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WILMA HELENA DE OLIVEIRA

AVALIAÇÃO DOS MECANISMOS DE AÇÃO DA METFORMINA SOBRE OS PROCESSOS DE NEUROINFLAMAÇÃO, NEURODEGENERAÇÃO E COGNIÇÃO EM MODELO DE ENCEFALOPATIA DIABÉTICA

Recife

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Tese apresentada ao programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos parciais para obtenção do título de Doutora em Ciências Biológicas.

Área de concentração: Biotecnologia.

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RESUMO

No cérebro, a diabetes pode causar alterações de memória, inflamação e ainda aumentar a expressão dos marcadores da Doença de Alzheimer, pTau e β amiloide. A metformina é um fármaco hipoglicemiante ativador de AMPK. Esse fármaco ainda possui potencial anti-inflamatório e melhora a neurogênese. O objetivo deste estudo foi avaliar os efeitos da metformina sobre a memória de longo prazo/prejuízo de aprendizado, sobre os marcadores da doença de Alzheimer em camundongos diabéticos e avaliar possíveis mecanismos. Foram utilizados 40 camundongos machos *Swiss* Webster distribuídos nos grupos experimentais: controle, estreptozotocina (STZ), STZ tratado com metformina na dose 100 mg/Kg (M100) e STZ tratado com metformina na dose de 200 mg/Kg (M200). A diabetes foi induzida com estreptozotocina. Após a confirmação da diabetes, os tratamentos com cloridrato de metformina por nove semanas foram iniciados. O peso e glicemia foram acompanhados semanalmente. Na última semana de tratamento, os animais foram submetidos ao teste de memória Barnes Maze, então foram eutanasiados. O soro foi coletado para dosagem de óxido nítrico e os cérebros processados para dosagem de óxido nítrico, imunoistoquímica, imunofluorêscencia e Western Blot. Os animais dos grupos STZ, M100 e M200 tiveram redução significativa de peso e elevação da glicemia de jejum durante todo o tratamento. Os animais do grupo STZ tiveram aumento significativo do tempo gasto para encontrar a caixa alvo no labirinto de Barnes, enquanto o grupo M200 reduziu significativamente o tempo gasto para encontrar a caixa alvo. A dosagem de óxido nítrico pelo método de Greiss no soro e no hemisfério cerebral mostrou significativa elevação dos níveis de nitrito no grupo STZ e redução no grupo M200. A imunoistoquímica para GFAP foi significativamente elevada no grupo STZ e reduzida no grupo M200 nas áreas do giro denteado e CA1. A imunofluorescência indicou aumento de p-Tau nas áreas do hipocampo e corpo caloso do grupo STZ e redução significativa no grupo M200. Ainda pela imunofluorescência, a diabetes reduziu a p-CREB e aumentou a formação de placas β amiloide no córtex cerebral, enquanto o tratamento com 200 mg/kg de metformina reverteu essas alterações. Quanto à expressão das proteínas avaliadas por Western Blot houve redução significativa da expressão das proteínas pAMPK e p-Akt no grupo STZ e aumento significativo no grupo M200, apenas na expressão de p-Akt. A expressão das proteínas pTAU, pERK, pGSK3, iNOS, nNOS, PARP, Citocromo C, IL-1 e GluN2A foram significativamente elevados no grupo STZ e reduzidos no grupo M200, exceto a expressão de IL-1, sem redução significativa, e a expressão de nNOS, a qual permaneceu elevada. A expressão de eNOS foi significativamente aumentada apenas no

grupo M200 e a expressão de PI3K, GluA1, GluA2 e GluN1 não foi alterada em nenhum dos grupos experimentais. Em conclusão, o tratamento com metformina contribuiu para a melhora da memória espacial em camundongos diabéticos que pode ser atribuída aos efeitos da metformina sobre a redução da fosforilação de Tau, redução da produção de óxido nítrico, modulação da expressão das sintases de óxido nítrico (eNOS, iNOS e nNOS) e pela redução da morte neuronal.

Palavras-chave: Diabetes. Memória. Inflamação.

ABSTRACT

In brain, diabetes can cause memory changes, inflammation and augment the expression of Alzheimer's Disease (AD) markers, pTau and amyoloid β. Metformin is hypoglycemic, AMPK-activating drug. It still has anti-inflammatory potential and can improve neurogenesis. The goal of this study was to evaluate the effects of metformin on long-term memory/impairment of learning, on AD's markers in diabetic mice and to assess the possible molecular/cellular mechanisms of this drug in this animal model. Forty Swiss Webster mice were distributed in the following experimental groups: control; streptozotocin (STZ); streptozotocin (STZ) + metformin treatment at a dose of 100 mg/kg (M100); streptozotocin (STZ) + metformin treatment at a dose of 200 mg/kg (M200). Diabetes was induced using streptozotocin. After of diabetes confirmation, the treatment with metformin hydrochloride was initiated and followed for nine weeks. The weight and fasting blood glucose were avaluated weeky. The memory task in the Barnes maze was performed in the last week of treatment, then the animals were anesthetized and euthanized. The serum was collected for nitric oxide dosage and the brains were processed to nitric oxide dosage, imunohistoquemistry, immunofluorescence and western blot. After nine weeks of treatment, STZ, M100 and M200 mice had significant decrease in body weight and increase in fasting glycemia throughout the entire treatment. STZ mice showed significant augmentation of time spent to reach the target box in the Barnes maze, while the M200 group was able to reduce the time spent to find the target box compared to STZ mice. Nitric oxide dosage of the serum and brain tissue by Greiss method showed increased nitrite levels in STZ mice and reduction in M200 mice. Immunohistochemistry for GFAP revealed augmented and diminished immunoreactivity in STZ and M200, respectively, in hippocampal dentate gyrus and CA1. Immunofluorescence for p-Tau indicated rise in phosphorylation of Tau in hippocampal areas and corpus callosum. CREB phosphorylation and amyloid β accumulation were detected on cerebral cortex by immunofluorescence. Diabetes reduced CREB phosphorylation and increased the formation of amyloid β plaque, whereas metformin treatment at a dose of 200mg/kg reversed these changes. The protein expression analysis by Western Blot demonstrated increase in the expression of p-AMPK and p-Akt in M200 mice and significant reduction in the other experimental groups. Protein expression of pTau, pERK, pGSK3, iNOS, nNOS, PARP, Cytochrome c, caspase 3, IL-1 e GluN2A were increased in STZ mice and reduced in M200 mice, except for the IL-1 expression, which had no significant reduction, and nNOS expression, which remained elevated. eNOS expression rose only in

M200 mice and PI3K, GluA1, GluA2 e GluN1 expression was not altered in any of the experimental groups. In conclusion, the treatment with metformin contributed to the improvement of spatial memory in diabetic mice, which can be attributed to the effects of metformin on reduction of Tau phosphorylation, reduction of nitric oxide production, modulation of nitric oxide's synthases expression (eNOS, iNOS e nNOS) and on decrease in cell death.

Key-words: Diabetes. Memory. Inflammation.

LISTA DE ILUSTRAÇÕES

Revisão de Literatura

Artigo 2

LISTA DE ABREVIATURAS E SIGLAS

AC	Adenilato ciclase
AD	Doença de Alzheimer
AKAP	*A kinase anchoring proteins*
AMPA	Ácido 2-amino-3hidroxi-5-metil-4-isoxazolproprionato
AMPc	Adenosina monofosfato cíclico
AMPK	Proteína quinase de Adenosina monofosfato
Aβ	β amiloide
BDNF	Fator neurotrófico derivado do cérebro
BHE	Barreira hematoencefálica
CaM	Calmodulina
COX-1	Cicloxigenase 1
COX-2	Cicloxigenase-2
CREB	Proteína de ligação do elemento de resposta ao AMPc
PSD	Densidade pós-sináptica
DG	Giro denteado
ERK	Proteína quinase regulada por sinalização extracelular
GFAP	Proteína fiblilar ácida glial
GLUT	Transportadores de glicose independentes de sódio
GMPc	Guanosina monofosfato cíclico
IGF1	Fator de crescimento semelhante à insulina 1
i.p	intraperitoneal
IL-1	Interleucina 1

Inos	Sintase de óxido nítrico induzível
IR	Receptor de insulina
LPS	Lipopolissacarídeos
NFκB	Fator nuclear kappa B
NMDA	N-metil-D-aspártico
nNOS	Sintase de óxido nítrico neuronal
NO	Óxido nítrico
p-Akt	Akt fosforilada
pAMPK	Proteína quinase de adenosina monofosfato fosforilada
PARP	Poli (ADP-ribose) polimerase
pCREB	Proteína de ligação do elemento de resposta ao AMPc fosforilada
pERK	Proteína quinase regulada por sinalização extracelular fosforilada
pGSK3	Glicogênio sintase quinase 3 fosforilada
PI3K	Fosfatidil inositol-3 quinase
PKA	Proteína quinase A
PKC	Proteína quinase C
PKG	Proteína quinase G
LTP	Potenciação de longa duração
LTD	Depressão de longa duração
MAPK	Proteína quinase ativada por mitógeno
CaMKII	Proteína quinase II dependente de Ca^{2+}/calmodulina
pTau	Tau fosforilada
ROS	Espécies reativas de oxigênio

SGLT	Transportadores de glicose dependentes de sódio
SNC	Sistema nervoso central
STZ	Estreptozotocina
IRS	Substratos de receptor de insulina
TNF-α	Fator de necrose tumoral

SUMÁRIO

1 INTRODUÇÃO

Hiperglicemia causada pela insuficiência da secreção da insulina ou insensibilidade do receptor à insulina endógena assinalam a patologia da Diabetes *mellitus*, uma desordem metabólica (ROLO; PALMEIRA, 2006). Segundo a Organização Mundial de Saúde, em 2014 haviam 422 milhões de pessoas diabéticas no mundo (WORLD HEALTH ORGANIZATION, 2016). No Brasil, segundo a Sociedade Brasileira de Diabetes o Brasil ocupa o 4º lugar em número de diabéticos no mundo com 12,5 milhões de pessoas diabéticas (SOCIEDADE BRASILEIRA DE DIABETES, 2017).

A diabetes *mellitus* pode ser classificada em diabetes tipo 1 ou tipo 2. Na diabetes *mellitus* tipo 1, o sistema imune ataca as células pancreáticas responsáveis pela produção e secreção do hormônio insulina, que promove a entrada de glicose nas células do corpo. Como resultado disto, há deficiência da insulina endógena necessária para o funcionamento metabólico. Por outro lado, na diabetes *mellitus* tipo 2 há secreção de insulina pelas células do pâncreas, e apesar disso, os receptores de insulina são resistentes à ação desta, tornando-a ineficaz. Nesse tipo de diabetes, o excesso de peso corporal, sedentarismo e alimentação estão entre as principais causas de diabetes tipo 2 (MARX, 2002).

Além de prejuízos no metabolismo energético das células do corpo, a diabetes está associada a complicações crônicas como nefropatia (UWAEZUOKE, 2017), neuropatia (VINCENT et al., 2004) e retinopatia diabética (CAPITÃO; SOARES, 2016) derivados do aumento do estresse oxidativo, há também alterações endoteliais e inflamação. No sistema nervoso central, além do aumento do estresse oxidativo, danos endoteliais e inflamação causados pela hiperglicemia (EL-AKABAWY; EL-KHOLY, 2014; JING et al., 2013; OLIVEIRA et al., 2016). A deficiência de insulina também é relevante nas alterações de aprendizado e memória em roedores (FRANCIS et al., 2008; JOLIVALT et al., 2008; KUHAD et al., 2009; PEARSON-LEARY et al., 2018).

Estudos epidemiológicos também mostram uma maior taxa de convulsão em diabéticos (MCCORRY et al., 2006; VERROTTI et al., 2012). Além disso, pacientes diabéticos apresentam incidência de duas a quatro vezes maior de sofrer um acidente vascular e após a isquemia, o prognóstico é pior do que em pacientes normais, pois a diabetes aumenta o dano neuronal e a área isquêmica (LI et al., 2004; MURANYI et al., 2003). Vários estudos *in vitro* e *in vivo* apontam a diabetes como causa ou agravante da doença de Alzheimer, pois a deficiência de insulina exacerba a formação de placas β amiloides e fosforilação da proteína

Tau (BISWAS et al., 2015; BLÁZQUEZ et al., 2014; DEVI et al., 2012; JOLIVALT et al., 2010; TAKEDA et al., 2011). As pesquisas sobre a incidência da doença de Alzheimer em pacientes diabéticos submetidos a tratamentos com hipoglicemiantes orais têm resultados variados (CHEN et al., 2017; GOODARZI, 2014; KADOHARA; SATO; KAWAKAMI, 2017; KUAN et al., 2017; MOORE et al., 2013, 2014). Apesar de estudos experimentais relacionarem positivamente a diabetes com alterações características da doença de Alzheimer, como dano cognitivo, hiperfosforilação da proteína Tau e placas β amiloides, poucos estudos avaliam potenciais tratamentos ou a interação da diabetes com Alzheimer sob o tratamento com hipoglicemiantes.

Existem vários modelos experimentais de diabetes tipo 1 ou tipo 2. O modelo utilizado neste trabalho foi um modelo de hiperglicemia sustentada induzida pela estreptozotocina (STZ). Estudos demonstram que além de danos na sinalização da insulina cerebral, os cérebros dos animais, com diabetes induzida por STZ, apresentam características da Doença de Alzheimer tais como aumento da fosforilação da proteína Tau, acúmulo de β amoilóide e danos de memória (JOLIVALT et al., 2008, 2010; KING et al., 2015). A estreptozotocina é uma molécula tóxica para as células β pancreáticas das ilhotas de Langehans de roedores o que causa danos irreversíveis nestas células, mantendo assim a hiperglicemia (KING, 2012). Dessa forma, é um modelo que reproduz a ausência de produção e secreção da insulina e hiperglicemia, características associadas à diabetes tipo 1 (LENZEN, 2008). É um modelo ainda bastante utilizado, apesar de recentemente está disponível modelos genéticos de diabetes que abrange mais características da diabetes tipo 1 (OLIVARES et al., 2017).

A metformina é o fármaco de primeira escolha para o tratamento da diabetes *mellitus* tipo 2 e no Brasil é distribuído gratuitamente pelo Sistema Único de Saúde. Seu mecanismo de ação é através da proteína quinase ativada por AMP (AMPK), que regula a homeostase energética (revisado em CORREIA et al., 2008). Pesquisas demonstram que a metformina possui ação anti-inflamatória (ISMAIEL et al., 2016) independente da ativação do AMPK (ŁABUZEK et al., 2010a), atividade antioxidante (KUKIDOME et al., 2006), capacidade de restaurar a disfunção endotelial reduzindo as complicações vasculares (CORREIA et al., 2008; MAJITHIYA; BALARAMAN, 2006) e promove a neurogênese (ARNOLD et al., 2014). Em camundongos diabéticos foi observado aumento dos marcadores inflamatórios, redução de neurônios maduros e baixo desempenho no teste de memória, alterações que foram revertidas pela metformina (OLIVEIRA et al., 2016). Assim, a metformina apresenta potencial farmacológico para reverter os danos neurodegenerativos e cognitivos causados pela

hiperglicemia, através da modulação do processo inflamatório, porém o seu mecanismo de ação necessita ser analisado.

Desta forma, a hipótese avaliada neste trabalho é que a hiperglicemia e ausência de insulina induzidas pela estreptozotocina, um modelo idiopático de diabetes tipo 1, causam prejuízos no processo de aprendizado e aquisição da memória, através do defeito na sinalização da insulina cerebral e neuroinflamação, ocasionando assim aumento dos marcadores da Doença de Alzheimer (fosforilação de Tau e β amiloide) e morte neuronal; e a metformina, por sua vez, é capaz de reverter tais alterações cerebrais e cognitivas induzidas pela estreptozotocina em camundongos *Swiss*.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Avaliar o mecanismo de ação da metformina sobre o processo de neuroinflamação, neurodegeneração e cognição em modelo de diabetes *mellitus* em camundongos machos *Swiss*.

1.2.2 Objetivos Específicos

a) Avaliar os efeitos da metformina no aprendizado e formação da memória espacial e de longo tempo;

b) Avaliar os efeitos da metformina sobre a sinalização da memória pelo fator de transcrição CREB;

c) Avaliar os efeitos da metformina sobre os marcadores da Doença de Alzheimer (Tau fosforilada e acúmulo de β amiloide) em camundongos *Swiss* diabéticos;

d) Avaliar os efeitos da metformina sobre as proteínas quinases de Tau GSK3, PKA e ERK;

e) Avaliar a expressão dos receptores de glutamato no cérebro de camundongos *Swiss* diabéticos;

f) Avaliar os efeitos da metformina na modulação da cascata de sinalização da insulina (Akt e GSK3);

g) Avaliar os efeitos da metformina sobre a inflamação através da Il-1 e iNOS no cérebro de camundongos *Swiss* diabéticos;

h) Avaliar os efeitos da metformina sobre os níveis de óxido nítrico no soro e no cérebro de animais *Swiss* diabéticos;

i) Caracterizar a expressão das proteínas sintases de óxido nítrico (iNOS, eNOS e nNOS) no cérebro de camundongos *Swiss* diabéticos;

j) Avaliar os efeitos da metformina sobre a expressão das proteínas sintases de óxido nítrico (eNOS e nNOS) no cérebro de camundongos *Swiss* diabéticos;

k) Avaliar os efeitos da metformina sobre a reatividade astrocitária hipocampais através do marcador GFAP no cérebro de camundongos *Swiss* diabéticos.

2 REVISÃO BIBLIOGRÁFICA

2.1 TRANSPORTE DE GLICOSE E O PAPEL DA INSULINA NO CÉREBRO

A entrada de glicose nas células ocorre através dos transportadores de glicose presentes na membrana plasmática celular. Os transportadores de glicose são divididos em duas classes. A primeira classe de transportadores de glicose independentes de sódio (GLUT) é composta por 14 proteínas com transporte facilitado sendo o GLUT-4, e possivelmente o GLUT-8 e GLUT-12, sensíveis à insulina. A insulina é um hormônio anabólico produzido pelas células β das ilhotas pancreáticas. Sua principal função é aumentar a taxa de captação de glicose nas células fornecendo uma fonte aumentada de energia (KUMAR et al., 2010). A segunda classe de transportadores de glicose dependentes de sódio (SGLT), com transporte ativo secundário, é composta por dez proteínas transportadoras SGLT-1 a SGLT-10 (SHAH; DESILVA; ABBRUSCATO, 2012).

A maioria das células expressa vários transportadores de glicose e o padrão de expressão varia de acordo com o tecido devido aos requisitos metabólicos específicos (SHAH; DESILVA; ABBRUSCATO, 2012). O cérebro consome metade do suprimento total de glicose devido à intensa atividade metabólica dos neurônios e pela ausência de reserva neuronal de carboidratos (ABBONDANTE et al., 2014; VILCHEZ et al., 2007). É bem estabelecido que nos órgãos periféricos o GLUT-4 é uma molécula chave na regulação da sinalização da insulina e entrada de glicose na célula (KAHN, 1996). Sugere-se que no cérebro a captação de glicose ocorre pela sinalização da insulina e pelo fator de crescimento semelhante à insulina 1 (IGF1) (VILCHEZ et al., 2007), mas o papel da sinalização da insulina na captação de glicose neuronal ainda não está claro (PEARSON-LEARY et al., 2018). Além disso, comparado com os órgãos periféricos, a disponibilidade de glicose e outros nutrientes para os tecidos neurais é limitada pelas propriedades restritivas da barreira hematoencefálica (BHE) (PRASAD et al., 2014; SHAH; DESILVA; ABBRUSCATO, 2012).

A glicose ultrapassa a BHE através do GLUT-1, principal transportador da BHE, que está presente nas células endoteliais microvasculares do cérebro e nos astrócitos (DUELLI; KUSCHINSKY, 2001). Nos neurônios, o GLUT-3 é considerado o principal transportador de glicose, embora o transportador sensível à insulina (GLUT-4) também seja altamente expresso em regiões específicas do cérebro (SHAH; DESILVA; ABBRUSCATO, 2012) sugerindo um papel importante para a insulina no metabolismo de glicose e lipídios do

cérebro, incluindo a regulação do desenvolvimento neuronal, memória e processos de aprendizagem (CARDOSO et al., 2009; GEROZISSIS, 2008; LIU et al., 2011).

Alguns estudos sugerem que os neurônios sintetizam a insulina (SCHECHTER et al., 1996; SCHECHTER; ABBOUD, 2001). No entanto, a maior parte da demanda de insulina do cérebro é cumprida pela insulina periférica que acessa as células cerebrais tanto através do líquido cefalorraquidiano atingindo regiões que não possuem BHE real, como na neuro-hipófise, e diretamente através da BHE através de receptores específicos de insulina que podem atuar como transportadores (PRASAD et al., 2014; SHAH; DESILVA; ABBRUSCATO, 2012).

As condições de glicemia alteradas, como as observadas em pacientes diabéticos, precedem as alterações estruturais da BHE (ACHARYA et al., 2013; HAWKINS et al., 2007; HORANI; MOORADIAN, 2003; MURESANU; SHARMA; SHARMA, 2010; SERLIN; LEVY; SHALEV, 2011; SIMPSON et al., 1999; STARR, 2003). Uma vez que a BHE exerce uma série de funções no controle da homeostase cerebral, tais como regulação do transporte de moléculas essenciais, e proteção do SNC de substâncias potencialmente nocivas, as alterações na estrutura da BHE durante a diabetes são relevantes para a patogênese de distúrbios cerebrais (DANEMAN, 2012). Apesar da existência de uma grande quantidade de dados sobre as complicações microvasculares induzidas pela diabetes no rim e na retina, o impacto da diabetes no nível do sistema cerebrovascular ainda é pouco compreendido e estudado (PRASAD et al., 2014).

Da mesma forma, os estudos sobre a expressão de GLUT na BHE em ratos e em humanos são conflitantes devido às diferenças metodológicas (PRASAD et al., 2014). Os resultados obtidos com estudos em ratos diabéticos induzidos por estreptozotocina (STZ) mostraram que a hiperglicemia crônica regula negativamente a expressão de mRNA e proteína de GLUT-1 e GLUT-3 independentemente da administração de baixas e altas doses de insulina. Segundo os autores, a regulação negativa de GLUT-1 e 3 em condições hiperglicêmicas pode ser uma reação adaptativa do corpo para evitar a exposição excessiva de glicose que pode causar dano celular (HOU et al., 2007). Por outro lado, em outros estudos não foram observadas alterações significativas na absorção de glicose e na expressão de GLUT-1 em ratos diabéticos induzidos por STZ (BADR et al., 2000; SIMPSON et al., 1999). Em humanos também não foram encontradas mudanças importantes no fluxo sanguíneo

cerebral global ou no metabolismo regional da glicose (incluindo a velocidade máxima de transporte de glicose) após hiperglicemia aguda (SEAQUIST et al., 2005).

A supressão da sinalização da insulina endógena em humanos resulta na diminuição da captação de glicose no cérebro e em cultura de neurônios do hipocampo, por sua vez, o tratamento com insulina facilita a translocação do GLUT-3 (UEMURA; GREENLEE, 2006). A captação de glicose no hipocampo é aumentada durante a alternância espontânea em labirinto, uma tarefa de memória de trabalho espacial mediada pelo hipocampo, e a administração de insulina exógena no hipocampo aumenta o desempenho dos animais na alternância espontânea (PEARSON-LEARY et al., 2018). A inibição da absorção de glicose mediada por GLUT-4 não altera o desempenho dos animais na alternância espontânea, mas impede a melhora observada quando a insulina exógena é administrada no hipocampo. Isso sugere que o processamento cognitivo inicial do hipocampo não requer o GLUT-4 funcional no hipocampo, enquanto o aprimoramento cognitivo mediado pela insulina é dependente do deste transportador (PEARSON-LEARY et al., 2018).

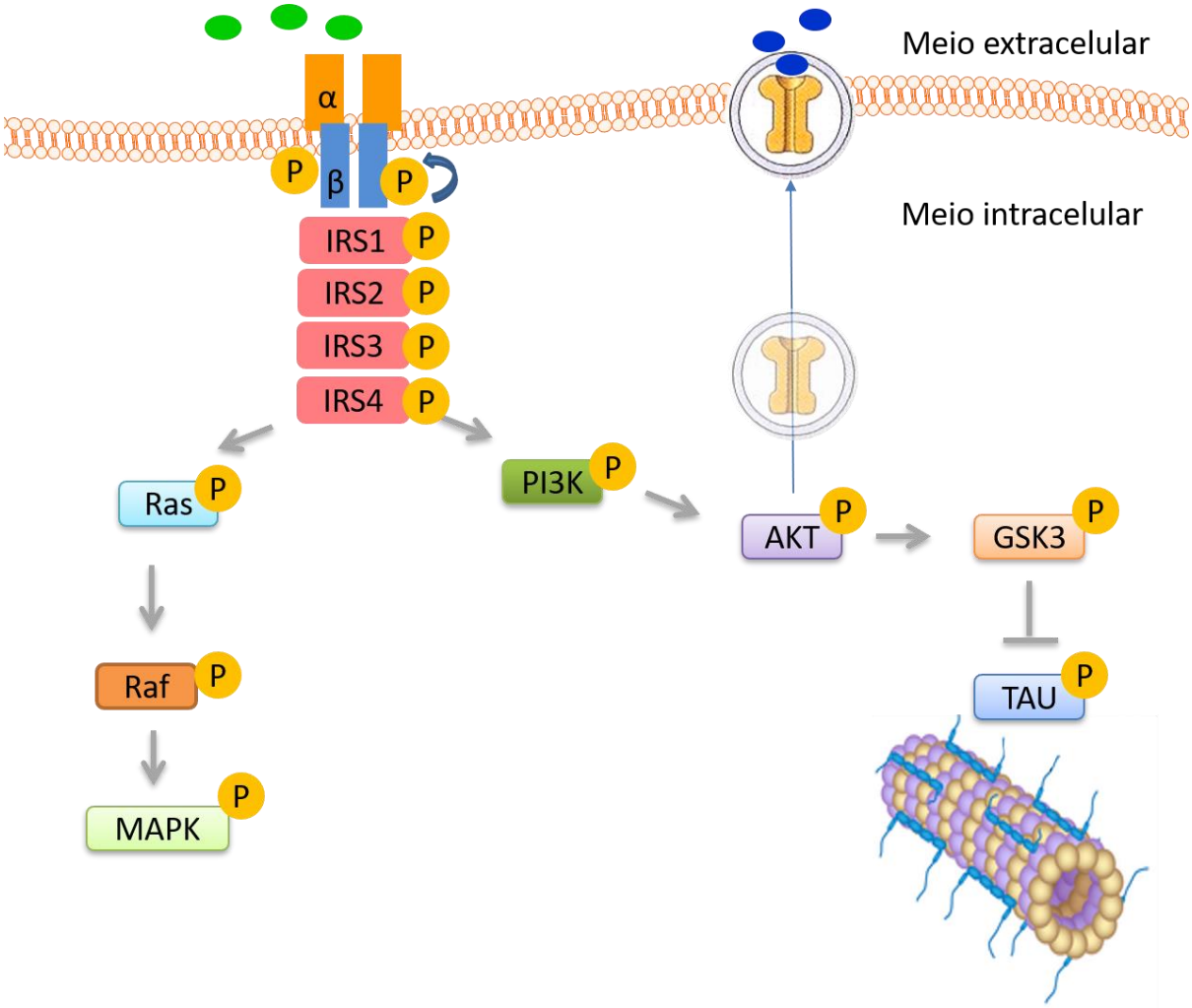
As alterações no metabolismo e na absorção de glicose estão presentes no cérebro de pacientes com a doença de Alzheimer (SHAH; DESILVA; ABBRUSCATO, 2012) e estudos experimentais confirmam que a diabetes tanto do tipo 1 como do tipo 2 promovem ou agravam as características da doença de Alzheimer (JOLIVALT et al., 2008, 2010; MAESAKO et al., 2012). Análises *post mortem* de cérebros de pacientes com Doença de Alzheimer mostram redução da expressão dos transportadores GLUT-1 no córtex e em microvasos (KALARIA; HARIK, 1989; MOORADIAN; GIRGIS; SHAH, 1997; SIMPSON et al., 1994), e GLUT-3 no córtex e giro denteado (DG – do inglês “dentate gyrus”), além de aumento da expressão de GLUT-2 (SHAH; DESILVA; ABBRUSCATO, 2012). Posteriormente, foi demonstrado em camundongos geneticamente modificados para a doença de Alzheimer, que o GLUT-1 era necessário para a manutenção estrutural da BHE, além da arquitetura dos vasos e do fluxo sanguíneo cerebral. Por outro lado, a redução de GLUT-1 acelerou o acúmulo da proteína β-amiloide causando disfunção neuronal progressiva, déficits comportamentais, perda neuronal e neurodegeneração que se desenvolveram após alterações cerebrais iniciais (WINKLER et al., 2015).

Ainda sobre a influência da diabetes na doença de Alzheimer, é bem mais aceito que as alterações da cascata de sinalização do receptor da insulina neuronal afetam a patogênese da doença de Alzheimer. O receptor de insulina (IR) é uma proteína tetramérica com duas

subunidades (α e β). As subunidades α estão voltadas para o meio extracelular. A insulina, ao se ligar na subunidade α, promove a ativação da tirosina quinase das subunidades β, localizadas na parte interna da membrana plasmática, resultando na autofosforilação do IR. Após a autofosforilação segue-se então uma cascata de fosforilação dos substratos de receptor de insulina (IRS) de 1 a 4 e 3-fosfatidil inositol quinase (PI3K) é recrutado para a membrana para induzir a inserção do transportador de glicose GLUT-4 na membrana plasmática via fosforilação da Akt/PKB (proteína quinase B) (KUMAR et al., 2010). Além disso, a Akt fosforila a proteína glicogênio sintase 3-quinase (GSK3) inativando-a. Nos neurônios, esta inativação diminui a fosforilação da proteína Tau permitindo a estabilização dos microtúbulos para o transporte de vesículas sinápticas e outros componentes celulares dos neurônios (figura 1) (CHO; JOHNSON, 2004). Desse modo, o desligamento da via de sinalização da insulina promove formação de redes neurofibrilares intracelulares, uma dos principais características da doença de Alzheimer, resultante da agregação de Tau hiperfosforilada (marcador da doença de Alzheimer) (JOLIVALT et al., 2008, 2010; TAKEDA et al., 2011).

Além disso, quando os IRS são fosforilados ocorre a fosforilação da proteína Ras, que por sua vez fosforila a Raf, recrutanto-a para a membrana (AVRUCH, 1998; SRIVASTAVA; PANDEY, 1998). A Raf é uma proteína quinase serina/treonina do tipo MAP3K que ao ser ativada, fosforila a proteína quinase ativada por mitógeno (MAPK) nos resíduos de serina/treonina, ativando a cascata de crescimento celular (ARTHUR; LEY, 2013; SRIVASTAVA; PANDEY, 1998). No cérebro, acredita-se que a cascata Ras-ERK desempenha um papel na plasticidade sináptica e na formação da memória. A proteína de ligação do elemento de resposta ao AMPc (CREB) é um alvo direto de ERK e é capaz de induzir mudanças estruturais associadas à formação de memória a longo prazo (SCOTT et al., 2002; TULLY et al., 2003), cujos mecanismos e formas de avaliação serão descritos no tópico a seguir.

Figura 1 – Via de sinalização da insulina neuronal. A insulina (representado pelos círculos verdes) se liga ao receptor da insulina nas subunidades α que estão voltadas para meio extracelular. Após a ligação da insulina ao seu receptor, ocorre uma autofosforilação das subunidades β e segue-se então uma fosforilação das IRS1, IRS2, IRS3 e IRS4. O PI3K é recrutado e ativado por fosforilação. Em seguida, a proteína Akt é fosforilada e ativada, o que promove a inserção do transportador da glicose na membrana plasmática para entrada de glicose (círculos azuis) no neurônio. Além de desempenhar esse papel na entrada de glicose, a Akt fosforila a proteína GSK3, inativando-a. Assim a fosforilação da Tau via GSK3 é reduzida e há estabilização dos microtúbulos neuronais.



Fonte: adaptado de OLIVEIRA (2015).

2.2 MEMÓRIA

Aprendizado e memória são termos frequentemente empregados como sinônimos. Entretanto segundo Kandel (2014) “o aprendizado refere-se a uma mudança no comportamento que resulta da aquisição de conhecimento acerca do mundo, e a memória é o processo pelo qual esse conhecimento é codificado, armazenado e posteriormente evocado”. Assim, o aprendizado é o processo de repetição que promove modificações no comportamento, enquanto a memória é a capacidade do indivíduo lembrar as experiências prévias (SHARMA; RAKOCZY; BROWN-BORG, 2010).

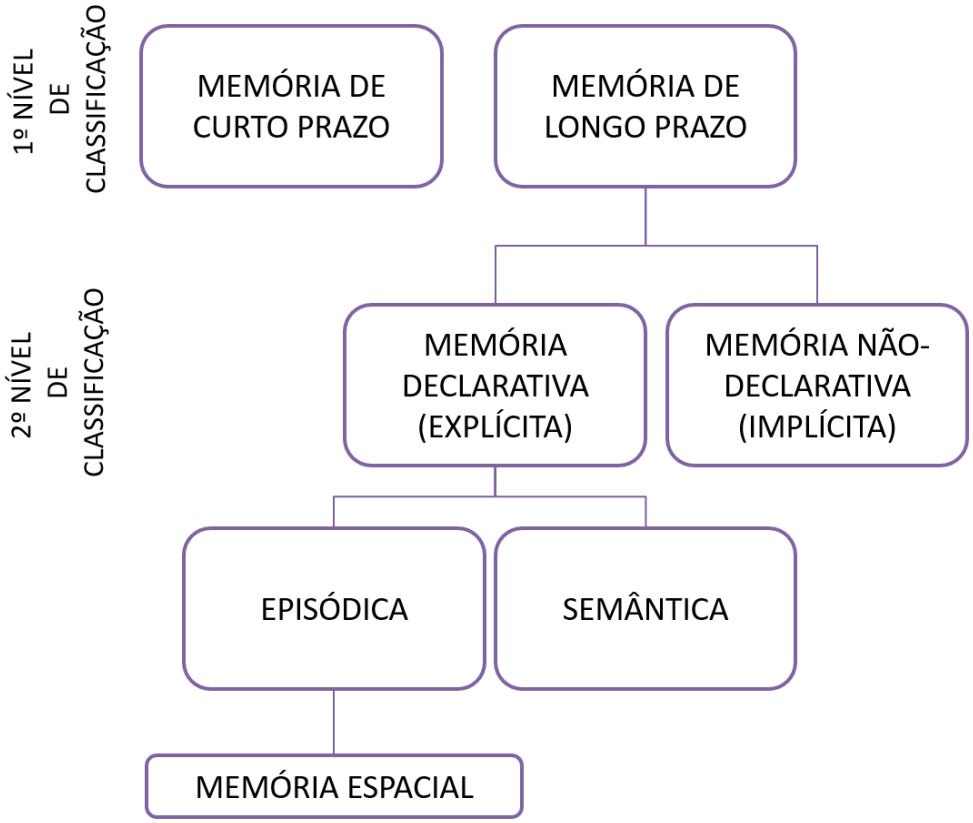
A memória pode ser classificada em dois níveis (figura 2). O primeiro nível refere-se ao tempo de armazenamento da memória, podendo assim ser classificado como memória de curto prazo e memória de longo prazo (KANDEL, et al., 2014). A memória de curto prazo do tipo memória de trabalho é limitada pela quantidade de informações que é capaz de armazenar e essas informações permanecem armazenadas apenas por alguns segundos ou minutos. Por outro lado, a memória de longo prazo armazena grandes quantidades de informação e com tempo ilimitado de armazenamento (SHARMA; RAKOCZY; BROWN-BORG, 2010).

