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Vanessa Angonesi Zborowski

Depressão e prejuízo de memória induzidos pela administração de estreptozotocina em camundongos: efeitos terapêuticos do disseleneto de *p*-clorodifenila

Santa Maria, RS
2020

Vanessa Angonesi Zborowski

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Tese de doutorado apresentado ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Doutor em Bioquímica Toxicológica**.

Orientador: Dra. Cristina Wayne Nogueira

Santa Maria, RS
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Vanessa Angonesi Zborowski

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“Só a experiência própria é capaz de tornar sábio o ser humano.”

Sigmund Freud

RESUMO

DEPRESSÃO E PREJUÍZO DE MEMÓRIA INDUZIDOS PELA ADMINISTRAÇÃO DE ESTREPTOZOTOCINA EM CAMUNDONGOS: EFEITOS TERAPÊUTICOS DO DISSELENETO DE *p*-CLORODIFENILA

AUTORA: Vanessa Angonesi Zborowski
ORIENTADORA: Cristina Wayne Nogueira

A pré-diabetes e diabetes são doenças metabólicas de etiopatologias heterogêneas incluindo defeitos na secreção e ação da insulina (ou em ambos), presença de hiperglicemia, alteração em testes de tolerância a glicose e a insulina, distúrbios do metabolismo de carboidratos, gorduras e proteínas, entre outros. Além dos efeitos metabólicos, são conhecidas as complicações dessas doenças no sistema nervoso central (SNC), tais como o prejuízo na memória, a alteração de humor e a depressão. Quando testado em modelos animais, o composto orgânico de selênio, disseleneto de *p*-clorodifenila (p-ClPhSe)$_2$, apresenta efeitos positivos sobre a memória e o fenótipo tipo-depressivo. Nesse sentido, o objetivo desta tese foi avaliar os efeitos da diabetes sob administração de estreptozotocina (ETZ) no prejuízo da memória e no fenótipo do tipo depressivo elucidando os possíveis mecanismos envolvidos em camundongos Swiss machos. Bem como investigar a ação neuroprotetora do (*p*-ClPhSe)$_2$ em camundongos diabéticos. Os resultados do **artigo 1** demonstraram que o tratamento com (*p*-ClPhSe)$_2$, na dose de 5mg/kg por 7 dias, apresentou efeito contra o dano de memória, demonstrado nos testes de reconhecimento e localização do objeto em animais diabéticos. Embora esse regime de tratamento tenha reduzido a hiperglicemia no dia 21 do protocolo experimental, o tratamento com o composto não reverteu todos os parâmetros metabólicos característicos do fenótipo diabético nos animais. Em contrapartida, apresentou efeitos positivos na modulação da via hipocampal do BDNF/TrkB e neuroproteção, contribuindo para os efeitos promissores do composto nos testes comportamentais. Com base nesses achados benéficos do composto sobre proteínas do hipocampo, investigou-se os efeitos do (*p*-ClPhSe)2 sobre o comportamento tipo-depressivo induzido por ETZ. Assim, os resultados do **artigo 2** mostraram que o composto na dose de 5 mg/kg por 7 dias apresentou efeitos do tipo antidepressivo nos testes de suspensão da cauda e de nado forçado nos animais diabéticos. Além disso, os camundongos diabéticos apresentaram aumento do estresse oxidativo, redução nos níveis das proteínas Keap1/Nrf2/HO-1, redução dos níveis de receptores de glicocorticoides no córtex cerebral, bem como o aumento do peso relativo da glândula adrenal. Interessantemente, após o tratamento com o (*p*-ClPhSe)$_2$ foi observado o efeito antioxidante deste composto orgânico de selênio e a modulação da via de sinalização Keap1/Nrf2/HO-1 no córtex cerebral de animais diabéticos. Entretanto, o composto não foi efetivo sobre os parâmetros do eixo hipotálamo-pituitária-adrenal. Ainda, com intuito de seguir as diretrizes do 3R's, aqueles animais, oriundos dos dois estudos acima descritos, que não apresentaram hiperglicemia, foram caracterizados como pré-diabéticos. De fato, estes camundongos apresentaram alterações metabólicas como moderada hiperglicemia, redução do peso corporal e da gordura epididimal e aumento da área sobre a curva dos testes de tolerância a glicose e a insulina quando comparados aos animais controle. Nesse contexto, o corpo de resultados encontrados no **manuscrito 1** revelou que os animais pré-diabéticos apresentaram prejuízo de memória e fenótipo do tipo depressivo. Esses resultados foram acompanhados pela redução dos níveis hipocampais de proteínas da via da insulina (IRS-1/Akt/GLUT4) e do BDNF/CREB. Em conjunto, os resultados desta tese contribuem para a compreensão dos mecanismos envolvidos na condição de pré-diabetes e no diabetes em um modelo experimental induzido com ETZ, assim como revela os efeitos terapêuticos do (*p*-ClPhSe)$_2$ no dano de memória e no fenótipo do tipo depressivo e sua ação em proteínas do SNC, indicando que este composto pode ser um candidato a alternativa terapêutica na díade memória-depressão associado ao diabetes.

Palavras-chave: Selênio. Memória. Depressão. ETZ. Estresse oxidativo. BDNF. Nrf2. GLUT4.

ABSTRACT

STREPTOZOTOCIN-INDUCED DEPRESSION AND MEMORY IMPAIRMENT IN MICE: THERAPEUTIC EFFECTS OF *p*-DICHLORODIPHENYL DISELENIDE

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Pre-diabetes and diabetes are metabolic diseases of heterogeneous etiopathology including defects in the secretion and action of insulin, or both, the presence of hyperglycemia, glucose and insulin intolerance, disorders of carbohydrate, fat, and protein metabolism, among others. In addition to the metabolic effects, complications of these diseases in the central nervous system (CNS) are known, such as impaired memory, mood disorders, and depression. In animal models, p-dichlorodiphenyl diselenide (*p*-ClPhSe)$_2$ has positive effects on memory impairment and the depressive-like phenotype. In this sense, this thesis aimed to evaluate the effects of streptozotocin (STZ) administration on memory impairment and the depressive-like phenotype, elucidating the possible mechanisms involved in male Swiss mice. Moreover, the neuroprotective action of (*p*-ClPhSe)$_2$ in diabetic mice was investigated. **Paper 1** demonstrated that (*p*-ClPhSe)$_2$ treatment, at a dose of 5 mg/kg for 7 days, was effective against memory impairment, which was demonstrated in the object recognition and object location tests in diabetic animals. Although this treatment regimen reduced hyperglycemia on day 21 of the experimental protocol, (*p*-ClPhSe)$_2$ did not reverse all metabolic parameters characteristics of the diabetic phenotype in mice. On the other hand, (*p*-ClPhSe)$_2$ had positive effects on the modulation of the hippocampal BDNF/TrkB pathway and neuroprotection, contributing to the promising effects of this compound in behavioral tests. Based on the (*p*-ClPhSe)$_2$ beneficial effects on hippocampal proteins, other possible effects of (*p*-ClPhSe)$_2$ concerning the depressive-like phenotype associated with diabetes were investigated. Thus, **paper 2** showed that (*p*-ClPhSe)$_2$ at a dose of 5 mg/kg for 7 days elicited an antidepressant-like effect on tail suspension and forced swimming tests in diabetic mice. Besides, diabetic mice showed oxidative stress and reduced levels of Keap1/Nrf2/HO-1 proteins in the cerebral cortex, as well as an increase in the adrenal gland relative weight and a reduction in the levels of glucocorticoid receptors in the cerebral cortex. The antioxidant effect of (*p*-ClPhSe)$_2$ and the modulation of the Keap1/Nrf2/HO-1 signaling pathway in the cerebral cortex of mice were also demonstrated. However, the compound was not effective against the parameters determined to characterize the hypothalamic-pituitary-adrenal axis. Besides, to preserve the 3R's guidelines, those animals, from the protocols described above, that did not present hyperglycemia, after intraperitoneal injection of STZ, were evaluated and characterized as pre-diabetic. These mice showed metabolic alterations such as mild hyperglycemia, reduced body weight and epididymal fat, and increased area under the curve in glucose and insulin tolerance tests when compared to control animals. In this context, the body of **manuscript 1** results revealed that pre-diabetic mice had impairment of memory and depressive-like phenotype and that these behaviors were accompanied by the reduction in the hippocampal levels of proteins of the insulin (IRS-1/Akt/GLUT4) and BDNF/CREB pathways. This thesis contributes to our understanding of the mechanisms involved in the pre-diabetes and diabetes in an experimental model induced by STZ. Moreover, it reveals the (*p*-ClPhSe)$_2$ promising effects on memory impairment and depressive-like phenotype and its actions on hippocampal and cerebral cortical proteins, indicating that this compound may be an alternative to treat the memory-depression dyad associated with diabetes.

Keywords: Selenium. Memory. Depression. STZ. Oxidative stress. BDNF. Nrf2. GLUT4.

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LISTA DE ABREVIATURAS E SIGLAS

AAP	Associação Americana de Psiquiatria
ACTH	Hormônio adrenocorticotrófico
ADA	Associação de Diabetes Americana
ADP	Adenosina difosfato
AGE	Produtos finais da glicação avançada
Akt	Proteina quinase B
AMP	Adenosina monofosfato
AMPK	Proteína quinase ativada por AMP
ANVISA	Agência Nacional de Vigilância Sanitária
ATP	Adenosina trifosfato
BB-DP	*Biobreeding - Diabetes Prone*
BDNF	Fator Neurotrófico do Encéfalo
CAMKII	Proteína quinase II dependente de Ca2+/calmodulina
CREB	Proteína de Ligação ao Elemento de Resposta ao cAMP
CRH	Hormônio liberador de corticotropina
DNA	Ácido desoxirribonucleico
ERA	Elementos de resposta antioxidante
ERK	Quinase regulada por sinal extracelular
EROS	Espécies Reativas de Oxigênio
ETZ	Estreptozotocina
GC	Glicocorticoide
GK	*Goto-Kakizaki*
GLUT2	Transportador de Glicose 2
GLUT4	Transportador de Glicose 4
GPx	Glutationa peroxidase
GR	Receptor de Glicocorticoide
GST	Glutationa-S-transferase
HO-1	Hemeoxigenase-1
HPA	Hipotálamo-pituitária-adrenal
Keap1	Proteína 1 associada à ECH tipo Kelch
LAM	Teste do labirinto aquático de *Morris*
LTP	Potenciação de longa duração
MAPK	Proteína-quinases ativadas por mitógenos
MR	Receptor de mineralocorticoide
NADPH	Fosfato de dinucleotídeo de adenina e nicotinamida
Nrf2	Fator nuclear eritroide 2-relacionado ao fator 2
OMS	Organização Mundial da Saúde
PCL-γ	Fosfolipase C Gamma
PI3K	Fosfoinositol-3-quinase
SBD	Sociedade Brasileira de Diabetes
SOD	Superóxido Dismutase
TrkB	Receptor de Tirosina Quinase B
Vs	Vesículas
ZDF	Zucker Diabetic Fatty

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1 INTRODUÇÃO

1.1 (PRÉ-)DIABETES

1.1.1 Histórico

Em 1889, von Mering e Minkowski, a partir de experimentos em cães, descobriram que a remoção do pâncreas causava sintomas semelhantes aos que apresentavam os pacientes que desconheciam a origem da doença diabetes (MINKOWSKI; VON MERING, 1890). Banting, Best e Collip, em 1921, em seus elegantes experimentos com animais, usaram extratos de insulina canina para reverter o quadro dos sintomas da doença, e concluíram que a deficiência de insulina era a causa do diabetes (BANTING et al., 1922). Nesse mesmo ano, Leonard Thompson foi o primeiro paciente a receber insulina e logo após a injeção, esforços foram feitos para aperfeiçoar o processo de extração, e a comercialização da insulina foi iniciada. Então, em 1923 a insulina passou a ser comercialmente disponibilizada nos Estados Unidos (WHITE, 2014).

Após essas descobertas, os estudos relacionados à doença foram iniciados e as definições e o refinamento dos conceitos de pré-diabetes e diabetes foram se desenvolvendo. Nesse contexto, termos variáveis foram utilizados para se referir a pessoas com níveis plasmáticos de glicose intermediários entre aquelas consideradas “normais” e aquelas na faixa de “diabetes”, os quais hoje designamos de pré-diabetes (ECHOUFFO-TCHEUGUI et al., 2018; FAJANS, 1973; JACKSON, 1959).

1.1.2 Diagnóstico e Classificação

Em 1979, o *National Diabetes Data Group* (NDDG) introduziu pela primeira vez o conceito de um estado metabólico intermediário entre a homeostase normal da glicose e o diabetes, chamado intolerância à glicose (BUYSSCHAERT; BERGMAN, 2011). Indivíduos com intolerância à glicose não atendiam aos critérios para o diagnóstico de diabetes, mas apresentavam níveis de glicose superiores aos considerados normais. Em 1997, o Comitê de Especialistas em Diagnóstico e Classificação do Diabetes Mellitus estendeu o conceito de pré-diabetes (ASSOCIATION, 1997), desde então, pacientes com pré-diabetes são definidos pela alteração dos níveis de glicemia de jejum (100 mg/dl- 125 mg/dl) e/ou prejuízo na tolerância a glicose e/ou A1c-hemoglobina glicada (5,7-6,4%). Essas alterações foram referidas como pré-diabetes e são consideradas fatores de risco substanciais para a progressão ao diabetes.

Estudos indicaram que algumas condições microvasculares, macrovasculares, doença periodontal, disfunção cognitiva, alterações da pressão arterial, doença hepática gordurosa e câncer podem estar presentes no início da desregulação glicêmica (BUYSSCHAERT et al., 2015; TABAK et al., 2012). O pré-diabetes não deve ser entendido como uma entidade clínica por si só, mas uma forma de aumentar o risco de desenvolvimento de diabetes e outras doenças associadas a esta (GARBER et al., 2008).

Já o termo diabetes descreve o grupo de distúrbios metabólicos caracterizados e identificados pela presença de hiperglicemia. A etiopatologia heterogênea inclui defeitos na secreção de insulina, ação da insulina, ou ambos, e também distúrbios do metabolismo de carboidratos, gorduras e proteínas (AAD, 2019). Os efeitos específicos em longo prazo do diabetes incluem retinopatia, nefropatia e neuropatia, entre outras complicações (AAD, 2019). O diabetes tem sintomas característicos como sede, poliúria, polifagia e perda de peso. As manifestações clínicas mais graves são a cetoacidose ou um estado hiperosmolar que pode levar à desidratação, coma e, na ausência de tratamento efetivo, à morte (WHO, 2019).

Atualmente, são recomendados quatro testes de diagnóstico para pré ou diabetes, incluindo a dosagem de glicemia de jejum; de glicemia após um teste de tolerância oral à glicose (pós-carga de 75 g por 2 horas); de HbA1c; e uma glicemia aleatória na presença de sinais e sintomas de diabetes (AAD, 2019). A figura 1 a seguir demonstra os níveis limítrofes para o diagnóstico de cada estágio da doença de acordo com a Sociedade Brasileira de Diabetes de 2018.

Figura 1. Critérios laboratoriais para diagnóstico de normoglicemia, pré-diabetes e diabetes, adotados pela SBD de 2018.

	Glicose de jejum (mg/dL)	**Glicose 2 h após sobrecarga com 75g de glicose (mg/dL)**	**Glicose ao acaso**	**HbA1c (%)**	**Observações**
Normoglicemia	< 100	< 140		< 5,7	OMS emprega valor de corte de 110mg/dL para normalidade da glicose de jejum.
Pré-diabetes ou risco aumentado para Diabetes	≥ 100 e < 126	≥ 140 e < 200		≥ 5,7 e < 6,5	Positividade de qualquer dos parâmetros confirma diganóstico de pré-diabetes.
Diabetes estabelecido	≥ 126	≥ 200	≥ 200 com sintomas inequívocos de hiperglicemia	≥ 6,5	Positividade de qualquer dos parâmetros confirma diganóstico de diabetes.

Fonte: Retirado das diretrizes da SBD 2017-2018.

Além disso, segundo a *ADA* de 2019, o diabetes pode ser classificado nas seguintes categorias gerais: Diabetes tipo 1 (devido à destruição de células por ação autoimune, levando geralmente à deficiência absoluta de insulina); Diabetes tipo 2 (devido a uma perda progressiva da secreção de insulina das células beta pancreáticas com frequência no contexto da resistência à insulina); Diabetes mellitus gestacional (diabetes diagnosticado no segundo ou terceiro trimestre de gravidez) e tipos específicos de diabetes devido a outras causas, por exemplo, síndromes monogênicas de diabetes, doenças do pâncreas exócrino (como fibrose cística e pancreatite) e diabetes induzido por produtos químicos (como com o uso de glicocorticoide, no tratamento do HIV ou após transplante de órgãos).

1.1.3 Epidemiologia

A transição do pré-diabetes para o diabetes pode levar alguns anos, mas também pode ocorrer de maneira rápida (FERRANNINI et al., 2004). Estudos epidemiológicos apontam que a maioria dos indivíduos (até 70%) com pré-diabetes, eventualmente, desenvolvem diabetes (ARODA; RATNER, 2008). O risco médio de desenvolver diabetes é de cerca de 5% a 10% ao ano em indivíduos com glicemia de jejum alterada e tolerância a glicose prejudicada, em comparação com aproximadamente 0,7% ao ano em indivíduos normoglicêmicos (NATHAN et al., 2007).

O número de pessoas com diabetes tem aumentando constantemente, subindo de 4,7% em 1980 para 8,5% em 2014, com a OMS estimando a existência de 422 milhões de adultos com diabetes em todo o mundo em 2014 (WHO, 2017). Nesse contexto, sem quaisquer intervenções para interromper o aumento do diabetes, haverá pelo menos 629 milhões de pessoas vivendo com diabetes até 2045 no mundo (CHO et al., 2018). Atualmente, no Brasil, há mais de 13 milhões de pessoas vivendo com diabetes, o que representa 6,9% da população. E esse número está crescendo, visto que, em alguns casos, o diagnóstico é demorado, favorecendo o aparecimento de complicações (SBD, 2017).

O aumento da prevalência do diabetes está associado a diversos fatores, tais como: rápida urbanização, transição epidemiológica, transição nutricional, maior frequência de estilo de vida sedentário e de excesso de peso, crescimento e envelhecimento populacional e, também, a maior sobrevida dos indivíduos com diabetes (WHO, 2017). A figura 2 a seguir, demonstra uma estimativa da doença para 2040 em 4 países com maiores índices de pessoas com diabetes.

Figura 2. Relação dos 10 países com maior número de pessoas com diabetes (20 a 79 anos) em 2015 e projeções para 2040.

Posição	País	Número de pessoas com diabetes (2015)	Posição	País	Número de pessoas com diabetes (2040)
1	China	109,6 milhões	1	China	150,7 milhões
2	Índia	69,2 milhões	2	Índia	123,5 milhões
3	Estados Unidos da América	29,3 milhões	3	Estados Unidos da América	35,1 milhões
4	Brasil	14,3 milhões	4	Brasil	23,3 milhões

Fonte: Federação Internacional de Diabetes de 2015.

1.2 MODELOS ANIMAIS PARA O ESTUDO DO (PRÉ-)DIABETES

1.2.1. Indutores químicos, genéticos e outros

A deficiência na produção de insulina característica do diabetes tipo 1 é obtida experimentalmente por diferentes mecanismos, desde a indução química nas células beta pancreáticas até roedores reprodutores que desenvolvem espontaneamente diabetes autoimune (AL-AWAR et al., 2016). A progressão das complicações diabéticas é afetada por vários fatores, incluindo a obesidade, a resistência à insulina, a hiperglicemia e a hiperlipidemia (CALCUTT et al., 2009).

A abordagem química da indução do diabetes tipo 1 em roedores envolve a administração de produtos químicos para destruir, especificamente, as células beta de Langerhans do pâncreas. Inicialmente, o aloxano foi utilizado como agente beta-citotóxico para induzir sintomas de diabetes em camundongos e ratos (ACHARJEE et al., 2013). No entanto, a toxicidade renal com aloxano foi um fator indesejável recorrente. Além disso, os camundongos tratados com aloxano podem apresentar recuperação espontânea de uma condição diabética crônica, o que torna extremamente difícil a interpretação da eficácia terapêutica de moléculas testadas como candidatas a novos medicamentos (ACHARJEE et al., 2013).

A ação da estreptozotocina (ETZ) é considerada semelhante à do aloxano, ambos os compostos são captados pelos transportadores de glicose, GLUT2, agem intracelularmente, e são seletivamente tóxicos para as células β produtoras de insulina (GRIEB, 2016). A alcalinização do DNA celular e subsequente ativação da poli-ADP ribose sintetase causam depleção rápida e letal de NAD nas células pancreáticas, com subsequente redução no nível

de ATP e posterior inibição da síntese e secreção da insulina (GRIEB, 2016). Em roedores suscetíveis, isso induz diabetes (RERUP, 1970). Administrados em diferentes doses, intervalos ou vias (por exemplo, intraperitoneal ou intravenoso), tanto a ETZ quanto o aloxano podem levar a distúrbios que se assemelham ao diabetes tipo 1, 2 ou pré-diabetes (FURMAN, 2015). Se altas doses são aplicadas, o modelo simula a diabetes tipo 1 humana uma vez que ocorre a perda irreversível de células β. Se doses mais baixas são administradas, obtém-se um comprometimento discreto da secreção de insulina, uma condição semelhante à diabetes tipo 2 (JAWERBAUM; WHITE, 2010).

Embora vários modelos animais que mimetizam a diabetes tipos 1 e 2 estejam disponíveis na literatura científica, o número de modelos animais de pré-diabetes e/ou resistência à insulina ainda é escasso (ISLAM; VENKATESAN, 2016). Alguns modelos de diabetes induzidos geneticamente ou espontaneamente são usados para estudar a pré-diabetes e a resistência à insulina tais como ratos pré-diabéticos SHROB (CHEN et al., 2011), ratos Zucker Diabetic Fatty (ZDF) (ELLIS et al., 2010), ratos Goto Kakizaki (GK) (MOVASSAT et al., 2008), ratos Otsuka Long Evan Tokushima (OLETF) (IWASE et al., 2002), modelo pré-diabético não obeso (BLUTH et al., 1999), ratos pré-diabéticos BB-DP (SPARRE et al., 2003) e hamster chinês pré-diabético (modelo não genético) (FRANKEL et al., 1984). No entanto, esses modelos são relativamente caros, não estão amplamente disponíveis em comparação com modelos não genéticos induzidos experimentalmente, portanto, não são adequados para a triagem farmacológica de rotina de agentes candidatos a drogas antidiabéticas (ISLAM; VENKATESAN, 2016).

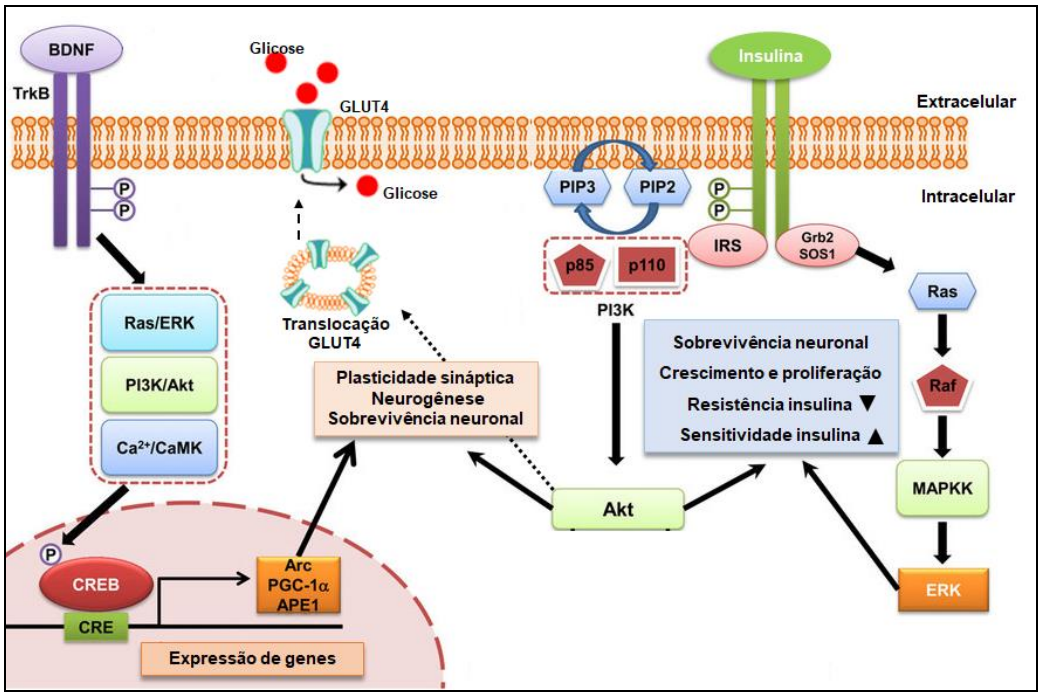
Além disso, modelos experimentais induzidos pela dieta têm sido usados para desenvolver pré-diabetes e resistência à insulina, como a dieta rica em gordura ou com aumento de calorias, administração de ETZ associada à dieta hipercalórica, dieta com alto teor de sacarose, dieta com alto teor de gordura saturada/colesterol/carboidratos, dieta com alto teor de frutose, modelos induzidos pela administração de dexametasona e modelos induzidos pela administração de zimosan. Esses modelos têm sido mais utilizados pelo fato de que reproduzem a pré-diabetes ou a resistência à insulina ou ambos, mas podem levar um maior tempo de protocolo experimental para desenvolver a patogênese da doença (ISLAM; VENKATESAN, 2016).

