Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-Graduação em Biologia Celular e Molecular

KATIUCIA MARQUES CAPIOTTI

Avaliação dos sistemas colinérgico e purinérgico em encéfalo de peixe-zebra (*Danio rerio*) adulto submetido a um modelo de hiperglicemia

Porto Alegre

2015

KATIUCIA MARQUES CAPIOTTI

Avaliação dos sistemas colinérgico e purinérgico em encéfalo depeixe-zebra (*Danio rerio*) adulto submetido a um modelo de hiperglicemia

Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Profª Drª Rosane Souza da Silva

Porto Alegre

2015

KATIUCIA MARQUES CAPIOTTI

Avaliação dos sistemas colinérgico e purinérgico em encéfalo de peixe-zebra (*Danio rerio*) adulto submetido a um modelo de hiperglicemia

Tese apresentada como requisito para obtenção do grau de Doutor pelo

Programa de Pós-Graduação em Biologia Celular e Molecular da

Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Aprovada em 10 de Junho de 2015.

BANCA EXAMINADORA:

____

Prof. Dra.Nadja Schroder– PUCRS

____

Profa. Dra.Vera Maria Melchiors Morsch– UFSM

____

Prof. Dra.Carina Rodrigues Boeck - UNIFRA

Porto Alegre
2015

AGRADECIMENTOS

Ao meu parceiro, amigo, namorado e noivo Gabriel Moser, por toda ajuda, amor, compreensão e paciência.

À minha orientadora, Drª. Rosane Souza Da Silva, por toda amizade, incentivo, teimosia, conselhos e orientações durante a minha trajetória acadêmica. Pelos cafés e lanchinhos durantes esses longos anos de caminhada.

Aos meus avós, Elisabeth e Edgar Capiotti, Dinah e Hernani Marques por sempre acreditarem em todas as minhas escolhas e darem os melhores exemplos de vida.

À minha irmã, Karine Capiotti, ao meu pai, Emival Severo Capiotti, minha mãe, Glória Maria Marques Capiotti que sempre estão por perto para qualquer desafio.

Ao meu grande amigo Fabiano Peres, por todas as discussões bioquímicas intermináveis, parcerias e ajudas durante todos esses anos.

Aos bolsistas: Régis Antoniolo Junior, Daiani Moraes e Ana Blazina por terem contribuído para a realização dos trabalhos dessa tese. Não tenho palavras para agradecer a vocês.

Aos meus colegas do Laboratório de Neuroquímica e Psicofarmacologia que compartilharam os resultados e colaboraram para meu crescimento profissional e pessoal, meu muito obrigada de coração!

Aos meus amigos por aliviarem as horas de estresse e estarem sempre por perto quando preciso.

Aos demais professores do programa, em especial, a profª. Carla Denise Bonan e Guendalina Turcato pela oportunidade de aprendizado.

RESUMO

O Diabetes Mellitus (DM) éuma doença crônica que atinge cerca de 387 milhões de pessoas no mundo, sendo caracterizado como um grupo heterogêneo de distúrbios metabólicos que apresentam em comum o sintoma de hiperglicemia.O peixe-zebra (*Danio rerio*) tem sido muito utilizadona pesquisapara compreender diferentes doenças, devido às características apresentadas por esta espécie, como o genoma e a descrição da organização geral e de circuitos neuronais muito semelhantes aos observados em mamíferos e a presença dos principais neurotransmissores, hormônios e receptores. Alguns modelos de doenças metabólicas já foram desenvolvidos em peixe-zebra, demonstrando que este é capaz de reproduzir sintomas importantes das disfunções encontradas em humanos. Neste estudo, nós caracterizamos um modelo de hiperglicemia em peixe-zebra e avaliamosparâmetros comportamentais e os efeitos sobre os sistemas purinérgico e colinérgico sob esta condição. O modelo de hiperglicemia foi desenvolvido através da imersão do peixe-zebra em 111 mM de glicose por 14 dias e 7 dias de *washout*. A glicação de proteínas, a sensibilidade à insulina, a resposta a drogas anti-diabéticas e a expressão gênica de receptores de insulina e transportadores de glicose foram avaliadas. Nossos resultados demonstraram que este modelo provocou um aumento dos níveis de glicose sanguínea, sendo capaz de diminuir a resposta à insulina, aumentar a glicação de proteínas da retina e a expressão dos níveis de RNAm dos receptores de insulina no músculo esquelético, tanto no grupo de 111 mM de glicose como após 7 dias de *washout*. Os tratamentos com glimepirida e metformina foram capazes de reverter a hiperglicemia. Estudos têm demonstrado que ossistemas colinérgico e purinérgico estão envolvidos nos mecanismos de declínio cognitivos relacionados ao DM. A capacidade mnemônica dos animais foi avaliada através de esquiva inibitória. Nossos resultados demonstraram quea hiperglicemia foi capaz de promover prejuízos na memória dos animais, o qual pode estar relacionadocomo aumento da atividade da AChE registrada em encéfalo de animais hiperglicêmicos. O tratamento com galantamina, um inibidor da AChE, foi capaz de reverter os efeitos sobre a memória causados pela hiperglicemia. Nossos dados também demonstraram que a hiperglicemia reduziu a atividade de hidrólise de nucleotídeos da adenina (ATP, ADP e AMP)e aumentou a atividade da adenosina desaminase (ADA), sugerindo que as modificações causadas podem estar contribuindo para a piora cognitiva induzida pelo DM.Estes achados podem contribuir para um melhor entendimento das vias de sinalização envolvidas nos mecanismos de ação do DM e apresentarem alvosalternativos para a utilização de fármacos que minimizem os efeitos da hiperglicemia sob o SNC.

Palavras-chave: acetilcolina, adenosina, diabetes mellitus, hiperglicemia, memória, peixe-zebra.

ABSTRACT

Diabetes Mellitus (DM) is a chronic disease that affects about 387 million people worldwide, being characterized as a heterogeneous group of metabolic disorders that have in common the symptom of hyperglycemia. The zebrafish (*Danio rerio*) has long been used in research to understand different diseases, due to characteristics shown by this species, as the genome and the description of the general organization and neuronal circuitry very similar to those observed in mammals and the presence of the main neurotransmitters, hormones, and receptors in this animal. Some metabolic disease models have already been developed using zebrafish, demonstrating that it is capable to reproduce important symptoms of human disorders. In this study, we characterized a hyperglycemia model in zebrafish and evaluated behavioral parameters and the effects on the purinergic and cholinergic systems under this condition. The hyperglycemia model was developed by immersion of adult zebrafish in 111 mM glucose for 14 days followed by 7 days of glucose washout. The protein glycation, the insulin sensitivity, the response to anti-diabetic drugs and the gene expression of insulin receptors and glucose transporters were evaluated. Our results showed that this model caused a rise in blood glucose levels, being able to reduce the response to insulin, increasing retinal protein glycation and the expression of mRNA levels of insulin receptors on skeletal muscle, in both groups of 111 mM glucose and after 7 days of glucose washout. Treatments with Glimepiride and Metformin were able to revert hyperglycemia. Studies have demonstrated that the cholinergic and purinergic systems are involved in the cognitive decrease mechanisms related to DM. The mnemonic capacity of the animals was assessed by inhibitory avoidance. Our results demonstrated that hyperglycemia was able to promote memory loss of the animals, which can be related to the increase of AChE activity. The therapy with galantamine, a AChE inhibitor, was able to reverse hiperglycemia-induced memory deficits. Our data also showed that hyperglycemia reduced the activity of hydrolysis of purine nucleotides (ATP, ADP and AMP) and increased the activity of adenosine deaminase (ADA), suggesting that these changes may be contributing to cognitive deterioration induced by DM. These findings may contribute to a better understanding of the signaling pathways involved in the cognitive impairment in DM and presenting alternative targets to the utilization of drugs that minimize the effects of hyperglycemia in the CNS.

Keywords: acetylcholine, adenosine, diabetes mellitus, hyperglycemia, memory, zebrafish.

LISTA DE ABREVIATURAS

Acetil-CoA - Acetilcoenzima A

ACh- Acetilcolina

AChE - Acetilcolinesterase

ADA - Adenosina Desaminase

ADAl - Adenosina Desaminase-like

ADGFs - Fatores de Crescimento relacionados a adenosina deaminase

ADP - Adenosina 5´ Difosfatado

AGEs - Produtos Finais de Glicação Avançada

AMP - Adenosina 5´ monofosfato

AMPc - Adenosina 5´ monofosfato cíclico

A_pnA - Diadenosina Polifosfato

ATP - Adenosina 5´ trifosfato

ChAT - Colina Acetiltransferase

DM - Diabetes Mellitus

DM1 - Diabetes Mellitus tipo 1

DM2 - Diabetes Mellitus tipo 2

E-NPP - Ectonucleotídeo Pirofosfatase/Fosfodiesterase

EHNA - erytro-9-(2-hidroxi-3-nonil)adenina)

GABA - Ácido Gama-Aminobutírico

GAD 65 - Antidescarboxilase do Ácido Glutâmico

GPI – Glicosilfosfatidilinositol

HbA1C - Hemoglobina Glicada

IA2 - Antitirosina-Fosfatases

LTP - Potencial de Loga Duração

mAchrs - Receptores Muscarínicos

MODY - *Maturity Onset Diabetes of the Young*

MPTP - 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

nAchrs - Receptores Nicotínicos

E-NTPDase - Ecto-nucleosídeo trifosfato difosfoidrolases

OMS - Organização Mundial de Saúde

P1 – Receptores Purinérgico 1

P2 - Receptores Purinérgico 2

PEPCK - Fosfenolpiruvato Carbolixase

PI3K – Quinase Fosfatidilinositol-3

ROS - Espécie Reativa de Oxigênio

SBD - Sociedade Brasileira de Diabetes

SNC - Sistema Nervoso Central

STZ - Estreptozotocina

ZNT - Antitransportador de Zinco

RI - Receptor de Insulina

LISTA DE FIGURAS

Figura 1: Número de pessoas com DM por região, segundo a Federação Internacional de Diabetes no ano de 2014..11

Figura 2: Representação esquemática de um neurônio colinérgico...........22

Figura 3: Representação esquemática do metabolismo purinérgicos........29

Sumário

1 INTRODUÇÃO ... 10

1.1 Diabetes Mellitus ... 10

1.2 Diabetes e Disfunções neurológicas ... 15

1.3 Sistema colinérgico ... 21

1.4 Diabetes e Sistema colinérgico ... 24

1.5 Sistema purinérgico ... 26

1.6 Diabetes e Sistema purinérgico ... 34

1.7 Peixe-zebra ... 36

2 OBJETIVOS ... 40

2.1 Objetivo geral ... 40

2.1.1 Objetivos específicos ... 40

3 RESULTADOS ... 41

3.1 CAPÍTULO I ... 41

3.2 CAPÍTULO II ... 51

3.3 CAPÍTULO III ... 59

4 DISCUSSÃO E CONCLUSÃO ... 83

REFERÊNCIAS ... 95

ANEXOS – Parecer de aprovação do projeto de pesquisa pelo CEUA ... 120

1 INTRODUÇÃO

1.1 Diabetes Mellitus

O diabetes mellitus (DM) não é uma única doença, mas um grupo heterogêneo de distúrbios metabólicos que apresenta em comum o sintoma de hiperglicemia, a qual é o resultado de defeitos na ação da insulina, na secreção de insulina ou ambos (American Diabetes, 2014b). Segundo a Federação Internacional de Diabetes (IDF), a prevalência de DM é de 8,3% da população mundial, afetando 387 milhões de pessoas(IDF, 2014) (Figura 1). Projeções demonstram que esse número tende a ser ainda maior, afetando até 10,1% da população, ou seja, 592 milhões de adultos diabéticos até 2035 (Aguiree et al., 2013).

O DM é um dos problemas de saúde mais desafiadores do século XXI, levando ao crescente número de casos anuais da doença. O Atlas de Diabetes publicado anualmente pelo IDF revela a prevalência e as projeções da doença no mundo todo. O Atlas aponta que mais de 80% das pessoas com DM vivem em países de baixa e média renda (Artola et al., 2002; Nam Han Cho et al., 2013).

O Brasil é o quarto país no ranking mundial com maior número de pessoas com diabetes entre 20 e 79 anos de idade, possuindo 8,7% da população nacional diabética(Artola et al., 2002). As diretrizes publicadas pela Sociedade Brasileira de Diabetes (SDB) estimamque hoje existam 11.623 pessoas diabéticas no Brasil(Milech et al., 2015). Números

alarmantes têm sido divulgados pela IDF em relação à mortalidade causada pela doença no mundo, chegando a 116.382,56 milhões de mortes em 2014.

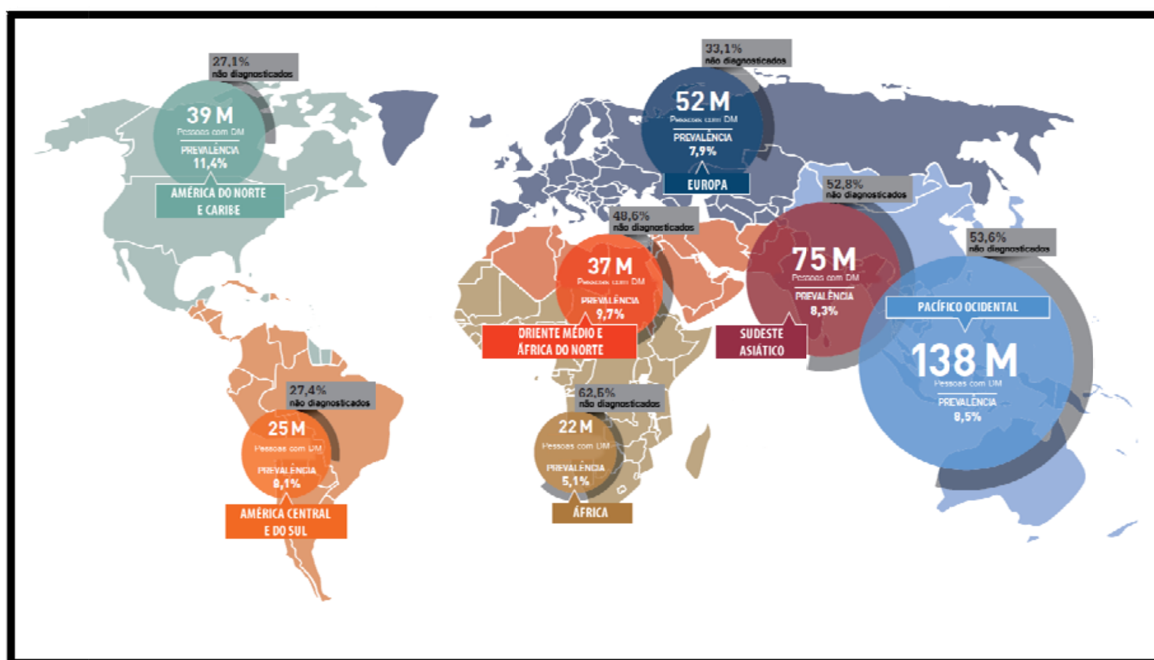


Figura 1: Número de pessoas com DM por região, segundo a Federação Internacional de Diabetes no ano de 2014. M: indica milhões.Fonte: IDF, 2014.

A classificação atual do DM baseia-se na etiologia, sendo proposta em 1999, pela Organização Mundial de Saúde (OMS) e em 2013 pela Associação Americana de Diabetes (ADA)como: DM tipo 1 (DM1), DM tipo 2 (DM2), DM gestacional e outros tipos específicos de DM. Entende-se o pré-diabetes e a tolerância à glicose diminuída como fatores de risco para o desenvolvimento de DM e de doenças cardiovasculares(Schwartz et al., 2010; Hirshberg e Raz, 2011).

O DM do tipo 1, presente em 5% dos casos, é causado por uma reação autoimune contra às célulasβ pancreáticas, que são as

responsáveis pela produção de insulina, gerando uma deficiência absoluta de insulina (Richardson et al., 2014). Na maioria dos casos, os marcadoresde autoimunidade utilizados são os autoanticorposanti-insulina(Palmer et al., 1983), antidescarboxilasedo ácido glutâmico (GAD 65)(Gorus et al., 1997),antitirosina-fosfatases (IA2 e IA2B)(Rabin et al., 1994) eantitransportador de zinco (Znt) (Baekkeskov et al., 1990). Existem casos em quenão há evidências de processo autoimune,sendo, portanto, referidos como forma idiopática de DM1. Esse tipo de DM normalmente é diagnosticado em crianças ou adultos jovens com menos de 30 anos de idade, mas pode ocorrer em qualquer idade (Szablewski, 2014).

O DM do tipo 2 é o tipo mais comum da doença, presente em 90 a 95% dos casos de diabetes, sendo caracterizada pela redução da sensibilidade da insulina nas células-alvo, seguida por uma compensação do aumento dasecreção desse hormônio pelo pâncreas(Artola et al., 2002). Esse tipo de DM é causado pela combinação de diferentes fatores, incluindo susceptibilidade genética, obesidade esedentarismo (Artola et al., 2002). O DM tipo 2 ocorre em qualquer idade, mas é geralmente diagnosticado após os 40 anos de idade (Artola et al., 2002).

O DM gestacional é definido por qualquer nível de intolerância a carboidratos resultando em hiperglicemia de intensidade variada, com inicio ou diagnóstico na gestação (Bellamy et al., 2009). Esse tipo de DM ocorre em torno da 24° semana de gravidez, provavelmente devido aos hormônios produzidos na placenta bloquearem a ação da insulina (Artola et al., 2002).

O DM gestacional associa-se tanto à resistência à insulina quanto à diminuição da função das células βpancreáticas (Kautzky-Willer et al., 1997; Kühl, 1998). Evidências sugerem quemulheres que desenvolveram DM gestacional, apresentam uma forte tendência em desenvolver DM tipo 2 tardiamente (Bellamy et al., 2009; Veeraswamy et al., 2012).

Existem alguns tipos mais específicos de DM, como os defeitos genéticos na ativação da produção de insulinanas células β pancreáticas, mais conhecidos como *Maturity Onset Diabetes of the Young*(MODY), doenças do pâncreas exócrino, endocrinopatias, alguns tipos induzidos por drogas e agentes químicos, infecções e, por fim,defeitos associados a síndromes genéticas(Nicole Campagnolo, Pablo Folha Dallapicola, Nádia Murussi, Luís Henrique Canani, Jorge Luiz Gross, 2004; Milech et al., 2015).

O termo pré-diabetes é utilizado para as pessoas que possuem uma menor tolerância à glicose e apresentam a glicemia de jejum alterada, ou seja, acima do nível de normoglicemia de jejum. Em 2009, chegou-se a um consenso sobre o uso da hemoglobina glicada (HbA1c) para diagnosticar o DM (WHO, 2011), sendo o pré-diabetes diagnosticado através dos níveis de HbA1c entre 5,7% até 6,4% (American Diabetes, 2014a).

A diminuição dos níveis de glicose no sangue é dependente de insulina e da sinalização dosreceptores de insulina (RI)no tecido adiposo e muscular. Sob condições não-patológicas, o excesso de glicose no sangue é depurado pelo fígado e depois armazenado como glicogênio ou ácidos

graxos. Sob condições patológicas, como obesidade e/ou DM, ocorre uma incapacidade de corrigir a glicemia de forma eficaz. A ineficiência da insulina em diminuir os níveis de glicose sanguínea é definida como resistência à insulina (McGarry, 2002). A resistência à insulina é a condição na qual, quantidades normais de insulina são insuficientes para produzir as respostas necessárias nos tecidos-alvo (Sesti, 2006; Bloomgarden, 2008). Pacientes com DM tipo 2, possuem hiperinsulinemia, ou seja, níveis de produção de insulina aumentados, enquanto a diminuição da sensibilidade à insulina esta mais relacionada com a obesidade e a hiperlipidemia (Muntoni e Muntoni, 2011).

A resistência à insulina afeta todas as ações metabólicas da insulina, que estão associadas ao sistema de transporte de glicose e ao próprio sistema de transdução de sinal da insulina, que é responsável por desencadear a translocação do transportador de glicose 4 (GLUT 4)(Richardson et al., 1991). A resistência à insulina afeta o metabolismo da glicose, através dos defeitos na sinalização dos RI (Mandarino et al., 1987; Saltiel e Kahn, 2001; Pirola et al., 2004). A insulina se liga ao seu receptor e promove a autofosforilação dos resíduos da tirosina quinase (Tyr) da sua subunidade β (Kim e Feldman, 2012). Após a autofosforilação do receptor de insulina Tyr e a fosforilação dos substratos de RI 1 a 4, ocorre a transdução do sinal de três diferentes vias de sinalização: via dependente da enzima fosfatidilinositol 3-quinase (PI3K), a qual medeia as respostas metabólicas, incluindo o metabolismo da glicose, dos lipídeos e de

proteínas (Kanai et al., 1993); o CAP / via Cbl é adicionalmente necessário para transportar a translocação do GLUT 4 e por último a via ativada pela proteína ativada por mitógeno (MAP quinase) que resulta na proliferação e diferenciação celular (Pirola et al., 2004).

1.2 Diabetes e Disfunções neurológicas

Estudos têm demonstrado que o DM afeta negativamente o Sistema Nervoso Central (SNC), revelando consequências neurológicas tanto em indivíduos com DM1 quanto com DM2 (McCall, 1992; Biessels et al., 1994, 2008; Cole et al., 2007; Lee et al., 2014; Tonoli et al., 2014). Diversas pesquisas revelam que pacientes com DM apresentam déficits cognitivos associados com o desempenho reduzido em diferentes domínios da função cognitiva(Kodl e Seaquist, 2008; Cholerton et al., 2013; Mayeda et al., 2015).A fisiopatologia dessas alterações causadas no cérebro de pacientes diabéticos ainda não está completamente esclarecida, mas é provável que a hiperglicemia e a resistência à insulina tenham significativa participação nesses processos (Kodl e Seaquist, 2008).

A hiperglicemia crônica é capaz de alterar os níveis de insulina cerebral e a atividade de seus receptores no cérebro, assim como é capaz de promover o aumento da oligomerização β-amilóide e induzir a hiperfosforilação da proteína Tau(Gasparini e Xu, 2003; Watson e Craft, 2004). A resistência à insulina também é capaz de acelerar o envelhecimento biológico, gerando a formação de produtos finais de

glicação avançada (AGE) e, consequentemente, espécies reativas de oxigênio(ROS)(Smith et al., 2003). Esses mecanismos podem contribuir para a disfunção no SNC, causando assim, mudanças na função cognitiva de pacientes diabéticos (Reagan, 2012).

A noção de que a insulina poderia cruzar a barreira hematoencefálica foi sugerida pela primeira vez por Margolis e Altszuler em 1967. Estes autores demonstraram que os níveis de insulina do fluído cérebro espinhal de ratos foram elevados após a infusão periférica desse hormônio, sugerindo que a insulina havia passado pela barreira hematoencefálica, possivelmente através do sistema de transporte saturável (Margolis RU, 1967).

A detecção de insulina no cérebro foi realizada por Havrankova e colaboradores em 1978 pelo uso da técnica de radioimunoensaio para determinar os níveis de insulina em extratos cerebrais. Os autores revelaram um aumento significativo de insulina no sangue e um pequeno aumento no fluído cerebroespinhal (Havrankova et al., 1978).Esses resultados confirmaram a correlação não linear entre os níveis de insulina no plasma e no fluído cerebroespinhal, proporcionando a primeira evidência concreta do sistema de transporte saturável de insulina a partir da circulação sistêmicapara o cérebro.Esses estudos támbémconfirmam a hipótese de que, pelo menos em parte, a insulina é produzida no SNC(Havrankova et al., 1978). No entanto, a insulina proveniente do

sistema periférico que atravessa a barreira hematoencefálica deve ser considerada.

A insulina presente no SNC de adultos é proveniente das células β-pancreáticas e é transportada pelo fluído cérebro-espinhal (Banks, 2004; Burns et al., 2007; Salkovic-Petrisic e Hoyer, 2007; Erol, 2008; Laron, 2009). Essa insulina atravessa a barreira hematoencefálica e, através de um transportador regulável, mediado por saturação e sensível à temperatura, é transportada para o encéfalo (Banks, 2004; Burns et al., 2007; Salkovic-Petrisic e Hoyer, 2007; Erol, 2008). Esse processo é limitado por um sistema de barreiras formado pelas junções de oclusão entre as células endoteliais (Schulingkamp et al., 2000). Alternativamente, a insulina periférica pode acessar diretamente o SNC, através da área póstrema e da região circumventricular como uma “fuga” da barreira hematoencefálica que permite a livre difusão de plasma nessa área (Schechter et al., 1992; Pardridge, 1993).

O efeito mais conhecido da insulina é a regulação do transporte de glicose e do metabolismo periférico, no entanto, demais efeitos já foram descritos, demonstrando que a insulina está envolvida na importante integração dos sinais periféricos hormonais e nutricionais mediados por um grupo de neurônios especializados do núcleo arqueado do hipotálamo, os chamados neurônios glicosensíveis (Porte et al., 2005). Esses neurônios respondem aos sinais periféricos que controlam o comportamento de ingesta alimentar e a energia basal (Porte et al., 2005). Essas evidências

demonstram que a insulina provoca uma resposta do eixo hipotálamo-fígado para regular a produção de glicose hepática (Girard, 2006). A insulina possui outras importantes funções no cérebro, como suporte a função neuronal, incluindo o processo de sinaptogênese, remodelação sináptica, e participação na modulação dos níveis de neurotransmissores (Craft et al., 2012).

A disfunção cognitiva em pacientes com DM foi descrita pela primeira vez em 1922, quando um estudo revelou que pacientes diabéticos demonstravam um comprometimento na memória e na atenção (Miles, W. R. & Root, 1922). O desenvolvimento da disfunção cognitiva em pacientes com DM ainda não é claro. Muitas hipóteses evidenciam essa interação citando como potenciais causadores a hiperglicemia, as doenças vasculares, a hipoglicemia, a resistência à insulina e a deposição de placas β-amilóide(McCrimmon et al., 2012). A causa da disfunção cognitiva em pacientes diabéticos pode ser a combinação desses fatores, dependendo do tipo de DM do paciente, das comorbidades, da terapia eda idade (Kodl e Seaquist, 2008).

O DM1 tem efeitos específicos sob um subconjunto de domínios cognitivos em adultos, como inteligência, atenção, velocidade psicomotora, flexibilidade cognitiva e percepção visual(McCrimmon et al., 2012; Cato et al., 2014). Estudos revelam que as disfunções cognitivas no DM1 são mais associadas com a velocidade psicomotora e com a eficiência mental (Brands et al., 2005; McCrimmon et al., 2012). As disfunções cognitivas no

DM1 surgem no início da doença (dentro de 2 anos do diagnóstico) (McCrimmon et al., 2012; Cato et al., 2014). Oencéfalo de crianças pode ser mais susceptível aos efeitos do DM do que o encéfalo de adultos, embora esse fato possa ocorrer devido à dificuldade de manter o controle glicêmico nessa idade(Ryan et al., 1985). Indivíduos que desenvolvem DM1 antes dos 7 anos de idade tem um risco maior de desenvolver déficits cognitivos severos do que os que desenvolvem a doença em idades mais tardias (Ryan, 2006). A aprendizagem e a memória, que pareciam ser os domínios cognitivos mais suscetíveis a doenças cerebrais precoces, não são afetadas mesmo quando os pacientes tem um longo histórico de mau controle glicêmico (Ryan, 2006).

O encéfalo é um órgão alvo tardio do DM2 e do pré-diabetes, porém as causas das disfunções cognitivas relacionadas com DM2 são difíceis de serem estabelecidas, devido à prevalência de comorbidades severas que podem estar afetando de forma conjunta à disfunção cognitiva (McCrimmon et al., 2012). Alguns mecanismos que corroboram para o declínio cognitivo têm sido propostos, como a desregulação dos níveis de insulina (Schulingkamp et al., 2000; Umegaki et al., 2008) e a hiperglicemia crônica (Bree et al., 2009; Cukierman-Yaffe et al., 2009), levando à disfunção neuronal (Baker et al., 2011), inflamação (Galasko e Montine, 2010), formação de AGEs (Yaffe et al., 2011), estresse oxidativo (Whitmer, 2007; Allan Butterfield et al., 2014), entre outros. Estudos demonstram déficits cognitivos específicos em pacientes com DM2, caracterizados pela

diminuição da velocidade psicomotora (Reaven et al., 1990; Kodl e Seaquist, 2008), da atenção (Fontbonne et al., 2001), da memória (Messier, 2005; Munshi et al., 2006; Caletti et al., 2015), da fluência verbal (Kanaya et al., 2004; Vincent e Hall, 2014), das funções executivas e o desenvolvimento de depressão (Bruce et al., 2003; Stuart e Baune, 2012; Haider et al., 2013; Gupta et al., 2014).

A hiperglicemia,a principal característica patológica do DM, pode ter efeitos tóxicos sob os neurônios no encéfalo através de insultos osmóticos e estresse oxidativo (Umegaki, 2012; Butterfield et al., 2014). As doenças neurodegenerativas são caracterizadas pela perda de neurônios que ocorre em uma região específica do SNC aliado a perda das funções normais apresentadas pelas células neuronais remanescentes (Doherty, 2011). Assim como as causas da neurodegeneração por si só são complexas, as causas do aumento do risco de neurodegeneração induzido pela hiperglicemia também podem ser múltiplas e de difícil compreensão. Existem estudos apontando que alguns fatores como a atrofia da vasculatura cerebral, o decréscimo na atividade de transporte de glicose e a piora na sinalização de insulina podem ter uma função crucial na redução do metabolismo cerebral em pacientes comDoença de Alzheimer (DA)(Moreira et al., 2009; Cholerton et al., 2013; Kleinridders et al., 2014).

Alguns estudos tem demonstrado a relação entre o DM e as disfunções em sistemas de neurotransmissão, tais como: dopaminérgico,

glutamatérgico, purinérgico e colinérgico (Artola et al., 2002; Rivera et al., 2005; Sherin et al., 2012; Elsherbiny e Al-Gayyar, 2013; Aung et al., 2014).

1.3 Sistema colinérgico

A acetilcolina (ACh) foi descrita em meados de 1920 e representa o primeiro neurotransmissor descoberto na história da neurociência (Brown, 2006). O sistema colinérgico participa de várias funções vitais (Mesulam et al., 2002), sendo a ACh o neurotransmissor mais importante desse sistema (Descarries et al., 1997). A ACh desempenha uma função fundamental no SNC e está relacionada à modulação da resposta neuronal por estímulos sensoriais (Murphy e Sillito, 1991), ao comportamento, à participação em circuitos neurais do controle do sono, ao aprendizado e memória (Shaked et al., 2008), à organização cortical do movimento e ao controle do fluxo sanguíneo cerebral(Moretto et al., 2004).Além de sua ação neurotransmissora, a ACh possui função neuromoduladora, pois seus níveis podem regular a concentração de outros neurotransmissores no encéfalo, como glutamato e ATP entre outros(Cooper e Roth, 1991).

A ACh é sintetizada nas extremidades dos axônios deneurônios colinérgicospré-sinápticos, a partir da acetilcoenzima A (acetil-CoA), e da colina, pela colina acetiltransferase (ChAT), enzima responsável por transferir um grupamento acetil da acetil-CoA para a colina (Oda, 1999; Clemente et al., 2004)(Figura 2).

A colina é captada do meio extracelular pela ação de transportadores específicos.Outraimportante fonte de ACh é a quebra de fosfatidilcolina. O passo limitante naformação de ACh é o transporte de acetil coenzima A, oriunda da membranainterna da mitocôndria após a via metabólica de transformação da glicose em piruvato.Após a síntese, a ACh é transportada dentro de vesículas para os terminais dos axônioscolinérgicos, onde é armazenada (Ferguson e Blakely, 2004; Amenta e Tayebati, 2008).