A memória de longo prazo pode ser subdivida em um segundo nível de classificação. Este considera a natureza da informação armazenada, sendo então classificadas como memória declarativa e memória não-declarativa. A memória declarativa, também chamada de explícita, refere-se à informação que é convencionalmente transmitida ou expressa, enquanto a memória não-declarativa (implícita) é referente às informações motoras ou perceptivas que não podem ser transmitidas pela linguagem (KANDEL, et al., 2014; SUNYER et al., 2007). A memória declarativa é ainda subdivida em memória episódica e semântica. A memória episódica é aquela em que a pessoa experimenta o evento pessoalmente em um contexto particular como lugar e tempo, enquanto a memória semântica é aquela em que a pessoa toma conhecimento de tais fatos independentemente da forma em que foram aprendidos (KOOPMANS et al., 2003; SUNYER et al., 2007).

A memória espacial é considerada um subtipo da memória episódica, pois a informação é armazenada dentro de um contexto espacial-temporal (O’KEEFE e NADEL, 1978). A princípio, pelo conceito de memória declarativa em que a memória espacial está inserida, é difícil aplicar estes termos aos animais, pois é uma classificação que considera características

inerentes aos humanos, como a fala e a escrita (PAUL; MAGDA; ABEL, 2009b). Entretanto, após os estudos do psicólogo Edward Tolman vários autores estudaram a aquisição da memória espacial experimentalmente utilizando os instintos básicos dos ratos.

Figura 2 – representação esquemática da classificação dos tipos de memória.



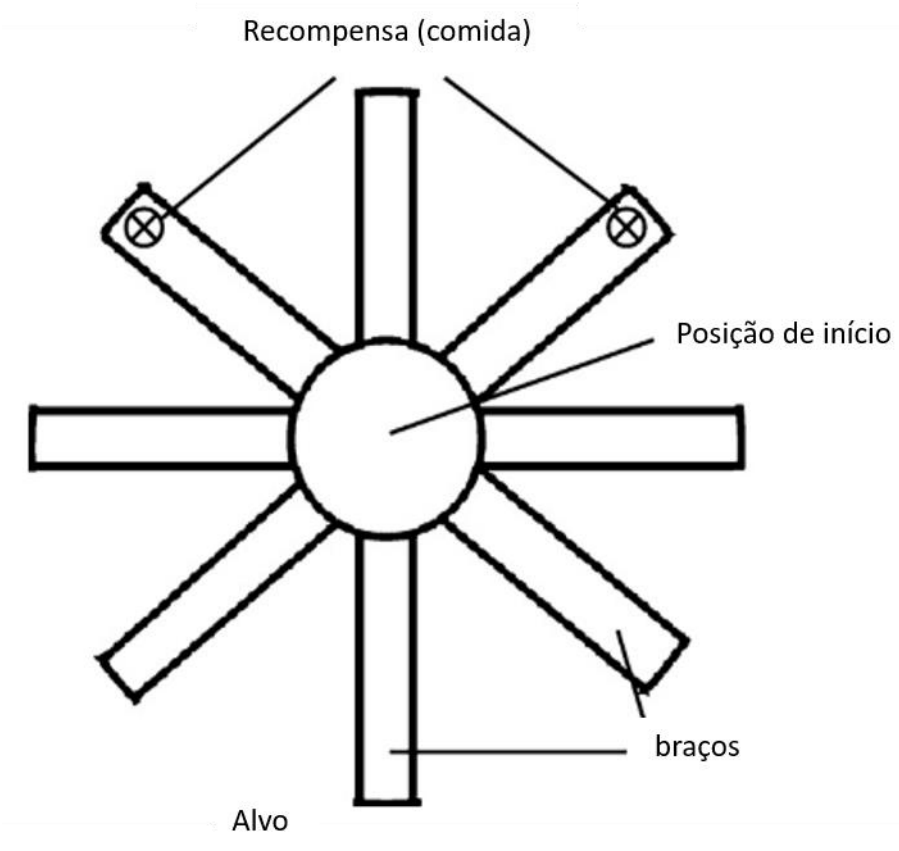
Fonte: A autora.

2.2.1 Testes de Aprendizado e Memória em Roedores

O sistema de memória espacial está presente nos mais diversos grupos animais e serve como um sistema de orientação para diferenciar objetos e localizar rotas específicas de comida baseado na experiência prévia do animal. Dessa forma, é um sistema importante na preservação e sobrevivência das espécies (PAUL; MAGDA; ABEL, 2009). Baseado nessas características, o psicólogo Edward Tolman foi um dos primeiros a analisar o aprendizado espacial em ratos. Nos experimentos realizados por ele, foi observado que os ratos são capazes de aprender locais alvo-específicos. Motivados pela privação de comida, os animais eram submetidos a um labirinto para encontrar alimento e conforme o teste era repetido, nos

dias seguintes, os animais alcançavam o alvo mais rapidamente (TOLMAN; GLEITMAN, 1949; TOLMAN; RITCHIE; KALISH, 1992). Posteriormente em 1979, Olton submeteu ratos famintos a um labirinto com braços dispostos de forma radial (figura 3). Ao final de alguns braços havia comida e a outra metade estava vazia. Após lesionar o hipocampo dos animais ele observou que os ratos visitavam novamente os braços que ele já tinha coletado a comida. Por outro lado, os animais visitavam menos os braços que nunca tiveram comida, sugerindo prejuízos na memória de trabalho, mas não na memória de longo prazo já adquirida (OLTON; PAPAS, 1979). A memória espacial no contexto da experimentação animal é subdivida em dois tipos de memória. o primeiro tipo é a memória de trabalho ou curto prazo a qual refere-se à memória dentro de uma sessão de teste ou treino. O segundo tipo é a memória de referência que refere-se a aquisição da memória em sessões de teste ou ensaios e está mais relacionada à memória de longo prazo (ROSENFELD; FERGUSON, 2014).

Figura 3 – Desenho esquemático do labirinto radial.



Fonte: adaptado de PAUL; MAGDA; ABEL (2009).

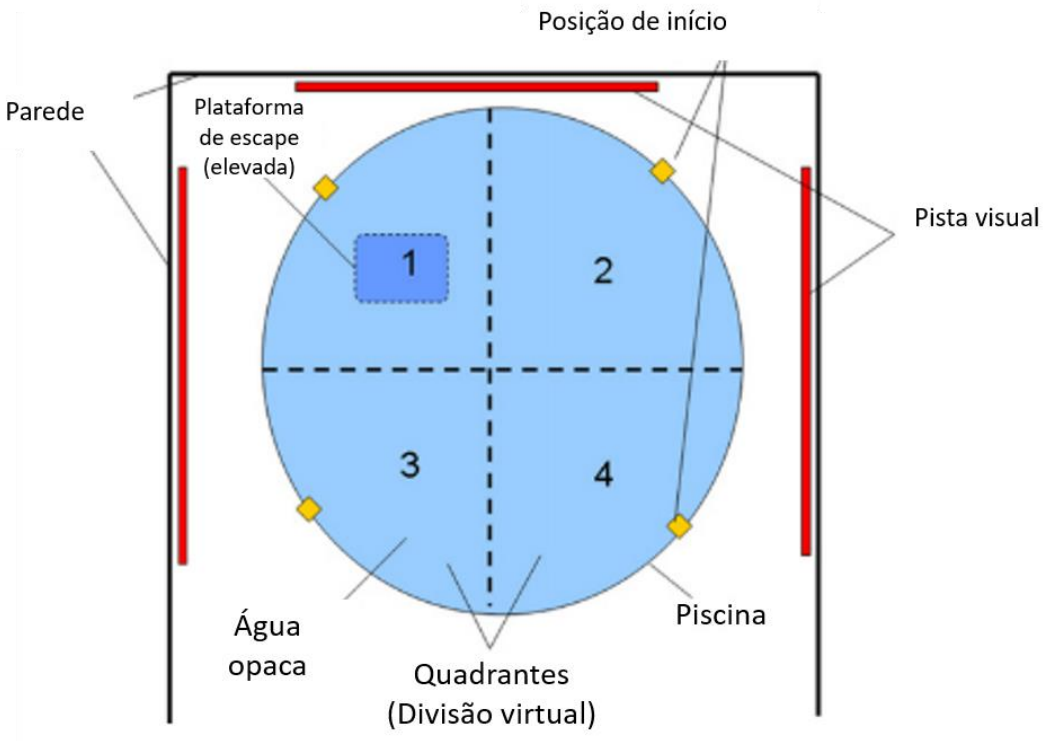
A partir dessa diferenciação de tipos de memória, posteriormente foram desenvolvidos vários testes comportamentais para analisar a memória de trabalho e/ou referência

(SHARMA; RAKOCZY; BROWN-BORG, 2010). A princípio os testes foram desenvolvidos para experimentação com ratos e para usá-los em camundongos é preciso fazer adaptações na estrutura física e nos fatores motivacionais (PAUL; MAGDA; ABEL, 2009).

2.2.1.1 Morris Water Maze

O labirinto usado por Olton no experimento citado acima, chamado de radial-arm-maze (labirinto de braço radial), foi proposto primeiramente por ele em 1976 (OLTON; SAMUELSON, 1976). Em 1981, Morris propôs o Morris water maze (labirinto aquático de Morris) (figura 4) como alternativa ao labirinto de braços radiais. Ao contrário deste, o Morris water maze não necessita de privação de comida e a motivação está na aversão que os ratos têm à água. O Morris water maze consiste numa piscina redonda com água esbranquiçada com leite ou tinta branca para ficar opaca. O animal aprende a localizar a plataforma escondida sob a água partindo de quatro pontos diferentes. Ao longo de uma série de ensaios, os animais aprendem a localização da plataforma oculta com base em pistas distais e, com o tempo, a latência para localizar a plataforma diminui. A aprendizagem é confirmada posteriormente retirando a plataforma oculta e a quantidade de tempo gasto na região onde estava a plataforma é medida (MORRIS, 1981, 1984).

Figura 4 – Desenho esquemático do labirinto aquático de Morris.



Fonte: adaptado de PAUL; MAGDA; ABEL (2009).

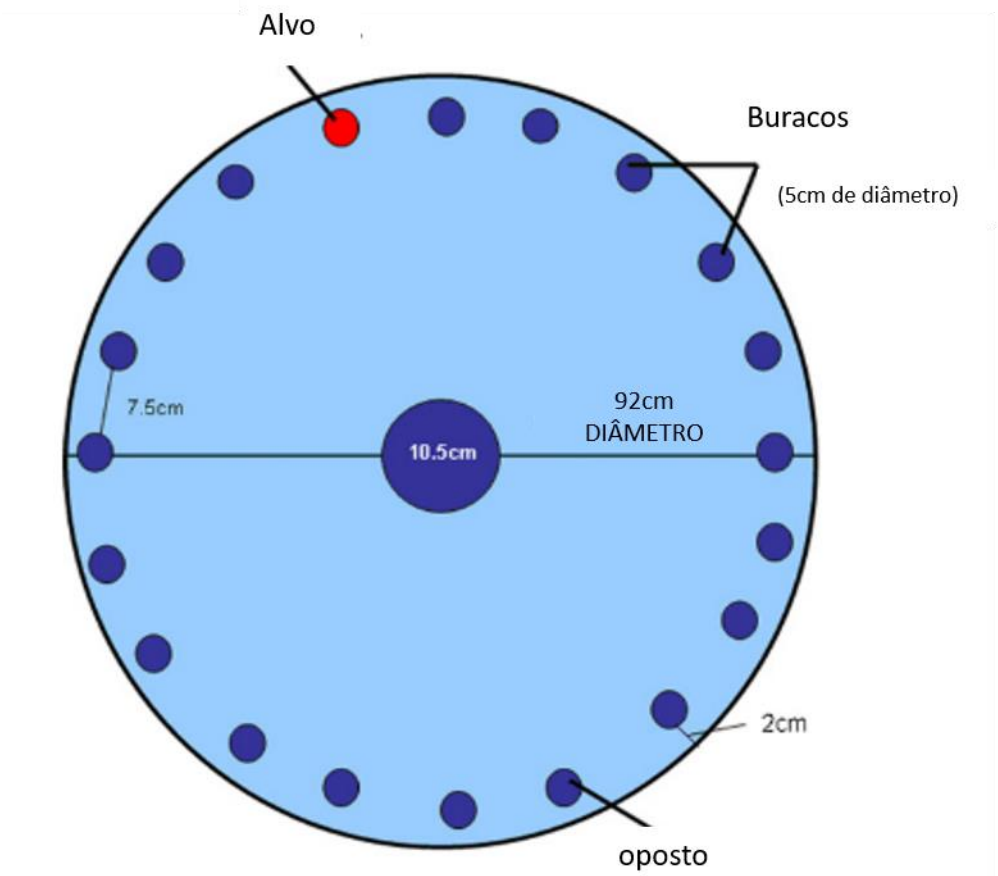
2.2.1.2 Barnes Maze

Com o intuito de diminuir o estresse causado pela natação no Morris water maze, Carol Barnes (1979) desenvolveu um labirinto de plataforma circular com uma altura de 1,4 metros, chamado de Barnes maze (figura 5), para ser uma alternativa menos estressante para analisar o aprendizado espacial de trabalho e a memória de referência. Ao longo de todo perímetro da plataforma há buracos igualmente distribuídos e em um dos buracos há uma caixa-alvo escura e os demais buracos são fechados. Ao redor da plataforma deve haver pistas visuais que servem de referência para o animal. Durante o teste, os animais recebem um estímulo/reforço, como por exemplo, luz, ruído e vento, para escapar da superfície da plataforma para dentro da caixa alvo e o tempo gasto é analisado (BARNES, 1979; SUNYER et al., 2007). As proporções iniciais da plataforma foram projetadas para avaliar a memória e aprendizado de ratos e há tamanhos adaptados para camundongos[1]. O Barnes maze foi considerado apropriado para camundongos, visto que eles têm facilidade de encontrar e fugir através de pequenos buracos e compartilharem da preferência dos roedores por ambientes escuros, enquanto a mesma empregabilidade não ocorre no Morris water maze (POMPL et al., 1999).

Vários estudos têm utilizado o Barnes maze com sucesso, porém a desvantagem deste labirinto é o aprendizado lento ou nulo em alguns casos, que podem ser explicados pelo estímulo de escolha não ser suficiente para ativar a resposta de fuga do animal permitindo seu comportamento exploratório no labirinto evitando que o animal desempenhe a tarefa de entrar no buraco. Uma solução para este problema é calcular o tempo gasto para encontrar o buraco (latência primária), a distância que o animal percorreu para encontrar o buraco (comprimento do caminho primário) e calcular o número de erros até encontrar o buraco (erros primários) (HARRISON et al., 2006).

[1]As dimensões da plataforma e detalhes metodológicos empregados com camundongos encontram se na metodologia do artigo 2 deste trabalho.

Figura 5 – desenho esquemático do labirinto de Barnes.



Fonte: adaptado de PAUL; MAGDA; ABEL (2009).

2.3 BASES MOLECULARES DO PROCESSO DE FORMAÇÃO DA MEMÓRIA

2.3.1 Receptores Ionotrópicos de Glutamato

O glutamato é um aminoácido que no sistema nervoso central é o principal neurotransmissor excitatório e está relacionado com a plasticidade sináptica e as funções cognitivas como o aprendizado e a memória (BROSNAN; BROSNAN, 2013). O glutamato é capaz de ativar dois tipos de receptores, os ionotrópicos e os metabotrópicos. Os receptores ionotrópicos formam canais de cátions dependentes de ligantes, enquanto os metabotrópicos são acoplados à proteína G. Os receptores metabotrópicos participam de respostas intracelulares que envolvem segundos mensageiros, sendo uma forma de transmissão sináptica mais lenta (KUBO; ITO, 2004). Dentre os ionotrópicos existem três tipos diferentes: os receptores NMDA, AMPA e cainato. Esses nomes são originados das moléculas agonistas

desses receptores, ácido N-metil-D-aspártico, ácido 2-amino-3hidroxi-5-metil-4-isoxazolproprionato e ácido caínico, respectivamente (EACK, 2015).

Os receptores AMPA são tetrâmeros que podem ser montados por subunidades GluA1-4. A variabilidade das subunidades presentes nesses receptores implica em mudanças nas características fisiológicas e farmacológicas do receptor. Os receptores AMPA frequentemente coexistem com os receptores NMDA e medeia a transmissão sináptica excitatória rápida no SNC. Os receptores AMPA estão presentes nos neurônios e também nos astrócitos (RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016).

Os receptores NMDA são os mais estudados e podem ser formados por sete subunidades as quais são GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, GluN3B (HANSEN et al., 2014). O receptor NMDA é formado por quatro subunidades variáveis contendo, na maioria, obrigatoriamente a subunidade GluN1 mais a subunidade GluN2B ou GluN2A ou uma mistura dos dois (ZHANG et al., 2016). A distribuição das subunidades do receptor de NMDA no cérebro é heterogênea e varia durante o desenvolvimento. Acredita-se essas mudanças na expressão das subunidades está relacionada com a manutenção da plasticidade sináptica ao longo da vida (LAU et al., 2009). A ativação do receptor de NMDA medeia o aprendizado e a memória sob condições normais e no hipocampo a expressão das subunidades GluN2A e GluN2B são críticas para a indução da potenciação de longa duração (LTP-*Long term potention*) e a depressão de longa duração (LTD- *Long term depression*) (MULLER; ALBRECHT; GEBHARDT, 2009). Entretanto a ativação e o aumento da expressão desse receptor também o relacionam com condições patológicas tais como a Doença de Alzheimer, esquizofrenia, doença bipolar e depressão (ZHANG et al., 2016).

2.3.2 A Transmissão e a Plasticidade Sináptica

As memórias são armazenadas em várias regiões do encéfalo e para alguns tipos de memória algumas áreas são mais relevantes do que outras (KANDEL, ERIC; SCHWARTZ, THOMAS; SIEGELBAUM, STEVEN; HUDSPETH, 2014). Acredita-se que o hipocampo é a estrutura cerebral crucial responsável pela aquisição de diferentes tipos de memória. Estudos com roedores indicam que o hipocampo é responsável pela memória espacial e em humanos acredita-se que o giro para-hipocampal direito é a região mais relacionada com o processamento espacial (LISMAN, 1989). As diferentes áreas cerebrais relacionadas com a memória estão conectadas por circuitos de memória e a capacidade de um neurônio excitar

outro reforça tais circuitos promovendo o armazenamento da memória. Dessa forma, o armazenamento das memórias depende da comunicação entre os neurônios, a qual é estabelecida ou reforçada por modificações físicas entre esses neurônios formando circuitos neurais (GAZZANIGA, 2018). As regiões de comunicação entre os neurônios são chamadas de sinapse e a comunicação em si é a transmissão sináptica, a qual pode ser elétrica ou química. Já as modificações físicas que ocorrem nos circuitos neuronais são denominadas plasticidade sináptica (LOPES et al., 1998).

As sinapses elétricas são as mais rápidas, pois ocorrem pela passagem de uma corrente elétrica de uma célula a outra e estão envolvidas em respostas fisiológicas imediatas como por exemplo as respostas de fuga. Por outro lado, as sinapses químicas apresentam sinalização variável, são capazes de mediar ações excitatórias e inibitórias nas células pós-sinápticas com duração de milissegundos a muitos minutos. Além disso, as sinapses químicas correspondem à maior parte das sinapses que ocorrem no encéfalo e estão envolvidas na produção de comportamentos mais complexos (KANDEL, ERIC; SCHWARTZ, THOMAS; SIEGELBAUM, STEVEN; HUDSPETH, 2014).

Na transmissão sináptica química, um potencial elétrico no terminal axonal da célula pré-sináptica promove a abertura de canais de Ca^{2+} e consequente influxo de Ca^{2+} na célula. Então, vesículas sinápticas contendo um neurotransmissor se fundem na membrana da célula pré-sináptica e o neurotransmissor é liberado na fenda sináptica (espaço compreendido entre o neurônio pré-sináptico e a célula pós-sináptica). Na fenda sináptica, o neurotransmissor pode ser recaptado (pelo neurônio pré-sináptico ou células gliais) ou se ligar ao seu receptor presente na membrana da célula pós-sináptica para desencadear seus efeitos dentro da célula. Após a ligação neurotransmissor-receptor, os canais iônicos da célula pós-sináptica se abrem ou fecham causando uma mudança na condutância do potencial elétrico da célula pós-sináptica (KANDEL et al., 2014). Nas sinapses excitatórias, o neurotransmissor promove uma mudança localizada na membrana da célula pós-sináptica que a leva a se despolarizar, promovendo a geração de um potencial elétrico. Nas sinapses inibitórias, a ligação do neurotransmissor ao receptor causa uma mudança na permeabilidade de íons, que reduzir a chance de disparar um potencial de ação na célula pós-sináptica por hiperpolarização de suas membranas (LOPES et al., 1998).

A plasticidade sináptica ocorre de modo independente nas sinapses, apresenta padrão dinâmico e varia ao longo do tempo em resposta a estímulos ambientais e as experiências

(LOPES et al., 1998). Existem vários tipos de plasticidade sináptica que ocorrem no sistema nervoso (KANDEL et al., 2014) as quais podem ser classificadas como de curta duração ou longa duração (RUGGIERO et al., 2011). A primeira ocorre quando um padrão de atividade neural gera uma alteração na transmissão sináptica por curto período de tempo (dezenas ou centenas de milissegundos) sendo possível observar inibição ou facilitação da resposta do neurônio pós-sináptico. Tanto a inibição quanto a ativação da resposta neuronal estão relacionadas com as respostas adaptativas rápidas a estímulos sensoriais, mudanças transitórias em estados comportamentais e formas curtas de memória (ZUCKER; REGEHR, 2002). Por outro lado, a plasticidade sináptica de longa duração gera alterações que duram horas ou até dias e pode ser estudada através da LTP e LTD (BLISS; LØMO, 1973). Acredita-se que os mecanismos neurais do hipocampo envolvem as mudanças de longa duração nas conexões sinápticas para aquisição da memória (KANDEL et al., 2014).

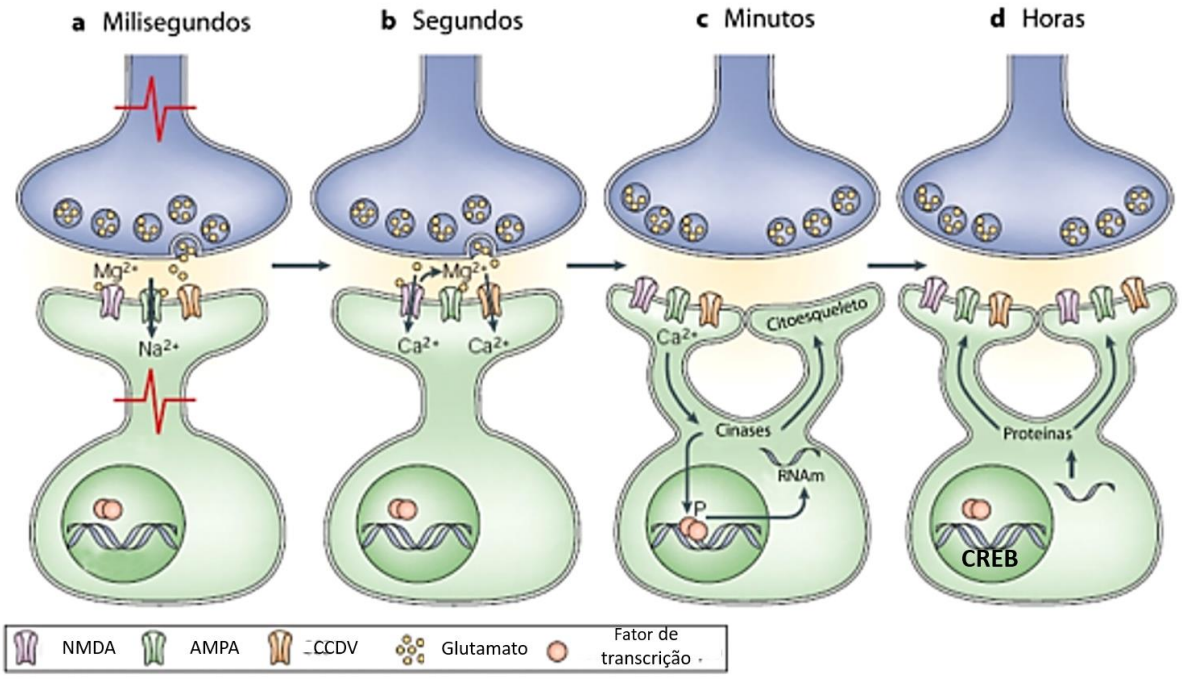
A LTP é uma medida eletrofisiológica estudada experimentalmente através de estímulos elétricos (BLISS; LØMO, 1973). A LTP ocorre após um curto disparo estimulador pré-sináptico de alta frequência que causa uma série de reações bioquímicas nos terminais pré e/ou pós-sinápticos, promovendo assim o aumento na eficácia ou reforço da comunicação sináptica. Enquanto a LTP aumenta a eficácia da sinapse, a LTD reduz o reforço sináptico (BLISS; COLLINGRIDGE, 1993; RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016) e ocorre após uma salva mais prolongada de estímulos de frequência menor (BLISS; COOKE, 2011; MASSEY; BASHIR, 2007). Tanto a LTP quanto a LTD são importantes para a formação de novas memórias, pois a LTP forma ou reforça conexões para a aquisição da memória e a LTD reduz outras conexões evitando assim a saturação das conexões sinápticas (KANDEL et al., 2014).

A LTP do hipocampo que ocorre na região CA1 é a forma de plasticidade mais estudada e é dependente de receptores glutamatérgicos do subtipo NMDA. Para ocorrer a ativação dos receptores NMDA as células pré e pós-sináptica devem receber um estímulo de alta frequência. Primeiramente, a célula pré-sináptica libera glutamato na fenda sináptica o qual se liga ao receptor de glutamato do subtipo AMPA, enquanto os receptores NMDA permanecem bloqueados pelo íon Mg^{2+}. Despolarizações pós-sináptica sustentadas promovidas pelo glutamato nos receptores AMPA removem o bloqueio do Mg^{2+} nos receptores NMDA (RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016). A abertura do canal do receptor NMDA ocorre apenas se o glutamato estiver ligado ao sítio ativo do NMDA e a célula pós-sináptica estiver suficientemente despolarizada para remover o bloqueio do Mg^{2+}. Quando

esses eventos ocorrem simultaneamente, os canais dos receptores NMDA se abrem e há influxo de Ca^{2+}na célula pós-sináptica e ativação de cascatas de sinalização dependentes de Ca^{2+} (KANDEL et al., 2014). Além da entrada de Ca^{2+} na célula, a concentração deste íon aumenta ainda mais pela abertura dos canais de Ca^{2+} dependentes de voltagem e pela liberação de Ca^{2+} dos estoques intracelulares promovida pelo influxo de Ca^{2+} (MALENKA, 1994). Na fase inicial da LTP, primeira hora, de acordo com CITRI E MALENKA (2008), há o envolvimento das cascatas moleculares dependentes de Ca^{2+} tais como fosfatases, quinases e sintase de óxido nítrico que juntamente com outras proteínas promovem a LTP (RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016).

Na fase tardia da LTP (até 24 horas) há mudanças na expressão gênica e esse é um processo crítico para o armazenamento de longa duração da memória (figura 6). É nesta etapa que ocorre a sinalização da via AMPc/PKA (KANDEL et al., 2014). A adenilato ciclase produz AMPc e o nível aumentado de AMPc intracelular ativa a proteína quinase A (PKA) que então recruta a proteína ligadora do elemento de resposta do AMPc (CREB, de cAMP response elemento binding protein) (PIERCE; PREMONT; LEFKOWITZ, 2002). Este fator além de atuar na LTP, é o regulador majoritário de muitas funções nas células neuronais adultas, como a sobrevivência neuronal (ALVAREZ-NÖLTING et al., 2012). A CREB é ativada por fosforilação na Ser133 e sua atividade está implicada na transcrição de genes (GONZALEZ; MONTMINY, 1989; NAQVI; MARTIN; ARTHUR, 2014) como o fator neurotrófico derivado do cérebro (BDNF), somatostatina e fator de crescimento do nervo (NGF) (MAYR; MONTMINY, 2001).

Figura 6 – Etapas da potenciação de longo tempo (LTP). (a) um estímulo curto de alta frequência no neurônio pré-sináptico promove a liberação do glutamato das vesículas sinápticas na fenda sináptica. O glutamato se liga aos receptores AMPA e NMDA presentes na membrana do neurônio pós-sináptico. O receptor NMDA permanece fechado pelo íon magnésio (Mg^{2+}). Por outro lado, a ligação do glutamato ao receptor AMPA abre esse canal iônico promovendo a entrada de sódio no neurônio pós-sináptico e então ocorre a despolarização do neurônio pós-sináptico. (b) a despolarização do neurônio pós-sináptico promove a abertura dos canais de cálcio dependentes de voltagem (CCDV), o bloqueio de Mg^{2+} no receptor NMDA é removido e então há influxo de cálcio (Ca^{2+}) no neurônio pós-sináptico pelos receptores NMDA e pelos CCDV. (c) o aumento da concentração de Ca^{2+} intracelular ativa proteínas quinases que promovem o remodelamento sináptico e a transcrição gênica. (d) horas após o estímulo curto de alta frequência é observado o remodelamento do dendrito do neurônio pós-sináptico, ativação do fator de transcrição CREB, e consequentemente ocorrem mudanças na expressão das proteínas no dendrito do neurônio pós-sináptico. Além disso, há aumento da quantidade dos receptores de glutamato inseridos na membrana do neurônio pós-sináptico.



Fonte: adaptado de Ruggiero (2011).

A ativação da CREB por fosforilação inclui vários mecanismos como a via de sinalização da MAPK/ proteína quinase regulada por sinalização extracelular (ERK) (YI et al., 2014), a via de sinalização dos nucleotídeos cíclicos monofosfatados AMPc e GMPc (GARCÍA-OSTA et al., 2012) e pela ligação do BDNF ao seu respectivo receptor na membrana plasmática. Esta última via leva a fosforilação da CREB, a qual inicia a expressão de diferentes genes-alvo (CHEN et al., 2015). A ativação da CREB promove a acetilação das histonas o que permite a transcrição dos genes-alvo que facilitam a formação da memória (KANDEL et al., 2014). E ainda, a ativação da CREB induz a síntese de novas proteínas, as quais aumentam o número de sinapses e a eficácia sináptica (formação de espículas dendríticas) (TULLY et al., 2003).

2.3.3 Sinalização Intracelular na Formação da Memória

Várias proteínas quinases e suas cascatas de sinalização estão envolvidas no processo de formação da memória, frequentemente pela modulação da CREB. A liberação de cálcio intracelular de modo breve estimula a fase inicial da LTP e períodos prolongados é que são capazes de fosforilar e ativar CREB na LTP tardia (LU; HAWKINS, 2002). Apesar da PKA ser a quinase mais importante da regulação da atividade da CREB, as proteínas quinases agem em conjunto para ativar a transcrição gênica e a síntese proteica durante a consolidação da memória. As bases moleculares do aprendizado e memória envolve proteínas quinases que podem atuar por dois mecanismos na transmissão sináptica. O primeiro é pela alteração das propriedades dos canais iônicos ou densidades sinápticas ou podem modificar a estrutura sináptica. O segundo é pela formação de sinapses novas (sinaptogênese) por atuarem na síntese de proteínas (GIESE; MIZUNO, 2013).

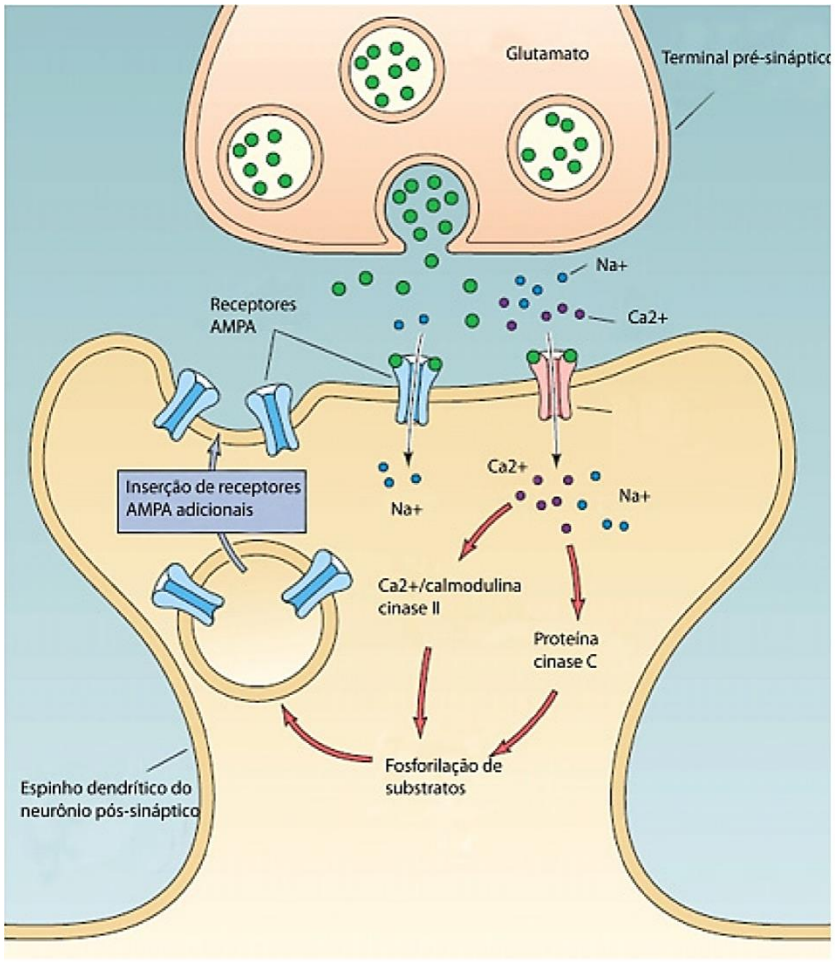
Na microscopia eletrônica observa-se próximo à membrana plasmática do neurônio pós-sináptico uma região eletrondensa que é chamada de densidade pós-sináptica. As moléculas que compõem essa estrutura podem ser alguns tipos de receptores, proteína quinase II dependente de Ca^{2+}/calmodulina (CaMKII) e a densidade pós-sináptica 95 (PSD95) entre outras. Essas proteínas podem estar acopladas à subunidade GluN2B (RAVEENDRAN et al., 2009). A PSD95 é uma importante proteína estrutural da membrana pós-sináptica que liga o receptor NMDA e as vias de sinalização intracelulares da plasticidade sináptica. Ela pode estar ligada a várias proteínas regulatórias como, por exemplo, a nNOS. Na densidade pós-sináptica, as subunidades GluN2A e GluN2B interagem diretamente com a PSD-95 e com outros membros da família das quinases de guanilato associadas à membrana. O receptor tirosina quinase B (TrkB-tyrosine receptor kinase B), um receptor de BDNF, regula a localização da PSD-95 pelas vias de sinalização da PI3K, fosfolipase Cγ e MAPK/ERK (YOSHII; CONSTANTINE-PATON, 2014).