1.3 MECANISMOS DA PATOGENIA DO (PRÉ-)DIABETES NO SNC

1.3.1 Via de sinalização do fator neurotrófico derivado do encéfalo (BDNF)

O BDNF é a neurotrofina mais abundante no cérebro adulto e regula a plasticidade neuronal através da morfogênese e arborização da coluna dendrítica (MARTINEZ-MORGA; MARTINEZ, 2017), sendo produzido como um peptídeo pré-pro e depois clivado, por proteases intracelulares e extracelulares, em sua forma madura (LU et al., 2008). Embora várias células não neuronais produzam o BDNF, incluindo o endotélio, as células mononucleares do sangue periférico e as células gliais, os neurônios corticais cerebrais continuam sendo a principal fonte de BDNF no cérebro adulto (JOSHI et al., 2019). O BDNF juntamente com seu receptor cognato, receptor de tirosina-quinase B (TrkB), são essenciais para várias funções neuronais e são conhecidos por regular o aprendizado e a memória, modulando a plasticidade sináptica (LU et al., 2008). A dimerização do TrkB, o principal receptor de BDNF no hipocampo e no córtex, ativa três cascatas de sinalização diferentes: a fosfolipase C-gama (PLC-γ), fosfatidilinositol-3-quinase (PI3K) e proteínas-quinases ativadas por mitógenos (MAPK) do tipo proteínas quinase regulada por sinal extracelular (ERK) (AUTRY; MONTEGGIA, 2012). A PLC-γ induz um aumento do cálcio intracelular, seguido pela ativação de elementos de resposta imediata ao cálcio intracelular, incluindo a proteína quinase dependente de Ca^{2+}/calmodulina (CAMKII) (Figura 3). As vias PI3K e ERK, por outro lado, induzem a expressão de BDNF e TrkB e a fosforilação de CREB, potencializando a sobrevivência neuronal e a potenciação de longa duração (LTP) em um *feedback* positivo (QUESSEVEUR et al., 2013) (Figura 3). O CREB é um componente crítico para a consolidação da memória, sendo conhecido como a “proteína da memória”, que está implicado na plasticidade sináptica (LONZE; GINTY, 2002). Nesse contexto, sabendo que o SNC é um órgão dependente de glicose, sua disfunção pode ocorrer devido a distúrbios no transporte neuronal de glicose e metabolismo na hiperglicemia, podendo ocorrer uma alteração da produção de BDNF (SUWA et al., 2006b).

Figura 3. Representação das vias de sinalização do BDNF e da Insulina no neurônio. O receptor TrkB e o receptor de insulina têm um domínio tirosina-quinase na sua região citoplasmática. A ligação do ligando resulta na dimerização do receptor e autofosforilação dos receptores que recrutam proteínas adaptadoras e ativam várias proteínas quinases.



Fonte: Adaptado de LEE et al. (2014). *Pharmacological reviews.*

Curiosamente, em um estudo usando camundongos pré-diabéticos db/db, o tratamento com BDNF impediu o aumento na concentração de glicose no sangue, sugerindo que o BDNF evita o desenvolvimento de diabetes, melhorando a ação da insulina nos tecidos periféricos e prevenindo a exaustão pancreática (YAMANAKA et al., 2008). Além disso, existem relatos de que a injeção periférica de BDNF exibe efeitos hipofágicos e hipoglicêmicos em animais obesos hiperglicêmicos, mas não em animais normais, indicando seus efeitos antiobesidade e antidiabéticos (NAKAGAWA et al., 2000; ONO et al., 1997; TONRA et al., 1999). Outro estudo mostrou que o BDNF também está associado à homeostase da glicose em humanos (KRABBE et al., 2007; SUWA et al., 2006a). Esses dados em conjunto sugerem que o BDNF tem um papel crítico não só na sua função fisiológica de manutenção no SNC, mas também no que se refere aos parâmetros metabólicos da homeostase glicêmica, tornando-se um alvo atrativo para estudos relacionados à memória e diabetes.

1.3.2 Via de sinalização da insulina

A insulina é um hormônio polipeptídico produzido pelas células β pancreáticas. Sua síntese e liberação no sangue é estimulada por um aumento no nível de glicose no sangue

circulante (HENQUIN, 2009) e seu papel mais conhecido é manter a glicose plasmática dentro de um intervalo fisiológico, promovendo a captação e inibindo a produção e liberação de glicose pelo fígado (HENQUIN, 2009).

Nas vias canônicas de sinalização de insulina, as subunidades β autofosforiladas dos receptores de insulina recrutam proteínas pertencentes à família substrato do receptor de insulina (IRS), bem como à família de proteínas transformadoras de SHC (Figura 3). A atividade da tirosina-quinase dos receptores de insulina fosforila os resíduos de tirosina no IRS-1 ou IRS-2, que ativa da ação da insulina e estimula a sinalização através da via AKT. O recrutamento de proteínas SHC por receptores de insulina também leva à ativação da via RAS/RAF/MAPK (LEE et al., 2014).

A via insulina/IRS/AKT é de especial interesse no diabetes, pois medeia a translocação do principal transportador de glicose, GLUT4, das vesículas intracelulares para a membrana plasmática das células, o que facilita a difusão da glicose para dentro da célula (SANO et al., 2007). Além disso, no cérebro, o GLUT4 é encontrado em neurônios de regiões específicas envolvidas na memória motora e episódica, como o cerebelo, o córtex motor e, principalmente, o hipocampo (ALQUIER et al., 2006). No entanto, o papel funcional do GLUT4 cerebral é pouco estudado, embora estudos recentes tenham demonstrado um papel do GLUT4 hipotalâmico na regulação metabólica (REN et al., 2015). Muitos dos reguladores do GLUT4 estão envolvidos nos processos de memória, sugerindo que este transportador de glicose pode contribuir diretamente para a memória (REN et al., 2015). Quando inativo, o GLUT4 é armazenado no citosol nas vesículas de armazenamento (VsA) do GLUT4. A translocação do GLUT4 das VsA para a superfície celular ocorre em resposta à insulina e a outras moléculas envolvidas nos processos de aprendizado e memória do hipocampo. Essa translocação também é regulada pela proteína quinase C-zeta, proteínas acetiladoras de histonas, proteína quinase A e Ca^{2+}/calmodulina quinase II, que também podem afetar o processamento da memória (PEARSON-LEARY; MCNAY, 2016).

A via insulina/IRS/AKT regula a fosforilação de muitas proteínas intracelulares, incluindo a serina/treonina-quinase-mTOR, a glicogênio-sintase-quinase 3 (GSK3), CREB e a óxido nítrico sintase e, portanto, influencia outros processos, como replicação do DNA e atividade do ciclo celular, síntese proteica, sobrevivência celular, metabolismo, angiogênese, captação de potássio, modificação lipídica e autofagia (HENI et al., 2014). Nesse sentido, sugere-se que melhorando a sinalização dessa via da insulina, consequentemente, melhore o fluxo de glicose nos neurônios facilitando processos como aprendizado e memória (AGRAWAL et al., 2011).

1.3.3 Neurodegeneração

As doenças neurodegenerativas, como o nome sugere, são um comprometimento progressivo de qualquer função cerebral que, como no diabetes, afeta predominantemente a população adulta e piora com o aumento da idade (AMTUL; ATTA UR, 2015). Podem ser causadas devido ao comprometimento de neurônios específicos ou um comprometimento neuronal geral (MOSCONI et al., 2008). Há evidências de que os processos neurodegenerativos são mais prevalentes em pacientes diabéticos, quando comparados à incidência na população em geral. Estudos clínicos sugerem o comprometimento do funcionamento neuropsicológico em pacientes diabéticos (COKER; SHUMAKER, 2003). Neste sentido, pacientes diabéticos têm maior prevalência de comprometimento cognitivo global e declínio cognitivo em comparação com indivíduos normoglicêmicos. Além disso, tem sido relatados déficits no desempenho da memória e na cognição baseados no hipocampo, além de redução no volume cerebral de indivíduos diabéticos (GOLD et al., 2007).

Nos modelos de roedores diabéticos induzidos por ETZ, foi observada encefalopatia com degeneração e inflamação neuronal (HAWKINS et al., 2007). Além disso, o metilglioxal (produto final da glicação avançada - AGE) pode ser um promotor da morte neuronal por meio da formação de AGE que desempenha um papel na resistência à insulina, afetando o metabolismo da glicose no cérebro. Outros estudos também demonstraram lesões na substância branca e atrofia cerebral em diabéticos (THORENS, 2011). Além disso, foi evidenciado que a hiperglicemia está envolvida com processos apoptóticos (CAI et al., 2002). Nesse contexto, vale ressaltar que a apoptose é a forma predominante de morte celular que, por sua vez, prejudica a função neuronal (GHAVAMI et al., 2014). A apoptose/ativação da cascata de sinalização apoptótica em neurônios desempenha um papel fundamental na neurodegeneração (GHAVAMI et al., 2014).

Com o diabetes, a hiperglicemia também leva diretamente à produção de espécies reativas de oxigênio (EROs), aumento do estresse celular e glicotoxicidade, causando a morte celular (JIANG et al., 1990). Nesse sentido, combater os mecanismos associados à morte celular mencionados anteriormente, poderiam reduzir a neurodegeneração relatada em modelos de diabetes.

1.3.4 Estresse oxidativo e o sistema Keap1/Nrf2

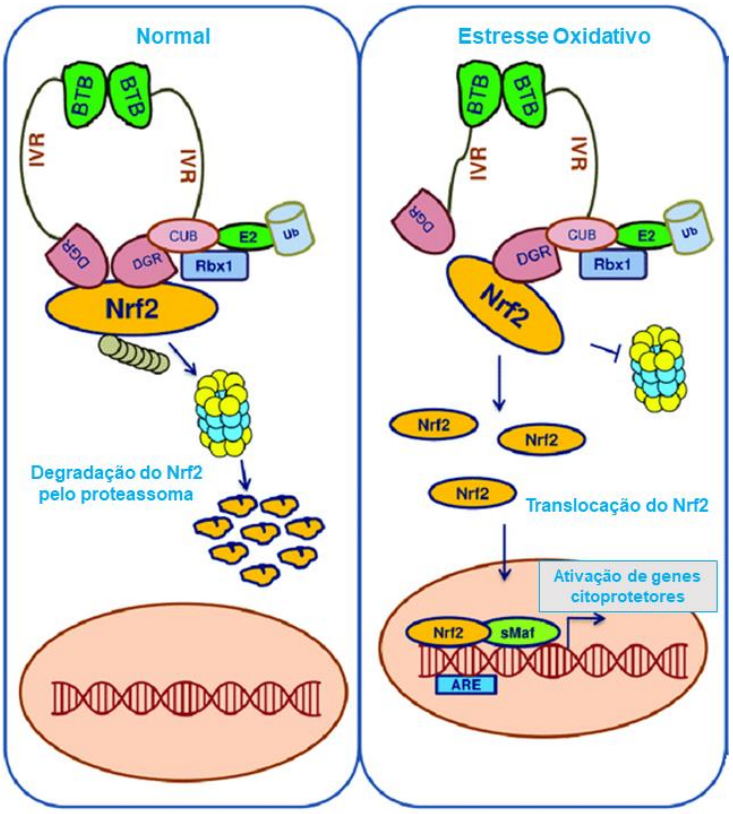
As EROs, como o ânion superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila (HO•), consistem em espécies de oxigênio formadas pela redução parcial do

oxigênio (RAY et al., 2012). Quando as EROs se sobressaem em relação ao sistema de defesa antioxidante celular, seja por meio de um aumento nos níveis de EROs ou por uma diminuição na capacidade antioxidante celular, ocorre o estresse oxidativo (RAY et al., 2012). O estresse oxidativo resulta em dano direto ou indireto, mediado por subprodutos tóxicos do metabolismo, em ácidos nucléicos, proteínas e lipídios, e tem sido implicado na carcinogênese (TRACHOOTHAM et al., 2009), neurodegeneração (SHUKLA et al., 2011), aterosclerose, diabetes (MURIACH et al., 2014) e envelhecimento (HAIGIS; YANKNER, 2010). Como o estresse oxidativo e a sinalização redox estão presentes nas células, organelas e vias moleculares, investigar a relação entre o estresse oxidativo e as funções de sinalização redox das EROs é importante para o desenvolvimento de novas estratégias terapêuticas para tratar doenças como o diabetes (HUANG; LI, 2020).

Nesse contexto, o cérebro é especialmente vulnerável a danos oxidativos como resultado de sua alta taxa de consumo de oxigênio, conteúdo lipídico abundante e escassez relativa de enzimas antioxidantes em comparação com outros tecidos (MURIACH et al., 2014). As células neuronais são sensíveis a insultos oxidativos e, portanto, as EROs estão envolvidas em muitos processos neurodegenerativos (DUGAN et al., 1995). Além disso, a hiperglicemia reduz os níveis de antioxidantes e aumenta concomitantemente a produção de EROs (MURIACH et al., 2014), assim esses efeitos contribuem para o dano tecidual no diabetes, levando a alterações no potencial redox da célula com a subsequente ativação de genes sensíveis a modulação redox (BONNEFONT-ROUSSELOT, 2002).

Uma das respostas biológicas ao estresse oxidativo é a ativação do sistema da Keap1/Nrf2 que através da via dos elementos de resposta antioxidante (ERA), regula a transcrição de genes antioxidantes, os quais preservam a homeostase celular, e os genes de desintoxicação, processando e eliminando substâncias cancerígenas e toxinas antes que possam causar danos as células (Figura 4) (TU et al., 2019). Quando as células são expostas ao estresse oxidativo ou eletrofílico, os resíduos de cisteína da Keap1 são modificados e a Keap1 perde sua capacidade de direcionar Nrf2 à ubiquitinização; dessa maneira, o Nrf2 se acumula dentro da célula e induz a expressão de seus genes-alvo. Esse sistema regulatório é denominado como o sistema da Keap1–Nrf2, e desempenha um papel central na proteção das células do estresse oxidativo (URUNO et al., 2015).

Figura 4. Via Keap1/Nrf2 no estado normal e de sob condições de estresse oxidativo. O Keap1 possui três domínios característicos: BTB, IVR e DGR. Na condição normal, Keap1 promove a degradação do Nrf2. Durante o estresse oxidativo, o Nrf2 é translocado para o núcleo para manter a homeostase redox.



Fonte: Adaptado de RAGHUNATH et al. (2018). *Cancers.*

O Nrf2 pode ativar a expressão gênica de uma série de proteínas antioxidantes e citoprotetoras, incluindo a heme oxigenase-1 (HO-1), a nicotinamida adenina dinucleotídeo NADPH desidrogenase quinona 1 (NQO1), glutationa peroxidase-1, glutationina S-transferase (GST), glutationa redutase e superóxido dismutase (SOD) (MAGESH et al., 2012). A HO-1 é uma enzima induzível que catalisa a liberação do ferro ligado ao grupo heme para formar a biliverdina. A biliverdina pode então ser reduzida pela biliverdina redutase à bilirrubina, liberando monóxido de carbono (CO) e exercendo seu efeito anti-inflamatório (JIAN et al., 2011).

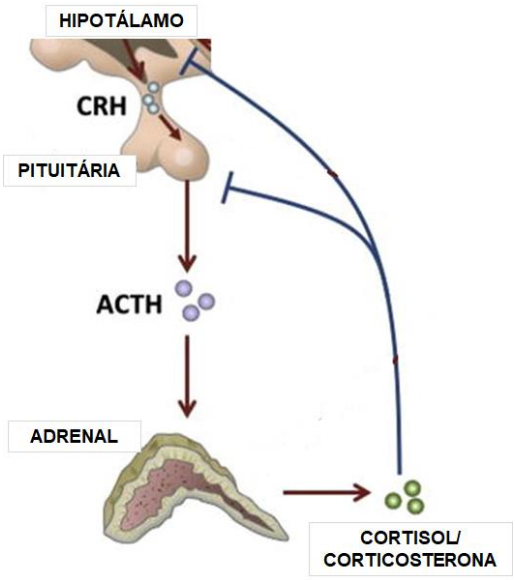
Nesse contexto é descrito que o estresse oxidativo está correlacionado com o comprometimento da regulação da glicose no sangue. Em particular, os achados de que ilhotas pancreáticas expressam poucas enzimas antioxidantes e possuem atividade antioxidante baixa, sugerem que o estresse oxidativo nas ilhotas pancreáticas desempenha um papel na patogênese e/ou progressão do diabetes (SINGAL; SINGAL, 1988). De fato, o estresse oxidativo mediado pela hiperglicemia tem sido relacionado com complicações

diabéticas em modelos animais (CERIELLO, 2003) e pacientes com diabetes (DANDONA et al., 1996). Nesse sentido, o papel do sistema Keap1/Nrf2 na regulação da expressão gênica relacionada ao metabolismo parece ser importante na manutenção da homeostase da glicose em animais diabéticos (CHARTOUMPEKIS; KENSLER, 2013).

1.3.5 Eixo hipotálamo-pituitária-adrenal (HPA)

O eixo HPA é ativado em resposta a estressores fisiológicos ou psicológicos, resultando na secreção do hormônio liberador de corticotropina (CRH) do hipotálamo, que estimula a hipófise anterior a liberar o hormônio adrenocorticotrópico (ACTH). O ACTH estimula a liberação de glicocorticoides pela glândula adrenal, resultando em uma cascata de eventos fisiológicos (Figura 5) (JOSEPH; GOLDEN, 2017). As ações dos glicocorticoides (GC) são mediadas principalmente pela ativação de dois receptores específicos: o receptor de mineralocorticoide (MR) e o de glicocorticoide (GR)(TASKER et al., 2006). O MR e o GR são receptores nucleares que medeiam as ações dos glicocorticoides, sendo eles a corticosterona em roedores e o cortisol no homem (PU et al., 2007; ZHOU; CIDLOWSKI, 2005).

Figura 5. Ativação o eixo HPA. CRH hipotalâmico estimula a hipófise, que, por sua vez, libera ACTH, levando à estimulação do córtex adrenal. O glicocorticóide liberado fornece feedback negativo sobre o eixo HPA através do hipotálamo e da hipófise.



Fonte: Adaptado de IWATA et al. (2013). *Brain, Behavior, and Immunity.*

O GR é encontrado em altas concentrações no hipocampo, nos neurônios aminérgicos no tronco cerebral e nos núcleos que fazem parte do eixo HPA, como o núcleo paraventricular hipotalâmico (DE KLOET et al., 2005; JOËLS et al., 2009). Os GRs exercem sua ação como monômeros ou como homodímeros, influenciando diretamente a transcrição do DNA, ou os GCs podem exercer efeitos não genômicos rápidos, por exemplo, interagindo com proteínas ligadas à membrana (DOREY et al., 2011; GROENEWEG et al., 2012). Essa ação produz uma resposta rápida de *feedback* negativo do eixo HPA que suprime sua própria hiperatividade induzida por estresse (KELLER-WOOD; DALLMAN, 1984).

Uma vez resolvido o estressor, a resposta é encerrada através de um *feedback* negativo, no qual o cortisol suprime a liberação adicional de ACTH e CRH (GOLDEN et al., 2011). O estresse crônico pode prejudicar os mecanismos de retroalimentação que retornam esses sistemas hormonais ao normal, resultando em elevação crônica dos níveis de cortisol, catecolaminas e marcadores inflamatórios. Nesse sentido, supõe-se que a associação biológica entre depressão e diabetes esteja relacionado a um eixo HPA desregulado e hiperativo, uma mudança no tônus do sistema nervoso simpático em direção a uma atividade simpática aumentada e um estado pró-inflamatório (CHAMPANERI et al., 2010).

Além disso, tem sido demonstrado que uma redução em mediadores pró-inflamatórios e uma melhora na função do eixo HPA, proporcionaram efeitos benéficos em roedores diabéticos com fenótipo tipo-depressivo (ASWAR et al., 2017). Ainda, foi relatado que um agonista seletivo do receptor de glicocorticoide bloqueia os efeitos imunoinflamatórios da diabetes induzida por múltiplas doses de ETZ em camundongos (SAKSIDA et al., 2014).

1.4 COMORBIDADES ASSOCIADAS AO (PRÉ-)DIABETES

Nos últimos anos, um interesse maior vem sendo dedicado ao efeito do diabetes no SNC. Sabe-se que juntamente com a doença cerebrovascular, o diabetes está implicado no desenvolvimento de outras comorbidades neurológicas (BITRA et al., 2015). Além disso, existem evidências da alta prevalência de prejuízo de memória, depressão e deficiências funcionais em pacientes com diabetes (BITRA et al., 2015).

1.4.1 Memória

O termo “memória” abrange à capacidade de adquirir uma informação, retê-la ou consolidá-la, para que possamos evocar a informação aprendida. Assim, a memória pode ser divida em fases: aquisição, consolidação e evocação (IZQUIERDO, 2018). A memória é uma modificação de certas essências temporais de incidentes passados, como a repetição de um

estímulo ou a coincidência de múltiplos estímulos (KUKUSHKIN; CAREW, 2017). Para a detecção e resposta a uma situação específica, o sistema nervoso usa sua capacidade de codificar e armazenar memória nos níveis molecular, celular, sináptico e de circuito (KUKUSHKIN; CAREW, 2017). Alterações na atividade e estrutura sináptica e sua eficiência de transmissão são chamadas de plasticidade sináptica (JOSHI et al., 2019). A pesquisa sobre plasticidade sináptica abre oportunidades para a compreensão dos mecanismos moleculares envolvidos na formação da memória (ABRAHAM et al., 2019).

Além disso, as memórias podem ser designadas de acordo com o tempo de sua duração, uma memória pode durar apenas alguns segundos, como guardar um número telefônico, esse tipo é designado como memória de trabalho (IZQUIERDO et al., 1998). Outras memórias podem durar alguns minutos, ou até algumas horas, e referem-se a memória de curta duração e, ainda, uma memória pode durar horas, dias e até anos, as quais são denominadas como memórias de longa duração. Uma memória de longa duração requer alterações na expressão gênica e na síntese proteíca para poder conservar essa nova informação que é armazenada em diferentes regiões cerebrais (IZQUIERDO et al., 1998). Nesse sentido, a formação da memória pode sofrer interferência em diferentes fases e não é dependente apenas da integridade funcional e bioquímica das estruturas envolvidas nos processos de memória. Os processos mnemônicos estão sob modulação de diversos sistemas de neurotrasmissão, função sináptica, quantidade e expressão proteica, entre outros (MELLO-CARPES et al., 2016; MELLO-CARPES; IZQUIERDO, 2013).

Nesse sentido, a disfunção cognitiva representa um problema sério e sua prevalência tem aumentado em todo o mundo, principalmente entre os idosos (PODDAR et al., 2019). Além disso, pacientes com diabetes tipo 1 e 2 apresentam déficit cognitivo (J et al., 2009). Estudos epidemiológicos também mostraram que o pré-diabetes e o diabetes estão relacionados ao maior risco de demência (LUCHSINGER, 2010). De fato, estudos prospectivos baseados na população, do tipo metanálise, demonstraram um risco aumentado de comprometimento cognitivo em pessoas com diabetes (BIESSELS et al., 2006; CHENG et al., 2012).

Em condições pré-diabéticas os tecidos do corpo são expostos a níveis anormalmente altos de insulina, que podem persistir por muitos anos (YAFFE et al., 2004), sendo que a resistência à insulina parece aumentar os produtos finais da AGE que, consequentemente, aumentam o estresse oxidativo pela formação de EROs, acelerando o processo de envelhecimento (DE LA MONTE; WANDS, 2005). O estresse oxidativo resultante pode levar ao aumento do dano ao DNA, disfunção mitocondrial e redução da produção de ATP.

Além disso, o comprometimento da sinalização da insulina cerebral sugere um elo no risco de declínio cognitivo e neurodegeneração na pré-diabetes (BITRA et al., 2015). Sabe-se que os fatores de crescimento semelhantes à insulina modulam as funções vitais, incluindo sobrevivência e crescimento de neurônios, expressão gênica, síntese de proteínas, formação de sinapse e plasticidade (KIM; FELDMAN, 2012). Consequentemente, o prejuízo na sinalização ou a resistência à insulina afetam adversamente uma ampla gama de funções neuronais e gliais (ARNOLD et al., 2018).

Recentemente, foi demonstrado que as disfunções cognitivas comumente observadas em pacientes com diabetes tipo 1 estão associadas à diminuição da velocidade do processamento de informações, eficiência psicomotora, atenção, memória, aprendizado, habilidades para resolver problemas, velocidade motora, entre outras (KIM, 2019). Ainda, sabe-se que a região cerebral de maior relevância para o desempenho dessas funções é o hipocampo, que é reconhecido há muito tempo como uma estrutura cerebral que evidencia múltiplas formas de memória (TAN et al., 2017).

1.4.2 Depressão

O risco de adquirir um problema de saúde mental ao longo da vida é de cerca de 50%, e isso está associado a uma queda no desempenho do emprego, na produtividade e nos salários (HEWLETT; MORAN, 2014). Como é definido pelo Manual Estatístico e Diagnóstico de Transtornos Mentais da Associação Americana de Psiquiatria (AAP), a depressão altera emoções, cognição e comportamentos (APA, 2013). De acordo com o AAP, os critérios de diagnóstico para um transtorno depressivo maior consistem em um sintoma central - humor diminuído/irritável ou interesse/prazer diminuído (anedonia) - ou ambos e pelo menos quatro dos seguintes sintomas: sentimento de culpa ou inutilidade, fadiga ou perda de energia, problemas de concentração, pensamentos suicidas ou pensamentos sobre morte, perda ou ganho de peso (alteração de 5% no peso), retardo ou ativação psicomotora (mudança de atividade), hipersonia ou insônia (alteração no sono) com duração de pelo menos duas semanas (APA, 2013).