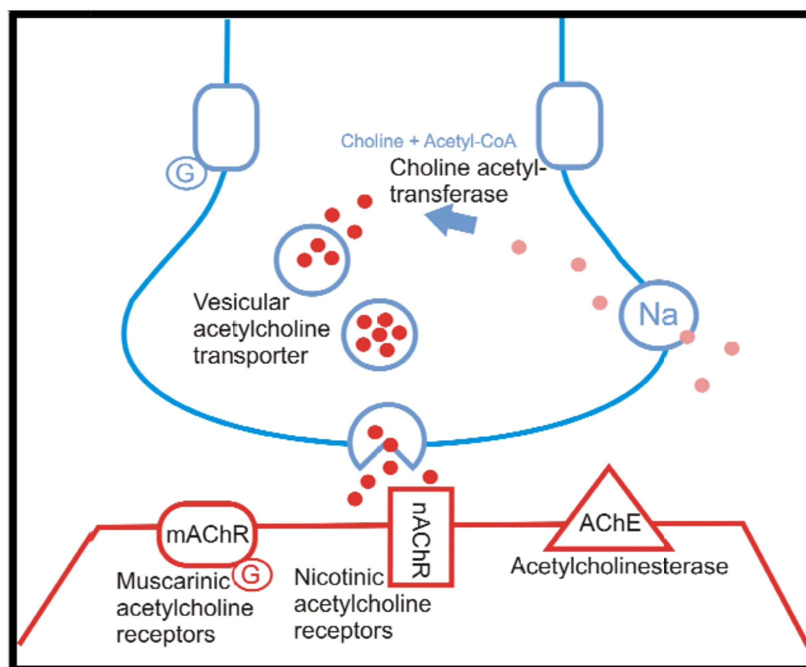


Figura 2:Representação esquemática de um neurônio colinérgico, demonstrando a síntese intracelular e degradação extracelular de acetilcolina. Fonte: Zimmermann (2007).

A liberação de ACh depende das variações no potencial elétrico da membrana plasmática do terminal nervosos e este processo é dependente da concentração de Ca^{2+} intracelular (Picciotto et al., 1998; Oda, 1999).Após um estímulo elétrico, a ACh é liberada na fenda sináptica e

exerce seus efeitos através da ativação de receptores nicotínicos e muscarínicos (Soreq e Seidman, 2001; Edwards et al., 2007; Park et al., 2008; Richetti et al., 2011).

Os receptores nicotínicos (nAChRs) consistem de cinco subunidades designadas α, β, γ e δ, sendo que a subunidade α é expressa em duas formas(Olivera-Bravo et al., 2006). A ACh se liga a subunidade α, produzindo mudanças conformacionais no receptor nicotínico que permitem a passagem de cátions, principalmente sódio (Na^{+}), potássio(K^{+}) e Ca^{+2}(Dani e Bertrand, 2007). A dessensibilização do receptor nicotínico aumenta quando o mesmo é fosforilado por proteína quinase dependente de AMP cíclico ou tirosina quinase (Diaz-Hernandez et al., 2002). Os receptores nicotínicos estão envolvidos em mecanismos de recompensa no SNC, isto explica em grande parte a dependência do uso de tabaco e de nicotina (Picciotto et al., 1998).

Os receptores muscarínicos (mAChRs) se associam à proteínas G e consistem em cinco tipos diferentes de receptores (M1-M5)(Bymaster et al., 2003; Wess, 2004). Assim como os nAChRs, um único neurônio colinérgico pode expressar mais de um tipo de subtipo de mAChR (Anagnostaras et al., 2003). Os mAChRs estão envolvidos na neurotransmissão e neuromodulação (Castillo et al., 1999; Ghatpande et al., 2006), memória olfatória (Ravel et al., 1994), aquisição de tarefas de discriminação de odores em roedores(De Rosa et al., 2001; Prediger et al., 2005; Edwards et al., 2007). Muitas evidências também os relacionam a processos de

aprendizado e memória, entre elas a observação de déficits cognitivos em ratos *knockout* para o gene do receptor muscarínico M1 (Anagnostaras et al., 2003).

Aacetilcolinesterase (AChE), enzima responsável pela degradação da Ach, pode ser usada como um marcador da função colinérgica, e mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (Soreq e Seidman, 2001). Esta enzima possui uma tríade catalítica composta por um ácido glutâmico, uma histidina e um resíduo de serina. O grupo carboxílico do ácido glutâmico ativa o grupamento hidroxila da serina que então hidrolisa as funções éster da ACh por ataque nucleofílico (Shafferman et al., 1992). Duas formas moleculares desta enzima são descritas: uma com forma assimétrica e outra com forma globular. A forma globular é encontrada preferencialmente em encéfalos de mamíferos com múltiplas subunidades, ficando algumas livres no citosol e outras ligadas à membrana (Heller e Hanahan, 1972; Taylor e Radić, 1994). Além disso, é também conhecida a importante função da AChE em doenças cuja incidência eleva com o aumento da idade, como aDA (Park et al., 2008; Casey et al., 2010).

1.4 Diabetes e Sistema colinérgico

Por ser uma das mais eficientes e conhecidas catálises biológicas, a ação da AChE tem sido investigada como um importante alvo terapêutico em várias doenças neurodegenerativas, sendo considerada uma

importante enzima regulatória e um bom indicador da atividade colinérgica (Appleyard, 1994; Szegletes et al., 1999; Das et al., 2001).Disfunções cognitivas observadas em pacientes humanos e modelos experimentais de DM tem sido relacionadas com alterações na atividade da AChE, o que pode indicar modificações na neurotransmissão colinérgica (Sánchez-Chávez e Salceda, 2000). Schmatzet al. (2009)demonstraram que ratos submetidos a um modelo de DM apresentam um aumento da atividade da AChE em diferentes regiões cerebrais, como: córtex cerebral, hipocampo, estriado, cerebelo e hipotálamo. Por esse motivo, agentes inibidores da atividade da AChE têm sido estudados com o objetivo de diminuir os efeitos hipocolinérgicos, observados quando a atividade da enzima aumenta (Das et al., 2001).

Alguns estudos também revelaram que anormalidades na manutenção da glicemia e resistência à insulina podem causar efeitos na síntese de ACh(Rivera et al., 2005; Sherin et al., 2012). Além disso,Gireeshet al. (2009) demonstraram que a expressão do receptor M1 foi diminuída no encéfalo de ratos em um modelo de DM induzido por estreptozotocina (STZ). Neste estudo, o tratamento com insulina foi capaz de reverter às alterações encontradas, demonstrando que o receptor muscarínico M1 pode ser afetado quando ocorre uma diminuição da concentração de insulina como, por exemplo, no DM.

Sherin et al.(2012)demonstraram que a hiperglicemia e a hipoglicemia, foi capaz de causar uma diminuição na neurotransmissão

colinérgica hipocampal em função de um aumento nos níveis de RNAm da AChE e uma redução nos níveis de RNAm dos receptores colinérgicos M1 e M3em modelo animal de ratos diabéticos, demonstrando assim, uma baixa estimulação dos receptores colinérgicos e portanto, uma piora cognitiva progressiva e outras disfunções neurológicas já vistas em pacientes diabéticos (Sherin et al., 2012).

1.5 Sistema purinérgico

A adenosina e o ATP são os principais agonistas endógenos do sistema purinérgico. A adenosina atua como neuromodulador endógeno da atividade neuronal, preservando e reestabelecendo a homeostase do SNC (Boison e Shen, 2010; Antonioli et al., 2013). O ATP atua como um rápido neurotransmissor excitatório e como neuromodulador, apresentando efeitos tróficosna proliferação, diferenciação e morte celular durante odesenvolvimento e a regeneração, assim como em condições patológicas(Burnstock e Verkhratsky, 2010; Burnstock e Novak, 2012).

A sinalização purinérgica no SNC atua através da regulação do balanço entre os efeitos do ATP, adenosina e das ectonucleotidases sobre a transmissão sináptica (Kato et al., 2004; Matsuoka e Ohkubo, 2004) (Figura 3). O ATP é presente em altas concentrações no encéfalo, variando de aproximadamente 2 mmol/kg no córtex a 4 mmol/kg no hipocampo (Kogure e Furones Alonso, 1978). O ATP exerce seus efeitos através da ativação de receptores purinérgicos do tipo P2, classificados em dois grupos, P2X e P2Y, de acordo com o mecanismo de ação, farmacologia e

clonagem molecular (Ralevic e Burnstock, 1998; Surprenant e North, 2009; Skaper et al., 2010). A família P2X consiste em receptores ionotrópicos que apresentam permeabilidade rápida e seletiva para cátions (Na^+, K^+ e Ca^{2+}) e está dividida em sete membros (P2X1-7), distribuídos em neurônios, células gliais e no músculo liso, os quais são ativados principalmente por ATP (Burnstock e Novak, 2012).

A família P2Y consiste em oito membros de receptores metabotrópicos funcionalmente descritos (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14), que apresentam uma ampla distribuição nos tecidos e sistemas, tais como: vascular, nervoso, imune, cardíaco e epitelial (Abbracchio et al., 2006; Erb et al., 2006; Burnstock, 2007).

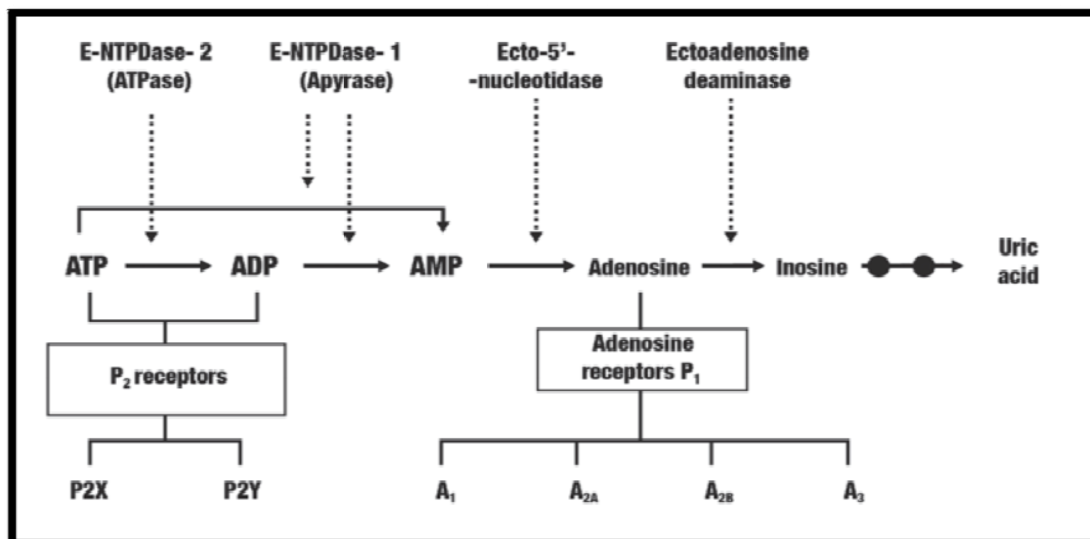
As ectonucleotidases são enzimas que controlam a disponibilidade e os níveis de nucleotídeos e nucleosídeos no meio extracelular, promovendo a ativação dos receptores P2 e P1, respectivamente (Robson et al., 2006; Miron et al., 2007; Zimmermann et al., 2012). As ectonucleotidases se encontram ancoradas em membrana celular, possuindo seu sítio ativo voltado para o meio extracelular ou presentes na forma solúvel no meio intersticial. Dentre elas, pode-se destacar a família das ectonucleosídeo trifosfato difosfohidrolase (E-NTPDases), que são responsáveis pela hidrólise extracelular de nucleotídeos trifosfatados e difosfatados, produzindo nucleotídeos monofostafatose fosfato inorgânico. As NTPDasessão compostas por oito membros (NTPDases 1-8) (Knowles, 2011) e são codificadas por oito genes diferentes chamados *entpd*. Quatro

destas enzimas estão localizadas na membrana celular com o sítio ativo voltado para o meio extracelular(NTPDases 1, 2, 3 e 8); duas estão localizadas intracelularmente em membranas de organelas(NTPDases 4 e 7) e duas são secretadas para o meio extracelular após expressão heteróloga (NTPDases 5 e 6) (Robson et al., 2006).

Outra família de ecto-enzimas envolvidas na hidrólise de nucleotídeos extracelulares é a da ectonucleotídeo pirofosfatase/fosfodiesterase (E-NPP) que é capaz de hidrolisar 3`,5`-AMPc, ATP, ADP, o NAD e a diadenosina polifosfato (Ap_nA) (Zimmermann, 2001).

A família da ecto-5`-nucleotidase, também pertence a este grupo de ecto-enzimas, sendo responsável pela hidrólise de nucleotídeos monofosfatados aos seus respectivos nucleosídeos(Zimmermann, 2001; Robson et al., 2006; Yegutkin, 2008; Zimmermann et al., 2012). A ecto-5-nucleotidase é responsável pela desfosforilação do nucleotídeo monofosfatado, AMP, gerando fosfato livre e adenosina.A ecto-5`-nucleotidase consiste em duas subunidades glicoproteícas, que estãoancoradas a membrana plasmática por uma molécula de glicosilfosfatidilinositol (GPI)(Zimmermann, 1996).Geralmente, o AMP é um nucleotídeo hidrolisado com maior eficiência, sendo que os valores de K_M para esta substância estão na faixa de micromolar (Zimmermann, 1992). A hidrólise de AMP pela ecto-5´-nucleotidaserepresenta um mecanismo extremamente importante no controledos níveis extracelulares de

adenosina (Knapp et al., 2012), sendo essa enzima marca-passo da cascata enzimática de degradação de nucleotídeos(Zimmermann, 1996; Cunha, 2001). Essa ecto-enzima também é conhecida como CD73 e



representa um marcador de maturação de linfócitos B e T (Hunsucker et al., 2005), podendo assim exercer uma variedade de funções, dependendo de sua expressão celular e tecidual (Zimmermann, 2001).

Figura 3- Representação esquemática do metabolismo dos ecto-nucleotídeos e ecto-nucleosídeos e os tipos de receptores purinérgicos. Fonte: Cieślak e Roszek, 2014.

A adenosina é um metabólito constituinte de todas as células, desenvolvendo diversas funções no organismo, tais como doadora de elementos para a síntese de ácidos nucléicos e aminoácidos e como molécula moduladora do estado metabólico da célula. No SNC, ela atua como neuromodulador, não sendo descrita como neurotransmissor por não ser armazenada em grânulos sinápticos ou liberada de forma quântica(Fredholm et al., 2005).

Além de ser formada a partir da hidrólise do ATP através da ação das ectonucleotidases, a adenosina pode ser produzida no meio intracelular e transportada para o meio extracelular através de transportadores específicos bidirecionais (ENTs), que mantém os níveis intracelulares e extracelulares de adenosina(King et al., 2006; Bonan, 2012). Os transportadores de nucleosídeos são divididos em duas categorias: os equilibrativose os concentrativos. Os transportadores equilibrativos transportam adenosina de acordo com seu gradiente de concentração (Bonan, 2012). Os transportadores concentrativos transportam a adenosina contra o seu gradiente de concentração de forma dependente de Na^+(Anderson et al., 1999; Sinclair et al., 2001; Visser et al., 2005).

Otransporte denucleosídeos afeta profundamente a neurotransmissão e tônus vascular. O sistema detransportadores é de suma importância para a regulação dos níveis endógenos de adenosina etambém para a regulação da estimulação de seus receptores (Cass et al., 1998; Anderson et al., 1999; Baldwin et al., 1999).

O nucleosídeo adenosina é uma molécula de sinalização que se acumula no meio intra e extracelular em resposta ao estresse metabólico, lesão tecidual ou inflamação (Antonioli et al., 2013). Esse nucleosídeo exerce seus efeitos através da ativação de receptores de membrana específicos do tipo P1 (Trincavelli et al., 2010), subdivididos em quatro subtipos: A_1, A_{2A}(de maiorafinidade pela adenosina), A_{2B} e A_3(de

menorafinidade pela adenosina), acoplados à proteína G e exibindo sete domínios transmembrana (Fredholm et al., 2005; Abbracchio et al., 2009).

A ação direta de agonistas de receptores adenosinérgicos é conhecida por oferecer neuroproteção, especialmente pelo bloqueio do influxo de Ca^{2+}, o qual resulta na inibição da liberação de glutamato e redução dos efeitos excitatórios deste aminoácido na pós-sinapse(Wardas, 2002). Outro mecanismo para promover neuroproteção mediada pela adenosina é relacionado à ativação dos receptores pós-sinápticos de adenosina, os quais atenuam a despolarização excessiva da membrana plasmática neuronal ao ativar canais de K^+ e aumentar o influxo deste íon levando à hiperpolarização (De Mendonça et al., 2000).

A concentração de adenosina pode ser controlada a partir de suahidrólise pela adenosina desaminase (ADA), responsável por catalisar a conversão da adenosina e da deoxiadenosina à inosina e deoxiinosina, respectivamente(Franco et al., 1998; Romanowska et al., 2007). Esta enzima possui uma função importante no sistema imune durante processos inflamatórios (Zavialov e Engström, 2005). Estudos têm sugerido que a inosina não é apenas um metabólito inativo, mas que pode ter ação neuroprotetora sob danos cerebrais (Tsuda, 2005).

A ADA pode ser encontrada como uma enzima citosólica e também pode ser expressa na superfície celular como uma ecto-enzima. Dois membros desta família já foram descritos, sendo eles denominados como ADA1 e ADA2 (Hirschhorn e Ratech, 1980; Zavialov e Engström, 2005;

Boison, 2012), além de um grupo similar desta família de proteínas denominado ADAL (*adenosine deaminase-like*)(Maier et al., 2005). Todos estes membros foram classificados como subfamílias pertencentes ao grupo das adenil-desaminases. Estudos tem demonstrado que a ecto-ADA pode estar co-localizada com receptores de adenosina do tipo P1 (A_1 e A_{2B}), contribuindo com a sinalização adenosinérgica (Ciruela et al., 1996; Ruiz et al., 2000; Herrera et al., 2001). A ADA1 esta localizada tanto no citosol como na membrana celular (Franco et al., 1997). Este membro da ADA tem importante função no sistema imunológico, controlando a inibição da proliferação das células T mediada pela adenosina (Gorrell et al., 2001).

A ADA2 é abundante no plasma humano, e possui diferentes caracteríticas cinéticas quando comparada a ADA1 (Iwaki-Egawa e Watanabe, 2002). Esse membro possui a habilidade de regular a proliferação celular, sendo considerado um fator de crescimento, denominando uma nova família de fatores de crescimento, os chamados fatores de crescimento relacionados à adenosina desaminase (ADGFs)(Zavialov e Engström, 2005; Zhang e Takeda, 2007).

Os aspectos funcionais da ADAL ainda não estão bem descritos na literatura, mas sabe-se quepor apresentar domínios catalíticos importantes relacionados à desaminação de adenosina e motivos conservados entre as subfamílias da ADA, é possível que a ADAL também realize a desaminação hidrolítica de adenosina à inosina (Maier et al., 2005; Sauer et al., 2012).

O peixe-zebra é um importante modelo para estudos neurológicos e comportamentais, sendo a sinalização adenosinérgica muito estudadaneste modelo. Estudos realizados em nosso laboratório identificaram e caracterizaram a atividade e expressão gênica das enzimas NTPDase e 5´-nucleotidase (Rico et al., 2003; Senger et al., 2004). Rosemberg et al. (2010)verificaram a presença de diferentes membros da família das NTPDases edemonstraram a expressão destas enzimas (NTPDases 1, 2, 3, 4, 5, 6 e 8) emamostras de encéfalo, coração e fígado. As NTPDase1 e NTPDase2 tambémforam encontradas em fotorreceptores, células horizontais e célulasganglionares em retina de peixe-zebra (Ricatti et al., 2009; Rosemberg et al., 2010). Outro estudo clonou e caracterizou o padrão de expressão da NTPDase 3 em peixe-zebra (Appelbaum et al., 2007), indicando a conservação e a expressão destes genes nos neurônios sensoriais.

Estudos já demonstraram a presença dos receptores de adenosina, em peixe-zebra, indicando a presença de duas formas de receptores A_{2A}de embriões de peixe-zebra e uma forma de A_{2B}, bem como a presença de RNAm de receptores A_1e sua expressão desde 24 horas apósa fertilização(Boehmler et al., 2009; Capiotti et al., 2011). Estesestudos também demonstraram que a exposição à cafeína é capaz de alterar a expressão desses receptores nas fases iniciais do desenvolvimento, bem como, das proteínas DARPP-32 e BDNF, além de promover proteção à exposição ao MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina), em um

modelo de lesão dopaminérgica(Boehmler et al., 2009; Capiotti et al., 2011).

Nosso laboratório realizou a identificação molecular e avaliou o padrão de expressão de diferentes genes relacionados à família da ADA em peixe-zebra (Rosemberg et al., 2007). Neste estudo, foi realizado um mapeamento dos diferentes genes da ADA no genoma no peixe-zebra, realizando um estudo filogenético e confirmando a presença dos diferentes genes relacionados à ADA (ADA1, ADAL e duas isoformas da ADA2).Os resultados demonstraram ainda que diferentes membros da família da ADA são expressos em diferentes tecidos, como encéfalo, brânquias, coração, fígado, músculo esquelético e rinsde peixe-zebra.

Nosso grupo também realizou acaracterização cinética da atividade da ADA, nas frações solúveis e de membrana em encéfalo de peixe-zebra indicando as condições ótimas para a atividade desta enzima edemonstrando uma inibição significativa na presença de 0,1 mM de EHNA, um inibidor clássico da ADA(Rosemberg et al., 2008).

1.6 Diabetes e Sistema purinérgico

O DM está associado com alterações cognitivas, estruturais e fisiológicas do SNC. Alterações nervosas resultantes da degeneração cerebral têm sido descritas no estadodiabético, possivelmente em decorrência de modificações na plasticidade sináptica,o que compromete o mecanismo de regulação da homeostase celular, e tem comoconsequência

a disfunção na atividade dos neurotransmissores na fenda sináptica(Biessels et al., 2002).

Um aumento crescente de evidências tem destacado o papel do sistema purinérgico como um fator importante na regulação da homeostase da glicose e da fisiopatologia do DM(Burnstock e Novak, 2012; Johnston-Cox et al., 2012; Csóka et al., 2014). Alterações nos níveis intracelulares de ATP/ADP podem gerarconsequências na sinalização e na sobrevivência celular de indivíduos diabéticos(Burnstock e Novak, 2012). Alguns autores sugerem que o DM é capaz dealterar o tônus purinérgico, através da indução de mudanças adaptativas na sensibilidade dos receptores de adenosina no encéfalo(Morrison et al., 1992; Duarte et al., 2006).Consequentemente, pode-se presumir que os níveis extracelulares de nucleotídeo/nucleosídeo da adenina poderiam também ser afetados (Burnstock e Novak, 2012). A disfunção da plasticidade sináptica em indivíduos diabéticos pode ser relacionada com a ineficiência da liberação de neurotransmissores, como dopamina, serotonina, glutamatoe acetilcolina (Guyot et al., 2001; Yamato et al., 2004; Sherin et al., 2012). Duarte et al. (2006) revelaram que ratos submetidos a um modelo de DM apresentaram uma redução na liberação de neurotransmissores e diferentes alterações nas proteínas pré-sinápticas associadas com a liberação vesicular de neurotransmissores.

Lunkes et al.(2004)demonstraram em um modelo animal deDM2uma alteração na hidrólise dos nucleotídeos ATP/ADP em sinaptossomas e

plaquetas, sendo estes resultados relacionados à expressão da NTPDase I aumentada. Donget al. (2001)demonstraram que a ativação de receptores de adenosina do tipo A_1 desenvolve uma função protetora contra a resistência à insulina.

1.7 Peixe-zebra

O peixe-zebra, *Danio rerio*, é um pequeno teleósteo de água doce pertencente à família Cyprinidae. O gênero Danio possui 44 espécies descritas que fazem parte da subfamília Raborinae e apresenta um padrão de coloração com listras horizontais claras e escuras alternadas (Spence et al., 2008). A distribuição natural do peixe-zebra é ao sul e sudeste da Ásia, principalmente Índia, Bangladesh e Nepal.

O peixe-zebra se tornou um modelo experimental amplamente utilizado nas mais diversas áreas devido a diversas características favoráveis, tais como: baixo custo, pouco espaço para manutenção, rápido desenvolvimento e ciclo biológico, fácilmanipulação e comportamentofacilmenteobservado em um ambiente controlado (Shin e Fishman, 2002; Lieschke e Currie, 2007).

O peixe-zebra apresentagrande sensibilidade a drogas, um rápido metabolismo (Goldsmith, 2004) e genes evolutivamente conservados que apresentam um alto grau de similaridade com os genes de humanos e de camundongos (Barbazuk et al., 2000).Recentemente, seu genoma foi completamentesequenciado, demonstrando que 70% dos genes deste teleósteo têm um ortólogo humanoidentificável (Howe et al., 2013).

O peixe-zebra é um importante modelo para estudos neurológicos e comportamentais (Egan et al., 2009; Piato et al., 2011; Jones e Norton, 2014; Stewart et al., 2014). O SNC do peixe-zebra apresenta organização geral, células neuronais especializadas, células gliais, mielina e circuitos neuronais semelhantes ao que é observado em mamíferos (Bandmann e Burton, 2010; Stewart et al., 2014). A ocorrência dos principais neurotransmissores encontrados em mamíferos, incluindo aminoácidos (glutamato, GABA, glicina), monoaminas (histamina, dopamina, norepinefrina, epinefrina, serotonina, melatonina) e acetilcolina, entre outros, bem como seus mecanismos de ação já foram descritos em peixe-zebra (Rinkwitz et al., 2011). Além disso, o peixe-zebra vem destacando-se como um importante modelo animal para estudos de numerosas doenças humanas, tais como obesidade, DM e neuropatias(Best e Alderton, 2008; Craig e Moon, 2011) e para a triagem e descoberta de novos fármacos (Chakrabarti e Freedman, 2008).

Este modelo animal tem sido cada vez mais utilizado para a análise de aprendizado e de memória(Colwill et al., 2005; Gerlai, 2011; Cognato et al., 2012), demonstrando que o peixe-zebra tem a capacidade deassociar uma variedade de estímulos condicionados e não condicionados(Sison e Gerlai, 2011; Zala e Määttänen, 2013; Fernandes et al., 2014), possui memória espacial (Spence et al., 2011; Cognato et al., 2012), memória aversiva (Blank et al., 2009) e visual (Avdesh et al., 2012). Nesse contexto, o peixe-zebra tem se tornado uma excelente ferramenta de pesquisa

damemória e da cognição, com possibilidade de realizar triagens genéticas e farmacológicas(Hicks et al., 2006; Gerlai, 2010).

Atualmente, muitos estudos são realizados em peixe-zebra para compreender as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (Guo, 2004). Modelos transgênicos já foram desenvolvidos nessa espécie expressando genes humanos mutados e modelos *knockout* que reproduzem patologias humanas, bem como técnicas para controle da expressão gênica (Bandmann e Burton, 2010) e indução de sintomas característicos de doenças metabólicas. Tais estudos evidenciam que o peixe-zebra é um modelo para compreender os mecanismos relacionados com algumas doenças humanas como, por exemplo, hiperargininemia (Capiotti et al., 2013), estresse (Siebel et al., 2010), obesidade (Oka et al., 2010; Craig e Moon, 2011) e DM(Gleeson et al., 2007; Jorgens et al., 2012).

Alguns estudos vêm sendo realizados utilizando o peixe-zebra como modelo para compreender os mecanismos pelos quais o DM atua. Glesson et al.(2007) e Alvarez et al.(2010), demonstraram um modelo de hiperglicemia através da imersão dos animais em diferentes concentrações de glicose. O modelo foi capaz de gerar sintomas de retinopatia, reproduzindo as fases iniciais da doença em humanos. Elo et al.(2007) demonstraram que a exposição transdermal de glicose em larvas de peixe-zebra é capaz de alterar a atividade da enzima fosfoenolpiruvato

carboxilase(PEPCK), sugerindo o peixe-zebra como um modelo para investigar o metabolismo da glicose, utilizando a enzima PEPCK como um marcador dos distúrbios da homeostasia da glicemia.

Olsen et al.(2010) também descreveram um modelo de DM em peixe-zebra adulto via utilização de STZ, a qual induz a morte das células pancreáticas, promovendo complicações diabéticas como níveis aumentados de glicose no sangue, glicação de proteínas séricas, retinopatia, diminuição dos níveis de insulina e piora da regeneração de áreas lesionadas. Oka et al. (2010) descreveram o modelo de obesidade em peixe-zebra adulto através do aumento da ingesta de alimentos ricos em gordura. O estudo revelou que a indução da obesidade é capaz de promover hiperglicemia e hipertrigliceridemia, como vias patofisiológicas semelhantes às encontradas em mamíferos (Oka et al., 2010). Powers et al.(2010) demonstraram um modelo de hiperglicemia por estresse em peixe-zebra, demonstrando a correlação dos altos níveis de glicose sanguínea com os níveis de cortisol. Nesse contexto, acreditamos que o modelo de hiperglicemia desenvolvido em peixe-zebra nesta tese, seja uma ferramenta muito importante para investigar os mecanismos que operam no SNC durante o estado de hiperglicemia.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar parâmetros bioquímicos e moleculares dos sistemas colinérgico e purinérgico e parâmetros comportamentais de peixe-zebra submetido a um modelo de hiperglicemia.

2.1.1 Objetivos específicos

- Caracterizar o modelo de hiperglicemia em peixe-zebra adulto através do registro da glicemia, do peso corporal, da glicação de proteínas, da sensibilidade à insulina e da expressão gênica de receptores de insulina em músculo esquelético e de transportadores de glicose em encéfalo;
- Avaliar o índice de retenção de memória através do teste de esquiva inibitória em peixe-zebra adulto submetido ao modelo de hiperglicemia;
- Avaliar a atividade enzimática e expressão gênica da acetilcolinesterase em encéfalo de peixe-zebra adulto submetido ao modelo dehiperglicemia;
- Avaliar a atividade enzimática e expressão gênica de ectonucleotidases e adenosina deaminase em encéfalo de peixe-zebra submetido ao modelo dehiperglicemia;

- Avaliar a expressão gênica de receptores adenosinérgicos em encéfalo de peixe-zebra submetido ao modelo de hiperglicemia.

3 RESULTADOS

3.1 CAPÍTULO I

ARTIGO CIENTÍFICO

Persistent impaired glucose metabolism in a zebrafish hyperglycemia model

Katiucia Marques Capiotti, Régis Antonioli Junior, Luiza Wilges Kist, Maurício Reis Bogo, Carla Denise Bonan, Rosane Souza da Silva.

Artigo publicado no periódico

Comparative Biochemistry and Physiology, Parte B 171:58-65. 2014

(doi: 10.1016/j.cbpb.2014.03.005)

Comparative Biochemistry and Physiology, Part B 171 (2014) 58–65

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Persistent impaired glucose metabolism in a zebrafish hyperglycemia model

Katiucia Marques Capiotti [a,c], Régis Antonioli Junior [a,c], Luiza Wilges Kist [b,c], Maurício Reis Bogo [b,c], Carla Denise Bonan [a,c], Rosane Souza Da Silva [a,c,*]

[a] Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUCRS, Porto Alegre, RS, Brazil
[b] Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUCRS, Porto Alegre, RS, Brazil
[c] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003 Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 9 November 2013
Received in revised form 12 March 2014
Accepted 27 March 2014
Available online 3 April 2014

Keywords:
Diabetes mellitus
Glucose
Insulin receptor
Metabolism
Zebrafish

ABSTRACT

Diabetes mellitus (DM) affects over 10% of the world's population. Hyperglycemia is the main feature for the diagnosis of this disease. The zebrafish (*Danio rerio*) is an established model organism for the study of various metabolic diseases. In this paper, hyperglycemic zebrafish, when immersed in a 111 mM glucose solution for 14 days, developed increased glycation of proteins from the eyes, decreased mRNA levels of insulin receptors in the muscle, and a reversion of high blood glucose level after treatment with anti-diabetic drugs (glimepiride and metformin) even after 7 days of glucose withdrawal. Additionally, hyperglycemic zebrafish developed an impaired response to exogenous insulin, which was recovered after 7 days of glucose withdrawal. These data suggest that the exposure of adult zebrafish to high glucose concentration is able to induce persistent metabolic changes probably underlined by a hyperinsulinemic state and impaired peripheral glucose metabolism.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by high levels of blood sugar, termed hyperglycemia (SBD, 2009). According to the International Diabetes Federation (IDF), in the year 2012, the estimated number of adults living with diabetes had soared to 371 million or 8.5% of the global adult population (IDF, 2012). New projected data suggest that until 2030 there will be 552 million people with diabetes; this means for that every 10 s approximately three more people will be diagnosed with diabetes (IDF, 2012).

Chronic blood sugar elevation is the major biochemical diagnostic parameter that is seen in the two major forms of diabetes: Type 1 diabetes or insulin-dependent, caused by autoimmune destruction of insulin producing pancreatic β-cells, and Type 2 diabetes or non-insulin dependent characterized by insulin insensitivity (Harris and Zimmet, 1997).