2.3.3.1 Papel da proteína quinase II dependente de Ca^{2+}/calmodulina

A CaMKII desempenha um papel relevante no processo de formação da memória. Durante a formação da memória há aumento da atividade da CaMKII e o seu bloqueio farmacológico, por outro lado, prejudica a formação da memória (GIESE; MIZUNO, 2013). O aumento da concentração de Ca^{2+} intracelular provoca a ligação do Ca^{2+} à calmodulina e

então ativa enzimas que modificam a sinapse. O complexo cálcio/calmodulina tem como principal alvo a CAMKII (GIESE; MIZUNO, 2013). Ao ser ativada, a CaMKII migra para a densidade pós-sináptica (WANG; PENG, 2016) e fosforila subunidades GluR1 aumentando a resposta ao glutamato, além de aumentar a expressão e translocação do receptor para o terminal pós-sináptico (MALENKA, 1994; SACKTOR et al., 1993). A fosforilação do receptor NMDA aumenta a sua permeabilidade ao Ca^{2+} enquanto a desfosforilação reduz (SANDERSON; GORSKI; DELL'ACQUA, 2016; SOBCZYK, 2005). Ainda, a CAMKII fosforila outras moléculas sinalizadoras da transdução de sinal que controlam a ativação gênica na célula pós-sináptica na fase tardia da LTP (Figura 7) (RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016; ZHOU et al., 2006).

Figura 7 – liberação de glutamato na fenda sináptica e a inserção do receptor AMPA no terminal sináptico pelas enzimas CaKMII e PKC. O influxo de sódio pela ativação do receptor AMPA promove a despolarização no neurônio pós-sináptico. O glutamato ligado ao receptor NMDA e a despolarização do neurônio pós-sináptico promove ativa o receptor NMDA. Esta ativação promove o influxo de Ca^{2+} no neurônio pós-sináptico. O Ca^{2+} ativa proteínas quinases como a Ca^{2+}/calmodulina quinase II e a proteína quinase C. A ativação dessas proteínas promove a translocação dos receptores AMPA para o terminal pós-sináptico.

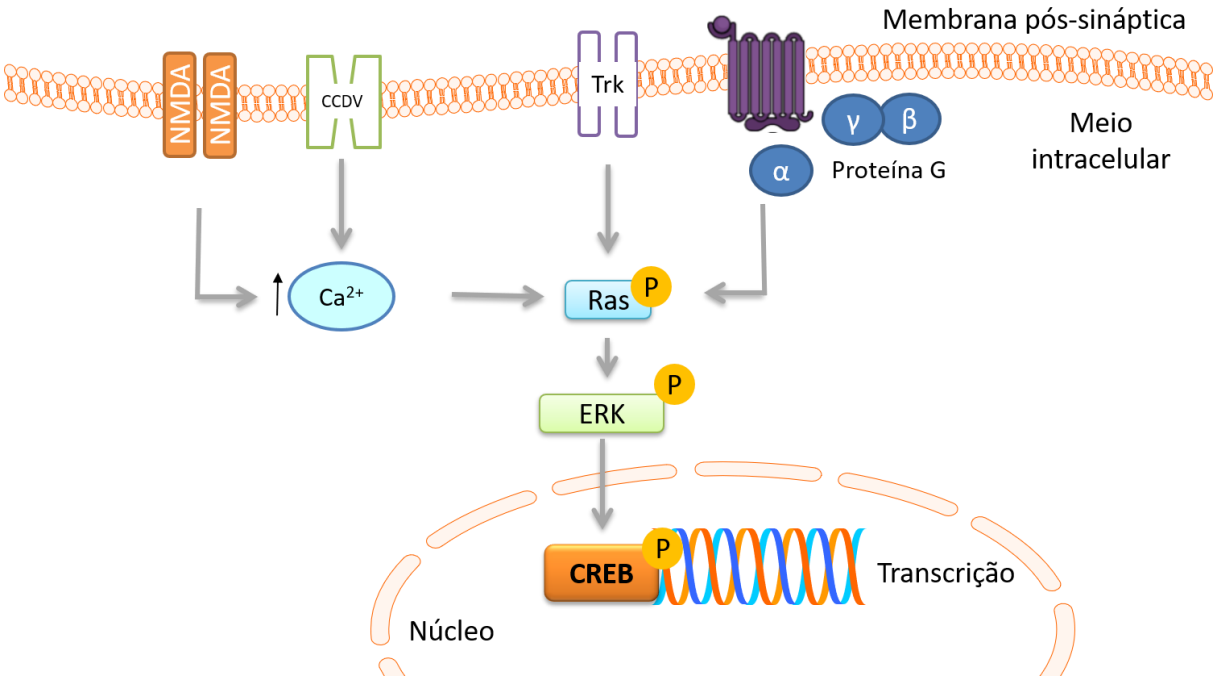


Fonte: Ruggiero (2011).

2.3.3.2 Importância da ativação da ERK1/2

A ERK exerce um relevante papel no processo de formação da memória. A ERK é uma proteína da família das proteínas quinases ativadas por mitógeno (*Mitogen Activated Protein Kinases*-MAPK) (KOGA et al., 2019). Outras proteínas da família MAPK, p38 e JNK (*c-Jun N-terminal kinase*-JNK) também estão envolvidas no processo de formação da memória, entretanto dentre as MAPK o papel da ERK1/2 é o mais caracterizado nesse processo (WANG; PENG, 2016). Durante o processo de formação da memória, a ERK 1/2 tem como principal função ativar a CREB, a qual promove a transcrição gênica na memória de longo prazo (GIESE; MIZUNO, 2013). Além de exercer um importante papel na transcrição de genes para indução da memória, a ativação da ERK1/2 também pode regular a síntese de proteínas dendríticas (KELLEHER et al., 2004). Após a ativação do receptor NMDA ou ativação dos canais de cálcio dependentes de voltagem, os níveis de Ca^{2+} aumentam no citoplasma ativando a ERK pela sinalização da Ras (ROSEN, 1994). Contudo, a sinalização Ras GTPases pode ser induzida por outros estímulos incluindo a ativação do receptor de tirosina quinase (receptor Trk) ou receptores acoplados à proteína G (CICCARELLI; GIUSTETTO, 2014). Além disso, a ativação simultânea do receptor NMDA e mGluR5 pode levar à fosforilação de ERK (YANG, 2004). A MAPK após ser ativada por fosforilação, pode fosforilar e assim ativar fatores de transcrição, levando à expressão de genes alvo na cascata de sinalização e síntese de novas proteínas (BARCO; ALARCON; KANDEL, 2002; PATTERSON et al., 2001). Veja na figura 8 as vias de ativação da ERK descritas acima e a ativação da CREB pela ERK.

Figura 8 – ativação da CREB pela ERK durante o processo de formação da memória. A ERK pode ser ativada através da sinalização do receptor NMDA, pelos canais de cálcio dependentes de voltagem (CCDV), pelo receptor de tirosina quinase (Trk) ou receptores acoplados à proteína G.



Fonte: A autora.

2.3.3.3 Contribuição da PKC

O bloqueio farmacológico de PKC na região CA1 do hipocampo prejudica a aquisição da memória, consolidação e reconsolidação da memória espacial bem como a indução da LTP (BONINI et al., 2007; MALINOW; SCHULMAN; TSIEN, 1989). A PKC pode ser ativada pelo Ca^{2+} dentre outras moléculas além de ativar de maneira recíproca a CaKMII (YAN et al., 2011). A PKC controla a adição de subunidades GluA1 no receptor AMPA na sinapse durante a LTP(JENKINS; TRAYNELIS, 2012). Por outro lado, a fosforilação da subunidade GluA2 pela PKC modifica sua ligação às proteínas estruturais o que parece ser essencial para a LTD (CAO et al., 2007). A PKC induz a ativação do receptor NMDA, atua no tráfego de proteínas e modula a atividade do receptor NMDA por fosforilar as subunidades GluN2A e GluN2B (GROSSHANS; BROWNING, 2001; YAN et al., 2011). A ativação da PKC ativa outras cascatas de sinalização, como a sinalização da PKA, e controla a ativação gênica ligada à CREB na célula pós-sináptica na fase tardia da LTP (ver figura 7) (RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016; ZHOU et al., 2006). Ainda, a PKC indiretamente ativa a CaKMII com a ativação do receptor NMDA e consequente aumento da concentração de Ca^{2+}

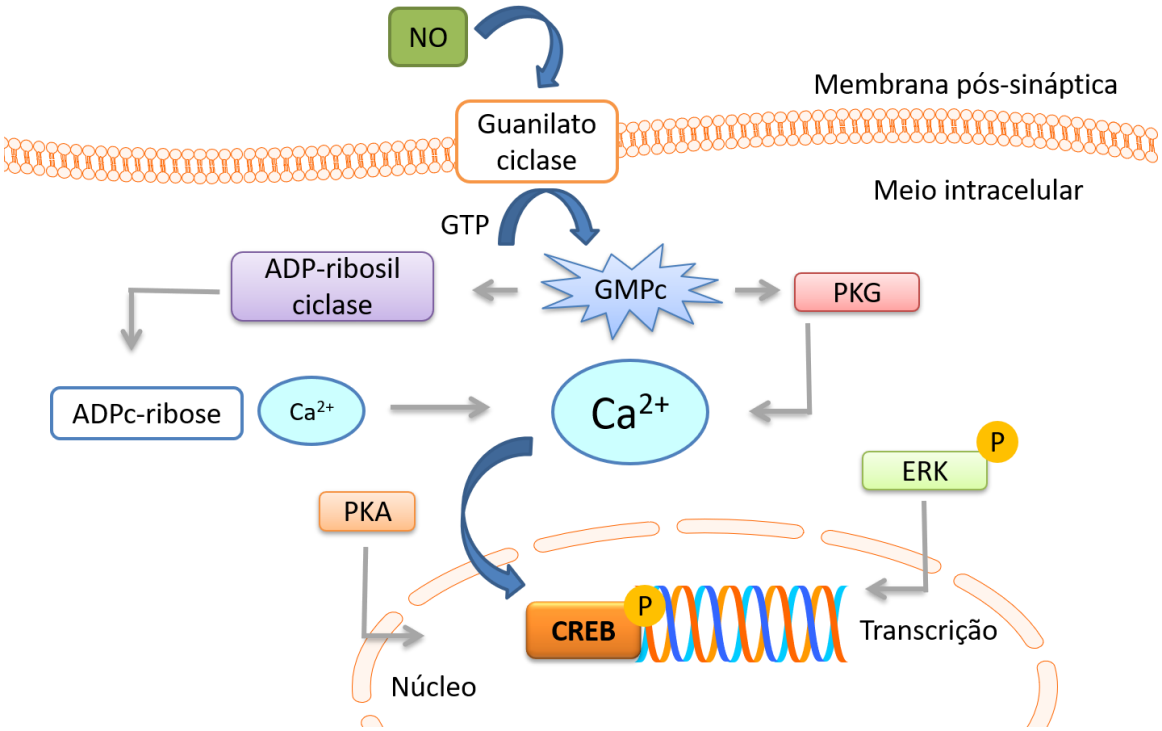
intracelular (YAN et al., 2011). Apesar da PKC ser ativada com a estimulação dos receptores AMPA e NMDA, o bloqueio desses receptores não impedem a ativação da CREB pela PKC, sugerindo que a sinalização PKC-CREB pode ocorrer independentemente da ativação desses receptores de glutamato (MAO, 2007).

2.3.3.4 Via de sinalização NO/GCs/GMPc/PKG

O GMPc é um segundo mensageiro mediador das ações do óxido nítrico e também modula algumas formas aprendizado e memória, incluindo o aprendizado espacial no Morris Water Maze, reconhecimento de objetos e discriminação condicional no teste Y-maze (MONTOLIU et al., 2010). Camundongos Knockout para a isoforma I da PKG apresentam prejuízos especificamente no processo de formação de medo de longo prazo (PAUL et al., 2008) e inibidores farmacológicos da PKG na amígdala exercem efeitos similares (VALTCHEVA et al., 2009). Em modelo de Alzheimer, a administração de sildenafil, um inibidor de fosfodiesterase-5 e ativador da via de sinalização NO/GMPc, reduz a hiperfosforilação da proteína Tau em áreas do cérebro envolvidas com o aprendizado e a memória e restaura a função da memória (CUADRADO-TEJEDOR et al., 2011). Além disso, a inibição da PKG prejudica a ativação da ERK e expressão de genes dirigidos à ERK na amigdala lateral e tálamo auditivo (OTA et al., 2010).

Os efeitos do GMPc e da proteína quinase dependente de GMPc (PKG) na plasticidade sináptica parecem estar indiretamente relacionados ao aumento dos níveis de cálcio intracelular (GARCÍA-OSTA et al., 2012). A PKG é ativada pelos níveis aumentados de GMPc produzido pela enzima guanilato ciclase solúvel após estimulação pelo óxido nítrico. Dessa forma, o aumento dos níveis de óxido nítrico estimula a atividade da PKG (GIESE; MIZUNO, 2013). O GMPc ativa PKG e ADP-ribosil ciclase, estimulando a produção de ADPc-ribose o qual age sinergicamente com o cálcio citoplasmático para promover o carregamento de cálcio de várias fontes (GARCÍA-OSTA et al., 2012). Quando a sinalização do cálcio está suficientemente grande, a CREB é fosforilada e inicia a LTP em paralelo com a sinalização da PKA e MAPK para ativar a CREB (PUZZO et al., 2009). Um resumo esquemático da via de sinalização do NO/GC/GMPc/PKG no neurônio pós-sináptico está representado na figura 9.

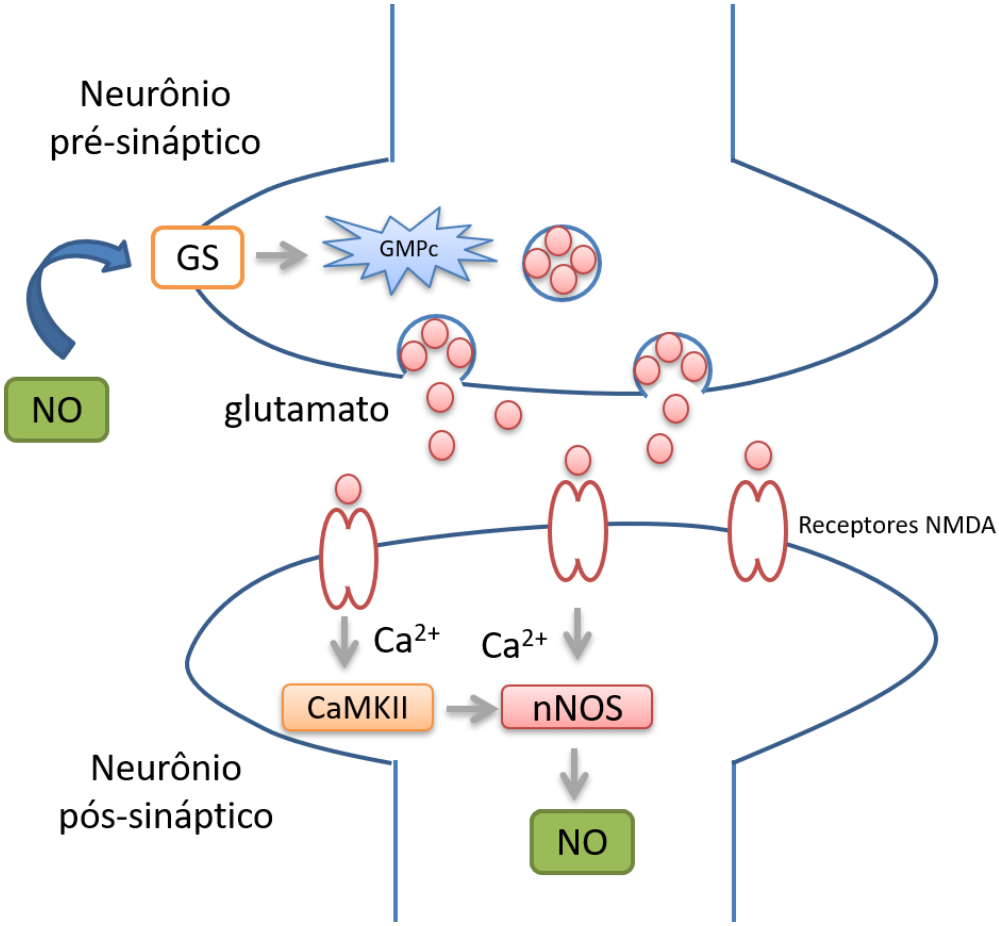
Figura 9 – ativação da CREB via óxido nítrico/guanilato ciclase/GMPc/Proteína quinase G no neurônio pós-sináptico.



Fonte: A autora.

A enzima sintase de óxido nítrico está acoplada próxima à ativação do receptor NMDA (GIESE; MIZUNO, 2013) e a estimulação desse receptor aumenta sua atividade e consequentemente aumenta os níveis de óxido nítrico no neurônio pós-sináptico. O NO desempenha um papel de sinalização retrógrada na formação da memória e está envolvido na fase inicial da LTP, em parte por ativar a guanilato ciclase e PKG. O aumento de Ca^{2+} intracelular no neurônio pós-sináptico, promovido pelo desbloqueio do receptor NMDA, promove a formação do complexo CaMKII que ativa a sintase óxido nítrico neuronal (nNOS) (SAGER, 2004). O óxido nítrico por ser um gás solúvel e hidrofóbico ultrapassa a membrana plasmática do neurônio pós-sináptico e pode ativar a produção de GMPc pela guanilato ciclase retrogradamente no citoplasma do neurônio pré-sináptico (HAWKINS; SON; ARANCIO, 1998). Além disso, no hipocampo o NO é capaz de estimular a liberação de glutamato (LONART; WANG; JOHNSON, 1992; SEGIETH et al., 1995), enquanto o bloqueio do receptor NMDA e NOS impedem a liberação de glutamato (NEI et al., 1996; SEGIETH et al., 1995). Em camundongos knockout para nNOS, a estimulação do receptor NMDA com o seu agonista apresentou menor liberação de glutamato (KANO et al., 1998). Dessa forma, a ativação do receptor NMDA ativa a nNOS, que então aumenta os níveis de NO e este por sua vez, promove a liberação de mais glutamato na fenda sináptica (figura 10).

Figura 10 – ativação do receptor NMDA e a sinalização retrógrada de óxido nítrico. A liberação de glutamato ativa os receptores NMDA. A abertura do canal do receptor NMDA permite a entrada de cálcio no meio intracelular. O Cálcio promove a formação do complexo CaMKII e ativação da nNOS. O complexo CaMKII também ativa a nNOS e há produção de óxido nítrico. Esse gás solúvel no neurônio pós-sináptico sinaliza retrogradamente no neurônio pré-sináptico, onde ativa a via de sinalização guanilato ciclase/GMPc. Em seguida, ocorre o aumento da liberação de glutamato na fenda sináptica.



Fonte: A autora.

2.3.3.5 Papel da via AMPc/PKA

Existem muitas vias de sinalização da CREB, contudo a sinalização da PKA é considerada a mais importante. A PKA é uma holoenzima tetramérica com dois sítios catalíticos e duas subunidades regulatórias. No estado basal a subunidade regulatória inibe a subunidade catalítica, formando o complexo tetramérico R_2C_2. O AMPc se liga a subunidade regulatória (R) e promove mudanças na conformação da enzima que se dissocia da holoenzima. Sabe-se que a PKA está envolvida no processo de formação da memória de curto e longo prazo e os estudos que avaliam a sinalização da PKA na formação da memória foram realizados com invertebrados (ABEL et al., 1997).

Nos mamíferos há quatro genes que codificam a subunidade regulatória e três genes que codificam a subunidade catalítica. É particularmente difícil avaliar os efeitos da PKA usando camundongos *knockout* para alguma das isoformas, pois os fenótipos são de difícil interpretação devido à compensação por outras isoformas (BRANDON; IDZERDA; MCKNIGHT, 1997). Além disso, avanços genéticos também possuem limitações como a falta de uma especificidade da região e/ou de tempo, além da causar a morte dos camundongos neonatos (NGUYEN; WOO, 2003).

Apesar disso, alguns estudos com manipulação genética da via de sinalização AMPc/PKA ajudaram na elucidação do papel dessa via no processo de formação da LTP tardia. A deleção da subunidade Riβ da PKA não altera a morfologia do cérebro ou a indução da LTP no CA1 (BRANDON et al., 1995). Em contraste, a deleção genética da subunidade Cβ1 resultou em uma deficiência seletiva da LTP tardia produzida por múltiplos trens de estimulação (QI et al., 1996). Então, parece haver um requerimento seletivo para expressão de certas subunidades durante a LTP tardia. Contudo, a atividade da PKA no cérebro inteiro não muda nos camundongos mutantes de PKA (BRANDON et al., 1995; QI et al., 1996).

Outras evidencias que suportam o papel da PKA na LTP são derivadas de estudos genéticos que manipularam a produção de AMPc pela adenilato ciclase (AC). Existem 10 isoformas diferentes de AC e cinco delas são sensíveis aos níveis de Ca^{2+}(WANG; ZHANG, 2012). Das muitas isoformas presentes no hipocampo, duas (AC1 e AC8) são dependentes de calmodulina (CaM) (WONG et al., 1999). Camundongos mutantes com deleção alvo de genes codificantes de AC1 mostraram LTP inicial normal na área do CA1, sugerindo que a AC1 estimulada pela CaM não é requerida para a LTP tardia (WU et al., 1995). No entanto, a deleção genética da AC1 e AC8 prejudicou a LTP tardia, enquanto o camundongo que tinha a deleção de apenas uma das AC (AC1 ou AC8) exibiu LTP tardia normal. Esses resultados sugerem que a expressão ou ativação de AC1 e AC8 parece ser um passo importante para a produção da sinalização de AMPc necessária para produzir a LTP tardia dependente de PKA (NGUYEN; WOO, 2003; WONG et al., 1999; WU et al., 1995).

A sinalização da cascata AMPc/PKA no hipocampo é principalmente ativada por dois mecanismos. O primeiro envolve cálcio e calmodulina. O influxo de cálcio induz a estimulação da adenilato ciclase sensível a CaKM (ELIOT et al., 1989). A ativação dos receptores NMDA na via sináptica, pode aumentar os níveis de AMPc na área CA1 do hipocampo (CHETKOVICH et al., 1991). Além da calmodulina, o aumento de Ca^{2+} induz a

ativação da guanilato ciclase e inibidores da calmodulina bloqueiam a produção de AMPc associado com a ativação do receptor NMDA (CHETKOVICH; SWEATT, 1993). Assim, a ativação do receptor NMDA pode levar a ativação de PKA pelo aumento dos níveis de cálcio intracelular (ROBERSON; SWEATT, 1996). O segundo mecanismo de ativação da sinalização AMPc/PKA envolve a ligação de transmissores químicos e hormônios aos seus respectivos receptores, seguido pela estimulação da adenilato ciclase pela proteína regulatória ligada a nucleotídeo (proteína G) (TANG; GILMAN, 1991). Essas proteínas G interagem com a adenilato ciclase no interior da superfície da membrana para ativar (ou em alguns casos, inibir) a produção de AMPc (NGUYEN; WOO, 2003).

Numerosas moléculas estruturais têm sido identificadas na PSD. Muitas dessas moléculas, são conhecidas por servir como proteínas de anexação, proteínas adaptadoras, proteínas quinases para diferentes alvos, incluindo PKA (PAWSON, 1997). A localização espacial de uma parte da PKA (SCOTT et al., 1990) é direcionada para a membrana por proteínas de anexação da proteína quinase A (AKAP- *A kinase anchoring proteins*). Acredita-se que a maioria da PKA celular é associada a alguns membros da família AKAP (RUBIN, 1994). Esta interação permite a exposição da PKA a gradientes de AMPc em um espaço confinado e permite a regulação seletiva de diferentes proteínas efetoras a altos níveis de especificidade enzimática e eficiência (NGUYEN; WOO, 2003).

Na densidade pós-sináptica, a AKAP medeia a ligação entre a PKA e receptores de glutamato. Uma AKAP direciona a PKA para o receptor glutamatérgico do tipo NMDA ligando a PKA e a proteína PSD-95 que fica ancorada à extremidade citoplasmática do receptor NMDA (KANDEL, ERIC; SCHWARTZ, THOMAS; SIEGELBAUM, STEVEN; HUDSPETH, 2014). Além de ligar a PKA ao receptor NMDA, as AKAPs também ligam proteínas fosfatases que tem ação oposta da quinase (COGHLAN et al., 1995; KLAUCK et al., 1996; WESTPHAL, 2000).

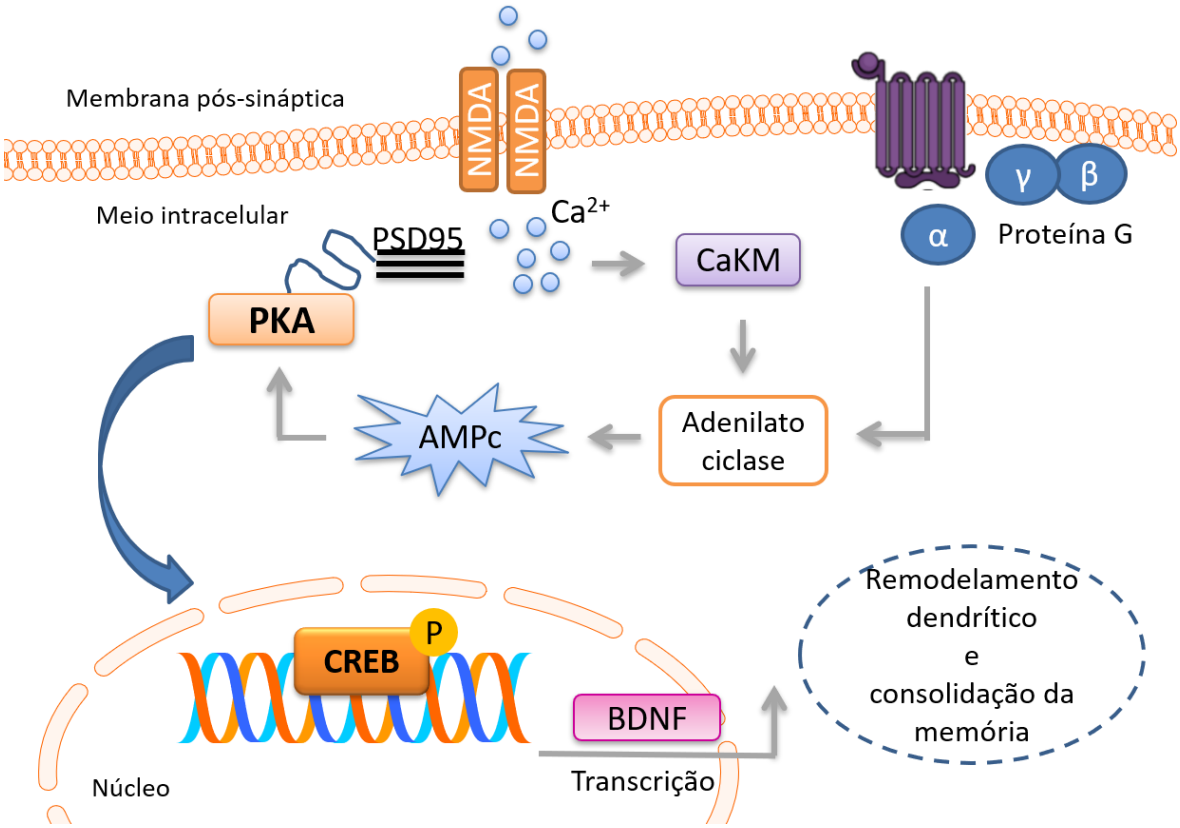
A LTP induzida pela estimulação de alta frequência induz um importante aumento nos níveis de AMPc (CHETKOVICH et al., 1991). Contudo, a LTP está associada com a ativação passageira de PKA que ocorre logo depois da indução, mas a atividade da PKA lentamente decai com o curso do tempo assim como o decaimento dos níveis de AMPc (ROBERSON; SWEATT, 1996). Portanto, a atividade persistente da PKA sozinha não causa diretamente a longevidade de algumas formas de LTP, tais como a fase tardia da LTP (L-LTP) (NGUYEN; WOO, 2003). A PKA interage com muitas outras vias de sinalização importantes como a

MAPK e proteínas fosfatases (NGUYEN; WOO, 2003). Após a estimulação que leva ao aumento do AMPc citosólico, subunidades de PKA podem translocar para dentro do núcleo e então fosforilar a CREB na serina 133 (BACSKAI et al., 1993) iniciando a transcrição de genes. De fato, a indução da LTP induzida por AMPc na região CA1 envolve a ativação rápida de receptores TrkB para o BDNF (PATTERSON et al., 2001). Assim, o AMPc pode provocar a liberação de BDNF, que por sua vez, contribui para o estabelecimento da LTP tardia (NGUYEN; WOO, 2003).

A sinalização AMPc/PKA é importante para alguns remodelamentos dendríticos. Em culturas de neurônios do hipocampo, a aplicação de estradiol aumentou a densidade do espinho dendrítico e aumentou a fosforilação da CREB (MURPHY; SEGAL, 1997). Entretanto, o aumento da densidade do espinho dendrítico foi bloqueado pelo antagonista de PKA e por um oligonucleotídeo antisense específico para CREB (MURPHY; SEGAL, 1997). A ativação da PKA também aumenta o número de sítios funcionais de liberação pré-sináptica em neurônios do hipocampo (NGUYEN; WOO, 2003).

Em resumo, a estimulação da atividade da enzima AC, seja pelo aumento de cálcio intracelular ou ativação de receptores ligados à proteína G, pode aumentar os níveis intracelulares de AMPc ativando a PKA. A PKA, por sua vez, é translocada para o núcleo onde fosforila a CREB e então ocorre a ativação da transcrição de genes para modificação dos espinhos dendríticos e dessa forma, possivelmente, acontece a consolidação da memória de longo prazo (figura 11).

Figura 11 – receptor NMDA e a consolidação da memória. A ativação do receptor NMDA aumenta a concentração de cálcio intracelular. O aumento do cálcio intracelular ativa a proteína cálcio calmodulina que ativa a adenilato ciclase sensível à CaKM. Além da CaKM, receptores acoplados à proteína G também podem ativar a adenilato ciclase. Então, a adenilato ciclase aumenta os níveis de AMPc causando assim a ativação da PKA. A PKA ao ser ativada pelo AMPc ativa a CREB que promove a transcrição de genes que codificam fatores neurotróficos como o BDNF. O aumento da expressão do BDNF tem sido relacionado com o remodelamento dendrítico e a consolidação da memória.



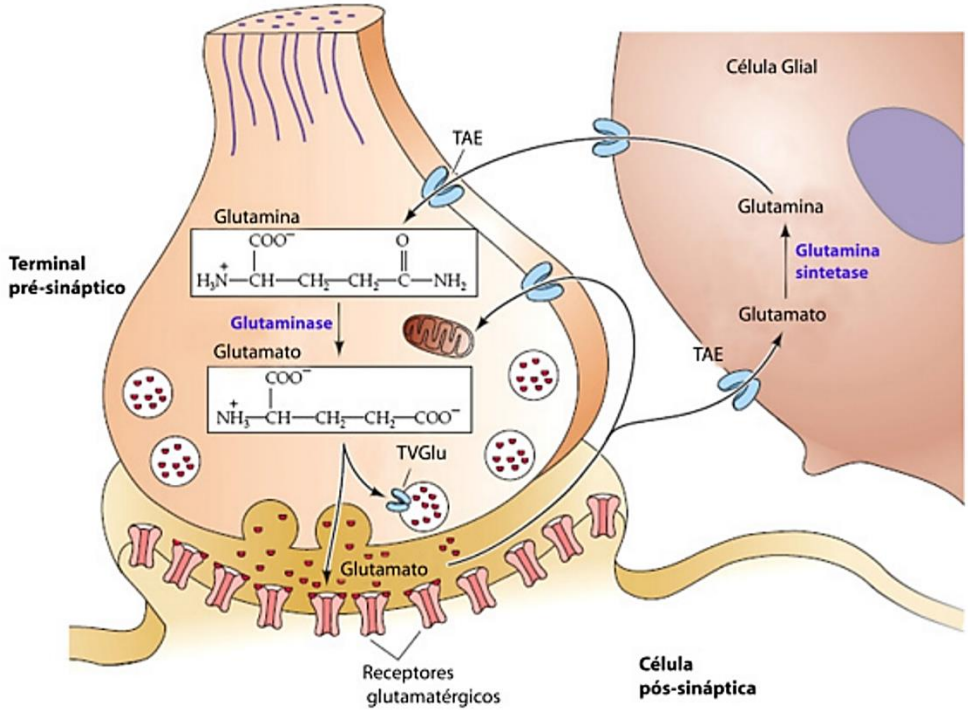
Fonte: A autora.