Há evidências de que a prevalência de depressão é moderadamente aumentada em pacientes pré-diabéticos e diabéticos não diagnosticados, e acentuadamente aumentada nos pacientes diabéticos previamente diagnosticados em comparação com indivíduos com o metabolismo da glicose normais (CHEN et al., 2016). As taxas de prevalência de depressão podem ser até três vezes maiores em pacientes com diabetes tipo 1 e duas vezes mais altas em

pessoas com diabetes tipo 2 em comparação com a população geral em todo o mundo (ROY; LLOYD, 2012).

Mezuk e colaboradores em um trabalho de metanálise forneceram evidências de que existe uma relação bidirecional entre diabetes e depressão (MEZUK et al., 2008), no qual pessoas com diabetes tipo 2 têm 15% mais risco de ter depressão quando comparadas com pessoas sem diabetes, e pessoas com depressão têm um risco aumentado de 60% de desenvolver diabetes tipo 2 (PAN et al., 2010). Outros estudos também apontaram que a associação entre diabetes e depressão é bidirecional (GENDELMAN et al., 2009), taxas de prevalência mais altas de sintomas depressivos em homens e mulheres com diabetes tipo 1 em comparação com indivíduos sem diabetes (homens: 25,5 vs. 11,6%; mulheres: 37,9 vs. 20,5%). Além disso, idosos com sintomas depressivos e com pré-diabetes têm um risco aumentado de progredir ao diabetes em comparação com aqueles com apenas um dos fatores de risco (GRAHAM et al., 2017).

Em suma, é possível que exista uma relação de compartilhamento de mecanismos biológicos entre a diabetes e a depressão e, portanto, o entendimento destes poderá proporcionar uma melhor abordagem terapêutica para estas doenças.

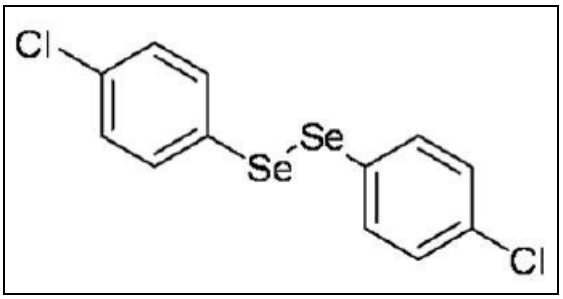
1.5 COMPOSTOS ORGÂNICOS DE SELÊNIO

O Selênio (Se) está presente na natureza e nos organismos em formas orgânicas e/ou inorgânicas. As principais formas orgânicas são a selenometionina e a selenocisteína. As formas inorgânicas são o selenito (SeO_3^{-2}), seleneto (Se_2-), selenato (SeO_4^{-2}) e o elemento Se (GRAHAM, 1991). O Se é usado nos campos da agricultura e da biologia, como suplemento em solos deficientes, em inseticidas e, ainda, na alimentação de animais (MEHDI et al., 2013). O Se como um componente integrante das selenoproteínas participa de toda uma série de processos fisiologicamente importantes (BARBOSA et al., 2017). A primeira selenoenzima comprovada foi a glutationa peroxidase (GPx), que é um componente indispensável do sistema antioxidante no organismo (ARTHUR, 2000; BARBOSA et al., 2017). O Se também é importante para a regulação das funções imunes (KOHRLE; GARTNER, 2009), desempenha um papel essencial na resposta imune inespecífica e seu baixo nível está relacionado ao sistema imunológico deprimido (SCHOMBURG, 2011). A deficiência de Se leva à diminuição da atividade da enzima GPx, assim as células se tornam mais suscetíveis a danos oxidativos (HOSNEDLOVA et al., 2017). Além disso, o Se está envolvido no crescimento e desenvolvimento, além de participar de processos de regulação relacionados às habilidades de produção e reprodução de animais (SONG et al., 2015;

SPEIGHT et al., 2012). Apesar da necessidade da ingesta de Se na dieta, sabe-se que os limites entre níveis essenciais e tóxicos podem ser estreitos, no Brasil a ANVISA determinou que a ingestão diária de Se é de 34 µg. Dentre as fontes dietéticas de Se estão a castanha-do-Pará, cebola, alho, brócolis, cogumelos, cereais, pescados, ovos e carnes (DUMONT et al., 2006).

Nesse sentido, os compostos contendo Se são alvo de interesse científico visto que estudos tem encontrado compostos orgânicos de selênio sintéticos estáveis com atividades biológicas promissoras (NOGUEIRA; ROCHA, 2011). Durante as duas últimas décadas, nosso grupo de pesquisa tem focado, sob o ponto de vista terapêutico e toxicológico, os disselenetos de diorganoila (NOGUEIRA; ROCHA, 2011). Considerando que pequenas mudanças na estrutura química de moléculas modificam o efeito de uma droga, nesta tese o foco do estudo dará ênfase a um derivado do disseleneto de difenila $(PhSe)_2$, que é o composto disseleneto de p-clorodifenila $(p\text{-ClPhSe})_2$ (Figura 6). Estudos com esse composto tem reportado efeitos promissores no SNC, relacionados com a neuroproteção em um modelo animal de estresse induzido por corticosterona (ZBOROWSKI et al., 2016), atividade antioxidante em cérebro de ratos e camundongos (MEOTTI et al., 2004), ação do tipo antidepressiva e melhora na memória em ratos velhos (BORTOLATTO et al., 2012). Além disso, este composto apresentou efeito anorexígeno, induzindo saciedade comportamental precoce em ratos, através de uma regulação no sistema serotoninérgico (BORTOLATTO et al., 2015a; BORTOLATTO et al., 2015b).

Figura 6. Estrutura química do $(p\text{-ClPhSe})_2$.



Fonte: próprio autor, 2020.

Ainda, tem sido demonstrado efeitos do $(p\text{-ClPhSe})_2$ no metabolismo periférico em roedores. De fato, Quines e colaboradores mostraram que o $(p\text{-ClPhSe})_2$ estimula o metabolismo dos carboidratos e modula alterações induzidas pela sobrecarga de ingesta de frutose em ratos (QUINES et al., 2017b). Além disso, o $(p\text{-ClPhSe})_2$ possui efeito

homeostático no metabolismo da glicose e reduz a hepatotoxicidade após a administração de glutamato monossódico em ratos, através de uma regulação hipotalâmica (QUINES et al., 2017a; QUINES et al., 2016; QUINES et al., 2018b). Recentemente, efeitos do tipo antidepressivo do (*p*-ClPhSe)$_2$ foram demonstrados em camundongos expostos a dexametasona, efeito este que foi associado a modulação do estresse oxidativo e da neurotransmissão glutamatérgica (HECK et al., 2019). Associando esses efeitos em diferentes regiões, tanto em tecidos periféricos quanto no SNC, seria interessante investigar os possíveis impactos biológicos do composto em questão no modelo de diabetes, visto que essa doença é uma doença multifatorial.

Embora os efeitos biológicos já apresentados pelo (*p*-ClPhSe)2 sejam de grande valor para continuação de pesquisas acerca de seus efeitos terapêuticos, não podemos deixar de mencionar os possíveis efeitos toxicológicos relatados desse composto (NOGUEIRA; ROCHA, 2011). É sabido que os compostos orgânicos de selênio podem interagir com grupos tióis para produzir dissulfetos *in vitro*, e isso pode ser responsável pela toxicidade *in vivo* (NOGUEIRA; ROCHA, 2010). Além disso, já foi observado que os disselenetos (por ex., (PhSe)$_2$ e (*p*-ClPhSe)$_2$) inibem a atividade das enzimas Na^+K^+ATPase e delta-aminolevulinato desidratase pela oxidação dos grupos tióis *in vitro* (BRUNING et al., 2009). Portanto, a investigação de eventuais efeitos tóxicos *in vivo* destes compostos continua dependendo de estudos futuros mais detalhados, para descoberta de uma janela terapêutica segura.

Entretanto, tendo em vista as propriedades do composto (*p*-ClPhSe)$_2$ já relatadas na literatura, tem-se o interesse em investigar seus efeitos visando minimizar possíveis danos de memória e o fenótipo do tipo depressivo, provenientes das complicações diabéticas. Uma hipótese seria associar o composto orgânico de selênio a medicamentos clássicos usados para o tratamento da diabetes, está hipótese é baseada no fato de que, atualmente, existem poucos medicamentos que poderiam tratar estas comorbidades associadas ao diabetes.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar os efeitos da administração de ETZ na memória e no fenótipo do tipo depressivo elucidando os possíveis mecanismos envolvidos em camundongos. Bem como investigar a ação neuroprotetora do composto disseleneto de *p*-clorodifenila (*p*-ClPhSe)$_2$ em animais diabéticos.

2.2 OBJETIVOS ESPECÍFICOS

- **Em camundongos administrados com ETZ avaliar se**:

➢ Os animais apresentam o prejuízo de memória;

➢ Os animais apresentam fenótipo do tipo depressivo;

➢ Altera o perfil metabólico dos animais;

➢ Modula os níveis hipocampais das proteínas da via de sinalização do BDNF/TrkB dos animais;

➢ Modula os níveis de proteínas do sistema Keap1/Nrf2 e o eixo HPA, bem como o envolvimento do estresse oxidativo no córtex cerebral dos animais;

➢ Ocorre alteração na neurodegeneração no SNC dos camundongos;

➢ Ocorre alteração do conteúdo das proteínas hipocampais das vias de sinalização do BDNF/CREB e IRS-1/Akt/GLUT4 dos animais.

- **Em camundongos tratados com (*p*-ClPhSe)$_2$ avaliar**:

➢ Se o tratamento com composto tem efeito terapêutico sobre o prejuízo de memória de animais diabéticos;

➢ Se o tratamento com composto tem efeito terapêutico sobre fenótipo do tipo depressivo de animais diabéticos;

➢ Se o tratamento com composto modula níveis de proteínas hipocampais de BDNF/TrkB de animais diabéticos;

➢ Se o tratamento com composto modula a via de sinalização Keap1/Nrf2 e o eixo HPA, bem como o estresse oxidativo no córtex cerebral de animais diabéticos;

Se o tratamento com composto modula a neurodegeneração no SNC de animais diabéticos.

3 DESENVOLVIMENTO

O desenvolvimento desta tese está apresentado sob a forma de 2 artigos científicos e 1 manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências bibliográficas encontram-se nos próprios artigos e manuscrito, os quais estão estruturados de acordo com as normas das revistas onde foram publicados e submetidos, respectivamente.

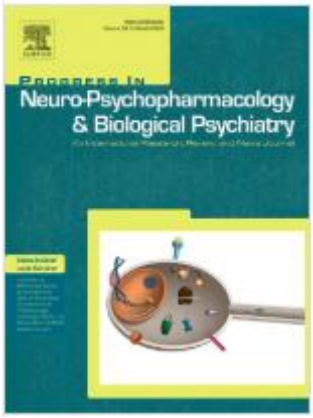
Em anexo a esta tese encontram-se a autorização da editora para reprodução dos artigos científicos, bem como a aprovação do projeto de pesquisa pela Comissão de Ética de Uso de Animais (CEUA) da Universidade Federal de Santa Maria.

3.1 ARTIGO 1

$(p\text{-}ClPhSe)_2$ modula a sinalização hipocampal do BDNF/Trk e reverte o prejuízo de memória induzido pelo diabetes em camundongos

$(p\text{-}ClPhSe)_2$ modulates hippocampal BDNF/TrkB signaling and reverses memory impairment induced by diabetes in mice

Vanessa Angonesi Zborowski, Suélen Osório Heck, Marcel Henrique Marcondes Sari, Nícolas Klummer Bastos, José Sebastião Neto, Cristina Wayne Nogueira



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$(p\text{-ClPhSe})_2$ modulates hippocampal BDNF/TrkB signaling and reverses memory impairment induced by diabetes in mice

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ABSTRACT

Diabetes is a metabolic disease characterized by hyperglycemia because of insulin resistance and/or insufficient insulin release. The most common diabetic brain complications include cognitive decline and depression. The present study investigated whether the 4–4′-dichlorodiphenyl diselenide $(p\text{-ClPhSe})_2$ is effective against memory impairment induced by diabetes in mice and the role of hippocampal BDNF/TrkB signaling in this effect. Male adult Swiss mice received an injection of streptozotocin (STZ) (200 mg/kg, i.p.) to induce diabetes. The results revealed that STZ injection in mice resulted in resilience (glycemia < 200 mg/dl) or diabetes (glycemia ≥ 200 mg/dl). The vehicle-control group received citrate buffer (5 ml/kg). The animals were subchronically treated with $(p\text{-ClPhSe})_2$ (1 or 5 mg/kg, i.g.) for 7 days. Mice performed a battery of well-validated behavior tests designated to evaluate memory, object recognition (ORT), object location (OLT), and *Morris* water maze (MWM). The hippocampal protein contents of the BDNF/TrkB pathway were determined in the samples of experimental groups. Fluoro Jade C (FJC) was used for staining degenerating neurons. The STZ administration resulted in memory impairment that was demonstrated in the mouse ORT, OLT, and MWM tests. The molecular findings indicate an increase in hippocampal protein levels of proBDNF and TrKB but a decrease in those of mBDNF and pCREB in diabetic mice. The number of FJC-positive cells was increased in the hippocampus of diabetic mice. $(p\text{-ClPhSe})_2$ at the dose of 5 mg/kg modulated the hippocampal BDNF/TrkB pathway, reduced FJC-positive cells and reversed memory impairment induced by STZ in mice. These findings demonstrate the effectiveness of $(p\text{-ClPhSe})_2$ against memory impairment caused by diabetes in mice. $(p\text{-ClPhSe})_2$ modulated the hippocampal BDNF/TrkB signaling pathway in diabetic mice.

1. Introduction

Diabetes is a metabolic disease, characterized by hyperglycemia because of insulin resistance or insufficient insulin release, which requires both continuous medical care as well as self-management (Nwosu and Chime, 2017). For these reasons, diabetes is among the diseases with high rates of complications which significantly lower the quality of life in patients (Hershey et al., 1999) representing a significant cause of death worldwide (Garcia-Caballero et al., 2012). The most common complications of the peripheral nervous system presented in diabetic patients are retinopathy, nephropathy, and peripheral neuropathy. In addition, diabetes may also have negative consequences on the central nervous system (McCrimmon et al., 2012).

From a clinical perspective, studies with diabetic patients report that they present a decrease in learning and memory abilities as well as damage in hippocampus neurons (Wrighten et al., 2009). In fact, the hippocampus is a brain region essential to perform learning and memory, which is highly sensitive to uncontrolled peripheral hyperglycemia (Ye et al., 2011). Although the pathogenesis of diabetes-related cognitive impairment is multifactorial, several lines of evidence indicate that the chronic hyperglycemia, insulin resistance, inflammation, oxidative stress, negative modulation of neurotrophins, and cell signaling proteins are involved in the development of cognitive impairment (McCrimmon et al., 2012; Nonomura et al., 2001).

Molecular basis of learning and memory processes involves neuronal synapses modification in response to electrical activity, a process termed synaptic plasticity (Marx and Gilon, 2012). Accumulating evidence suggests that Brain-derived Neurotrophic Factor (BDNF) plays an important role in neuronal survival and growth, serving as a neurotransmitter modulator, and participating in neuronal plasticity, which

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is essential for learning and memory (Bathina and Das, 2015). The BDNF signaling activates TrkB receptors and triggers different intracellular pathways, including PI3K/Akt, Ras-Raf-MEK-ERK, PLCγ, transactivation of EGFR, Jak/STAT, NF-kB, Wnt/β-catenin and VEGF, and the phosphorylation of cAMP response element-binding protein (CREB) transcriptional factor among others (Meng et al., 2019; Yan et al., 2016). CREB activation plays an important role in the long-term memory formation (Paramanik and Thakur, 2013). Thus, BDNF signaling pathways regulate expression of genes encoding proteins involved in neural plasticity (Bathina and Das, 2015).

New therapeutic approaches are needed to prevent or delay the progression of diabetes complications. This way, the organoselenium compounds have emerged as an important subject of research worldwide because of the pharmacological properties assigned to these molecules (Nogueira and Rocha, 2011). According to our previous studies, 4,4′-dichlorodiphenyl diselenide (p-ClPhSe)$_2$, a lipophilic molecule, has been reported to have promising effects in different models of metabolic disease. In fact, (p-ClPhSe)$_2$ was effective against metabolic disorders in a rat model of neuroendocrine obesity (Quines et al., 2018) and in the high-fructose intake in rats (Quines et al., 2017). In addition, (p-ClPhSe)$_2$ has an anorectic action by modulating hypothalamic pathways (Bortolatto et al., 2017). The blood-brain barrier compatible property characterizes this compound as a neurotherapeutic target that interacts with neurotransmitter systems (Zborowski et al., 2016). Regarding the (p-ClPhSe)$_2$ effects on memory, studies from our research group have reported that (p-ClPhSe)$_2$ was effective against memory impairment in aged rats and exposed to corticosterone in mice (Bortolatto et al., 2012; Zborowski et al., 2016). Therefore, the neuroprotector and memory enhancing properties of (p-ClPhSe)$_2$ stimulate the search for new mechanistic targets by which this compound could act to enhance memory.

Considering the potential pharmacological actions of (p-ClPhSe)$_2$, the aim of this study was to investigate whether (p-ClPhSe)$_2$ is effective against memory impairment induced by diabetes in mice and the role of hippocampal BDNF/TrkB signaling given that the effects of (p-ClPhSe)$_2$ on this pathway are unknown.

2. Materials and methods

2.1. Animals

The experiments were carried out by using 2-month old (~35 g) male Swiss mice from our own breeding colony. Swiss out bred mice were obtained from the Central Animal Facility of the University of Santa Maria (Rio Grande do Sul, Brazil). The animals were housed in cages with free access to food and tap water. Eight animals were allocated in each cage (70 cm^2 and 12.7 cm height). They were kept in a separate animal room, on a 12-h light/12-h dark cycle; the lights were turned on every day at 7:00 a.m., in a controlled temperature environment (22 ± 2 °C). The manipulations were carried out between 08:00 a.m. and 06:00 p.m. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil (#2964150317/2017). The procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.2. Chemicals

4–4′-Dichlorodiphenyl diselenide (p-ClPhSe)$_2$ was prepared and characterized in our laboratory according to a previously described method (Paulmier, 1986). Analyses of the 1HNMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of studied compound (99.9%) was determined by gas chromatography–mass spectrometry.

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Development of diabetes

After 4 h of fasting, the mice received a single intraperitoneal injection of STZ at a dose of 200 mg/kg, prepared in citrate buffer (pH 4.5, 0.5 M), to induce diabetes (Gupta et al., 2014). The vehicle control-group received an equal volume (5 ml/kg, i.p.) of citrate buffer. Diabetes was confirmed after 72 h of STZ injection, the fasting blood tail glucose levels of mice were estimated by Accu-Check Active glucometer®. Hyperglycemic mice (blood glucose ≥200 mg/dl) were considered diabetic and those with glycemia below 200 mg/dl were classified as resilient (18%). The resilient animals were not used in the present study.

2.4. Treatment schedule

After STZ administration, the mice were randomized into six experimental groups (n = 8 animals/group):

I) Control = citrate buffer (vehicle) + mineral oil (vehicle).

II) Diabetic = STZ + mineral oil (vehicle).

III and IV) Control- treated = citrate buffer (vehicle) + (p-ClPhSe)$_2$ (1 or 5 mg/kg).

V and VI) Diabetic- treated = STZ + (p-ClPhSe)$_2$ (1 or 5 mg/kg).

The mice from groups I and II received the vehicle of compound (mineral oil, 10 ml/kg) and those from groups III - VI received (p-ClPhSe)$_2$ at the dose of 1 or 5 mg/kg, by the intragastric route, once a day, during the last week of the experimental protocol (Fig. 1). The dose range of (p-ClPhSe)$_2$ treatment was chosen based on our previously published study (Zborowski et al., 2016).

At day 17 of the experimental protocol, mice were divided in two sets to perform the behavior tests (Fig. 1). The first set of animals performed the object recognition test (ORT), the object location test (OLT) and locomotor profile (LP). The second set of animals performed the *Morris* water maze (MWM) test. After a battery of behavior tests, the subsequent analyzes were carried out with (p-ClPhSe)$_2$ only at the dose of 5 mg/kg because this was the most effective dose in memory tests.

For the fluorescence technique, first set of animals were deeply anaesthetized with ketamine/xylazine (150/10 mg/kg). They were perfused through the left cardiac ventricle with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. After perfusion, the brains were removed, post-fixed in the same fixative solution by 24 h for later Fluoro Jade C (FJC) staining, the analysis was made in the dentate gyrus (DG) of the hippocampus.

The second set of animals was died by cervical dislocation, the brains were removed and the hippocampus samples were immediately dissected on a cold plate. The samples were stored at −80 °C until the Western Blot analyses.

2.5. STZ -induced diabetes in mice

2.5.1. General parameters

The individual body weight gain was calculated by the difference between the baseline body weight, obtained before the beginning of treatment, and the body weight at the end of treatment. One h after the behavior tests, the mice from both experimental sets were individually placed in metabolic chambers for 2 h, they were not repeatedly exposed to the metabolic chamber. The results were expressed using an average for all the days. The amount of food and water intake, and the volume of urine excreted were recorded (n = 6 animals/group).

2.5.2. Measurement of metabolic parameters

Glycemia was measured from blood taken from the tail, after 4 h of fasting, using a blood glucose meter (Accu-chek active glucometer) at days 3, 7, 14 and 21 after STZ administration (n = 6 animals/group). At

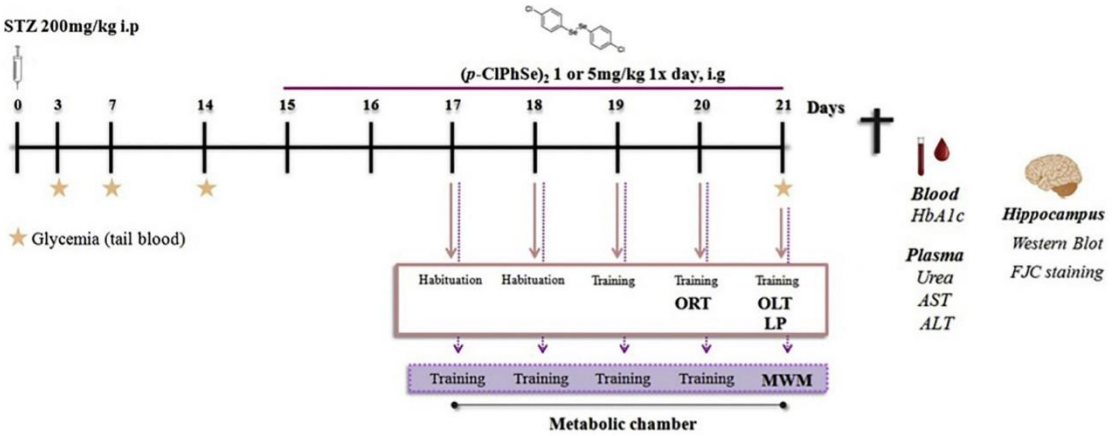

Fig. 1. Schematic representation of the experimental design. A battery of behavior tests started at 17th day of protocol. Object recognition test (ORT), object location test (OLT) and locomotor profile (LP). Another set of animals performed the Morris water maze (MWM) test. Ex vivo assays were carried out after the last dose of (*p*-ClPhSe)$_2$.

22 day, glycated hemoglobin (HbA1c) was measured in the total blood to identify the average plasma glucose concentration through commercial kit (n = 6 animals/group) (NycoCard Cicla®, Brazil). A1c reflects the proportion of hemoglobin (Hb) that is glycated and, because of the lifespan of erythrocytes, it usually represents the average glucose over the last 3 months (Sarnowski and Hivert, 2018). The glycemia was expressed as (mg/dl) and HbA1c was expressed as (%).

The physical and chemical properties of urine were evaluated using urinalysis reagent strips (Sensi 10®) and semiquantitative parameters, such as density, pH, leukocytes, nitrites, proteins, glucose, ketones, urobilinogen, bilirubin, and blood.

2.6. Hepatic and renal toxicity

In order to investigate hepatic and renal toxic effects caused by (*p*-ClPhSe)$_2$ and/or STZ treatments, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and the levels of urea were determined in plasma of mice by commercial kits ($n = 8$ animals/group) (Labtest Diagnostica, MG, Brazil). To obtain plasma samples, blood samples of mice were centrifuged at 4000 $\times g$ for 10 min.

AST catalyzes specifically the transfer of the amino group of aspartic acid to a ketoglutarate yielding glutamate and oxaloacetate. The oxaloacetate is reduced to malate by the enzyme malate dehydrogenase, and nicotinamide adenine dinucleotide (NADH) is oxidized to NAD^+. The absorbance reduction at 340 nm, as a consequence of NADH oxidation, is determined spectrophotometrically and is directly proportional to the AST activity in the sample. ALT catalyzes the transfer of the amino group from alanine to ketoglutarate, yielding glutamate and pyruvate. The pyruvate is reduced to lactate by the action of the lactate dehydrogenase, which oxidizes NADH to NAD^+. The reduction of absorbance at 340 nm due to oxidation of NADH is spectrophotometrically monitored, and it is proportional to the ALT activity in the sample. Enzyme activity was expressed as U/l.

The plasma urea level may suggest impaired renal function. Therefore, the levels of urea were determined through photometric reaction: ammonia reacts with 2-ketoglutarate and NADH in a reaction catalyzed by glutamate dehydrogenase, promoting oxidation of NADH to NAD; the consequent reduction of absorbance measured at 340 nm is proportional to the urea concentration in the sample. The urea level was expressed as mg/dl.

2.7. Behavior tests

For behavior tests with objects, the apparatus was made of plywood and surrounded by walls of 30 cm height; the floor had 45 cm length and 45 cm width. This apparatus was useful to familiarize the mouse with the arena as a context habituation trial for memory tests.