Zebrafish has been established as a good animal model to understand physiological and pathological conditions in vertebrates (Maddison and Chen, 2012; Asaoka et al., 2013; Stewart et al., 2014). Olsen et al. (2010) described the diabetes model in adult zebrafish via streptozocin which induces pancreactic cell death, promoting diabetic complications such as fasting elevated blood glucose values, increased glycation of serum protein, retinopathy, decreased serum insulin levels and impaired regeneration in damaged areas (Oka et al., 2010; Olsen et al., 2010). Other studies described an obesity model in adult zebrafish, revealing that the induction of obesity is able to promote hypertriglyceridemia and hyperglycemia, as common pathophysiological pathways similar with those found in mammalians (Gleeson et al., 2007; Oka et al., 2010). Adult zebrafish easily absorb molecules from water, due to an ability to regulate their internal water and total solute concentrations (Moyle and Cech, 2000). They operate hyperosmotically, a strategy of osmoregulation that involves the continuous gain of water as a result of a higher internal concentration of salt compared to their freshwater environment. The constant influx of water results in the uptake of molecules from their environment. In this way, an easy model of hyperglycemia was proposed by immersing zebrafish in a glucose solution, being capable of generating diabetic retinopathy similar to humans (Ferraris and Ahearn, 1984; Bogé and Péres, 1990; Gleeson et al., 2007; Tseng et al., 2009; Alvarez et al., 2010). In zebrafish, the glucose absorption/uptake occurs through a glucose transporter, called GLUT, expressed in the

* Corresponding author at: Faculdade de Biociências, PUCRS, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil. Tel.: +55 51 3320 3500x4158; fax: +55 51 3320 3612.
E-mail addresses: katucapiotti@gmail.com (K.M. Capiotti), regis.antonioli@acad.pucrs.br (R. Antonioli), lwkist@gmail.com (L.W. Kist), mbogo@pucrs.br (M.R. Bogo), cbonan@pucrs.br (C.D. Bonan), rosane.silva@pucrs.br (R.S. Da Silva).

http://dx.doi.org/10.1016/j.cbpb.2014.03.005
1096-4959/© 2014 Elsevier Inc. All rights reserved.

gills (GLUT 1–3, 6, 8, and 10–13) and intestine (GLUT 5 and 9) (Tseng et al., 2009). Here we described that glucose overload in living water of fish is able to induce symptoms related to the pathophysiology of DM, which are persistent after a glucose withdrawal period and sensitive to anti-diabetic drugs.

2. Material and methods

2.1. Animals

Zebrafish (*Danio rerio*) adult wild type (Tübingen background; 3–5 cm) of both sexes were acclimated for at least 14 days in the experimental room. Animals were housed in groups of 15 fish in 5-L thermostated (28 ± 2 °C) tanks, kept under constant chemical, biological and mechanical water filtration and aeration (7.20 mg O_2/L). Fish were maintained under a 14 h/10 h day/night photoperiod cycle, fed three times a day with commercial flakes containing 48% protein, 8% fat, and 2% fiber (TetraMin™, NC, USA) (Siccardi et al., 2009) and supplemented with live brine shrimp. All fish used in these experiments were randomly chosen from different clusters. The animals were submitted to hypothermia by exposure to ice water, flocked, and followed by decapitation as the endpoint. Anesthesia with Tricaine MS-222 was done only in experiments using intraperitoneal injection as described below. All protocols were approved by the Institutional Animal Care Committee (12/00310 — CEUA PUCRS) and followed Brazilian legislation, the guidelines of the Federal Council of Veterinary Medicine (CFMV), and the Canadian Council for Animal Care (CCAC) "Guide on the Care and Use of Fish in Research, Teaching, and Testing".

2.2. Drugs

Treatment concentrations were as follows: Tricaine MS-222 (0.1 g/mL; ethyl 3-aminobenzoate methanesulfonate salt, Fluka™); glucose in three concentrations: 55.5, 111 and 166 mM (w/v) (Nuclear™); human insulin (0.01, 0.1 and 1 U/kg; Novolin™); metformin chloridate (10 μM; Merck™) and glimepiride (100 μM; Merck™).

2.3. Induction of hyperglycemia

Groups of 15 adult animals were placed in 5-L aquaria containing different glucose solutions (55.5, 111 and 166 mM), which were diluted in water and maintained during 14 days at room temperature. The feeding schedule and general maintenance procedures were as already described in the previous section. The solutions of glucose were exchanged three times per week to avoid contamination with opportunistic microorganisms. The animals were placed in each solution and were monitored for signs of stress such as difficulty of swimming or excessive gill movement (Pedroso et al., 2012). Control and 55.5 and 111 mM glucose-treated groups showed no signs of stress during all treatment with low mortality (30% and 20%, respectively), while 166 mM glucose-treated animals appeared to be more affected by the treatment, demonstrating 40% mortality. Control group animals were maintained in 5-L aquariums with normal water for the same period and conditions as the glucose-treated groups. For the following experiments, we chose the 111 mM glucose solution since the survival was the highest and the profile of blood glucose levels was similar to those published by Gleeson et al. (2007).

2.4. Withdrawal of glucose

In order to verify whether the effects caused by 111 mM glucose solution treatment would be persistent, we kept a group of 15 fish in glucose-free water for an additional 7 days (washout group, no glucose added). During this period the animals were maintained at the same conditions of welfare described in the previous section. Control group animals were maintained in 5-L aquariums with water for the same period and were handled as a washout group.

2.5. Determination of blood glucose levels

For all tests with determination of blood glucose levels, before the beginning of blood collection procedures, fish were fasted for 12 h and placed for 15 min in an aquarium with water without glucose to avoid contamination of the glucometer strip (Gleeson et al., 2007). The procedure of euthanasia occurred after hypothermia induction, which contributes to reduce the variability of blood glucose analyses (Eames et al., 2010). Immediately, the tail was cut and blood-glucose readings were taken by placing a glucometer test strip (One-Touch Ultra, Accu Check) directly on the docked tail. The blood glucose levels were measured throughout the treatment for 14 days at all concentrations tested (55.5, 111 and 166 mM glucose-treated groups) and after 7 days of glucose withdrawal (washout group) (Gleeson et al., 2007).

2.6. Determination of glycated proteins from zebrafish eyes

One of the methods to monitor glycemic control in humans is through the levels of non-enzymatic glycation of proteins, such as the determination of fructosamine levels (Armbruster, 1987). We utilized the enzyme based fructosamine quantification assay (Biotechnical™, RS, Brazil), with minor adaptation, to document the presence of non-enzymatic glycation products from zebrafish eyes from all groups. Zebrafish eyes were removed and washed in water and afterwards placed in 500 μL of saline and homogenized. Following the manufacturer's instructions, 50 μL samples were added to the 1 mL of reagent containing 200 nmol/L carbonate buffer, and 0.25 nmol/L Blue nitrotetrazol which were incubated for 10–15 min at 37 °C. The quantification of fructosamine was determined spectrophotometrically at 520 nm and the concentration was expressed as μmol/L.

2.7. Treatment with anti-diabetic metformin and glimepiride drugs

Glucose-treated and washout animals were submitted to the anti-diabetic metformin or glimepiride. We dissolved metformin chloridate in water of fish to a final concentration of 10 μM. The collection of blood samples was performed after 4 days of treatment (Elo et al., 2007; Polakof et al., 2011; Polakof and Comte, 2012). For treatment with glimepiride, we dissolved glimepiride in DMSO (final concentration 0.0005%) and added to water to a final concentration of 100 μM. The collection of blood samples was performed after 24 h of treatment, due to the results found by Elo et al. (2007). Blood glucose levels were measured with a blood glucometer as described. Saline was used as control for the metformin group and 0.0005% (v/v) DMSO solution was used as control for the glimepiride group. Metformin, glimepiride, DMSO and saline were freshly prepared on the experimental day.

2.8. Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

The expression of isoforms of insulin receptor genes (*insra-1*, *insra-2*, *insrb-1* and *insrb-2*) was analyzed by a quantitative real time reverse transcription polymerase chain reaction (RT-qPCR) assay. TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) was employed to isolate total RNA from zebrafish skeletal muscle in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for reference genes *EF1α*, *Rpl13α* and *insra-1* and 1:100 for *insra-2*, *insrb-1*, *insrb-2*), containing SYBR® Green I 0.2 times diluted (Invitrogen), 100 μM dNTP, 1 × PCR buffer, 3 mM $MgCl_2$, 0.25 U

Platinum® Taq DNA polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing, and 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System sequence detection software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR version 2012.3 software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α*, and *Rpl13α* (*M-value*) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Oggier et al., 2010; Nery et al., 2011).

2.9. Response to insulin

Glucose and washout groups were checked for the responsiveness to insulin. Fifteen animals of each group (control, 111 mM glucose-treated and washout) were placed in water without glucose for 24 h before the beginning of the experiment, in order to prevent interference on the fasting regimen. After that, fish were anesthetized in 0.1 mg/mL of Tricaine MS-222 prior the collection of blood. Fish were considered to be anesthetized when they presented lack of motor coordination, reduced respiration rate and no response to external stimuli (Phelps et al., 2009). We utilized Novolin™ Insulin Human 100 U/mL in three different doses: 0.01 U, 0.1 U and 1 U with volume adjusted to the fish body weight (injection volume was 10 μL) in a 20 mL/kg regimen. Intraperitonial injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31 G short needles (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. (2009). The needle was inserted parallel to the spine into the midline of the abdomen, posterior to the pectoral fins. The injection procedure was conducted to guarantee that the animals did not spend more than 10 s out of the water. After the injection, the animals were placed in a separate tank with highly aerated unchlorinated tap water (28 ± 2 °C) to facilitate animal recovery from anesthesia. Saline was used as control. Both insulin and saline were freshly prepared on the experimental day. All the animals had recovered after 2–3 min following the injection. The glycemia was accessed 4 h after insulin injection as suggested by previous studies using zebrafish (Olsen et al., 2010). The insulin-treated animals and controls had its tail cut and the blood collected (Pedroso et al., 2012). Animals were gently placed in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen) (Phelps et al., 2009).

2.10. Statistical analysis

Data were expressed as mean ± standard error of mean. The data from insulin response experiments were analyzed by student's *t*-test. All other data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's considering $P < 0.05$ as statistically different for biochemical and mRNA expression experiments in adult zebrafish.

3. Results

3.1. Chronic treatment with glucose induces persistent hyperglycemia

Our results demonstrated that the immersion in all glucose solution tested (Fig. 1) provoked up to a 4–5 times increase in blood glucose levels until the 14th day of treatment in relation to control animals (2.99 ± 0.18 mM) [55.5 mM − $F_{(6;\ 100)} = 25.39$, 111 mM − $F_{(6;\ 100)} = 25.57$, 166 mM − $F_{(6;\ 101)} = 34.72$]. We observed that 20% of the animals treated with 166 mM glucose died during the treatment, and the next analyses were made with only the 111 mM glucose treated animals, which is in accordance to the zebrafish model of hyperglycemia described by Gleeson et al. (2007). In order to verify if the effects caused by treatment of 14 days of immersion in 111 mM glucose solution would be persistent, we performed a glucose withdrawal. Our results demonstrated that after 7 days of glucose withdrawal, there was a strong reduction (68%) on blood glucose levels (from 13.52 ± 1.29 mM to 4.26 ± 0.31 mM) when compared to the 111 mM glucose group, but still almost 2 times higher than the control group (2.48 ± 0.17 mM) [$F_{(2;\ 43)} = 69.03$, $p < 0.001$] (Fig. 2). These data show that hyperglycemia can be induced and maintained in zebrafish.

3.2. Hyperglycemia increases glycation of proteins from zebrafish eyes

In order to demonstrate that treatment with glucose for 14 days was causing persistent glycation of proteins, we analyzed the levels of fructosamine from zebrafish eyes during glucose treatment and after glucose withdrawal. Our results demonstrated that the treatment with 111 mM glucose for 14 days was able to increase (41%) fructosamine levels from zebrafish eyes and also demonstrated that this effect was prolonged up to 7 days of glucose withdrawal, demonstrating an increase (40%) in the fructosamine levels from zebrafish eyes in comparison to the control group (80.78 ± 8.54 μmol/L) [$F_{(2;\ 17)} = 6.369$, $p < 0.05$] (Fig. 3).

3.3. Anti-diabetic drugs reduce blood glucose levels from hyperglycemic zebrafish

To evaluate if hyperglycemic zebrafish would be responsive to the anti-diabetic drugs, we treated the animals with glimepiride

Table 1
Primer sequences for RT-qPCR experiments included in the study.

Sequence	Forward	Reverse	Accession number (mRNA)	Amplicon size (bp)
EF1α[*]	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′	ENSDART00000023156	86
Rpl13α[*]	5′-TCTGGAGGACTGTAAGAGGTATGC-3′	5′-AGACGCACAATCTTGAGAGCAG-3′	NM_212784.1	147
insra-1[**]	5′-CAACATGCCCCCTCACCACT-3	5′-CGACACACATGTTGTTGTG-3′	NM_001142672	215
insra-2[**]	5′-GGAGCCCCACTCGTCTAACAAA-3′	5′-CGCCGTTGTGAATGACGTATTC-3′	–	193
insrb-1[**]	5′-GACTGATTACTATCGCAAGGG-3′	5′-TCCAGGTATCCTCCGTCCAT-3′	NM_001123229	190
insrb-2[**]	5′-CCACCGCCAACCCTAAAGGA-3′	5′-TTGCGATAGTAATCAGTC TCGTAAAT-3′	–	170

[*] Tang et al., (2007).
[**] Toyoshima et al., (2008).

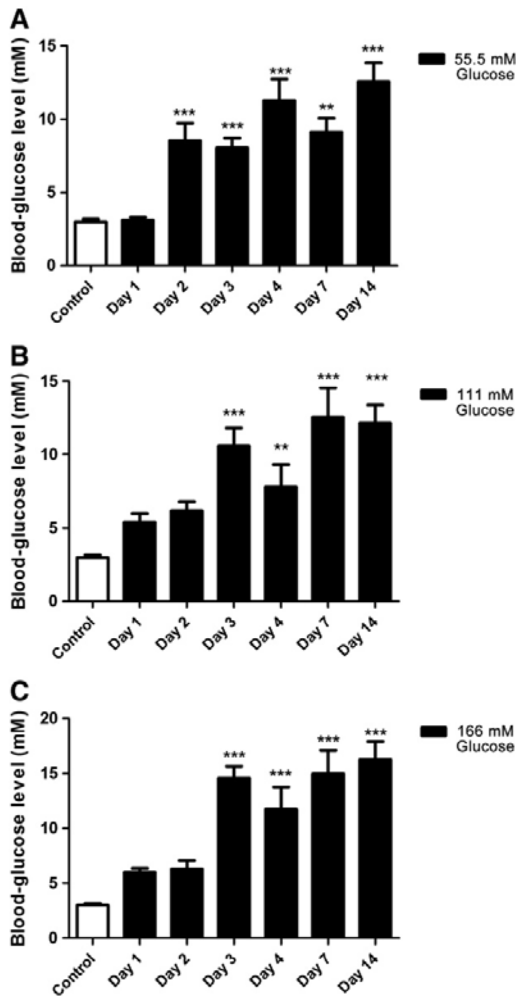


Fig. 1. Effects of glucose treatment up to 14 days on blood glucose levels. (A) 55.5 mM glucose, (B) 111 mM glucose and (C) 166 mM glucose. At least nine zebrafish were used for each group. Data are expressed as mean ± SEM of nine independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. ***p < 0.001 and **p < 0.01.

and metformin. Our results demonstrated that the treatment with 100 μM glimepiride was able to reduce 64% of the blood glucose levels provoked by 111 mM glucose treatment (from 8.71 ± 2.22 mM to 4.43 ± 0.56 mM) [$F_{(3;\ 13)} = 63.68$; $p < 0.001$] (Fig. 4A), and 63% in the glucose washout group (from 5.23 ± 0.32 mM to 1.94 ± 0.16 mM) [$F_{(3;20)} = 53.48$; $p < 0.001$] (Fig. 4B). The treatment with 10 μM of metformin was able to reduce 51% of the blood glucose levels provoked by 111 mM glucose treatment (from 9.21 ± 1.12 mM to 4.69 ± 1.77 mM) [$F_{(3,49)} = 99.72$; $p < 0.0001$], and 45% in the glucose washout group (from 5.20 ± 0.55 mM to 2.36 mM ± 0.66 mM) [$F_{(3,39)} = 47.77$; $p < 0.0001$] (Fig. 4C and D, respectively). The anti-diabetic drugs alone did not affect the control glycemia (Fig. 4).

3.4. Effect of hyperglycemia on insulin receptors expression in skeletal muscles of zebrafish

To evaluate if the hyperglycemia could alter the relative gene expression of insulin receptors in skeletal muscles, real-time quantitative

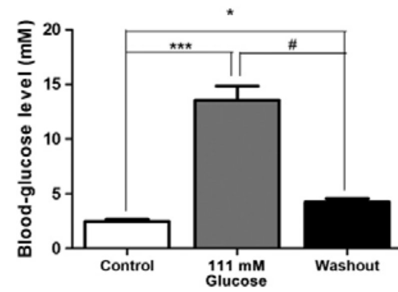


Fig. 2. Effect of glucose washout on hyperglycemic zebrafish. At least thirteen zebrafish were used for each group. Data are expressed as mean ± SEM of ten independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. The # represents a significant difference from the 111 mM glucose group. *p < 0.05 and ***p < 0.001 and #p < 0.001.

PCR analyses were performed. Our results demonstrated that the expression levels of insulin receptor subunit genes (*insra-1*, *insrb-1* and *insrb-2*) were increased (*insra-1* (97.5%) [$F_{(2;\ 17)} = 8.040$; $p < 0.05$], *insrb-1* (48%) [$F_{(2;16)} = 6.288$; $p < 0.01$] and *insrb-2* (44.5%) [$F_{(2;16)} = 5.92$; $p < 0.05$]) in the washout group when compared to the control group (Fig. 5 A, C and D). However, there were no statistical relevant changes in *insra-2* gene (Fig. 5B).

3.5. Impaired response to insulin

As the above analyses assemble possible information on insulin response impairment, we submitted glucose-treated animals to an injection of insulin in three different doses: 0.01 U/kg, 0.1 U/kg and 1 U/kg. Our results demonstrated that 0.01 U/kg and 0.1 U/kg doses were unable to reverse the hyperglycemic effect caused by the treatment with 111 mM glucose solution or to reduce glucose blood level of the control group (data not shown). However, the 1 U/kg dose was able to decrease 20–32% of the basal blood glucose levels of the control group (Fig. 6A: from 2.80 ± 0.18 mM to 2.27 ± 0.09 mM and Fig. 6B: from 2.01 ± 0.14 mM to 1.36 ± 0.1 mM) [$p < 0.05$ and $p < 0.01$, respectively], while the same dose was unable to decrease the hyperglycemia from 111 mM glucose treated animals (Fig. 6A). In the washout group (Fig. 6B), the 1 U/kg dose was able to decrease 39% of the blood glucose levels (from 4.01 ± 0.32 mM to 2.58 ± 0.32 mM) [$p < 0.01$]. This protocol requires a prior anesthesia with tricaine, which could be the reason for the variability of the blood glucose levels from 111 mM glucose-treated animals (4.53 ± 0.68 mM) as discussed by Eames et al. (2010).

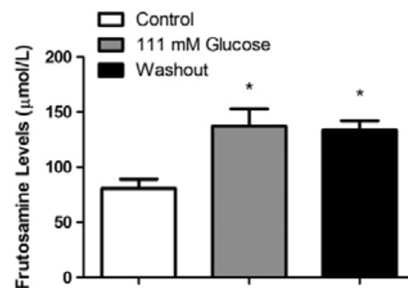


Fig. 3. Effect of 111 mM glucose treatment for 14 days and glucose washout of 7 days on fructosamine levels in zebrafish's eyes. A pool of three zebrafish's eyes were used for each group. Data are expressed as mean ± SEM of five independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. *p < 0.05.

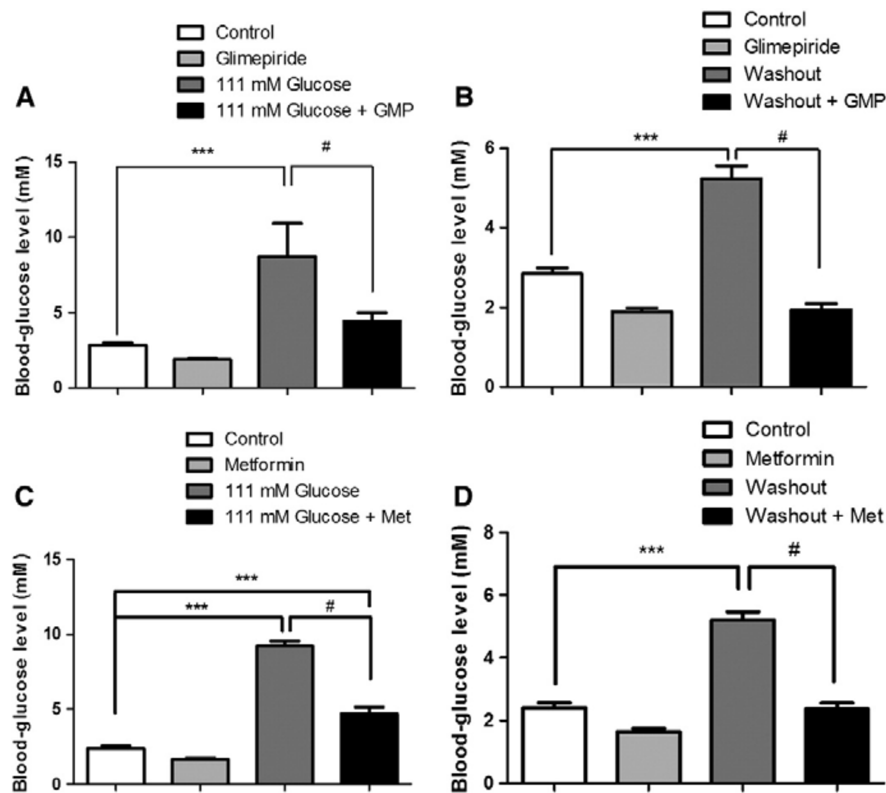


Fig. 4. Effect of anti-diabetic drug administration on 2% glucose treated and washout groups. Effect of glimepiride (100 μM) (A) and metformin (10 μM) (C) on blood glucose levels of zebrafish exposed to 111 mM glucose. Effect of glimepiride (100 μM) (B) and metformin (10 μM) (D) on blood glucose levels of zebrafish after a glucose washout period. At least ten zebrafish were used for each group. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. The # represents a significant difference from the 2% glucose group. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ and #$p < 0.01$.

4. Discussion

In the present study, we described persistent features of impaired glucose metabolism in adult zebrafish exposed to a solution of 111 mM glucose during 14 days. The lowest mortality was seemed in animals treated with 111 mM of glucose (20%). However, the high mortality observed in 55.5 and 166 mM glucose-treated animals can be related to reduced oxygenation and/or osmotic stress.

In this work we reached similar high blood glucose levels as the former work designed by Gleeson et al. (2007). Here, all glucose solutions promoted high blood glucose levels after 48–72 h after exposure. After the glucose withdrawal by 7 days, the blood glucose levels were still elevated, but lower than the 111 mM glucose group. However, as the blood glucose levels of the washout group remained significantly higher than the control group, a long time course observation should be interesting to observe when/if levels return to normal.

In order to verify if persistent hyperglycemia could change a classical parameter of DM diagnostic, as glycosylation of proteins, we evaluated the fructosamine levels from zebrafish eyes. Our results demonstrated that there is an increase of fructosamine levels from zebrafish eyes treated with 111 mM glucose, which persists in the washout group. Although, traditionally glycosylated hemoglobin (HbA1c) has been used to monitor glycemic control in DM, unfortunately we could not analyze the HbA1c levels in blood zebrafish considering the needed volume of blood for this procedure. However, several studies have demonstrated that fructosamine and HbA1c levels have been shown as an appropriate index of glycemic control in DM patients (Juraschek et al., 2012; Lee et al., 2013).

To assist in defining the mechanisms involved in persistent hyperglycemia developed for this model, we used two normoglycemic drugs with different mechanisms: metformin chloridate (biguanide) and glimepiride (sulphonylurea). Our results demonstrated that the metformin treatment by 4 days was able to decrease the blood glucose levels in hyperglycemic zebrafish. Metformin is often recommended as the first line drug in patients with type 2 diabetes (Nathan et al., 2009; ADA, 2013), because this oral medication has an effect on the regulation of glucose uptake, gluconeogenesis, glycolysis and glycogen synthesis (McIntyre et al., 1991; Shaw et al., 2005). Metformin increases the activity of the insulin receptor and enhances glucose uptake via increased translocation of glucose transporters, such as GLUT-1 and GLUT-4 to the plasma membrane (Gunton et al., 2003; Yang and Holman, 2006). As a result, metformin enhances the insulin-mediated suppression of gluconeogenesis (Bailey and Turner, 1996). However the progressive nature of type 2 diabetes, leads to a decline in endogenous insulin secretion, thus the use of a second drug, such as glimepiride, is suggested.

The glimepiride treatment, proved to be effective for lowering blood glucose levels in zebrafish after 24 h of exposure. The main mechanism of action of glimepiride is on the activation of ATPase-dependent potassium channels in β cells of the pancreas which stimulates insulin release (Campbell, 1998), leading to a decrease in hexokinase binding to porin proteins and an increase in the expression of hexokinase mRNA (Hällsten et al., 2002). Several studies demonstrated that glimepiride is able to stimulate insulin secretion, but these studies also demonstrated important extrapancreatic effects, such as activation of glucose transport and glucose-metabolizing enzymes leading to stimulation of

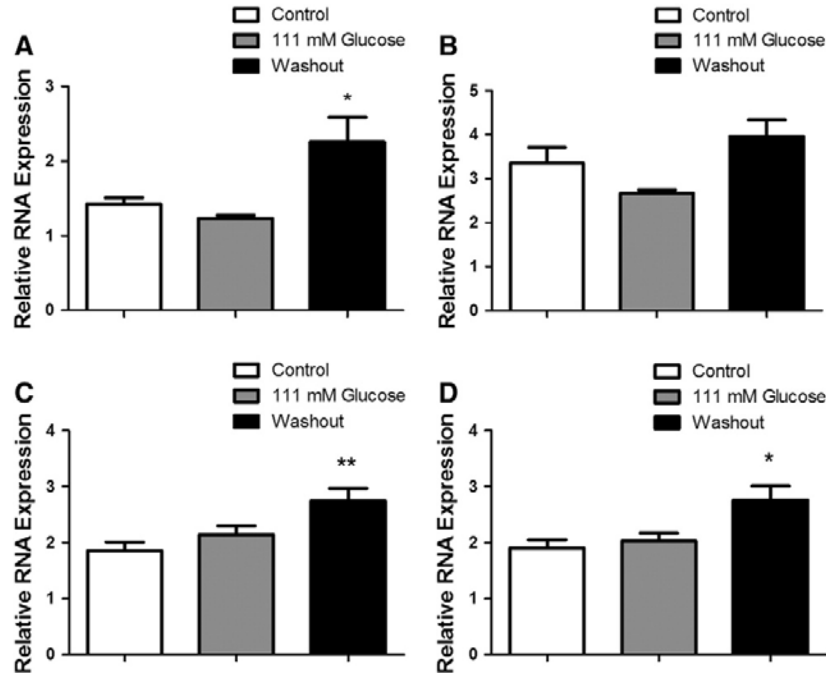


Fig. 5. Effect of 111 mM glucose treatment for 14 days and washout of 7 days on (A) *insra-1*, (B) *insra-2*, (C) *insrb-1* and (D) *insrb-2* gene expression in the skeletal muscle of zebrafish. At least four zebrafish were used for each group. Data are expressed as mean ± SEM of experiments performed in quadruplicate. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. The * represents a significant difference from the control group. *p < 0.05 and **p < 0.01.

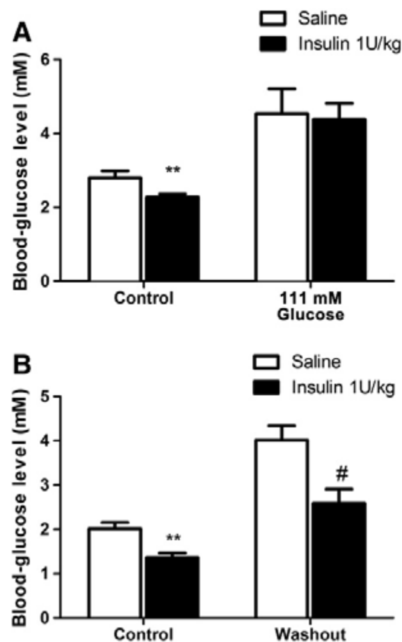


Fig. 6. Effect of insulin administration (1 U/kg) on blood glucose levels from the 111 mM glucose treated group (A) and the washout group (B). At least eleven zebrafish were used for each group. Data are expressed as mean ± SEM. Data were analyzed by *t*-test. The * represents a significant difference from the control–saline group. The # represents difference from the washout–saline group. **p < 0.01 and #p < 0.01.

glycogen and lipid synthesis, downregulation of the cAMP regulatory cascade, and inhibition of lipolysis (Briscoe et al., 2010). According to Muller et al. (1995), glimepiride seems to exert pleiotropic effects via two independent mechanisms: the stimulation of specific protein phosphatases responsible for the regulation of GLUT-4 translocation and glycogen synthase/glycerol3-phosphate acyltransferase activity; and the stimulation of cAMP-specific phosphodiesterase (Muller and Wied, 1994; Muller et al., 1995; Muller and Geisen, 1996).

If the successful effects of glimepiride were related to increased insulin secretion, this lowering of blood glucose levels should be mimicked by exogenous insulin. We injected insulin in the control, 111 mM glucose-treated and washout animals. Exogenous insulin was able to reduce blood glucose levels in all groups except in those from the glucose-treated group. The insulin resistance state is related to an impaired insulin action and it is believed that this resistance leads to hyperinsulinemia when β-islets produce a large amount of insulin in an effort to control the blood glucose level (Pessin and Saltiel, 2000; Ye, 2013). Even though an insulin level measurement should be mandatory in this approach, we failed in the collection of the required blood sample using an immunoenzymatic assay for quantitative determination of insulin from serum (Insulin SYM Kit, Symbiosis Diagnostica LTDA, São Paulo, Brazil). Gathering such information, the glimepiride effects observed could be more related to extrapancreatic effects than its well-known effect of stimulating insulin secretion.

The insulin responsive tissues express insulin receptors (IRs) at the cell surface plasma membrane. After the IR has been activated, they generate second messengers that activate a cascade of phosphorylation–dephosphorylation reactions resulting in the stimulation of intracellular glucose metabolism (Kahn et al., 1993; Whitehead et al., 2000). Dysfunctions of the IR and components of signaling cascade result in insulin resistance that leads to type 2 diabetes mellitus (Tseng et al., 2009). The

IRs of zebrafish were identified and characterized by Toyoshima et al., who demonstrated two isoforms of the IRs in zebrafish, *Insra* and *Insrb*, which are homologous to human IRs (Toyoshima et al., 2008). Several proteins have been identified in zebrafish as components of glucose metabolism, as glucose transports (GLUTs) and phosphoenolpyruvate carboxykinase (PEPCK), which have similar regulation patterns and activity as seen in mammalian counterparts (Taniguchi et al., 2006; Elo et al., 2007).

Interestingly, our results demonstrated that the mRNA expression levels of *insra-1*, *insrb-1* and *insrb-2* were significantly increased in the washout group, promoting a classical mechanism known as up-regulation, which could explain the partial reduction of glycemia in the washout group and the rescue of response to exogenous insulin. Other studies demonstrated that the kinetics of the insulin signaling via *insra* is similar to the mammalian insulin signaling cascades, suggesting that the signaling pathways downstream of the zebrafish IR are the same as found in other vertebrates (Stumvoll et al., 1995; Toyoshima et al., 2008). During early development of zebrafish the two IRs appear to play differently, whereas *insra* is critical for brain development and for general growth, *insrb* is essential for proper heart development (Stumvoll et al., 1995; Toyoshima et al., 2008).