2.3.4 Excitotoxicidade Glutamatérgica e a diabetes

Estudos *in vitro* demonstraram que exposições, mesmo curtas, a grandes quantidades de glutamato levam à morte neuronal, processo chamado de excitotoxicidade (MELLO; QUINCOZES-SANTOS; FUNCHAL, 2012). A concentração de glutamato intracelular é maior do que a disponível na fenda sináptica, entretanto o aumento da concentração de Ca^{2+} intracelular promove a liberação de mais glutamato na fenda sináptica, levando a uma retroalimentação positiva da excitabilidade celular (AMARA; FONTANA, 2002; EMERIT; EDEAS; BRICAIRE, 2004; KANNER, 1993; MARAGAKIS; ROTHSTEIN, 2004; MILLAN; CHAPMAN; MELDRUM, 1993). A regulação dos níveis de glutamato na fenda sináptica tem grande importância para evitar a morte neuronal. O glutamato do SNC é

originado principalmente do ciclo de Krebs ou da glutamina. A glutamina é sintetizada pelos astrócitos e captada pelos neurônios através do transportador da glutamina presente na membrana dos neurônios. A glutamina nos neurônios é convertida em glutamato pela enzima glutaminase. Uma parte do glutamato produzido pelo neurônio é captada por outros neurônios e outra parte é captada pelos astrócitos pelo transportador de aminoácido excitatório. Os astrócitos por sua vez reciclam o glutamato através da conversão do glutamato em glutamina pela ação da enzima glutamina sintase. A glutamina então é transportada novamente para os neurônios para síntese de glutamato (RANG, H; RITTER, J; FLOWER, R; HENDERSON, 2016) (figura 12).

Figura 12 – Liberação de glutamato na fenda sináptica e a recaptação de glutamato pelos astrócitos bem como a conversão de glutamato em glutamina e vice-versa.



Fonte: Ruggiero (2011).

A estimulação excessiva do receptor NMDA pelo glutamato pode exercer efeitos prejudiciais na sinapse causando alterações na memória e aprendizado, e ainda neurodegeneração (PALOP; CHIN; MUCKE, 2006). Em condições normais de ativação do NMDA, a concentração de Ca^{2+} citoplasmático é controlada pela bomba de efluxo de Ca^{2+} na membrana mitocondrial e no retículo endoplasmático. A sobrecarga de Ca^{2+} atrapalha a produção de ATP, uma função da mitocôndria. Assim, a retirada de Ca^{2+}do meio intracelular pela bomba de cálcio, um processo que requer ATP, é prejudicada. Da mesma forma, o

desbalanço energético mitocondrial que ocorre durante a diabetes, por exemplo, também reduz a capacidade da bomba de Ca^{2+} em retirar o Ca^{2+} do meio intracelular pelo déficit energético (ZHANG et al., 2016). A estimulação excessiva dos receptores de glutamato aumenta a concentração de Ca^{2+} intracelular no terminal pós-sináptico que juntamente com o prejuízo dos mecanismos intracelulares de retirada de Ca^{2+} promove a concentração de Ca^{2+} dentro da célula além do limiar de ativação dos mecanismos regulatórios ativando os mecanismos intracelulares de excitotoxicidade que conduzem à morte neuronal (SATTLER; TYMIANSKI, 2000).

O principal mecanismo de redução dos níveis de glutamato na fenda sináptica é através da recaptação do glutamato pelos astrócitos (MOUSSA et al., 2007), entretanto algumas condições patológicas podem provocar disfunção dos astrócitos e levar ao aumento da disfunção e até morte neuronal (BÉLANGER; ALLAMAN; MAGISTRETTI, 2011; NEDERGAARD; DIRNAGL, 2005). Tais alterações podem ser avaliadas pela expressão de S100B e da a proteína ácida fibrilar glial (GFAP-*glial fibrilar acid protein*). O S100B é um peptídeo de ligação ao cálcio, é encontrado principalmente em astrócitos e pode ser usado como marcador da ativação e/ou morte dos astrócitos em distúrbios do sistema nervoso central como a AD (YARDAN et al., 2011). A GFAP é um marcador de filamentos intermediários nos astrócitos. Durante doenças neurodegenerativas, inflamação e após traumas, pode ocorrer a reatividade astrocitária que é a alteração morfológica e funcional dos astrócitos. Uma vez que a GFAP tem expressão aumentada durante a gliose reativa, a GFAP é comumente utilizada como marcador da alteração morfológica e funcional dos astrócitos (HOL; PEKNY, 2015).

A hiperglicemia prejudica o tráfego de metabólitos (GISPEN; BIESSELS, 2000) e a sinalização de moléculas entre os astrócitos, neurônios e células endoteliais contribuindo com alterações das funções cerebrais (GANDHI et al., 2010). Estudos *in vitro* sugerem que os níveis elevados e crônicos de glicose afetam a atividade astrocítica, pois há redução da proteína S100B e de glutationa, sugerindo redução da capacidade dos astrócitos em recaptar o glutamato, uma vez que a glutationa é a principal enzima de defesa antioxidante dos astrócitos e um dos destinos bioquímicos do glutamato (DRÖGE, 2002; MILLAN; CHAPMAN; MELDRUM, 1993; NARDIN et al., 2007; PUSKAS et al., 2007).

No hipocampo de animais com diabetes tipo 1 a atividade da glutamina sintetase encontra-se reduzida, enquanto ocorre aumento dos níveis de glutamato, espécies reativas de

oxigênio (ROS- *reactive oxygen species*) e espécies reativas de nitrogênio (RNS- *reactive oxygen species*). O aumento da produção de ROS/RNS pode ser a causa da baixa atividade da glutamina sintetase. Ao mesmo tempo, o acúmulo do glutamato pode ser associado como uma consequência da menor atividade da glutamina sintetase. No mesmo estudo, os resultados *in vitro* indicaram que há aumento da expressão da GFAP quando as células foram tratadas com um nível elevado de glicose. Entretanto, quando as células foram cultivadas com variação da concentração de glicose presente no meio de cultura, houve redução da expressão de GFAP (SON et al., 2015).

Em pacientes diabéticos, a variação dos níveis de glicose, principalmente a hipoglicemia, contribuem também para o quadro de danos cognitivos (GISPEN; BIESSELS, 2000). Em contrapartida, na diabetes tipo 1 e 2 experimental a ativação de astrócitos e micróglia, avaliadas respectivamente pela expressão de GFAP e Iba-1, apontam que no início da diabetes não há ativação dessas células gliais, embora há outras alterações que modificam a função neuronal (NAGAYACH; PATRO; PATRO, 2014; VAN DER HARG et al., 2015). Assim, a gliose reativa pode estar associada aos estágios mais avançados de danos cerebrais induzidos pela diabetes. E ainda, os astrócitos são mais resilientes do que os neurônios (BÉLANGER; ALLAMAN; MAGISTRETTI, 2011; NEDERGAARD; DIRNAGL, 2005), o que explica alguns achados em que a diabetes atrapalha a função neuronal, mas não há gliose reativa.

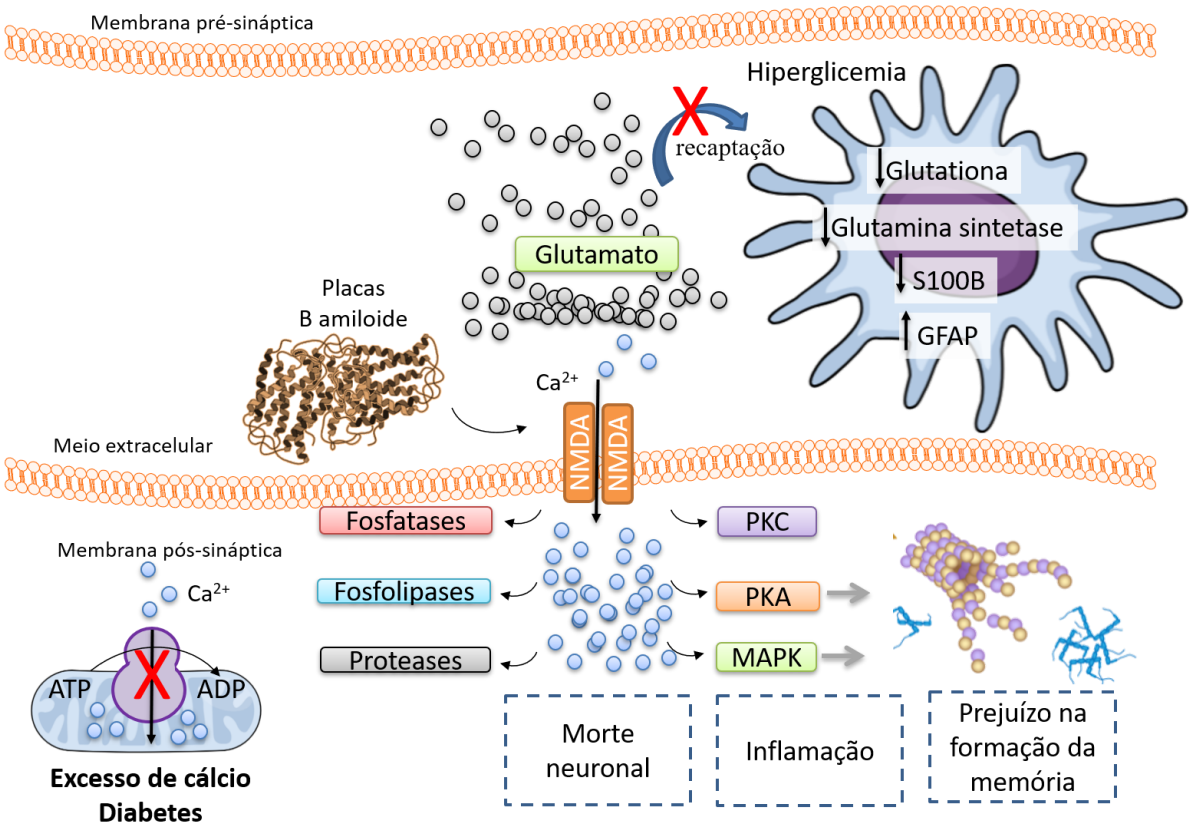
Na doença de Alzheimer, o acúmulo da proteína β amiloide (Aβ), um dos marcadores moleculares da doença de Alzheimer, pode ativar os receptores NMDA nos estágios iniciais da doença (PARAMESHWARAN; DHANASEKARAN; SUPPIRAMANIAM, 2008). Em neurônios corticais cultivados, o tratamento com Aβ ativa as subunidades GluN2B dos receptores NMDA e promove o aumento dos níveis de cálcio no meio intracelular (FERREIRA et al., 2012). Da mesma forma, oligômeros de Aβ mostram prejudicar a indução da LTP de maneira dependente de NMDA na região CA1 do hipocampo e no giro denteado (DANYSZ; PARSONS, 2012). Dessa forma, é possível que o prejuízo da LTP induzido por Aβ na doença de Alzheimer seja causado pela ativação excessiva do receptor NMDA (DANYSZ; PARSONS, 2012).

Uma vez que na excitotoxicidade há excesso de glutamato na fenda sináptica e Ca^{2+} intracelular, o aumento da concentração de Ca^{2+} intracelular ativa as enzimas dependentes de Ca^{2+} como proteases e consequente produção de radicais livres, a proteína quinase C,

fosfatases, fosfolipases levando à destruição dos fosfolipídeos da membrana neuronal, e óxido nítrico neuronal (nNOS) (EMERIT; EDEAS; BRICAIRE, 2004; MELLO; QUINCOZES-SANTOS; FUNCHAL, 2012; MILLIGAN; WATKINS, 2009). As vias de sinalização intracelular do glutamato, como a sinalização da PKA e MAPK podem apresentar efeito de regulação negativo na formação da LTP (WANG; PENG, 2016) como em condições de estimulação excessiva pelo glutamato. A PKA regula processos vitais por fosforilar de modo reversível algumas proteínas envolvidas no metabolismo celular, a expressão de genes, o desenvolvimento de tecidos e células, a morfogênese, a excitabilidade neuronal, condutividade de íons e motilidade celular. Assim, devido ao envolvendo da via AMPc-PKA em diversos processos fisiológicos, a sinalização aberrante através da via AMPc-PKA pode causar danos de várias maneiras (STRATAKIS; CHO-CHUNG, 2002).

No estudo realizado por Van Der Harg (2017), a diabetes tipo 1 induzida por estreptozotocina e a ausência de insulina em cultura de neuroblastoma humano apresentaram aumento da fosforilação da proteína Tau a qual foi associada com o aumento da atividade da PKA. A análise *pos mortem* de cérebros de pacientes com a doença de Alzheimer revelou que a atividade da PKA encontrava-se aumentada nos pacientes que apresentavam os estágios iniciais da doença e os níveis mais baixos da PKA estava relacionada com os estágios mais avançados da doença (VAN DER HARG et al., 2017). Em modelo genético duplo de diabetes tipo 2 e AD, os animais apresentaram prejuízos de memória de longo prazo, ativação da via de sinalização AMPc/PKA e apoptose neuronal pelo aumento da expressão de proteínas pró-apoptóticas. Além disso, o uso de um agonista de AMPc induziu a apoptose neuronal, enquanto a inibição de PKA pelo tratamento com H-89 mostrou reduzir a apoptose neuronal. Esses resultados sugerem que a diabetes do tipo 2 acelera o processo patológico na AD causando acúmulo dos marcadores da doença de Alzheimer (Tau e Aβ) pela ativação da via de sinalização AMPc/PKA (LI et al., 2018). Em um outro estudo, a infusão bilateral de Aβ no hipocampo de ratos induziu prejuízos de memória, ativação de NFκB, aumento da expressão da proteína pró-apoptótica caspase-3, estresse oxidativo, enquanto a inibição da PKA pelo H-89 bloqueou o prejuízo na retenção da memória mediada pela Aβ. Além desse efeito na memória, o H-89 reduziu a inflamação pela redução da atividade do NFκB e ativou a defesa antioxidante, indicando que a inibição da PKA equilibra o comprometimento antioxidante celular causado pela depleção de glutationa (EFTEKHARZADEH et al., 2012). Veja um desenho esquemático na figura 13.

Figura 13 – Excitotoxicidade glutamatérgica e a diabetes. Em condições normais a mitocôndria retira do meio intracelular o excesso de cálcio pela bomba de cálcio, um processo que ocorre com gasto energético. Entretanto, durante a diabetes, o déficit energético prejudica a retirada de cálcio do meio extracelular pela mitocôndria e promove a formação de placas amiloides. Paralelamente, a hiperglicemia prejudica a recaptação de glutamato pelos astrócitos e há ativação do receptor NMDA. O aumento do influxo de cálcio causado pela ativação do NMDA e o prejuízo da bomba de cálcio mitocondrial permitem o acúmulo de cálcio no meio intracelular induz a ativação aberrante dos mecanismos intracelulares de fosfatases, fosfolipases, proteases, PKC, PKA e MAPK causando inflamação, prejuízo na formação da memória e morte neuronal.



Fonte: A autora.

Nas sinapses neuronais o aumento dos níveis de Ca^{2+} nos astrócitos promove a liberação de glutamato (MAZZANTI; SUL; HAYDON, 2001; SANTELLO; VOLTERRA, 2009). Em cultura de células, a sinalização de cálcio dos astrócitos chega às células endoteliais e o aumento de cálcio nos astrócitos também promove sinalização nos vasos (LEYBAERT et al., 1998). Dessa maneira, a atividade neuronal pode elevar os níveis de cálcio nos astrócitos e a sinalização do cálcio pode chegar até o endotélio. O óxido nítrico endotelial por sua vez, também pode elevar o cálcio astrocítico (ZHU et al., 2013). Os estudos realizados por Charles 1999 e Patel et al. 1999, observaram que a sinalização do óxido nítrico no endotélio do fígado potencializou a sinalização do cálcio nos hepatócitos adjacentes. Uma vez que o cálcio

astrocítico elevado leva à liberação de glutamato, é possível que a sinalização de óxido nítrico da circulação proporcione o aumento de cálcio dos astrócitos e então leve a liberação de glutamato (CHARLES, 1999; MAZZANTI; SUL; HAYDON, 2001; PATEL et al., 1999).

2.4 SINTASES DE ÓXIDO NÍTRICO

O óxido nítrico é um gás solúvel produzido a partir da ação das enzimas óxido nítrico sintase - NOS (endotelial, neuronal e induzível) sobre a L-arginina (KUMAR et al., 2010). As isoformas endotelial e neuronal são ativadas de maneira dependente de cálcio e a induzível é completamente ativada em concentrações basais de cálcio intracelular, sendo sua ativação independente de cálcio (CALABRESE et al., 2007).

A sintase óxido nítrico induzível (iNOS) ou NOS-2 é encontrada em macrófagos, incluindo micróglia, e a liberação de óxido nítrico por essa enzima é induzida por citocinas pró-inflamatórias e produtos microbianos (KUMAR et al., 2010). Em células pulmonares a iNOS foi encontrada continuamente expressa sob condições fisiológicas (GUO et al., 1995). Em camundongos *knockout* para iNOS houve prejuízo no processo de cicatrização cutânea (KITANO et al., 2017), e desmielinização e inflamação exagerada no modelo de cuprizona (ARNETT et al., 2002; RAPOSO et al., 2013). Em camundongos geneticamente modificados para superexpressar a proteína precursora da amiloide (APP- *amiloid percussor protein*) e *knockout* para o gene da iNOS, a deleção genética promoveu a redução da maior fonte produtora de óxido nítrico para o modelo da Doença de Alzheimer. Nos mesmos camundongos, a redução do óxido nítrico da iNOS promoveu o aumento da fosforilação de Tau e aumento da produção de Aβ, neurodegeneração e ativação da caspase 3 (COLTON et al., 2006). Todas essas evidências sugerem que o óxido nítrico produzido pela iNOS também pode exercer um importante papel na regeneração tecidual e na regulação da inflamação sendo um componente, por vezes, protetor nos tecidos.

A sintase de óxido nítrico endotelial (eNOS), ou NOS-3, é expressa nos endotélios e é responsável por grande parte do NO endotelial, sendo sua expressão constitutiva e crucial para o funcionamento normal do sistema cardiovascular. Apesar de ser apontada como uma enzima constitutiva, fatores como hipóxia, dieta e exercício podem aumentar a liberação de NO através das células endoteliais. Da mesma forma, o estresse oxidativo e doenças vasculares, como diabetes e hipertensão, podem diminuir o NO liberado pela eNOS

(VANHOUTTE et al., 2009), indicando que a expressão da eNOS, em algumas situações, pode ser “induzida” (MICHEL, 1999).

A sintase de óxido nítrico neuronal (nNOS) ou NOS-1, está presente principalmente em neurônios tanto imaturos quanto maduros, também é encontrada em astrócitos, miócitos cardíacos e vasos cerebrais de ratos. Assim como a NOS endotelial, também é considerada constitutiva. Contudo também pode ser induzida durante a isquemia (HUANG et al., 1994; KHAN et al., 2015) e sob condições de estresse mental agudo (KHAN et al., 2017).

2.4.1 Óxido Nítrico e a Excitotoxicidade Glutamatérgica

Os mecanismos moleculares envolvidos na excitotoxicidade glutamatérgica é mediada em grande parte pelo óxido nítrico (DAWSON; DAWSON, 2018). Embora o NO derivado da nNOS seja uma molécula crítica na mediação da plasticidade sináptica e sinalização neuronal, ele pode mudar de neuromodulador fisiológico para um fator neurotóxico quando produzido em excesso (ZHOU; ZHU, 2009). O GMPc exerce um importante papel como mediador das ações do óxido nítrico (NO) e a via de sinalização NO/GMPc tem sido também reconhecida como mediadora de fenômenos neuropatológicos em condições como epilepsia, derrame e desordens neurodegenerativas. Nessas condições, a via pode contribuir para a morte celular excitotóxica e dano celular por neuroinflamação (SZABÓ, 1996). No estudo realizado por Parathath e colaboradores (2007) para determinar a relevância de cada isoforma da sintase de óxido nítrico indicou que a deleção de nNOS reduziu a neurodegeneração causada pelo óxido nítrico, enquanto a deleção de iNOS e eNOS não produziu danos neuronais, mas a deleção de eNOS preveniu danos na barreira hematoencefálica. Esses resultados indicam que a nNOS é uma isoforma crítica capaz de induzir excitotoxicidade e a quebra da barreira hematoencefálica pode ser um processo separado do dano neuronal (PARATHATH; GRAVANIS; TSIRKA, 2007).

Sob condições isquêmicas, a sinalização intracelular da ativação do receptor NMDA está relacionada com o processo de neurodegeneração. Um dos mecanismos mais avaliados é o mecanismos de acoplamento da nNOS pelo receptor NMDA através da proteína PSD-95 (ARUNDINE; TYMIANSKI, 2004; GARTHWAITE; CHARLES; CHESS-WILLIAMS, 1988; LAI; ZHANG; WANG, 2014; MO et al., 2016) e o bloqueio farmacológico da PSD-95-nNOS protege os neurônios da excitotoxicidade (MO et al., 2016). Por esse mecanismo a entrada de cálcio pelo receptor NMDA também é um componente importante de indução da

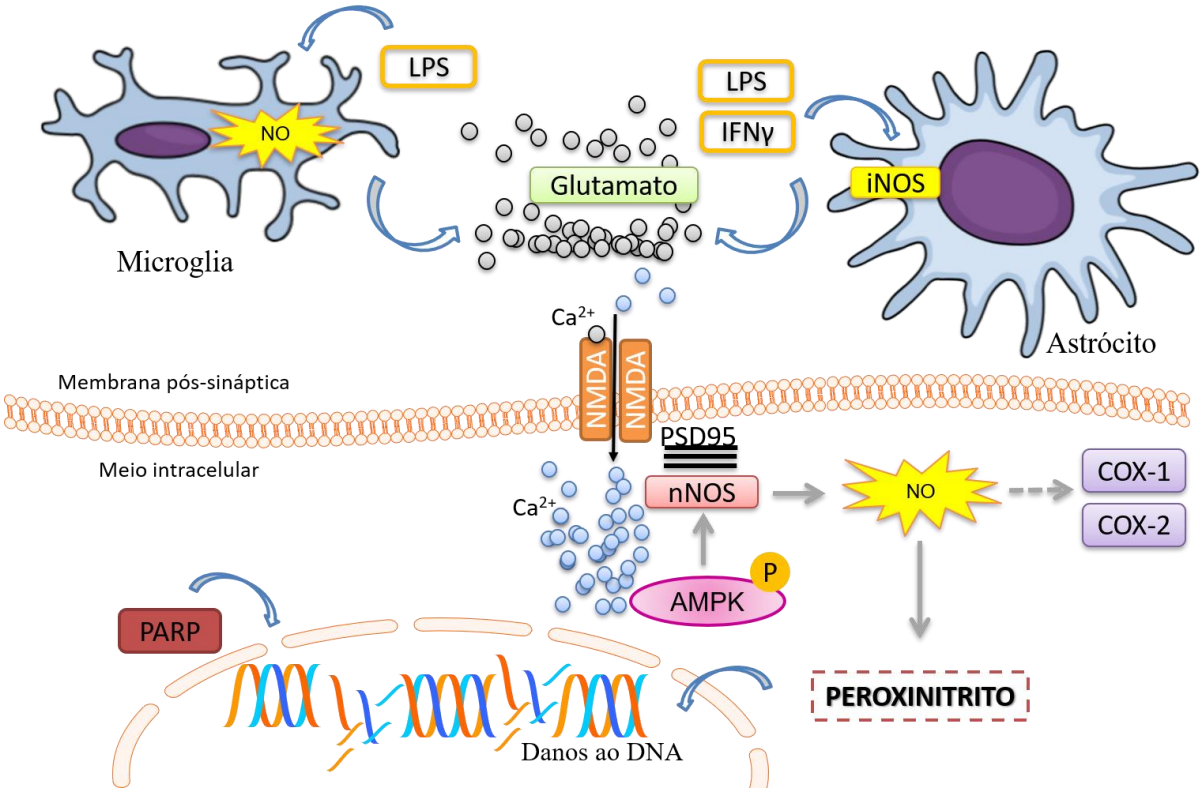
excitotoxicidade (DAWSON; DAWSON, 2018). Em modelo de lesão cerebral isquemia-reperfusão, a ativação de AMPK ativa a nNOS levando a produção de óxido nítrico. Esse óxido nítrico forma peroxinitrito que causa a morte neuronal. O óxido nítrico ainda neste processo, induz a ativação do receptor NMDA que ativa a nNOS pelo influxo de cálcio formando um ciclo vicioso. A redução da produção de NO pela inibição da nNOS reduz os efeitos tóxicos da ativação da via nNOS/peroxinitrito/AMPK (KHAN et al., 2015).

A formação de peroxinitrito tem papel chave na mediação dos efeitos tóxicos do NO. O peroxinitrito pode causar danos ao DNA causando a morte neuronal (CALABRESE et al., 2007; DAWSON; DAWSON, 2018). A enzima poli (ADP-ribose) polimerase-1 (PARP) é ativada para reparar o DNA (DAWSON; DAWSON, 2018). Em camundongos a inibição farmacológica e a deleção genética da PARP-1 impediu a excitotoxicidade do glutamato após a indução do acidente vascular cerebral (Dawson, 1994; Eliasson, 1997). O dano neuronal após o acidente vascular cerebral é associado com a superativação da PARP-1 em animais e em humanos (DAWSON; DAWSON, 2018). Por outro lado, além de causar danos ao DNA, o NO é capaz de ativar a cicloxigenase 1 (COX-1) e cicloxigenase-2 (COX-2). Em células cerebrais, durante o processo inflamatório a expressão dessas proteínas pode estar aumentada. Estudos experimentais ou clínicos indicam que em doenças que geram neurodegeneração como diabetes, Alzheimer, Parkinson e acidente vascular cerebral mostram o aumento da expressão da COX-2 (LIU et al., 2014; MANCUSO, 2007; OJHA et al., 2016; WANG et al., 2012b). Dessa maneira, a indução da expressão de COX-2 nessas patologias também pode ser considerada uma via indireta de excitotoxicidade mediada pelo NO (CALABRESE et al., 2007).

Além de promover a liberação de glutamato nos neurônios, o NO também é capaz de induzir a liberação de glutamato nas células gliais. Em astrócitos ativados por LPS e interferon γ, o aumento da expressão de iNOS aumentou o efluxo de glutamato, sugerindo que a inflamação ativa a produção de NO pela iNOS nos astrócitos e e então promove a liberação de glutamato (BAL-PRICE; MONEER; BROWN, 2002). A microglia também é capaz de estimular a liberação de glutamato dependente de NO. Foi observado que em células da micróglia de rato, ativadas por LPS, poderia aumentar a liberação de glutamato via produção de NO, enquanto o uso de inibidores de NOS nas células estimuladas não induziu a liberação de glutamato (NAKAMURA et al., 2003).

Em resumo, doenças com características inflamatórias aumentam a expressão de citocinas como a Il-1 e o TNF-α, o que pode induzir a produção de óxido nítrico nas células gliais. Esse óxido nítrico, por sua vez, pode estimular a liberação de glutamato das células gliais ou neuronais que em um ciclo vicioso que causa neurodegeneração (figura 14).

Figura 14 – Inflamação e a neurodegeneração. Nos astrócitos, LPS e IFNγ induzem o aumento da expressão de iNOS. Em microglia o LPS aumenta a liberação de NO. O NO liberado pelos astrócitos e microglia em processos inflamatórios induz o acúmulo de glutamato na fenda sináptica. O glutamato se liga ao receptor NMDA aumentando o influxo de cálcio no neurônio pós-sináptico. A ativação do NMDA e do AMPK aumentam a expressão de nNOS que por sua vez produz NO e indiretamente o óxido nítrico induz a expressão de COX-1 e COX-2. A formação de peroxinitrito a medeia o papel tóxico do NO causando danos ao DNA. Para reparar o DNA ocorre aumento da atividade da PARP.



Fonte: A autora.

2.5 MODELO EXPERIMENTAL

A Estreptozotocina (STZ) [2-deoxi-(3-(metil-3-nitrosoureido)-D-glicopiranose] é uma substância produzida pela bactéria *Streptomyces achromogenes* e tem sido empregada como um agente quimioterápico no tratamento de câncer de pâncreas em humanos. Essa substância tem sido empregada na pesquisa como agente indutor da diabetes (LENZEN, 2008). Variações na dose e idade do animal pode induzir experimentalmente a diabetes tipo 1, com

destruição das células β pancreáticas e consequente redução da produção de insulina e hiperglicemia, e a diabetes tipo 2 com hiperinsulinemia e hiperglicemia (KING, 2012).

Em ratos a dose usual é 65 mg/Kg, a qual pode ser administrada pela veia caudal ou via intraperitoneal (i.p). Os camundongos são mais resistentes à ação da STZ, sendo necessária a administração de altas doses e apenas via i.p. Em linhagens de camundongos que são heterogênicas, alguns animais da amostra experimental podem apresentar resistência à indução da diabetes pela STZ. Uma única dose de 200 mg/Kg produz a ação diabetogênica em camundongos (KING, 2012), contudo a padronização no nosso Laboratório de Ultraestrutura (IAM/Fiocruz) indicou uma alta taxa de mortalidade. A indução da diabetes em duas doses de 90 mg/Kg em dois dias consecutivos apresenta uma baixa taxa de mortalidade (10%) em experimentos com duração de oito semanas (JOLIVALT et al., 2010), sendo esta metodologia adotada nos nossos modelos experimentais de diabetes utilizando camundongos.

Dentre os tipos de indução química da diabetes, a STZ é mais usada principalmente na avaliação de alterações no SNC. Além do seu efeito diabetogênico, a STZ em cultura de neurônios imaturos tem efeito tóxico o que pode ser associado a prejuízos da neurogênese (ISAEV et al., 2018). Essa substância também exerce uma ação tóxica para o cérebro. A injeção intracerebroventricular da STZ em animais induz o modelo idiopático da doença de Alzheimer caracterizado pela resistência à insulina no cérebro, danos de memória e deficiência progressiva colinérgica, astrogliose, estresse oxidativo, dentre outras características que são compatíveis com aquelas que ocorrem na doença de Alzheimer esporádica em humanos (PARK, 2011; SALKOVIC-PETRISIC et al., 2013). Em camundongos *Swiss* Webster, Jolivalt et al. (2008) induziram diabetes com STZ na dose de 90mg/kg, via intraperitoneal em dois dias consecutivos e observaram que os animais apresentaram deficiência na aquisição da memória de longo prazo, redução da fosforilação/sinalização da insulina PIK3-Akt-GSK3 e aumento dos marcadores para DA (fosforilação da proteína Tau e acúmulo de β amiloide) no hemisfério cerebral após nove semanas de diabetes.

O mecanismo de ação da STZ para indução da diabetes tipo 1 é pela destruição das células β do pâncreas geralmente de modo irreversível. A STZ tem efeito tóxico nessas células principalmente por formar danos pela adição de radicais metil à estrutura do DNA (BENNET; PEGG, 1981) e secundariamente pela formação de ROS e peroxinitrito (TURK et al., 1993). Os danos nas células β pancreáticas, então elevam a glicose e reduzem os níveis de insulina

circulantes, características da diabetes tipo 1 (KING, 2012). A STZ é captada pelas células β pancreáticas pelo receptor de glicose GLUT2, portanto é necessário um prévio jejum *overnight* para diminuir a competição com a glicose pelo receptor e a indução ter êxito. A baixa expressão do GLUT2 confere resistência à STZ. Em humanos, a STZ não exerce efeito diabetogênico devido à baixa expressão do receptor GLUT2 (SCHNEDL et al., 1994). Após a administração da STZ, ocorre variação nos níveis de glicose plasmática até o estabelecimento da hiperglicemia. A primeira fase é o aumento da glicemia após uma hora da injeção e redução da insulina plasmática. Na segunda fase, há hipoglicemia pelo aumento dos níveis de insulina depois de 4-8 horas e esse quadro pode permanecer por várias horas. Por último, é estabelecida a hiperglicemia e hipoinsulinemia permanentemente após 48 horas, e morfologicamente as células secretoras de insulina encontram-se alteradas (LENZEN, 2008).

3 RESULTADOS

3.1 ARTIGO 1 - AMPK ACTIVATION: ROLE IN THE SIGNALING PATHWAYS OF NEUROINFLAMMATION AND NEURODEGENERATION

O primeiro artigo que compõe parte dos resultados dessa tese foi publicado na revista Experimental Neurology em 2017 com o título "AMPK Activation: Role in the Signaling Pathways of Neuroinflammation and Neurodegeneration". O artigo encontra-se no apêndice A dessa tese.

3.2 ARTIGO 2 - EFFECTS OF METFORMIN ON LONG-TERM MEMORY AND HALLMARKS OF ALZHEIMER'S DISEASE IN DIABETIC MICE

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ABSTRACT

Besides initial symptoms of diabetes, which are hyperglycemia and insulin deficiency or resistance, it can alter the brain causing memory changes, inflammation and augment the expression of Alzheimer's Disease (AD) markers, pTau and amyloid β. This study aimed to evaluate the effects of metformin on long-term memory/impairment of learning and on hallmarks of AD in diabetic mice. *Swiss* Webster mice, which were distributed in the following experimental groups: control; streptozotocin (STZ); STZ + metformin 100 mg/kg (M100); STZ + metformin 200 mg/kg (M200). STZ mice showed significant augmentation of time spent to reach the target box in the Barnes maze, while MT200 mice were able to reduce it. STZ mice showed increased nitrite levels in serum and brain tissues, whereas M200 mice had significantly reduced levels. Moreover, M200 group reduced GFAP immunoreactivity in hippocampal dentate gyrus and CA1 compared with STZ group. STZ mice showed high p-Tau levels, reduced p-CREB and accumulation of amyloid β plaques in hippocampal areas and corpus callosum, whereas all these changes were reversed in the M200 group. Protein expression of pTau, pERK, pGSK3, iNOS, nNOS, PARP, Cytochrome c, caspase 3 and GluN2A were increased in STZ mice and significantly reduced in M200 mice. M200 mice showed also significant high levels of eNOS, AMPK and p-AKT expression. In conclusion, the treatment with metformin improved spatial memory in diabetic mice, which can be attributed to reduction of p-Tau and amyloid β plaques, modulation of nitric oxide's synthases expression (eNOS, iNOS e nNOS) and inhibition of neuronal death.

Keywords: Alzheimer's disease, diabetic encephalopathy, amyloid β, nitric oxide, AMPK, glutamate, neuroinflammation.

1. INTRODUCTION

Diabetes is a common and growing metabolic disorder in modern life style (WHITING et al., 2011). Besides initial symptoms of diabetes, which are hyperglycemia and deficiency or resistance of insulin, there are others related complications, such as retinopathy (WANG; LO, 2018), nephropathy (BJORNSTAD; CHERNEY; MAAHS, 2014), peripheral neuropathy (IQBAL et al., 2018), depression (ALZOUBI et al., 2018) and dementia (FOLCH et al., 2018). These diabetic complications are due to oxidative stress, inflammation and impaired mitochondrial function (PICKERING et al., 2018).