The exploration time of the objects was defined as sniffing or touching with the nose and/or forepaws. The objects were positioned 10 cm from the walls of apparatus. All objects presented similar textures, colors, and sizes, but distinctive shapes. Periods in which the mice climbed over or sat on the objects were not recorded. The animals were allowed to explore the objects for 10 min, in the training sessions, and 6 min in the test sessions, during this period the total time that mice spent exploring each of two objects was recorded by an experimenter using a stopwatch. After that, the mouse was brought back to its home cage. Between trials, the objects were cleaned with 10% ethanol solution.

2.7.1. Object recognition test (ORT)

All animals were subjected to a protocol of habituation comprised of two sessions in two consecutive days, in which they were allowed to freely explore the arena for 10 min. No objects were placed in the arena during the habituation trials (Rosa et al., 2003). Twenty-four h after the last habituation session, two training trials were carried out in two consecutive days by placing a single mouse for 10 min in the field (Fig. 1), in which two identical objects (objects A and A'; Lego bricks, length and height of 6 cm) were positioned in two adjacent corners. For the short-term memory (STM) test, given 1.5 h after the last training session, the mice explored the arena for 6 min in the presence of one familiar (A) and one novel (B) object. The results were expressed as exploratory preference; the recognition index was calculated for each animal by the ratio of TB/(TA + TB) [TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B].

2.7.2. Object location test (OLT)

Twenty-four h after the ORT, the mice performed one training session of OLT in the same apparatus used in the ORT. The training session was carried out by placing one mouse into the field where two objects (objects C and D; Lego bricks colorful toy) were positioned in two adjacent corners, as described by Takahashi et al. (2009) with some modifications. Thus, 2 h after the training session, the object D was moved to a location that was diagonally opposite to object C, and the

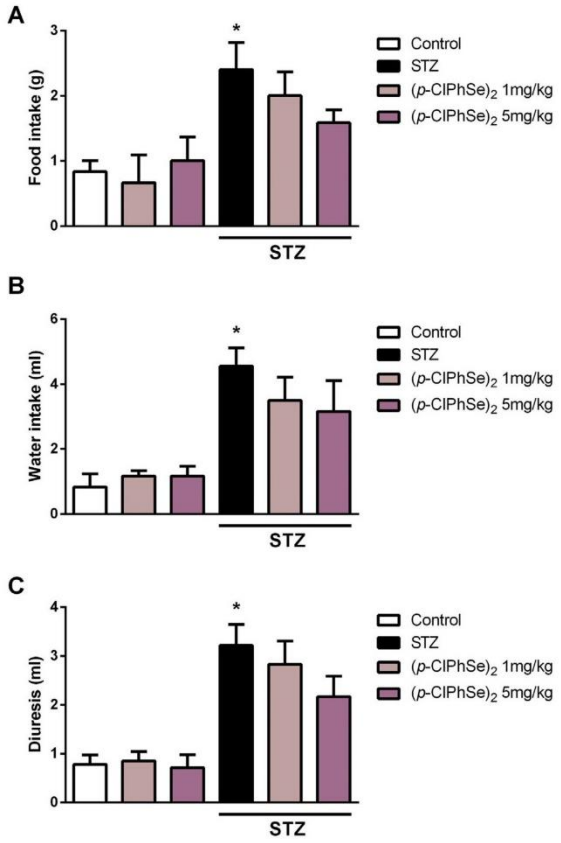

Fig. 2. Effects of (p-ClPhSe)$_2$ (1 and 5 mg/kg) in the food (A) and water (B) intake, and diuresis (C) of mice during 2 h in the metabolic chamber. Each column represents the mean ± S.E.M. of 6 animals/group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test. *$p < 0.05$ as compared with the control group.

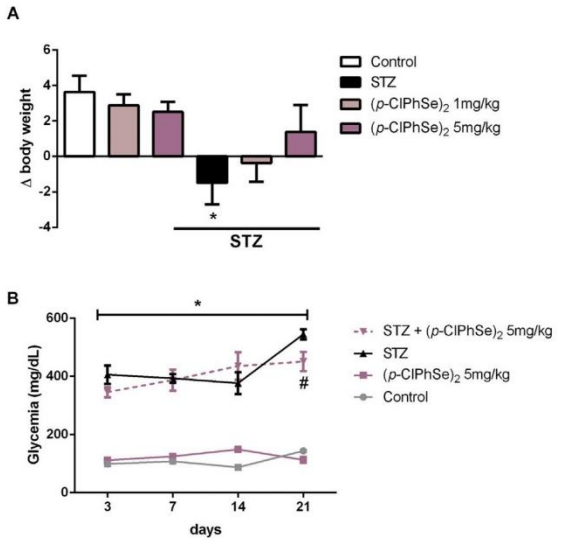

Fig. 3. Effects of (p-ClPhSe)$_2$ in the Δ body weight (A) and glycemia (B) on different days of experimental protocol. Each column and row represents the mean ± S.E.M. of 6 animals/group. *p < 0.05 as compared with the control group; #p < 0.05 as compared with the diabetic group. Data were analyzed through two-way ANOVA (A) and two-way ANOVA repeated measures (B) followed by the Newman-Keuls test.

Table 1
Effect of (p-ClPhSe)$_2$ on glycated hemoglobin (HbA1c) in diabetic mice.

	HbA1c (%)
Control	≥4.0 ± 0.0
(p-ClPhSe)$_2$ 5 mg/kg	4.0 ± 0.0
STZ	6.4 ± 0.2*
STZ + (p-ClPhSe)$_2$ 5 mg/kg	6.1 ± 0.2*

Data are reported as mean ± S.E.M of 6 animals/group. *p < 0.05 as compared with the control group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

Table 2
Effects of (p-ClPhSe)$_2$ on hepatic and renal markers of toxicity in diabetic mice.

	Urea (mg/dL)	ALT (U/L)	AST (U/L)
Control	24.5 ± 0.3	59.6 ± 3.0	74.4 ± 1.1
(p-ClPhSe)$_2$ 1 mg/kg	24.4 ± 1.5	56.1 ± 7.1	73.7 ± 6.9
(p-ClPhSe)$_2$ 5 mg/kg	22.1 ± 1.2	54.0 ± 3.2	66.6 ± 2.6
STZ	36.6 ± 1.2*	87.8 ± 1.7*	158.1 ± 6.6*
STZ + (p-ClPhSe)$_2$ 1 mg/kg	31.7 ± 1.4#	59.3 ± 4.3#	108.3 ± 9.1#
STZ + (p-ClPhSe)$_2$ 5 mg/kg	29.3 ± 2.1#	47.1 ± 4.5#	102.0 ± 8.6#

Data are reported as mean ± S.E.M of 8 animals/group. *$p < 0.05$ as compared with the control group, #p < 0.05 as compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

animals were allowed to explore the objects during 6 min (Fig. 1). The results were expressed as the location index according to the equation: TD/(TC + TD) [TC = time spent exploring the familiar object C; TD = time spent exploring the novel object D].

2.7.3. Morris water maze test (MWM)

Spatial learning and memory were accessed using the MWM test according to the method described by Morris (1984). The water-maze consisted of a swimming pool (110 cm × 40 cm) made of black plastic and filled with water (26 ± 2 °C) at a height of 30 cm. The pool was placed in a room with extra maze visual cues. For the acquisition phase, mice were placed next to and facing the wall successively in north, south, east and west positions. The escape platform was hidden 1 cm below the water level in the middle of the northwest quadrant. The water was made opaque by adding a nontoxic dye. Behaviors were videotaped via camera connected to the computer. The experimenter was hidden from the view of the animals, but it was able to follow their swimming trajectories on a video monitor, in which the pool was previously separated into four equally spaced quadrants and the platform location was designated.

The latency to reach the submerged platform was measured in four trial sessions during 4 days. The mice remained on the platform for at least 60 s after each trial. Whenever the mice failed to reach the escape platform within the 1 min cut-off period, they were directed to the platform and placed on it for 40 s. Twenty-four h after the last day of acquisition phase; a probe trial was conducted by removing the platform and placing the mice in the opposite quadrant of the platform. The distance travelled in the quadrant, where was the platform, were measured for a single 1 min test in the probe phase. The results were analyzed using a software Any-maze® for Windows.

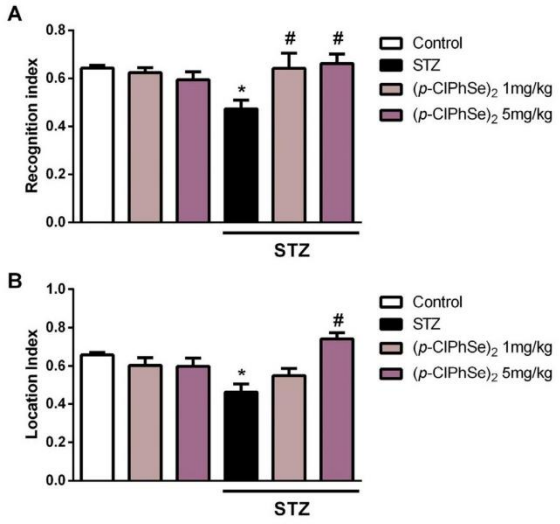

Fig. 4. Effects of (p-ClPhSe)$_2$ (1 and 5 mg/kg) on the recognition index (A) in the object recognition test (ORT) and on the location index (B) in the object location test (OLT). Each column represents the mean ± S.E.M. of 8 animals/group. *p < 0.05 as compared with the control group and #p < 0.05 as compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

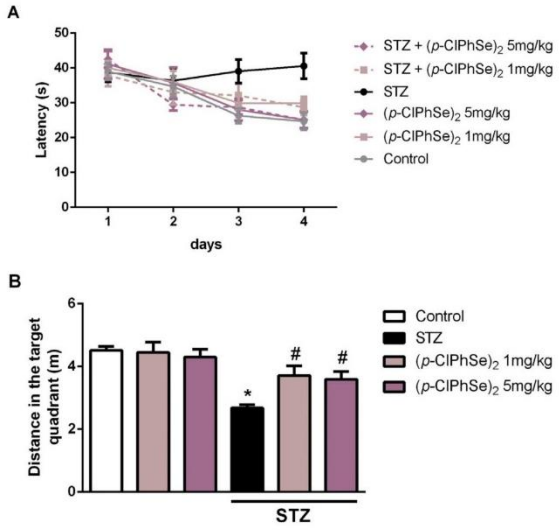

Fig. 5. Effects of (p-ClPhSe)$_2$ (1 and 5 mg/kg) on latency to reach the platform in the training period (A), distance travelled in the target quadrant (B) in the Morris water maze (MWM) test. Each column represents the mean ± S.E.M. of 8 animals/group. *p < 0.05 as compared with the control group and #p < 0.05 as compared with the diabetic group. Data were analyzed through two-way ANOVA repeated measures (A) and two-way ANOVA followed by the Newman-Keuls test (B).

2.7.4. Locomotor profile (LP)

The locomotor profile of mice was carried out in the last day of treatment, 1 h after the OLT. The apparatus consisted in a clear acrylic chamber (500 × 480 × 500 mm) equipped with 16 infrared sensors for the automatic recording of horizontal activity (Model EP149, Insight Instruments Ltda, SP, Brazil). General locomotor activity and the mouse's position in the chamber were detected by breaks of the photocell beams, which were recorded by the software. Animals were placed in the center of the apparatus, where they were allowed to freely explore it during 4 min (York et al., 2013). At this time, the number of crossings (number of segments crossed with the four paws) and total distance travelled (mm) were recorded.

Table 3
Effects of (p-ClPhSe)$_2$ on locomotor and exploratory activities in diabetic mice.

	Crossings	Distance (mm)
Control	579.9 ± 40.6	10,217 ± 289.8
(p-ClPhSe)$_2$ 1 mg/kg	594.7 ± 26.26	11,123 ± 446.1
(p-ClPhSe)$_2$ 5 mg/kg	563.7 ± 55.7	10,014 ± 1035.0
STZ	523.7 ± 31.7	8745 ± 814.3
STZ + (p-ClPhSe)$_2$ 1 mg/kg	601.3 ± 40.5	9677 ± 1118.0
STZ + (p-ClPhSe)$_2$ 5 mg/kg	506.8 ± 55.2	8377 ± 603.6

Data are reported as mean ± S.E.M of 6 animals/group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

2.8. Western blot assay

The samples of hippocampus were manually homogenized in a glass–glass potter containing ice-cold RIPA buffer (Sigma-Aldrich Co., St. Louis, Missouri, USA) in the presence of proteases and phosphatases inhibitor cocktail (Sigma- Aldrich Co., St. Louis, Missouri, USA). The samples of hippocampus were diluted to a final protein concentration of 2 µg/µl in sample buffer. The samples (20 µg of protein) and prestained molecular weight standards (Sigma-Aldrich Co., St. Louis, Missouri, USA) were separated by 10% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (0.45 µm, Bio-Rad) using Transfer-Blot® Turbo™ Transfer System (1.0 A; 35 min) and equal protein loading was confirmed by Ponceau S staining. After blocking with 3% bovine serum albumin solution, the blots were incubated overnight at 4 °C with rabbit anti-BDNF (1:1000, Abcam), rabbit anti-TrkB (tropomyosin kinase B) (1:5000, Cell Signaling), rabbit anti-Akt (protein kinase B) (1:1000, Cell Signaling), rabbit anti-p-Akt (1:1000, Cell Signaling), rabbit anti-ERK (extracellular signal–regulated kinase) (1:1000, Cell Signaling), mouse anti-p-ERK (1:1000, Santa Cruz), rabbit anti-CREB and anti-p-CREB (1:1000, Cell Signaling). Mouse anti-β-actin (1:1000, Cell Signaling) was stained as additional control of the protein loading.

After the primary antibody incubations, membranes were washed and incubated for 1 h at room temperature with anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:5000, Bio-Rad Laboratories, Hercules, CA, USA). For protein detection, we used chemiluminescence kit (Amersham, SP, Brazil) and the signals were captured with Amersham Imager 600 (GE health care life sciences). The optical density (O.D.) of western blot bands was quantified using Image J (NIH, Bethesda, MD, USA) software for Windows. Each value was derived from the ratio between arbitrary units obtained by the protein band and the β-actin band. The results were expressed as ratio/β-actin.

2.9. Fluorescence analysis

The Fluoro Jade C (FJC) staining was carried out because it is a marker of degenerating cells (Schmued et al., 2005). In this assay, the mice were deeply anaesthetized with ketamine/xylazine (150/10 mg/kg); subsequently, they were perfused with saline solution followed by cold 4% paraformaldehyde (PFA). Brains were removed, fixed in 4% PFA and embedded in paraffin. Hippocampal sections (8 µm) were cut in a microtome (Thermo Fisher Scientific, HM 325, USA) and mounted on Super Frost-Plus glass slides (Thermo 213 Scientific, Rockford, IL, USA). Hippocampus slices were deparaffinized, rehydrated, and boiled three times in 10 mM citrate buffer, pH 6. Sections were blocked for 1 h

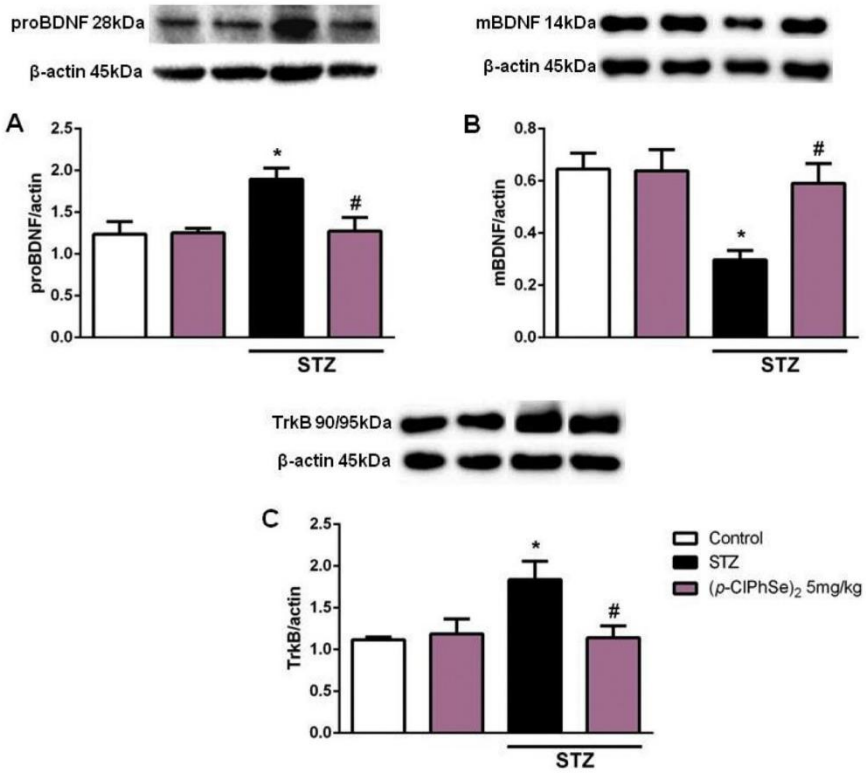

Fig. 6. Effects of $(p\text{-ClPhSe})_2$ (5 mg/kg) on proBDNF (A), mBDNF (B) and TrkB (C) levels in the hippocampus of mice. Representative qualitative Western blotting analysis is at the top of figure. Each column represents the mean ± S.E.M. of 6 animals/group. *$p < 0.05$ as compared with the control group; #$p < 0.05$ as compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

with sodium phosphate buffer (PBS) containing 0.1% (v/v) Triton X-100 (PBS-Tx) and 10% (v/v) normal donkey serum at room temperature. After that, the sections were washed three times in PBS and incubated with 5 μg/ml DAPI (Invitrogen) for 5 min. The sections were washed with PBS and incubated with 0.0001% (v/v) FJC staining (Merck Millipore, USA) for 10 min. Subsequently, the sections were washed three times in PBS and mounted on slides, covered with coverslips. The images of hippocampal sections were obtained on fluorescence microscope. The FJC-positive cells in the dentate gyrus of the hippocampus were counted in ten different fields.

2.10. Statistical analysis

To test a Gaussian distribution, a D'Agostino-Pearson normality test was used. In order to compare experimental groups, two-way analysis of variance (ANOVA) followed by the Newman–Keuls test was used. MWM and glycemia data were analyzed by repeated measure ANOVA followed by the Newman–Keuls test. Statistical comparisons for FJC staining data were performed using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Data were analyzed using one-sample *t*-test with theoretical mean = 50% to determine object preference in the ORT. Data are expressed as mean ± standard error of the mean (S.E.M). All analyses of data were performed using Statistic version 7 for Windows software. Probability value of $p < 0.05$ was considered to be significant.

3. Results

3.1. $(p\text{-ClPhSe})_2$ did not reverse characteristic signs of diabetes in mice

The mice administered with STZ showed an increase in the characteristic signs of diabetes, such as food (Fig. 2A; Table 3S) and water (Fig. 2B; Table 3S) intake, diuresis (Fig. 2C; Table 3S), hyperglycemia (Fig. 3B; Table 3S), an increase in glycated hemoglobin (Table 1; Table 3S); and the presence of glucose, ketones and protein in urine (Table 1S). In addition, the diabetic mice showed a reduction in the Δ body weight (Fig. 3A; Table 3S) and urine pH (Table 2S; Table 3S). Besides, no significant changes in others physical and chemical properties of urine (Table 2S; Table 3S).

Both doses of $(p\text{-ClPhSe})_2$ showed no effect on food intake (Fig. 2A; Table 3S), water intake (Fig. 2B; Table 3S), diuresis (Fig. 2C; Table 3S), glycated hemoglobin (Table 1; Table 3S), Δ body weight (Fig. 3A; Table 3S) and and urine pH (Table 2S; Table 3S) in diabetic mice. Furthermore, treatment with $(p\text{-ClPhSe})_2$ at the dose of 5 mg/kg attenuated hyperglycemia at day 21 after induction with STZ (Fig. 3B; Table 3S). This dose of $(p\text{-ClPhSe})_2$ reduced the presence of ketones and protein in urine of diabetic mice (Table 1S).

3.2. $(p\text{-ClPhSe})_2$ reversed hepatic and renal toxicity in diabetic mice

The diabetic mice had AST and ALT activities increased when compared to those of control group (Table 2; Table 4S). Both doses of $(p\text{-ClPhSe})_2$ were effective against the increase of AST and ALT

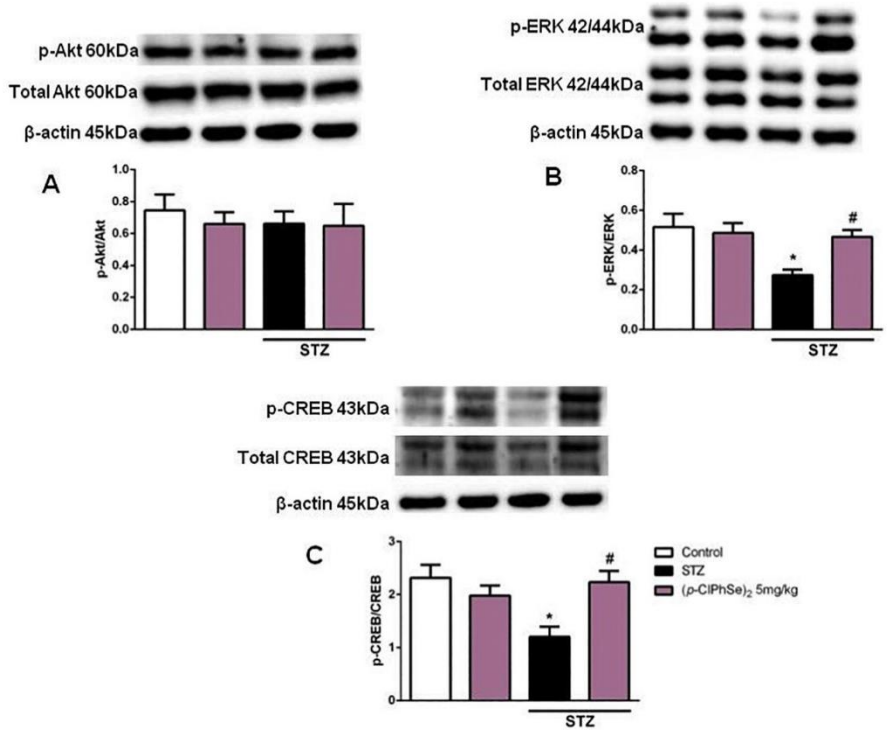

Fig. 7. Effects of $(p\text{-ClPhSe})_2$ (5 mg/kg) on the phosphorylation ratio of proteins Akt (A), ERK (B) and CREB (C) in the hippocampus of mice. Representative qualitative Western blotting analysis is at the top of figure. Each column represents the mean ± S.E.M. of 6 animals/group. *p < 0.05 as compared with the control group; #p < 0.05 as compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

activities induced by diabetes in mice.

Diabetic mice had increased urea levels when compared to those of the control group (Table 2; Table 4S). Treatments with $(p\text{-ClPhSe})_2$, at both doses, were effective against the increase of urea levels caused by diabetes in mice.

3.3. $(p\text{-ClPhSe})_2$ reversed memory impairment of diabetic mice in the ORT and OLT

During the ORT and OLT training sessions, mice of all groups spent similar time exploring the identical objects (data not shown, $p > 0.05$).

The diabetic mice showed a decrease in the recognition index when compared to those of the control group in the ORT (Fig. 4A; Table 5S). Treatment with both doses of $(p\text{-ClPhSe})_2$ was effective against the decrease in the recognition index of diabetic mice in the ORT. Regarding the OLT, the diabetic mice showed a decrease in the location index when compared to those of the control group (Fig. 4B; Table 5S). Only at the highest dose, $(p\text{-ClPhSe})_2$ reversed the decrease in the location index of diabetic mice in the OLT.

In the ORT, mice in the control group and those in $(p\text{-ClPhSe})_2$-treated groups explored the novel object longer than the familiar (one-sample Student's *t*-test with theoretical mean = 50%). Furthermore, diabetic mice treated with both doses of $(p\text{-ClPhSe})_2$ also explored the novel object longer than the familiar one (one-sample Student's t-test with theoretical mean = 50%), which indicates the preservation of memory (Table 6S). In contrast, diabetic mice spent the same amount of time exploring the novel and the familiar objects, which suggests that they do not have intact memory (one-sample Student's t-test with theoretical mean = 50%) (Table 6S).

3.4. $(p\text{-ClPhSe})_2$ reversed memory impairment of diabetic mice in the MWM test

Fig. 5A shows that the latency to reach the platform in the 4 days of MWM training was similar between experimental groups. There was no statistically significant difference between groups with respect to the latency to reach the platform in the 4 days of MWM training (Table 5S).

Diabetic mice decreased the travelled distance in the target quadrant when compared to that of the control group (Fig. 5B; Table 5S). Both doses of $(p\text{-ClPhSe})_2$ were effective against the decrease of travelled distance in the target quadrant induced by diabetes in the MWM test (Fig. 5B; Table 5S).

3.5. Treatments did not alter the mouse spontaneous behavior

The locomotor and exploratory activities were similar among mice from all experimental groups (Table 3, Table 5S).

3.6. $(p\text{-ClPhSe})_2$ modulated the BDNF/TrkB signaling in diabetic mice

Fig. 6A and C show that the treatment with $(p\text{-ClPhSe})_2$ was effective against the increase in the levels of proBDNF and TrkB in the hippocampus of diabetic mice (Table 7S).

Treatment with $(p\text{-ClPhSe})_2$ reversed the decrease in the levels of mBDNF, the p-ERK/ERK ratio, and the p-CREB/CREB ratio induced by diabetes in the hippocampus of mice (Fig. 6B, 7B and C; Table 7S).

Fig. 7A shows that the p-Akt/Akt ratio was similar among the experimental groups (Table 7S).

