ratos diabéticos.

Quando avaliamos a captação de glicose em fatias hipocampais de ratos diabéticos não obtivemos alterações em relação aos ratos controles, porém observamos um aumento no conteúdo de GLUT-1 nos animais diabéticos. Nosso resultado é intrigante, embora o significado e mecanismo que levam ao aumento do GLUT-1 nos animais diabéticos sejam desconhecidos, este dado corrobora com Simpson e colaboradores (1999), que observaram um aumento no RNAm de GLUT-1 na microvasculatura cortical e não obtiveram alterações na captação de glicose no hipocampo e no córtex cerebral de ratos com DM. Um aumento no GLUT-1 também foi observado em linhagem de células endoteliais da BHE humana expostas a um meio com elevados níveis de glicose (Prasad et al., 2015). Além disso, Wang e colaboradores (2012) também não encontraram alterações no transporte de glicose através da BHE em ratos diabéticos até 10 semanas após a indução. Sabe-se que a expressão do transportador GLUT-1 é aumentada por hipoglicemia (Simpson et al., 1999) e estudos *in vitro* sugerem uma possível regulação inflamatória deste transportador (revisado por Jurcovicova, 2014). Portanto, a ativação de RAGE observada em nossos animais e as vias inflamatórias desencadeadas por essa ativação poderia nos ajudar a explicar esse aumento de GLUT-1 em ratos diabéticos.

Os astrócitos estão envolvidos em várias complicações relacionadas com o DM, incluindo neuropatia e neurodegeneração (Chu et al., 2014). Além disso, estas células estão intimamente associadas com os neurônios e a ativação de astrócitos pode estar relacionada com a formação de placas senis e a morte neuronal (Costa et al., 2012). A proliferação e hipertrofia dos astrócitos é um processo complexo conhecido como astrogliose, a qual pode ter efeitos benéficos ou prejudiciais dependendo da situação, além de contribuir para a liberação de citocinas pró e anti-

inflamatórias como o fator de transformação de crescimento beta (TGF-β), fator de necrose tumoral alfa (TNF-α), interferon gama (IFN-γ), interleucinas 1 e 6 (IL-1 e IL-6) que modulam a inflamação e mecanismos secundários ao dano (Karimi-Abdolrezaee e Billakanti, 2012).

A astrogliose é benéfica geralmente no início do dano ao SNC, onde produz elevados níveis de antioxidantes, como a glutationa, promove a revascularização e produz substratos energéticos e fatores tróficos para neurônios e oligodendrócitos. Quando a astrogliose ocorre tardiamente, ela inibe a regeneração neuronal e contribui para manter a inflamação no SNC (Lee e MacLean, 2015).

Dois marcadores astrogliais têm sido investigados no DM: as proteínas S100B e GFAP, as quais são marcadoras das funções astrocitárias (Nagayach et al., 2014; Revsin et al., 2005). Além disso, parâmetros relacionados como a captação de glutamato são utilizados para verificar a atividade astrocitária. Em nosso trabalho a proteína GFAP não foi alterada em ratos diabéticos, no entanto, quando analisamos a S100B, obtivemos uma diminuição no seu conteúdo hipocampal e um aumento na sua secreção em fatias agudas hipocampais de ratos diabéticos. Quando analisamos seu conteúdo no soro e LCR, houve uma diminuição desta proteína no soro, entretanto, no LCR, não houve alteração. A captação de glutamato se mostrou diminuída nos animais diabéticos, mas o conteúdo dos transportadores GLAST e GLT-1 não foi modificado.

Os resultados de medida de GFAP no DM presentes na literatura são controversos. Alguns autores relataram um aumento significativo nesta proteína (Baydas et al., 2003; Saravia et al., 2006; El-Akabawy e El-Kholy, 2014), enquanto outros indicaram uma diminuição desta proteína no hipocampo de animais diabéticos (Coleman et al., 2004; Coleman et al., 2010; de Senna et al., 2011; Amin et al., 2013).