In the last decade many articles had focused on elucidating the mechanisms by which diabetes promotes dementia and hallmarks of Alzheimer disease (AD). It is known that the formation of neurofibrillary tangles (NFTs) of hyperphosphorylated TAU protein, extracellular amyloid-beta (Aβ) plaques (senile plaques) and neuronal loss are related to the switching off of insulin signaling in brain areas involved with cognition (BLÁZQUEZ et al., 2014; CHAMI et al., 2016; FOLCH et al., 2018; LEE et al., 2018). However, there are only few studies that investigated hypoglycemic drugs that act in the insulin pathways in the diabetic brain with positive hallmarks Azheimer's disease.

Since the insulin signaling activates phosphatidil-inositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, it plays a role in cognition by promoting neuronal growth, survival and differentiation (BANKS; OWEN; ERICKSON, 2012). Furthermore, the PIK3/Akt pathway phosphorylates and inhibits the protein glycogen synthase kinase 3 β (GSK3β) (SCHUBERT et al., 2004). When GSK3 protein is in its activated form, it can hyperphosphorylate TAU protein (ISHIGURO et al., 1992), impairing the stabilization of neuronal microtubules and the axonal transport (GRUNDKE-IQBAL et al., 1986, 1987).

Metformin is the drug more prescribed for type 2 diabetes mellitus treatment, though it also exerts beneficial effects in polycystic ovarian treatment (TSILCHOROZIDOU; BATTERHAM; CONWAY, 2008). Metformin is known to activate AMP-activated protein kinase (AMPK) (CORREIA et al., 2008). However, some studies have shown that it can also activate atypical pathways, such as PKC-PCB, to promote neurogenesis and improve spatial memory (WANG et al., 2012a) and exert AMPK-independent anti-inflammatory effects *in vitro* (ŁABUZEK et al., 2010). Additionally, metformin exerts neuroprotective and anti-inflammatory effects, restores dopamine depletion in the animal model of Parkinson's disease

(ISMAIEL et al., 2016) and presents antioxidant activity in the brain (CLARK; PANDYA; LAU-CAM, 2017). Our previous study demonstrated that metformin reversed the inflammation and short-term memory impairments in streptozotocin (STZ)-induced diabetic mice (OLIVEIRA et al., 2016).

Although experimental studies positively correlate diabetes with hallmarks of Alzheimer's disease, such as cognitive damage, TAU protein hyperphosphorylation and β-amyloid plaques, few studies have evaluated potential treatments or the interaction of diabetes with Alzheimer's disease under treatment with hypoglycemic agents. Thus, the aim of the present study was to analyze whether the metformin long-treatment prevents memory/learning impairment and reduces the hallmarks of AD in diabetic brain, as well as evaluate possible molecular/cellular mechanisms of this drug.

2. EXPERIMENTAL PROCEDURES

2.1. ANIMALS

The study was performed using forty Swiss Webster male mice, aged 12 weeks and weighting 35-42 g. Three to four animals were housed per cage with standard food and water *ad libitum* and were kept at a temperature of 22 °C and a light/dark cycle of 12 h. All experimental procedures were approved by the Aggeu Magalhães Institute Ethics Committee/Oswaldo Cruz Foundation (81/2015- CEUA/FIOCRUZ).

2.2. EXPERIMENTAL DESIGN

Forty animals were distributed equally in the following four groups: control; streptozotocin (STZ); streptozotocin treated with 100 mg of metformin/kg/day (STZ+M100); streptozotocin treated with 200 mg of metformin/kg/day (STZ+M200). At 12 weeks old, diabetes was induced in animals of STZ, STZ+M100 and STZ+M200 groups according to a previously described protocol (OLIVEIRA et al., 2016). On the fourth day after the second injection and after four hours of fasting, a tail puncture was performed to confirm diabetes induction by using a One Touch Ultra Lifescan (J & J) glucometer and the treatment with metformin hydrochloride initiated (twice a day). After 9 weeks of treatment the animals were anesthetized and euthanized. The evaluation of the glycemia was repeated once weekly and

the animals with glycemia <270 mg/dL in three consecutive weeks were excluded from the study.

2.3. BARNES MAZE

The used Barnes circular maze consists of a white circular platform with 20 equally spaced holes (5 cm diameter) and located 2 cm from the perimeter. A black escape box was placed under one of the holes. Ten days before the euthanasia (between the 7^{th} and 8^{th} weeks of diabetes), the animals were trained (learning) to entry in the escape box on Barnes maze, during five consecutive days (Monday to Friday), once a day. In acquisition phase, the animals were placed in a black, cylindrical container in the middle of the maze and after 10 seconds were allowed to explore the maze until they entered in the escape box or at the end of 5 minutes. Then, the light was turned off and the animals were placed or maintained in the escape box for 1 minute. After the learning phase, the animals were untested for 3 days and tested at day 9 for the memory phase of the test. The animals were placed in a black, cylindrical container in the middle of the maze and after 10 seconds were allowed to explore. The time to find the escape box during the acquisition phase and probe memory was analyzed.

2.4. IMMUNOHISTOCHEMISTRY

After anesthesia, we withdrew 1 mL of blood by cardiac puncture and then the animals were transcardially perfused with physiological saline (20 ml), followed by 4% paraformaldehyde (Sigma–Aldrich) (20 ml) in 0.1M phosphate (sodium phosphate monobasic and dibasic heptahydrated – Sigma–Aldrich) buffered saline, pH 7.2. The brain of each mouse was immediately removed and post-fixed overnight in the same fixative solution. The samples were dehydrated in a series of ethanol rinses, cleared in xylene and embedded in paraffin as routine. Sections with a thickness of 5 mm were cut on an RM 2035 microtome (Reichert S, Leica), re-hydrated, and treated with 20mM citrate buffer, pH 6.0, at 100 °C, for 30 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) and the sections were blocked with 1% bovine serum albumin (Sigma Aldrich, USA) for 1 h at room temperature. The samples were incubated with the following rabbit polyclonal primary antibodies: GFAP (Novus Biologicus, NB300-141) at 1:1000 dilution, overnight at 4 °C. After washing, the sections were overlaid with a biotin-conjugated secondary antibody for 1 h (DakoCytomation, Biotinylated Link Universal HRP; catalog number: K0690, CA, USA), and

visualized with 30-3-diaminobenzidine (DAB) as the chromogen. The slices were counter-stained with Carazzi's hematoxylin and mounted in Entellan (Merck, catalog number: 1079610100, USA). The pictures were captured by using an optic microscopy (Leica ICC50 HD). Pixel density of five pictures of dentate gyrus (DG) and CA1 region was measured by using the GIMP 2.8.18 software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia).

2.5. WESTERN BLOT

The hemibrains (without cerebellum) were rapidly freezer in liquid nitrogen and then four hemibrains of different animals per group were homogenized in an extraction solution containing protease inhibitor cocktail (10mM EDTA, Amresco, Solon, USA; 2mM phenylmethane sulfonyl-fluoride, 100mM NaF, 10mM sodium pyrophosphate, 10mM $NaVO_4$, 10 mg of aprotinin/ml and 100mM Tris, pH 7.4 – Sigma–Aldrich). The samples were mixed and homogenized to form a pool from each group. Homogenates were centrifuged and frozen at -80 °C. Protein levels were determined by using the Bradford method, with bovine serum albumin as the standard (BRADFORD, 1976) and 30 µg of the total protein were loaded into each well in an electrophoresis gel, separated and then electrophoretically transferred onto nitrocellulose membranes. After blocking for 1 h with 3% Bovine albumin Serum, 0.01% tween 20 and Phosphate Buffer Saline, the membranes were incubated at 4º C overnight with the following antibodies: anti PI3K p85 (cell signaling, 4257), phospho-CREB (cell signaling, 9198), CREB (cell signaling, 9197), iNOS (Abcam, ab3523), AMPK (Abcam, ab32047), phospho-AMPK (cell signaling, 2535), Guanylate cyclase (Sigma, G4425), Tau phospho ser 416 (cell signaling, 15013), Tau (cell signaling, 4019), ERK1/2 (Santa Cruz, sc-292838), phospho-ERK1/2 (Santa Cruz, sc-16982), AKT1/2/3 (Abcam, ab106693), AKT-Pan-phospho (Abcam, ab38449), GSK3β phospho ser9 (cell signaling, 9323), GSK3β (cell signaling, 12456), PKA (Abcam, 59253), eNOS (Abcam, 66127), nNOS (cell signaling, 4231), PARP (Abcam, 6079), IL-1β (Genway, 18-732-292194), cytochrome c (Santa Cruz, 13156), GluA1 (Alomone, AGC-004), GluA2 (Alomone, AGC-005), GluN1 (Alomone, AGC-001), GluN2A (Alomone, AGC-002) and antibody β-actin (Sigma, A2228). All the primary antibodies were diluted in blocking solution at a 1:1000 dilution, except β-actin, PARP and Cytochrome c which was used at a 1:3000, 1:400 and 1:500 dilution, respectively. Following washing, the membranes were incubated with horseradish peroxidase- conjugated

(HRP) anti-rabbit (Abcam, catalog number: ab6721, UK), anti-mouse (Sigma-Aldrich, catalog number: A0168, USA) or anti-goat (Sigma Aldrich, A5420) secondary antibodies in 1:8000 dilution when necessary. Chemiluminescence reagent (Milipore, catalog number: WBLUF0100) was added for protein band visualization and the reaction was detected using LI-COR equipment. For quantification, densitometry values were obtained by measuring the pixel density of each band using Image J 1.38 software (NIH, MD, USA).

2.6. NITRIC OXIDE MEASUREMENT

The nitrite levels in serum and brain tissue were determined by a method based on the Griess reaction (GREEN et al., 1982) and deproteinized according to a previously published method (MOSHAGE et al., 1995). Four hemibrain per group (no cerebellum) were prepared using an extraction solution containing protease inhibitor cocktail (as in western blot) and the homogenates were centrifuged. The supernatant and serum (50 µL) were diluted in 150 µL of distilled water and deproteinized by adding of 10 µL zinc sulfate (0,3 g/mL) (MOSHAGE et al., 1995). After, 50µL of sample were loaded in 96-well ELISA plate, followed by the same volume of Griess reagent, which is composed of 1% sulfanilamide diluted in 2.5% H_3PO_4 (solution A) and N-1-naphtyl-ethtylenodiamina, also diluted in 2.5% H_3PO_4 (solution B). The standard curve was performed by using sodium nitrite serially diluted in water. After incubation for 10 minutes in the dark, a reading was performed by the spectrophotometer at 490 nm. The absorbance of different samples was compared with the standard curve and the results were expressed as the mean ± SD (ROCHA et al., 2014).

2.7. IMMUNOFLUORESCENCE

The animals were anesthetized and submitted to euthanasia as described in section 1.4. The brain was embedded in paraffin as routine described in section 2.4. Sections with a thickness of 3 µm were cut on an RM 2035 microtome (Reichert S, Leica) re-hydrated, and treated with 20mM citrate buffer, pH 6.0, at 100 °C, for 30 min. After, they were permeabilized with 0.5% Triton X-100 and incubated for 1 h with blocking solution (3% BSA plus 0.2% Tween 20 in Tris buffered saline). Subsequently, the sections were incubated with anti-TAU antibody (Cell signaling, catalog number 15013, dilution of 1:50), amyloid β (Cell signaling, catalog number 8243, dilution of 1:100) and p-CREB (Cell signaling, catalog

number 9198, dilution of 1:100). The primary antibodies were incubated overnight and then incubated with polyclonal fluor 546-conjugated secondary antibody (Alexa, catalog number A10040) against rabbit immunoglobulin for 1 h. The slices were washed and mounted in gelatin medium and observed under a fluorescence microscope (Leica DM2500) coupled with a digital camera (Leica DFC345 FX). Pixel density was measured in five stained areas using the GIMP 2.6.11 software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia).

2.8. STATISTICAL ANALYSIS

The statistical analysis of the data was performed using the program Graphpad prism v 6.01. The non-parametric data were expressed by median ± interquartile range and analyzed by Kruskal-Wallis followed by post test Dunn's. The parametric data were analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc test and represented by mean ± SD.

3. RESULTS

3.1. METFORMIN IMPROVES THE PERFORMANCE OF HYPERGLYCEMIC MICE ON BARNES MAZE TASK

Four days after induction of diabetes, the 1st fasting glycemia test was performed. After confirming the hyperglycemic status, the treatment with metformin was initiated. The animals with glycemia <270 mg/dL in two analyses consecutives were excluded from the experiment. Then, until the 4th week one STZ mice and two STZ+M200 mice were excluded. From the 5th week until the 9th week all animals maintained high blood glucose levels (270 mg/dL) (figure 1).

The deficiency of insulin causes symptoms like polyuria, polydipsia, increases food intake and loss of body weight. During the experimental procedures, polyuria, polydipsia and increased food intake were observed indirectly by increased replacement of water, feed and shavings, respectively (data not shown). On the other hand, the body weight was accompanied weekly and the STZ ($p<0.001$), STZ+M100 ($p<0.0001$) and STZ+M200 ($p<0.001$) mice had significantly reduced body weight at the end of study compared to their initial weight (figure 1).

Regarding behavioral assays, the Barnes maze test was performed on the 8th experimental week. On the first day of learning acquisition phase, the animals of all groups spent a similar time to find the escape (group median: control= 221, STZ= 201, M100= 227 and M200= 211). In contrast, on the second day the control mice spent 229 s (median) and the others STZ, STZ+M100 and STZ+M200 mice did not find the escape box. On the fifth day of learning acquisition phase, the control mice had significantly reduced ($p<0.05$ compared to second day of control group) time to find the escape box (median 38 s), suggesting learning and memory. However, STZ and STZ+M100 mice had no significant reduction of the time to find the escape box on the 5th day (median 274 s and 218 s respectively) compared to values of the second day, suggesting significant impairment in learning and/or memory. In contrast, the STZ+M200 mice had significantly reduced ($p<0.05$ compared to second day of STZ+M200 group) time to find the escape box (median 161 s). Then, the animals were left untested for three days and after that were tested again to analyse the spatial memory acquisition. On the 9th day of the Barnes maze test, the spatial memory was evaluated. The control mice spent 38 s (median) to find the escape box, while the STZ (median 150 s, $p<0.01$ compared to control) and STZ+M100 (median 150 s) mice did not have reduced time to find the escape box. Conversely, the STZ+M200 mice had significantly reduced time to find the escape box (median 47 s), compared with STZ mice ($p<0.05$). All these data of Barnes maze task indicate that type 1 diabetes induced spatial learning and memory impairments, while treatment with 200 mg/kg/day of metformin prevented cognitive deficits in diabetic mice (Figure 2).

3.2. EFFECTS OF METFORMIN ON AMPK/PI3K/Akt PATHWAY IN DIABETIC BRAIN

Since the treatment with metformin at dose of 100 mg/Kg/day did not improve the performance of animals on the Barnes maze task, this group was no longer analyzed. The expression of AMPK phosphorylated at Thr 172 (activated form) was significantly reduced in STZ ($p<0.0001$) mice and increased in STZ+M200 mice ($p<0.05$). These data indicated that diabetes impairs the energetic balance and metformin at dose of 200 mg/kg/day increased the levels of p-AMPK in diabetic brain (Figure 3).

The insulin cascade signaling involves a sequence of phosphorylation in proteins such as PI3K and Akt. The present results showed no significant difference among groups in

relation to PI3K p85 subunit (figure 3). In contrast, The STZ mice showed a significantly reduced expression of p-Akt when compared with the control mice ($p<0.05$) (figure 3).

2.3 EFFECTS OF METFORMIN ON TAU PHOSPHORYLATION AND AMYLOID β LEVELS IN DIABETIC BRAIN

Alzheimer's disease is a disorder characterized by dementia, hyperphosphorylation of TAU protein and deposition of amyloid-β**.** In cerebral cortex of the STZ and M200 mice, the amyloid β was significantly increased ($p<0.001$ and $p<0.05$, respectively) (Figure 4). However, amyloid plaques were only observed in diabetic animals (not shown). The STZ mice showed phosphorylated TAU in hippocampal CA1 region, cortex and corpus callosum, whereas the M200 mice showed reduced pTAU expression (Figure 5) confirmed by western blot (Figure 6) ($p<0.05$).

2.4 EFFECTS OF METFORMIN ON TAU KINASES

GSK3 is a TAU protein kinase downstream of PI3K-Akt pathway. It is inactivated by phosphorylation on the Ser 9 in the isoform β. Surprisingly, although the Akt protein levels were significantly reduced in STZ mice ($p<0.05$) (Figure 3), no difference was observed in p-GSK3 expression among groups (Figure 6).

Extracellular signal-regulated kinase (ERK) is involved in pro-inflammatory signaling pathways, memory consolidation and can also phosphorylate TAU. In diabetic animals the phosphorylated ERK expression was significantly increased ($p<0.0001$), whereas the metformin treatment significantly reduced p-ERK ($p<0.0001$) (Figure 6).

PKA is another protein kinase that can phosphorylate TAU protein. The expression of protein kinase A (PKA) was significantly increased in diabetic mice when compared to control mice ($p<0.05$) and significantly reduced in metformin-treated mice when compared to STZ mice ($p<0.05$).

2.5 EFFECTS OF METFORMIN ON NITRIC OXIDE MODULATION

The NO levels were detected by total nitrites measurement. In serum, the levels of nitrites (μM) were significantly elevated ($p<0.01$) in diabetic mice (102±3,536) compared to

control mice (49±0.7071), while diabetic mice treated with metformin at 200 mg/Kg/day showed reduced (88±4.95) levels ($p<0.01$) (Figure 7A). Similarly, the levels of nitrites (Figure 7B), detected in µg/mg protein, were significantly elevated ($p<0.05$) in the brain of diabetic mice (14.93±0.8066) compared to control mice (13.17±0.2733) and significantly reduced ($p<0.01$) in STZ+M200 mice (12.33±1.662).

In order to identify which nitric oxide synthase was related to the elevated NO levels observed in STZ mice, we performed western blot analysis for the expression of nitric oxide synthases. In STZ mice expression of iNOS was significantly increased compared to control mice ($p<0.05$). In contrast, STZ+M200 mice showed a significantly decreased iNOS levels when compared to STZ mice ($p<0.05$). On the other hand, the nNOS expression was increased both in STZ mice ($p<0.05$) and in metformin treated mice ($p<0.01$) when compared to control mice (figure 12). The eNOS produces NO in nanomolar levels that have a potential anti-inflammatory effect. In the present study, eNOS expression was significantly elevated after metformin treatment, when compared with STZ ($p<0.05$) and control ($p<0.01$) mice.

2.6 EFFECTS OF METFORMIN ON CELLULAR SURVIVAL AND INFLAMMATION

The Poly (ADP-ribose) polymerase (PARP) is a protein involved in numerous processes such as DNA repair and cell death. In diabetic group the levels of PARP were significantly elevated compared with control mice ($p<0.05$), while the treatment with metformin significantly reduced the PARP levels in diabetic brain compared to STZ mice ($p<0.05$) (Figure 8).

The interleukin-1β is one of the first protein expressed during acute inflammation and is also related to excitotoxicity (Harron and Miller et al., 2016). STZ-diabetes induced mice showed significantly high levels of IL-1β when compared with the control mice ($p<0.05$) (Figure 8).

In addition, the cytochrome c is a protein present in mitochondrial membrane and is elevated in pathological conditions triggering cellular apoptosis. The cytochrome C expression in STZ mice was significantly elevated when compared with the control mice ($p<0.01$), whereas the treatment with metformin significantly reduced its expression ($p<0.05$) (Figure 8). The caspase 3 is a protein downstream of cytochrome C pathway that promotes cellular apoptosis. The diabetic mice showed increased expression of activated caspase 3

($p<0.01$), while the diabetic mice treated with metformin had significantly reduced caspase 3 levels ($p<0.01$).

The phosphorylation of CREB activates the transcription of neurotrophic factors, which plays a role in memory and is neuroprotective to neurons. Immunofluorescence for phosphorylated CREB was detected in neurons of the cerebral cortex in control mice. The diabetic mice exhibited reduced p-CREB expression ($p<0.001$), whereas diabetic mice treated with metformin had restored p-CREB levels ($p<0.001$) (Figure 9).

2.7 EFFECTS OF METFORMIN ON GLUMATATERGIC EXCITOTOXICITY

The levels of AMPA receptor 1 (GluA1) (Figure 10A), AMPA receptor 2 (GluA2) (Figure 10B) and NMDA receptor 1 (GluN1) (Figure 10C) in the STZ mice did not significantly differ when compared to control mice. In contrast, the NMDA receptor 2A (GluN2A) expression was significantly elevated in STZ mice ($p<0.01$) (Figure 10D). Nevertheless, the M200 group showed significantly reduced expression of GluN2A when compared to STZ mice ($p<0.05$).

2.8 METFORMIN REDUCES HIPPOCAMPAL ASTROGLYOSIS

GFAP is an astrocytes marker that allows evaluation of reactive astrogliosis by morphological hallmarks. Hippocampal astrocytes in control mice presented thin extensions. The STZ mice displayed numerous astrocytes with thick extensions, whereas STZ+M200 mice had less numerous astrocytes exhibiting thin extensions. In quantitative analysis, the GFAP was significantly increased both in the dentate gyrus (DG) ($p<0.001$) and CA1 ($p<0.05$) areas in STZ mice. Comparatively, STZ+M200 mice had significantly decreased expression of GFAP in DG ($p<0.01$) and CA1 ($p<0.05$) (Figure 11).

3 DISCUSSION

Many studies have investigated the effects of diabetes in the brain. Most of them concluded that the deficiency of insulin or insulin resistance can switch off the insulin signaling in hemibrain or isolated hippocampus, leading to cognitive impairment and TAU hyperphosphorylation via increased activity of GSK3. Additionally, diabetes exacerbates β-amyloid accumulation in animal models of Alzheimer's disease (BEDSE et al., 2015; BLÁZQUEZ et al., 2014; DEVI et al., 2012; JOLIVALT et al., 2008, 2010). Although there are evidences that the impairment of insulin signaling in the brain exacerbates the hallmarks of Alzheimer's disease, there are few studies that analyzed the mechanism that link the insulin signaling, memory impairment, the hallmarks of Alzheimer's disease and propose a pharmacological alternative.

The present results showed that the diabetic animals had impaired memory acquisition, analyzed by Barnes maze task, and the treatment with metformin at a dose of 200 mg/kg/day prevented the effects of hyperglycemia in memory and learning. Similarly, Jolivalt et al. (2008) analyzed the memory and learning of STZ-diabetic Swiss Webster in Barnes maze and observed long-term memory impairment. In contrast, another study showed that C57BL/6J mice fed with high fat diet and treated with metformin for four months had improved long-term memory in Morris water maze without affecting the fasting glucose levels (ALLARD et al., 2016). Previously, we have demonstrated that STZ-induced diabetic mice (C57BL/6) treated with metformin at a dose of 200 mg/kg had improved work memory, when analyzed by T-maze, without reducing blood glucose (OLIVEIRA et al., 2016). Thus, the present results confirm and extend that metformin can to improve long-term memory in STZ-induced diabetic mice independent of glycemic control.

AMPK is an energetic sensor which is activated by increased AMP and decreased ATP, thus when there is an energetic depletion AMPK activation occurs (CORREIA et al., 2008). In diabetes the insulin signaling is switched off in the brain, which affects the energetic support of neurons that have receptor sensitive to insulin, specially the hippocampal neurons (CHAMI et al., 2016). Additionally, Peng and colls (2016) observed that in diabetic brains the reduced levels of AMPK and Akt phosphorylation in neurons were preceded by impaired mitochondrial function. Our results showed that the lack of insulin in hemibrain of STZ-induced diabetic mice trended to reduce the levels of PI3Kp85 and significantly reduced the

phosphorylated Akt. These results confirm previous findings obtained by Jolivalt et al. (2008) using the same experimental model. Besides, other authors showed that in scopolamine-induced amnesic model, treatment with 100 mg/kg of metformin induced memory improvement in rats as evaluated by Morris water maze, and these findings were related to phosphorylated Akt (MOSTAFA; ISMAIL; GHAREEB, 2016). In the present study, the phosphorylation of Akt by metformin trended to increase in association with the improvement of memory performance in Barnes maze.

The disbalance in kinases and/or phosphatases of TAU causes hyperphosphorylation in TAU protein. The main TAU kinases are GSK3, Cdk5, MAPK/ERK and JNK (PLANEL et al., 2007). Since GSK3 is downstream in PI3K/Akt pathway, we hypothesized that GSK3 could be activated in the brain of diabetic mice. Surprisingly, p-GSK3β (the inactivated form) levels were elevated in STZ mice, as well as in metformin-treated mice. In contrast, STZ mice exhibited high MAPK/ERK and PKA expression, and reduced expression of these proteins were observed in metformin-treated mice. Our results are in accordance with those previously obtained by Planel et al., (2007) and Van der Harg et al. (2017) that demonstrated no relationship between the phosphorylation of TAU and the activity of GSK3 in the brain of STZ mice. Besides, van der Harg et al. (2017) also demonstrated that the defficiency of insulin activates PKA, which was associated with TAU hyperphosphorylation. Thus, since GSK3 can be inactivated by PI3K/Akt signalling or PKA (ZHOU et al., 2013), the phosphorylation of GSK3 observed in STZ mice in the present work coud be due to PKA activity. Jing et al. (2013) observed that the activation of AMPK reduced the levels of phosphorylated ERK in the brain of STZ-induced diabetic rats treated with resveratrol. In addition, the activation of AMPK by metformin in hepatocytes induces the acummulation of AMP nucleotide causing reduction in cAMP and consequently decreasing the activated PKA levels (HE et al., 2016; MILLER et al., 2013). Here, it was observed for the first time that metformin exerted similar effects on AMPK/PKA signaling in the brain of diabetic mice.

Nitric oxide is a soluble gas produced by enzymes such as nitric oxide synthase inducible (iNOS), endothelial (eNOS) and neuronal (nNOS). In general, an excessive amount of nitric oxide produced by iNOS plays a role in inflammatory process, while the NO produced by eNOS and nNOS released in nanomolar levels is considered to play a protective role (KNOWLES; MONCADA, 1994; MALINSKI, 2007). Particularly, in neuronal tissue nitric oxide produced by nNOS acts like a neurotransmitter. However, in pathological

conditions its excess can be toxic to neurons (CALABRESE et al., 2007; GARTHWAITE; BOULTON, 1995).

The nitric oxide released by eNOS plays a role as vasodilator and is associated with the improvement of vascular function (XIA; FÖRSTERMANN; LI, 2014). Interestingly, in hyperglycemic conditions the vascular damage can be associated to altered eNOS and vascular endothelial growth factor (VEGF) levels (ADVANI et al., 2013; VANHOUTTE et al., 2009). Additionally, there is a positive feedback between AMPK and eNOS, since they can active each other reciprocally (REIHILL et al., 2007). Our results indicated that in diabetic condition the decreased AMPK phosphorylation did not affect the eNOS expression, while the pharmacological activation of AMPK by metformin significantly elevated the levels of eNOS. One limitation of the present study is the absence of an AMPK antagonist, such as compound C, in order to discard the possibility of elevated expression of eNOS by metformin itself, and not by its indirect effect by AMPK activation. It is also important to point that metformin have anti-inflammatory effects that are AMPK activation-independent (ŁABUZEK et al., 2010b).

In addition, evidences suggest that neurotoxicity involves activation of NMDA receptors by glutamate, production of NO by nNOS and iNOS, oxidative injury to DNA, and activation of the DNA damage-sensing enzyme poly (ADP-ribose) polymerase (PARP) (ARRICK et al., 2007; DAWSON; DAWSON, 2018; JANGRA; DATUSALIA; SHARMA, 2014; MANUCHA, 2017). Our results indicate that the production of nitric oxide in the brain during diabetes is elevated and is due to iNOS and nNOS expression. In contrast, the treatment with metformin significantly reduced the NO levels and iNOS expression in diabetic mice, indicating that the modulation of iNOS by metformin plays a central role in reversal of nitrosative stress. Besides, hyperglycemic conditions induces the production of AGE, stimulating the inflammatory response mediated by NFκB (EVANS et al., 2003), one of the main pro-inflammatory transcription factor. Once NFκB is activated, it induces the expression of iNOS and pro-inflammatory cytokines, such as TNFα, MIP1α, IL-1 and IFNα (HAYDEN; GHOSH, 2008). In human neural stem cells (hNSCs) under AGE effects, there is increased iNOS expression and the treatment with metformin reduced iNOS levels by activation of AMPK (CHUNG et al., 2017). Likewise, previous studies related that metformin exerted similar effects to those found here on the eNOS and nNOS in brain of obese rats (DERKACH et al., 2015) and in others tissues (KIM et al., 2007; ZHANG et al., 2011).

Likewise, cytokines and β-amyloid and others inflammatory mediators induce high levels of NO due to expression of iNOS in astrocytes and microglia, inducing an excess of glutamate release in synaptic cleft and, consequently, excitotoxicity (MURPHY, 2000). Astrocytes are a type of glial cell that help the maintenance of the blood-brain barrier, play a role in energy support for neurons, induce neuronal synapses, besides regulating the glutamate neurotransmitter levels by uptake (PEKNY; WILHELMSSON; PEKNA, 2014) and, therefore, a dysfunctional activity of astrocytes can cause neuronal death (BÉLANGER; ALLAMAN; MAGISTRETTI, 2011; NEDERGAARD; DIRNAGL, 2005). On the other hand, the neurons contribute to total amyloid β load, which consequently activates astrocytes and microglia. By its turn, activated microglia releases pro-inflammatory cytokines that can activate astrocytes, which can produce amyloid β (FROST; LI, 2017). The brain of diabetic animals exhibit increased GFAP immunoreactivity, memory impairment, glutamatergic excitotoxicity and worsening of Alzheimer's disease markers (JAYANARAYANAN et al., 2013; JOLIVALT et al., 2010; NAGAYACH; PATRO; PATRO, 2014; WANG et al., 2010). Here, the STZ-induced diabetes caused memory impairment and elevated the expression of iNOS, NMDA receptor (GluN2A), GFAP (astrogliosis) and β amyloid, while metformin treatment counteracted these changes.

During the excitotoxicity glutamate activates the NMDA receptor and causes neuronal death through of translocation of a mitochondrial protein apoptosis inducing factor (AIF) from mitochondria to the nucleus and this translocation is dependent of PARP (BA; GARG, 2011; DAWSON; DAWSON, 2018). Besides, the NMDA receptor allows the Ca^{2+} influx, increases the nNOS expression and nitric oxide release. The peroxynitrite from a reaction between NO and superoxide anion impairs the mitochondrial function releasing the cytochrome C protein, which is an important apoptosis initiator (DAWSON; DAWSON, 2004; MANUCHA, 2017). In turn, the nitrosative stress causes damage in DNA and activates PARP, an enzyme responsible for repairing breaks in DNA. Data indicate that the pharmacological PARP inhibition also inhibits NFκB activation, suggesting a relationship between PARP activation and inflammation mediated by NFκB (GIANSANTI et al., 2010). Studies indicate that during STZ-induced diabetes, the expression of IL-1 and PARP is elevated on brain and is associated to alterations on memory or behavior (JANGRA; DATUSALIA; SHARMA, 2014; OLIVEIRA et al., 2016; SEUNG et al., 2018; SIMA et al., 2009). Neurons are cells that spend high amount of energy. However, they are not able to stock energy and are thus dependent of the supply of peripheral glucose. The mitochondria are

the organelle responsible for ATP production in neurons (AMATO; MAN, 2011). In diabetic conditions, the mitochondrial function is damaged, causing increased influx of Ca^{2+} from the extracellular milieu to neurons and reduction of the antioxidant defense, resulting in oxidative stress (SHOKRZADEH et al., 2018). Cytochrome c is a protein present in the mitochondrial membrane and in pathological conditions that causes damage to the mitochondria there is translocation of cytochrome c from mitochondria to cytoplasm, deflagrating cellular apoptosis by caspase-3 activation. Besides, cytochrome c can also activate PARP (AULSTON et al., 2013; DAWSON; DAWSON, 2004).

Here, the levels of PARP, cytochrome c, caspase 3 and IL-1β were increased in diabetic mice, which can be correlated to the low expression of CREB in the cerebral cortex and the memory impairment observed in Barnes maze test. Conversely, treatment with 200 mg/kg of metformin reversed all these changes inhibiting neuronal apoptosis, as well as increasing CREB levels and cognition function in diabetic mice. Thus, since metformin reduced the hallmarks of AD, this molecule is a possible therapeutic alternative to prevent AD in diabetic patients or to be as adjunctive therapy with standard drugs used in the AD treatment.

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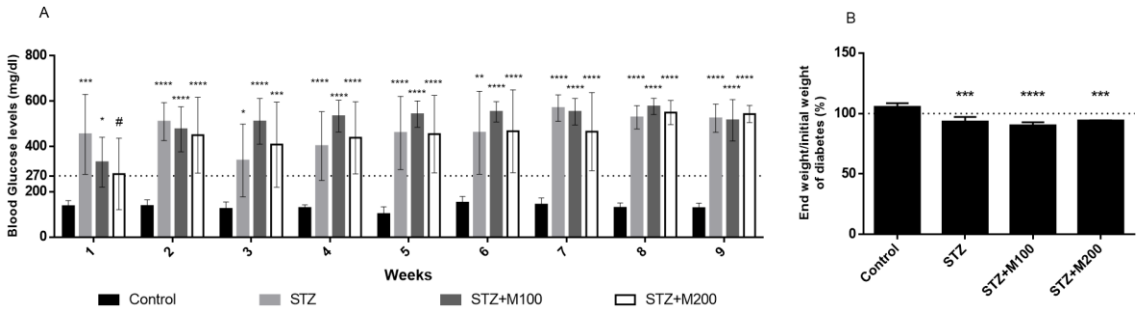

Figure 1. Effects of metformin on fasting glycemia after 4 hours of fasting and administration of metformin (A) and on body weight at the end of study related to its beginning. Data are presented as mean ± SD. * p<0.05, *** p<0.001 and **** p<0.0001 when compared to control group and # p<0.05 when compared to STZ group using analysis of variance (ANOVA) followed by Tukey's post hoc test.