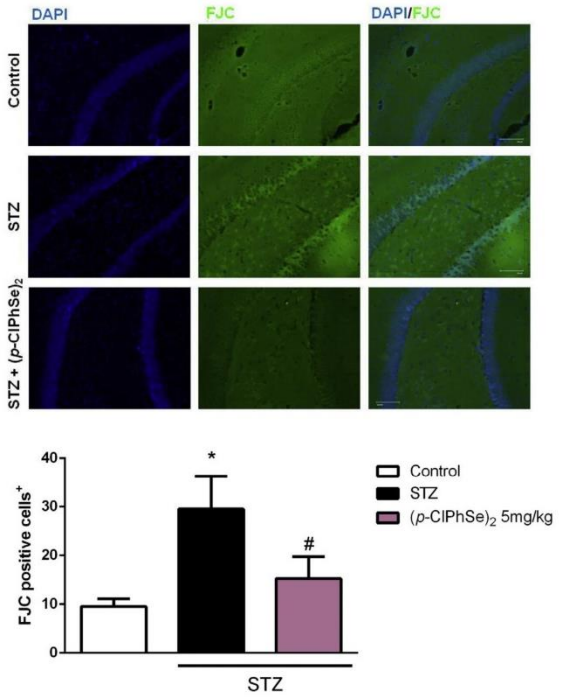

Fig. 8. Effects of (p-ClPhSe)$_2$ (5 mg/kg) on the Fluoro-Jade C staining for degenerative neuronal cells in the hippocampal DG of diabetic mice. Representative images for each group at the top of figure. Each column represents the mean ± S.E.M. of 8 animals/group. *p < 0.05 as compared with the control group; #p < 0.05 as compared with the diabetic group. Data were analyzed through one-way (ANOVA) followed by the Newman-Keuls test.

3.7. (p-ClPhSe)$_2$ reduced FJC-positive cells staining in the hippocampal DG of diabetic mice

FJC-positive cells were increased in the hippocampal DG of diabetic when compared to control mice (Fig. 8; Table 8S). The treatment with (p-ClPhSe)$_2$ was effective against the statistically significant increase of FJC-positive cells in the hippocampal DG of diabetic mice.

4. Discussion

The main findings of our study indicate memory impairment in diabetic mice, which was demonstrated in well-validated behavior tests designated to evaluate memory, ORT, TLO, and MWM (Wolf et al., 2016). Moreover, this study provided evidence that treatment with (p-ClPhSe)$_2$ reversed memory impairment induced by diabetes, which was accompanied by the modulation of hippocampal proteins of the BDNF/TrkB pathway signaling (Fig. 9).

STZ systemic administration has been widely studied as an animal model of type 1 diabetes (Yang et al., 2018). STZ is a toxin that destroys the insulin secreting pancreatic beta cells, which causes a subsequent hyperglycemia. In the present study, the STZ model of diabetes, which was characterized by persistent hyperglycemia (Fig. 3B), increased HbA1c (Table 1), the presence of ketone bodies, protein, and glucose in urine (supplementary data, Table 1S). Furthermore, the food and water intake, as well as diuresis were increased (Fig. 2A, B and C), but the body weight (Fig. 3A) and urine pH (Table 2S) were reduced in diabetic mice. Although the treatment with (p-ClPhSe)$_2$ was effective against diabetes-induced hyperglycemia, it was ineffective against all other parameters of the diabetic phenotype. Therefore, it is possible that would be necessary a more prolonged treatment with compound to achieve metabolic homeostasis.

A study performed with a model of diabetes revealed cognitive impairment, neuroinflammation, and neuronal synaptic loss in rats administered with STZ (Zhang et al., 2013). Further, it was reported that hyperglycemia induced hippocampal and cortical neuronal damage, resulting in the spatial learning and memory impairment of the animals (Zhang et al., 2008). Thus, our results corroborate with previously published data by Chen et al. (2018), in which diabetic mice had memory impairment in the hippocampal-dependent spatial memory tests, MWM and OLT, and in a non-spatial memory test, such as ORT (de Senna et al., 2015). Our results also indicate that (p-ClPhSe)$_2$ reversed memory impairment in diabetic mice, and that the beneficial effects on memory were not parallel to treatment-dependent effects on peripheral metabolism. This because the treatment with (p-ClPhSe)$_2$ was not effective against most metabolic phenotype characteristics altered in diabetic animals.

Regarding the modulation of BDNF/TrkB pathway, (p-ClPhSe)$_2$ treatment positively modulated mBDNF and TrkB levels, and it promoted phosphorylation of key proteins, such as ERK and CREB, reversing memory impairment induced by diabetes. It is possible that (p-ClPhSe)$_2$ up-regulates the BDNF pathway by promoting the downstream induction of proteins, such as ERK and CREB, contributing to the memory enhancer effects (Table 9S). However, to support the hypothesis that (p-ClPhSe)$_2$ directly affects the BDNF pathway other experiments would be required. Despite this, our data allow the interpretation that the BDNF pathway contributes to the beneficial effects of (p-ClPhSe)$_2$ on memory impairment in diabetic mice. Furthermore, our research group have reported that the unsubstituted diphenyl diselenide, a parent compound of (p-ClPhSe)$_2$, increased the mBDNF levels in the hippocampus of aged rats (Cechella et al., 2018; Leite et al., 2016), strengthening the idea of link between the effects of organoselenium compounds on BDNF levels and in the memory.

It is well established that BDNF is one of the neurotrophic factors that supports differentiation, maturation, and survival of neurons in the central nervous system (Acheson et al., 1995; Binder and Scharfman, 2004). The proBDNF is cleaved by a convertase enzyme to form the biologically active mBDNF (Schildt et al., 2013) that binds to its TrkB receptor, resulting in the recruitment of proteins that activate different signal transduction cascades. In the present study, a decrease in the hippocampal BDNF/TrkB signaling pathway, which plays an essential role in memory processes, was found in diabetic mice. A decrease of BDNF levels was demonstrated in experimental models of diabetes (Yang and Gao, 2017) and that a restoration of BDNF content may be an alternative to prevent complications associated to diabetes, and possibly the development of neurodegenerative diseases (Yamanaka et al., 2008). It is also well reported that acute and chronic interference with the BDNF/TrkB signaling damage long-term potentiation in hippocampus of mouse (Schildt et al., 2013).

It has become clear over the last decade that diabetes is a pathology that modifies the morphological integrity of the hippocampus, which contributes to the risk of developing cognitive problems in this disease (Ho et al., 2013). A growing body of evidence suggests that neurodegenerative processes play critical roles in the pathophysiology of diabetes (Wang et al., 2014). Although the neurodegenerative process was not the main focus of our present study, increased FJC-positive cells were found in the hippocampal DG of diabetic mice. Regarding the (p-ClPhSe)$_2$ effects on FJC, the neuroprotective action of (p-ClPhSe)$_2$ was highlighted, but future experiments should be designed to investigate its mechanism of action.

Concerning signs of hepatic and renal toxicity, both doses of (p-ClPhSe)$_2$ reversed damage induced by diabetes (Table 2). In this regard, (p-ClPhSe)$_2$ is an organoselenium compound that did not cause toxicity in mice (Zborowski et al., 2016).

In conclusion, the present study demonstrates that (p-ClPhSe)$_2$ was

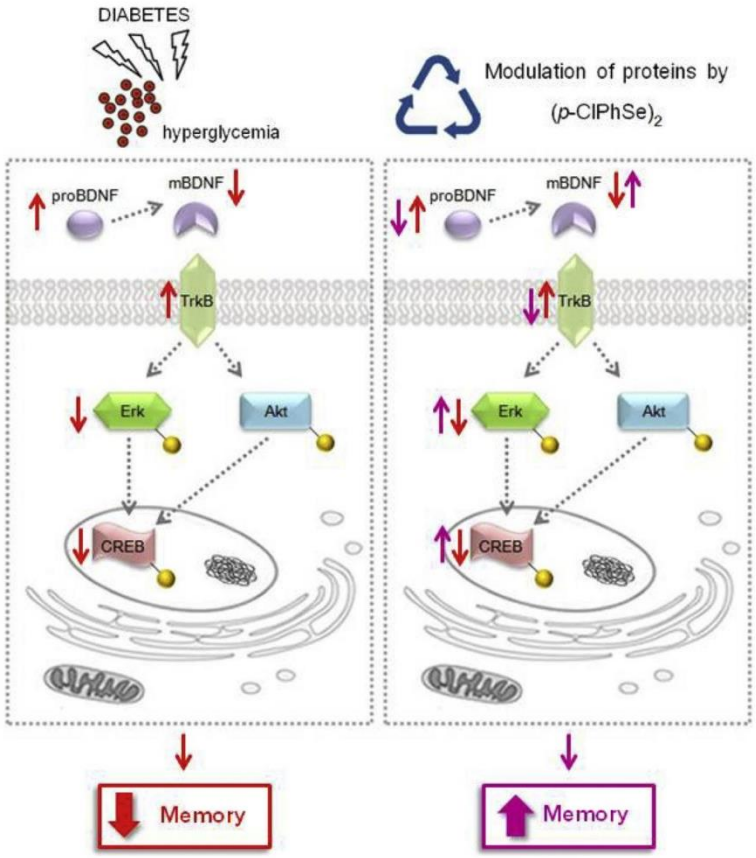

Fig. 9. Overview of neurotoxicity induced by STZ and modulatory effects of $(p\text{-ClPhSe})_2$ on the BDNF/TrkB pathway signaling in the hippocampus of diabetic mice. Red arrows indicate effects in diabetic mice. Violet/Red arrows indicate effects of $(p\text{-ClPhSe})_2$ in diabetic mice. BDNF - brain-derived neurotrophic factor, TrkB - Tropomyosin receptor kinase B, ERK - extracellular signal–regulated kinases, Akt - Protein kinase B, CREB - cAMP response element-binding protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effective against memory impairment induced by diabetes, even without reversing most of metabolic parameters characteristic of diabetes phenotype. The modulation of the hippocampal BDNF/TrkB signaling pathway and neuroprotection (Fig. 9) contributes to the beneficial effects of $(p\text{-ClPhSe})_2$ treatment on memory.

Conflict of interests

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2019.109660.

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Supporting File

(*p*-ClPhSe)$_2$ modulates hippocampal BDNF/TrkB signaling and reverses memory impairment induced by diabetes in mice

Progress in Neuro-Psychopharmacology & Biological Psychiatry

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Supplementary Table 1 Effects of (*p*-ClPhSe)$_2$ on protein, glucose and ketones in urine of diabetic mice.

Supplementary Table 2 Effects of (*p*-ClPhSe)$_2$ on physical and chemical properties of urine in diabetic mice.

Supplementary Table 3 Effect of (*p*-ClPhSe)$_2$ on parameters of the diabetic phenotype in mice.

Supplementary Table 4 Effect of (*p*-ClPhSe)$_2$ on hepatic and renal toxicity in diabetic mice.

Supplementary Table 5 Effect of (*p*-ClPhSe)$_2$ on the object recognition test (ORT), object location test (OLT), Morris water maze test (MWM) and locomotor profile in diabetic mice.

Supplementary Table 6 Effect of (*p*-ClPhSe)$_2$ on single sample t-test against 50% of the TRO in mice.

Supplementary Table 7 Effect of (*p*-ClPhSe)$_2$ on hippocampal proteins of the BDNF/TrkB pathway signaling in mice.

Supplementary Table 8 Effect of (*p*-ClPhSe)$_2$ on the Fluoro-Jade C staining for degenerative neuronal cells in the DG of hippocampus in mice.

Supplementary Table 9 Pearson correlation between distance travelled in the target quadrant of MWM test and BDNF pathway-related proteins.

Supplementary Figure 1 The track plot of the Morris water maze test that were performed on the fifth day, without the target platform.

Table 1S Effects of (*p*-ClPhSe)$_2$ on protein, glucose and ketones in urine of diabetic mice.

	Protein			
	Negative	+	++	+++
Control	**6/6**			
(*p*-ClPhSe)$_2$ 1mg/kg	**6/6**			
(*p*-ClPhSe)$_2$ 5mg/kg	**6/6**			
STZ		**6/6**		
STZ + (*p*-ClPhSe)$_2$ 1mg/kg	**5/6**	1/6		
STZ + (*p*-ClPhSe)$_2$ 5mg/kg	**6/6**			

	Glucose			
	Negative	+	++	+++
Control	**6/6**			
(*p*-ClPhSe)$_2$ 1mg/kg	**6/6**			
(*p*-ClPhSe)$_2$ 5mg/kg	**6/6**			
STZ				**6/6**
STZ + (*p*-ClPhSe)$_2$ 1mg/kg				**6/6**
STZ + (*p*-ClPhSe)$_2$ 5mg/kg				**6/6**

	Ketones			
	Negative	+	++	+++
Control	**6/6**			
(*p*-ClPhSe)$_2$ 1mg/kg	**6/6**			
(*p*-ClPhSe)$_2$ 5mg/kg	**6/6**			
STZ	1/6	**5/6**		
STZ + (*p*-ClPhSe)$_2$ 1mg/kg	**6/6**			
STZ + (*p*-ClPhSe)$_2$ 5mg/kg	**6/6**			

Representative numbers of 6 animals/group. The statistical analysis was not performed.

Table 2S Effects of (p-ClPhSe)$_2$ on physical and chemical properties of urine in diabetic mice.

	Leucocytes (WBC/µL)	Urobilinogen (mg/dL)	Bilirrubin (mg/dL)	Blood (RBC/µL)	Nitrite	pH	Density
Control	-	-	-	-	-	6.75 ± 0.24	1015 ± 1.82
(p-ClPhSe)$_2$ 1mg/kg	-	-	-	-	-	6.25 ± 0.11	1010 ± 2.23
(p-ClPhSe)$_2$ 5mg/kg	-	-	-	-	-	6.66 ± 0.16	1010 ± 3.41
STZ	-	-	-	-	-	5.67 ± 0.21*	1012 ± 2.10
STZ + (p-ClPhSe)$_2$ 1mg/kg	-	-	-	-	-	6.08 ± 0.27	1010 ± 1.82
STZ + (p-ClPhSe)$_2$ 5mg/kg	-	-	-	-	-	6.33 ± 0.16	1011 ± 1.53

Values are expressed as the mean ± S.E.M of 6 animals/group. *$p<0.05$ as compared with the control group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

Table 3S Effects of (*p*-ClPhSe)$_2$ on parameters of the diabetic phenotype in mice.

Signs of diabetes	**Variables two-way ANOVA**	**SS**	**DF**	**MS**	**F**	**p value**
Food intake	(*p*-ClPhSe)$_2$ factor	0.75	2	0.37	0.55	0.5842
	STZ factor	12.13	1	12.13	17.69	0.0002
	STZ x (*p*-ClPhSe)$_2$	1.58	2	0.79	1.15	0.3287
Water intake	(*p*-ClPhSe)$_2$ factor	1.76	2	0.88	0.44	0.6490
	STZ factor	64.96	1	64.96	32.32	0.0000
	STZ x (*p*-ClPhSe)$_2$	5.03	2	2.51	2.52	0.3004
Diuresis	(*p*-ClPhSe)$_2$ factor	1.99	2	0.99	1.34	0.2758
	STZ factor	34.32	1	34.41	46.58	0.0000
	STZ x (*p*-ClPhSe)$_2$	1.45	2	0.72	0.98	0.3856
HbA1c	(*p*-ClPhSe)$_2$ factor	0.06	1	0.06	0.32	0.5763
	STZ factor	30.83	1	30.83	165.73	0.0000
	STZ x (*p*-ClPhSe)$_2$	0.17	1	0.17	0.89	0.3551
Δ body weight	(*p*-ClPhSe)$_2$ factor	6.79	2	3.39	0.40	0.6757
	STZ factor	120.33	1	120.33	14.02	0.0005
	STZ x (*p*-ClPhSe)$_2$	32.04	2	16.02	1.87	0.1672
Urine pH	(*p*-ClPhSe)$_2$ factor	0.79	2	0.39	1.59	0.2202
	STZ factor	2.50	1	2.50	10.08	0.0034
	STZ x (*p*-ClPhSe)$_2$	1.43	2	0.71	2.88	0.0719
Urine density	(*p*-ClPhSe)$_2$ factor	79.17	2	39.58	1.31	0.2839
	STZ factor	6.25	1	6.25	0.20	0.6521
	STZ x (*p*-ClPhSe)$_2$	29.17	2	14.58	0.48	0.6211
Glycemia	**Variables two-way ANOVA repeated measures**	**SS**	**DF**	**MS**	**F**	**p value**
	Groups factor	2176690.0	3	725563.0	318.21	0.0000
	Days factor	72850.0	3	24283.0	7.15	0.0003
	Groups x Days	92101.0	9	10233.0	3.01	0.0049

Data were analyzed through two-way ANOVA or two-way ANOVA repeated measures followed by the Newman-Keuls test.

Table 4S Effects of (p-ClPhSe)$_2$ on hepatic and renal toxicity in diabetic mice.

Blood biomarkers	**Variables two-way ANOVA**	**SS**	**DF**	**MS**	**F**	**p value**
AST	(p-ClPhSe)$_2$ factor	9095.3	2	4547.6	13.19	0.0000
	STZ factor	31445.5	1	31445.5	91.24	0.0000
	STZ x (p-ClPhSe)$_2$	6341.3	2	3170.7	9.20	0.0004
ALT	(p-ClPhSe)$_2$ factor	4500.7	2	2250.3	14.83	0.0000
	STZ factor	810.2	1	810.2	5.34	0.0258
	STZ x (p-ClPhSe)$_2$	2610.6	2	1305.3	8.60	0.0007
Urea	(p-ClPhSe)$_2$ factor	249.20	2	124.60	9.44	0.0004
	STZ factor	798.95	1	798.95	60.55	0.0000
	STZ x (p-ClPhSe)$_2$	95.62	2	47.81	3.62	0.0353

Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

Table 5S Effects of (p-ClPhSe)$_2$ on behavior tests of diabetic mice.

Behavior	**Variables two-way ANOVA**	**SS**	**DF**	**MS**	**F**	**p value**
ORT	(p-ClPhSe)$_2$ factor	0.056	2	0.028	2.42	0.1009
	STZ factor	0.009	1	0.009	0.82	0.3687
	STZ x (p-ClPhSe)$_2$	0.125	2	0.063	5.46	0.0078
OLT	(p-ClPhSe)$_2$ factor	0.112	2	0.056	5.18	0.0097
	STZ factor	0.014	1	0.014	1.31	0.2582
	STZ x (p-ClPhSe)$_2$	0.232	2	0.116	10.70	0.0001
MWM	(p-ClPhSe)$_2$ factor	1.98	2	0.99	2.14	0.1305
	STZ factor	14.30	1	14.30	30.73	0.0000
	STZ x (p-ClPhSe)$_2$	3.32	2	1.66	3.57	0.0370
Latency to reach the platform (MWM)	**Variables two-way ANOVA repeated measures**	**SS**	**DF**	**MS**	**F**	**p value**
	Groups factor	1215.8	5	243.2	2.95	0.0227
	Days factor	3309.8	3	1103.3	17.93	0.0000
	Groups x Days	1466.6	15	97.8	1.59	0.0856
Locomotor profile	**Variables two-way ANOVA**	**SS**	**DF**	**MS**	**F**	**p value**
Crossing	(p-ClPhSe)$_2$ factor	33865.9	2	16932.9	1.13	0.3300
	STZ factor	15108.8	1	15108.8	1.01	0.3192
	STZ x (p-ClPhSe)$_2$	10639.7	2	5319.8	0.35	0.7014
Distance	(p-ClPhSe)$_2$ factor	12680431.1	2	6340215.5	1.31	0.2805
	STZ factor	27664033.3	1	27664033.3	5.71	0.0213
	STZ x (p-ClPhSe)$_2$	84804.1	2	42402	0.00	0.9912

Data were analyzed through two-way ANOVA and two-way ANOVA repeated measures followed by the Newman-Keuls test. Object recognition test (ORT), object location test (OLT) and Morris water maze test (MWM).

Table 6S The single sample t-test against 50% of the TRO.

	Single sample t-test against 50% in TRO		
	t	**df**	**p value**
Control	t=12.51	df=14	< 0.0001
(*p*-ClPhSe)$_2$ 1mg/kg	t=5.90	df=14	< 0.0001
(*p*-ClPhSe)$_2$ 5mg/kg	t=2.80	df=14	0.0142
STZ	t=0.74	df=14	0.4676
STZ + (*p*-ClPhSe)$_2$ 1mg/kg	t=2.22	df=14	0.0429
STZ + (*p*-ClPhSe)$_2$ 5mg/kg	t=4.03	df=14	0.0012

Data were analyzed by an unpaired Student's test.

Table 7S Effects of (*p*-ClPhSe)$_2$ on the hippocampal BDNF/TrkB pathway proteins of diabetic mice.

Western Blot	Variables two-way ANOVA	SS	DF	MS	F	p value
mBDNF	(*p*-ClPhSe)$_2$ factor	0.123	1	0.123	4.74	0.0415
	STZ factor	0.236	1	0.236	9.09	0.0068
	STZ x (*p*-ClPhSe)$_2$	0.135	1	0.135	5.22	0.0332
proBDNF	(*p*-ClPhSe)$_2$ factor	0.554	1	0.554	5.26	0.0327
	STZ factor	0.694	1	0.694	6.58	0.0184
	STZ x (*p*-ClPhSe)$_2$	0.615	1	0.615	5.83	0.0254
TrkB	(*p*-ClPhSe)$_2$ factor	0.589	1	0.589	3.76	0.0664
	STZ factor	0.673	1	0.673	4.30	0.0511
	STZ x (*p*-ClPhSe)$_2$	0.877	1	0.877	5.61	0.0280
p-Akt/Akt	(*p*-ClPhSe)$_2$ factor	0.014	1	0.014	0.23	0.6329
	STZ factor	0.013	1	0.013	0.22	0.6415
	STZ x (*p*-ClPhSe)$_2$	0.008	1	0.008	0.13	0.7218
p-ERK/ERK	(*p*-ClPhSe)$_2$ factor	0.041	1	0.041	3.00	0.0984
	STZ factor	0.103	1	0.103	7.66	0.0118
	STZ x (*p*-ClPhSe)$_2$	0.075	1	0.075	5.59	0.0281
p-CREB/CREB	(*p*-ClPhSe)$_2$ factor	0.731	1	0.731	2.71	0.1153
	STZ factor	1.110	1	1.110	4.11	0.5560
	STZ x (*p*-ClPhSe)$_2$	2.827	1	2.827	10.48	0.0041

Data were analyzed through two-way ANOVA followed by the Newman-Keuls test.

Table 8S Effects of (*p*-ClPhSe)$_2$ on the Fluoro-Jade C staining in the DG of hippocampus in diabetic mice.

	Variables one-way ANOVA	**SS**	**DF**	**MS**	**F**	**p value**
FJC staining	between groups	1696.0	2	848.2	4.68	0.0208
	within groups	3802.0	21	181.0		

Data were analyzed through one-way ANOVA followed by the Newman-Keuls test.

Table 9S Pearson correlation between travelled distance in the target quadrant of MWM test and BDNF pathway-related proteins.

		Pearson's coefficient	
		r	p value
Distance in the target quadrant of MWM	mBDND	0.4886	0.0154
	proBDNF	-0.4955	0.0138
	TrkB	-0.492	0.0146
	ERK	0.6727	0.0003
	CREB	0.4329	0.0346

The Pearson's correlation coefficient was used for correlation analysis.

Figure 1S

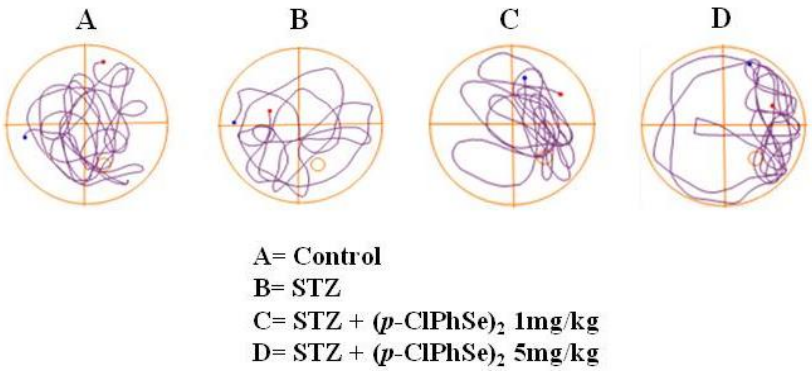


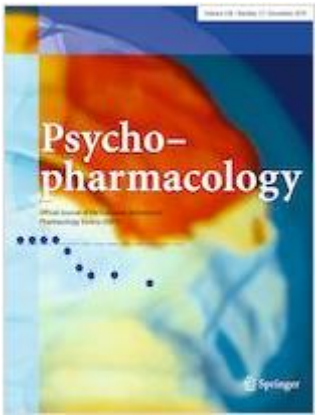
Fig 1S The track plot of the Morris water maze test that were performed on the fifth day (probe trial), without the target platform.

3.2 ARTIGO 2

Via de sinalização Keap1/Nrf2/HO-1 contribui para ação do tipo antidepressiva do *p*-clorodifenil disseleneto em camundongos diabéticos

Keap1/Nrf2/HO-1 signaling pathway contributes to *p*-chlorodiphenyl diselenide antidepressant-like action in diabetic mice

Vanessa Angonesi Zborowski, Suélen Osório Heck, Marina Vencato, Simone Pinton, Luiza Souza Marques, Cristina Wayne Nogueira



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ORIGINAL INVESTIGATION

Keap1/Nrf2/HO-1 signaling pathway contributes to *p*-chlorodiphenyl diselenide antidepressant-like action in diabetic mice

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Abstract

Rationale The association between depression and diabetes has been recognized for many years, but the nature of this relationship remains uncertain.