Estas discrepâncias podem ser devido a diferenças metodológicas nos modelos animais, heterogeneidade das células gliais, tempo das análises e ensaios para a medida de GFAP. Também é importante enfatizarmos que, embora a GFAP seja o marcador mais comum de astrogliose, a ativação astroglial não necessariamente envolve aumento no conteúdo desta proteína, já que as células astrocitárias podem estar com sua morfologia alterada e não ter uma maior produção de GFAP (Liberto et al., 2004).

A medida da S100B no soro é proposta como um marcador de ativação de células gliais ou de danos encefálicos (Rothermundt et al., 2003), mas diversas fontes extracerebrais, tais como o tecido adiposo, podem contribuir para alterar os níveis de S100B no soro (Goncalves et al., 2010). Steiner e colaboradores (2010) observaram uma correlação entre os níveis séricos de S100B e o índice de massa corporal em humanos. Nossos resultados mostram uma redução nos níveis séricos de S100B nos ratos diabéticos, o que está de acordo com alguns relatos clínicos (Hovsepyan et al., 2004; Celikbilek et al., 2014). Conforme verificamos, esta é a primeira vez que a S100B é dosada em modelo animal de DM induzido por STZ. Além disso, a diminuição da S100B no soro não foi acompanhada por alterações nos níveis desta proteína no LCR. É importante citarmos que, em outro estudo, onde a STZ foi administrada no espaço intracerebroventricular (para induzir demência), observou-se uma diminuição da S100B no LCR, sem mudanças na S100B sérica (Rodrigues et al., 2010a). Em conjunto, esses dados reforçam a ideia de que mudanças nos conteúdos de S100B no LCR e no soro não estão necessariamente relacionadas (Goncalves et al., 2008).

A S100B é utilizada como parâmetro de ativação ou morte glial em diversas desordens encefálicas e/ou rompimento da BHE. Os dados da literatura são

controversos, enquanto diversos autores não relatam alterações no número de células S100B positivas em ratos diabéticos (apesar de observarem outras alterações gliais) (Coleman et al., 2004; Collino et al., 2009; de Senna et al., 2011), outros autores observaram um aumento transitório das células S100B positivas no hipocampo na primeira semana após a administração de STZ (Lebed et al., 2008). Em nosso trabalho observamos uma diminuição no conteúdo da S100B no hipocampo e um aumento da secreção desta proteína em fatias agudas de hipocampo. Utilizamos fatias agudas hipocampais porque este modelo apresenta a circuitaria neuroglial parcialmente preservada e poderia ajudar a esclarecer a relação entre astrócitos e neurônios, a qual desempenha importantes papéis em lesões encefálicas (Nardin et al., 2009). É importante enfatizarmos que, assim como os AGEs, a S100B extracelular se liga à RAGE e, nesta condição, poderia funcionar como um mediador de feedback positivo, reforçando a sinalização inflamatória.

A explicação para o aumento da secreção de S100B observada em nossos resultados se baseia em publicação anterior do nosso grupo que demonstrou um aumento na secreção de S100B por citocinas pró-inflamatórias em fatias hipocampais agudas (de Souza et al., 2009). Estas citocinas pró-inflamatórias, tais como a IL-1β e o TNF-α, poderiam resultar de um aumento na sinalização AGE-RAGE, o que poderia levar a ativação do NF-κB que, por sua vez, induziria a expressão de tais citocinas (Berbaum et al., 2008). Portanto, embora não tenhamos encontrado níveis elevados de S100B no LCR, o estado inflamatório poderia explicar o aumento basal na secreção de S100B em fatias agudas de hipocampo de ratos diabéticos. Por outro lado, obtivemos uma diminuição na captação de glutamato pelos astrócitos e sabemos que níveis extracelulares elevados de glutamato podem reduzir a secreção de S100B em fatias do hipocampo (Buyukuysal, 2005; Nardin et al., 2009).