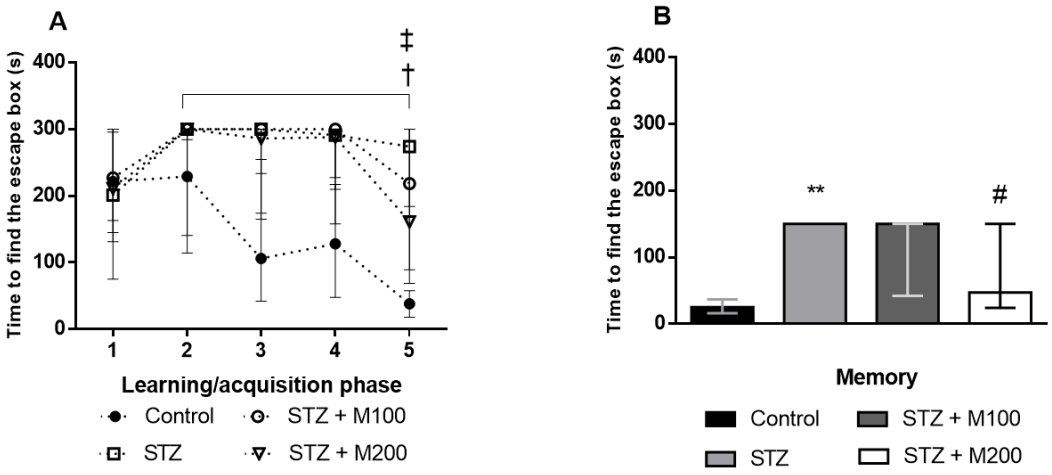

Figure 2. Effects of metformin on learning and spatial memory. Data are presented as median ± interquartile range. A) Analysis of time to find the escape Box (s) on Barnes Maze task during the learning/acquisition phase. Non-parametric data analyzed by Kruskal-Wallis followed by post test Dunn's. † p<0.05 when control vs. control group on the second day. ‡ p<0.05 when STZ+M200 vs. STZ+M200 group on the second day. ** p<0.01 and # p<0.05 when compared to control group and STZ, respectively.

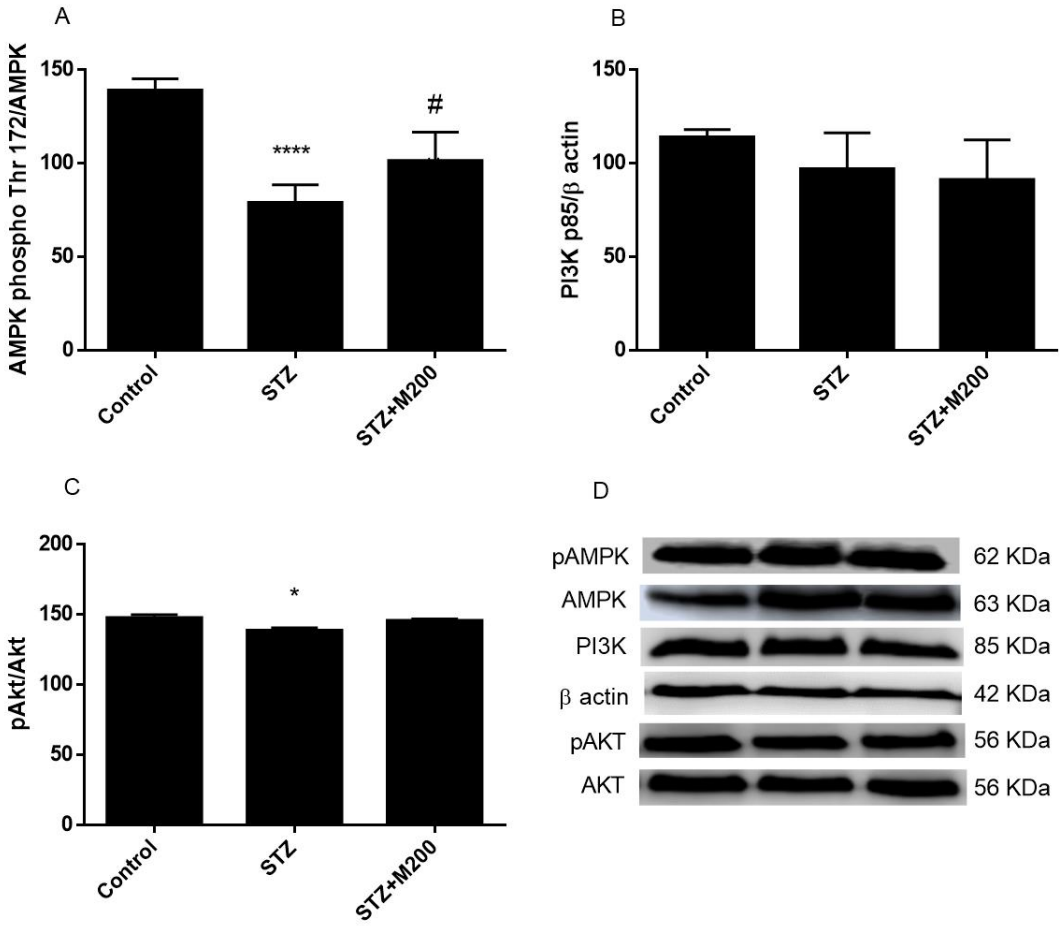

Figure 3. Western blot for (A) PI3K p85. (B) phosphorylated Akt and (C) p-AMPK. * $p<0.05$ when compared to control, **** $p<0.0001$ when compared to control and # $p<0.05$ when compared to STZ group using analysis of variance (ANOVA) followed by Tukey's post hoc test. All the data are presented as mean ± SD.

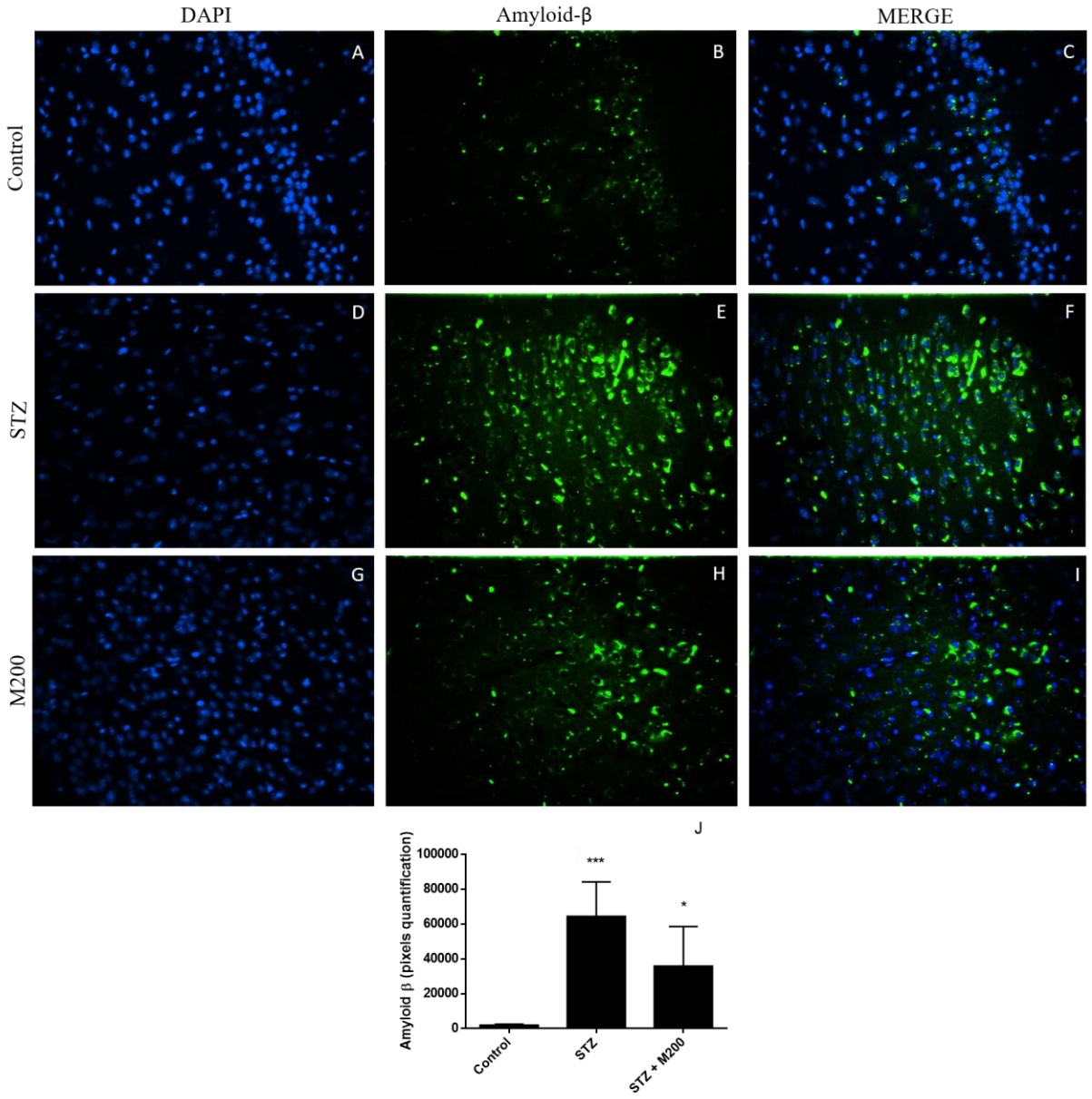

Figure 4. Immunofluorescence for amyloid β. Data are presented as mean ± SD. ***p<0.001 and * p<0.05 when compared to control group. Magnification of 40x.

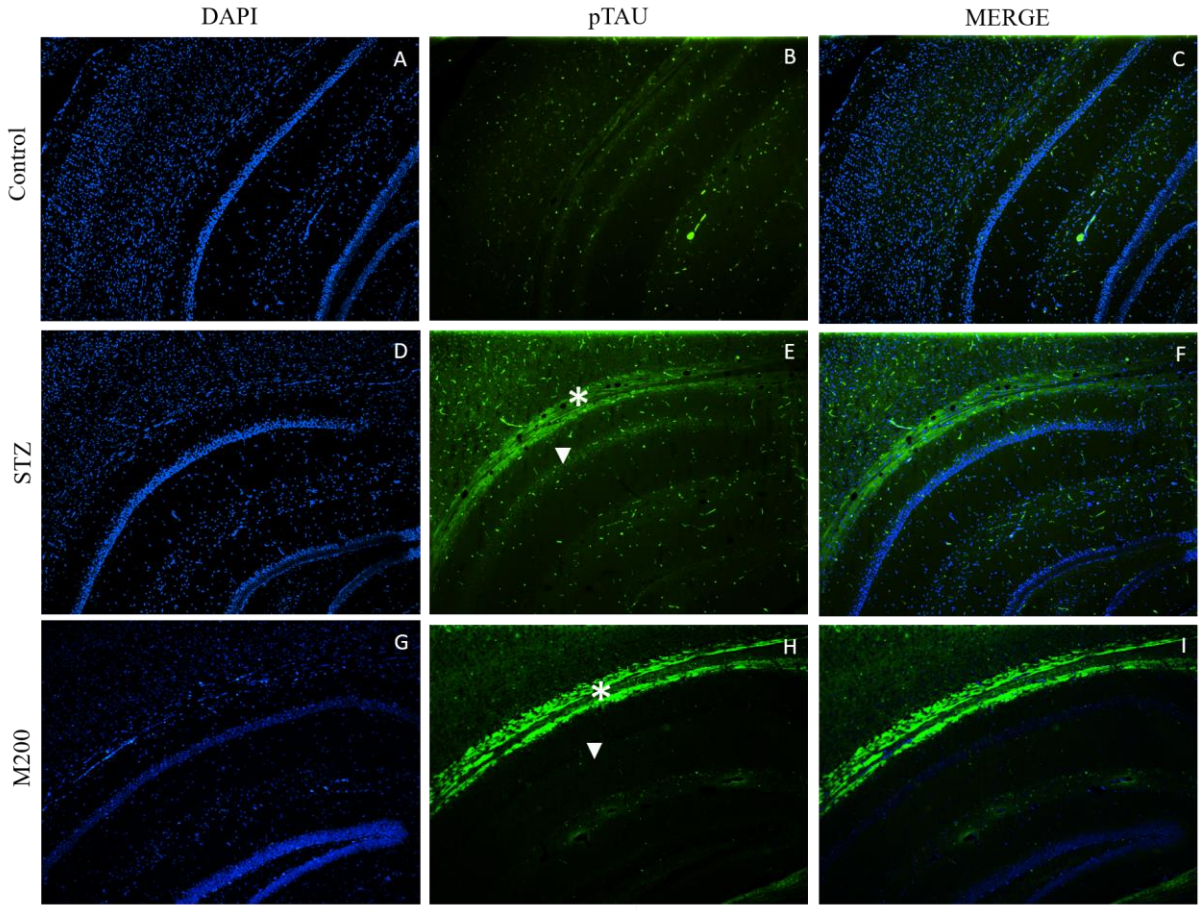

Figure 5. Immunofluorescence for pTAU. Data are presented as mean ± SD. ***p<0.001 and * p<0.05 when compared to control group. Asterisk indicates corpus callosum and arrowhead indicates CA1 areas. Magnification of 10x.

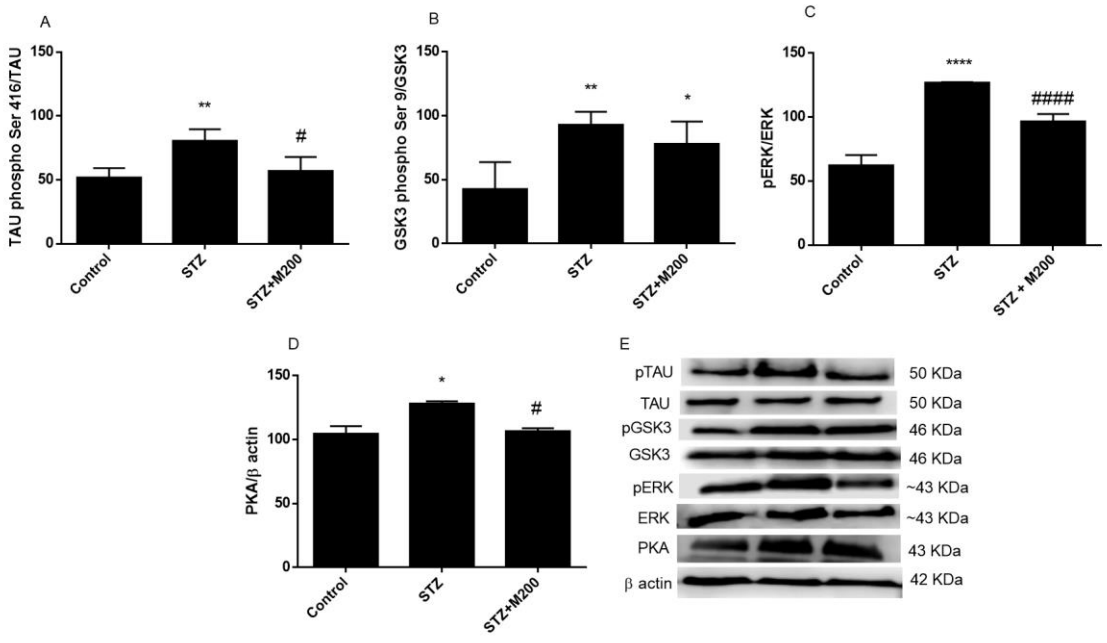

Figure 6. Effects of metformin on TAU kinases: pGSK3 (A), pERK (B) and PKA (C) assessed by immunoblot. *p<0.05, **p<0.01 and **** p<0.0001 when compared to control. #p<0.05 and ####p<0.0001 when compared to STZ group. Analysis of variance (ANOVA) followed by Tukey's post hoc test. All the data are presented as mean ± SD.

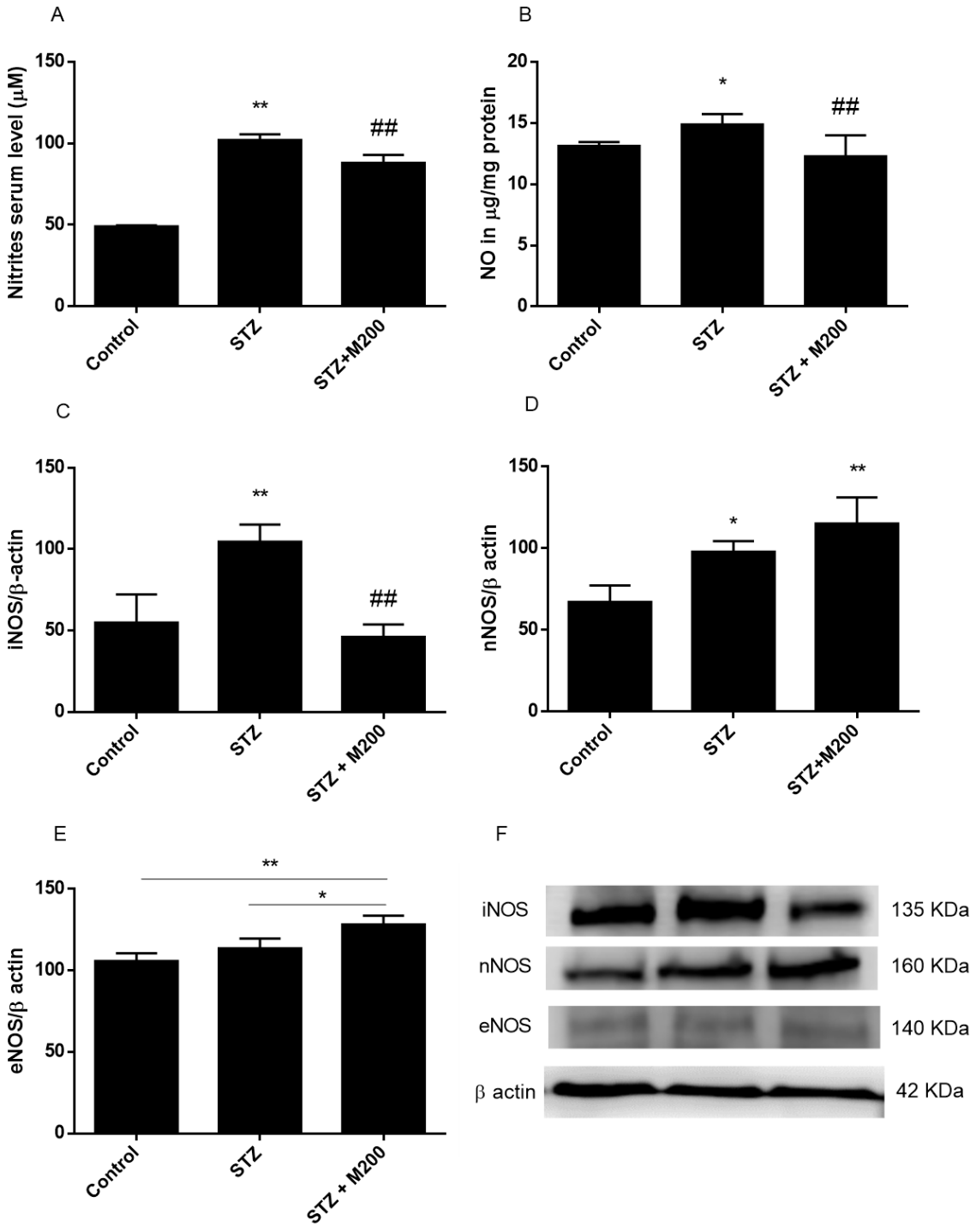

Figure 7. Effects of metformin on nitric oxide in serum (A) and brain (B) through of the measure of total nitrite metabolites, and on iNOS (C), nNOS (D), eNOS (E) assessed by

immunoblot (F). * p<0.05 and ** p<0.01 when compared to control group and ## p<0.01 when compared to STZ group using analysis of variance (ANOVA) followed by Tukey's post hoc test. All the data are presented as mean ± SD.

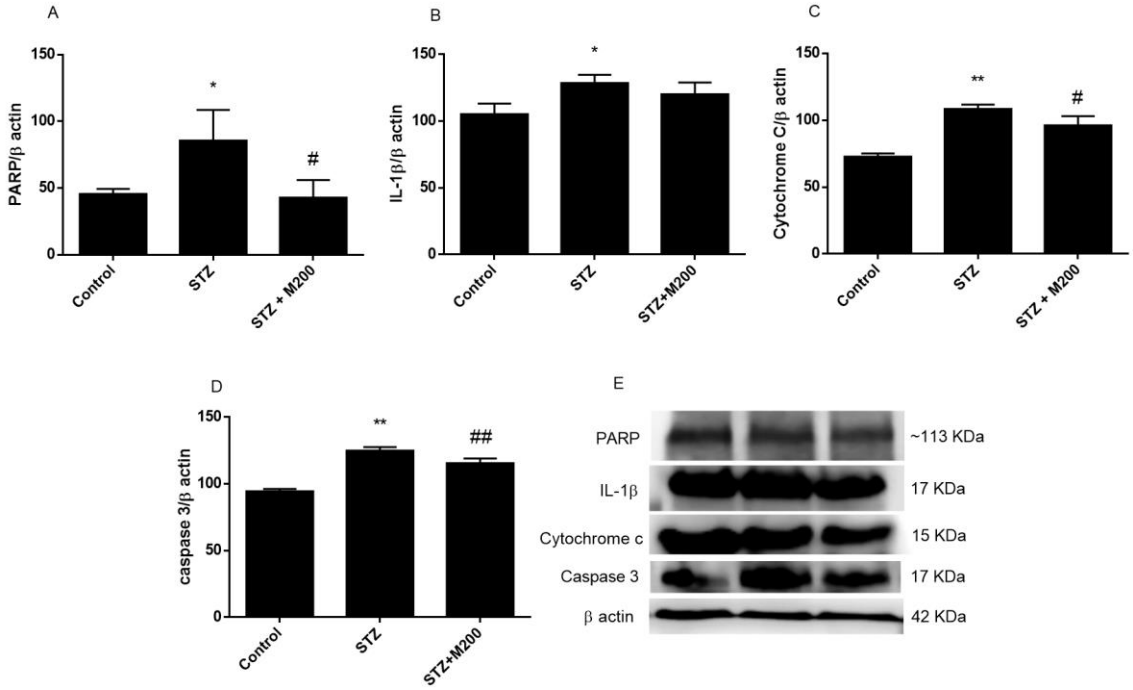

Figure 8. Western blot (E) for PARP (A), IL-1β (B), cytochrome c (C) and caspase 3 (D). * p<0.05 and **p<0.01 when compared to control group. #p<0.05 and ##p<0,01 when compared to STZ group. Analysis of variance (ANOVA) followed by Tukey's post hoc test. All the data are presented as mean ± SD.

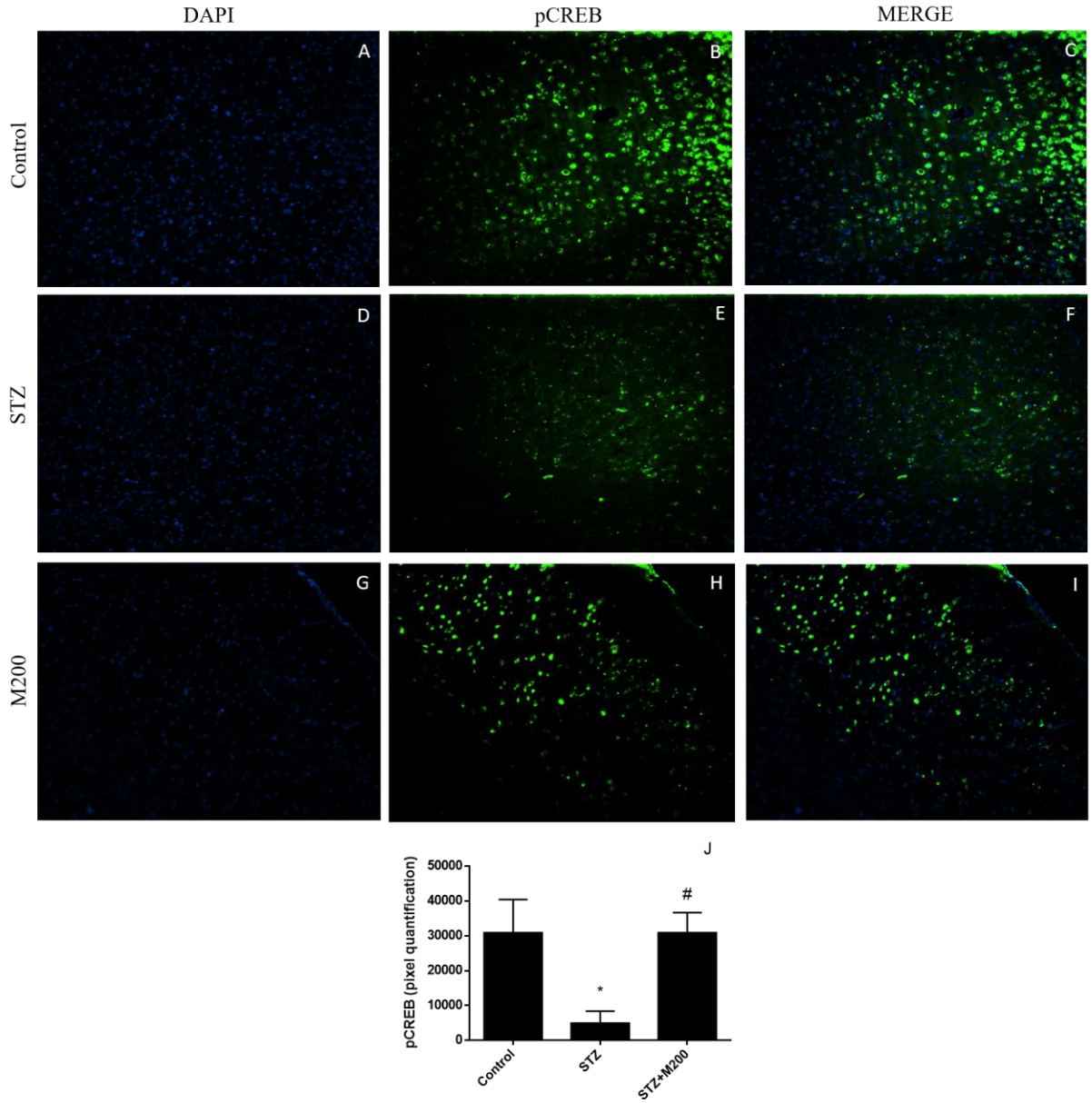

Figure 9. Immunofluorescence for p-CREB in cerebral cortex of control group (A-C), STZ group (D-F), M200 group (G-I) and pixels quantification (J). *** $p<0.001$ when compared to control group and ### $p<0.001$ when compared to STZ group using analysis of variance (ANOVA) followed by Tukey's post hoc test. Data are presented as mean ± SD.

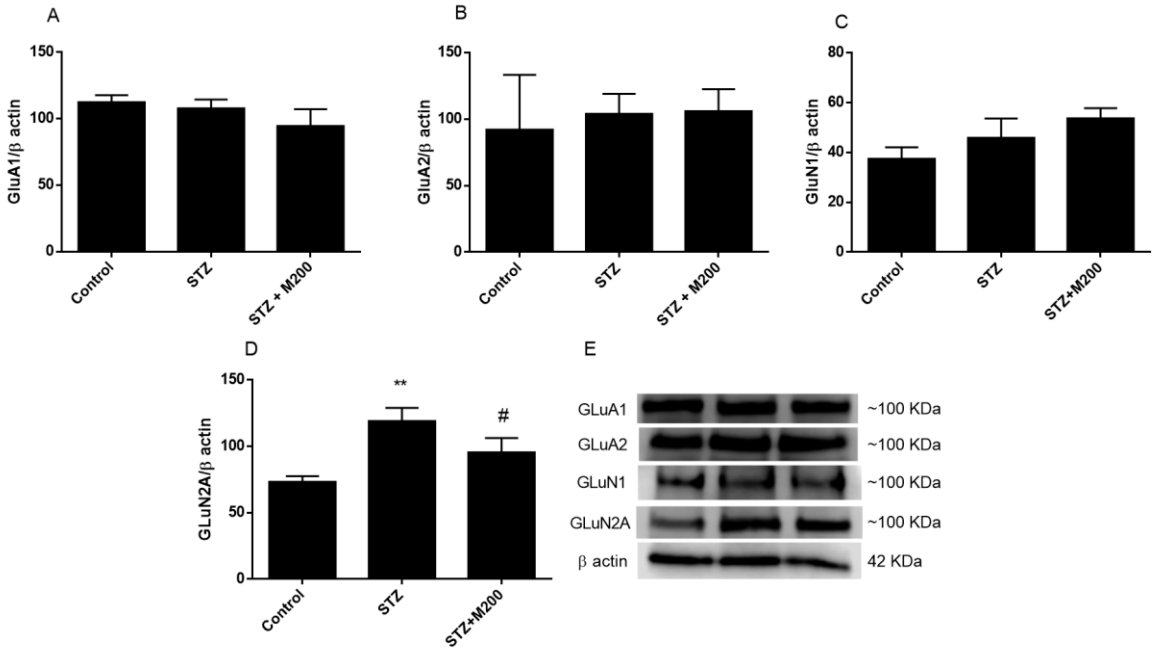

Figure 10. Western blot for glutamatergic receptors. Expression of GluA1 (A), GluA2 (B), GluN1 (C) and GluN2A (D). Immunoblot bands (E). Data are presented as mean ± SD. **$p<0.01$ when compared to control group and $^{\#}p<0.05$ when compared to STZ group. Analysis of variance (ANOVA) followed by Tukey's post hoc test.

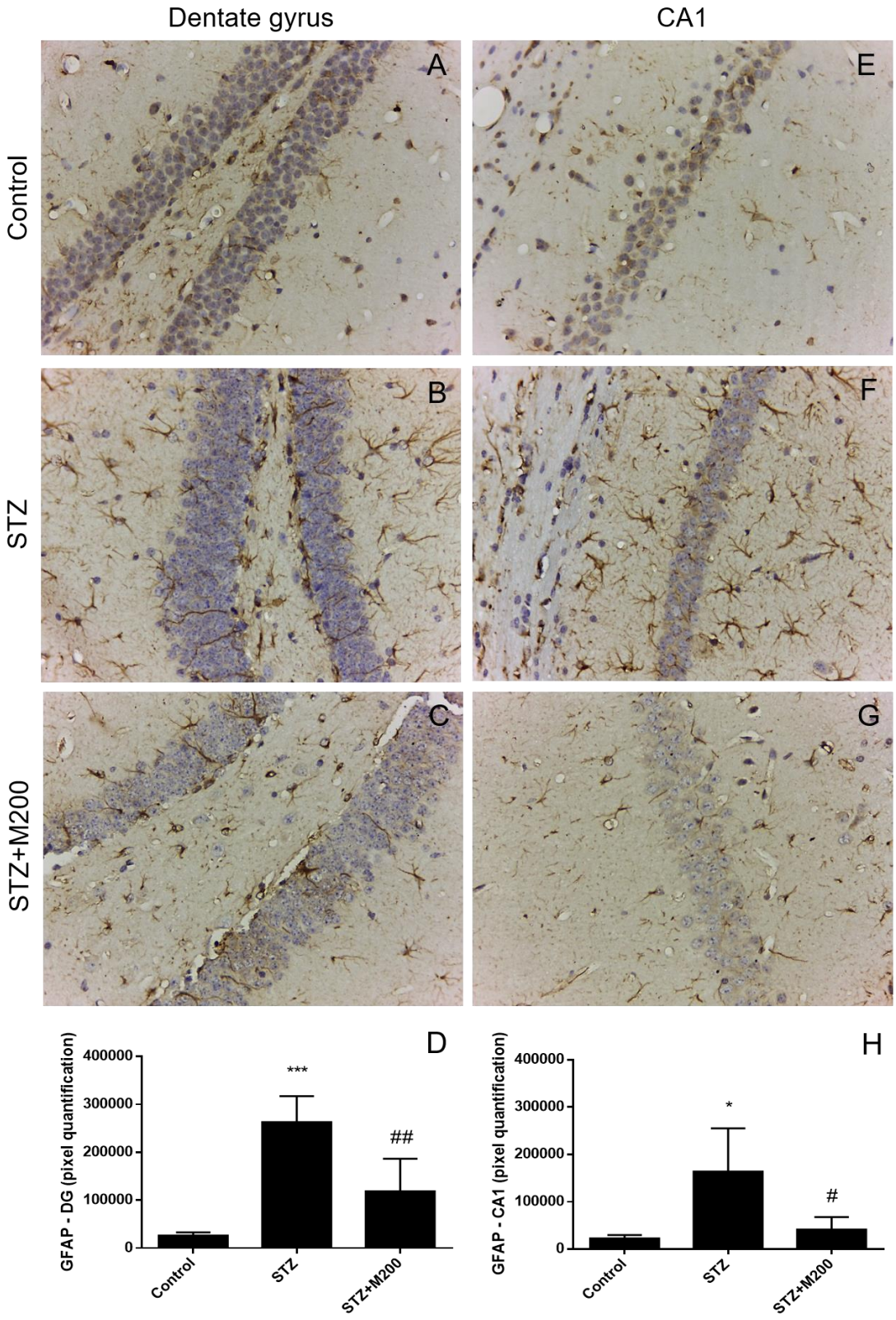

Figure 11. Effects of metformin on hippocampal astrocytes. Immunohistochemistry for GFAP in dentate gyrus (A, B and C) and CA1 (E, F and G) with magnification of 400x. Analysis of variance (ANOVA) followed by Tukey's post hoc test for the pixel quantification of GFAP in

dentate gyrus and CA1 are represented in D and H, respectively. Data are presented as mean ± SD. $^{*}p<0.05$ and $^{***}p<0.001$ when compared to control group, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ when compared to STZ group.