5. Conclusions

In summary, our data demonstrated that the exposure of zebrafish to glucose diluted in water during 14 days promoted persistent high blood glucose levels, glycation of proteins of the eyes and impaired response to exogenous insulin. The hyperglycemic state was counteracted by biguanide and sulphonylurea drugs, probably by the increase of peripheral tissue response to insulin and not by pancreatic secretion of insulin, since exogenous insulin was ineffective. The glucose withdrawal of 7 days was able to partially recover some consequences of glucose exposure, probably by the recovery of insulin response, through an increase of insulin receptors in muscle. This study attempts to establish zebrafish as a valuable model for the study of targets applicable to diabetes. Zebrafish may be an ideal model to study the different mechanisms affected by hyperglycemia, as well as to understand the link between the central nervous system function and the alterations in the endocrine system, such as in DM.

Acknowledgments

This work was supported by DECIT/SCTIE-MS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. 10/0036-5, conv. n. 700545/2008 — PRONEX). K.M.C. is a recipient of a FAPERGS/Capes fellowship (Proc. no. 550992/2010-3).

References

Alvarez, Y., Chen, K., Reynolds, A.L., Waghorne, N., O'Connor, J.J., Kennedy, B.N., 2010. Predominant cone photoreceptor dysfunction in a hyperglycaemic model of non-proliferative diabetic retinopathy. Dis. Model. Mech. 3–4, 236–245.

Armbruster, D.A., 1987. Fructosamine: structure, analysis, and clinical usefulness. Clin. Chem. 33, 2153–2163.

Asaoka, Y., Terai, S., Sakaida, I., Nishina, H., 2013. The expanding role of fish models in understanding non-alcoholic fatty liver disease. Dis. Model. Mech. 6, 905–914.

Association American Diabetes, 2013. Standards of medical care in diabetes—2013. Diabetes Care 36, S11–S66.

Bailey, C.J., Turner, R.C., 1996. Metformin. N. Engl. J. Med. 334, 574–579.

Bogé, G., Péres, G., 1990. Chloride requirements of sodium cotransporter systems. Comp. Physiol. 186–215.

Briscoe, V.J., Griffith, M.L., Davis, S.N., 2010. The role of glimepiride in the treatment of type 2 diabetes mellitus. Expert. Opin. Drug Metab. Toxicol. 6, 225–235.

Campbell, R.K., 1998. Glimepiride: role of a new sulfonylurea in the treatment of type 2 diabetes mellitus. Ann. Pharmacother. 32, 1044–1052.

Eames, S.C., Philipson, L.H., Prince, V.E., Kinkel, M.D., 2010. Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. Zebrafish 7, 205–213.

Elo, B., Villano, C.M., Govorko, D., White, L.A., 2007. Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds. J. Mol. Endocrinol. 38, 433–440.

Ferraris, R.P., Ahearn, G.A., 1984. Sugar and aminoacid transport in fish intestine. Comp. Biochem. Physiol. 3, 397–413.

Gleeson, M., Connaughton, V., Arneson, L.S., 2007. Induction of hyperglycaemia in zebrafish (*Danio rerio*) leads to morphological changes in the retina. Acta Diabetol. 44, 150–163.

Gunton, J.E., Delhanty, P.J., Takahashi, S., Baxter, R.C., 2003. Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2. J. Clin. Endocrinol. Metab. 88, 1323–1332.

Hällsten, K., Virtanen, K.A., Lonnqvist, F., 2002. Rosiglitazone but not metformin enhances insulin- and exercise-stimulated skeletal muscle glucose uptake in patients with newly diagnosed Type 2 diabetes. Diabetes 51, 3479–3484.

Harris, M., Zimmet, P., 1997. Classification of diabetes mellitus and other categories of glucose intolerance, In: Alberti, K., Zimmet, P., Defronzo, R. (Eds.), International Textbook of Diabetes Mellitus, Second edition. John Wiley and Sons Ltd., Chichester, pp. 9–23.

International Diabetes Federation (IDF), 2012. Diabetes Atlas, 5th edition. Russels, Belgium.

Juraschek, S.P., Steffes, M.W., Miller III, E.R., Selvin, E., 2012. Alternative markers of hyperglycemia and risk of diabetes. Diabetes Care 35, 2265–2270.

Kahn, S.E., Prigeon, R.L., McCulloch, D.K., Boyko, E.J., Bergman, R.N., Schwartz, M.W., Neifing, J.L., Ward, W.K., Beard, J.C., Palmer, J.P., et al., 1993. Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function. Diabetes 42, 1663–1672.

Lee, S.-Y., Chen, Y.-C., Tsai, I.-C., Yen, C.-J., Chueh, S.-N., et al., 2013. Glycosylated hemoglobin and albumin-corrected fructosamine are good indicators for glycemic control in peritoneal dialysis patients. PLoS ONE 8, e57762.

Maddison, L.A., Chen, W., 2012. Nutrient excess stimulates β-cell neogenesis in zebrafish. Diabetes 61, 2517–2524.

McIntyre, H.D., Ma, A., Bird, D.M., Paterson, C.A., Ravenscroft, P.J., Cameron, D.P., 1991. Metformin increases insulin sensitivity and basal glucose clearance in type 2 (non-insulin dependent) diabetes mellitus. Aust. N. Z. J. Med. 21, 714–719.

Moyle, P., Cech, J., 2000. Fishes: An Introduction to Ichthyology. Prentice Hall, Upper Saddle River, USA.

Muller, G., Geisen, K., 1996. Characterization of the molecular mode of action of the sulphonylurea, glimepiride at adipocytes. Horm. Metab. Res. 28, 469–487.

Muller, G., Wied, S., 1994. The sulphonylurea drug, glimepiride, stimulates glucose transport, glucose transport translocation, and dephosphorylation in insulin-resistance rat adipocytes in vitro. Diabetes 5, 985–996.

Muller, G., Satoh, Y., Geisen, K., 1995. Extrapancreatic effects of sulphonylureas — a comparison between glimiperide and conventional sulphonylureas. Diabetes Res. Clin. Pract. 28, S115–S137.

Nathan, D.M., Buse, J.B., Davidson, M.B., Ferranninni, E., Holman, R.R., Sherwin, R., Zinman, B., 2009. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy. Diabetes Care 32, 193–203.

Nery, L.R., Rodrigues, M.M., Rosemberg, D.B., Bogo, M.R., De Farias, C.B., Abujamra, A. L., Schwartsmann, G., Roesler, R., Vianna, M.R., 2011. Regulation of E-cadherin expression by growth factor receptors in cancer cells. J. Surg. Oncol. 104, 220–221.

Oggier, D.M., Weisbrod, C.J., Stoller, A.M., Zenker, A.K., Fent, K., 2010. Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish *Danio rerio*. Environ. Sci. Technol. 44, 7685–7691.

Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., Umemoto, N., Kuroyanagi, J., Nishimura, N., Tanaka, T., 2010. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. BMC Physiol. 21, 10–21.

Olsen, A.S., Sarras Jr., M.P., Intine, R.V., 2010. Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. Wound Repair Regen. 18, 532–542.

Pedroso, G.L., Hammes, T.O., Escobar, T.D., Fracasso, L.B., Forgiarini, L.F., da Silveira, T.R., 2012. Blood collection for biochemical analysis in adult zebrafish. J. Vis. Exp. 26, e3865.

Pessin, J.E., Saltiel, A.R., 2000. Signaling pathways in insulin action: molecular targets of insulin resistance. J. Clin. Invest. 106, 165–169.

Phelps, H.A., Runft, D.L., Neely, M.N., 2009. Adult Zebrafish Model of Streptococcal Infection. Current Protocols in Microbiology 13 9D.1.1–9D.1.27.

Polakof, S., Comte, B., 2012. Glucose-induced insulin secretion: nutritional prevention and novel avenues for therapy. Adv. Exp. Med. Biol. 771, 319–339.

Polakof, S., Moon, W., Aguirre, P., Skiba-Cassy, S., Panserat, S., 2011. Glucose homeostasis in rainbow trout fed a high-carbohydrate diet: metformin and insulin interact in a tissue-dependent manner. Am. J. Physiol. Regul. Integr. Comp. Physiol. 300, R166–R174.

Shaw, R.J., Lamia, K.A., Vasquez, D., Koo, S.H., Bardeesy, N., Depinho, R.A., Montminy, M., Cantley, L.C., 2005. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 310, 1642–1646.

Siccardi III, A.J., Garris, H.W., Jones, W.T., Moseley, D.B., D'Abramo, L.R., Watts, S.A., 2009. Growth and survival of zebrafish (*Danio rerio*) fed different commercial and laboratory diets. Zebrafish 3, 275–280.

Sociedade Brasileira de Diabetes, 2009. Consenso Brasileiro Sobre Diabetes, Classificação Etiológica do Diabetes Mellitus3ª Edição. (São Paulo).

Stewart, A.M., Nguyen, M., Wong, K., Poudel, M.K., Kalueff, A.V., 2014. Developing zebrafish models of autism spectrum disorder (ASD). Prog. Neuropsychopharmacol. Biol. Psychiatry 50, 27–36.

Stumvoll, M., Nurjhan, N., Perriello, G., Dailey, G., Gerich, J.E., 1995. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. N. Engl. J. Med. 31, 550–554.

Tang, R., Dodd, A., Lai, D., Mcnabb, W.C., Love, D.R., 2007. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim. Biophys. Sin. 39, 384–390.
Taniguchi, C.M., Emanuelli, B., Kahn, C.R., 2006. Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell Biol. 7, 85–96.
Toyoshima, Y., Monson, C., Duan, C., Wu, Y., Gao, C., Yakar, S., Sadler, K.C., LeRoith, D., 2008. The role of insulin receptor signaling in zebrafish embryogenesis. Endocrinology 149, 5996–6005.
Tseng, Y.C., Chen, R.D., Lee, J.R., Liu, S.T., Lee, S.J., Hwang, P.P., 2009. Specific expression and regulation of glucose transporters in zebrafish ionocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297, R275–R290.
Whitehead, J.P., Clark, S.F., Urso, B., James, D.E., 2000. Signaling through the insulin receptor. Curr. Opin. Cell Biol. 12, 222–228.
Yang, J., Holman, G.D., 2006. Long-term metformin treatment stimulates cardiomyocyte glucose transport through an AMP-activated protein kinase-dependent reduction in GLUT4 endocytosis. Endocrinology 147, 2728–2736.
Ye, J., 2013. Mechanisms of insulin resistance in obesity. Front. Med. 7, 14–24.

3.2 CAPÍTULO II

ARTIGO CIENTÍFICO

Hyperglicemia induces memory impairment linked to increased acetilcholinesterase activity in zebrafish (*Danio rerio*).

Katiucia Marques Capiotti, Daiani Almeida De Moraes, Fabiano Peres Menezes, Luiza Wilges Kist, Maurício Reis Bogo, Rosane Souza Da Silva.

Artigo publicado no periódico

Behavioural Brain Reasearch, 274:319-25. 2014.

(doi: 10.1016/j.bbr.2014.08.033)

Behavioural Brain Research 274 (2014) 319–325

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Hyperglycemia induces memory impairment linked to increased acetylcholinesterase activity in zebrafish (*Danio rerio*)

Katiucia Marques Capiotti [a], Daiani Almeida De Moraes [a], Fabiano Peres Menezes [a], Luiza Wilges Kist [b,c], Maurício Reis Bogo [b,c], Rosane Souza Da Silva [a,c,*]

[a] *Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUCRS, Porto Alegre, RS, Brazil*
[b] *Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUCRS, Porto Alegre, RS, Brazil*
[c] *Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003 Porto Alegre, RS, Brazil*

HIGHLIGHTS

- The hyperglycemia promoted memory deficit in adult zebrafish.
- Memory deficit is linked to increased acetylcholinesterase activity.
- Galantamine reverses memory deficit caused by hyperglycemia.

ARTICLE INFO

Article history:
Received 11 July 2014
Received in revised form 12 August 2014
Accepted 16 August 2014
Available online 23 August 2014

Keywords:
Cholinergic system
Diabetes mellitus
Glucose
Hyperglycemia
Memory
Locomotor activity

ABSTRACT

Diabetes mellitus, which causes hyperglycemia, affects the central nervous system and can impairs cognitive functions, such as memory. The aim of this study was to investigate the effects of hyperglycemia on memory as well as on the activity of acethylcholinesterase. Hyperglycemia was induced in adult zebrafish by immersion in glucose 111 mM by 14 days. The animals were divided in 4 groups: control, glucose-treated, glucose-washout 7-days and glucose-washout 14-days. We evaluated the performance in inhibitory avoidance task and locomotor activity. We also determined acethylcholinesterase activity and gene expression from whole brain. In order to counteract the effect of hyperglycemia underlined by effects on acethylcholinesterase activity, we treated the animals with galantamine (0.05 ng/g), an inhibitor of this enzyme. Also we evaluated the gene expression of insulin receptor and glucose transporter from zebrafish brain. The hyperglycemia promoted memory deficit in adult zebrafish, which can be explained by increased AChE activity. The *ache* mRNA levels from zebrafish brain were decrease in 111 mM glucose group and returned to normal levels after 7 days of glucose withdrawal. Insulin receptors (*insra-1*, *insra-2*, *insrb*-1 and *insrb*-2) and *glut-3* mRNA levels were not significantly changed. Our results also demonstrated that galantamine was able to reverse the memory deficit caused by hyperglycemia, demonstrating that these effects involve modulation of AChE activity. These data suggest that the memory impairment induced by hyperglycemia is underlined by the cholinergic dysfunction caused by the mechanisms involving the control of acetylcholinesterase function and gene expression.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Diabetes mellitus (DM) is a complex disorder that can cause damage to multiple organs in the body, due to changes caused by dysfunctional glucose metabolism, as hyperglycemia and hypoglycemia [1]. The DM itself has been recognized as an independent risk factor for development of cognitive impairment [2,3], and has been suggested by several authors that this diabetes-related cognitive dysfunction is largely a consequence of changes within the central nervous system (CNS) that are secondary to chronic hyperglycemia [4,5]. Kodl and Seaquist [1] also addressed the causes

* Corresponding author at: Faculdade de Biociências, PUCRS, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil. Tel.: +55 51 3320 3500x4158; fax: +55 51 3320 3612.
E-mail addresses: katucapiotti@gmail.com (K.M. Capiotti), daiani.moraes@acad.pucrs.br (D.A. De Moraes), fabiano.menezes@acad.pucrs.br (F.P. Menezes), lwkist@gmail.com (L.W. Kist), mbogo@pucrs.br (M.R. Bogo), rosane.silva@pucrs.br (R.S. Da Silva).

http://dx.doi.org/10.1016/j.bbr.2014.08.033
0166-4328/© 2014 Elsevier B.V. All rights reserved.

of cognitive dysfunction in diabetic patients as a combination of different factors such as the type of DM, comorbidities, age, and effectiveness of therapy utilized.

Some studies have demonstrated the relationship between DM and dysfunction on neurotransmission systems, such as dopaminergic [6], glutamatergic [7], purinergic [8] and cholinergic [9,10]. The cholinergic system is very important and modulates many vital functions in the CNS, as sleep control, learning and memory [11]. In diabetic rats, Sherin and collaborators [10] showed an increase in acethycholinesterase (AChE) mRNA levels and a reduction on mRNA levels of cholinergic receptors M1 and M3. These results demonstrated a low stimulation of cholinergic receptors, and hence a progressive worsening of cognition and other neurological dysfunction as those already seen in diabetic patients [10]. In addition, some studies using animal models of diabetes have found an increase in AChE activity which may indicate alterations in cholinergic neurotransmission and consequently be associated to cognitive impairments observed in DM [12,13].

The zebrafish is an animal model that has been used to mimic symptoms of human disease, as DM. DM type 1 can be modeled in zebrafish by streptozocin (STZ) injection [14]. Similar symptoms of DM type 2, such as protein glycation, persistent hyperglycemia and impaired insulin response can be reached by immersion of animals in solution of glucose [15,16].

Memory assessment in zebrafish has been receiving strongly contributions from avoidance task paradigm as a way to reveal robust and stable memory traces evolutionary conserved [17,18]. Therefore, the aim of this study is evaluate the effect of persistent hyperglycemia on memory using an inhibitory avoidance task and correlates to AChE activity and gene expression in zebrafish model of hyperglycemia.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) wild type (Tübingen background; 3–5 cm) of both sexes were obtained from a specialized commercial supplier (Redfish, RS, Brazil). The fish were acclimatized for at least 14 days in the experimental room. Animals were housed in groups of 15 fish in 5-L thermostated (28 ± 2 °C) tanks, kept under constant chemical, biological and mechanical water filtration and aeration (7.20 mg O_2/L). Fish were maintained under a 14–10 h day/night photoperiod cycle, fed three times a day with commercial flakes (TetraMin™, NC, USA) and supplemented with live brine shrimp. All fish used in these experiments were randomly chosen from different clusters. Briefly, anesthesia of the animals prior to the injection to galantamine experiments was obtained by its immersion in a tricaine MS-222 solution 100 mg/L until the animal shows lack of motor coordination and reduced respiration rate. The animals were submitted to hypothermia by exposure to ice water flocked followed by decapitation as endpoint. All protocols were approved by the Institutional Animal Care Committee (12/00310–CEUA PUCRS) and followed Brazilian legislation, the guidelines Federal Council of Veterinary Medicine (CFMV), and the Canadian Council for Animal Care (CCAC) "Guide on the Care and Use of Fish in Research, Teaching, and Testing".

2.2. Drugs

Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine MS-222) was purchased from Fluka Analytical (St Louis, USA). Glucose was purchased from Nuclear™ (São Paulo, Brazil). Galantamine hydrobromide (Reminyl®) was purchased from Janssen Ortho LLC (Gurabo, Porto Rico–EUA). All other reagents were of analytical grade.

2.3. Induction of hyperglycemia

Groups of 15 adult animals were placed in 5-L aquariums containing 111 mM glucose solution, which were diluted in water and maintained during 14 days at room temperature as described in previous section according to [16].

2.4. Withdrawal of glucose

In order to verify if the effects caused by 111 mM glucose solution treatment would be persistent, we kept a group of 15 fish in glucose-free water by additional 7 or 14 days (washout group, no glucose added) [16]. During this period the animals were maintained at the same conditions of welfare describe in previous section. Control group animals were maintained in aquariums with water for the same period and were handled as washout group.

2.5. Treatment with Galantamine

The AChE inhibitor, Galantamine hydrobromide (Reminyl®), was dissolved in 0.9% saline in three doses (0.5 mg/g, 0.05 mg/g and 0.05 ng/g) adjusted to the fish body weight (mean injection volume was 10 μL) in a 20 mL/kg regimen in order to determine the dose to be used in the following experiments. The animals injected with 0.5 mg/g of galantamine did not survive after five minutes of treatment and animals treated with 0.05 mg/g of galantamine showed difficulties in swimming, precluding evaluation of locomotor activity, because this we choose the dose 0.05 ng/g. Galantamine hydrobromide was injected (0.05 ng/g) in all tested groups (glucose-free, glucose-treated, and 7 and 14 days glucose washout group). A control group injected with 0.9% saline was performed. In the glucose group, all animals were placed in water without glucose 24 h before the start of experiment. The animals were anesthetized as described and gently put in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). Intraperitoneal injections were conducted using a 3/10-ml U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31G Short Needles (Becton Dickinson and Company, NJ, USA) according to the protocol previously established [19]. The needle was inserted parallel to the spine into the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted to guarantee that the animal do not spend more than 10 s out of the water. After the injection the animals were placed in a separate tank with highly aerated unchlorinated tap water (28 ± 2 °C) to facilitate the animal recovery from the anesthesia. Saline was used as control. Both galantamine and saline were prepared freshly in the experimental day. All the animals have recovered after 2–3 min following the injection. Animals were tested on activity locomotor and inhibitory avoidance after 2 h of galantamine injection.

2.6. Behavioral analysis

2.6.1. Inhibitory avoidance task

After treatments adult zebrafish were individually trained and tested for long-term memory in the inhibitory avoidance paradigm as previously described in detail [20,18]. Briefly, an 18 cm L × 9 cm W × 7 cm H glass tank divided in two equally sized compartments, designated hereon as dark and white, separated by a sliding guillotine-type partition (9 cm × 7 cm) was used. The tank water level was 3 cm and the partition raised 1 cm above the tank floor to allow zebrafish to swim freely from one side of the tank to the other. Two electrodes extending through the wall height and placed on each end side of the dark walls attached to an 8 V stimulator administered a final 3 ± 0.2 V AC shock, when manually

Table 1
Primer sequences for RT-qPCR experiments included in the study.

Sequence	Forward	Reverse	Accession number (mRNA)	Amplicon size (bp)
EF1α[a]	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′	ENSDART00000023156	86
Rpl13α[a]	5′-TCTGGAGGACTGTAAGAGGTATGC-3′	5′-AGACGCACAATCTTGAGAGCAG-3′	NM_212784	147
insra-1[b]	5′-CAACATGCCCCCTCACCACT-3	5′-CGACACACATGTTGTTGTG-3′	NM_001142672	215
insra-2[b]	5′-GGAGCCCCACTCGTCTAACAAA-3′	5′-CGCCGTTGTGAATGACGTATTC-3′	–	193
insrb-1[b]	5′-GACTGATTACTATCGCAAGGG-3′	5′-TCCAGGTATCCTCCGTCCAT-3′	NM_001123229	190
insrb-2[b]	5_-CCACCGCCAACCCTAAAGGA-3′	5′-TTGCGATAGTAATCAGTC TCGTAAAT-3′	–	170
Glut3[c]	5′-TCGTCAATGTCTTGGCTCTG-3′	5′-CAACATACATTGGCGTGAGG-3′	ENSDART00000016197	141
ache[d]	5′-GCTAATGAGCAAAAGCATGTGGGCTTG-3′	5′-TATCTGTGATGTTAAGCAGACGAGGCAGG-3′	NP_571921	121

According to [23[d]], [24[c]], [25[b]], [26[a]].

activated. On training session, animals were placed in the white side of the tank while the partition between compartments was closed. After 1 min of familiarization with the new environment, the partition was raised, allowing fish to cross to the dark side of the tank. When animals entered the dark side with their entire body, the sliding partition was closed and a pulsed electric shock administered for 5 s. Fish were then removed from the apparatus and placed in the dedicated temporary tank. Animals were tested 24 h after training. The test session repeated the training protocol except that no shock was administered and animals immediately removed from the dark compartment. The latency to completely enter the dark compartment was measured on both sessions and the test latencies used as an index of memory retention. The animals treated with galantamine were injected 2 h before training session.

2.6.2. Locomotor activity assessment

After treatment all animals of groups (control, glucose-treated and 7 and 14 days washout) were individually placed in the experimental tank (30 cm L × 15 cm H × 10 cm W) where were first habituated to the tank for 30 s, as previously described [21]. There was no drug exposure during locomotor activity assessment experiments. The animals' locomotor activity was recorded on video for 5 min after the habituation period and simultaneously analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The tank was virtually divided into equal sections with four vertical lines and one horizontal line, and the following locomotion parameters were measured: distance traveled (m) and mean speed (m/s).

2.7. Determination of acetylcholinesterase (AChE) activity

Zebrafish were euthanized as already described and their whole brains were removed by dissection. The brains (five whole brains for each sample) were homogenized on ice in 60 vol. (v/w) of 50 mM Tris–HCl, pH 8.0, in a glass-Teflon homogenizer. Acetylcholinesterase activity was measured as the method described previously [22] determining the rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 ml assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Samples containing protein (10 μg) and the reaction medium described above were preincubated during 10 min at 25 °C followed by starting of reaction with addition of substrate. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm every 30 s for 2.5 min. The linearity of absorbance toward time and protein concentration was previously determined [23]. AChE activity was expressed as micromoles of thiocholine (SCh) released per hour per milligram of protein. All enzyme assays were performed in at least four different experiments, each one performed in triplicate. Protein was measured by the Coomassie blue method [24] and bovine serum albumin was used as standard.

2.8. Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

The expression of *ache*, *glut-3* and isoforms of insulin receptor gene (*insra*-1, *insra-2*, *insrb*-1 and *insrb*-2) was analyzed by a quantitative real time reverse transcription polymerase chain reaction (RT-qPCR) assay. TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was employed to isolate total zebrafish brain RNA in accordance with manufacturer's instructions. The total RNA was quantified by spectrophotometer (A260/280 nm) and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for *EF1α*, *Rpl13α* and 1:100 for *glut-3*, *insra*-1, *insra-2*, *insrb*-1, *insrb*-2 and *ache*), containing SYBR® Green I 0.2 times diluted (Invitrogen, Carlsbad, CA, USA), 100 μM dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers [25–28] (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 minutes at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR version 2012.3 Software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α* and *Rpl13α* (*M*-value) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method [29,30].

2.9. Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M). Inhibitory avoidance data were analyzed by Kruskal–Wallis non-parametric analysis of variance followed by Dunn's for comparisons among groups. Data of the locomotion, biochemical and molecular assessment were analyzed by one-way ANOVA test followed by Tukey post hoc test, considering treatment as a factor. Significance was set at $p < 0.05$.

3. Results

As confirmed before by Capiotti et al. [16], the treatment with 111 mM glucose increased the blood glucose concentration after 14 days of treatment (control: 4.015 ± 0.3158 mM; Glucose-treated: 8.83 ± 0.663 mM; $p < 0.0001$).

3.1. Inhibitory avoidance task

In order to evaluate if hyperglycemia could be causing a deficit on memory, we submitted the animals to the inhibitory avoidance paradigm. All animals groups had their memory tested 24 h after training. Importantly, no differences were found between training latencies for all groups. Our results demonstrated that animals exposed to 111 mM glucose had a decrease on the latency on the test session ($p < 0.0001$) (Fig. 1). Therefore, to ascertain that hyperglycemia continues to affect the memory even after 7 or 14 days of glucose washout, we performed the same experiment of inhibitory avoidance with washout groups. Our results demonstrated that washout groups (7 and 14 days) also demonstrated a decrease of latency on test session ($p < 0.0001$ and $p < 0.05$, respectively) (Fig. 1). However, these 14 days of glucose withdrawal seem to promote an improvement in memory compared to the 7 days of glucose withdrawal (Fig. 1).

3.2. Locomotor activity assessment

To evaluate if hyperglycemia could alter locomotor parameters, we firstly observed the locomotor activity of animals treated in 111 mM glucose and washout group (7 days). Our results demonstrated that there are no differences in distance traveled [$F_{(2,39)} = 0.2772$; $p = 0.7594$] and mean speed [$F_{(2,39)} = 0.1729$; $p = 0.8418$] between control, 111 mM glucose-treated and washout group (data not showed).

3.3. Effect of hyperglycemia on acetylcholinesterase (AChE) activity

After verify that hyperglycemia modifies memory parameters in zebrafish, we observed if the glucose treatment was able to induce changes on AChE activity in brain from adult zebrafish. Our results demonstrated that AChE activity was significantly increased in 111 mM glucose group (15%) [$F_{(2,25)} = 16.35$; $p = 0.0001$] when compared to control group (Fig. 2). There are no changes on AChE activity in glucose-washout 7 days group when compared to control group, but when compared to 111 mM Glucose group there was a reduction of AChE activity (25%), [$F_{(2,25)} = 16.35$ $p = 0.0001$].

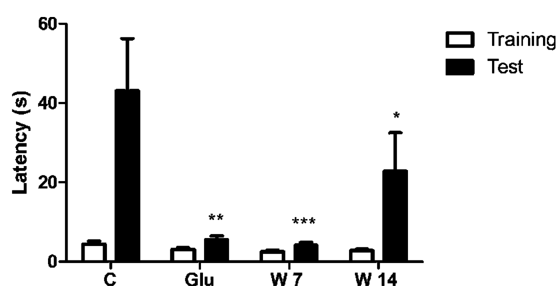


Fig. 1. 111 mM glucose and washout (7 and 14 days) treatment induces long-term memory deficits in inhibitory avoidance task. C: control-treated animals, Glu: 111 mM glucose-treated animals, W 7: Washout 7 days – treated animals, W 14: Washout 14 days – treated animals. Control animals were manipulated similarly but exposed to water only. Bars indicate animals' mean latencies + S.E.M to cross compartment (in seconds) in training (TR) and test (TT) sessions. At least eleven zebrafish were used for each group. *p < 0.05, **p < 0.01 and ***p < 0.0001 indicate statistically significant difference between latencies test sessions for control group animals.

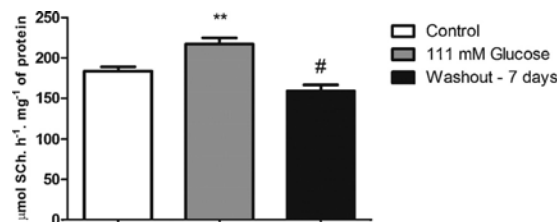


Fig. 2. Effect of 111 mM glucose and glucose-washout (7 days) treatment on acetylcholine hydrolysis. ** indicates significantly difference when compared to the control group. # indicates significantly difference when compared to the 111 mM glucose group. Data represent means ± S.E.M of four different experiments, each one performed in triplicate. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post hoc test, considering a p < 0.05 significant. The specific enzyme activity is reported as micromole of thiocholine released per hour per milligram of protein.

3.4. Effect of galantamine treatment on hyperglycemic zebrafish

3.4.1. Inhibitor avoidance task

In order to evaluate if galantamine could reverse the deficit on memory caused by hyperglycemia, we submitted the animals to the inhibitory avoidance paradigm 2 h after galantamine treatment. Our results demonstrated that galantamine was able to reverse the memory deficit caused by hyperglycemia ($p < 0.001$) (Fig. 3). Importantly, no differences were found between training latencies for all groups.

3.4.2. Locomotor activity assessment

To evaluate if galantamine could alter locomotor parameters, we observed the locomotor activity of animals treated in control, galantamine 0.05 ng/g, 111 mM glucose and 111 mM glucose + galantamine group. Our results demonstrated that the treatment with galantamine decreased the distance traveled [$F_{(3,28)} = 7.145$; $p = 0.0013$] (Fig. 4A) and mean speed [$F_{(3,30)} = 4.693$; $p = 0.0072$] (Fig. 4B) in comparison to 111 mM glucose group. Galantamine by itself showed no statistical difference from the saline group.

3.5. Effect of hyperglycemia on ache, insulin receptors, and glut-3 expression in brain of zebrafish

To evaluate whether the treatment with 111 mM glucose and 7 days of washout could alter the gene expression of AChE, real-time

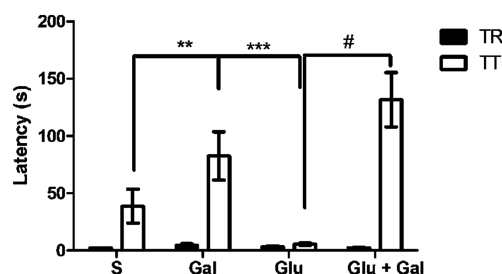


Fig. 3. Hyperglycemia caused by 111 mM glucose treatment by 14 days caused cognitive deficits that were reversed by galantamine (0.05 ng/g). S: saline-treated animals, Gal: Galantamine-treated animals, Glu: 111 mM glucose-treated animals, Glu + Gal: 111 mM Glucose + Galantamine-treated animals. At least nine zebrafish were used for each group. Animals received single intraperitoneal injection galantamine (0.05 ng/g) 2 h before the training session. ***p < 0.001 and *p < 0.05 indicate statistically significant difference between latencies on test sessions to control group animals and #p < 0.001 indicates difference between latencies on test session to glucose group.

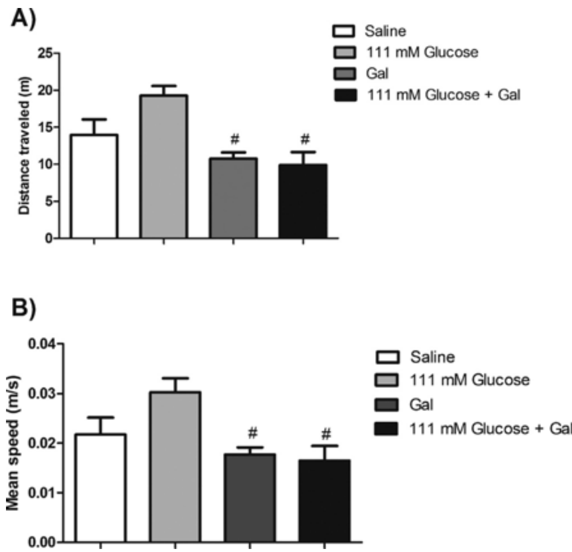


Fig. 4. Effect of 111 mM glucose, galantamine (Gal – 0.05 ng/g), 111 mM glucose + galantamine (Gal – 0.05 ng/g) on distance traveled (A) and mean speed (B), during 5 min of video recording. Data were expressed as mean ± S.E.M. At least seven zebrafish were used for each group and analyzed by one-way ANOVA followed by Tukey post hoc test. $^{\#}p < 0.001$ denotes a significant difference from 111 mM glucose group.