A presença de mediadores inflamatórios, como a IL-1β e TNF-α também pode estar envolvida com a diminuição da captação de glutamato observada em nosso trabalho, como descrito por Tilleux e Hermans 2007. No entanto, um outro mecanismo de diminuição da captação de glutamato em ratos diabéticos que envolve AGEs, mas independente de RAGE não pode ser descartado (Hansen et al., 2015). Apesar desta redução na captação de glutamato, não encontramos alterações nas quantidades dos transportadores de glutamato presentes em astrócitos, GLT-1 e GLAST. Independentemente do mecanismo, a diminuição da captação de glutamato resultaria em níveis extracelulares aumentados de glutamato e elevada excitotoxicidade. De acordo, a subunidade GluN1 do receptor N-metil-D-Aspartato (NMDA), que é regulada negativamente por excitotoxicidade crônica (Gascon et al., 2005) se mostrou diminuída em nossos ratos diabéticos.

É importante mencionarmos que a excitotoxicidade do glutamato já foi associada com diversas doenças neurológicas, como epilepsia, acidente vascular encefálico e desordens neurodegenerativas, e a disfunção nos transportadores de glutamato é muitas vezes o evento inicial que leva a estas doenças (Wang e Qin, 2010).

A BHE é uma estrutura heterogênea na qual a vasculatura é mais vulnerável ao desacoplamento neurovascular em determinadas regiões do encéfalo (Huber et al., 2006). Complicações no SNC induzida pelo DM são multifatoriais e o comprometimento das funções da BHE e BHL possivelmente constitui alguma dessas alterações. A patologia vascular tem sido caracterizada em pacientes diabéticos e modelos experimentais de DM. Contudo, ainda não está esclarecido se o dano vascular leva à ruptura da BHE, e, ainda está em debate, a possibilidade de ocorrer ruptura da BHE e suas associações com o comprometimento cognitivo (Mogi e

Horiuchi, 2011; Prasad et al., 2014).

Neste trabalho nós verificamos se a patologia vascular do DM acomete estruturalmente e funcionalmente a BHE e a BHL e se estas alterações contribuem para o prejuízo cognitivo observado em pacientes diabéticos e modelos experimentais de DM. De acordo com essa hipótese, nossos animais diabéticos apresentaram um aumento do déficit cognitivo e da permeabilidade da BHE e BHL, além de alterações nas proteínas que formam as junções oclusivas destas barreiras.

Nossos resultados mostram um aumento na relação albumina LCR/soro e uma forte coloração para *Evans blue* em ratos diabéticos 60 dias após a administração de STZ, indicando dano na BHE. Este resultado está de acordo com Hawkins e colaboradores (2007) e Mooradian e colaboradores (2005), que encontraram um aumento na permeabilidade da BHE em modelo animal de DM induzido por STZ. É importante ressaltarmos que 30 dias após a indução do DM nós observados sinais metabólicos compatíveis com o DM, como diminuição do peso corporal e aumento da glicemia. No entanto, a funcionalidade das barreiras encefálicas e o desempenho cognitivo não foram alterados, sugerindo que estas alterações são dependentes do tempo de duração da doença.

A BHE tem como função inibir a difusão paracelular de moléculas solúveis em água devido às junções oclusivas que interconectam as células endoteliais (Engelhardt e Sorokin, 2009). Nós obtivemos uma diminuição no conteúdo das proteínas claudina-5 e ocludina, que são responsáveis pela formação das junções oclusivas na BHE, o que poderia explicar o aumento na permeabilidade da BHE observado no nosso modelo de DM.

A diminuição no conteúdo de ocludina está de acordo com a literatura e foi descrita em outros trabalhos com ratos diabéticos (Chehade et al., 2002; Hawkins et

al., 2007), assim como em cultura de células endoteliais da microvasculatura cerebral humana (HBMEC) expostas a meio de cultura com elevados níveis de glicose (Shao e Bayraktutan, 2013). A diminuição nos níveis de claudina-5 também tem sido descrita em ratos diabéticos (VanGilder et al., 2009; Sun et al., 2015) e em HBMEC expostas a AGEs (Shimizu et al., 2013). No entanto, o conteúdo de ZO-1 não alterou em nossos animais diabéticos, o que corrobora com Chehade et al. (2002), enquanto que Hawkins et al. (2007) e VanGilder et al. (2009) encontraram níveis diminuídos de ZO-1 no DM.