Objectives This study investigated the antidepressant-like effect of $(p\text{-ClPhSe})_2$ on mice made diabetic by streptozotocin (STZ) and the contribution of cerebral cortical Keap1/Nrf2/HO-1 signaling pathway for this effect.

Methods Male adult Swiss mice received streptozotocin (STZ, 200 mg/kg, i.p.) to induce diabetes (glycemia ≥ 200 mg/dl) or citrate buffer (5 ml/kg, control group). The mice were treated with $(p\text{-ClPhSe})_2$ at the dose of 5 mg/kg, i.g., for 7 days. Mice performed behavior tests, tail suspension (TST), and forced swimming tests (FST), to evaluate depressive-like phenotype.

Results Diabetic mice showed an increase in immobility time in the TST and FST when compared to the control group. The protein contents of Keap1/Nrf2/HO-1 pathway were decreased in the cerebral cortex of diabetic mice. Diabetic mice had an increase in the relative adrenal weight and a decrease in the protein content of glucocorticoid receptor. The levels of TBARS and RS and SOD activity were found altered in the cerebral cortex of diabetic mice. The number of FJC-positive cells was increased in the cerebral cortex of diabetic mice. Treatment with $(p\text{-ClPhSe})_2$ was effective against depressive-like phenotype, oxidative stress, and FJC-positive cells of diabetic mice. $(p\text{-ClPhSe})_2$ did not reverse the parameters of HPA axis evaluated in this study. $(p\text{-ClPhSe})_2$ modulated the cerebral cortical Keap1/Nrf2/HO-1 pathway in diabetic mice.

Conclusions This study demonstrates the contribution of cerebral cortical Keap1/Nrf2/HO-1 pathway in the $(p\text{-ClPhSe})_2$ antidepressant-like action in diabetic mice.

Keywords Depressive-like · Organoselenium · Selenium · Keap1/Nrf2/HO-1 · Diabetes

Introduction

The prevalence of clinical depression and the presence of depressive symptoms are higher among people with diabetes compared with the general population (Talbot and Nouwen 2000). In experimental models, it has been reported that diabetic animals administered with streptozotocin (STZ) present depressive-like phenotype (Lenart et al. 2019; Zhou et al. 2017). Although the association between depression and diabetes has been recognized for many years (Anderson et al. 2001), the nature of this relationship remains uncertain.

The occurrence of both pathologies could be attributed to a variety of factors, but the underlying mechanisms concerning the high occurrence of these two diseases are not fully understood (Reus et al. 2017). Different lines of evidence suggest that hyperglycemia in diabetes is a chronic metabolic stressor and, consequently, this endocrine disorder may produce its effects on the central nervous system (CNS) through apoptosis and astrogliosis (Shi et al. 2018), oxidative stress and inflammation (Reus et al. 2016), alterations in the neuronal structure (Castillo-Gomez et al. 2015), and increase of hypothalamo-pituitary-adrenocortical (HPA) activity (Chan et al. 2001).

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Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and endogenous antioxidant enzymes, resulting in damage to biomolecules, including DNA, proteins, and lipids (Muriach et al. 2014). Thus, agents that attenuate oxidative stress or associated factors, such as inflammation and energy metabolism impairment, could be important therapeutic targets for treatment of diabetes and depression (Reus et al. 2016). Because oxidative stress is associated with diabetes and depression pathological processes (Reus et al. 2017) and 4-4′-dichlorodiphenyl diselenide (p-ClPhSe)$_2$ is an antioxidant (Prigol et al. 2009), we hypothesized that boosting nuclear factor erythroid-2-related factor 2 (Nrf2) antioxidant capacity with (p-ClPhSe)$_2$ would contribute to its antidepressant-like action in a STZ-diabetic model.

In fact, the Nrf2 is an important factor in the inducible expression of cellular defense enzymes (Suzuki and Yamamoto 2015). Besides, the Kelch-like ECH-associated protein 1 (Keap1) plays a sensory role for detecting oxidative and electrophilic stresses (Uruno et al. 2015). Thus, the Keap1 causes Nrf2 to be degraded through the ubiquitin–proteasome pathway and ensures that Nrf2 is suppressed under unstressed conditions (Uruno et al. 2015). However, under stressful conditions, Nrf2 transcribes many neuro-protective genes having antioxidant response element (ARE) in their promoter region (Yin et al. 2015). These include mainly heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase 1, γ-glutamyl cysteine ligase (γ-GCL), and superoxide dismutase (SOD) etc. These downstream pathways together maintain the redox status of cells (Satoh et al. 2006).

(p-ClPhSe)$_2$, an organoselenium compound that belongs to the class of disubstituted diaryl diselenides, has been reported to have a number of peripheral and central pharmacological properties in rodents. Among these properties, we highlight the anorectic action in rats (Bortolatto et al. 2017), the regulation of metabolic alterations induced by high fructose load in rats (Quines et al. 2017), memory enhancer in aged rats (Bortolatto et al. 2012), and in corticosterone-exposed mice (Bortolatto et al. 2012; Zborowski et al. 2016) as well as antidepressant in aged rats (Bortolatto et al. 2012).

In the present study, we investigated the effects of (p-ClPhSe)$_2$ on the depressive-like phenotype induced by systemic administration of STZ in mice. The contribution of cerebral cortical Keap1/Nrf2/HO-1 signaling pathway for the antidepressant-like action of (p-ClPhSe)$_2$ was investigated in mice made diabetic by the STZ administration.

Materials and methods

Animals

The experiments were carried out by using male adult Swiss mice (60 days old, 25–35 g) from our own breeding colony. Swiss out bred mice were obtained from the Central Animal Facility of Federal University of Santa Maria (Rio Grande do Sul, Brazil). The animals were housed in cages with free access to food and water. They were kept in a separate animal room on a regular cycle of 12 h light/12 h dark, in a constant temperature environment (22 ± 2 °C). All experimental procedures were approved by the Ethical Research Committee of Federal University of Santa Maria/Brazil (#2964150317/2017), affiliated to the Council for Control of Animal Experiments (CONCEA). The procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines (record number 10684).

Chemicals

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-4′-Dichlorodiphenyl diselenide (p-ClPhSe)$_2$ was prepared and characterized in our laboratory according to the method previously described by Paulmier (1986). Analysis of the 1HNMR and 13C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of studied compound (99.9%) was determined by gas chromatography–mass spectrometry.

Experimental protocol

After a week of acclimatization in the animal room, all animals were randomly separated into four experimental groups (n = 6 animals/group for all analyses), as follows: (I) control (STZ vehicle and mineral oil); (II) diabetic (STZ and mineral oil); (III) (p-ClPhSe)$_2$ (STZ vehicle and 5 mg/kg compound); and (IV) diabetic + (p-ClPhSe)$_2$ (STZ and 5 mg/kg compound).

Mice from groups II and IV received STZ at a single dose of 200 mg/kg, prepared in 0.5 M citrate buffer pH 4.5, by the intraperitoneal route to induce diabetes (Gupta et al. 2014). The STZ solution was freshly prepared in citrate buffer adjusted to pH 4.5 because the maximum stability is found in pH 4 (de la Garza-Rodea et al. 2010; Grieb 2016; Nayak et al. 2014). In order to minimize discomfort, the volume of intraperitoneal administration was reduced to 5 ml/kg rather than 10 ml/kg, as commonly used. Therefore, we believe that abdominal discomfort of mice was minimal. In addition, we did not observe physical demonstration of pain after the citrate buffer injection. Moreover, to reduce any confound factor that the STZ vehicle injection could cause in mice, all control animals (non-diabetic and non-diabetic compound-treated groups) received the i.p. injection of the STZ vehicle solution. After 14 days of STZ injection, mice with blood glucose above ≥ 200 mg/dl were considered diabetic. Animals that did not develop hyperglycemia were not used in this study.

Mice from groups III and IV received an intragastric (i.g.) administration of $(p\text{-ClPhSe})_2$, once a day, during the last week of experiment (Fig. 1). Furthermore, the animals from groups I and II were administered with the compound vehicle (mineral oil, 10 ml/kg). The dose of $(p\text{-ClPhSe})_2$ treatment was chosen based on our previously published study (Zborowski et al. 2016).

At day 21 of the experimental protocol, mice performed the behavioral tests (Fig. 1), locomotor profile (LP), tail suspension test (TST), and forced swimming test (FST).

For ex vivo assays, the animals were divided into two sets to process the brain samples: for the first set, the animals were killed by cervical dislocation, the brains were removed and samples of whole cerebral cortex were immediately dissected on a cold plate, these samples were designated to Western blot and oxidative stress assays. Furthermore, the adrenal glands were removed and weighed. The results were expressed as relative weight [adrenal weight/body weight (g)].

For the second processing batch, mice were deeply anesthetized with ketamine/xylazine (150/10 mg/kg) and perfused through the left cardiac ventricle with 0.9% saline solution, followed by cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. After perfusion, the brains were removed, post-fixed in the same fixative solution by 24 h, and these samples were used for the Fluoro Jade C assay.

Glycemia

At days 14 and 21 after STZ induction, glycemia was measured in blood from the tail of 4-h-fasted mice using a blood glucose meter (Accu-chek active glucometer). The blood glucose measurement was performed 4 h after the behavioral tests.

Behavioral tests

Tail suspension test (TST)

The TST was performed in a quiet experimental room according to the method reported by Steru et al. (1985). Each mouse was suspended by its tail to a horizontal wooden bar located approximately 50 cm above the floor. The mouse was secured to the bar by adhesive tape placed 1 cm from the tip of the tail. The trial was conducted for 6 min during which a blinded observer scored the total duration of immobility by using a stopwatch. The mouse was considered immobile only when it hung passively and completely motionless.

Forced swimming test (FST)

The procedure used in this study was based on that previously described by Porsolt et al. (1979). Mice were gently placed in an inescapable cylindrical container (10 × 25 cm^2) that was filled with water (19 cm, 25 ± 1 °C) and their escape related mobility behavior (total duration of floating) was measured by a blinded observer during a 6-min period by using a stopwatch. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

Locomotor profile (LP)

To discard non-specific effects of treatments, the spontaneous locomotor activity of mice was performed in the locomotor activity monitor (LMA). LMA is a Plexiglas cage (45 × 45 × 45 cm^3) surrounded by a frame consisting of 32 photocells mounted on opposite walls (16 L × 16 W, spaced 2 cm apart)

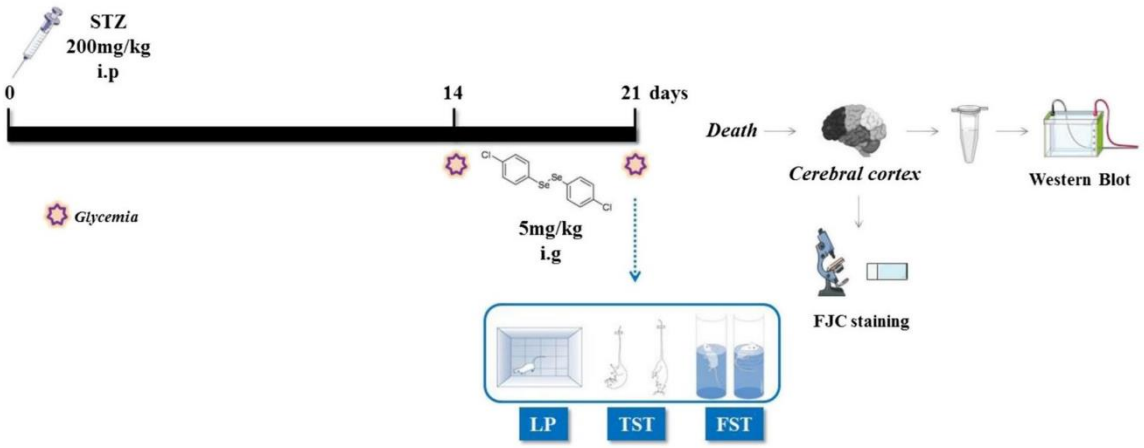

Fig. 1 Schematic representation of the experimental design. On day 21 of the experimental protocol, mice performed the behavior tests, locomotor profile (LP), tail suspension test (TST), forced swimming test (FST). Ex vivo assays were carried out after the last dose of $(p\text{-ClPhSe})_2$

that continuously tracks the animal movement. The animals were placed in the center of the apparatus and allowed to freely explore the arena during 4 min. Motor activity was monitored with the Insight® Monitor Activity System. The number of crossings and total distance traveled (dm) were recorded.

Oxidative damage markers

It has been reported that cerebral oxidative stress may play a role in the pathogenesis of depression (Bajpai et al. 2014). Therefore, parameters of oxidative stress were determined in the whole cerebral cortex of mice from all experimental groups. The samples of cerebral cortex were homogenized (1:5, *w*/*v*) in 50 mM of Tris-HCl at pH 7.4. The homogenates were then centrifuged at 2500×*g* for 10 min at 4 °C and the low-speed supernatant was used to determine the levels of thiobarbituric acid reactive substances (TBARS) and reactive species (RS), and activities of superoxide dismutase (SOD) and catalase (CAT).

Lipid peroxidation

TBARS, a measure of lipid peroxidation, were determined in supernatant according to the method described by Ohkawa et al. (1979). An aliquot of supernatant (200 μl) was added to the reaction mixture containing 500 μl TBA (0.8%), 200 μl sodium dodecyl sulfate (SDS, 8.1%), and 500 μl acetic acid (pH 3.4) with subsequent incubation at 95 °C for 1 h. The reaction product was determined at 532 nm and the results were expressed as nmol MDA/g tissue.

RS

RS levels were measured as reactive species to 2′, 7′-dichlorofluorescein diacetate (DCHF-DA). DCHF-DA is used as a fluorescent probe to measure RS levels, as it is easily oxidized to fluorescent dichlorofluorescein (DCF) (Hempel et al. 1999). The oxidation of DCHF-DA to DCF was determined at 488 nm for excitation and 525 nm for emission. An aliquot of 1 mM DCHF-DA in ethanol was added to a mixture containing 10 μl of supernatant and 3 ml of 10 mM Tris-HCl pH 7.4, incubated for 1 h at 37 °C, protected from light. DCF fluorescence intensity was expressed as fluorescence intensity (arbitrary units)/mg protein.

Antioxidant enzymes

Aliquots of supernatant were diluted 1:10 (*v*/*v*) to determine the activity of SOD, according to the method described by Misra and Fridovich (1972) with the following modifications: supernatant aliquots (6, 12, and 18 μl) of each sample were added in 260 μl of a 57.7 mM Na_2CO_3 buffer (pH 10.3). Enzymatic reaction was started adding 30 μl of 6 mM epinephrine. The colored reaction product was measured spectrophotometrically at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. The enzymatic activity was expressed as U/mg protein.

The CAT activity was determined in the supernatant according to Aebi (1984) by the reaction of 100 μl of supernatant and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.5. The enzymatic activity was measured at 240 nm and expressed as U/mg protein (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7 at 25 °C).

Protein content

The protein content in each sample was estimated using the Bradford method (Bradford 1976) and bovine serum albumin (1 mg/ml) as a standard.

Western blot

Samples of whole cerebral cortex were manually homogenized in a glass–glass potter in ice-cold with RIPA buffer (Sigma-Aldrich Co., St. Louis, Missouri, USA) in the presence of protease and phosphatase inhibitors cocktail (Sigma-Aldrich Co., St. Louis, Missouri, USA). The samples of whole cerebral cortex were diluted to a final protein concentration of 2 μg/μl in sample buffer. The samples (20 μg of protein) and prestained molecular weight standards (Sigma-Aldrich Co., St. Louis, Missouri, USA) were separated by 10% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (0.45 μm, Bio-rad) using Transfer-Blot® Turbo™ Transfer System (1.0 A; 45 min) and equal protein loading was confirmed by Ponceau S staining. After blocking with 3% bovine serum albumin solution, the blots were incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: rabbit anti-glucocorticoid receptor (GR) (1:250, Santa Cruz), mouse anti-heme oxygenase 1 (HO-1-1) (1:1000, Abcam), goat anti-Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1 (Keap1) (1:1000, Santa Cruz), rabbit anti-nuclear factor (erythroid 2-derived)-like 2 (Nrf2) (1:1000, Santa Cruz). Mouse anti-β-actin (1:1000, Cell Signaling) was stained as additional control of the protein loading.

After the primary antibody incubations, membranes were washed and incubated for 1 h at room temperature with anti-mouse, anti-goat, or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:5000, Bio-Rad Laboratories, Hercules, CA, USA). For protein detection, we used chemiluminescence kit (Amersham, São Paulo, Brazil) and the signals were captured with Amersham Imager 600 (GE health care life sciences). Optical density (O.D. of Western blotting bands was quantified using Image J (NIH, Bethesda, MD, USA) software for Windows. Each value was

derived from the ratio between arbitrary units obtained by the protein band and the β-actin band. The results were expressed as ratio/β-actin.

Fluorescence analysis

FJC staining was carried out because it is a marker of degenerating neurons (Schmued et al. 2005). Cortex sections (8 μm) were cut in a microtome (Thermo Fisher Scientific, HM 325, USA) and mounted on Super Frost-Plus glass slides (Thermo 213 Scientific, Rockford, IL, USA). Cortex slices were deparaffinized, rehydrated, and boiled three times in 10 mM citrate buffer, pH 6. Sections were blocked for 1 h with PBS containing 0.1% (*v*/*v*) Triton X-100 (PBS-Tx) and 10% (*v*/*v*) normal donkey serum at room temperature. After that, the sections were washed three times in PBS, incubated with 5 μg/ml DAPI (Invitrogen) for 5 min. Then, sections were washed with PBS and incubated with 0.0001% (*v*/*v*) FJC (Millipore) for 10 min. Subsequently, the sections were washed three times in PBS, mounted on slides, and covered with coverslips. Images of slides were obtained from cerebral cortex region in which FJC-positive cells were counted in ten random fields in a blinded fashion. Quantitative analysis of marked cells was made using the Image J software and results expressed as FJC positive cells.

Statistical analysis

To test a Gaussian distribution, a Kolmogorov-Smirnov normality test was used. The effects of STZ and $(p\text{-ClPhSe})_2$ were analyzed by two-way ANOVA followed by the Newman–Keuls test. Statistical comparisons for glycemia were performed using repeated measure two-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Main effects are presented only when the first order interaction was non-significant. Statistical comparisons for FJC staining was performed using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Pearson's correlation coefficient was used for the estimation of correlation between parameters analyzed. Data are expressed as the mean ± standard error of the mean (S.E.M). All analyses were performed using GraphPad Prism software version 6 for Windows. Probability values less than 0.05 ($p < 0.05$) were considered to be significant.

Results

$(p\text{-ClPhSe})_2$ reversed depressive-like phenotype behavior of diabetic mice

The two-way ANOVA of the immobility time in the TST and FST revealed a significant STZ and $(p\text{-ClPhSe})_2$ interaction [TST $F_{(3,20)} = 22.98$, $p = 0.0001$ and FST $F_{(3,20)} = 12.38$, $p = 0.0021$]. Post hoc comparisons showed that the immobility time of diabetic mice was significantly higher than that of control mice. Furthermore, the treatment with $(p\text{-ClPhSe})_2$ was effective against the increase in the immobility time when compared to that of diabetic mice, at both behavioral tests (Fig. 2a and b). Besides, the Pearson's correlation coefficient demonstrated that glycemia was positively correlated with immobility time in the TST and FST ($r = 0.4581$, $p = 0.0244$ and $r = 0.6524$, $p = 0.0006$, Table 1). These findings suggest that this organoselenium compound has a potential antidepressant-like effect.

Treatments did not alter the mouse spontaneous behavior

The two-way ANOVA of spontaneous behavior data revealed no significant STZ and/or $(p\text{-ClPhSe})_2$ interaction (data not shown, $p > 0.05$). STZ and/or $(p\text{-ClPhSe})_2$ did not cause motor abnormality in mice of all experimental groups.

$(p\text{-ClPhSe})_2$ reduced hyperglycemia of diabetic mice

The systemic administration of STZ in mice caused hyperglycemia, at days 14 and 21. Two-way repeated measures ANOVA of glycemia revealed a significant STZ and $(p\text{-ClPhSe})_2$ interaction at day 21 [$F_{(3,20)} = 2.71$, $p = 0.044$]. Post hoc comparisons showed that diabetic mice increased glycemia compared to that of the control group. Moreover, $(p\text{-ClPhSe})_2$ was effective against the hyperglycemia of diabetic mice after 7 days of treatment (Fig. 3).

$(p\text{-ClPhSe})_2$ counteracted cortical oxidative damage caused by diabetes

The two-way ANOVA data for TBARS and RS levels in the cerebral cortex revealed a significant STZ and $(p\text{-ClPhSe})_2$ interaction [TBARS $F_{(3,20)} = 5.47$, $p = 0.0297$ and RS $F_{(3,20)} = 5.23$, $p = 0.0331$]. Post hoc comparisons showed that TBARS and RS levels in the cerebral cortex of diabetic mice were significantly higher than those of control mice. Furthermore, the two-way ANOVA data for SOD activity in the cerebral cortex revealed a significant STZ and $(p\text{-ClPhSe})_2$ interaction [$F_{(3,20)} = 39.22$, $p = 0.0126$]. Post hoc comparisons demonstrated that SOD activity was significantly reduced in the cerebral cortex of diabetic mice when compared to control mice. In contrast, two-way ANOVA of CAT activity was not significantly altered in all experimental groups (Fig. 5b, $p > 0.05$).

Moreover, the treatment with $(p\text{-ClPhSe})_2$ abolished the increase of TBARS and RS levels (Fig. 4a and b) in the cerebral cortex of diabetic mice. Furthermore, $(p\text{-ClPhSe})_2$ was effective against the decrease in the SOD activity in the cerebral cortex caused by diabetes (Fig. 5a). The Pearson's correlation coefficient revealed that glycemia was positively

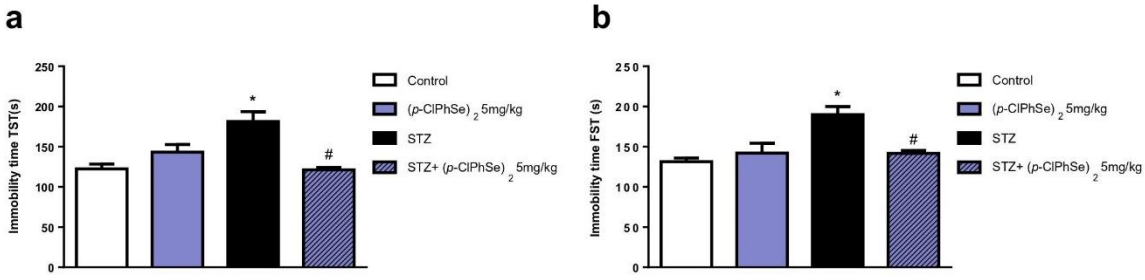

Fig. 2 Effects of $(p\text{-ClPhSe})_2$ on the immobility time in the tail suspension test (TST, **a**) and in the forced swimming test (FST, **b**) of diabetic mice. Each column represents the mean ± SEM of six animals/group. $^*p < 0.05$ when compared with the control group and $\#p < 0.05$ when compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman-Keuls test

correlated with TBARS and RS levels ($r = 0.6270$, $p = 0.0010$ and $r = 0.6300$, $p = 0.0010$, Table 1) and negatively correlated with SOD activity ($r = -0.8367$, $p < 0.0001$, Table 1). These data suggest that the already reported antioxidant property of $(p\text{-ClPhSe})_2$ contributed to the antidepressant-like action of this compound in diabetic mice.

$(p\text{-ClPhSe})_2$ did not reverse the increase in adrenal gland relative weight and the decrease in GR content induced by diabetes

The two-way ANOVA of adrenal gland relative weight revealed a significant main effect of STZ [$F_{(3,20)} = 0.36$, $p = 0.5540$]. The adrenal gland relative weight of diabetic and $(p\text{-ClPhSe})_2$-treated diabetic mice was increased when compared to that of the control mice (Fig. 6a). In addition, a positive correlation was found between glycemia and the adrenal gland relative weight ($r = 0.8700$, $p < 0.0001$, Table 1). The two-way ANOVA of GR content in the cerebral cortex revealed a significant main effect of STZ [$F_{(3,20)} = 0.02$, $p = 0.8851$]. The GR content in the cerebral cortex of diabetic and $(p\text{-ClPhSe})_2$-treated diabetic mice was reduced when compared to that of control mice (Fig. 6b). However, Pearson's correlation analysis revealed a negative correlation between glycemia and GR levels ($r = -0.7226$, $p < 0.0001$, Table 1).