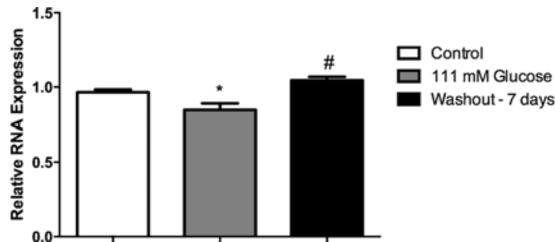


Fig. 5. Effect of 111 mM glucose treatment by 14 days and washout of 7 on *ache* gene expression in zebrafish brain. At least four zebrafish were used for each group. Data are expressed as mean ± S.E.M of experiments performed in quadruplicate. Data were analyzed by one-way ANOVA followed by Tukey post hoc test. The $^{*}p < 0.05$ represents a significant difference from the control group. $^{\#}p < 0.05$ represents a significant difference from the 111 mM glucose group.

quantitative PCR analyses were performed when kinetic alterations were observed. We also evaluate if hyperglycemia was able to alter the gene expression of insulin receptors and *glut-3* in zebrafish brain. The results demonstrated that the AChE expression levels were significantly decreased after treatment with 111 mM glucose [$F_{(2,21)} = 10.93$; $p = 0.0006$] (Fig. 5). However, there were no changes in washout group (7 days) when compared to control group. There were no changes in insulin receptors (*insra*-1, *insra-2*, *insrb*-1 and *insrb*-2) and *glut-3* genes expression of animals treated with 111 mM glucose or from washout group when compared to control group (Data not show).

4. Discussion

In the present study, we demonstrated that hyperglycemia was able to induce deficit memory linked to AChE activity increase in brain of adult zebrafish. We induced hyperglycemia by immersing zebrafish in a 111 mM glucose solution for 14 days, and evaluated the acquisition of memory and locomotion parameters. We also evaluated the same parameters after glucose withdrawal. After, we performed the evaluation of AChE activity and molecular analysis of insulin receptors, *glut-3* and *ache* in the brain of adult zebrafish. Furthermore, we used an inhibitor of AChE, galantamine, in order to understand whether the effects on memory acquisition of hyperglycemic animals could be dependent on AChE activity.

The present study demonstrated that the hyperglycemia is able to induce memory impairment. This result corroborates with the data found in literature, which demonstrate that DM is a risk factor for cognitive impairment and dementia [4,31]. The hyperglycemia has been proposed as a mechanism that may contribute to the association between DM and reduced cognitive function [32]. Kanaya et al. [33], demonstrated that chronic hyperglycemia may mediate the relationship between DM and cognitive decline, and that improved glycemic control may prevent cognitive decline, as well as data found by [34] and [35]. In a prior study, Capiotti et al. [16] showed that the chronic treatment with glucose in adult zebrafish induced persistent hyperglycemia, insulin resistance and an increase of glycation of proteins from zebrafish eyes, demonstrating that high glucose concentration is able to induce persistent metabolic changes. In this paper, our results showed that animals treated with 111 mM glucose by 14 days had memory impairment in inhibitory avoidance task, demonstrating that this effect seems to be persistent as blood glucose levels are still high. As glucose metabolism can affect several cellular functions, the memory impairment seems after 7 days of glucose withdrawal, which was exactly in the same magnitude of glucose-treated animals, and the partially impaired memory after 14 days of glucose withdrawal could be a response of changes in synaptic plasticity and transmission [36,37], as a consequence of synthesis and degradation of neurotransmitters and neuromodulators, such as acetylcholine. However, in this study acetylcholine degradation returned to control levels after glucose withdrawal, suggesting that another persistent mechanism are playing some role in the memory dysfunction. In fact, the use of scavenger to recover cognitive effects of diabetes have being suggested the role of reactive oxygen species in this context [38].

The control of acetylcholine (Ach) levels executed by AChE enzyme plays a crucial role in learning and memory process [39–42]. An increased level of Ach in the synaptic cleft are observed in several brain areas during a performance in behavioral task, demonstrating the involvement of this system in learning processes and memory [42]. Some authors suggested that alterations in the lipid membrane observed during the diabetic state could be a decisive factor in the modification of the conformational state of the AChE molecule and would explain changes in the activity of this enzyme [43,44]. According to Schmatz et al. [45] and Mushtaq et al. [46], AChE activation found in diabetic rats may be mediated by free radical production and consequent oxidative stress in the different brain regions. Our results showed that AChE activity was increased in animals treated with 111 mM glucose, suggesting a minor amount of Ach in the synaptic cleft, which could be associated with the cognitive worsening observed.

The alterations promoted by 111 mM glucose in AChE activity could be a consequence of transcriptional control. Our results demonstrated that the mRNA expression levels of AChE were decreased in 111 mM glucose group probably as a consequence of the continuous control of transcription machinery to adjust gene expression profile of the cell, a phenomenon known as negative feedback loop [47,48]. This complex kind of control of signaling system is situated at the interface of genetic and metabolic networks, and could explain the concomitant increase of Ach hydrolysis and the decrease of AChE mRNA levels in zebrafish brain treated with 111 mM glucose.

Our results also demonstrated that AChE activity was decreased in washout (7 days) group while the AChE mRNA levels increased

after glucose withdrawal. However, this rearrangement was not able to reverberate in memory rescue, at least after 7 days of glucose withdrawal, but after additional 7 days of glucose withdrawal the index of memory retention increased, but not enough to reach similar results as the control.

Some studies have found an increased AChE activity in brain associated to cognitive impairments in diabetics [45,46] which is supported by observation in the present study that galantamine, an AChE enzyme inhibitor, reverted the memory dysfunction induced by hyperglycemia. Galantamine has a unique dual mode of action, combining the reversible, competitive inhibition of AChE with positive allosteric modulation of nicotinic receptors [49–51]. At low doses, galantamine binds allosterically to nicotinic receptors and, at high dose, the main effect of galantamine is mediated through its inhibitory action on nicotinic receptors [52]. Our results showed that galantamine was able to recover the memory deficit caused by hyperglycemia, demonstrating that AChE enzyme is highly involved in the worsening of memory caused by hyperglycemia. Meanwhile, we cannot rule out the possible unspecific effects of galantamine, such as a scavenger of reactive oxygen species, which was already demonstrated by in vitro and in vivo investigations [53].

5. Conclusions

In summary, our data demonstrated that the exposure of zebrafish to glucose diluted in water during 14 days induced a prolonged memory impairment linked to increased AChE activity increase. The galantamine treatment, reverted the effect caused by hyperglycemia on memory, demonstrating that cognitive worsening observed is directly related with AChE activity. Different mechanisms may be operating in the CNS and induce changes in cognitive function in diabetic patients beyond the cholinergic dysfunction, in this way more studies on the level of basic cellular mechanism are needed to understand the CNS dysfunction caused by hyperglycemia.

Conflict of interest

No competing financial interests exist.

Acknowledgments

This work was supported by DECIT/SCTIE-MS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. 10/0036-5, conv. n. 700545/2008 – PRONEX). K.M.C is recipient of FAPERGS/Capes fellowship.

References

[1] Kodl CT, Seaquist ER. Cognitive dysfunction and diabetes mellitus. Endocr Rev 2008;29:494–511.
[2] Chen Z, Zhong C. Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. Prog Neurobiol 2013;108:21–43.
[3] Luchsinger JA. Type 2 diabetes and cognitive impairment: linking mechanisms. J Alzheimers Dis 2012:30.
[4] Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. Lancet Neurol 2006;5:64–74.
[5] McCall AL. Cerebral glucose metabolism in diabetes mellitus. Eur J Pharmacol 2004;490:147–58.
[6] Aung MH, Park HN, Han MK, Obertone TS, Abey J, Aseem F, et al. Dopamine deficiency contributes to early visual dysfunction in a rodent model of type 1 diabetes. J Neurosci 2014;34:726–36.
[7] Gardoni F, Kamal a, Bellone C, Biessels GJ, Ramakers GMJ, Cattabeni F, et al. Effects of streptozotocin-diabetes on the hippocampal NMDA receptor complex in rats. J Neurochem 2002;80:438–47.
[8] Elsherbiny N, Al-Gayyar M. Adenosine receptors new therapeutic targets for inflammation in diabetic nephropathy. Inflamm Allergy-Drug Targets 2013;12:153–61.
[9] Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. J Alzheimers Dis 2005;8:247–68.
[10] Sherin A, Anu J, Peeyush KT, Smijin S, Anitha M, Roshni BT, et al. Cholinergic and GABAergic receptor functional deficit in the hippocampus of insulin-induced hypoglycemic and streptozotocin-induced diabetic rats. Neuroscience 2012;202:69–76.
[11] Shaked I, Zimmerman G, Soreq H. Stress-induced alternative splicing modulations in brain and periphery: acetylcholinesterase as a case study. Ann NY Acad Sci 2008;1148:269–81.
[12] Sánchez-Chávez G, Salceda R. Effect of streptozotocin-induced diabetes on activities of cholinesterases in the rat retina. IUBMB Life 2000;49:283–7.
[13] Kuhad A, Chopra K. Lycopene ameliorates thermal hyperalgesia and cold allodynia in STZ-induced diabetic rat. Indian J Exp Biol 2008;46:108–11.
[14] Olsen AS, Sarras MP, Intine RV. Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. Wound Repair Regen 2010;18:532–42.
[15] Gleeson M, Connaughton V, Arneson LS. Induction of hyperglycaemia in zebrafish (*Danio rerio*) leads to morphological changes in the retina. Acta Diabetol 2007;44:157–63.
[16] Capiotti KM, Antonioli R, Kist LW, Bogo MR, Bonan CD, Da Silva RS. Persistent impaired glucose metabolism in a zebrafish hyperglycemia model. Comp Biochem Physiol B Biochem Mol Biol 2014;171:58–65.
[17] Schoel M. Instrumental learning in animals: parameters of reinforcement, 1,2; 1970. p. 2.
[18] McGaugh JL. Memory – a century of consolidation. Science 2000;287:248–51.
[19] Phelps HA, Runft DL, Neely MN. Adult zebrafish model of streptococcal infection. Curr Protoc Microbiol 2009. Chapter 9: Unit 9D.1.
[20] Blank M, Guerim LD, Cordeiro RF, Vianna MRM. A one-trial inhibitory avoidance task to zebrafish: rapid acquisition of an NMDA-dependent long-term memory. Neurobiol Learn Mem 2009;92:529–34.
[21] Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav 2000;67:773–82.
[22] Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
[23] Rico EP, Rosemberg DB, Senger MR, de Bem Arizi M, Bernardi GF, Dias RD, et al. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol Teratol 2006;28:489–96.
[24] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[25] Pereira VM, Bortolotto JW, Kist LW, Azevedo MBde, Fritsch RS, Oliveira R, da L, et al. Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (*Danio rerio*). Neurotoxicology 2012;33:469–75.
[26] Tseng Y-C, Chen R-D, Lee J-R, Liu S-T, Lee S-J, Hwang P-P. Specific expression and regulation of glucose transporters in zebrafish ionocytes. Am J Physiol Regul, Integr Comp Physiol 2009;297:R275–90.
[27] Toyoshima Y, Monson C, Duan C, Wu Y, Gao C, Yakar S, et al. The role of insulin receptor signaling in zebrafish embryogenesis. Endocrinology 2008;149:5996–6005.
[28] Tang R, Dodd A, Lai D, McNabb WC, Love DR. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin 2007;39:384–90.
[29] Oggier DM, Weisbrod CJ, Stoller AM, Zenker AK, Fent K. Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish *Danio rerio*. Environ Sci Technol 2010;44:7685–91.
[30] Nery LR, Rodrigues MM, Rosemberg DB, Bogo MR, De Farias CB, Abujamra AL, et al. Regulation of E-cadherin expression by growth factor receptors in cancer cells. J Surg Oncol 2011;104:220–1.
[31] Exalto LG, Whitmer RA, Kappele LJ, Biessels GJ. An update on type 2 diabetes, vascular dementia and Alzheimer's disease. Exp Gerontol 2012;47:858–64.
[32] Cox DJ, Kovatchev BP, Gonder-Frederick LA, Summers KH, McCall A, Grimm KJ, et al. Relationships between hyperglycemia and cognitive performance among adults with type 1 and type 2 diabetes. Diabetes Care 2005;28:71–7.
[33] Kanaya AM, Barrett-Connor E, Gildengorin G, Yaffe K. Change in cognitive function by glucose tolerance status in older adults: a 4-year prospective study of the Rancho Bernardo study cohort. Arch Intern Med 2004;164:1327–33.
[34] Yaffe K, Falvey C, Hamilton N, Schwartz AV, Simonsick EM, Satterfield S, et al. Diabetes, glucose control, and 9-year cognitive decline among older adults without dementia. Arch Neurol 2012;69:1170–5.
[35] Launer LJ, Miller ME, Williamson JD, Lazar RM, Gerstein HC, Murray AM, et al. Effects of intensive glucose lowering on brain structure and function in people with type 2 diabetes (ACCORD MIND): a randomised open-label substudy. Lancet Neurol 2011;10:969–77.
[36] Biessels GJ, Kamal A, Ramakers GM, Urban IJ, Spruijt BM, Erkelens DW, et al. Place learning and hippocampal synaptic plasticity in streptozotocin-induced diabetic rats. Diabetes 1996;45:1259–66.
[37] Kamal A, Biessels GJ, Hendrik W, Ramakers GMJ. Synaptic transmission changes in the pyramidal cells of the hippocampus in streptozotocin-induced diabetes mellitus in rats. Brain Res 2006;4:2–6.

[38] Yin Q-Q, Pei J-J, Xu S, Luo D-Z, Dong S-Q, Sun M-H, et al. Pioglitazone improves cognitive function via increasing insulin sensitivity and strengthening antioxidant defense system in fructose-drinking insulin resistance rats. PLOS ONE 2013;8:e59313.
[39] Hasselmo ME. The role of acetylcholine in learning and memory. Curr Opin Neurobiol 2006;16:710–5.
[40] Deutsch JA. The cholinergic synapse and the site of memory. Science (New York, NY) 1971;174:788–94.
[41] Yakel JL. Cholinergic receptors: functional role of nicotinic ACh receptors in brain circuits and disease. Pflugers Arch Eur J Physiol 2013;465:441–50.
[42] Pepeu G, Grazia M, Bracco L. effect of cholinesterase inhibitors on attention. Chem-Biol Interact 2013;203:361–4.
[43] Das A, Dikshit M, Nath C. Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. Life Sci 2001;68:1545–55.
[44] Aldunate R, Casar JC, Brandan E, Inestrosa NC. Structural and functional organization of synaptic acetylcholinesterase. Brain Res Brain Res Rev 2004;47:96–104.
[45] Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Corrêa M, et al. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. Eur J Pharmacol 2009;610:42–8.
[46] Mushtaq N, Schmatz R, Pereira LB, Ahmad M, Stefanello N, Vieira JM, et al. Rosmarinic acid prevents lipid peroxidation and increase in acetylcholinesterase activity in brain of streptozotocin-induced diabetic rats. Cell Biochem Funct 2014;32:287–93.
[47] Salgado H, Santos-Zavaleta A, Gama-Castro S, Millán-Zárate D, Díaz-Peredo E, Sánchez-Solano F, et al. RegulonDB (version 3.2): transcriptional regulation and operon organization in *Escherichia coli* K-12. Nucleic Acids Res 2001;29:72–4.
[48] Keseler IM, Collado-Vides J, Gama-Castro S, Ingraham J, Paley S, Paulsen IT, et al. EcoCyc: a comprehensive database resource for *Escherichia coli*. Nucleic Acids Res 2005:33.
[49] Thomsen T, Bickel U, Fischer JP, Kewitz H. Stereoslectivity of cholinesterase inhibition by galanthamine and tolerance in humans. Eur J Clin Pharmacol 1990;39:603–5.
[50] Albuquerque EX, Santos MD, Alkondon M, Pereira EF, Maelicke A. Modulation of nicotinic receptor activity in the central nervous system: a novel approach to the treatment of Alzheimer disease. Alzheimer Dis Assoc Disord 2001;15(Suppl. 1):S19–25.
[51] Maelicke A, Hoeffle-Maas A, Ludwig J, Maus A, Samochocki M, Jordis U, et al. Memogain is a galantamine pro-drug having dramatically reduced adverse effects and enhanced efficacy. J Mol Neurosci 2010;40:135–7.
[52] Samochocki M, Höffle A, Fehrenbacher A, Jostock R, Ludwig J, Christner C, et al. Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. J Pharmacol Exp Ther 2003;305:1024–36.
[53] Traykova M, Traykov T, Hadjimitova V, Krikorian K, Bojadgieva N. Antioxidant properties of galantamine hydrobromide. Z Naturforsch/J Biosci 2003;58:361–5.

3.3 CAPÍTULO III

ARTIGO CIENTÍFICO

Hyperglycemia alters E-NTPDases, ecto-5'-nucleotidase, ecto and cytosolic adenosine deaminase activities and expression from encephala of adult zebrafish (*Danio rerio*).

Katiucia Marques Capiotti, Anna Maria Siebel, Luiza Wilges Kist, Maurício Reis Bogo, Carla Denise Bonan, Rosane Souza Da Silva.

Artigo submetido no periódico

Purinergic Signalling

Purinergic Signalling

Hyperglycemia alters E-NTPDases, ecto-5'-nucleotidase and ecto and cytosolic adenosine deaminase activities and expression from encephala of adult zebrafish (Danio rerio)

--Manuscript Draft--

Manuscript Number:	PUSI-D-15-00026
Full Title:	Hyperglycemia alters E-NTPDases, ecto-5'-nucleotidase and ecto and cytosolic adenosine deaminase activities and expression from encephala of adult zebrafish (Danio rerio)
Article Type:	Original Research
Keywords:	Adenosine deaminase; Hyperglycemia; E-NTPDase; ecto-5'-nucleotidase; Zebrafish.
Corresponding Author:	Katiucia Marques Capiotti, Msc. Faculdade de Biociências, PUCRS Brazil, Porto Alegre BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Faculdade de Biociências, PUCRS
Corresponding Author's Secondary Institution:	
First Author:	Katiucia Marques Capiotti, Msc.
First Author Secondary Information:	
Order of Authors:	Katiucia Marques Capiotti, Msc.
	Anna Maria Siebel, Dr.
	Luiza Wilges Kist, Dr.
	Maurício Reis Bogo, Dr.
	Carla Denise Bonan, Dr.
	Rosane Souza Da Silva, Dr.
Order of Authors Secondary Information:	
Funding Information:	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Edital n. 10/2011) — Dr. Katiucia Marques Capiotti

Title: Hyperglycemia alters E-NTPDases, ecto-5'-nucleotidase and ecto and cytosolic adenosine deaminase activities and expression from encephala of adult zebrafish (*Danio rerio*)

Authors full name:

Katiucia Marques Capiotti[a]; Anna Maria Siebel [c, d]; Luiza Wilges Kist [b, c]; Maurício Reis Bogo [b, c];

Carla Denise Bonan[a,c]; Rosane Souza Da Silva[a, c*]

Institutional Affiliations:

[a] Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[b] Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUCRS, Porto Alegre, RS, Brazil.

[c] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil.

[d] Programa de Pós-graduação em Ciências Ambientais, Universidade Comunitária da Região de Chapecó, Chapecó, SC, Brazil.

Keywords: Adenosine deaminase; Hyperglycemia; E-NTPDase; ecto-5'-nucleotidase; Zebrafish.

***Contact details of the corresponding author**

Postal Address: Faculdade de Biociências, PUCRS.

Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil

Phone/ Fax: + 55 51 3353 4158/+ 55 51 3320 3612

e-mail: rosane.silva@pucrs.com

E-mail addresses of authors

Katiucia Marques Capiotti: katucapiotti@gmail.com

Anna Maria Siebel: annasiebel.siebel@gmail.com

Luiza Wilges Kist: lwkist@gmail.com

Maurício Reis Bogo: mbogo@pucrs.br

Carla Denise Bonan: cbonan@pucrs.br

Rosane Souza Da Silva: rosane.silva@pucrs.br

Abstract

Hyperglycemia is the main feature for the diagnosis of Diabetes Mellitus (DM). The DM has been recognized as an independent risk factor for development of cognitive impairment. Some studies have demonstrated the relationship between DM and dysfunction on neurotransmission systems, such as the purinergic system. In this study, we evaluated the extracellular nucleotide hydrolysis and adenosine deamination activities from encephalic membranes of hyperglycemic zebrafish. A significant decrease in ATP, ADP, and AMP hydrolysis was observed at 111 mM glucose-treated group, which returned to normal levels after 7 days of glucose withdrawal. A significant increase in ecto-adenosine deaminase (ecto-ADA) activity was observed in 111 mM glucose group, which remain elevated after 7 days of glucose withdrawal. The soluble-adenosine deaminase (soluble-ADA) activity was significantly increased just after 7 days of glucose withdrawal. We also evaluated the gene expression of E-NTPDases, ecto-5'-nucleotidase, ADA and adenosine receptors from encephala of adult zebrafish. The *entpd* 2a.1, *entpd* 2a.2, *entpd* 3 and *entpd* 8 mRNA levels from encephala of adult zebrafish were decreased in 111 mM glucose-treated and glucose withdrawal group. The gene expression of adenosine receptors ($adora_{1}$, $adora_{2a.1}$, $adora_{2a.2}$ and $adora_{2b}$) was decreased in 111 mM glucose-treated and glucose withdrawal group. The gene expression of ADA (*ada* 2a.1) was decreased in glucose withdrawal group. These findings demonstrated that hyperglycemia might affect the ectonucleotidase and adenosine deaminase activities and gene expression in zebrafish, suggesting that the modifications caused on purinergic system may also contribute to the diabetes-induced progressive cognitive impairment.

Introduction

The incidence of Diabetes Mellitus (DM) is increasing rapidly worldwide, reaching approximately 382 million people [1]. According to International Diabetes Federation (2013) [1], there is an alarming estimate that this number will raise to 582 million people by 2035. DM is a chronic disease, characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action or both [2, 3]. The effects of this disease are being strongly associated with neurophysiological, neurochemical and behavioral modifications in brain of patients, that are also observed in animal model of DM [4–8].

Several studies have shown that dysfunction of synaptic plasticity in diabetic subjects may be related to the inefficiency of neurotransmitter release [9, 10]. One possible strategy to correct the changes in synaptic efficiencies caused by DM can be through of analysis of the presynaptic neuromodulation systems, as purinergic system [9, 10]. Adenine nucleotides represent an important class of extracellular molecules involved in modulation of signaling pathways that are essential to the normal functioning of the central nervous system (CNS) [11].

Purine nucleotides, ATP and ADP, and the nucleoside, adenosine, act as extracellular messengers and their levels are controlled by a cascade of ecto-enzymes, including ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases), ecto-nucleotide pyrophosphohydrolases/phosphodiesterases (ecto-NPPs), ecto-alkaline phosphatases (APs), and ecto-5'nucleotidase [12]. The hydrolysis of ATP to AMP is catalyzed mainly by a family of ENTPDases. The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of an ecto-5′-nucleotidase (CD73, EC 3.1.3.5) [13–15]. Extracellular concentrations of adenosine are also regulated by the interplay of equilibrative and concentrative nucleoside transporter with enzymes of adenosine metabolism, as adenosine deaminase [16].

Some authors have suggested that diabetic conditions may alter the purinergic tonus by inducing adaptive changes of the sensibility of adenosine receptors in the brain [17, 18]. Also, Schmatz et al.(2009)[19] demonstrated that ATP, ADP, and AMP hydrolysis were increased in synaptosomes from STZ-induced diabetic rats, as well as, Lunkes et al. (2004)[20], demonstrated that diabetic rats show an increase in NTPDase and 5'-nucleotidase activities.

Considering the importance to control nucleotides and nucleosides levels to the properly neurotransmission in CNS functioning and that cognitive impairment is a long-term consequence of

Diabetes Mellitus, the aim of the present study was to evaluate the activity and expression of these enzymes and adenosine receptors in adult zebrafish submitted to a hyperglycemia model.

Material and Methods

Animals

Adult zebrafish (*Danio rerio*) wild type (Tübingen background; 3 – 5 cm) of both sexes were purchased from commercial suppliers and acclimated for at least 14 days in the experimental room. Animals were housed in groups of 15 fish in 5-L thermostated (28 ± 2 °C) tanks, kept under constant chemical, biological and mechanical water filtration and aeration (7.20 mg O_2/L). Fish were maintained under a 14-10 h day/night photoperiod cycle, fed three times a day with commercial flakes (TetraMin™, NC, USA) and supplemented with live brine shrimp. All fish used in these experiments were randomly chosen from different clusters. The animals were submitted to hypothermia by exposure to ice water flocked followed by decapitation as endpoint. All protocols were approved by the Institutional Animal Care Committee (12/00310–CEUA PUCRS) and followed Brazilian legislation, the guidelines Federal Council of Veterinary Medicine (CFMV), and the Canadian Council for Animal Care (CCAC) "Guide on the Care and Use of Fish in Research, Teaching, and Testing".

Drugs

Glucose was purchased from Nuclear™ (São Paulo, Brazil). Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). All other reagents used were from analytical grade.

Hyperglycemia model

Groups of 15 adult animals were placed in 5-L aquariums containing 111 mM glucose solution, which were diluted in water and maintained during 14 days at room temperature as described

in previous section according to Capiotti et al. (2014a)[8]. The solutions of glucose were exchanged three times per week to avoid contamination with opportunistic microorganisms. Control group animals were maintained in 5-L aquariums with normal water for the same period and conditions as the glucose-treated groups. The animals were placed in 111 mM glucose and were monitored for signs of stress as such as difficulty for swimming or excessive gill movement [21].

Withdrawal of glucose

In order to verify if the effects caused by 111 mM glucose solution treatment would be persistent, a group of 15 fish was kept in glucose-free water by additional 7 days (washout group, no glucose added) [8]. During this period, the animals were maintained at the same conditions of welfare described in previous section. Control group animals were maintained in aquariums with water for the same period and were handled as washout group.

Preparation of soluble and membrane fractions from zebrafish encephalon

The animals were cryoanesthetized, euthanized by decapitation, and encephalon were dissected [22]. Encephalic samples were prepared as previously described and each independent experiment was performed using biological preparations consisting of a "pool" of five encephalon [23–25]. Following the dissection, the whole zebrafish encephalon were homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For E-NTPDase and ecto-5′-nucleotidase assays, zebrafish encephalon were homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ecto-ADA experiments, encephalon were homogenized in 20 vol. (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The encephalic membranes were obtained as previously described [26]. The homogenates were centrifuged at 800 ×g for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40.000 ×g. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers (to ensure the lysis of the encephalic vesicle membranes) and centrifuged for 20 min at 40.000 ×g. The final pellets were resuspended and used for assays to ectonucleotidases and ecto-ADA. All samples were maintained at

2–4 °C throughout preparations. Protein was measured by the Coomassie blue method [27] using bovine serum albumin as a standard.

Ectonucleotidase assays

E-NTPDase and ecto-5′-nucleotidase assays were performed following previous described methods [23, 25]. Zebrafish encephalic membranes (3–5 μg protein) were added to the reaction medium containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the E-NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the ecto-5′-nucleotidase activity) at a total volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was started by the addition of the substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was terminated after 30 min by the addition of 200 μL trichloroacetic acid at a final concentration of 5%, and then the samples were chilled on ice for 10 min. The colorimetric reagent composed by 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added (1 mL) in order to determine the inorganic phosphate released (Pi) [28]. The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of Pi $.min^{-1}$ $.mg^{-1}$ of protein. Controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. All enzyme reactions were performed with triplicate samples. At least seven independent experiments were performed.

Adenosine deaminase assays

Ecto- and soluble-ADA activities were determined as previously described [24]. The encephalic fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively. The samples were preincubated for 10 min at 37 °C and the reaction was started by the addition of substrate (adenosine) to a final concentration of 1.5 mM, in a final volume of 200 μL. The reaction was stopped by the addition of 500 μL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml) after 75 min (soluble fraction) and 120 min (membrane fraction). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time interval using a Berthelot reaction according to [29]. The reaction mixtures were mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine,

in 0.6 M NaOH) and vortexed. Samples were incubated at 37 °C for 15 min and the colorimetric assay was carried out at 635 nm. The ADA activity was expressed as nmol of NH_3 .min^{-1} .mg^{-1} of protein. Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct the substrates' non-enzymatic hydrolysis. All enzyme reactions were performed with triplicate samples. At least five independent experiments were performed.

Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

The gene expression of ADA subfamilies (*ada1, ada2.1, ada2.2*) including an alternative splicing isoform (*adaasi)* and, an adenosine deaminase like related gene (*adal*), adenosine receptor subtypes ($adora_1$, $adora_{2a.1}$, $adora_{2a.2}$ and $adora_{2b}$), ectonucleotidases (*entpd1, entpd2a.1, entpd2a.2, entpd2-like, entpd3* and *entpd8*) and ecto-5`-nucleotidase (*nt5e*) were determined. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) from 1 µg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA, containing SYBR® Green I 0.2 times diluted (Invitrogen), 100 µM dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1) [30–33]. The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95°C, 40 cycles of 15 s at 95°C for denaturation, 35 s at 60 °C for annealing and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C and showed in all cases one single peak. *ef1α* and *rpl13α* were used as reference genes for normalization. Relative expression levels were determined with 7500 and 7500 Fast Real-Time PCR Systems Software v.2.0.6 (Applied Biosystems, California, USA). The efficiency *per* sample was calculated using LinRegPCR version 2012.3 Software (http://LinRegPCR.nl). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method[34, 35].

Statistical Analysis

The data are shown as mean ± S.E.M. of at least seven (Ectonucleotidase assays), five (Adenosine deaminase assays) and four (molecular analysis) independent experiments. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test or Student T Test when appropriated. $p < 0.05$ were considered as significant.

Results

Blood glucose levels and mortality

As confirmed before by Capiotti et al. (2014b) [36] the profile of blood glucose levels from the experimental groups showed an increase of 117% in the 111 mM glucose-treated animals in relation to control group [p<0.0001] (control: 4.015 ± 0.3158 mM; Glucose-treated: 8.83 ± 0.663 mM; p<0.0001), while after 7 days of glucose withdrawal the animals kept 50% of increase of blood glucose levels in relation to the specific control [p=0.02]. The 111 mM de glucose-treated groups showed mortality of 20%.

Nucleotide hydrolysis from encephalic membrane of zebrafish after hyperglycemia

We evaluated the influence of hyperglycemia on ATP, ADP and AMP hydrolysis activities from encephalic membrane of adult zebrafish. The figures 1A-C show that adult zebrafish exposed to 111 mM glucose significantly decreased the ATP (29%) [$F_{(2;28)} = 7.212$; p = 0.003], ADP (58%) [$F_{(2;24)} = 11.59$; p = 0.0003] and AMP (32%) [$F_{(2;27)} = 4.991$; p = 0.0143] hydrolysis when compared to control groups. After 7 days of glucose withdrawal, the nucleotide hydrolysis returned to levels similar to control group (Figure 1A-C).

Nucleoside hydrolysis from encephalic membrane of zebrafish after hyperglycemia

After verifying that hyperglycemia modifies the ATP, ADP and AMP hydrolysis from encephala of zebrafish, we observed whether the hyperglycemia was able to promote changes on ecto-ADA and soluble-ADA activities from encephalic membrane of adult zebrafish. Our results demonstrated that ecto-ADA activity was increased (61%) in 111 mM glucose-treated animals (Figure 2A) [$F_{(2;17)} = 5.594$; p = 0.0136], when compared to control group. After 7 days of glucose withdrawal, the ecto-ADA activity remained increased (62%) [$F_{(2;17)} = 5.594$; p = 0.0136] (Figure 2A). We also evaluated soluble-ADA activity. Our results demonstrated that glucose-treated animals had their

soluble-ADA activity unaltered while, after 7 days of glucose withdrawal, the soluble-ADA activity was significantly increased (38%) [$F_{(2;19)} = 53.87$; $p < 0.0001$], when compared to control group (Figure 2B).