Sabe-se que a permeabilidade da BHE pode ser alterada em diversos eventos patológicos do SNC, como por exemplo, na hipóxia-isquemia e em doenças nas quais mecanismos inflamatórios afetam a BHE, como a encefalopatia séptica, demência induzida por HIV, esclerose múltipla e doença de Alzheimer (Redzic, 2011). Além disso, alterações nas funções e integridade da BHE podem ter um forte impacto sobre a patogênese e progressão das principais doenças neurológicas, e o dano na BHE é considerado por alguns autores como um dos passos chave na encefalopatia diabética (VanGilder et al., 2009; Hawkins et al., 2007).

Além das alterações na BHE, observamos em nosso modelo de DM prejuízos na função e integridade da BHL. As células epiteliais nos plexos coróides produzem o LCR e estão cercadas por células endoteliais fenestradas, além disso possuem junções oclusivas que mantém as células epiteliais ligadas para exercerem a função de barreira (Arlt et al., 2002). O ácido ascórbico desempenha importantes papéis como cofator na biossíntese de catecolaminas e hormônios peptídicos, atua como antioxidante e na detoxificação de radicais livres no encéfalo (Minamizono et al., 2006). Devido a suas funções, os níveis de ácido ascórbico estão aproximadamente 5 vezes maiores no LCR do que no soro e estes níveis elevados no LCR se mantém

devido a um mecanismo de transporte ativo na BHL (Angelow et al., 2003). Em vista disso, nós medimos a taxa de ácido ascórbico LCR/soro para avaliar a função da BHL e encontramos uma diminuição nos animais diabéticos. Também observamos uma redução no nível de claudina-2, uma proteína relacionada com as junções oclusivas da BHL. Assim, até onde sabemos, demonstramos pela primeira vez que a integridade bioquímica da BHL é prejudicada em ratos diabéticos. Os dados do nosso estudo corroboram com Egleton e colaboradores (2003) que observaram um aumento no efluxo do ^{86}Rb nos plexos coróides de ratos diabéticos induzidos por STZ.

Embora não podemos afirmar quais os mecanismos que provocam danos na BHE e BHL em nossos animais diabéticos, possivelmente o acúmulo de AGEs, a disfunção astrocitária e a excitotoxicidade decorrente da diminuição da captação de glutamato estejam envolvidos.

Como mencionamos anteriormente, o aumento nos níveis de AGE-RAGE observado em nosso trabalho poderia estar acionando mecanismos inflamatórios, como a ativação de NF-kB e a produção de citocinas pró-inflamatórias, e estes poderiam estar modulando as proteínas das junções oclusivas e aumentando a permeabilidade da BHE e BHL (Hoffman et al., 2009).

Um crescente número de evidências sugerem que condições patológicas caracterizadas por neuroinflamação, como a doença de Alzheimer, esclerose múltipla, DM e retinopatia de prematuridade compartilham uma característica comum: o dano na BHE (Goncalves et al., 2013). Além disso, já é bem estabelecido na literatura que no DM ocorre o aumento do estresse oxidativo, e este, por sua vez, também pode estar diminuindo a expressão das proteínas das junções oclusivas e aumentando a permeabilidade de ambas, BHE e BHL (Krizbai et al., 2005; Goncalves et al., 2013).

Ainda não está claro se o dano nas barreiras encefálicas dependente do DM pode afetar seriamente o SNC e resultar nos sintomas de comprometimento cognitivo e psiquiátricos observados em pacientes diabéticos (Serlin et al., 2011). A BHL protege o SNC das constantes alterações bioquímicas na corrente sanguínea, bem como de infecções e toxinas, mantendo assim a homeostase encefálica (Tietz e Engelhardt, 2015). A BHE, por sua vez, é extremamente importante para a patogênese de doenças cerebrais, devido ao fato dela atuar como uma guardiã do encéfalo, com diversas funções inter-relacionadas, incluindo a proteção do SNC de substâncias potencialmente nocivas, regulação do transporte de moléculas essenciais, manutenção da homeostase encefálica e regulação de funções imunológicas (Prasad et al., 2014). Quando temos um comprometimento nas funções da BHE e BHL, o encéfalo fica exposto às toxinas e substâncias danosas, podendo levar ao prejuízo de funções gliais e neuronais que podem provocar sérios danos ao SNC (Tietz e Engelhardt, 2015). Os prejuízos das funções astrocitárias observados em nossos resultados podem ser devido ao aumento da produção de AGEs e suas consequências, como mencionamos anteriormente. Além disso, os danos nas funções da BHE e BHL decorrentes do DM poderiam estar contribuindo para a perda das funções dos astrócitos.