Table 1 Pearson correlation of glycemia with behavioral and biochemical parameters

Behavioral/biochemical parameters		Correlations	
		Pearson *r*	*p* value
Glycemia	TST	0.4581	0.0244
	FST	0.6524	0.0006
	TBARS	0.6270	0.0010
	RS	0.6300	0.0010
	SOD	− 0.8367	< 0.0001
	Adrenal weight	0.8700	< 0.0001
	GR	− 0.7226	< 0.0001
	Keap1	− 0.6731	0.0003
	Nrf2	− 0.5801	0.0030
	HO-1	− 0.8197	< 0.0001
	FJC	0.5310	0.0234

The Pearson's correlation coefficient was used for correlation analysis

$(p\text{-ClPhSe})_2$ modulated Keap1/Nrf2/HO-1 signaling pathway in diabetic mice

The two-way ANOVA of Keap1, Nrf2, and HO-1 levels in the cerebral cortex revealed a significant STZ and $(p\text{-ClPhSe})_2$ interaction (Keap1 [$F_{(3,20)} = 5.30$, $p = 0.0.3219$], Nrf2 [$F_{(3,20)} = 9.23$, $p = 0.0065$], and HO-1 [$F_{(3,20)} = 9.12$, $p = 0.0067$]). Post hoc comparisons showed a decrease in Keap1, Nrf2, and HO-1 levels in the cerebral cortex of diabetic mice when compared to those of the control mice. Moreover, treatment with $(p\text{-ClPhSe})_2$ was effective against the decrease in the levels of Keap1, Nrf2, and HO-1 (Fig. 7a–c). Besides, a negative correlation was found when glycemia was analyzed with Keap1 ($r = -0.6731$, $p = 0.0003$), Nrf2 ($r =$

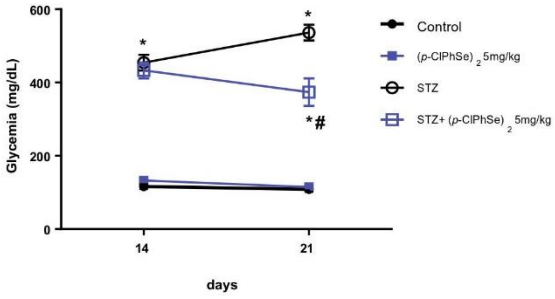

Fig. 3 Effects of $(p\text{-ClPhSe})_2$ on the mouse glycemia in different days of the experimental protocol. Each column represents the mean ± SEM of six animals/group. $^*p < 0.05$ when compared with the control group and $\#p < 0.05$ when compared with the diabetic group. Data were analyzed through repeated measure two-way analysis of variance (ANOVA) followed by the Newman–Keuls test

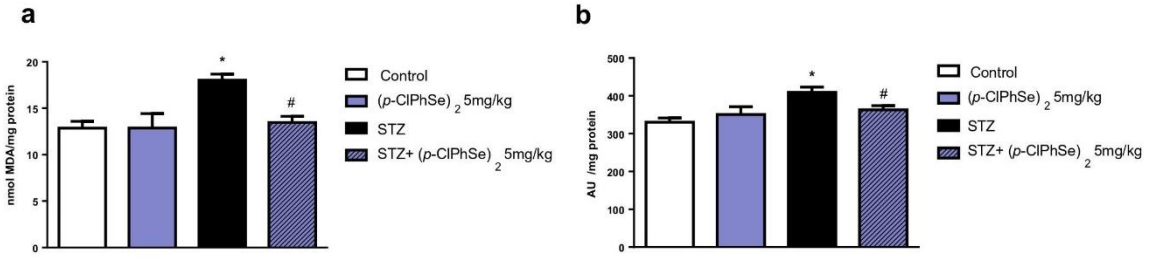

Fig. 4 Effects of $(p\text{-ClPhSe})_2$ on TBARS (**a**) and RS (**b**) levels in the cerebral cortex of diabetic mice. Each column represents the mean ± SEM of six animals/group. $*p < 0.05$ when compared with the control group and $\#p < 0.05$ when compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test

− 0.5801, $p = 0.0030$), and HO-1 levels ($r = -0.8197$, $p < 0.0001$) (Table 1). These results show that this signaling pathway contributes to the depressive-like effects in diabetic mice.

$(p\text{-ClPhSe})_2$ reduced FJC positive cells in the cerebral cortex of diabetic mice

The one-way ANOVA of FJC-positive cells revealed a significant difference among the groups [$F_{(2,15)} = 7.12$, $p = 0.0067$]. Post hoc test showed that the number of FJC-positive cells was increased in the cerebral cortex of diabetic mice when compared to that of the control mice. A statistically significant decrease in the number of FJC-positive cells was found in the cerebral cortex of diabetic mice treated with $(p\text{-ClPhSe})_2$ (Fig. 8). Besides, Pearson's correlation coefficient showed a positive correlation between glycemia and FJC-positive cells ($r = 0.5310$, $p = 0.0234$, Table 1). These findings suggest that this compound may have a neuroprotective effect.

Discussion

The present study demonstrates that Keap1/Nrf2/HO-1 signaling pathway contributes to the $(p\text{-ClPhSe})_2$ antidepressant-like action in diabetic mice. In fact, the organoselenium compound $(p\text{-ClPhSe})_2$ suppressed depressive-like phenotype, in the TST and FST, without changing spontaneous locomotor activity of diabetic mice. In addition, the organoselenium compound reversed hyperglycemia, reduced oxidative stress and FJC positive cell marking, and it modulated the Keap1/Nrf2/HO-1 signaling pathway in the cerebral cortex of mice made diabetic after the administration of systemic STZ (Fig. 9). Despite these positive effects, $(p\text{-ClPhSe})_2$ was ineffective against markers of the HPA axis in diabetic mice.

STZ is a synthetic substance used for the induction of experimental models of diabetes in rodents (Skovso 2014). Since the initial report of its diabetogenic properties described by Rakieten et al. (1963), the diabetic animal models have been very useful in elucidating the mechanisms of diabetic pathogenesis and in screening artificial chemicals, natural products, and pharmacological agents that are potentially capable of lowering blood glucose levels (Kumar et al. 2012).

In the present study, diabetic mice had depressive-like phenotype, hyperglycemia, and the dysregulation of the HPA axis. However, the neurobiological bases of the neuro–psycho-endocrine interaction between depression and diabetes seem to be complex and multifactorial (Reagan 2012). Accordingly, evidence has been found to suggest that poorly controlled diabetes shares HPA axis hyperactivity (Stranahan et al. 2008), induces the mitochondrial production of free radicals (Lee et al. 2017), oxidative stress, neurodegenerative processes (Wang et al. 2014), and increases the risk of developing depression

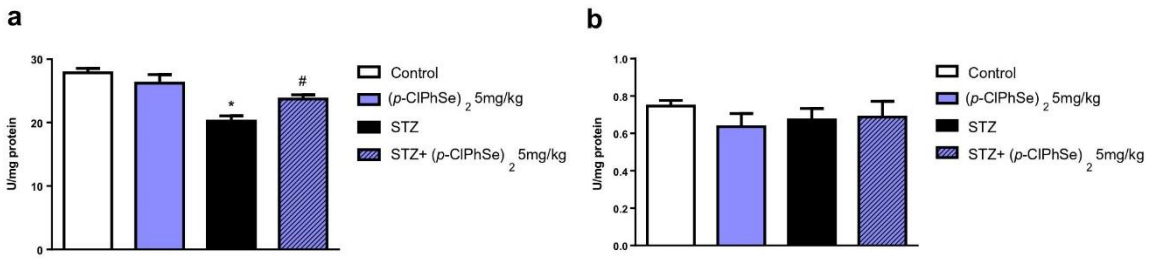

Fig. 5 Effects of $(p\text{-ClPhSe})_2$ on SOD (**a**) and CAT (**b**) activities in the cerebral cortex of diabetic mice. Each column represents the mean ± SEM of six animals/group. $*p < 0.05$ when compared with the control group and $\#p < 0.05$ when compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test

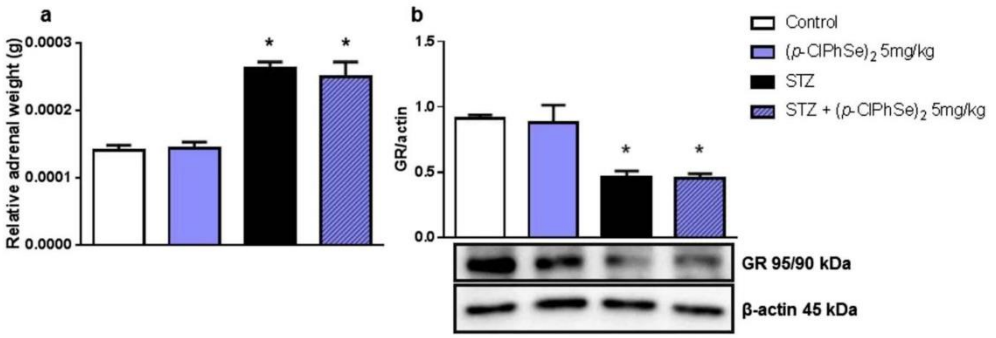

Fig. 6 Effects of $(p\text{-ClPhSe})_2$ on relative adrenal weight (**a**) and GR levels (**b**) in the cerebral cortex of diabetic mice. Representative qualitative Western blotting analysis is at the base of figure. Each column represents the mean ± SEM of six animals/group. $^*p < 0.05$ when compared with the control group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test

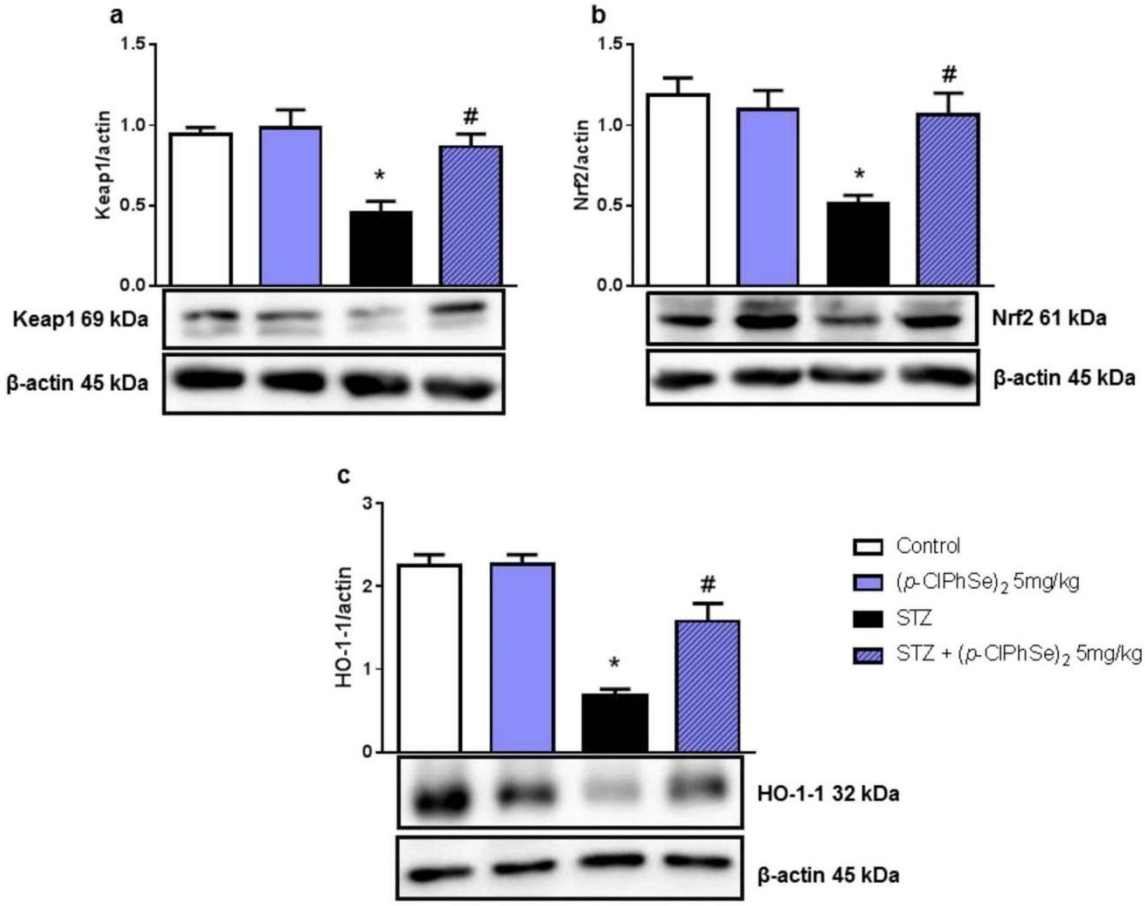

Fig. 7 Effects of $(p\text{-ClPhSe})_2$ on Keap1 (**a**), Nrf2 (**b**), and HO-1 (**c**) levels in the cerebral cortex of diabetic mice. Representative qualitative Western blotting analysis is at the base of figure. Each column represents the mean ± SEM of six animals/group. $^*p < 0.05$ when compared with the control group and $\#p < 0.05$ when compared with the diabetic group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test

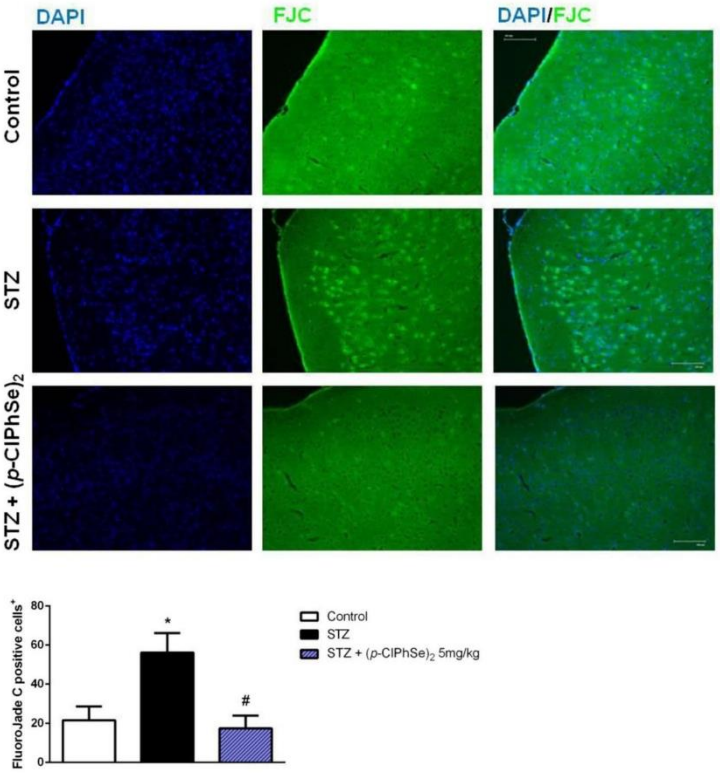

Fig. 8 Effects of $(p\text{-ClPhSe})_2$ on the Fluoro-Jade C staining for degenerative neuronal cells in the whole cerebral cortex in diabetic mice. Representative images for each group are at the top of figure. Each column represents the mean ± SEM of six animals/group. $^*p < 0.05$ when compared with the control group and $\#p < 0.05$ when compared with the diabetic group. Data were analyzed through one-way (ANOVA) followed by the Newman-Keuls test

symptoms (Castillo-Gomez et al. 2015) among other events that play a role in the pathophysiology of diabetes.

Moreover, it has been shown that the disproportion in the activity of HPA axis is one of the common etiological factors between diabetes and depression (Revsin et al. 2009). The results on the HPA markers presented here indicate the dysregulation of this axis, in which the hypothetical increase in corticosterone levels could activate the negative feedback and decrease the expression of glucocorticoid receptors in the cerebral cortex of diabetic mice. Although we acknowledge that the levels of corticosterone and ACTH were not determined in this experimental protocol, data on scientific literature indicate that the basal plasma levels of corticosterone were increased (Chan et al. 2001) whereas those of ACTH were decreased in diabetic mice (Revsin et al. 2009), what is in agreement with and supports the hypothesis of this study.

Chronic hyperglycemia has been reported to have detrimental effects on various brain functions (Richa et al. 2017) such as cerebral metabolism, vascular reactivity, and increased oxidative stress in mice (Valko et al. 2007). In fact, it has been suggested that oxidative stress plays an important role in the pathogenesis of diabetic complications (Richa et al. 2017). Oxidative stress, a condition that may appear as a result of disproportion between reactive oxygen species (ROS) generation and their neutralization by means of antioxidants mediated pathways (Patar et al. 2018), was characterized in the cerebral cortex of diabetic mice by the increase in lipid peroxidation markers, the decrease in the SOD activity and of Keap1–Nrf2-HO-1 signaling pathway. Accordingly, the combination of ROS and TBARS overproduction and concomitant downregulation of the antioxidant enzymes activity, such as SOD, alters the redox homeostasis and leads to oxidative stress in diabetes (Rababa'h et al. 2019).

There is a wide variety of factors associated with the cellular response to oxidative stress (Kaspar et al. 2009). In this respect, it should be noted that the Keap1–Nrf2 system has emerged as an important research topic in the pathogenesis of diabetes mellitus and in the development of its complications (Uruno et al. 2015). This because the Nrf2 is a transcription factor with a central role in cellular defense against oxidative and electrophilic insults (Ma 2013). It binds to antioxidant response elements (ARE) located in the promoter region of genes encoding many phase II detoxifying or antioxidant enzymes and related stress-responsive proteins (Suzuki and Yamamoto 2015). During oxidative stress, Nrf2 is derepressed and activates the transcription of cytoprotective and antioxidant enzyme genes such as HO-1 (Suzuki and Yamamoto 2015), SOD and CAT, among others. Moreover,

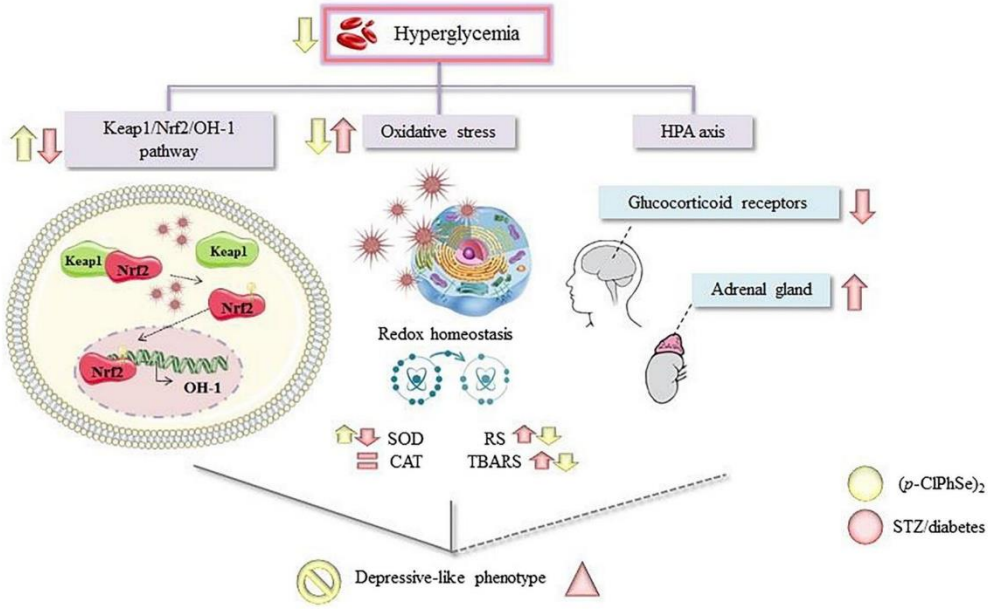

Fig. 9 Overview of changes induced by STZ over Keap1/Nrf2/HO-1 signaling pathway, oxidative stress, and HPA axis in cerebral cortex of mice; together with modulatory effects of $(p\text{-ClPhSe})_2$ on the Keap1/Nrf2/HO-1 signaling pathway and oxidative stress in the cerebral cortex of diabetic mice. Red arrows indicate effects in diabetic mice. Yellow arrows indicate effects of $(p\text{-ClPhSe})_2$ in diabetic mice. Keap1(Kelch-like ECH-associated protein 1); Nrf2 (nuclear factor erythroid-2-related factor 2); HO-1 (heme oxygenase-1); SOD (superoxide dismutase); CAT (catalase); RS (reactive species); TBARS (thiobarbituric acid reactive substances)

there are accumulating lines of evidence indicating that Nrf2 directly regulates genes related to cellular metabolism (Mitsuishi et al. 2012), and that the activation of this protein suppresses the onset and/or progression of insulin resistance by enhancing AMPK phosphorylation and glucose uptake, suppressing gluconeogenesis and leading to the protection of the body against diabetes (Uruno et al. 2015).

Brain tissue is highly sensitive to energy metabolism impairment and oxidative stress, which are important events that have been related to the pathogenesis of diseases affecting the central nervous system (Streck et al. 2013). This way, the cerebral cortex is a critical brain region for emotional regulation, decision making, learning, and cognition (McEwen and Morrison 2013). In the present experimental protocol, mice made diabetic by the STZ administration showed an increase in the number of FJC-positive cells in the cerebral cortex, which indicates a process of neuronal degeneration. In this regard, rats at 4 months after STZ injection also showed increased number of FJC-positive cells in the cerebral cortex, hypothalamus, and hippocampus (Wang et al. 2014).

The results obtained with $(p\text{-ClPhSe})_2$ reveal the potential beneficial effects of this treatment on the comorbidity of diabetes and depression in a mouse model of diabetes induced by systemic administration of STZ. In the present experimental protocol, $(p\text{-ClPhSe})_2$ reversed depressive-like phenotype, in the TST and FST, and hyperglycemia in diabetic mice. Furthermore, the $(p\text{-ClPhSe})_2$ effects on the reversion of depressive-like phenotype were accompanied by the modulation of the Keap1/Nrf2/HO-1 signaling pathway, which counteracted oxidative stress in the cerebral cortex of diabetic mice. Corroborating our findings, the antioxidant action of $(p\text{-ClPhSe})_2$ in aged rats was demonstrated by Bortolatto et al. (2012). The well-known neuroprotective effects of $(p\text{-ClPhSe})_2$ (Bortolatto et al. 2015; Bortolatto et al. 2017; Quines et al. 2018; Zborowski et al. 2016) were reproduced in the cerebral cortex of diabetic mice. Multiple encephalic regions, such as the hippocampus, prefrontal cortex, amygdala, ventral striatum, and hypothalamus, can contribute to the onset and development of depression (Liu et al. 2017). Furthermore, changes in neural plasticity as neurogenesis, apoptosis, and energetic metabolism have been related to events that can happen in different brain regions of depressive-like animals (Liu et al. 2017; Pittenger and Duman 2008). Certainly, it would be interesting to understand the effect of $(p\text{-ClPhSe})_2$ in other brain regions. In the hippocampus in addition to its antioxidant action, $(p\text{-ClPhSe})_2$ modulated 5-HT(1A) and 5-HT(3) receptors in a model of aged-induced depression in rats (Bortolatto et al. 2012).

Despite the positive results obtained with $(p\text{-ClPhSe})_2$ treatment in diabetic mice, this organoselenium compound was not effective against the dysregulation of HPA axis, suggesting that its antidepressant-like action might not be

explained by only one mechanism, which is presumably not related to this axis. Moreover, because women have a higher rate of depression than do men (Kendler and Gardner 2014), we acknowledge the use of only male mice as a limitation of our study. Understanding how genetic difference confers sexual differences in predisposition to mental illness is a complex, multilevel puzzle that remains to be studied (Albert 2015).

In summary, the findings of the present study demonstrate that $(p\text{-ClPhSe})_2$ elicited an antidepressant-like action and that cerebral cortical Keap1/Nrf2/HO-1-1 signaling pathway contributed for this action in diabetic mice.

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Compliance with ethical standards

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

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3.3 MANUSCRITO 1

Prejuízo de memória e fenótipo do tipo depressivo são acompanhados pela *downregulation* das vias de sinalização hipocampais da insulina e do BDNF em camundongos pré-diabéticos

Memory impairment and depressive-like phenotype are accompanied by the downregulation of hippocampal insulin and BDNF signaling pathways in prediabetic mice

Vanessa Angonesi Zborowski, Suélen OsórioHeck, Luiza Souza Marques, Nícolas Klummer Bastos, Cristina Wayne Nogueira

Em fase de Redação

Memory impairment and depressive-like phenotype are accompanied by downregulation of hippocampal insulin and BDNF signaling pathways in prediabetic mice

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Abstract

Prediabetes is the stage before diabetes in which not all the symptoms or signs required to diagnose diabetes are present, but blood glucose is abnormally high. This study aimed to investigate whether prediabetes induces memory impairment and depressive-like phenotype in mice as well as the contribution of hippocampal insulin and BDNF signaling to these effects. Male adult Swiss mice received streptozotocin (ETZ, 200 mg/kg, ip) to induce prediabetes. Control mice were given citrate buffer (5 ml/kg, ip). To characterize prediabetes status, metabolic parameters were determined in mice. The behavioral test battery to assess memory of mice consisted of object recognition (ORT), object location (OLT), and Morris water maze (MWM) tests. The mouse depressive-like phenotype was investigated using the forced swimming (FST) and tail suspension (TST) tests. IRS-1/Akt/GLUT4 and BDNF/CREB protein contents were determined in the hippocampus of mice. Prediabetic mice showed mild hyperglycemia, a reduction in the body weight gain, and an increase of glucose and insulin tolerance tests (AUCs). Prediabetic mice had smaller recognition and location indexes, in the ORT and OLT, than those in the control group. Prediabetic mice showed hippocampus-dependent spatial memory dysfunction in the MWM test. Prediabetic mice showed an increase in immobility time in the TST and FST when compared to those in the control group. The molecular findings indicate the downregulation of hippocampal insulin and BDNF/CREB signaling in prediabetic mice. In conclusion, memory impairment and depressive-like phenotype were accompanied by the downregulation of hippocampal IRS-1/Akt/GLUT4 and BDNF/CREB signaling in prediabetic mice.

Keywords: memory, depressive-like, ETZ, prediabetes, BDNF, insulin

1 Introduction

Prediabetes is the stage before diabetes in which not all of the symptoms required to diagnose diabetes are present, but blood glucose rises to abnormally high levels (Chen et al. 2018). The diagnostic criteria for this disease include one or more of the following: elevated fasting plasma glucose, impaired glucose tolerance or increased hemoglobin A1c; additionally, insulin resistance is also already present in the prediabetic stage (Tabak et al. 2012). Moreover, the *International Diabetes Federation* reported that in 2017 there were 425 million people with diabetes and 352 million adults with impaired glucose tolerance, which puts them at high risk of developing the disease (Atlas 2017). It has been demonstrated that people with prediabetes, when compared with a normoglycemic population, are found to be at an increased risk of cognitive decline (J et al. 2009). A population-based cohort study revealed an association of cognitive decline with prediabetes and diabetes (Marseglia et al. 2019). Furthermore, some of the existing longitudinal studies on psychological distress and the risk of diabetes have focused on depressive symptoms (Pouwer et al. 2010). In this regard, it has been demonstrated that prediabetes and depression interact synergistically to increase diabetes risk (Deschenes et al. 2016). Moreover, prediabetes, as an impaired metabolic condition, affects cognitive performance and causes heightened anxiety in rats, as well as significantly reduces the hippocampal dendritic spine density (Fan et al. 2019).