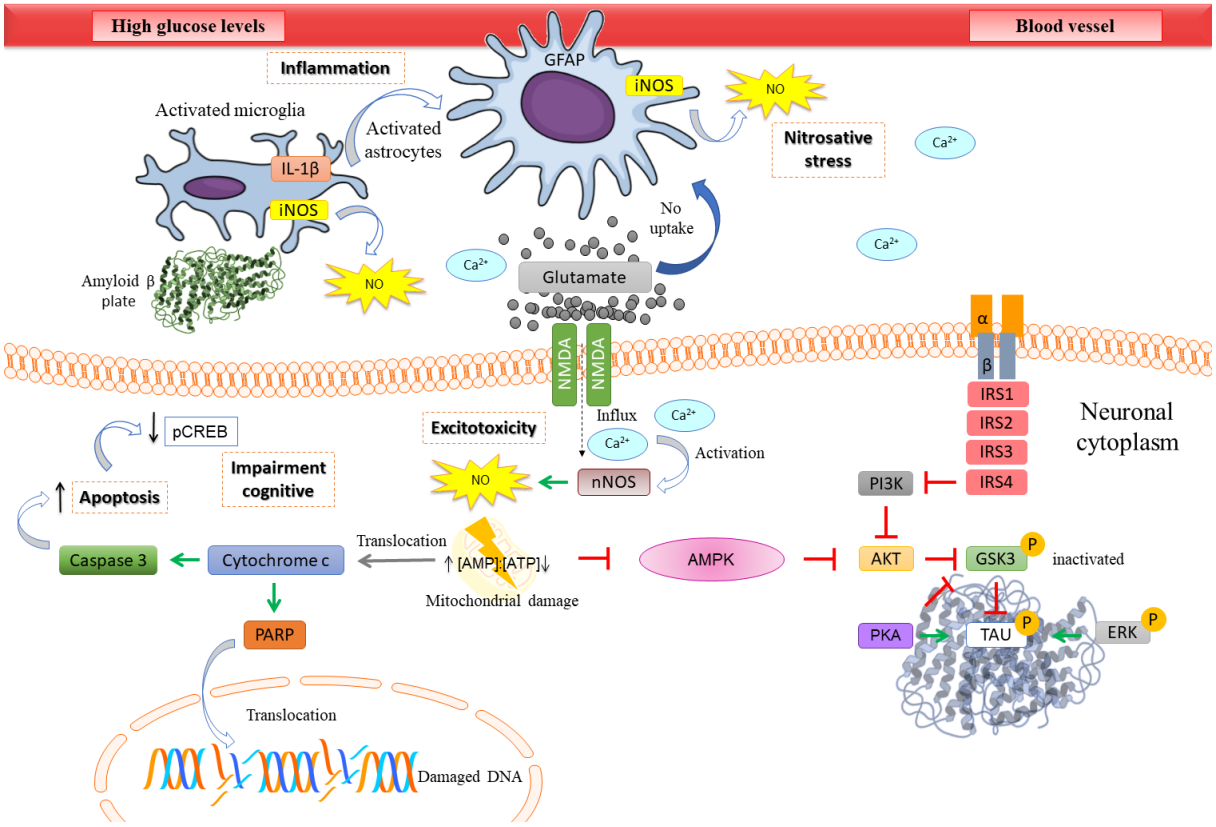

Figure 12. Schematic summarizing the effects of STZ-induced diabetes in the brain. Green arrows indicate activation and red bar-headed line indicates inhibition (see text for details).

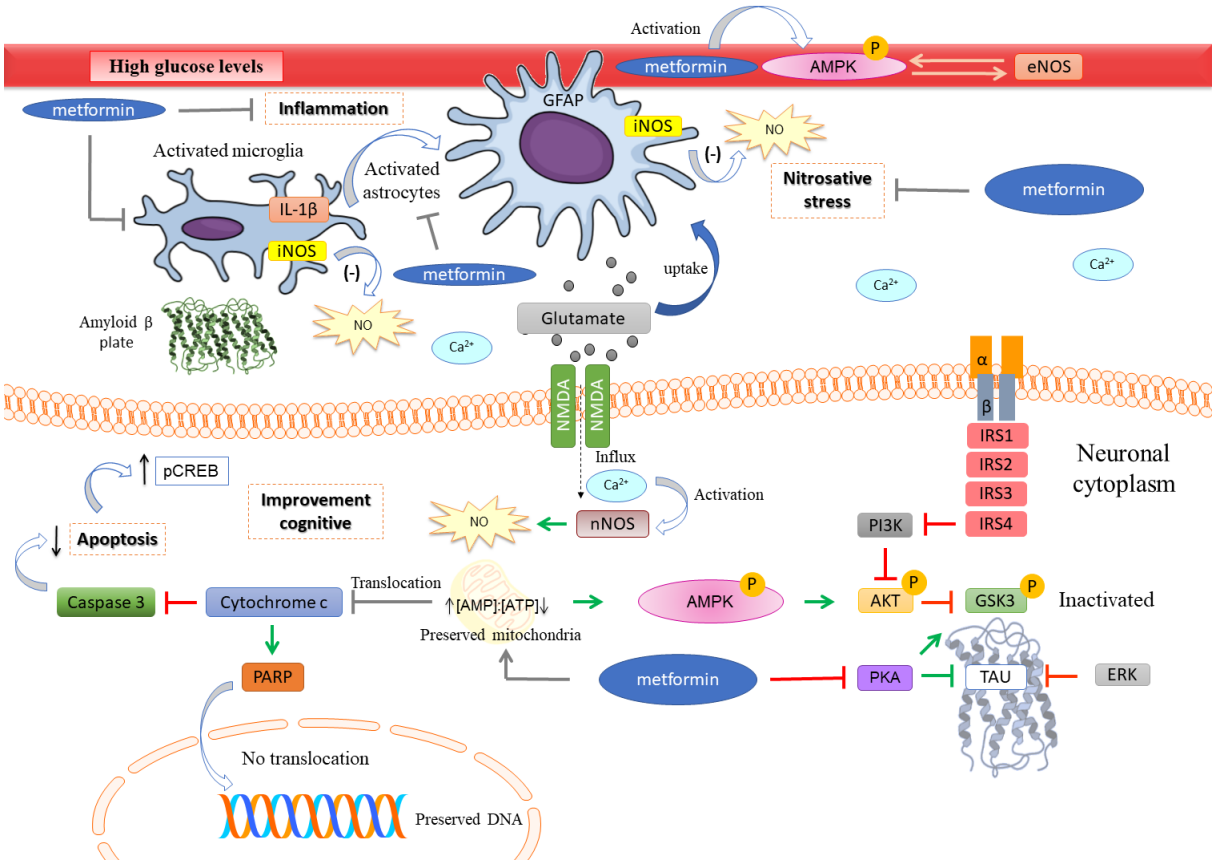

Figure 13. Scheme showing the effects of metformin in the brain of STZ-induced diabetic mice. Green arrows indicate activation and red bar-headed lines indicates inhibition (see text for details).

4. CONCLUSÃO

Após nove semanas de diabetes foi observado que os cérebros dos camundongos apresentaram aumento da expressão dos marcadores da doença de Alzheimer, Tau fosforilada e β amiloide, além de danos no aprendizado e na memória espacial. Também foi observado dano na cascata de sinalização da insulina cerebral (PIK3/Akt), entretanto a deficiência desta sinalização não foi a causa da hiperfosforilação da proteína Tau uma vez que a quinase de Tau presente na cascata de sinalização da insulina, GSK3β, apresentou atividade reduzida. Por sua vez, a atividade das proteínas quinases da Tau (ERK e PKA) apresentaram expressão aumentada. A sinalização da ERK e PKA no cérebro é importante para o processo de formação da memória, contudo pelo efeito que elas exercem sobre a fosforilação da Tau, a superexpressão dessas proteínas passa de benéfico para prejudicial no processo de formação da memória em camundongos diabéticos.

Como consequência do acúmulo de placas β amiloides, houve aumento da atividade glial com liberação de proteínas inflamatórias (Il-1 e iNOS). O óxido nítrico é um segundo mensageiro que participa do processo de formação da memória, entretanto seus níveis elevados como os observados neste estudo, indicou que o óxido nítrico produzido pela iNOS e nNOS causou danos neuronais. E ainda, a produção de óxido nítrico aumentou a liberação de glutamato, a atividade dos receptores NMDA com consequente aumento de cálcio intracelular e causou danos nas mitocôndrias e no DNA (PARP).

Durante a diabetes foi observado que o dano mitocondrial, além de prejudicar o fornecimento de energia para a célula, prejudicando assim a atividade neuronal, ativou a sinalização da apoptose celular (Citocromo c e caspase-3), reduzindo marcação da CREB fosforilada.

Em contrapartida, o tratamento com metformina ativou o AMPK e foi capaz de promover melhora da aquisição da memória, reduziu os danos neuronais pela inibição da apoptose e aumento da marcação da CREB fosforilada, reduziu a produção de óxido nítrico pela inibição da iNOS, mesmo sem reduzir a atividade da nNOS, e ativando a eNOS. Além disso, diminuiu a atividade do receptor NMDA, reduziu os níveis de Tau fosforilada pela inibição da atividade da PKA e ERK, e reduziu a imunolocalização da β amiloide. Dessa forma, o tratamento com metformina pode ser considerado como uma alternativa terapêutica

preventiva da progressão dos danos de memória ou da progressão para a doença de Alzheimer em pacientes diabéticos.

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APÊNDICE A – ARTIGO 1 - AMPK ACTIVATION: ROLE IN THE SIGNALING PATHWAYS OF NEUROINFLAMMATION AND NEURODEGENERATION

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Review Article

AMPK activation: Role in the signaling pathways of neuroinflammation and neurodegeneration

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ABSTRACT

Adenosine monophosphate-activated protein kinase (AMPK) is an evolutionarily conserved sensor of cellular energy status and has been reported to be involved in chronic inflammatory disorders. AMPK is expressed in immune cells, such as dendritic cells, macrophages, lymphocytes and neutrophils, and is an important regulator of inflammatory responses through the regulation of complex signaling networks in part by inhibiting downstream cascade pathways, such as nuclear factor kB, which is a key regulator of innate immunity and inflammation, as well as acting as a negative regulator of toll-like receptors. Recent data suggest that AMPK dysregulation may participate in neurodegenerative diseases, such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and neuropathies. However, there are conflicting reports on the benefits or detrimental effects of AMPK in distinct pathological conditions. This paper offers a review of the recent literature on the pharmacological modulation of the AMPK system as a potential molecular target in the management of neurodegenerative diseases.

1. AMPK: structure, functions and activators

Adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase that plays a central role in the regulation of energy metabolism. AMPK is a heterotrimeric protein consisting of three subunits: a catalytic α subunit and regulatory β and γ subunits. Different α (α1 and α2), β (β1 and β2) and γ (γ1- γ2- γ3) isoforms are expressed in different mammalian tissues (Hardie, 2015, 2007; Lage et al., 2008), with the ubiquitous expression of the α1-β1-γ1 complex in many tissues (Kim et al., 2016).

Under conditions of energy deprivation (ATP depletion and increase in AMP), AMPK acts as an "adenylate charge" regulatory kinase that inhibits anabolic pathways that consume ATP, such as lipid and protein synthesis, and stimulates catabolic pathways that produce ATP, such as fatty acid oxidation and mitochondrial oxidative phosphorylation (Steinberg and Kemp, 2009). In ATP-depleted conditions, the increased concentration of AMP binds to the AMPK γ-subunit and activates kinase activity in the α-catalytic subunit (Hardie et al., 2003; Xiao et al., 2013). The allosteric activation of AMPK by AMP makes the enzyme a much better substrate for upstream AMPK kinases (AMPKKs) and worse substrate for protein phosphatases. The phosphorylation of threonine residue (Thr172) of the α-catalytic subunit is a more potent modulator of AMPK activity than allosteric activation alone, increasing it nearly 100-fold. Three AMPKKs can phosphorylate Thr172: the upstream tumor suppressor liver kinase B1 (LKB1) (Alessi et al., 2006; Hawley et al., 2003), the Ca2 + /calmodulin-dependent protein kinase kinaseβ (CaMKKβ) (Hurley et al., 2005) and the transforming growth factor-β-activated kinase 1 (Tak1 kinase) (Momcilovic et al., 2006). AMP also regulates the rate of AMPK activation by directly inhibiting the protein phosphatases PP2A and PP2C, which are responsible for removing phosphate from the Thr172 site and maintaining AMPK in its inactive state (Carling et al., 2012; Sanders et al., 2007).

During exercise and fasting, AMPK promotes glucose uptake through the phosphorylation of Akt and other enzymes necessary to the translocation of glucose transporter type 4 (GLUT4) and plays a complex role in increasing insulin sensitivity. AMPK also stimulates fatty acid oxidation by phosphorylating and inactivating acetyl CoA carboxylase (ACC), which converts acetyl-CoA into malonyl-CoA, which is the regulator of the switch between fatty acid synthesis and oxidation (Foster, 2012; Kahn et al., 2005). Therefore, the inactivation of ACC by

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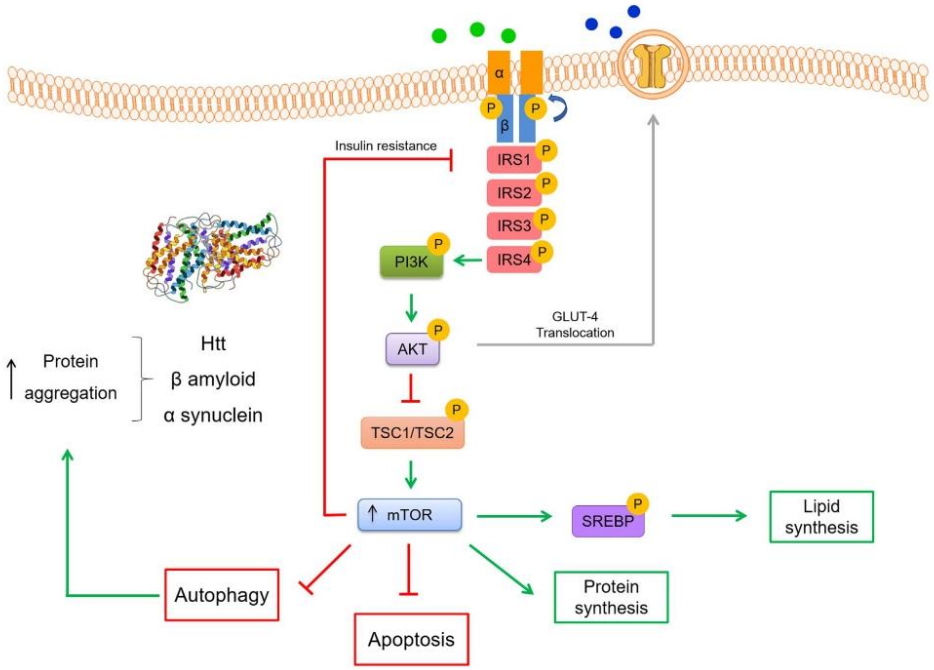

Fig. 1. Schematic summarizing insulin-induced mTOR regulation. Green arrows indicate activation and red bar-headed line indicates inhibition (see text for details). Green circles represent insulin and blue circles represent glucose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

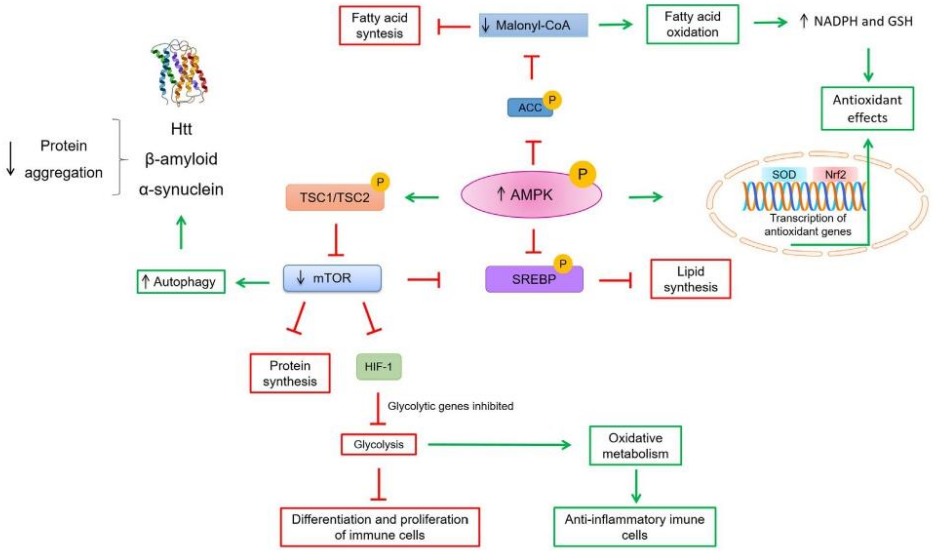

Fig. 2. Schematic representation of AMPK-dependent metabolic pathways. Arrows indicate activation; bar-headed line indicates inhibition.

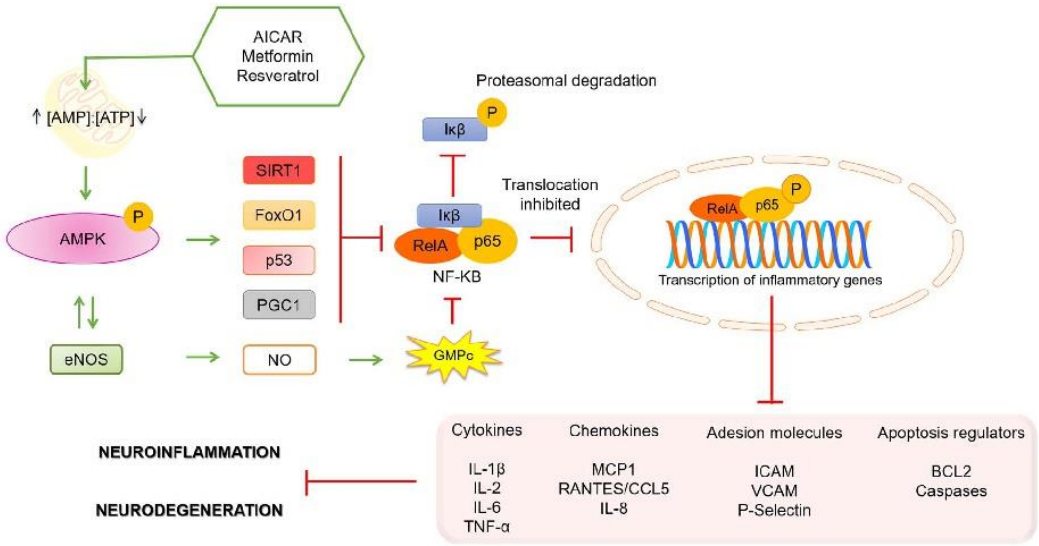

Fig. 3. AMPK role in NF-κB signaling pathway. Green arrows indicate activation; red bar-headed line indicates inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AMPK results in increased fatty acid transport into mitochondria and subsequent oxidation, which maintains NADPH and GSH levels (antioxidant defense). Moreover, AMPK upregulates several antioxidant genes (superoxide dismutase, uncoupling protein 2) by activating the nuclear factor E2-related factor 2 (Nrf2), which is a master regulator of the antioxidant response (Jeon, 2016). AMPK also phosphorylates and decreases the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c), thereby reducing the expression of lipogenic genes (Steinberg and Kemp, 2009).

In addition, AMPK reduces protein synthesis and stimulates apoptotic and autophagic pathways through the inhibition of the mechanistic target of rapamycin (mTOR), which regulates cellular metabolic homeostasis, insulin secretion, insulin resistance, autophagy and apoptosis (Maiese, 2016). mTOR is the central component of the protein complexes mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is activated by insulin, growth factors and nutrients, resulting in increased translation. Insulin induces mTORC1 activity by inhibiting the tumor suppressor complex (TSC1/TSC2), which is an endogenous mTORC1 repressor (Fig. 1). In contrast, AMPK inhibits mTORC1 signaling by phosphorylating TSC1/TSC2 as well as phosphorylating and promoting the dissociation of the protein raptor, which is one of the mTORC1 components (Foster, 2012; Hasenour et al., 2013; Steinberg and Kemp, 2009). During regular exercise, AMPK mediates fat oxidation by inhibiting ACC and decreases both lipogenesis and protein synthesis through the inhibition of SREBP-1c and mTORC1, respectively. Interestingly, autophagy is involved in the degradation and removal of aggregated proteins, the impairment of which causes neuronal cell death. The inhibition of constitutive autophagy leads to neurodegeneration and mTORC1 has been implicated as having a detrimental role in the autophagic process associated with several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) (Kiriyama and Nochi, 2015). Consequently, AMPK could emerge as a therapeutic target for these neurodegenerative diseases also by acting as a modulator of the autophagic process (Fig. 2).

AMPK is also thought to play an important role in inflammation and insulin resistance and, consequently, the pathogenesis of type 2 diabetes, nonalcoholic fatty liver disease and hypertension. An association has been found between the activation of inflammatory genes and a decrease in AMPK activity due to elevated oxidative stress in adipose tissue of insulin-resistant patients (Xu et al., 2012). Moreover, AMPK activation in patients with obesity or type 2 diabetes is attenuated after exercise, indicating that chronic metabolic syndrome may result in AMPK dysregulation (Sriwijitkamol et al., 2007).

Clinical studies indicate that higher glucose levels are associated with an increased risk of dementia, even at the lowest end of the glucose spectrum among individuals who have not been diagnosed with diabetes (Crane et al., 2013). Other studies provide evidence of an association between insulin resistance in type 2 diabetes and an increased incidence of both dementia (Qiu et al., 2014) and Alzheimer's disease (Schrijvers et al., 2010). Recently, elegant studies have demonstrated that patients with preclinical Alzheimer's exhibit dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes (Kapogiannis et al., 2015). All these data demonstrate that degeneration in the nervous system may be the result of impaired cellular metabolism similar to what occurs in patients with diabetes mellitus.

Direct and indirect AMPK activators have been proposed as novel therapeutic tools for metabolic syndrome, type 2 diabetes, atherosclerosis and cancer. The direct AMPK activators [thienopyridone (A-769662), benzimidazole (compound 911), compound-13 and salicylate derivatives] lead to activation, possibly through a direct interaction with a specific subunit of AMPK. Indirect AMPK activators (metformin, thiazolidinediones, polyphenols and α-lipoic acid) cause changes in the cellular AMP/ATP ratio by inhibiting complex I of the mitochondrial respiratory chain or mitochondrial F1F0-ATPase/ATP synthase (Kim et al., 2016; Luengo et al., 2014). However, little is known regarding potential AMPK activators, such as anti-inflammatory drugs, in neurodegenerative disorders.

2. Role of AMPK in inflammation signaling

Pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), activate Ikβ kinase (IKKβ), which phosphorylates IκBα, triggering the degradation of proteasomal IκB. This liberates active nuclear factor kB (NF-kB) to translocate into the nucleus and promote inflammatory target gene expression in a positive feedback loop that leads to a further increase in inflammation. Interestingly,

several studies have demonstrated that AMPK indirectly inhibits NF-kB activation through multiple downstream pathways that suppress the expression of inflammatory genes, including the activation of sirtuin 1 (SIRT1), FOXO and PGC1α (Jeon, 2016) (Fig. 3).

SIRT1 is a class of NAD + dependent histone deacetylases that regulates critical physiological processes, including glucose/lipid metabolism, fatty acid oxidation, autophagy/apoptosis and senescence, through its deacetylase action on many signaling proteins and histones, promoting chromatin condensation and thereby silencing gene transcription (Chung et al., 2010; Xie et al., 2013). SIRT1 has a crosstalk with AMPK in the regulation of oxidative metabolism and inflammation. SIRT1 promotes the deacetylation of LKB1, which consequently triggers AMPK activation. In turn, AMPK increases cellular NAD + levels, inducing the activation of SIRT1, which directly inhibits NF-κB signaling through the deacetylation of p65 (Chung et al., 2010; Salminen et al., 2008; Xie et al., 2013).

AMPK/p53/NF-κB and AMPK/FoxO/NF-κB signaling are other possible anti-inflammatory pathways. Both p-53 and FoxO are transcription factors involved in the regulation of energy metabolism, cell growth and inflammation (Peng, 2008; Salminen and Kaarniranta, 2011). AMPK can activate the function of these factors through direct phosphorylation, which consequently inhibits NF-κB signaling (Greer et al., 2007; Maclaine and Hupp, 2009; Salminen and Kaarniranta, 2011).

AMPK also modulates the NF-kB pathway by phosphorylating eNOS at Ser1177, which contributes an increase in NO production. A positive feedback loop between AMPK and eNOS has also been described, in which the phosphorylation of eNOS implies the activation of AMPK (Ewart and Kennedy, 2011; Hattori et al., 2008; Zhang et al., 2006).

Activated pro-inflammatory immune cells mainly generate energy through glycolysis, whereas anti-inflammatory immune cells predominantly generate energy through oxidative phosphorylation. AMPK is critical to the switch between oxidative *versus* glycolytic metabolism and has therefore been implicated in regulating metabolic processes that direct immune function. AMPK/LKB1 acts as a key metabolic control in activated T cells by suppressing nutrient uptake, energy production (glycolysis) and biosynthesis (mTOR), which may regulate the transition from CD8 + T effector cells to CD8 + T memory cells as well as from CD4 + T effector cells (Th1, Th2, and Th17) to CD4 + regulatory T cells. Moreover, the silencing of LKB1 or AMPK expression promotes the development of pro-inflammatory Th cells (see review in Blagih et al., 2012). One possible mechanism involved in this transition from glycolytic to mitochondrial metabolism is the inhibition of mTORC1 by AMPK, which attenuates the hypoxia-inducible factor 1 (HIF-1)-mediated transcription of glycolytic genes, promoting oxidative metabolism (Fullerton and Steinberg, 2013) (Fig. 2).

There is evidence that AMPK regulates the metabolic processes that direct immune function in macrophages, T cells and dendritic cells. Interesting studies using RNAi or adenovirus expressing dominant negative and constitutively active AMPKα1 have demonstrated that AMPK activation prevents the lipopolysaccharide (LPS)-induced and fatty acid-induced production of inflammatory cytokines. Galic et al. (2011) found that the genetic deletion of AMPKβ1 reduced macrophage AMPK activity, ACC phosphorylation and mitochondrial enzyme content, resulting in increased macrophage lipid accumulation and inflammation. Similarly, the adoptive transfer of $\beta1^{-/-}$ bone marrow into wild type (WT) recipient mice resulted in the activation of adipose tissue macrophages, leading to systemic inflammation, hyperinsulinemia and hyperglycemia. These studies demonstrate that AMPK is crucial to suppressing lipid-induced inflammation and the development of obesity-induced insulin resistance.

RNA interference assays have shown that the inhibition of AMPK expression leads to increases in TNF-α, IL-6 and cyclooxygenase-2 (COX-2) levels after LPS stimulus, whereas the transfection of macrophages with a constitutively active form of AMPKα1 results in decreased LPS-induced TNF-α and IL-6 production as well as the heightened production of IL-10. AMPK acts as a potent counter-regulator of macrophage inflammatory function and promoter of macrophage polarization toward an anti-inflammatory phenotype. Furthermore, anti-inflammatory activity of AMPK in macrophages is associated with reduced IκB degradation, enhanced Akt activity and the inactivation of glycogen synthase kinase 3 β (GSK3-β). It is thought that GSK3-β inhibition allows the cAMP response element binding (CREB) protein to compete for the nuclear coactivator protein CBP (CREB-binding protein) required for NF-κB function, which reduces the expression of pro-inflammatory genes (Sag et al., 2008).

Other data indicate that AMPK acts as a negative regulator of toll-like receptor-induced inflammatory function. Carroll et al. (2013) found that macrophages and dendritic cells generated from AMPKα1-deficient mice exhibited heightened inflammatory function and an enhanced capacity for antigen presentation, thereby stimulating Th1 and Th17 responses. In antigen presentation assays, bone marrow-derived dendritic cells and macrophages generated in AMPKα1-deficient mice induced significantly higher T cell-produced IL-17 and interferon (IFN)-γ levels. Moreover, CD40 stimulation in dendritic cells deficient for AMPKα resulted in the increased phosphorylation of extracellular signal-regulated kinases (ERK) 1 and 2, p38 and NF-kB p65 and decreased activation of the anti-inflammatory Akt-GSK3β-CREB pathway. These results suggest that the dysregulation of AMPK could play an important role in the development of autoimmune diseases as the result of increased interactions during initial antigen-presenting cell (APC) events as well as the T cell activation of myeloid cells at inflammation sites.

3. Role of AMPK in neuroinflammation

Some studies indicate that AMPK activation has a potential therapeutic effect on neuroinflammation of the central nervous system. Meares et al. (2013) demonstrated that AMPK activation blocks IFN-γ-induced gene expression, including CCL2, TNF-α, CXCL10 and inducible nitric oxide synthase (iNOS), in primary astrocytes and microglia through the modulation of signal transducer and activator of transcription 1 (STAT1). Likewise, the deletion of AMPKα1 and AMPKα2 in primary astrocytes enhances STAT1 expression, leading to the production of pro-inflammatory cytokines and chemokines. The authors also found that AMPK signaling in experimental autoimmune encephalomyelitis (EAE) is downregulated in the brain at the onset and peak of the disease, which is correlated with the increased expression of IFN-γ and CCL2 in the central nervous system. These results highlight the interaction between AMPK and STAT1 and provide evidence of how bioenergetics and inflammation are related.

Other studies have shown that high levels of AMPK are present in embryonic hippocampal neurons *in vivo* and in cell cultures. In hippocampal neuron cultures, the AMPK-activating agent AICAR protected neurons against death induced by glucose deprivation, chemical hypoxia and exposure to glutamate and amyloid β-peptide. Suppression of the expression of the AMPK α1 and α2 subunits using antisense oligonucleotides resulted in enhanced neuronal death and abolished the neuroprotective effect of AICAR. Based on these results, AMPK protects neurons against metabolic and excitotoxic glutamate insults related to neurodegenerative conditions (Culmsee et al., 2001).

Chen et al. (2014) demonstrated that ENERGI-F704, which is a direct AMPK agonist, exerts inhibitory activity on LPS-induced inflammation. Treatment of LPS-stimulated microglia BV2 with ENERGI-F704 decreased activated nuclear translocation and the protein level of NF-κB and consequently reduced pro-inflammatory mediators, such as IL-6, TNF-α, iNOS and COX-2. Another *in vitro* study evaluated the anti-inflammatory effects of (+)-catechin in LPS-stimulated microglia, the effects of which were linked to the attenuation of NF-kB activation through AMPK (Syed Hussein et al., 2015). Resveratrol, which is a natural AMPK activator, directly reduces morphine tolerance by inhibiting microglial activation through AMPK signaling (Han et al.,

2014). Other studies have also shown that treatment with resveratrol inhibits glial activation as well as suppresses neuroinflammation and cancer pain in a model of tibial bone tumor cells implanted in rats (Song et al., 2015).

Recently, Kamoshita et al. (2016) evaluated a mouse model of retinal neuronal disturbance with the intraperitoneal injection of LPS and found that treatment with AICAR suppressed the reduction of conical function and decreased mRNA levels of TNF-α as well as improved mRNA levels of the mitochondrial biogenesis regulator PGC-1α. Moreover, 24 h after the injection of LPS, treatment with AICAR suppressed the expression of the glial fibrillary acidic protein (GFAP). However, other studies performed on neuronal cell lines support the hypothesis that AMPK activation is detrimental, as AICAR promoted apoptosis in undifferentiated human neuroblastoma cells (SH-SY5Y) through an increase in caspase-3 activity (Garcia-Gil et al., 2003). Similar results were found in mouse Neuro 2a neuroblastoma cells (Eun et al., 2004).

Kainic acid is a potent agonist of glutamate receptors that induces excitotoxicity and apoptosis in hippocampal neurons. Ullah et al. (2014) found that treatment with kainic acid significantly decreased cell viability, elevated the generation of radical oxygen species (ROS), increased intracellular Ca2 + levels and led to the loss of mitochondrial membrane potential. Kainic acid also induced the upregulation of Bax, decreased Bcl-2 levels, released cytochrome-c and activated caspase-3. All these events were accompanied by sustained phosphorylation and the activation of AMPK, possibly due a bioenergetic dysregulation.

These conflicting results regarding the role of AMPK may reflect a dual function of this enzyme in regulating cell death and survival depending on the type of stress (Ramamurthy and Ronnett, 2006). Moreover, the role of AMPK *in vivo* is very likely different from that observed *in vitro*, especially in neurons. Neurons are postmitotic cells that have low energy reserves and are intolerant of cell stress, especially when examined without the supportive glial cells and vasculature that characterize the *in vivo* condition. The metabolic functions of neurons and astrocytes are distinct and there are complex interactions between these two types of cells during energy depletion. Therefore, analyses regarding the role of AMPK in neuroinflammation should take into account the cell-specific context.

4. Parkinson's disease

Parkinson's disease is characterized by the degeneration of dopaminergic neurons of the *substantia nigra pars compacta*, the presence of Lewy bodies (α-synuclein) and motor alterations (Stocchi and Olanow, 2003). The etiology of Parkinson's disease is unknown, although some genes are associated to familiar Parkinson's disease (Tansey et al., 2007; Tansey and Goldberg, 2010). Recent studies suggest that intestinal inflammation may contribute to the development of neurodegenerative conditions. Individuals with Parkinson's exhibit inflammation and oxidative stress in the gut characterized by constipation, intestinal permeability, dysbiosis and increased levels of potentially pathogenic forms of enteric αSYN. Synucleinopathy exacerbates inflammation, inducing chronic systemic immune responses that, among other consequences, can increase the permeability of the blood-brain barrier. Moreover, aggregated αSYN can be transmitted from the gut to the brain *via* the vagus nerve, where it activates microglia, accelerating the timeline by which neuroinflammation induces degeneration of the central nervous system (see review in Houser and Tansey, 2017).

The use of inhibitors of complex 1 mitochondrial, such as 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) and rotenone, as well as genetic mouse models of mitochondrial dysfunction to develop Parkinson's symptoms, lend support to the hypothesis that mitochondrial activity is relevant to the development of Parkinson's disease (Hang et al., 2015). Several studies indicate that energetic homeostasis is crucial to dopaminergic neurons; however, the neuronal effects of AMPK activation are controversial, as activation may be either protective or detrimental (Choi et al., 2010; McCullough et al., 2005).

Evidence shows that glial activation increases the expression of cytokines and chemokines in Parkinson's disease, which precede the degeneration of the *substance nigra* (Halliday and Stevens, 2011; Tansey and Goldberg, 2010). The use of non-steroidal anti-inflammatory drugs (except aspirin) can prevent or delay the onset of Parkinson's (see review in Rocha et al., 2015) and the use of natural molecules with antioxidant and anti-inflammatory properties can protect dopaminergic neurons (Magalingam et al., 2015; Ojha et al., 2016; Sandoval-Avila et al., 2016).

Recently, Bayliss et al. (2016) found that circulating ghrelin, which is elevated during caloric restriction, plays a protective role in the nigrostriatal system *via* enhanced AMPK activity. According to the authors, caloric restriction in ghrelin WT mice attenuated the MPTP-induced loss of *substantia nigra* dopamine neurons and striatal dopamine turnover, but these effects were abolished in ghrelin knockout (KO) mice, demonstrating that ghrelin has a neuroprotective effect. Moreover, treatment with ghrelin elevated p-AMPK and ACC levels in the striatum of AMPK WT, but not KO mice, suggesting that AMPK is a target for the ghrelin-induced neuroprotective effect. Likewise, chronic ghrelin treatment to AMPK WT, but not KO mice reduced the activation of microglia and astrocytes.