Os mecanismos subjacentes aos efeitos da hiperglicemia crônica e níveis elevados de AGEs sobre as barreiras do encéfalo e suas correlações estruturais-funcionais ainda são desconhecidos. Estes poderiam estar associados com a resposta inflamatória induzida por níveis elevados de glicose (Huang et al., 2012) e a idade (Yamagishi et al., 2005), o que modula a rotação de proteínas das junções oclusivas e AQPs. Além disso, outros mecanismos de regulação, como a fosforilação de proteínas, poderiam estar envolvidos (Harhaj e Antonetti, 2004, Gunzel e Yu, 2013).

A determinação da expressão de AQPs é importante em diversas doenças neurológicas e pode contribuir para uma melhor compreensão das funções das AQPs associadas com as barreiras encefálicas (Badaut et al., 2014).

Nós observamos uma diminuição dos níveis de AQP4 e AQP1 no hipocampo de ratos diabéticos. Essa diminuição da AQP4 no hipocampo está em conformidade com outro estudo em que foi avaliada a região do estriado no DM induzido por STZ e não foram encontradas alterações na AQP4 (de Senna et al., 2015), sugerindo que o insulto no SNC induzido pelo DM é dependente da região encefálica avaliada. De acordo com os nossos resultados, Koves et al. (2012) mostraram uma diminuição na expressão do RNAm de AQP4 em linhagem celular de neuroblastoma exposta a um meio hiperosmolar. Por outro lado, Deng et al. (2014) relataram um aumento nos níveis de AQP4 após edema cerebral induzido por hipoglicemia em tecido cortical de rato. Isso é relevante uma vez que episódios de hipoglicemia são frequentes em pacientes diabéticos.

Até onde sabemos, o nosso trabalho é o primeiro a analisar os níveis de AQP1 em cérebros de ratos diabéticos e a diminuição nos níveis de AQP1 poderia refletir em alterações na BHL destes animais. No entanto, esta proteína está possivelmente envolvida em outras funções encefálicas, incluindo a plasticidade celular (Nesic et al., 2008), assim como a AQP4 (Iliff et al., 2012; Nagelhus e Ottersen, 2013). Embora as contribuições precisas das AQPs 1 e 4 no encéfalo ainda não estejam completamente esclarecidas, elas provavelmente desempenham papéis na neuroproteção e, dessa forma, uma redução em seus níveis poderia estar relacionada com complicações no SNC provocadas pelo DM. O prejuízo nas funções das barreiras encefálicas e a presença de mediadores inflamatórios poderia explicar as alterações obtidas nos níveis das AQPs 1 e 4. Alterações na AQP4 ocorrem durante o processo inflamatório,

sugerindo mudanças no movimento da água relacionado com a neuroinflamação (Badaut et al., 2014). No entanto, os resultados quanto a regulação da AQP4 frente a citocinas pró-inflamatórias ainda não estão bem elucidados e mais estudos são necessários para esclarecer o papel da AQP4 durante processos inflamatórios.

Diversas pesquisas têm demonstrado uma associação entre DM e prejuízo na aprendizagem e memória, o que corrobora com nosso resultado. O déficit cognitivo observado no DM está associado, entre outros fatores, com mudanças da plasticidade sináptica no hipocampo (Gispen e Biessels, 2000). Neste trabalho, observamos uma diminuição na captação de glutamato e no conteúdo de GluN1. Nossos resultados suportam a hipótese de que a transmissão glutamatérgica é alterada no DM. De acordo, foram relatadas alterações pós-translacionais na subunidade GluN1 em ratos diabéticos induzidos por STZ (Rondon et al., 2010).