Effect of hyperglycemia on entpdases, nt5e, ada and adora gene expression from encephala of adult zebrafish

Quantitative RT-PCR experiments were performed to verify whether the hyperglycemia could alter the expression of ectonucleotidases, ADA-related genes (*ada1*, *ada2.1*, *ada2.2*, *adaasi* and *adal*) and adenosine receptors ($adora_1$, $adora_{2a.1}$, $adora_{2a.2}$ and $adora_{2b}$). Our results showed a decrease of the *entpd 2a.1*, *entpd 2a.2* and *entpd 3* and *entpd 8* genes expression in both groups analyzed: 111 mM glucose and washout group. Nevertheless, the *entpd 1*, *entpd 2-like* and *nt5e* genes expression did not change (Figure 3). Regarding ADA-related genes expression, we observed a decrease of the *ada2.1* gene expression, while no changes were observed in the other isoforms of *ada* (Figure 4). Furthermore, the evaluation of adenosine receptors in encephala of zebrafish demonstrated that *adora1, adora2a.1, adora2a.2* and *adora2b* were decreased in 111 mM glucose group. After 7 days of glucose withdrawal, only $adora_1$ adenosine receptor, returned to levels similar to control group (Figure 5).

Discussion

In the present study, we evaluated the E-NTPDases, ecto-5'-nucleotidase and ADA activities from encephala in a model of hyperglycemia using zebrafish. We also evaluated the same biochemical parameters after 7 days of glucose withdrawal. After, we performed the evaluation of the *entpdases*, *ada*-related and *adora* gene expression from encephala of adult zebrafish. The blood glucose levels from glucose-treated and washout animals demonstrated the same profile already developed in previous works [8, 36].

Nucleotides are important extracellular messengers in both physiological and pathological conditions [9]. After its release in the synaptic cleft, ATP can be catabolized to ADP, AMP, and adenosine. E-NTPDases and ecto-5′-nucleotidase action regulates the concentrations of ATP, ADP, and AMP by increasing/decreasing their hydrolysis with a consequent increase/decrease in adenosine levels [13]. Extracellular concentrations of adenosine might be regulated by adenosine uptake via bi-

directional transporters [37]. Adenosine uptake followed by its phosphorylation to AMP by adenosine kinase or deamination to inosine by ecto-adenosine deaminase are two possible mechanisms able to promote the inactivation of adenosine signaling [38–40].

Previous studies demonstrated that purinergic signaling was altered in rats submitted to diabetes mellitus. Schmatz et al.(2009) [19] showed that extracellular nucleotide hydrolysis was increased in synaptosomes from the cerebral cortex of rats submitted to streptozotocin-induced diabetes. Lunkes et al. (2004) and Miron et al. (2007) [20, 41] also demonstrated an increase on the activities of the enzymes E-NTPDase and ecto-5′-nucleotidase in platelets and synaptosomes from the cerebral cortex of rats with alloxan-induced diabetes. In contrast to these results, our findings showed that hyperglycemia reduces significantly ATP, ADP, and AMP hydrolysis promoted by E-NTPDases and ecto-5′-nucleotidase in zebrafish encephalic membranes. Likewise, Duarte et al. (2007)[42] demonstrated that ATP catabolism was decreased in diabetic rats, possibly due to decreased activity of ectonucleotidases.

The effect observed in ATP hydrolysis in encephalic membranes of zebrafish suggests that specific extracellular nucleotide-hydrolyzing enzymes may be involved in the effects observed. While we don't have specific information about the catalytic properties of ectonucleotidase isoforms expressed in zebrafish, assuming data from mamals and amphibian such as *Xenopus laevis*, the decrease in ATP hydrolysis seems here could involve NTPDase 2, due to hydrolysis ratio 30:1 (ATP:ADP) that contributes more effectively to ATP hydrolysis when compared with other NTPDases [13, 43]. In fact, the expression of E-NTPDase 2 isoforms (*entpd2a.1* and *entpd2a*) was significantly decreased in glucose-treated zebrafish. In this same way, the decrease in ADP hydrolysis could indicate a response to *enptd3 and entpd8* reduced expression in glucose-treated animals. Interestingly, the gene expression levels of *nt5e* have not changed, suggesting that the decrease of AMP hydrolysis observed is probably not directly related to *nt5e* gene expression control but possible by stoichiometric mechanism, since ADP and ATP are strong inhibitors of ecto-5'-nuleotidase [44]. Nucleotides hydrolyzes were fully recovered after 7 days of glucose withdrawal, probably in function of the partial recovery of glycemic control.

ADA is located both in the cytosol and in the cell membrane and the regulation of brain adenosine levels might be promoted by distinct *ada* members (*ada1, ada2* and *adal*) [24, 45, 46]. Evidences suggest that ecto-ADA (ADA1) can be anchored to the cell membrane by the A_1R adenosine

receptor as well, in order to down regulate the signal produced by adenosine [47]. Our results demonstrated that ecto-ADA activity was significantly increased in 111 mM glucose group, suggesting a decrease in adenosine levels in synaptic cleft. There is some consensus that a decrease of adenosine levels could be related with cognitive impairment observed in diabetic rats [8, 48, 49], which also could be suggested be occurring in the zebrafish hyperglycemic model.

At least in human plasma, the soluble-ADA has a high K_M value for the reaction of deamination, suggesting that this enzyme requires a high adenosine concentration for its activity [50]. Previous studies have reported that the regulation of adenosine levels in intracellular and extracellular fractions in the zebrafish encephala might be promoted by distinct *ada* members (*ada1, ada2* and *adal*), which have diverse gene expression patterns and activity properties [24, 46] and might contribute for the regulation of adenosine levels in different manners.

The results demonstrated that the relative gene expression levels of *ada* (*ada2.1)* was significantly decreased in 111 mM glucose, suggesting that the increase of adenosine hydrolysis promoted by soluble-ADA is probably not directly related to a reduced *ada* gene expression. The transcription machinery is continuously controlled by a complex signaling system, creating a set of signals able to adjust gene expression profile of the cell. The signal transduction can be exerted by proteins, products of enzyme reactions or even toxins able to regulate transcription factors [51]. The phenomenon known as negative feedback loop [51, 52], which is situated at the interface of genetic and metabolic networks, could explain, at least in part, the simultaneous increase of adenosine hydrolysis and the decrease of *ada2.1* transcripts in zebrafish encephala.

Regarding the relative gene expression of adenosine receptors, we verified a decrease in mRNA transcripts of $adora_{1}$, $adora_{2a.1}$, $adora_{2a.2}$ and $adora_{2b}$ receptors in encephala of hyperglycemic zebrafish. Previous studies reported a change of adenosine sensitivity in the hippocampus of diabetic rats [17, 18, 53], suggesting that diabetic conditions may also induce an adaptive change of the density of adenosine receptors in the brain. Faulhaber-Walter et al. (2001)[54], demonstrated that the decrease of $adora_1$ adenosine receptor signaling is strongly linked to impaired glucose tolerance and insulin resistance.

Previous studies from our group demonstrated that hyperglycemic zebrafish has impaired memory, probably by the incapacity to keep proper cholinergic signaling as a result of increased acetylcholinesterase activity [36]. As cholinergic system is closely related to purinergic system, based

on the co-release of acetylcholine and ATP, and in the adenosine control of acetylcholine release [55], the alteration on nucleotide and nucleoside metabolism detected after the hyperglycemic status could contribute to the neurophysiological disturbance that could implicate in the development of cognitive impairment.

Conclusions

The present study provides evidences that the purinergic signaling system is compromised in the central nervous system of zebrafish treated with 111 mM glucose. Molecular changes are observed even after 7 days glucose free. The modifications could lead to alterations in the modulation of neurotransmission, which may also contribute to the diabetes-induced progressive cognitive impairment.

Conflict of interest

The authors declare not potential conflict of interests relevant to this article.

Disclosure Statement

The authors declare no competing financial interests exist.

Acknowledgments

This work was supported by DECIT/SCTIE-MS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. 10/0036-5, conv. n. 700545/2008 – PRONEX). K.M.C is recipient of FAPERGS/Capes fellowship.

References

1. Aguiree F, Brown A, Cho N, Dahlquist G (2013) IDF Diabetes Atlas. International Diabetes Federation

2. Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539–553. doi: 10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S

3. Harris MI (1988) Classification and diagnostic criteria for diabetes mellitus and other categories of glucose intolerance. Prim Care 15:205–225.

4. Motta M, Sorace S, Restuccia S, et al. (1996) Cognitive impairment in the elderly diabetics. Arch Gerontol Geriatr 22 Suppl 1:43–46.

5. Kodl CT, Seaquist ER (2008) Cognitive dysfunction and diabetes mellitus. Endocr Rev 29:494–511.

6. Launer LJ, Miller ME, Williamson JD, et al. (2011) Effects of intensive glucose lowering on brain structure and function in people with type 2 diabetes (ACCORD MIND): A randomised open-label substudy. Lancet Neurol 10:969–977.

7. Chen Z, Zhong C (2013) Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. Prog Neurobiol 108:21–43. doi: 10.1016/j.pneurobio.2013.06.004

8. Capiotti KM, Antonioli R, Kist LW, et al. (2014) Persistent impaired glucose metabolism in a zebrafish hyperglycemia model. Comp Biochem Physiol B Biochem Mol Biol 171:58–65. doi: 10.1016/j.cbpb.2014.03.005

9. Burnstock G, Novak I (2013) Purinergic signalling and diabetes. Purinergic Signal 9:307–24. doi: 10.1007/s11302-013-9359-2

10. Stefanello N, Schmatz R, Pereira LB, et al. (2014) Effects of chlorogenic acid, caffeine, and coffee on behavioral and biochemical parameters of diabetic rats. Mol Cell Biochem 388:277–86. doi: 10.1007/s11010-013-1919-9

11. Gibb A.J., Halliday F.C. (1996) Fast purinergic transmission in the central nervous system. Sem Neurosc 8:225–232.

12. Zimmermann H, Zebisch M, Sträter N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8:437–502. doi: 10.1007/s11302-012-9309-4

13. Robson SC, Sévigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal 2:409–430.

14. Ernst PB, Garrison JC, Thompson LF (2010) Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology. J Immunol 185:1993–8. doi: 10.4049/jimmunol.1000108

15. Augusto E, Matos M, Sévigny J, et al. (2013) Ecto-5'-nucleotidase (CD73)-mediated formation of adenosine is critical for the striatal adenosine A2A receptor functions. J Neurosci 33:11390–9. doi: 10.1523/JNEUROSCI.5817-12.2013

16. Sala-Newby GB, Skladanowski a. C, Newby a. C (1999) The Mechanism of Adenosine Formation in Cells: CLONING OF CYTOSOLIC 5'-NUCLEOTIDASE-I. J Biol Chem 274:17789–17793. doi: 10.1074/jbc.274.25.17789

17. Morrison PD, Mackinnon MW, Bartrup JT, et al. (1992) Changes in adenosine sensitivity in the hippocampus of rats with streptozotocin-induced diabetes. Br J Pharmacol 105:1004–1008.

18. Duarte JM, Oliveira CR, Ambrósio a F, Cunha R a (2006) Modification of adenosine A1 and A2A receptor density in the hippocampus of streptozotocin-induced diabetic rats. Neurochem Int 48:144–150. doi: 10.1016/j.neuint.2005.08.008

19. Schmatz R, Mazzanti CM, Spanevello R, et al. (2009) Ectonucleotidase and acetylcholinesterase activities in synaptosomes from the cerebral cortex of streptozotocin-induced diabetic rats and treated with resveratrol. Brain Res Bull 80:371–376. doi: 10.1016/j.brainresbull.2009.08.019

20. Lunkes GIL, Lunkes DS, Morsch VM, et al. (2004) NTPDase and 5'-nucleotidase activities in rats with alloxan-induced diabetes. Diabetes Res Clin Pract 65:1–6. doi: 10.1016/j.diabres.2003.11.016

21. Pedroso GL, Hammes TO, Escobar TDC, et al. (2012) Blood collection for biochemical analysis in adult zebrafish. J Vis Exp e3865. doi: 10.3791/3865

22. Wilson JM, Bunte RM, Carty AJ (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). J Am Assoc Lab Anim Sci 48:785–789.

23. Rico EP, Senger MR, Fauth M da G, et al. (2003) ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci 73:2071–2082. doi: 10.1016/S0024-3205(03)00596-4

24. Rosemberg DB, Rico EP, Senger MR, et al. (2008) Kinetic characterization of adenosine deaminase activity in zebrafish (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol 151:96–101. doi: 10.1016/j.cbpb.2008.06.001

25. Senger MR, Rico EP, Dias RD, et al. (2004) Ecto-5'-nucleotidase activity in brain membranes of zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol 139:203–207. doi: 10.1016/j.cbpc.2004.07.011

26. Barnes JM1, Murphy PA, Kirkham D, Henley JM. (1993) Interaction of guanine nucleotides with [3H] kainate and 6-[3H] cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. J Neurochem 61:1685–91.

27. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

28. Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+ -stimulated ATPase activity. Anal Biochem 157:375–380.

29. Weisman MI, Caiolfa VR, Parola AH (1988) Adenosine deaminase-complexing protein from bovine kidney. Isolation of two distinct subunits. J Biol Chem 263:5266–5270.

30. Pereira VM, Bortolotto JW, Kist LW, et al. (2012) Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (Danio rerio). Neurotoxicology 33:469–475.

31. Tang R, Dodd A, Lai D, et al. (2007) Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin (Shanghai) 39:384–390.

32. Capiotti KM, Menezes FP, Nazario LR, et al. (2011) Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (Danio rerio). Neurotoxicol Teratol 33:680–685.

33. Tseng Y-C, Chen R-D, Lee J-R, et al. (2009) Specific expression and regulation of glucose transporters in zebrafish ionocytes. Am J Physiol Regul Integr Comp Physiol 297:R275–R290. doi: 10.1152/ajpregu.00180.2009

34. Nery LR, Rodrigues MM, Rosemberg DB, et al. (2011) Regulation of E-cadherin expression by growth factor receptors in cancer cells. J Surg Oncol 104:220–1. doi: 10.1002/jso.21898

35. Oggier DM, Weisbrod CJ, Stoller AM, et al. (2010) Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish Danio rerio. Environ Sci Technol 44:7685–7691. doi: 10.1021/es100980r

36. Capiotti KM, De Moraes DA, Menezes FP, et al. (2014) Hyperglycemia induces memory impairment linked to increased acetylcholinesterase activity in zebrafish (Danio rerio). Behav Brain Res 274:319–25. doi: 10.1016/j.bbr.2014.08.033

37. Pinto-Duarte A, Coelho JE, Cunha RA, et al. (2005) Adenosine A2A receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus. J Neurochem 93:595–604.

38. Latini S, Pedata F (2001) Adenosine in the central nervous system: Release mechanisms and extracellular concentrations. J Neurochem 79:463–484.

39. Fredholm BB, Chen J-F, Masino SA, Vaugeois J-M (2005) Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. Annu Rev Pharmacol Toxicol 45:385–412.

40. Boison D, Shen H-Y (2010) Adenosine kinase is a new therapeutic target to prevent ischemic neuronal death. Open Drug Discov J 2:108–118.

41. Miron VR, Bauermann L, Morsch ALB, et al. (2007) Enhanced NTPDase and 5'-nucleotidase activities in diabetes mellitus and iron-overload model. Mol Cell Biochem 298:101–7. doi: 10.1007/s11010-006-9357-6

42. Duarte JMN, Oses JP, Rodrigues RJ, Cunha R a. (2007) Modification of purinergic signaling in the hippocampus of streptozotocin-induced diabetic rats. Neuroscience 149:382–391. doi: 10.1016/j.neuroscience.2007.08.005

43. Kukulski F, Lévesque SA, Lavoie É G, et al. (2005) Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. Purinergic Signal 1:193–204.

44. Sträter N (2006) Ecto-5′-nucleotidase: Structure function relationships. Purinergic Signal 2:343–350.

45. Maier S a, Galellis JR, McDermid HE (2005) Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol 61:776–94. doi: 10.1007/s00239-005-0046-y

46. Rosemberg DB, Rico EP, Guidoti MR, et al. (2007) Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (Danio rerio). Life Sci 81:1526–34. doi: 10.1016/j.lfs.2007.09.019

47. Franco R, Valenzuela A, Lluis C, Blanco J (1998) Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. Immunol Rev 161:27–42.

48. Cox DJ, Kovatchev BP, Gonder-Frederick LA, et al. (2005) Relationships Between Hyperglycemia and Cognitive Performance Among Adults With Type 1 and Type 2 Diabetes. Diabetes Care 28:71–77. doi: 10.2337/diacare.28.1.71

49. Duarte JMN, Agostinho PM, Carvalho R a., Cunha R a. (2012) Caffeine consumption prevents diabetes-induced memory impairment and synaptotoxicity in the hippocampus of nonczno10/ltj mice. PLoS One 7:e21899. doi: 10.1371/journal.pone.0021899

50. Zavialov A V, Engström A (2005) Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. Biochem J 391:51–7. doi: 10.1042/BJ20050683

51. Salgado H, Santos-Zavaleta A, Gama-Castro S, et al. (2001) RegulonDB (version 3.2): transcriptional regulation and operon organization in Escherichia coli K-12. Nucleic Acids Res 29:72–74.

52. Keseler IM, Collado-Vides J, Gama-Castro S, et al. (2005) EcoCyc: A comprehensive database resource for Escherichia coli. Nucleic Acids Res. 33:

53. Artola A, Kamal A, Ramakers GMJ, et al. (2002) Synaptic plasticity in the diabetic brain: Advanced aging? Prog. Brain Res. pp 305–314

54. Faulhaber-Walter R, Jou W, Mizel D, et al. (2011) Impaired glucose tolerance in the absence of adenosine A1 receptor signaling. Diabetes 60:2578–87. doi: 10.2337/db11-0058

55. Zimmermann H (2008) ATP and acetylcholine, equal brethren. Neurochem Int 52:634–648.

Figure 1

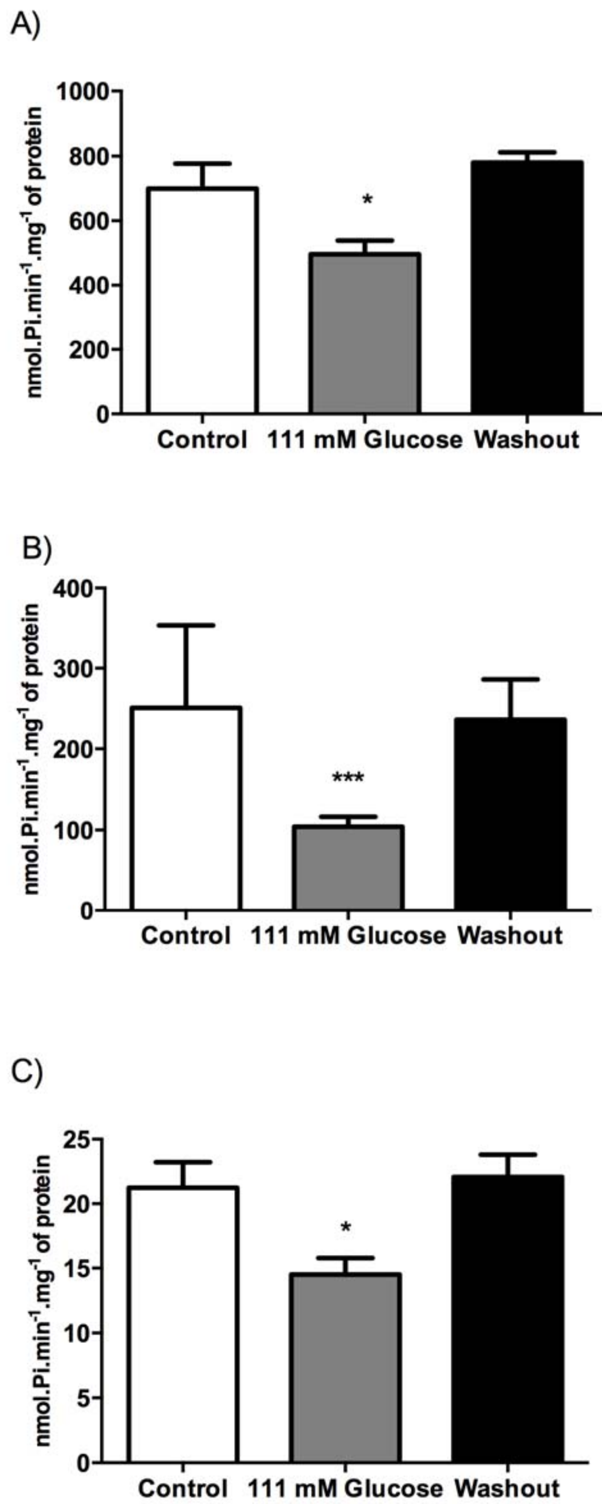


Figure 2

A)

B)

Figure 3

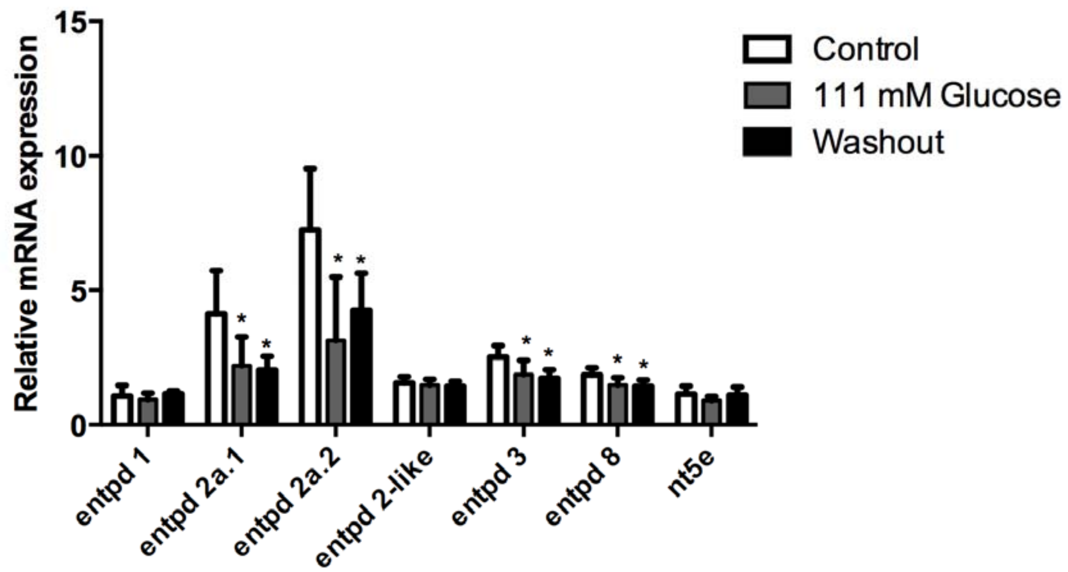


Figure 4

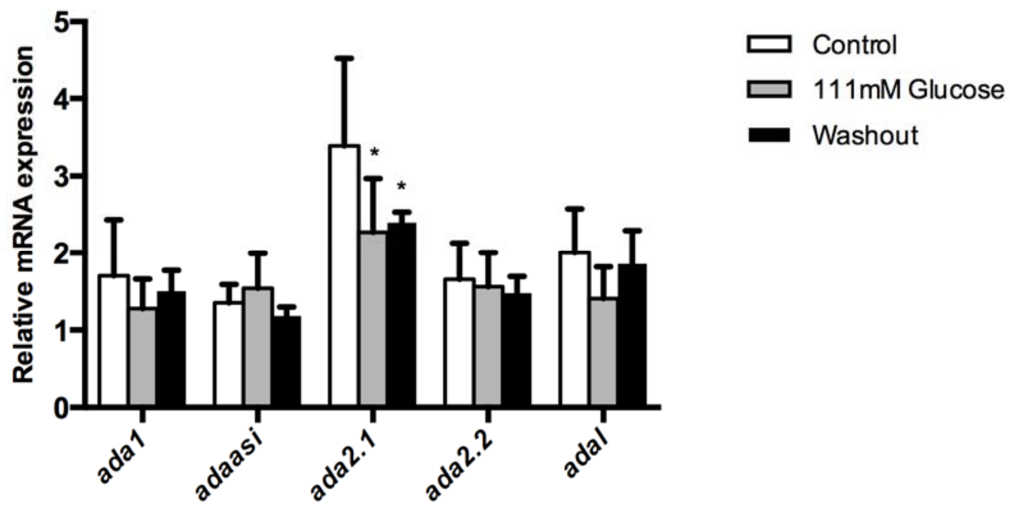
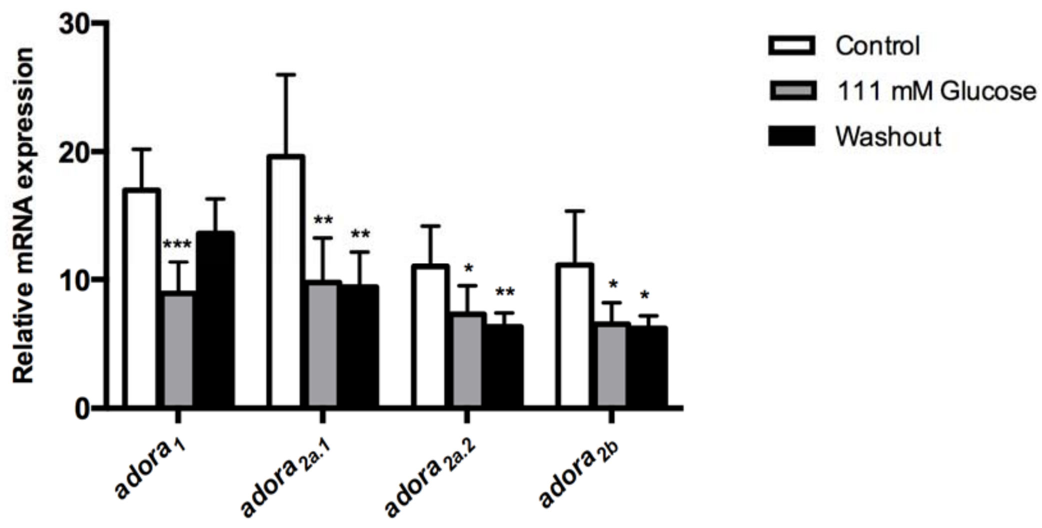


Figure 5



LEGEND OF FIGURES

Figure 1: Effect of hyperglycemia model on ATP (A), ADP (B), and AMP (C) hydrolysis in zebrafish brain membranes. Bars represent the mean ± S.E.M. of at least seven independent experiments performed in triplicate. The symbol (*) represents a significant difference from control group (one-way ANOVA, followed by Tukey test as post hoc, $P \leq 0.05$). The specific enzyme activity is reported as nmol of Pi $.min^{-1}$ $.mg^{-1}$ of protein.

Figure 2: Effect of hyperglycemia model on membrane-bound (A) and soluble (B) ADA activity from zebrafish brain. Bars represent the mean ± S.E.M. of at least five independent experiments performed in triplicate. The symbol (*) represents a significant difference from control group (one-way ANOVA, followed by Tukey test as post hoc, $P \leq 0.05$). The specific enzyme activity is reported as nmol of NH_3 $.min^{-1}$ $.mg^{-1}$ of protein.

Figure 3: Effect of hyperglycemia on *entpd1*, *entpd2a.1, entpd 2a.2, entpd2-like, entpd3*, *entpd8* and *nt5e* gene expression in 111 mM glucose and glucose washout. Data are expressed as mean ± SEM of four independent experiments performed in quadruplicate.

Figure 4: Effect of hyperglycemia on *ada* gene expression pattern in 111 mM glucose and glucose washout group. Data are expressed as mean ± S.E.M. of four independent experiments performed in quadruplicate.

Figure 5: Effect of hyperglycemia on *adora*gene expression pattern in 111 mM glucose and glucose washout group. Data are expressed as mean ± S.E.M. of four independent experiments performed in quadruplicate.

4 DISCUSSÃO E CONCLUSÃO

Os resultados apresentados nessa tese demonstram a caracterização de um modelo de hiperglicemia induzido por exposição à glicose por 14 dias em peixe-zebra adulto. Tal modelo é capaz de causar alterações mnemônicas que podem ser correlacionáveis com efeitos sobre ossistemascolinérgicoe purinérgico.

O DM é considerado uma epidemia mundial, atingindo em torno de 8,3% da população, ou seja, 387 milhões de pessoas (Mena et al., 2014).O mesmo levantamento realizado pela IDF (2014) demonstrou que no Brasil 8,7% da população mundial apresenta DM, chegando a proporção de 1 a cada 12 pessoas com a doença.Os pacientes com DM apresentam hiperglicemia crônica como o principal sintoma das duas formas de diabetes mais comuns, DM1e DM2 (Sociedade Brasileira de Diabetes, 2014).

No DM1, ocorre a destruição crônica das células β pancreáticas, por meio de mecanismos autoimunes, mediados por células como linfócitos T e macrófagos(Richardson et al., 2014). O processo de autodestruição se inicia meses a anos antes do diagnóstico clínico da doença e, dependendo da idade do diagnóstico, cerca de 70 a 90% das células β já foram destruídas após os primeiros sintomas de hiperglicemia (Lebastchi e Herold, 2012; Levetan et al., 2013; Atkinson et al., 2014).

No DM2, o principal fenômeno fisiopatológico é à resistência à ação da insulina, diminuindo a captação de glicose nos tecidos

insulinodependente(Bergman et al., 1981; Saisho, 2015). No início da doença, em resposta aresistência à insulina, ocorre a hiperinsulinemia compensatória, evitandoque ocorra a hiperglicemia. A função das células β pancreáticas também é diminuída em DM2 (Jensen et al., 2002; Defronzo, 2009; DeFronzo et al., 2013), chegando a 80%de perda da funcionalidade das células. Esses estudos demonstram que a síntese e a secreção de insulina ficam comprometidas desde as fases iniciais do DM(Jensen et al., 2002; Defronzo, 2009;DeFronzo et al., 2013).

Em nosso modelo de hiperglicemia, a exposição dos animais a 111 mM de glicose por 14 dias, provocou um aumento de 117% nos níveis de glicose sanguínea dos animais tratados, assim comoelevou em 41% os níveis de glicação da proteína frutosamina nos olhos dos animais. A hiperglicemia e o aumento da glicação de proteínas são consequências da disfunção do metabolismo da glicose e são utilizados como principais indicadores para o diagnóstico de intolerância à glicose e DM (American Diabetes, 2014b).

O modelo de hiperglicemia tambémrevelou que os efeitos da exposição à glicose por 14 dias foram prolongados, demonstrando que mesmo após 7 dias de *washout*, os níveis de glicose sanguínea e de glicação de proteínas ainda permaneciam elevados. Portanto, podemos sugerir que o modelo de hiperglicemia foi capaz de causar alterações significativas e duradouras no metabolismo da glicose dos animais, sendo

capaz de reproduzir sintomas semelhantes àqueles apresentados pelos indivíduos diabéticos.

Os medicamentos constituem a principal opção no tratamento do DM, não levando a cura da doença, mas evitando o agravamento de suas consequências. Alguns medicamentos reduzem a produção hepática de glicose e aumentam a sensibilidade periférica à insulina, outros estimulam a secreção de insulina (Tripathi e Srivastava, 2006). A fim de compreender as vias pelos quais a hiperglicemia induzida estava atuando, utilizamos dois medicamentos muito comuns no tratamento do DM em humanos, os quais possuem diferentes mecanismos de ação: a metformina e a glimepirida.

A metformina é um fármaco oral de primeira escolha para o tratamento do DM2 (Krentz e Bailey, 2005), que promove a oxidação dos ácidos graxos, o aumento da fosforilação dosreceptores de insulina (RI) no fígado e no músculo esquelético, assim como a diminuição da produção hepática de glicose (Tripathi e Srivastava, 2006). Alguns estudos demonstram que o uso da monoterapia desse fármaco em pacientes diabéticos reduz em torno de 1,5% os níveis de HbA1C (DeFronzo e Goodman, 1995; Bailey e Turner, 1996; Viollet et al., 2011; Sumitani et al., 2014).

A glimepirida por sua vez é uma sulfoniluréia que promove a estimulação da secreção de insulina, a ativação do transporte de glicose e

das enzimas que metabolizam a glicose, levando a estimulação da síntese de glicogênio e lípideos (Briscoe et al., 2010). De acordo com Mulleret al. (1995), a glimepirida parece exercer efeitos pleiotrópicos através de dois mecanismos independentes: a estimulação de proteínas fosfatases específicas responsáveis pela regulação da translocação deGLUT4; a estimulação da glicogênio sintase/ acetiltransferase glicerol-3-fosfato e a estimulação da fosfodiesterase específica do AMPc (Muller e Wied, 1993; Muller et al., 1995; Müller e Geisen, 1996).