On the other hand, MPTP-induced neuronal damage was accompanied by an increase in AMPK, but AMPK overexpression by transfection into SH-SY5Ycells exhibited a neuroprotective function (Choi et al., 2010). Equally, pAMPK was elevated in cytotoxicity rotenone-induced in Mes 23.5 cells (dopaminergic cell line), but the inhibition of AMPK lowered the accumulation of rotenone-induced synuclein and promoted cell death (Hou et al., 2015). These results could be related to pAMPK up-regulation in neurons that are damaged in a compensatory protective loop as a strategy to increase the energy supply and neuronal viability.

More recently, Lu et al. (2016) used a MPTP plus probenecid (MPTP/p) mouse model of Parkinson's to explore the therapeutic effect of metformin on dopaminergic neuronal degeneration. The results demonstrated that metformin inhibited microglial activation as well as decreased both NF-κBp65 nuclear translocation and the activation of NLRP3 inflammasome. Moreover, metformin reduced the transcription of pro-inflammatory cytokines (TNF-α and IL-6), restored the transcription of anti-inflammatory cytokines [IL-10, transforming growth factor-β (TGF-β) and IL-4], preserved the dopaminergic system and attenuated α-synuclein accumulation and motor symptoms. The reduction in the symptoms of Parkinson's disease was attributed to AMPK activation. *In vitro*, MPP^+ promoted cell death and increased levels of pAMPK and ROS. Since MPTP or MPP + are mitochondrial complex I inhibitors, these molecules could also promote the increase in AMPK levels through a compensative auto-regulatory mechanism. Moreover, co-treatment with MPP + and metformin enhanced AMPK levels even more and also elevated both AMPK-mediated autophagy and mitochondrial ROS clearance as well as attenuated dopaminergic neuron apoptosis. These results were confirmed by the suppression of the protective effects of metformin in SH-SY5Y cells after Compound C treatment. Thus, metformin can enhance the AMPK-autophagy pathway and, in turn, reduces ROS production and inhibits the activation of inflammasome.

However, some authors have hypothesized that treatment with metformin may be considered as a risk factor for the development of Parkinson's disease. In a study conducted by Ismaiel et al. (2016), metformin reduced microglial activation as well as TNF-α, IL-1β and iNOS mRNAs levels, but did not prevent the negative effect of MPTP on dopaminergic neurons, as evaluated by TH staining as well as the level of dopamine and its metabolite DOPAC measured in the striatum. In fact, metformin exacerbated dopaminergic harm in response to MPTP. Moreover, *in vitro* experiments on the viability of N27 dopaminergic neurons using the MTT assay showed that metformin not only failed to protect neurons, but even increased the harm. The authors suggest that

neuronal death is attributed to the inhibition of the mitochondrial complex I by both MPTP and metformin. However, a point to consider is that metformin also can exert effects in an AMPK-independent manner, which makes its evaluation more complex (Łabuzek et al., 2010). Furthermore, the absence of AMPK inhibitors in the study impedes the inference that cell damage occurred in an AMPK-dependent manner.

Kim et al. (2013) found that 6-hydroxydopamine (6-OHDA) promoted the atrophy of dopaminergic neurons associated with the activation of poly (ADP-ribose) polymerase (PARP), the translocation of apoptosis inductor factor (AIF) and the depletion of ATP. An increase in p-AMPK and pS6K (upstream kinase of the mTOR pathway) accompanied ATP depletion from the third day to the 14th day. These neurotoxicity effects of 6-OHDA were abolished in PARP KO mice. Treatment with metformin did not cause harm to dopaminergic neurons in control animals, but reduced the survival of dopaminergic neurons, deceased TH-positive expression and increased both p-c-Jun and AIF translocation in the 6-OHDA-PD model. Moreover, adenovirus carrying dominant-negative mutant AMPKα2 cDNA blocked the inhibition of ACC, restored the expression of TH as well as induced c-Jun phosphorylation and AIF translocation, thereby preventing 6-OHDA-induced atrophic changes in dopaminergic neurons (Kim et al., 2013).

It is well-know that glial activation and inflammation contribute to neuronal dysfunction and play an important role in the pathophysiology of Parkinson's (Tansey et al., 2007; Tansey and Goldberg, 2010). Thus, AMPK could represent a possible therapeutic target. However, the effects of AMPK on dopaminergic neurons are contradictory. It is not yet possible to conclude whether the elevation in pAMPK levels causes harm to dopaminergic neurons or can promote neuronal survival, since experimental models that mimetic Parkinson's pathophysiology act by deregulating the activity of mitochondrial complex I, which indeed increases AMP/ATP and ADP/ATP ratios and ultimately activates AMPK, making the results unclear.

5. Diabetic encephalopathy and Alzheimer's disease

Diabetic encephalopathy, which is characterized by cognitive decline and dementia, commonly occurs in elderly patients with long-standing diabetes and is also considered a risk factor for Alzheimer's disease (Abdelhafiz et al., 2015). The pathological hallmarks associated with Alzheimer's disease are hyperphosphorylated tau protein, which causes intracellular neurofibrillary tangles, and extracellular deposits of β-amyloid (Aβ), generating senile plaques (Querfurth and LaFerla, 2010; Montine et al., 2012), which are related to late-onset sporadic Alzheimer's. There is also familial early-onset Alzheimer's, which is correlated to mutations in genes encoding for amyloid precursor protein (APP) and/or presenilin 1 and 2 (PS1 and 2) (Goate et al., 1991; Levy-Lahad et al., 1995; Schellenberg et al., 1992).

Metabolic defects correlate diabetic encephalopathy and Alzheimer's, since insulin modulates the metabolism of amyloid peptides, which are derived from sequential proteolytic cleavages of full-length amyloid precursor protein by β-secretase (BACE1) and γ-secretase in neurons, thereby decreasing the intracellular accumulation of Aβ peptides (Chen et al., 2009). The disruption of insulin signaling is associated with the two neuropathological hallmarks of Alzheimer's: senile plaques and neurofibrillary tangles. Peripheral hyperinsulinemia and insulin resistance characterizes type 2 diabetes, whereas type 1 diabetes is associated with systemic insulin deficiency. Under conditions of hyperinsulinemia, insulin competes with Aβ for the action of the insulin degrading enzyme. Thus, excess insulin reduces the degradation of Aβ peptides. Moreover, GSK-3β, which is a protein kinase of tau (Ishiguro et al., 1992), is phosphorylated and inactivated by the insulin signaling pathway. Thus, impairment of the insulin/insulin receptor potentiates the hyperphosphorylation of tau and the formation of neurofibrillary tangles (reviewed in Correia et al., 2012).

The hippocampus of db/db mice (type 2 diabetes model) exhibits an increase in tau protein kinase (JNK), phosphorylated tau (p-tau) at S396 and total tau (Sadeghi et al., 2017). When these animals were treated with metformin, no beneficial or detrimental effects were found on spatial learning or memory; however, metformin reduced p-tau, total tau and JNK levels and led to the recovery of synaptophysin expression, which is a synaptic protein (Li et al., 2012).

Similarly, results obtained in our lab using experimental diabetic encephalopathy induced by streptozotocin (STZ) in C57BL-6 mice showed that the hippocampus of the diabetic animals exhibited reactive gliosis, neuronal loss, NF-kB signaling activation as well as high levels of IL-1β and vascular endothelial growth factor (VEGF). T-maze test scores of these animals revealed a significantly poorer performance. Treatment with metformin reduced the expression of astrocyte and microglial markers (GFAP and Iba-1, respectively) and inflammation markers (p-Iκβ, IL-1 and VEGF), enhanced p-AMPK and eNOS levels and increased neuronal survival (Fox-1 and neuronal nuclear protein-NeuN). Treatment with metformin also improved the spatial memory scores of diabetic animals. In summary, metformin reduced neuroinflammation and decreased the loss of neurons in the hippocampus of diabetic animals, which could be related to the subsequent improvements in spatial memory (Oliveira et al., 2016).

In contrast, a population-based, case-control study suggests that diabetic patients taking long-term metformin have a slightly increased risk of developing Alzheimer's than those taking other anti-diabetic drugs (Imfeld et al., 2012). Another epidemiological study also suggests that the administration of metformin in diabetic patients increases the risk of cognitive impairment (Moore et al., 2013). However, the evidence from this study was not sufficiently robust to establish that metformin causes cognitive deficit in diabetic patients, as the authors did not evaluate the duration and severity of diabetes, the length of treatment and the use of other hypoglycemic agents (Goodarzi, 2014).

Barini et al. (2016) found that chronic treatment with metformin reduced tau phosphorylation in the cortex and hippocampus *via* AMPK/mTOR and PP2A in a P301S transgenic mouse model of tauopathy. However, metformin increased insoluble tau species (including tau oligomers) and the number of inclusions with β-sheet aggregates in the brain of P301S mice. These findings suggest that metformin may promote tau aggregation *in vivo* and indicate a possible risk for tauopathy in elderly diabetic patients.

Jing et al. (2013) found that resveratrol attenuated hippocampal neurodegeneration in STZ-induced diabetic rats. Oral resveratrol treatment increased hippocampal p-AMPK levels and diminished both neurodegeneration and astrocytic activation. Resveratrol also reduced TNF-α and IL-6 transcripts as well as the expression of NF-kB, p38 and ERK1/2 phosphorylation/activation. According to the authors, this polyphenol treatment also reduced blood vessel permeability and VEGF expression and led to the recovery of capillary basement membrane thickness, thereby promoting the integrity and functionality of the brain-blood barrier (Jing et al., 2013).

Recently, Moussa et al. (2017) described the effects of resveratrol on the brain-blood barrier in Alzheimer's patients. The authors examined banked cerebrospinal fluid and plasma samples from a subset of patients with mild to moderate Alzheimer's treated with resveratrol or a placebo and found a reduction in MMP9 reduction, suggesting that resveratrol may decrease central nervous system permeability, thereby limiting the infiltration of leukocytes and other inflammatory agents into the brain. Moreover, resveratrol-treated patients exhibited an increase in macrophage-derived chemokine (MDC) levels, which may facilitate the intracerebral homing of specific Th2 leukocytes and thus promote neuroprotection by mediating an adaptive immune response. Resveratrol also attenuated cognitive and functional declines (evaluated through mental state examination scores) as well as stabilized Aβ42 and Aβ40 levels in the cerebrospinal fluid. Moreover, resveratrol reduced plasma levels of inflammatory markers, such as IL-1R4, IL-12P40, IL-12P70, TNF-α and RANTES (CCL22).

Using familial Alzheimer's double-transgenic mice (AβPPswe/

PS1dE9), Porquet et al. (2014) found that treatment with resveratrol prevented memory loss (as measured by the object recognition test), reduced the amyloid burden and increased mitochondrial complex IV protein levels. These effects were mainly mediated by the increased activation of SIRT-1 and AMPK pathways.

Linagliptin is a recently approved dipeptidyl peptidase-4 (DPP-4) inhibitor and widely considered as first-line treatment for patients with type 2 diabetes. DPP-4 is responsible for the inactivation of incretin hormones, such as glucagon-like peptide 1 (GLP-1). GLP-1 is released by the intestine after food intake and stimulates insulin secretion by the pancreas. Thus, by inhibiting DPP-4, Linagliptin allows GLP-1 to act longer, thereby releasing more insulin. In studies, linagliptin significantly protected against Aβ-induced cytotoxicity and prevented the activation of GSK3β and tau hyperphosphorylation by restoring downstream insulin signaling. Furthermore, linagliptin alleviated Aβ-induced mitochondrial dysfunction and intracellular ROS generation, which may be due to the activation of AMPK-SIRT1 signaling. These results indicate that DPP-4 inhibitors have therapeutic potential for reducing Aβ-induced impairment of insulin signaling and neurotoxicity in the pathogenesis of Alzheimer's (Kornelius et al., 2015).

Some studies also indicate that AMPK has a neuroprotective effect and may be a potential target for preventing/counteracting diabetic encephalopathy. Marein, which is a major compound from the hypoglycemic plant *Coreopsis tinctoria*, protected against methylglyoxal (MG), which is an endogenous toxic compound that plays an important role in diabetic complications that affect cells. Marein attenuated PC12 cell damage induced by MG through AMPK signaling. Marein enhanced phospho-AMPKα (Thr172) and Bcl-2 expression and diminished the activation of Bax, caspase-3 and inhibitor of caspase-activated deoxyribonuclease (ICAD). Molecular docking simulation showed that marein interacted with the γ subunit of AMPK (Jiang et al., 2016). Another study showed that chikusetsusaponin IVa (CHS) increased the production of adiponectin, which subsequently activated AdipoR1. Activated AdipoR1 promoted the phosphorylation of LKB/AMPK, inducing the inhibitory phosphorylation of GSK-3β. These effects suppressed apoptosis, inflammation and oxidative stress, thereby protecting against cerebral I/R injury (Duan et al., 2016).

The findings indicate that AMPK has beneficial effects against the development of Alzheimer's disease. However, conflicting data have been found, especially with regard to treatment with metformin, which known to have AMPK-independent effects.

6. Demyelinating diseases

Multiple sclerosis is a chronic, inflammatory, autoimmune disease that compromises the central nervous system and is characterized by demyelinating lesions associated with inflammatory infiltrate, the activation of glial cells as well as the rupture of the blood-brain barrier (Chiaravalloti and DeLuca, 2008). This demyelinating disease involves an autoimmune reaction by myelin-specific $CD4^+$ Th1 and Th17 cells, which initiate the neuropathology (Procaccini et al., 2015). According to the new paradigm, the IL-23/Th17/IL-17A axis is the autoimmune pathogenesis of the central nervous system. Th17 cells activated by IL-23 enter the central nervous system, where they secrete IL-17A, which affects the blood-brain barrier, causing the circulating immune cells to enter the central nervous system, where they stimulate astrocytes and microglia to produce inflammatory mediators (Jones et al., 2016; Rostami and Ciric, 2013).

According to Nath et al. (2005), AMPK signaling attenuates the inflammatory response in an experimental autoimmune encephalomyelitis (EAE) model by protecting neurons and modulating T cell immune function. Treatment with AICAR decreased clinical symptoms and inflammatory infiltrate, reducing the expression of Th1 cytokines (IFN-γ, TNF-α, IL-1β and IL-6) and NO levels, which are associated with the severity of EAE. Moreover, treatment with AICAR induced the expression of anti-inflammatory Th2 cytokines (IL-4 and IL-10).

Nath et al. (2009a) examined the activity of AMPK isoforms at the peak of symptoms in the EAE model and found a significant decrease in AMPK and its subunits when compared to normal mice. Knockouts for AMPKα1 −/− and AMPKα2 −/− were used to analyze the activity of these isoforms in the EAE model. The authors found that symptoms and inflammatory infiltrate were greater in AMPKα1 −/− animals when compared to wild animals. However, AMPKa2 −/− mice showed no worsening of symptoms, suggesting that this isoform does not play an important role in the development of EAE. In another study, the same authors found that metformin attenuated the progression of symptoms and inflammatory infiltrate in the EAE model by inhibiting inflammatory cytokines and chemokines in central nervous system as well as the expression of iNOS and MMP-9 (Nath et al., 2009b).

Platelet-derived growth factor (PDGF) and ciliary neurotrophic factor (CNTF) are expressed by glial cells and are important to neuron survival and the maintenance of oligodendrocytes. These neurotrophic factors contribute to the remyelination process by increasing the proliferation of oligodendrocyte precursor cells (Linker et al., 2002; Vana et al., 2007). Paintlia et al. (2013) provide evidence that AMPK signaling is crucial to the protection of oligodendrocytes, restoring the integrity of the central nervous system and functions in animals with EAE. Treatment with metformin enhanced the expression of the signatory genes of oligodendrocyte lineage cells, CNTF and PDGF. Moreover, treatment with metformin abolished the expression of inflammatory mediators (TNF-α and iNOS), attenuated oxidative stress and malondialdehyde levels as well as promoted antioxidative defenses in oligodendrocytes exposed to cytokines *via* AMPK activation.

Another demyelinating experimental model regards the use of cuprizone, which is a copper quelant that induces the degeneration of the myelin sheath and the death of oligodendrocytes without affecting the peripheral immune system (Matsushima and Morell, 2001). Analyzing the neuroprotective and anti-inflammatory action of sildenafil, Nunes et al. (2015) demonstrated protective action of the pAMPK-IKBα-NFκB pathway in a CPZ demyelination model. After treatment with sildenafil, both pAMPK and eNOS levels were significantly elevated, suggesting reciprocity between AMPK and eNOS. Treatment also reduced NFκB and increased IKβα. These data indicate that sildenafil activates AMPK, which, in turn, induces an increase in eNOS expression and NO production. The hypothesis is that NO starts the feedback loop, further activating AMPK and inhibiting NFκB.

In another study using the CPZ demyelinating model for eight weeks, the authors evaluated the protective action of the *Areca catechu* extract (ANE), which promoted improvements in cognition and social activity and protected myelin by promoting the differentiation of oligodendrocyte precursor cells. Contradictorily, the results also showed that the chronic administration of CPZ increased the phosphorylation level of AMPK*α*, which was suppressed by ANE treatment (Adilijiang et al., 2015). The conflicting AMPK results obtained could be related to the stage of the disease. However, the non-use of AMPK antagonists hinders the evaluation of the specific role of AMPK.

7. Neuropathies

In diabetic neuropathy, demyelination and neuron damage lead to motor and sensory deficits. Some studies have indicated metformin as potential therapy for painful diabetic neuropathy. Ma et al. (2015) found that metformin attenuated diabetic hyperalgesia and allodynia. The authors report that metformin reduced levels of malondialdehyde and glycation end-products as well as enhanced superoxide dismutase activity. Moreover, metformin activated the phosphorylation of AMPK and its target genes in the sciatic nerve of diabetic rats, which may be associated with its anti-oxidative and neuroprotective effects. Through electrophysiological studies and behavioral analyses, Ma et al. (2016) also report that metformin exerts beneficial effects on nerve regeneration and functional recovery after sciatic nerve crush injury in diabetic

rats.

Interestingly, AMPK also exerts anti-inflammatory and neuroprotective effects on diabetes-induced neuropathy. Treatment with metformin reduced blood glucose levels, inflammatory markers (such as IL-6, C-reactive protein and TNF-α) and enhanced motor nerve conduction velocities (MNCV) of the sciatic nerve, which is an electrophysiological marker for peripheral nerve damage. Co-administration of Compound C with metformin counteracted these effects. The results indicate that the anti-inflammatory effects of metformin in diabetic neuropathy may be associated with the AMPK signaling pathway (Hasanvand et al., 2016).

In another study, Yerra and Kumar (2016) used streptozotocin to induce diabetic neuropathy in rats. Mechanical/thermal hyperalgesia, motor/sensory nerve conduction velocities (MNCV and SNCV) and sciatic nerve blood flow (NBF) were evaluated. *In vitro* studies of Neuro2a (N2A) cell culture were performed under a condition of high glucose for the analysis of mitochondrial dysfunction. Treatment for two weeks with a potent allosteric activator of AMPK (A769662) attenuated pain hypersensitivity, restored MNCV and SNCV, decreased ROS, malondialdehyde and glutathione (GSH) and induced higher levels of GSH, reversing oxidative stress. Treatment with A769662 of high glucose-exposed N2A cells and peripheral nerves in STZ-induced rats reduced the number of positive cells on the TUNEL test as well as levels of inflammatory markers. Mitochondrial biogenesis and autophagy were also stimulated with the AMPK activator, demonstrating the beneficial effect of AMPK as a possible treatment for diabetic neuropathy.

According to Yuan et al. (2013), resveratrol has a protective effect against the toxicity induced by ethanol on Schwann cells (SCs) *in vitro*, improving cell viability and reducing apoptosis. Resveratrol increased the mRNA and protein levels of BDNF and glial cell line-derived neurotrophic factor (GDNF) in the ethanol-treated SCs, possibly protecting these cells from ethanol-induced cell death. Moreover, when the SCs culture was subjected to specific inhibitors of AMPK (Compound C) and SIRT1 (nicotinamide), the beneficial effect of resveratrol was reversed, indicating that resveratrol may be exerting its neuroprotective effects through the AMPK-SIRT1 pathway.

Huntington's disease is a hereditary, neurodegenerative disease associated with 36 or more CAG repeats in the huntingtin (Htt) gene. The major characteristic of Huntington's disease is neuronal loss in the striatum and cortex, which leads to movement disorders and dementia. According to Chou et al. (2005), transgenic Huntington's mice (R6/2) submitted to a daily administration with CGS21680 (CGS), which is an agonist selective for adenosine A2A receptor (A2A-R), induced a delay in the progressive deterioration of motor performance and prevented a reduction in brain weight. Treatment with CGS reduced levels of choline expression in the striatum, the size of the ubiquitin-positive neuronal intranuclear inclusions and mutant Htt aggregation. Moreover, chronic treatment with CGS diminished glucose levels and the overactivation of AMPK in the striatum of R6/2 mice. Since AMPK is crucial sensor of energetic metabolism, the beneficial effects of CGS may be correlated with the modulation of the energetic dysfunction caused by Htt mutation.

Activation of the AMPKα1 isoform occurred in striatal neurons of humans and mice with Huntington's. R6/2 transgenic mice exhibit overactivation of AMPKα1 in the striatum and are characterized by brain atrophy, decreased brain weight, neuronal loss and an increased formation of Htt aggregates. Ju et al. (2011) found a higher nuclear accumulation of pAMPKα in the neurons of the nucleus striatum and cortex of Huntington's patients and R6/2 transgenic mice, whereas pAMPKα was found in the neuronal cytoplasm in non-Huntington's subjects and non-diseased mice. Treatment of transgenic animals with AICAR increased the neuropathological characteristics of Huntington's, such as enlargement of the ventricles followed by a progressive reduction in brain weight, increased pAMPKα and AMPKα1 expression, activated the caspase-3 protein and reduced Bcl-2 expression in the striatum. Moreover, Compound C greatly reduced the nuclear localization of AMPKα1 in R6/2 mice provoked by AICAR.

In another study, Ju et al. (2014) found that elevated oxidative stress caused abnormal activation of AMPKα1 and subsequently induced neurotoxicity in a striatal progenitor cell line and the striatum of R6/2 transgenic mouse model, suggesting positive feedback between ROS and AMPKα1 in Huntington's striatal neurons. Chronic treatment with the antioxidant *N*-acetyl-cysteine suppressed the activation of AMPKα1, reduced neuronal toxicity and ventricle enlargement, increased neuronal density and improved motor dysfunction.

Interestingly, metabolic dysfunction is also observed in amyotrophic lateral sclerosis (ALS). Patients and mouse models of familial ALS exhibit higher levels of resting energy expenditure and lower fat-free mass, indicating a hypermetabolic phenotype. Lim et al. (2012) found that reducing AMPK activity, either pharmacologically or genetically, protects against motor neuron degeneration and muscle pathology observed in genetic models of motor neuron disease. Similarly, Liu et al. (2015) found that cytoplasmic mislocalization of TAR DNA-binding protein-43 (TDP-4) inclusions (a hallmark of ALS) is associated with an increased activation of AMPK in motor neurons in patients with ALS.

The analysis of the role of AMPK in neuropathies is complex and seems to be metabolic dependent. Reports have indicated an anti-inflammatory and neuroprotective role of AMPK in diabetic neuropathy. However, contrasting results are found in Huntington's and ALS, the bioenergetic abnormalities of which are critical to the development of motor neuron diseases. Since AMPK is a key sensor of cellular energy status, higher levels of active AMPK in Huntington's and ALS may be a metabolic consequence of the genetically induced energy imbalance, rather than the cause of the pathogenic development.

8. Concluding remarks

AMPK signaling is complex and one must avoid drawing simplistic conclusions based on conflicting reports. First of all, there are at least 12 types of possible AMPK trimeric complexes and little is known about which specific complex is preferentially formed within in a distinct cell, its exact subcellular localization or precise targeting set of substrates and whether there is the differential expression of AMPK isoforms in different pathological conditions (Dasgupta and Chhipa, 2016). When analyzing the seemingly discordant reports on the AMPK response, the experimental model (type of mouse strain and knockouts and type of cell cultures used), the stage of disease development and the specific cellular energy status of each neurodisease must be taken into consideration. In neuroinflammation disorders, the crucial challenge that warrants investigation is the possible benefits of AMPK agonists or antagonists in specific neurological contexts. Such efforts will certainly provide an opportunity to establish a rationale for the development of therapeutic strategies.

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APÊNDICE B – PUBLICAÇÕES NO PERÍODO DE 2015 A 2019

Artigos completos publicados em periódicos

1. RODRIGUES, GABRIEL BARROS; OLIVEIRA, ELQUIO ELEAMEN; JUNIOR, FRANCISCO JAIME BEZERRA MENDONÇA; SANTOS, LAISE ALINE MARTINS DOS; **OLIVEIRA, WILMA HELENA DE**; FRANÇA, MARIA EDUARDA ROCHA DE; LÓS, DENIELE BEZERRA; GABÍNIO, BRENNDA MARTINS; PEIXOTO, CHRISTINA ALVES
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2. SANTOS, LAISE ALINE MARTINS DOS; RODRIGUES, GABRIEL BARROS; MOTA, FERNANDA VIRGÍNIA BARRETO; FRANÇA, MARIA EDUARDA ROCHA DE; DE SOUZA BARBOSA, KARLA PATRÍCIA; **OLIVEIRA, WILMA HELENA DE**; ROCHA, SURA WANESSA SANTOS; LÓS, DENIELE BEZERRA; SILVA, AMANDA KAROLINA SOARES; SILVA, TERESINHA GONÇALVES DA; PEIXOTO, CHRISTINA ALVES
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3. DUARTE-SILVA, EDUARDO; ARAÚJO, SHYRLENE MEIRY DA ROCHA; **OLIVEIRA, WILMA HELENA**; LÓS, DENIELE BEZERRA DE; FRANÇA, MARIA EDUARDA ROCHA DE; BONFANTI, AMANDA PIRES; PERON, GABRIELA; THOMAZ, LIVIA DE LIMA; VERINAUD, LIANA; NUNES, ANA KAROLINA DE SANTANA; PEIXOTO, CHRISTINA ALVES
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5. RODRIGUES, GABRIEL BARROS; OLIVEIRA, ELQUIO ELEAMEN; JUNIOR, FRANCISCO JAIME BEZERRA MENDONÇA; SANTOS, LAISE ALINE MARTINS DOS; **OLIVEIRA, WILMA HELENA DE**; FRANÇA, MARIA EDUARDA ROCHA DE; LÓS, DENIELE BEZERRA; GABÍNIO, BRENNDA MARTINS; DE LIRA, FÁBIA CRISTIANE MELO LEITE; PEIXOTO, CHRISTINA ALVES
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6. FRANÇA, MARIA EDUARDA ROCHA DE; ROCHA, SURA WANESSA SANTOS; **OLIVEIRA, WILMA HELENA**; SANTOS, LAISE ALINE; DE OLIVEIRA, ANNE GABRIELLE VASCONCELOS; BARBOSA, KARLA PATRÍCIA SOUSA; NUNES, ANA KAROLINA SANTANA; RODRIGUES, GABRIEL BARROS; LÓS, DENIELE BEZERRA; PEIXOTO, CHRISTINA ALVES
Diethylcarbamazine attenuates the expression of pro-fibrogenic markers and hepatic stellate cells activation in carbon tetrachloride-induced liver fibrosis. INFLAMMOPHARMACOLOGY. , v.1, p.1 - , 2017.

7. **OLIVEIRA, WILMA HELENA**; NUNES, ANA KAROLINA; FRANÇA, MARIA EDUARDA ROCHA; SANTOS, LAISE ALINE; LÓS, DENIELE BEZERRA; ROCHA, SURA WANESSA; BARBOSA, KARLA PATRÍCIA; RODRIGUES, GABRIEL BARROS; PEIXOTO, CHRISTINA ALVES
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8. LUNA, RAYANA LEAL; VASCONCELOS, ANNE GABRIELLE; SANTANA NUNES, ANA KAROLINA; **DE OLIVEIRA, WILMA HELENA**; BARBOSA, KARLA PATRICIA DE SOUSA; PEIXOTO, CHRISTINA ALVES
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9. DE SANTANA NUNES, ANA KAROLINA; RAPÔSO, CATARINA; **DE OLIVEIRA, WILMA HELENA**; THOMÉ, RODOLFO; VERINAUD, LIANA; TOVAR-MOLL, FERNANDA; PEIXOTO, CHRISTINA ALVES
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10. KHALAJ, KASRA; LUNA, RAYANA LEAL; FRANÇA, MARIA EDUARDA ROCHA DE; **OLIVEIRA, WILMA HELENA DE**; PEIXOTO, CHRISTINA ALVES; TAYADE, CHANDRAKANT
RNA binding protein, tristetraprolin in a murine model of recurrent pregnancy loss. Oncotarget. , v.7, p.72486 - , 2016.

11. RODRIGUES, GABRIEL BARROS; ROCHA, SURA WANESSA SANTOS; SANTOS, LAISE ALINE MARTINS DOS; **DE OLIVEIRA, WILMA HELENA**; GOMES, FABIANA OLIVEIRA DOS SANTOS; DE FRANÇA, MARIA EDUARDA DA ROCHA; LÓS, DENIELE BEZERRA; PEIXOTO, CHRISTINA ALVES
Diethylcarbamazine: Possible therapeutic alternative in the treatment of alcoholic liver disease in C57BL/6 mice. Clinical and Experimental Pharmacology & Physiology. , v.42, p.369 - 379, 2015.

Trabalhos publicados em anais de eventos (resumo)

1. BRAGA, C. F.; **DE OLIVEIRA, WILMA HELENA**; PEIXOTO, CHRISTINA ALVES
Estudo sobre a ação da metformina na encefalopatia diabética In: XXV RAIC
XXV RAIC. , 2017.

2. BRAGA, C. F.; SILVA, E. P. D.; **DE OLIVEIRA, WILMA HELENA**; PEIXOTO, CHRISTINA ALVES
Estudo sobre a ação da metformina na encefalopatia diabética: neurodegeneração e cognição In: XXIV RAIC
XXIV RAIC. , 2016.

Trabalhos publicados em anais de eventos (resumo expandido)

1. FRANCA, M. E. R.; **OLIVEIRA, WILMA HELENA**; NUNES, A. K. S.; RAMOS, R. B.; PEIXOTO, C. A.
AVALIAÇÃO DOS EFEITOS DA ATIVAÇÃO DE AMPK SOBRE A NEUROINFLAMAÇÃO NO MODELO DE DIABETES INDUZIDA POR ESTREPTOZOTOCINA EMCAMUNDONGOS C57BL/6 In: IV Simpósio de Cognição Imune e Neural, 2015
IV Simpósio de Cognição Imune e Neural. , 2015.

2. **DE OLIVEIRA, WILMA HELENA**; FRANCA, M. E. R.; NUNES, A. K. S.; SILVA, E. P. D.; PEIXOTO, CHRISTINA ALVES
EFEITOS DA ATIVAÇÃO DE AMPK PELA METFORMINA SOBRE AS CÉLULAS GLIAIS E A MEMÓRIA ESPACIAL DURANTE A DIABETES INDUZIDA POR ESTREPTOZOTOCINA EM CAMUNDONGOS C57BL/6 In: 4º Simpósio sobre Cognição Imune e Neural, Natal.
4º Simpósio sobre Cognição Imune e Neural. , 2015.

APÊNDICE C – ORIENTAÇÃO DE MONOGRAFIA NO PERÍODO DE 2015 A 2019

Orientações e supervisões

Orientações e supervisões concluídas

Trabalhos de conclusão de curso de graduação

1. Clarissa Figueredo Braga. **Avaliação dos efeitos da metformina sobre a ativação de células gliais e liberação de óxido nítrico em modelo de encefalopatia diabética**. 2017. Curso (Farmácia) - Universidade Federal de Pernambuco

2. Millena Kallyne do Nascimento Silva. **Avaliação dos Efeitos do Tadalafil Sobre os Processos de Neuroinflamação e Cognição em Modelo de Encefalopatia Diabética**. 2017. Curso (Biomedicina) - Universidade Federal de Pernambuco

3. Eduardo Pereira Duarte da Silva. **Avaliação dos Efeitos da Metformina Sobre a Memória e o Processo Neurodegenerativo em Modelo Experimental de Encefalopatia Diabética**. 2015. Curso (Licenciatura Plena em Ciências Biológicas) - Universidade Federal Rural de Pernambuco

ANEXO A – CERTIFICADO DE APROVAÇÃO DE USO DE ANIMAIS

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado "**AVALIAÇÃO DOS MECANISMOS DE AÇÃO DA METFORMINA SOBRE OS PROCESSOS DE NEUROINFLAMAÇÃO, NEURODEGENERAÇÃO E COGNIÇÃO EM MODELO DE ENCEFALOPATIA DIABÉTICA**" Protocolado sob nº 81/2015 pela pesquisadora **CHRISTINA ALVES PEIXOTO** Está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz (CEUA/CPqAM) em 02/02/2016. Na presente versão, este projeto está licenciado e tem validade até fevereiro de 2020.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Camundongo *Swiss webster*	242 (machos)
TOTAL	**242**

We certify that project "**AVALIAÇÃO DOS MECANISMOS DE AÇÃO DA METFORMINA SOBRE OS PROCESSOS DE NEUROINFLAMAÇÃO, NEURODEGENERAÇÃO E COGNIÇÃO EM MODELO DE ENCEFALOPATIA DIABÉTICA**" (CEUA Protocol nº 81/2015), coordinated by **CHRISTINA ALVES PEIXOTO** is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz on 02/02/2016. In present verson this project is licensed and valid until February 2020.

Recife (PE, BRAZIL) 02 february 2016

Sheilla Andrade de Oliveira

Drª Sheilla Andrade de Oliveira
Coordenadora CEUA/CPqAM

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ANEXO B – COMPROVANTE DE SUBMISSÃO DO ARTIGO 2

Gmail **Wilma Helena Oliveira <wilmah.oliveira@gmail.com>**

Track your recent Co-Authored submission to NEUPSY

1 mensagem

European Neuropsychopharmacology <eesserver@eesmail.elsevier.com> 11 de fevereiro de 2019 12:00
Responder a: European Neuropsychopharmacology <ENP@elsevier.com>
Para: wilmah.oliveira@gmail.com

*** Automated email sent by the system ***

Dear Dr. Wilma Oliveira,

You have been listed as a Co-Author of the following submission:

Journal: European Neuropsychopharmacology
Title: Effects of metformin on long-term memory and hallmarks of Alzheimer's disease in diabetic mice.
Corresponding Author: Christina Alves Peixoto
Co-Authors: Wilma H Oliveira; Clarissa F Braga; Deniele B Lós; Shyrlene M Araújo; Maria Eduarda R França; Eduardo P Duarte-Silva; Gabriel B Rodrigues; Sura W Santos Rocha;

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Thank you,

European Neuropsychopharmacology