Dados na literatura sugerem que o prejuízo cognitivo ocorre em pacientes diabéticos e em modelos animais, sendo que o DM tipo 1 é frequentemente associado com decréscimos leves a moderados na função cognitiva. A velocidade psicomotora, flexibilidade mental, atenção e inteligência, em geral, são os fatores mais afetados pelo DM tipo 1 (revisado por Moheet et al., 2015), no entanto, a patofisiologia do encéfalo durante o DM não é bem compreendida. Técnicas avançadas de imagem mostraram anormalidades microestruturais em pequenos vasos de pacientes diabéticos, e essas alterações podem ser subjacentes à disfunção cognitiva associada ao DM (Biessels e Reijmer, 2014). Além disso, a exposição crônica à hiperglicemia (Ahmad, 2013), doença vascular (Taguchi, 2009), desregulação da via de sinalização da insulina (Jolivalt et al., 2008) e mecanismos inflamatórios (Takeda et al., 2010) estão associados tanto com o comprometimento cognitivo no DM quanto com a doença de Alzheimer, o que sugere que estas patologias podem compartilhar alguns

aspectos patogênicos (Biessels et al., 2002; de Senna et al., 2011, Ahmad, 2013). Os estudos ainda não são completamente esclarecedores, mas acredita-se que o DM aumente em 50-100% o risco de desenvolvimento de doença de Alzheimer e em 100-150% o risco de demência vascular (Xu et al., 2009; Sinclair et al., 2014; Umegaki, 2014).

O DM tipo 1 é comumente diagnosticado durante a infância ou adolescência. Este é um período de rápidas mudanças no desenvolvimento do SNC e trabalhos sugerem que o cérebro jovem pode ser mais suscetível aos extremos de glicemia. Além disso, a idade de início do DM e a presença de complicações microvasculares são fatores de risco para o declínio cognitivo (Moheet et al., 2015).

As estratégias terapêuticas padrões utilizadas no DM, como a administração de insulina em pacientes insulinodependentes, parecem ser insuficientes para prevenir ou reduzir o declínio cognitivo e comorbidades psiquiátricas a longo prazo observado nestes pacientes. Os nossos resultados demonstraram que a EX-4, um agonista dos receptores de GLP-1, foi capaz de reverter a disfunção cognitiva em ratos diabéticos, bem como restaurar as alterações na permeabilidade e nas proteínas da BHE e BHL. Além disso, essa droga foi capaz de reverter a diminuição na captação de glutamato causada pelo DM, diminuindo, portanto, o conteúdo de glutamato extracelular e a excitotoxicidade. Surpreendentemente, a EX-4 não restaurou aos níveis normais a glicose sanguínea, a Hb1Ac e o peptídeo C, o que nos possibilita afirmar que os efeitos da EX-4 observados em nosso trabalho não estão relacionados com alterações da glicose circulante.

Trabalhos relatam uma recuperação da glicemia em pacientes com DM tipo 1 com níveis detectáveis de peptídeo C, o que possibilita afirmar que estes pacientes apresentam uma função residual das células beta pancreáticas (Ghazi et al., 2014).

Nossos animas diabéticos não apresentaram níveis detectáveis de peptídeo C, então provavelmente este foi o motivo da EX-4 não ter sido capaz de recuperar a glicemia. E, por esta razão, a atividade da enzima GLO 1, bem como os níveis de AGEs no soro e LCR, também não foram recuperados pela EX-4.

Os agonistas dos receptores de GLP-1 possuem uma variedade de efeitos pancreáticos e podem melhorar o controle glicêmico de forma independente dos seus efeitos sobre as células beta (Pettus et al., 2013). Além disso, diversos efeitos extra-pancreáticos têm sido relatados na literatura (Seufert e Gallwitz, 2014). Recentemente, os miméticos de GLP-1 têm sido associados com ações neurotróficas (Li et al., 2010), neuroprotetoras (Holscher, 2014) e anti-inflamatórias (Shiraki et al., 2012).