Nossos estudos indicaram que tanto a metformina quanto a glimepirida, foram capazes de reduzir a glicemia em animais submetidos ao modelo de hiperglicemia, sendo a glimepirida, o fármaco com o maior percentual de redução da glicemia. O tratamento com metformina é capaz de melhorar a supressão da gliconeogênese mediada por insulina (Bailey e Turner, 1996; Nathan, 2007), porém com o passar do tempo o controle glicêmico provocado pela utilização de metformina deteriora-se, requerendo a adição de outro fármaco (Ferreira et al., 2014).A progressão do DM leva a diminuição da secreção de insulina endógena, na qual passa a ser mais adequada a utilização da glimepirida.

Hipóteses correntes sugeremque as monoterapias com metformina e glimepirida, reduzem os níveis de glicose sanguínea de forma muito eficiente, principalmente através do aumento da secreção de insulina, da translocação de GLUT 4 e da fosforilação de importantes enzimas do

metabolismo da glicose, como a PEPCK.Elo et al. (2007) demonstraram que a metformina é capaz de reduzir a expressão da PEPCK em peixe-zebra, demonstrando que a monoterapia é capaz de diminuir a hiperglicemia e a gliconeogênese. Requist et al.(2013)também demonstraram que o tratamento com metformina foi capaz de aumentar a sensibilidade à insulina em larvas de peixe-zebra. Maddison et al.(2015), descreveram um modelo de intolerância à glicose, utilizando um inibidor de IGF-1 e insulina em peixe-zebra. O estudo demonstrou que a captação da glicose no músculo esquelético é estimulada pela insulina, mas de forma menos eficiente que em roedores. Inicialmente ocorreu um aumento compensatório de células β e o controle da glicemia. Ao longo do tempo, o número de células β diminuiu e os primeiros sinais de intolerância à glicose surgiram através do aumento dos níveis de glicose em jejum.

O modelo de hiperglicemia caracterizadonessa tese foi capaz de gerar resistência à insulina nos animais expostos a 111 mM glicose, uma vez que quando tratados com 1U/kg de insulina não demonstraram redução significativa nos níveis de glicose sanguínea quando comparados ao controle. Após 7 dias de *washout*, a expressão de RNAm paraRI no músculofoi aumentada, demonstrando um efeito tardio de recuperação dos danos causados pela hiperglicemia, a fim de buscar a homeostase da glicemia.Considerando os resultados de resposta à insulina exógena e aos fármacos hipoglicemiantes, as alterações metabólicas vistas no modelo aqui caracterizado, provavelmente são um reflexo ao comprometimento da

resposta periférica à insulina e não por alterara secreção pancreática de insulina.

Os neurotransmissores do SNC tem um papel crucial na regulação da homeostase da glicose (Sherin et al., 2012), revelando que oSNC é um alvo da DM, gerando consequências neurológicas tanto no DM1 como no DM2 (McCall, 1992; Biessels et al., 1994). Embora océrebro seja um órgão alvo tardio do DM e do pré-diabetes, as causas das disfunções cognitivas relacionadas com DM são difíceis de serem estabelecidas, devido à prevalência de comorbidades severas que podem estar afetando a disfunção cognitiva (McCrimmon et al., 2012).Os mecanismos exatos pelos quais a disfunção neuronal ocorre ainda permanecem desconhecidos, porémalgumas pesquisas sugerem que os altos níveis de glicose podem ter efeitos tóxicos sobre os neurônios, através de danososmóticos, estresse oxidativo e desregulação dos níveis de neurotransmissores (Umegaki, 2012; Yamagishi, 2012; Butterfield et al., 2014; Rochette et al., 2014).

Alguns investigadores tem revelado que a Doença de Alzheimerpoderia ser um “Estado cerebral resistente à insulina”, ou então um diabetes tipo 3 (Steen et al., 2005; De La Monte et al., 2006; Moreira et al., 2009), devido a integração de diferentes fatores como idade, a diminuição dos níveis de proteína e de RNAm de insulina e dos RI. Estudos demonstraram que a sinalização regular da insulina no SNC previne a formação de oligômeros de β-amilóide, ou seja, a sinalização inadequada

da insulina, como na patologia do DM, poderia contribuir para a DA(Umegaki, 2012).

Estudos já demonstraram que o DM é um fator de risco independente para o desenvolvimento de déficits cognitivos (Luchsinger, 2012; Cato et al., 2014), sendo sugerido por vários autores como uma consequência de mudanças no SNC secundárias aos efeitos da hiperglicemia crônica (McCall, 2004; Biessels et al., 2006). Nossos estudos demonstraram que a hiperglicemia foi capaz de causaruma redução na retenção da memória dos animais hiperglicêmicos. Esses resultados corroboram com os dados encontrados na literatura, que demonstram que o DM é fator de risco para disfunção cognitiva e demência (Biessels et al., 2006; Exalto et al., 2012). Kanaya et al.(2004) demonstraram que a hiperglicemia crônica é capaz de mediar a relação entre o DM e o declínio cognitivo, e que a melhora do controle da glicemia pode prevenir o declínio cognitivo, assim como dados encontrados por Launeret al.(2011)e Yaffe et al.(2012).

Nossos dados demonstraram que o tratamento com galantamina, reverteu o efeito causado pela hiperglicemia na memória, demonstrando que a piora cognitiva observada esta diretamente relacionada com a atividade da enzima AChE. A galantamina é um alcalóide que atua como inibidor seletivo da AChE, melhorando a transmissão colinérgica através da modulação alostérica dos receptores nicotínicos (Wilkinson et al., 2004;

Maelicke et al., 2010). Existem muitos dados controversos entre pacientes que apresentam déficits cognitivos, DA e DM, e ainda mais estudos precisam ser realizados a fim que se conheçam os mecanismos pelos quais possa ocorrer essa interação mais especificamente.

Nossos resultados demonstraram que houve um aumento da atividade da AChE causada pelo modelo dehiperglicemia em peixe-zebra, sugerindo uma redução na eficiência da neurotransmissão colinérgica, devido a uma diminuição dos níveis de AChna fenda sináptica. Os dados apresentados nesse trabalho corroboram com os demais achados da literatura que demonstram uma deterioração cognitiva progressiva e outras disfunções neurológicas em pacientes diabéticos (Pepeu et al., 2013).

O aumento dos níveis de ACh na fenda sináptica éobservado nas áreas cerebrais durante a realizaçãodas tarefas comportamentais, demonstrando o envolvimento desse sistema nos processos de aprendizagem e memória (Pepeu et al., 2013). Outros estudos tem demonstrado o aumento da atividade da AChE no cérebro de roedores e pacientes diabéticos que apresentam déficits cognitivos (Schmatz et al., 2009b; Mushtaq et al., 2014). Sánchez-Chavez e Salceda (2000) também observaram o aumento significativo na atividade da AChE em diferentes estruturais cerebrais de ratos diabéticos induzidos por STZ,como: córtex cerebral, estriado e hipocampo.Esses resultadossugerem que a ativação da AChE leva a uma degradação rápida da ACh e uma baixa estimulaçãosubsequente dos receptores de ACh, causando efeitos

deletérios sobre as funções cognitivas (Mushtaq et al., 2014), o que possivelmente esteja ocorrendo em nosso modelo.

A adenosina desempenhaum papel importante como neuromodulador,controlando a excitabilidade neuronal e, consequentemente, modulando condic ões fisiológicas e patológicas no SNC (Boison, 2012; Chen e Zhong, 2013; Dias et al., 2013). No DM já se tem dados revelando que há um aumento da incidência de complicações neurológicas (Gispen e Biessels, 2000; Cox et al., 2005; Burnstock e Novak, 2013) que envolvem alterações do sistema purinérgico (Robson et al., 2006; Duarte et al., 2007).Estudos anteriores têm demonstrado que a eficiência dos receptores P2 esta diminuída em condições de DM (Ralevic e Burnstock, 1998; Sugiyama et al., 2004, 2006; Cieślak & Roszek, 2014). Assim, surge a hipótese de que o DM pode causar modificações no sistema purinérgico no cérebro, bem como, pode levar a um prejuízo das funções fisiológicas exercidas pelo ATP.

Durante nossos estudos, verificamos que a indução de hiperglicemia em peixe-zebra provoca uma diminuição na hidrólise de ATP, ADP e AMP pelas ectonucleotidases. Esses dados corroboram com a hipótese de que uma deficiência na sinalização pode contribuir para as disfunções da memória observadas no DM. Estudos anteriores já indicaram mudanças na eficiência dos receptores do tipo P2 no DM (Ralevic et al., 1993; Sugiyama et al., 2006). O ATP extracelular desempenha importante função no desenvolvimento do potencial de longa duração (LTP), através da ativação

dos receptores do tipo P2(Duarte et al., 2007), assim,a redução da hidrólise de ATP, ADP e AMP observados em nossos resultados, corroboram com a hipótese de que uma alteração na sinalização desse sistema esta ocorrendo e que possivelmente esteja contribuindo para as disfunções observadas.

Alguns estudos já demonstram os efeitos do DM sobre a atividade da ADA (Hoshino et al., 1994; Kurtul et al., 2004). A ADA é uma enzima que desamina irreversívelmente o nucleosídeo adenosina até inosina, contribuindo para a regulação da concentração intra e extracelular de adenosina(Latini e Pedata, 2001; Bonan, 2012). Nossos dadosdemonstraram que a atividade da ADA esta significativamente aumentada nos animais expostos a 111 mM de glicose, sugerindo uma redução dos níveis de adenosina na fenda sináptica.

A insulina esta envolvida na regulação da atividade da ADA em modelos animais de DM utilizando STZ (Rutkiewicz and Go´rski, 1990; Kurtul et al., 2004;Bopp et al., 2009; Lee et al., 2011). Calgaroto et al. (2015) tambémdemonstraramque a atividade da ADA foi aumentada em modelo animal com STZ, revelando que a administração de insulina é capaz de reduzir a atividade dessa enzima nos tecidos. Estes dados corroboram com a hipótese que a administração de insulina parece regular a atividade da ADA(Hoshino et al., 1994; Bottini e Gloria-Bottini, 1999; Kurtul et al., 2004; Vanitha Gowda et al., 2012). Nesse contexto, nós sugerimos que o aumento da atividade da ADA observado em nosso

estudo, pode ser explicado pela diminuição da atividade das NTPDases e pela resistência à insulina observada no modelo de hiperglicemia desta tese.

Nossos resultados ainda demonstraram que os receptores de adenosina tem a expressão gênica alterada quandoos animais foram expostos a altos níveis de glicose.Diversos estudos sugerem que condições de DM podem induzir mudanças adaptativas na densidade desses receptores no cérebro (Morrison et al., 1992; Dong et al., 2001; Artola et al., 2002; Duarte et al., 2006). Em roedores, a hiperglicemia foi atenuada pelo agonismo dos receptores A_1 e A_3(Németh et al., 2007).Donget al. (2001) demonstraram que a ativação de receptores de adenosina A_1 desenvolve uma função protetora contra a resistência à insulina, concordando com os achados de Faulhaber-Walter et al. (2011) que revelaram que a ausência do receptor A_1 prejudica o controle da glicemia em roedores.

De forma conjunta, os resultados apresentados nos tre s capítulos desta tese demonstram que as vias de sinalização colinérgica e purinérgica estão relacionadas com as alterações da hiperglicemia em peixe-zebra. Nossos resultados mostraram que a hiperglicemia é capaz de gerar déficits cognitivos significativos associados a redução dos níveis de ACh, assim como alterações na atividade das ectonucleotidases cerebrais, diminuindo a hidrólisede ATP, ADP e AMP.

A alteração demonstrada na atividade da ADA indica uma redução dos níveis de adenosina na fenda sináptica, sugerindo que a hiperglicemia diminui os níveis deste nucleosídeo, contribuindo assim para os déficits cognitivos apresentados no modelo.

Estes achados indicam o potencial do modelo de hiperglicemia em peixe-zebra para o estudo dos mecanismos envolvidos no DM e sua aplicabilidade para a avaliação de medicamentos para esta patologia. Adicionalmente, podemos contribuir para um maior entendimento das vias de sinalização cerebral envolvidas no declínio cognitivo desenvolvido nesta patologia, bem como, evidenciaro potencial farmacológico dos sistemas colinérgico e purinérgico nesta condic ão.

REFERÊNCIAS

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006;58:281–341.

Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. Trends Neurosci. 2009. p. 19–29.

Aguiree F, Brown A, Cho N, Dahlquist G. IDF Diabetes Atlas [Internet]. Guariguata L, Nolan T, Beagley J, Linnenkamp U, Jacqmain O, organizadores. International Diabetes Federation; 2013.

Allan Butterfield D, Di Domenico F, Barone E. Elevated risk of type 2 diabetes for development of Alzheimer disease: A key role for oxidative stress in brain. Biochim Biophys Acta. 2014;1842:1693–1706.

Alvarez Y, Chen K, Reynolds AL, Waghorne N, O'Connor JJ, Kennedy BN. Predominant cone photoreceptor dysfunction in a hyperglycaemic model of non-proliferative diabetic retinopathy. Dis Model Mech. 2010;3:236–245.

Amenta F, Tayebati SK. Pathways of acetylcholine synthesis, transport and release as targets for treatment of adult-onset cognitive dysfunction. Curr Med Chem. 2008;15:488–498.

American Diabetes A. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014a;37:81–90.

American Diabetes A. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014b;37 Suppl 1:S81–90.

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ. Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nat Neurosci. 2003;6:51–58.

Anderson CM, Xiong W, Geiger JD, Young JD, Cass CE, Baldwin SA, Parkinson FE. Distribution of equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporters (ENT1) in brain. J Neurochem. 1999;73:867–873.

Antonioli L, Blandizzi C, Pacher P, Haskó G. Immunity, inflammation and cancer: a leading role for adenosine. Nat Rev Cancer. 2013;13:842–57.

Appelbaum L, Skariah G, Mourrain P, Mignot E. Comparative expression of p2x receptors and ecto-nucleoside triphosphate diphosphohydrolase 3 in hypocretin and sensory neurons in zebrafish. Brain Res. 2007;1174:66–75.

Appleyard ME. Non-cholinergic functions of acetylcholinesterase. Biochem Soc Trans. 1994;22:749–755.

Artola A, Kamal A, Ramakers GMJ, Gardoni F, Di Luca M, Biessels GJ, Cattabeni F, Gispen WH. Synaptic plasticity in the diabetic brain: Advanced aging? Prog Brain Res. 2002. p. 305–314.

Atkinson M a, Eisenbarth GS, Michels AW. Type 1 diabetes. Lancet. Elsevier Ltd; 2014;383:69–82.

Aung MH, Park HN, Han MK, Obertone TS, Abey J, Aseem F, Thule PM, Iuvone PM, Pardue MT. Dopamine deficiency contributes to early visual dysfunction in a rodent model of type 1 diabetes. J Neurosci. 2014;34:726–36.

Avdesh A, Martin-Iverson MT, Mondal A, Chen M, Askraba S, Morgan N, Lardelli M, Groth DM, Verdile G, Martins RN. Evaluation of color preference in zebrafish for learning and memory. J Alzheimer's Dis. 2012;28:459–469.

Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature. 1990;347:151–156.

Bailey C, Turner R. Metformin. N Engl J Med. 1996;334:574–579.

Baker LD, Cross DJ, Minoshima S, Belongia D, Watson GS, Craft S. Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. Arch Neurol. 2011;68:51–57.

Baldwin SA, Mackey JR, Cass CE, Young JD. Nucleoside transporters: Molecular biology and implications for therapeutic development. Mol. Med. Today. 1999. p. 216–224.

Bandmann O, Burton EA. Genetic zebrafish models of neurodegenerative diseases. Neurobiol. Dis. 2010. p. 58–65.

Banks W a. The source of cerebral insulin. Eur J Pharmacol. 2004;490:5–12.

Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun E, Bedell JA, McPherson JD, Johnson SL. The syntenic relationship of the zebrafish and human genomes [1]. Genome Res. 2000. p. 1351–1358.

Bellamy L, Casas J-P, Hingorani AD, Williams D. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. Lancet. 2009;373:1773–1779.

Bergman RN, Phillips LS, Cobelli C. Physiologic Evaluation of Factors Controlling Glucose Tolerance in Man. J Clin Invest. 1981;68:1456–1467.

Best JD, Alderton WK. Zebrafish: An in vivo model for the study of neurological diseases. Neuropsychiatr. Dis. Treat. 2008. p. 567–576.

Biessels GJ, Deary IJ, Ryan CM. Cognition and diabetes: a lifespan perspective. Lancet Neurol. 2008;7:184–190.

Biessels GJ, Kappelle AC, Bravenboer B, Erkelens DW, Gispen WH. Cerebral function in diabetes mellitus. Diabetologia. 1994;37:643–650.

Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: A systematic review. Lancet Neurol. 2006. p. 64–74.

Biessels GJ, Van der Heide LP, Kamal A, Bleys RLAW, Gispen WH. Ageing and diabetes: Implications for brain function. Eur. J. Pharmacol. 2002. p. 1–14.

Blank M, Guerim LD, Cordeiro RF, Vianna MRM. A one-trial inhibitory avoidance task to zebrafish: Rapid acquisition of an NMDA-dependent long-term memory. Neurobiol Learn Mem. 2009;92:529–534.

Bloomgarden ZT. Cardiovascular disease in diabetes. Diabetes Care. 2008;31:1260–1266.

Boehmler W, Petko J, Woll M, Frey C, Thisse B, Thisse C, Canfield VA, Levenson R. Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expr Patterns. 2009;9:144–151.

Boison D. Adenosine augmentation therapy for epilepsy. Epilepsia. 2012;51:97.

Boison D, Shen H-Y. Adenosine kinase is a new therapeutic target to prevent ischemic neuronal death. Open Drug Discov J. 2010;2:108–118.

Bopp A, De Bona KS, Bellé LP, Moresco RN, Moretto MB. Syzygium cumini inhibits adenosine deaminase activity and reduces glucose levels in hyperglycemic patients. Fundam Clin Pharmacol. 2009;23:501–507.

Bottini E, Gloria-Bottini F. Adenosine deaminase and body mass index in non-insulin-dependent diabetes mellitus. Metabolism. 1999;48:949–951.

Brands AMA, Biessels GJ, de Haan EHF, Kappelle LJ, Kessels RPC. The effects of type 1 diabetes on cognitive performance: a meta-analysis. Diabetes Care. 2005;28:726–735.

Bree AJ, Puente EC, Daphna-Iken D, Fisher SJ. Diabetes increases brain damage caused by severe hypoglycemia. Am J Physiol Endocrinol Metab. 2009;297:E194–E201.

Briscoe VJ, Griffith ML, Davis SN. The role of glimepiride in the treatment of type 2 diabetes mellitus. Expert Opin Drug Metab Toxicol. 2010;6:225–235.

Brown DA. Acetylcholine. Br J Pharmacol. 2006;147 Suppl :S120–S126.

Bruce DG, Casey GP, Grange V, Clarnette RC, Almeida OP, Foster JK, Ives FJ, Davis TME. Cognitive impairment, physical disability and depressive symptoms in older diabetic patients: The Fremantle Cognition in Diabetes Study. Diabetes Res Clin Pract. 2003;61:59–67.

Burns JM, Donnelly JE, Anderson HS, Mayo MS, Spencer-Gardner L, Thomas G, Cronk BB, Haddad Z, Klima D, Hansen D, Brooks WM. Peripheral insulin and brain structure in early Alzheimer disease. Neurology. 2007;69:1094–1104.

Burnstock G. Purine and pyrimidine receptors. Cell. Mol. Life Sci. 2007. p. 1471–1483.

Burnstock G, Novak I. Purinergic signalling in the pancreas in health and disease. J. Endocrinol. 2012. p. 123–141.

Burnstock G, Novak I. Purinergic signalling and diabetes. Purinergic Signal. 2013;9:307–24.

Burnstock G, Verkhratsky A. Long-term (trophic) purinergic signalling: purinoceptors control cell proliferation, differentiation and death. Cell Death Dis. 2010;1:e9.

Bymaster FP, McKinzie DL, Felder CC, Wess J. Use of M1-M5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. Neurochem Res. 2003;28:437–442.

Caletti G, Almeida FB, Agnes G, Nin MS, Barros HMT, Gomez R. Antidepressant dose of taurine increases mRNA expression of GABAA receptor α2 subunit and BDNF in the hippocampus of diabetic rats. Behav Brain Res. 2015;283:11–15.

Calgaroto NS, da Costa P, Cardoso AM, Pereira LB, Vieira JM, Dalenogare D, Pelinson LP, Baldissarelli J, Morsch VM, Schetinger MRC. Vitamin D3 prevents the increase in ectonucleotidase activities and ameliorates lipid profile in type 1 diabetic rats. Mol Cell Biochem. 2015;

Capiotti KM, Fazenda L, Nazario LR, Menezes FP, Kist LW, Bogo MR, Da Silva RS, Wyse AT, Bonan CD. Arginine exposure alters ectonucleotidase activities and morphology of zebrafish larvae (Danio rerio). Int J Dev Neurosci. International Society for Developmental Neuroscience; 2013;31:75–81.

Capiotti KM, Menezes FP, Nazario LR, Pohlmann JB, de Oliveira GMT, Fazenda L, Bogo MR, Bonan CD, Da Silva RS. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (Danio rerio). Neurotoxicol Teratol. 2011;33:680–685.

Casey DA, Antimisiaris D, O'Brien J. Drugs for Alzheimer's disease: are they effective? P T. 2010;35:208–211.

Cass CE, Young JD, Baldwin SA. Recent advances in the molecular biology of nucleoside transporters of mammalian cells. Biochem Cell Biol. 1998;76:761–770.

Castillo PE, Carleton A, Vincent JD, Lledo PM. Multiple and opposing roles of cholinergic transmission in the main olfactory bulb. J Neurosci. 1999;19:9180–9191.

Cato MA, Mauras N, Ambrosino J, Bondurant A, Conrad AL, Kollman C, Cheng P, Beck RW, Ruedy KJ, Aye T, Reiss AL, White NH, Hershey T. Cognitive Functioning in Young Children with Type 1 Diabetes. J Int Neuropsychol Soc. 2014;20:238–247.

Chakrabarti S, Freedman JE. Dipyridamole, cerebrovascular disease, and the vasculature. Vascul Pharmacol. 2008;48:143–149.

Chen Z, Zhong C. Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. Prog Neurobiol. Elsevier Ltd; 2013;108:21–43.

Cholerton B, Baker LD, Craft S. Insulin, cognition, and dementia. Eur. J. Pharmacol. 2013. p. 170–179.

Ciruela F, Saura C, Canela EI, Mallol J, Lluis C, Franco R. Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett. 1996;380:219–223.

Clemente D, Porteros Á, Weruaga E, Alonso JR, Arenzana FJ, Aijón J, Arévalo R. Cholinergic elements in the zebrafish central nervous system: Histochemical and immunohistochemical analysis. J Comp Neurol. 2004;474:75–107.

Cognato G de P, Bortolotto JW, Blazina AR, Christoff RR, Lara DR, Vianna MR, Bonan CD. Y-Maze memory task in zebrafish (Danio rerio): The role of glutamatergic and cholinergic systems on the acquisition and consolidation periods. Neurobiol Learn Mem. 2012;98:321–328.

Cole AR, Astell A, Green C, Sutherland C. Molecular connexions between dementia and diabetes. Neurosci. Biobehav. Rev. 2007. p. 1046–1063.

Colwill RM, Raymond MP, Ferreira L, Escudero H. Visual discrimination learning in zebrafish (Danio rerio). Behav Processes. 2005;70:19–31.

Cox DJ, Kovatchev BP, Gonder-Frederick LA, Summers KH, McCall A, Grimm KJ, Clarke WL, Larke WILC. Relationships Between Hyperglycemia and Cognitive Performance Among Adults With Type 1 and Type 2 Diabetes. Diabetes Care 2005 p. 71–77.

Craft S, Cholerton B, Baker LD. Insulin and Alzheimer's disease: Untangling the web. Adv. Alzheimer's Dis. 2012. p. 263–275.

Craig PM, Moon TW. Fasted Zebrafish Mimic Genetic and Physiological Responses in Mammals: A Model for Obesity and Diabetes? Zebrafish. 2011. p. 109–117.

Csóka B, Koscsó B, Töro G, Kókai E, Virág L, Németh ZH, Pacher P, Bai P, Haskó G. A2B Adenosine receptors prevent insulin resistance by inhibiting adipose tissue inflammation via maintaining alternative macrophage activation. Diabetes. 2014;63:850–866.

Cukierman-Yaffe T, Gerstein HC, Williamson JD, Lazar RM, Lovato L, Miller ME, Coker LH, Murray A, Sullivan MD, Marcovina SM, Launer L. Relationship between baseline glycemic control and cognitive function in individuals with type 2 diabetes and other cardiovascular rIsk factors the action to control cardiovascular risk in diabetes-memory in diabetes (ACCORD-MIND) trial. Diabetes Care. 2009;32:221–226.

Cunha RA. Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem Res. 2001;26:979–991.

D. Bonan C. Ectonucleotidases and Nucleotide/Nucleoside Transporters as Pharmacological Targets for Neurological Disorders. CNS Neurol. Disord. - Drug Targets. 2012. p. 739–750.

Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol Toxicol. 2007;47:699–729.

Das A, Dikshit M, Nath C. Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. Life Sci. 2001;68:1545–55.

De la Monte SM, Tong M, Lester-Coll N, Plater M, Wands JR. Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease. J Alzheimers Dis. 2006;10:89–109.

De Mendonça A, Sebastião AM, Ribeiro JA. Adenosine: Does it have a neuroprotective role after all? Brain Res. Rev. 2000. p. 258–274.

De Rosa E, Hasselmo ME, Baxter MG. Contribution of the cholinergic basal forebrain to proactive interference from stored odor memories during associative learning in rats. Behav Neurosci. 2001;115:314–327.

Defronzo RA. From the triumvirate to the ominous octet: A new paradigm for the treatment of type 2 diabetes mellitus. Diabetes. 2009. p. 773–795.

DeFronzo RA, Eldor R, Abdul-Ghani M. Pathophysiologic approach to therapy in patients with newly diagnosed type 2 diabetes. Diabetes Care. 2013;36 Suppl 2:S127–38.

DeFronzo RA, Goodman AM. Efficacy of metformin in patients with non-insulin-dependent diabetes mellitus. The Multicenter Metformin Study Group. N. Engl. J. Med. 1995.

Descarries L, Gisiger V, Steriade M. Diffuse transmission by acetylcholine in the CNS. Prog. Neurobiol. 1997. p. 603–625.

Dias RB, Rombo DM, Ribeiro JA, Henley JM, Sebastião AM. Adenosine: Setting the stage for plasticity. Trends Neurosci. 2013. p. 248–257.

Diaz-Hernandez M, Cox JA, Migita K, Haines W, Egan TM, Voigt MM. Cloning and characterization of two novel zebrafish P2X receptor subunits. Biochem Biophys Res Commun. 2002;295:849–853.

Doherty GH. Obesity and the ageing brain: Could leptin play a role in neurodegeneration? Curr Gerontol Geriatr Res. 2011;2011:708154.

Dong Q, Ginsberg HN, Erlanger BF. Overexpression of the A1 adenosine receptor in adipose tissue protects mice from obesity-related insulin resistance. Diabetes, Obes Metab. 2001;3:360–366.

Duarte JM, Oliveira CR, Ambrósio a F, Cunha R a. Modification of adenosine A1 and A2A receptor density in the hippocampus of streptozotocin-induced diabetic rats. Neurochem Int. 2006;48:144–150.

Duarte JMN, Oses JP, Rodrigues RJ, Cunha R a. Modification of purinergic signaling in the hippocampus of streptozotocin-induced diabetic rats. Neuroscience. 2007;149:382–391.

Edwards JG, Greig A, Sakata Y, Elkin D, Michel WC. Cholinergic innervation of the zebrafish olfactory bulb. J Comp Neurol. 2007;504:631–645.

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff A V. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res. 2009;205:38–44.

Elo B, Villano CM, Govorko D, White L a. Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds. J Mol Endocrinol. 2007;38:433–440.

Elsherbiny N, Al-Gayyar M. Adenosine Receptors: New Therapeutic Targets for Inflammation in Diabetic Nephropathy. Inflamm Allergy-Drug Targets. 2013;12:153–161.

Erb L, Liao Z, Seye CI, Weisman GA. P2 receptors: Intracellular signaling. Pflugers Arch. Eur. J. Physiol. 2006. p. 552–562.

Erol A. An integrated and unifying hypothesis for the metabolic basis of sporadic Alzheimer's disease. J Alzheimers Dis. 2008;13:241–253.

Exalto LG, Whitmer RA, Kappele LJ, Biessels GJ. An update on type 2 diabetes, vascular dementia and Alzheimer's disease. Exp Gerontol. 2012;47:858–864.

Faulhaber-Walter R, Jou W, Mizel D, Li L, Zhang J, Kim SM, Huang Y, Chen M, Briggs JP, Gavrilova O, Schnermann JB. Impaired glucose

tolerance in the absence of adenosine A1 receptor signaling. Diabetes. 2011;60:2578–87.

Ferguson SM, Blakely RD. The choline transporter resurfaces: new roles for synaptic vesicles? Mol Interv. 2004;4:22–37.

Fernandes Y, Tran S, Abraham E, Gerlai R. Embryonic alcohol exposure impairs associative learning performance in adult zebrafish. Behav Brain Res. 2014;265:181–187.

Ferreira VA, Marques S, Campos BDE. AVANÇOS FARMACOLÓGICOS NO TRATAMENTO DO DIABETES TIPO 2 ADVANCES IN PHARMACOLOGICAL TREATMENT OF TYPE 2 DIABETES. 2014;8:72–78.

Fontbonne A, Berr C, Ducimetiere P, Alperovitch A. Changes in cognitive abilities over a 4-year period are unfavorably affected in elderly diabetic subjects: Results of the epidemiology of vascular aging study. Diabetes Care. 2001;24:366–370.

Franco R, Casadó V, Ciruela F, Saura C, Mallol J, Canela EI, Lluis C. Cell surface adenosine deaminase: Much more than an ectoenzyme. Prog. Neurobiol. 1997. p. 283–294.

Franco R, Valenzuela A, Lluis C, Blanco J. Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. Immunol. Rev. 1998. p. 27–42.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM. Adenosine and Brain Function. Int Rev Neurobiol. 2005;63:191–270.

Galasko D, Montine TJ. Biomarkers of oxidative damage and inflammation in Alzheimer's disease. Biomark Med. 2010;4:27–36.

Gasparini L, Xu H. Potential roles of insulin and IGF-1 in Alzheimer's disease. Trends Neurosci. 2003. p. 404–406.

Gerlai R. High-throughput behavioral screens: The first step towards finding genes involved in vertebrate brain function using zebrafish. Molecules. 2010;15:2609–2622.

Gerlai R. Associative Learning in Zebrafish (Danio rerio). Methods Cell Biol. 2011.

Ghatpande AS, Sivaraaman K, Vijayaraghavan S. Store calcium mediates cholinergic effects on mIPSCs in the rat main olfactory bulb. J Neurophysiol. 2006;95:1345–1355.

Girard J. Insulin's effect on the liver: "direct or indirect?" continues to be the question. J Clin Invest. 2006;116:302–304.

Gireesh G, Kumar TP, Mathew J, Paulose C. Enhanced muscarinic M1 receptor gene expression in the corpus striatum of streptozotocin-induced diabetic rats. J Biomed Sci. 2009;16:38.

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

Gleeson M, Connaughton V, Arneson LS. Induction of hyperglycaemia in zebrafish (Danio rerio) leads to morphological changes in the retina. Acta Diabetol. 2007;44:157–163.

Goldsmith P. Zebrafish as a pharmacological tool: The how, why and when. Curr. Opin. Pharmacol. 2004. p. 504–512.

Gorrell MD, Gysbers V, McCaughan GW. CD26: A multifunctional integral membrane and secreted protein of activated lymphocytes. Scand. J. Immunol. 2001. p. 249–264.

Gorus FK, Goubert P, Semakula C, Vandewalle CL, De Schepper J, Scheen A, Christie MR, Pipeleers DG. IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. Diabetologia. 1997;40:95–99.

Guo S. Linking genes to brain, behavior and neurological diseases: What can we learn from zebrafish? Genes, Brain Behav. 2004. p. 63–74.