Pesquisas científicas já demonstraram que o tratamento com EX-4 diminuiu fatores inflamatórios, tais como a IL-1β (Huang et al., 2012). Esta, por sua vez, pode modificar a via da proteína cinase A (PKA), que é capaz de regular a fosforilação de proteínas da BHE e BHL e, consequentemente, a funcionalidade das barreiras cerebrais. Além disso, o tratamento com EX-4 foi capaz de reduzir os níveis dos mediadores inflamatórios COX-1, COX-2, CD45 e NF-κB frente à uma situação de hiperglicemia (Long-Smith et al., 2013). De fato, em nosso trabalho a EX-4 reverteu os danos na BHE e BHL e isto poderia ser atribuído à diminuição de fatores ou mediadores pró-inflamatórios que levam a danos nas barreiras encefálicas. Estes achados reforçam nossa hipótese que a EX-4 poderia estar agindo na resposta inflamatória e, desta maneira, melhorando os parâmetros analisados no DM.

Além de recuperar as barreiras encefálicas, os agonistas dos receptores de GLP-1 podem estar agindo diretamente em astrócitos e neurônios, melhorando seu funcionamento (Long-Smith et al., 2013; Ji et al., 2015; Chen et al., 2012). No SNC,

os agonistas dos receptores de GLP-1 aumentam a atividade da AMPc/PKA promovendo a fosforilação da proteína ligante de resposta ao AMPc (CREB) e a transcrição gênica nuclear, o que finalmente resultará em aumento da aprendizagem e memória (Han et al., 2012). Outras vias de sinalização, como da proteína cinase ativada por mitógenos (MAPK) e da fosfatidilinositol-3-cinase/serina treonina cinase (PI3K/Akt) também estão envolvidas nos efeitos protetores mediados por GLP-1 nas células neuronais (Perry et al., 2002).

Nossos resultados mostraram que a EX-4 recuperou a diminuição na captação de glutamato causada pelo DM, evitando assim a excitotoxicidade e suas consequências deletérias. Ainda, a EX-4 recuperou os níveis do da subunidade GluN1 do receptor NMDA, cujo envolvimento com a memória e aprendizagem é um papel bem estabelecido na literatura científica (Wang et al., 2014; Cercato et al., 2014). Devido a isso, a melhora na cognição observada nos ratos diabéticos tratados com EX-4 também pode ser atribuída ao aumento da captação de glutamato e recuperação dos níveis de GluN1.

No entanto, mais estudos são necessários para esclarecer o mecanismo pelo qual a EX-4 recupera os sérios danos causados pelo DM ao SNC.

CONCLUSÕES

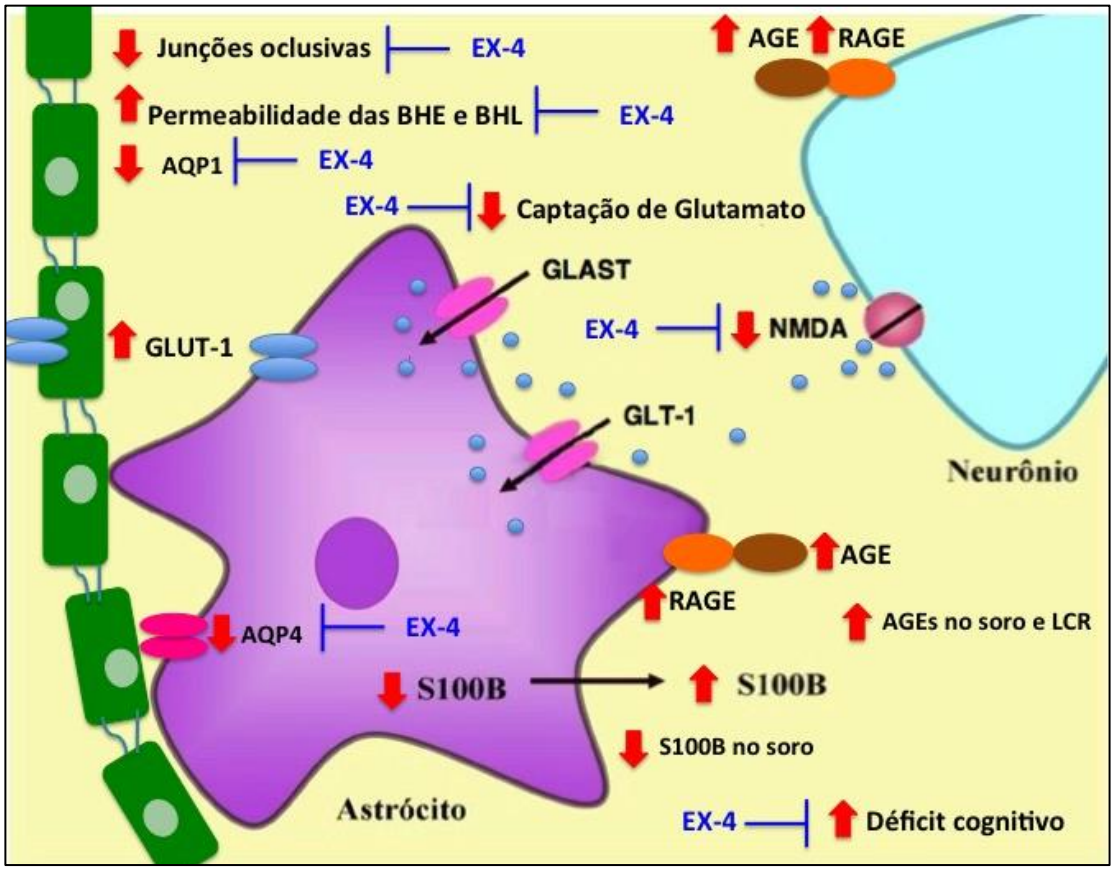

Figura 4. Representação esquemática dos principais resultados obtidos nesta tese

Os resultados apresentados nesta tese mostram claramente prejuízos no SNC decorrentes do DM e contribuem para os debates atuais sobre a encefalopatia diabética. A hiperglicemia é responsável pela excessiva produção de AGEs e estes, por sua vez, podem ativar vias inflamatórias e modular a função e expressão de diversas proteínas. O prejuízo em funções astrocitárias e os danos nas barreiras encefálicas observados neste trabalho podem ser decorrentes da ativação de vias de sinalização como da IL-1β e NF-κB desencadeadas pelos elevados níveis de AGEs no soro e no LCR. Além disso, o prejuízo no comportamento cognitivo observado neste

trabalho, provavelmente possa ser atribuído aos danos astrocitários e na BHE e BHL, observados nos animais diabéticos.

A EX-4 foi capaz de restaurar a captação de glutamato, o dano nas barreiras encefálicas e o comportamento cognitivo prejudicado pelo DM, sem ter eficácia sobre a glicemia e formação de AGEs. Sugerindo o papel da EX-4 como estratégia terapêutica na prática clínica para tratar a disfunção cognitiva associado ao DM.

O efeito da EX-4 na melhora cognitiva dos animais diabéticos possivelmente seja devido a quatro fatores principais: ao aumento da captação de glutamato, diminuindo assim a excitotoxicidade; ao aumento do conteúdo de GluN1, melhorando a cognição; à restauração das funções da BHE e BHL, deixando o encéfalo menos vulnerável a toxinas; à ativação pela EX-4 de vias relacionadas com a aprendizagem e memória, como AMPc/PKA, PI3K/Akt e MAPK. Além disso, a EX-4 pode estar diminuindo a expressão de mediadores inflamatórios, como IL-1β, NF-κB, COX-1 e COX-2, atenuando assim os danos causados pela hiperglicemia e pelo acúmulo de AGEs. No entanto, mais estudos são necessários para estabelecer o mecanismo de ação da EX-4 na recuperação dos danos causados pelo DM no SNC.

PERSPECTIVAS

- Avaliar as vias inflamatórias ativadas no modelo de DM incluindo os níveis dos mediadores inflamatórios envolvidos e o possível efeito da EX-4 na regulação dessas vias;
- Avaliar o conteúdo da AQP9 no modelo de DM, já que ela parece estar relacionada com o metabolismo energético encefálico;
- Avaliar o mecanismo de regulação das proteínas das barreiras encefálicas (BHE e BHL) dos animais diabéticos;
- Investigar os prováveis mecanismos e/ou vias de sinalização envolvidos no efeito da EX-4 sobre o SNC.
- Avaliar o efeito da EX-4 em estágios mais precoces do DM, ou seja, quando os animais diabéticos ainda apresentarem níveis detectáveis de peptídeo C.

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