Gupta D, Kurhe Y, Radhakrishnan M. Antidepressant effects of insulin in streptozotocin induced diabetic mice: Modulation of brain serotonin system. Physiol Behav. Elsevier B.V.; 2014;129C:73–78.

Guyot LL, Diaz FG, O'Regan MH, Song D, Phillis JW. The effect of streptozotocin-induced diabetes on the release of excitotoxic and other amino acids from the ischemic rat cerebral cortex. Neurosurgery. 2001;48:385–391.

Haider S, Ahmed S, Tabassum S, Memon Z, Ikram M, Haleem DJ. Streptozotocin-induced insulin deficiency leads to development of behavioral deficits in rats. Acta Neurol Belg. 2013;113:35–41.

Havrankova J, Schmechel D, Roth J, Brownstein M. Identification of insulin in rat brain. Proc Natl Acad Sci U S A. 1978;75:5737–5741.

Heller M, Hanahan DJ. Human erythrocyte membrane bound enzyme acetylcholinesterase. Biochim Biophys Acta. 1972;255:251–272.

Herrera C, Casadó V, Ciruela F, Schofield P, Mallol J, Lluis C, Franco R. Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol Pharmacol. 2001;59:127–134.

Hicks C, Sorocco D, Levin M. Automated analysis of behavior: A computer-controlled system for drug screening and the investigation of learning. J Neurobiol. 2006;66:977–990.

Hirschhorn R, Ratech H. Isozymes of adenosine deaminase. Isozymes Curr Top Biol Med Res. 1980;4:131–157.

Hirshberg B, Raz I. Impact of the U.S. food and drug administration cardiovascular assessment requirements on the development of novel antidiabetes drugs. Diabetes Care. 2011.

Hoshino T, Yamada K, Masuoka K, Tsuboi I, Itoh K, Nonaka K, Oizumi K. Elevated adenosine deaminase activity in the serum of patients with diabetes mellitus. Diabetes Res. Clin. Pract. 1994.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R,et al,. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496:498–503.

Hunsucker SA, Mitchell BS, Spychala J. The 5′-nucleotidases as regulators of nucleotide and drug metabolism. Pharmacol. Ther. 2005. p. 1–30.

Iwaki-Egawa S, Watanabe Y. Characterization and purification of adenosine deaminase 1 from human and chicken liver. Comp Biochem Physiol - B Biochem Mol Biol. 2002;133:173–182.

Jack R. Cooper, Robert H. Roth FEB. The Biochemical Basis of Neuropharmacology. 6th ed. Oxford University Press, USA; 1991.

Jensen CC, Cnop M, Hull RL, Fujimoto WY, Kahn SE. ??-cell function is a major contributor to oral glucose tolerance in high-risk relatives of four ethnic groups in the U.S. Diabetes. 2002;51:2170–2178.

Johnston-Cox H, Koupenova M, Yang D, Corkey B, Gokce N, Farb MG, LeBrasseur N, Ravid K. The A2b adenosine receptor modulates glucose homeostasis and obesity. PLoS One. 2012;7.

Jones LJ, Norton WHJ. Using zebrafish to uncover the genetic and neural basis of aggression, a frequent comorbid symptom of psychiatric disorders. Behav Brain Res. 2014;

Jorgens K, Hillebrands J-L, Hammes H-P, Kroll J. Zebrafish: A model for understanding diabetic complications. Exp Clin Endocrinol Diabetes. 2012;120:186–187.

Kanai F, Ito K, Todaka M, Hayashi H, Kamohara S, Ishii K, Okada T, Hazeki O, Ui M, Ebina Y. Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. Biochem Biophys Res Commun. 1993;195:762–768.

Kanaya AM, Barrett-Connor E, Gildengorin G, Yaffe K. Change in cognitive function by glucose tolerance status in older adults: a 4-year prospective study of the Rancho Bernardo study cohort. Arch Intern Med. 2004;164:1327–1333.

Kato F, Kawamura M, Shigetomi E, Tanaka J-I, Inoue K. ATP- and adenosine-mediated signaling in the central nervous system: synaptic purinoceptors: the stage for ATP to play its “dual-role”. J Pharmacol Sci. 2004;94:107–111.

Kautzky-Willer A, Prager R, Waldhäusl W, Pacini G, Thomaseth K, Wagner OF, Ulm M, Streli C, Ludvik B. Pronounced insulin resistance and inadequate β-cell secretion characterize lean gestational diabetes during and after pregnancy. Diabetes Care. 1997;20:1717–1723.

Kim B, Feldman EL. Insulin resistance in the nervous system. Trends Endocrinol Metab. Elsevier Ltd; 2012;23:133–141.

King AE, Ackley MA, Cass CE, Young JD, Baldwin SA. Nucleoside transporters: from scavengers to novel therapeutic targets. Trends Pharmacol. Sci. 2006. p. 416–425.

Kleinridders A, Ferris HA, Cai W, Kahn CR. Insulin action in brain regulates systemic metabolism and brain function. Diabetes. American Diabetes Association Inc.; 2014. p. 2232–2243.

Knapp K, Zebisch M, Pippel J, El-Tayeb A, Müller CE, Sträter N. Crystal structure of the human ecto-5′-nucleotidase (CD73): Insights into the regulation of purinergic signaling. Structure. 2012;20:2161–2173.

Knowles AF. The GDA1_CD39 superfamily: NTPDases with diverse functions. Purinergic Signal. 2011. p. 21–45.

Kodl CT, Seaquist ER. Cognitive dysfunction and diabetes mellitus. Endocr Rev. 2008;29:494–511.

Kogure K, Furones Alonso O. A pictorial representation of endogenous brain ATP by a bioluminescent method. Brain Res. 1978;154:273–284.

Krentz AJ, Bailey CJ. Oral antidiabetic agents: Current role in type 2 diabetes mellitus. Drugs. 2005. p. 385–411.

Kühl C. Etiology and pathogenesis of gestational diabetes. Diabetes Care. 1998;21 Suppl 2:B19–B26.

Kurtul N, Pence S, Akarsu E, Kocoglu H, Aksoy Y, Aksoy H. Adenosine deaminase activity in the serum of type 2 diabetic patients. Acta Medica (Hradec Kralove). 2004;47:33–35.

Laron Z. Insulin and the brain. Arch Physiol Biochem. 2009;115:112–116.

Latini S, Pedata F. Adenosine in the central nervous system: Release mechanisms and extracellular concentrations. J. Neurochem. 2001. p. 463–484.

Launer LJ, Miller ME, Williamson JD, Lazar RM, Gerstein HC, Murray AM, Sullivan M, Horowitz KR, Ding J, Marcovina S, Lovato LC, Lovato J, Margolis KL, O'Connor P, Lipkin EW, Hirsch J, Coker L, Maldjian J, Sunshine JL, Truwit C, Davatzikos C, Bryan RN. Effects of intensive glucose lowering on brain structure and function in people with type 2 diabetes (ACCORD MIND): A randomised open-label substudy. Lancet Neurol. 2011;10:969–977.

Lebastchi J, Herold KC. Immunologic and metabolic biomarkers of β-cell destruction in the diagnosis of type 1 diabetes. Cold Spring Harb Perspect Med. 2012;2.

Lee J-G, Kang DG, Yu JR, Kim Y, Kim J, Koh G, Lee D. Changes in Adenosine Deaminase Activity in Patients with Type 2 Diabetes Mellitus and Effect of DPP-4 Inhibitor Treatment on ADA Activity. Diabetes Metab J. 2011;35:149–158.

Lee JH, Choi Y, Jun C, Hong YS, Cho HB, Kim JE, Lyoo IK. Neurocognitive Changes and Their Neural Correlates in Patients with Type 2 Diabetes Mellitus. Endocrinol Metab. 2014;29:112.

Levetan C, Pozzilli P, Jovanovic L, Schatz D. Proposal for generating new beta cells in a muted immune environment for type 1 diabetes. Diabetes. Metab. Res. Rev. 2013. p. 604–606.

Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 2007;8:353–367.

Luchsinger JA. Type 2 diabetes and cognitive impairment: Linking mechanisms. J. Alzheimer's Dis. 2012.

Lunkes GIL, Lunkes DS, Morsch VM, Mazzanti CM, Morsch ALB, Miron VR, Schetinger MRC. NTPDase and 5'-nucleotidase activities in rats with alloxan-induced diabetes. Diabetes Res Clin Pract. 2004;65:1–6.

Maddison LA, Joest KE, Kammeyer RM, Chen W. Skeletal muscle insulin resistance in zebrafish induces alterations in β-cell number and glucose tolerance in an age and diet dependent manner. Am J Physiol - Endocrinol Metab. 2015;ajpendo.00441.2014.

Maelicke A, Hoeffle-Maas A, Ludwig J, Maus A, Samochocki M, Jordis U, Koepke AKE. Memogain is a galantamine pro-drug having dramatically reduced adverse effects and enhanced efficacy. J Mol Neurosci. 2010. p. 135–137.

Maier S a, Galellis JR, McDermid HE. Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol. 2005;61:776–94.

Mandarino LJ, Wright KS, Verity LS, Nichols J, Bell JM, Kolterman OG, Beck-Nielsen H. Effects of insulin infusion on human skeletal muscle pyruvate dehydrogenase, phosphofructokinase, and glycogen synthase. Evidence for their role in oxidative and nonoxidative glucose metabolism. J Clin Invest. 1987;80:655–663.

Margolis RU AN. Insulin in the cerebrospinal fluid. Nature. 1967;215:1375–6.

Matsuoka I, Ohkubo S. ATP- and adenosine-mediated signaling in the central nervous system: adenosine receptor activation by ATP through rapid and localized generation of adenosine by ecto-nucleotidases. J Pharmacol Sci. 2004;94:95–99.

Mayeda ER, Whitmer RA, Yaffe K. Diabetes and cognition. Clin Geriatr Med. 2015;31:101–15, ix.

McCall AL. The impact of diabetes on the CNS. Diabetes. 1992. p. 557–570.

McCall AL. Cerebral glucose metabolism in diabetes mellitus. Eur J Pharmacol. 2004;490:147–158.

McCrimmon RJ, Ryan CM, Frier BM. Diabetes and cognitive dysfunction. Lancet. 2012;379:2291–2299.

McGarry JD. Banting Lecture 2001:Dysregulation of Fatty Acid Metabolism in the Etiology of Type 2 Diabetes. Diabetes. 2002;51:7–18.

Mena NA, Sea EA, Lucia S. IDF Diabetes Atlas 6th edn. 2014 update. 2014;

Messier C. Impact of impaired glucose tolerance and type 2 diabetes on cognitive aging. Neurobiol Aging. 2005.

Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience. 2002;110:627–639.

Milech A, Angelucci AP, Golbert A, Carrilho AJF, Ramalho AC, Aguiar ACB de, Cardoso AMC, Seyffarth AS, Sachs A, Lerario AC, Pires AC, Ferreira AR, Chacra AR, Netto AP, Tschiedel B, Pereira BG, et al,. Diretrizes-Sbd-2015. 2015º ed. Oliveira JEP de, Vencio S, organizadores. Rio de Janeiro; 2015.

Miles, W. R. & Root HF. Psychologic tests applied to diabetic patients. Arch Intern Med. 1922;30:767–77.

Miron VR, Bauermann L, Morsch ALB, Zanin RF, Corrêa M, da Silva AC, Mazzanti C, Morsch VM, Lunkes GI, Schetinger MRC. Enhanced NTPDase and 5’-nucleotidase activities in diabetes mellitus and iron-overload model. Mol Cell Biochem. 2007;298:101–7.

Moreira PI, Cardoso SM, Pereira CM, Santos MS, Oliveira CR. Mitochondria as a Therapeutic Target in Alzheimer ’ s Disease and Diabetes. 2009;492–511.

Moretto MB, Lermen CL, Morsch VM, Bohrer D, Ineu RP, Da Silva AC, Balz D, Schetinger MRC. Effect of subchronic treatment with mercury chloride on NTPDase, 5′-nucleotidase and acetylcholinesterase from cerebral cortex of rats. J Trace Elem Med Biol. 2004;17:255–260.

Morrison PD, Mackinnon MW, Bartrup JT, Skett PG, Stone TW. Changes in adenosine sensitivity in the hippocampus of rats with streptozotocin-induced diabetes. Br J Pharmacol. 1992;105:1004–1008.

Müller G, Geisen K. Characterization of the molecular mode of action of the sulfonylurea, glimepiride, at adipocytes. Horm Metab Res. 1996;28:469–487.

Muller G, Satoh Y, Geisen K. Extrapancreatic effects of sulfonylureas - A comparison between glimepiride and conventional sulfonylureas. Diabetes Res Clin Pract. 1995.

Muller G, Wied S. The sulfonylurea drug, glimepiride, stimulates glucose transport, glucose transporter translocation, and dephosphorylation in insulin-resistant rat adipocytes in vitro. Diabetes. 1993;42:1852–1867.

Munshi M, Grande L, Hayes M, Ayres D, Suhl E, Capelson R, Lin S, Milberg W, Katie Weinger E. Cognitive dysfunction is associated with poor diabetes control in older adults. Diabetes Care. 2006;29:1794–1799.

Muntoni S, Muntoni S. Insulin resistance: Pathophysiology and rationale for treatment. Ann. Nutr. Metab. 2011. p. 25–36.

Murphy PC, Sillito AM. Cholinergic enhancement of direction selectivity in the visual cortex of the cat. Neuroscience. 1991;40:13–20.

Mushtaq N, Schmatz R, Pereira LB, Ahmad M, Stefanello N, Vieira JM, Abdalla F, Rodrigues M V, Baldissarelli J, Pelinson LP, Dalenogare DP, Reichert KP, Dutra EM, Mulinacci N, Innocenti M, Bellumori M, Morsch VM, Schetinger MR. Rosmarinic acid prevents lipid peroxidation and increase in acetylcholinesterase activity in brain of streptozotocin-induced diabetic rats. Cell Biochem Funct. 2014;32:287–93.

Nam Han Cho DW, Leonor Guariguata, Pablo Aschner Montoya N, Forouhi, Ian Hambleton, Rui Li AM, Jean Claude Mbanya, Ayesha Motala KMV, Narayan, Ambady Ramachandran W, Rathmann, Gojka Roglic, Jonathan Shaw M, Silink, D.R.R. Williams PZ. IDF DIABETES ATLAS IDF Diabetes Atlas – [Internet]. 6th ed. Leonor Guariguata, Tim Nolan JB, Ute Linnenkamp OJ, organizadores. 2013.

Nathan DM. Finding New Treatments for Diabetes — How Many, How Fast . . . How Good? N. Engl. J. Med. 2007. p. 437–440.

Németh ZH, Bleich D, Csóka B, Pacher P, Mabley JG, Himer L, Vizi ES, Deitch EA, Szabó C, Cronstein BN, Haskó G. Adenosine receptor activation ameliorates type 1 diabetes. FASEB J. 2007;21:2379–2388.

Nicole Campagnolo, Pablo Folha Dallapicola, Nádia Murussi, Luís Henrique Canani, Jorge Luiz Gross SPS. Aspectos clínicos e moleculares do Maturity-Onset Diabetes of the Young (MODY) / Clinical and molecular aspects of Maturity-Onset Diabetes of the Young (MODY). Rev HCPA Fac Med Univ Fed Rio Gd do Sul. 2004;24:51–59.

Oda Y. Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. Pathol Int. 1999;49:921–937.

Oka T, Nishimura Y, Zang L, Hirano M, Shimada Y, Wang Z, Umemoto N, Kuroyanagi J, Nishimura N, Tanaka T. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. BMC Physiol. 2010;10:21.

Olivera-Bravo S, Ivorra I, Morales A. Quaternary ammonium anticholinesterases have different effects on nicotinic receptors: is there a single binding site? J Mol Neurosci. 2006;30:205–208.

Olsen AS, Sarras MP, Intine R V. Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. Wound Repair Regen. 2010;18:532–42.

Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science. 1983;222:1337–1339.

Pardridge WM. Transport of insulin-related peptides and glucose across the blood-brain barrier. Ann N Y Acad Sci. 1993;692:126–137.

Park E, Lee Y, Kim Y, Lee CJ. Cholinergic modulation of neural activity in the telencephalon of the zebrafish. Neurosci Lett. 2008;439:79–83.

Pepeu G, Grazia M, Bracco L. Chemico-Biological Interactions Effect of cholinesterase inhibitors on attention. 2013;203:361–364.

Piato AL, Rosemberg DB, Capiotti KM, Siebel AM, Herrmann AP, Ghisleni G, Vianna MR, Bogo MR, Lara DR, Bonan CD. Acute restraint stress in zebrafish: Behavioral parameters and purinergic signaling. Neurochem Res. 2011;36:1876–1886.

Picciotto MR, Zoli M, Rimondini R, Léna C, Marubio LM, Pich EM, Fuxe K, Changeux JP. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature. 1998;391:173–177.

Pirola L, Johnston AM, Van Obberghen E. Modulation of insulin action. Diabetologia. 2004. p. 170–184.

Porte D, Baskin DG, Schwartz MW. Perspectives in Diabetes. 2005;54.

Powers JW, Mazilu JK, Lin S, McCabe ERB. The effects of hyperglycemia on adrenal cortex function and steroidogenesis in the zebrafish. Mol Genet Metab. Elsevier Inc.; 2010;101:421–422.

Prediger RDS, Batista LC, Takahashi RN. Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats: Involvement of adenosine A1 and A2A receptors. Neurobiol Aging. 2005;26:957–964.

Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, Goldstein DE, Rae PM. Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. J Immunol. 1994;152:3183–3188.

Ralevic V, Belai A, Burnstock G. Impaired sensory-motor nerve function in the isolated mesenteric arterial bed of streptozotocin-diabetic and ganglioside-treated streptozotocin-diabetic rats. Br J Pharmacol. 1993;110:1105–1111.

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998;50:413–492.

Ravel N, Elaagouby A, Gervais R. Scopolamine injection into the olfactory bulb impairs short-term olfactory memory in rats. Behav Neurosci. 1994;108:317–324.

Reagan LP. Diabetes as a chronic metabolic stressor: Causes, consequences and clinical complications. Exp Neurol. 2012;233:68–78.

Reaven GM, Thompson LW, Nahum D, Haskins E. Relationship between hyperglycemia and cognitive function in older NIDDM patients. Diabetes Care. 1990;13:16–21.

Renquist BJ, Zhang C, Williams SY, Cone RD. Development of an assay for high-throughput energy expenditure monitoring in the zebrafish. Zebrafish. 2013;10:343–52.

Ricatti MJ, Alfie LD, Lavoie Ế G, Sévigny J, Schwarzbaum PJ, Faillace MP. Immunocytochemical localization of NTPDases1 and 2 in the neural retina of mouse and zebrafish. Synapse. 2009;63:291–307.

Richardson JM, Balon TW, Treadway JL, Pessin JE. Differential regulation of glucose transporter activity and expression in red and white skeletal muscle. J Biol Chem. 1991;266:12690–12694.

Richardson SJ, Morgan NG, Foulis AK. Pancreatic pathology in type 1 diabetes mellitus. Endocr. Pathol. Humana Press Inc.; 2014. p. 80–92.

Richetti SK, Rosemberg DB, Ventura-Lima J, Monserrat JM, Bogo MR, Bonan CD. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. Neurotoxicology. 2011;32:116–122.

Rico EP, Senger MR, Fauth M da G, Dias RD, Bogo MR, Bonan CD. ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci. 2003;73:2071–2082.

Rinkwitz S, Mourrain P, Becker TS. Zebrafish: An integrative system for neurogenomics and neurosciences. Prog. Neurobiol. 2011. p. 231–243.

Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. J Alzheimers Dis. 2005;8:247–268.

Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal. 2006;2:409–430.

Rochette L, Zeller M, Cottin Y, Vergely C. Diabetes, oxidative stress and therapeutic strategies. Biochim Biophys Acta - Gen Subj. Elsevier; 2014;1840:2709–2729.

Romanowska M, Ostrowska M, Komoszyński MA. Adenosine ecto-deaminase (ecto-ADA) from porcine cerebral cortex synaptic membrane. Brain Res. 2007;1156:1–8.

Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD, Bogo MR. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (Danio rerio). Life Sci. 2007;81:1526–34.

Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD, Souza DO, Bonan CD, Bogo MR. NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol. 2010;155:230–40.

Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD, Souza DO. Kinetic characterization of adenosine deaminase activity in

zebrafish (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol. 2008;151:96–101.

Ruiz MA, Escriche M, Lluis C, Franco R, Martín M, Andrés A, Ros M. Adenosine A(1) receptor in cultured neurons from rat cerebral cortex: colocalization with adenosine deaminase. J Neurochem. 2000;75:656–664.

Ryan C, Vega A, Drash A. Cognitive deficits in adolescents who developed diabetes early in life. Pediatrics. 1985;75:921–927.

Ryan CM. Diabetes and brain damage: More (or less) than meets the eye? Diabetologia. 2006. p. 2229–2233.

Saisho Y. β-cell dysfunction: Its critical role in prevention and management of type 2 diabetes. World J Diabetes. 2015;6:109.

Salkovic-Petrisic M, Hoyer S. Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: An experimental approach. J. Neural Transm. Suppl. 2007. p. 217–233.

Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature. 2001;414:799–806.

Sánchez-Chávez G, Salceda R. Effect of streptozotocin-induced diabetes on activities of cholinesterases in the rat retina. IUBMB Life. 2000;49:283–287.

Sauer A, Brigida I, Carriglio N, Aiuti A. Autoimmune dysregulation and purine metabolism in adenosine deaminase deficiency. Front Immunol. 2012;3.

Schechter R, Whitmire J, Holtzclaw L, George M, Harlow R, Devaskar SU. Developmental regulation of insulin in the mammalian central nervous system. Brain Res. 1992;582:27–37.

Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Maldonado PA, Corrêa M, da Rosa CS, Becker L, Bagatini M, Gonçalves JF, Jaques JDS, Schetinger MR, Morsch VM. Ectonucleotidase and acetylcholinesterase activities in synaptosomes from the cerebral cortex of streptozotocin-induced diabetic rats and treated with resveratrol. Brain Res Bull. 2009a;80:371–376.

Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres JJ, Corr??a M, da Rosa MM, Rubin MA, Chitolina Schetinger MR, Morsch VM, Corrêa M, da Rosa MM, Rubin MA, Chitolina Schetinger MR, Morsch VM. Resveratrol prevents memory deficits and the increase in

acetylcholinesterase activity in streptozotocin-induced diabetic rats. Eur J Pharmacol. 2009b;610:42–48.

Schulingkamp R, Pagano T, Hung D, Raffa R. Insulin receptors and insulin action in the brain: review and clinical implications. Neurosci Biobehav Rev. 2000;24:855–872.

Schwartz EA, Koska J, Mullin MP, Syoufi I, Schwenke DC, Reaven PD. Exenatide suppresses postprandial elevations in lipids and lipoproteins in individuals with impaired glucose tolerance and recent onset type 2 diabetes mellitus. Atherosclerosis. 2010;212:217–222.

Senger MR, Rico EP, Dias RD, Bogo MR, Bonan CD. Ecto-5'-nucleotidase activity in brain membranes of zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol. 2004;139:203–207.

Sesti G. Pathophysiology of insulin resistance. Best Pract. Res. Clin. Endocrinol. Metab. 2006. p. 665–679.

Shafferman A, Kronman C, Flashner Y, Leitner M, Grosfeld H, Ordentlich A, Gozes Y, Cohen S, Ariel N, Barak D, Harel M, Silman I, Sussman JL, Velan B. Mutagenesis of human acetylcholinesterase. Identification of residues involved in catalytic activity and in polypeptide folding. J Biol Chem. 1992;267:17640–17648.

Shaked I, Zimmerman G, Soreq H. Stress-induced alternative splicing modulations in brain and periphery: Acetylcholinesterase as a case study. Ann N Y Acad Sci. 2008. p. 269–281.

Sherin a., Anu J, Peeyush KT, Smijin S, Anitha M, Roshni BT, Paulose CS. Cholinergic and GABAergic receptor functional deficit in the hippocampus of insulin-induced hypoglycemic and streptozotocin-induced diabetic rats. Neuroscience. Elsevier Inc.; 2012;202:69–76.

Shin JT, Fishman MC. From Zebrafish to human: modular medical models. Annu Rev Genomics Hum Genet. 2002;3:311–340.

Siebel AM, Rico EP, Capiotti KM, Piato AL, Cusinato CT, Franco TMA, Bogo MR, Bonan CD. In vitro effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (Danio rerio) brain. Toxicol Vitr. 2010;24:1279–1284.

Sinclair CJ, Powell AE, Xiong W, LaRivière CG, Baldwin SA, Cass CE, Young JD, Parkinson FE. Nucleoside transporter subtype expression: effects on potency of adenosine kinase inhibitors. Br J Pharmacol. 2001;134:1037–1044.

Sison M, Gerlai R. Behavioral performance altering effects of MK-801 in zebrafish (Danio rerio). Behav Brain Res. 2011;220:331–337.

Skaper SD, Debetto P, Giusti P. The P2X7 purinergic receptor: from physiology to neurological disorders. FASEB J. 2010;24:337–345.

Smith SR, Bai F, Charbonneau C, Janderová L, Argyropoulos G. A promoter genotype and oxidative stress potentially link resistin to human insulin resistance. Diabetes. 2003;52:1611–1618.

Sociedade Brasileira de Diabetes. Diretrizes da Sociedade Brasileira de Diabetes. Rio Janeiro. 2014.

Soreq H, Seidman S. Acetylcholinesterase--new roles for an old actor. Nat. Rev. Neurosci. 2001. p. 294–302.

Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, Danio rerio. Biol. Rev. 2008. p. 13–34.

Spence R, Magurran AE, Smith C. Spatial cognition in zebrafish: The role of strain and rearing environment. Anim Cogn. 2011;14:607–612.

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

Stewart AM, Braubach O, Spitsbergen J, Gerlai R, Kalueff A V. Zebrafish models for translational neuroscience research: From tank to bedside. Trends Neurosci. 2014. p. 264–278.

Stuart MJ, Baune BT. Depression and type 2 diabetes: Inflammatory mechanisms of a psychoneuroendocrine co-morbidity. Neurosci. Biobehav. Rev. 2012. p. 658–676.

Sugiyama T, Kobayshi M, Kawamura H, Li Q, Puro DG. Enhancement of P2X7-induced pore formation and apoptosis: An early effect of diabetes on the retinal microvasculature. Investig Ophthalmol Vis Sci. 2004;45:1026–1032.

Sugiyama T, Oku H, Komori A, Ikeda T. Effect of P2X7 receptor activation on the retinal blood velocity of diabetic rabbits. Arch Ophthalmol. 2006;124:1143–1149.

Sumitani S, Morita S, Deguchi R, Hirai K, Mukai K, Utsu Y, Miki S, Sato B, Nakamura H, Kasayama S, Koga M. Metformin decreases glycated albumin

to glycated haemoglobin ratio in patients with newly diagnosed type 2 diabetes. Ann Clin Biochem. SAGE Publications; 2014;0004563214522984.

Surprenant A, North RA. Signaling at purinergic P2X receptors. Annu Rev Physiol. 2009;71:333–359.

Szablewski L. Role of immune system in type 1 diabetes mellitus pathogenesis. Int Immunopharmacol. Elsevier B.V.; 2014;22:182–191.

Szegletes T, Mallender WD, Thomas PJ, Rosenberry TL. Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis. Substrate inhibition arises as a secondary effect. Biochemistry. 1999;38:122–133.

Taylor P, Radić Z. The cholinesterases: from genes to proteins. Annu Rev Pharmacol Toxicol. 1994;34:281–320.

Tonoli C, Heyman E, Roelands B, Pattyn N, Buyse L, Piacentini MF, Berthoin S, Meeusen R. Type 1 diabetes-associated cognitive decline: A meta-analysis and update of the current literature 1型糖尿病相▪的认知能力下降：一项对最新文献的 meta 分析. J Diabetes. 2014;6:499–513.

Trincavelli ML, Daniele S, Martini C. Adenosine receptors: what we know and what we are learning. Curr Top Med Chem. 2010;10:860–877.

Tripathi BK, Srivastava AK. Diabetes mellitus: complications and therapeutics. Med Sci Monit. 2006;12:RA130–A147.

Tsuda K. Inosine, calcium channels, and neuroprotection against ischemic brain injury. Stroke. 2005. p. 1823; author reply 1823.

Umegaki H. Neurocognitive dysfunction in old diabetes: management and treatment. Adv Exp Med Biol. 2012;771:465–70.

Umegaki H, Kawamura T, Mogi N, Umemura T, Kanai A, Sano T. Glucose control levels, ischaemic brain lesions, and hyperinsulinaemia were associated with cognitive dysfunction in diabetic elderly. Age Ageing. 2008. p. 458–461.

Vanitha Gowda MN, Vasudha KC, Reshma S, Sujatha KJ. Serum Adenosine deaminase activity in type 2 diabetes mellitus patients. Int J Diabetes Dev Ctries. 2012;32:176–181.

Veeraswamy S, Vijayam B, Gupta VK, Kapur A. Gestational diabetes: The public health relevance and approach. Diabetes Res. Clin. Pract. 2012. p. 350–358.

Vincent C, Hall PA. Executive Function in Adults With Type 2 Diabetes: A Meta-Analytic Review. Psychosom Med. 2014;

Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. Clin. Sci. 2011. p. 253–270.

Visser F, Zhang J, Raborn RT, Baldwin SA, Young JD, Cass CE. Residue 33 of human equilibrative nucleoside transporter 2 is a functionally important component of both the dipyridamole and nucleoside binding sites. Mol Pharmacol. 2005;67:1291–1298.

Wardas J. Neuroprotective role of adenosine in the CNS. Pol J Pharmacol. 2002;54:313–326.

Watson GS, Craft S. Modulation of memory by insulin and glucose: Neuropsychological observations in Alzheimer's disease. Eur. J. Pharmacol. 2004. p. 97–113.

Wess J. Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. Annu Rev Pharmacol Toxicol. 2004;44:423–450.

Whitmer RA. Type 2 diabetes and risk of cognitive impairment and dementia. Curr. Neurol. Neurosci. Rep. 2007. p. 373–380.

Wilkinson DG, Francis PT, Schwam E, Payne-Parrish J. Cholinesterase inhibitors used in the treatment of Alzheimer's disease: the relationship between pharmacological effects and clinical efficacy. Drugs Aging. 2004;21:453–478.

Yaffe K, Falvey C, Hamilton N, Schwartz A V, Simonsick EM, Satterfield S, Cauley JA, Rosano C, Launer LJ, Strotmeyer ES, Harris TB. Diabetes, glucose control, and 9-year cognitive decline among older adults without dementia. Arch Neurol. 2012;69:1170–5.

Yaffe K, Lindquist K, Schwartz A V., Vitartas C, Vittinghoff E, Satterfield S, Simonsick EM, Launer L, Rosano C, Cauley JA, Harris T. Advanced glycation end product level, diabetes, and accelerated cognitive aging. Neurology. 2011;77:1351–1356.

Yamagishi S. Potential Clinical Utility of Advanced Glycation End Product Cross-Link Breakers in Age- and Diabetes-Associated Disorders. Rejuvenation Res. 2012. p. 120924061356008.

Yamato T, Misumi Y, Yamasaki S, Kino M, Aomine M. Diabetes mellitus decreases hippocampal release of neurotransmitters: an in vivo microdialysis study of awake, freely moving rats. Diabetes Nutr Metab. 2004;17:128–136.

Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim. Biophys. Acta - Mol. Cell Res. 2008. p. 673–694.

Zala SM, Määttänen I. Social learning of an associative foraging task in zebrafish. Naturwissenschaften. 2013;100:469–472.

Zavialov A V, Engström A. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. Biochem J. 2005;391:51–7.

Zhang J, Takeda M. Molecular characterization of MbADGF, a novel member of the adenosine deaminase-related growth factor in the cabbage armyworm, Mamestra brassicae: The functional roles in the midgut cell proliferation. Insect Mol Biol. 2007;16:351–360.

Zimmermann H. 5’-Nucleotidase: molecular structure and functional aspects. Biochem J. 1992;285:345–365.

Zimmermann H. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog. Neurobiol. 1996. p. 589–618.

Zimmermann H. Ectonucleotidases: Some recent developments and a note on nomenclature. Drug Dev Res. 2001. p. 44–56.

Zimmermann H, Zebisch M, Sträter N. Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal. 2012;8:437–502.

ANEXOS – Parecer de aprovação do projeto de pesquisa pelo CEUA