Although it is reported that prediabetes causes damage to the central nervous system, a robust evidence base of how impaired glucose metabolism, as it happens in the prediabetic stage, affects memory and depression is still lacking in the literature. Changes in insulin levels might affect neuronal glucose uptake and metabolism via the translocation of glucose transporter 4 (GLUT4) in response to insulin/IRS-1/Akt signaling in the brain regions, important for cognitive and emotional function (Arnold et al. 2018). Insulin receptor substrate-1 (IRS-1) activates phosphoinositide 3-kinases (PI3K) *via* an interaction with its regulatory subunit, resulting in the subsequent phosphorylation of Akt, which is a central intermediate for many of the metabolic actions of insulin (Xu et al. 2018). The PI3K/Akt activation increases the glucose uptake, by stimulating the movement of GLUT4 isoform to the plasma membrane (Saltiel and Kahn 2001). Because the density of insulin receptors is high in limbic cortical and subcortical regions and that the insulin signaling pathway affects mood, motivation and other aspects of psychiatric functioning, it is suggested that this pathway may be involved with depressive behavior (Arnold et al. 2018). Furthermore, insulin resistance, the downregulation of the protein kinase B (Akt) pathway (Xiang et al. 2015) and, the consequent, decrease of hippocampal GLUT4 translocation in neurons (Patel et al. 2016) have

been well reported and are related to cognitive impairment in animal models of diabetes; however, still little is known about this mechanism in the prediabetic status.

Brain-derived neurotrophic factor (BDNF), a growth factor that exerts insulin-like effects, stimulates the synaptic plasticity, memory formation, and neurogenesis (Noble et al. 2011). The activation of BDNF increases the phosphorylation of both Akt and cAMP-responsive element-binding (CREB) (Clarkson et al. 2015; Massa et al. 2010), and CREB, in turn, leads to the transcription of genes crucial for neuronal survival, including the BDNF (Dong et al. 2018). Besides, evidence has been found to suggest that reduced levels of BDNF were found in not only the brain but also the serum of depressive patients (Cunha et al. 2006). Thus, we hypothesize that metabolic alterations, such as mild hyperglycemia, may interfere in the hippocampal protein contents of prediabetic mice. Therefore, the present study attempts to investigate whether prediabetes status induces memory impairment and depressive-like phenotype in mice. Moreover, the modulation of hippocampal insulin and BDNF signaling pathways in prediabetic mice was also investigated.

2 Materials and Methods

2.1 Animals

The experimental protocol of this study was conducted using male adult Swiss mice (60 day-old, 25-35g) obtained from the Central Animal Facility of the Federal University of Santa Maria (Rio Grande do Sul, Brazil). The mice were maintained in a separate animal room at a controlled temperature of 22 - 24 °C, 50 - 60% relative humidity. The light/dark cycle was 12 h/12 h with the lights turned on every day at 7:00 a.m. The mice were separated into groups and housed in cages with free access to food and tap water. The experimental protocol followed the mandatory guidelines for general animal care of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil (#2964150317/2017). The procedures of this study were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines (record number 10684).

2.2 Experimental design

2.2.1 Streptozotocin (ETZ) administration

Mice fasted for 4h received a single intraperitoneal administration of ETZ (Sigma-Aldrich) at the dose of 200 mg/kg, prepared in cold 0.5M citrate buffer (pH 4.5). The control mice received an equal volume (5 ml/kg) of citrate buffer (vehicle). Three days after ETZ

administration, glycemia was measured by the glucometer (Accu-Check Active glucometer®) in samples obtained from the tail-tip of mice (Figure 1). This endpoint was used to separate diabetic mice, which were selected based on the blood glucose levels ≥ 200 mg/dl. Diabetic mice were not included in the present study; they were used in another study published by us (Zborowski et al. 2019).

Mice with glycemia below 200 mg/dl were the subjects of this experimental protocol. Because after ETZ administration there are a significant number of animals that does not present hyperglycemia, which are generally discharged from the experimental protocol and to respect the three Rs (Replacement, Reduction, and Refinement) principle, we investigated whether mice with glycemia between 84 to 183 mg/dl could be classified as prediabetic. To further characterize the prediabetic status, glycemia in tail-tip blood samples of mice was monitored at days 7, 14 and 21 after ETZ administration (Figure 1).

Immediately after the end of behavioral tests, the mice of the first and second sets were individually placed in metabolic chambers for 2 h to record the amount of food and water consumption, and the volume of urine excreted. The physical-chemical properties of urine were determined using urinalysis reagent strips (Sensi 10®), in which semiquantitative parameters, such as density, pH, leukocytes, nitrites, proteins, glucose, ketones, urobilinogen, bilirubin, and blood, were recorded. The results were expressed as the mean of mice that were exposed to the chamber only once.

Insulin and glucose tolerance tests were carried out at the 22nd day of a protocol (Figure 1), to characterize the glucose response profile in prediabetic mice. Mice 4h fasted were challenged to the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) as previously described by Kuai et al. (2016), with some modifications. In the OGTT, tail-tip blood samples of mice were used to measure glucose, which were taken before oral 2 g/kg glucose administration and after glucose challenge (0 to 120 min). For the ITT, mice received recombinant human insulin (1 UI/kg, abdominal sc) and glucose was measured in the tail-tip blood from basal to 120 min. Each mouse performed only once for each test.

The individual body weight gain (g) was calculated by the difference between the baseline body weight, obtained before the beginning of treatment, and the body weight at the end of the experimental protocol. Visceral epididymal adipose fat was isolated, removed, and weighed. The results were expressed as a relative weight (epididymal fat weight/body weight (g)).

2.2.2 Experimental groups

The mice were divided into Control group (n = 6, for each assessment), mice administered with a cold 0.5M citrate buffer (ETZ vehicle) and Prediabetes group (n=6, for each assessment), mice received a single ETZ administration, developed mild hyperglycemia (84 to 183 mg/dl), glucose intolerance, and insulin resistance.

2.2.3 Experimental batches

At day 17 of the experimental protocol, mice were divided into three batches to perform the behavioral tests (Figure 1). In the first and second sets, the animals performed tests to assess memory performance; in the first one, mice were subjected to the object recognition test (ORT) and the object location test (OLT). In the second set, they performed the Morris water maze (MWM) test.

In the third set, the animals were subjected to a battery of tests, in the following order: the spontaneous locomotor profile (LP), tail suspension test (TST), and forced swimming test (FST). Mice were challenged in the TST and FST to evaluate a depressive-like phenotype.

2.2.3.1 Behavioral Tests

Object Recognition Test (ORT)

The ORT was performed according to the method previously described Rosa et al. (2003). This test was performed in a square open field arena 45 × 45 × 30 cm. A short habituation phase was performed in this protocol, which consisted of 2-d exposure to the apparatus, without objects, for 10 min. Twenty-four hours after the last habituation session, two identical objects were placed in the arena, 5 cm away from the walls, and mice performed 2-d training sessions (10 min each). The objects (A and A') were towers of Lego bricks (6 x 6 cm). After the familiarization session, the objects and the open field arena were cleaned with 70% (vol/vol) ethanol to minimize olfactory cues. A test session was carried out 1.5 h after the last training session, in which mice freely explored the arena for 6 min in the presence of a known object (A) and a new object (B) (Figure 1 A). For this session, the two familiar objects were replaced in the arena, one with the triplicate copy (to ensure that there are no residual olfactory cues on the previously used object) and the other by a novel object. They were placed at the same location, 5 cm away from the walls. The exploration time was defined as smelling or touching the nose and/or front paws. Results were expressed as the recognition index, which was calculated as the following: TB/(TA + TB) where TA means time spent exploring a known object (A) and TB means time spent exploring a new object (B).

Object Location Test (OLT)

The OLT was carried out in the same apparatus used in the ORT. The training was performed by placing an animal in the arena, where two identical objects (objects C and D) were placed in parallel, as described by Takahashi et al. (2009) with some modifications. The objects (C and D) were towers of Lego bricks (6 x 6 cm). Animals should explore the training arena completely and spend time exploring both objects equally. Mice performed the test session 2h after the training, object D was moved to a location diagonally opposite to the object C, and animals were allowed to explore the objects for 6 min.

Results were expressed as the location index, which was calculated as the following: TD/(CT + TD) where CT means time spent exploring a known object C and TD means time spent exploring the diagonally opposite object D.

Morris Water Maze Test (MWM)

Spatial memory and learning were assessed in the MWM test, in which mice were challenged according to procedures previously described by Morris (1984). Animals were placed in a black plastic pool 110 × 40 cm filled with colored opaque water (26 ºC) at a height of 30 cm. Because they were in opaque water, mice cannot see the platform; instead, they must rely on external/extra-maze cues. In the training phase, the animals were left in the 4 different quadrants (north, south, east, or west) to find the secure platform, which was hidden 1 cm below the water level in the middle of the northwest quadrant. Mice freely swam/searched for the platform for a maximum of 1 min. Latency to reach the submerged platform in 4-d training was recorded on videotape. After finding the secure platform, the mice remained on the platform for at least 1 min on each attempt. If the animals did not find the secure platform within 1 min, they were led to the platform where they remained for 1 min. After the animals being trained, they performed the test session, in which the platform was removed. Animals were placed in a novel start position in the maze, facing the tank wall in the opposite quadrant of the platform. The use of a novel start position during the probe trial is important to ensure that their spatial preference is a reflection of the memory of the goal location rather than for a specific swim path. The distance traveled by animals in the quadrant, where was the platform, was measured for 1 min. Results were analyzed using Any-maze® software for Windows.

Spontaneous Locomotor Profile

The locomotor activity was acquired in a transparent acrylic box (500 × 480 × 500 mm) equipped with 16 infrared sensors for automatic recording of horizontal activity (model EP149, Insight Instruments Ltda, SP, Brazil). Mice were placed in the center of the box and allowed to explore freely for 4 min. The parameters recorded were the number of crossings (number of segments crossed with all four legs), total distance traveled (mm), and the speed (mm/s).

Tail Suspension Test (TST)

In the TST the mice were suspended by their tails following the procedures previously described by Steru et al. (1985). Each animal was suspended in a horizontal bar kept at 50 cm over the floor. The observer scored for 6 min the total duration of mouse immobility, using a stopwatch. The mouse was considered immobile only when it was completely motionless.

Forced Swimming Test (FST)

The FST consisted of individually placing the mouse into an inescapable cylindrical container (10 × 25 cm^2) filled with clean water at 25 ± 1 ^oC (19 cm) (Porsolt et al. (1979). The mouse floating (immobile) behavior was measured by an observer for 6 min by using a stopwatch. Each animal was considered motionless when it stopped swimming and remained floating motionless in the water, making only the necessary movements to keep its head out of the water.

2.2.3.2 Designation of samples

A and B- Samples from mice of the first and second sets batches

Mice were killed by cervical dislocation and samples of the hippocampus were immediately dissected on a cold plate and stored at -80 ^oC until processed in the Western-blot assay (Figure 1). This technique was performed according to our previous study (Zborowski et al. 2019). The antibodies used in the study are listed in Table 1S.

Cell Fractionation: Hippocampal samples were homogenized following the established practice by Quines et al. (2018) in order to determine the levels of glucose transporter (GLUT4) in the membrane and cytosol fractions.

After protein detection, a chemiluminescence kit was used (Amersham, SP, Brazil), and protein signals were captured by the Amersham Imager 600 (GE life sciences) equipment. The optical density of the bands was quantified using Image J software (NIH, Bethesda, MD,

USA) for Windows. Each value was quantified the ratio between arbitrary units of the protein band and the β-actin band. Results were expressed as ratio/β-actin or tubulin.

C- Samples from mice of the third set batch

Mice were anesthetized for blood collection by cardiac puncture. The glycated hemoglobin (HbA1c) was determined in the total blood (NycoCard Cicla®, Brazil) and expressed as (%). Blood samples of mice were centrifuged at 4000×g for 10 min to obtain plasma samples, which were designated to determine the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and the levels of urea (Labtest Diagnostica, MG, Brazil).

2.3 *Statistical Analysis*

To test a Gaussian distribution, a Shapiro-Wilkov normality test was used. Data were analyzed by an unpaired Student's test. Statistical comparisons for data obtained from glycemia, OGTT, ITT, and latency of the MWM test were analyzed by repeated measure ANOVA followed by the Bonferroni test. Data are expressed as the mean ± standard deviation (S.D). All analyses were performed using GraphPad PRISM® version 7 for Windows software. Probability values less than 0.05 ($p < 0.05$) were considered significant.

3 Results

3.1 Prediabetic mice have metabolic alterations

Prediabetic mice had an increase in glycemia at day 14 (group x glycemia $F_{(1,10)}=5.09$, $p=0.0476$; Fig 2A), a reduction in the body weight gain ($t_{(5)}=3.38$, $p=0.0070$; Fig 2B) and in the relative epididymal fat weight ($t_{(5)}=9.44$, $p<0.0001$; Fig 2C) when compared to the control mice.

Figure 3A shows an increase in the OGTT of prediabetic mice at 60 min when compared to the control mice (group x glycemia $F_{(1,10)}=3.65$ $p=0.0068$). Despite the changes in glycemia and OGTT, prediabetic mice did not show a significant statistical difference in the ITT (Fig 3B) when compared to the control mice (group x glycemia $F_{(1,10)}=1.97$, $p=0.0991$). However, when the data were evaluated considering the area under the curve, prediabetic mice showed a significant statistical increase in both tests, OGTT and ITT, when compared to the control mice ($t_{(5)}=3.09$, $p=0.0114$ and $t_{(5)}=2.46$, $p=0.0332$; inset Fig 3A and 3B).

Prediabetic and control mice did not have a significant statistical difference in the following parameters: food consumption ($t_{(5)}$=0.41, p=0.6892; Table 2S), water intake ($t_{(5)}$=0.85, p=0.4105; Table 2S), diuresis ($t_{(5)}$=0.97, p=0.3506; Table 2S), % hemoglobin glycated (4.0% for control versus 4.1% for prediabetic, $t_{(5)}$=1.00, p=0.3409), and in the most of urine physical chemical parameters (Tables 3S). However, prediabetic mice tested positive for protein, glucose, and ketones in urine (Table 4S).

Regarding the hepatic and renal functionality (Table 5S), prediabetic mice showed an increase in the AST activity when compared to the control mice ($t_{(5)}$=2.86, p=0.0167), but the ALT activity ($t_{(5)}$=1.27, p=0.2319) and urea levels ($t_{(5)}$=1.98, p=0.0755) were similar among the experimental groups.

3.2 Prediabetic mice have impairment in the object recognition and location memory

In the ORT and OLT training sessions, mice spent a similar time exploring the identical objects (Fig 4A and 5A, p>0.05).

Figures 4B and 5B show that the recognition and location indexes of prediabetic mice were reduced when compared to those of the control mice in the ORT ($t_{(5)}$=4.97, p=0.006) and OLT ($t_{(5)}$=3.40, p=0.0067).

3.3 Prediabetic mice have hippocampal-dependent spatial memory impairment

Figure 6A shows no significant difference between groups in the latency to reach the platform in the 4 days of MWM training (p>0.05).

Prediabetic mice showed a statistically significant decrease of traveled distance in the target quadrant when compared to that of the control mice ($t_{(5)}$=2.95, p=0.0144; Fig 6B).

3.4 Prediabetic mice elicit a depressive-like phenotype

Prediabetic mice showed an increase in immobility time in the TST ($t_{(5)}$=2.54, p=0.0296; Fig 7A) and in the FST ($t_{(5)}$=4.96, p=0.0006; Fig 7B) when compared to that of the control mice.

The locomotor and exploratory activities were similar among mice from all experimental groups (Table 6S, p>0.05).

3.5 The hippocampal insulin and BDNF signaling pathways are downregulated in prediabetic mice

A statistically significant decrease of p-Akt/Akt ($t_{(5)}$=3.37, p=0.0071; Fig 8B) ratio and GLUT4 ($t_{(5)}$=3.16, p=0.0100; Fig 8C) content were found in the hippocampus of prediabetic mice when compared to those of the control mice. Although there was no statistically significant difference between groups in the hippocampal IRS-1 phosphorylation, it was found a tendency to decrease this protein content in prediabetic mice when compared to the control mice ($t_{(5)}$=2.03, p=0.0696; Fig 8A).

Fig 9A shows a statistically significant decrease of mBDNF ($t_{(5)}$=2.65, p=0.0241) levels and the p-CREB/CREB ($t_{(5)}$=2.44, p=0.0348; Fig 9B) ratio in the hippocampus of prediabetic mice when compared to those of the control mice.

4 Discussion

The present study demonstrates that prediabetic mice, induced by the peripheral ETZ administration, had memory impairment and a depressive-like phenotype that were accompanied by the downregulation of the hippocampal insulin and BDNF signaling pathways. The prediabetic state was characterized in mice by mild hyperglycemia, a reduction of weight gain, a decrease in the relative epididymal fat, an increase of OGTT and ITT AUCs, as well as positive protein, glucose, and ketones in urine despite of normal food and water consumption, diuresis, and HbA1c levels.

In spite of some differences in the experimental protocols, the findings of this study corroborate with those of Chen et al. (2018), who established that a single ETZ injection in mice induces β-cell apoptosis, mild hyperglycemia, mild pancreatic islet damage, being considered a prediabetic model (Chen et al. 2018). In this context, many factors can influence the variability to ETZ-sensitivity such as strain, gender, diet, circadian rhythm, and ETZ-solution (Deeds et al. 2011; Hayashi et al. 2006).

It is important to mention that the effects shown in mice are due to metabolic alterations caused by the peripheral ETZ injection and not a consequence of the cerebral administration. Thus, it is implicitly assumed that all central nervous system effects of ETZ given peripherally are indirect, i.e., there is no evidence that ETZ crosses the blood-brain barrier (Gotsch 1978; Grieb 2016). This way, it is a challenge to further understand the link between memory impairment/depression-like phenotype and the prediabetic stage in mice.

In peripheral tissues, GLUT4-mediated glucose uptake and utilization by tissues is a canonical effect regulated by insulin (Vannucci et al. 1998a) and, even though, the expression of GLUT4 in the hippocampus is already known, further studies are need to clarify its functions in neuronal metabolism (Witczak et al. 2008). It is possible that insulin affects

glucose transport and hence fuel supply in the brain through GLUT4. The present study demonstrated the downregulation of IRS-1/Akt/GLUT4 protein contents in the hippocampus of prediabetic mice. Although to the best of our knowledge there are no other studies relating these proteins to a prediabetic stage, we suggest that the impairment of insulin signaling is interfering with memory performance and the depressive-like phenotype in pre-diabetic animals. This because the hippocampus-mediated memory formation is known to be sensitive to glucose supply and metabolism (McNay et al. 2000; Gold et al. 1986). Furthermore, experimental evidence has been found to suggest that diabetes decreased the GLUT4 expression in different brain areas of rodents (Vannucci et al. 1998b) and caused cognitive dysfunction in mice (Zhuang et al. 2019; Jeon et al. 2012). Thus, we hypothesized that all metabolic alterations may interfere in the hippocampal protein contents of prediabetic mice. In the present study, prediabetic mice showed memory impairment and a depressive-like phenotype in different behavioral tests. Our present data also corroborate with those found by Guo et al. (2015), who showed that aged prediabetic rats had an increased immobility time in the FST and memory impairment when evaluated in the MWM. Regarding patients, individuals with high blood glucose levels even those who do not have diabetes may have memory impairment (Cherbuin et al. 2012; Crane et al. 2013).

BDNF has been reported to play a role in neurogenesis, neuroprotection, and synaptic plasticity in the brain (Begni et al. 2017). BDNF is produced by CREB activation, which acts as a transcription factor and plays an important function in neuronal survival (Shin et al. 2019). It is well-known that mBDNF through signaling cascades leads to positive regulation of learning and memory (Sasi et al. 2017), whereas its precursor proBDNF promotes increased neuronal cell death and negative regulation of learning and memory (Dincheva et al. 2016). CREB can induce the expression of BDNF (Wang et al. 2017), while on the other hand, BDNF promotes the activation of CREB through tropomyosin receptor kinase (Trk) B receptors (Wang et al. 2018), forming a positive feedback ring. In this study, prediabetic mice showed a decrease of mBDNF levels and p-CREB/CREB ratio in the hippocampus.

Moreover, in experimental models of diabetes, such as the Goto-Kakizaki rats and ETZ, downregulation of Akt/CREB/BDNF signaling was related to the impairment of learning and memory (Xiang et al. 2015). Likewise, a close relationship between BDNF levels and depressive-like phenotype has been reported in rodents and it has been proposed that restored BDNF signaling may contribute to the alleviation of depressive symptoms (Lenart et al. 2019). Therefore, we hypothesized that memory impairment added to

depressive-like phenotype were accompanied by the downregulation of CREB/BDNF signaling in the hippocampus of prediabetic mice.

Although further detailed studies will be needed to unveil the exact molecular mechanism of memory impairment and depressive-like phenotype in prediabetic mice, this study revealed that the downregulation of hippocampal insulin and BDNF signaling pathways were accompanied the behavioral phenotypes of prediabetic mice. More detailed investigation aimed at understanding better the long-term outcomes of prediabetes is needed to clarify potential targets of this pathology and advance the understanding about the prevalence of depression and memory impairment reported in prediabetes.

5 Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure Captions

Fig 1 Schematic representation of the experimental design. A battery of behavior tests started at 17th day of protocol. Two sets of different animals performed the object recognition test (ORT), object location test (OLT) and Morris water maze (MWM) test. Another set of animals performed the spontaneous locomotor profile (SLP), tail suspension test (TST) and forced swimming test (FST) at 21 days. Ex vivo assays were carried out after the 23 days

Fig 2 Effects of prediabetes on (A) glycemia, (B) body weight gain, and (C) relative epididymal fat in mice. Data were analyzed by an unpaired Student's test (B and C) and two-way analysis of variance (ANOVA) followed by the Bonferroni test (A). *$p < 0.05$ as compared to the control mice

Fig 3 Effects of prediabetes on (A) oral glucose tolerance teste-OGTT and (B) insulin tolerance test-ITT in mice. Insets (A) AUC_{OGTT} and (B) AUC_{ITT}. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni test (A and B) and by an unpaired Student's test (insets A and B). *$p < 0.05$ as compared to the control mice

Fig 4 Effects of prediabetes on the performance of mice in the object recognition test (ORT), (A) the exploration index of similar objects and (B) the recognition index. Data were analyzed by an unpaired Student's test. *$p < 0.05$ as compared to the control mice

Fig 5 Effects of prediabetes on the performance of mice in the object location test (OLT), (A) the exploration index of similar objects and (B) the location index. Data were analyzed by an unpaired Student's test. *$p < 0.05$ as compared to the control mice

Fig 6 Effects of prediabetes on the performance of mice in the Morris water maze (MWM) test, (A) latency to reach the platform in the 4 days of training and (B) traveled distance in the target quadrant. Data were analyzed by two-way analysis of variance (ANOVA)(A) and by an unpaired Student's test (B). *$p < 0.05$ as compared to the control mice

Fig 7 Effects of prediabetes on the performance of mice in immobility time (A) in the tail suspension test (TST) and (B) in the forced swimming test (FST). Data were analyzed by an unpaired Student's test. *$p < 0.05$ as compared to the control mice

Fig 8 Effects of prediabetes on (A) IRS-1 phosphorylation, (B) Akt ratio and (C) GLUT4 content in the hippocampus of mice. Representative qualitative Western blotting analysis is on the right side of the graphics. Data were analyzed by an unpaired Student's test. *$p < 0.05$ as compared to the control mice

Fig 9 Effects of prediabetes on (A) mBDNF levels and (B) CREB ratio in the hippocampus of mice. Representative qualitative Western blotting analysis is on the right side of the graphics. Data were analyzed by an unpaired Student's test. *$p < 0.05$ as compared to the control mice

Figures

Figure 1

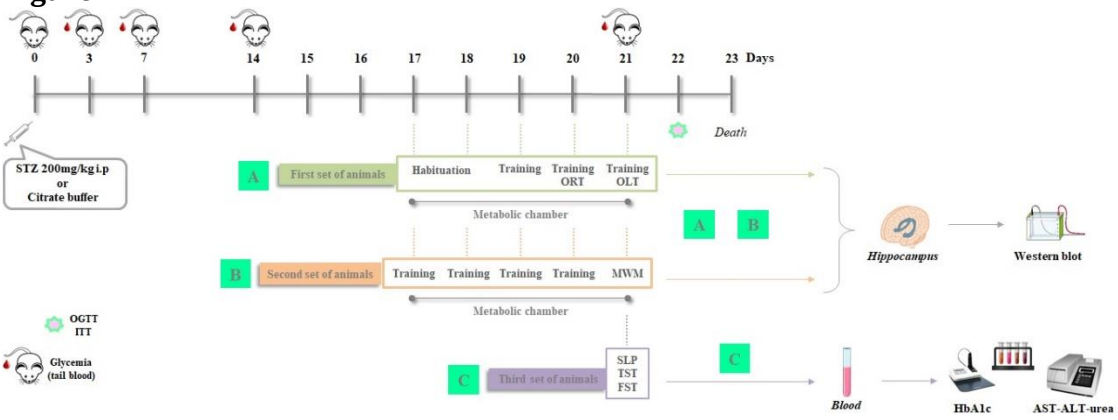


Figure 2

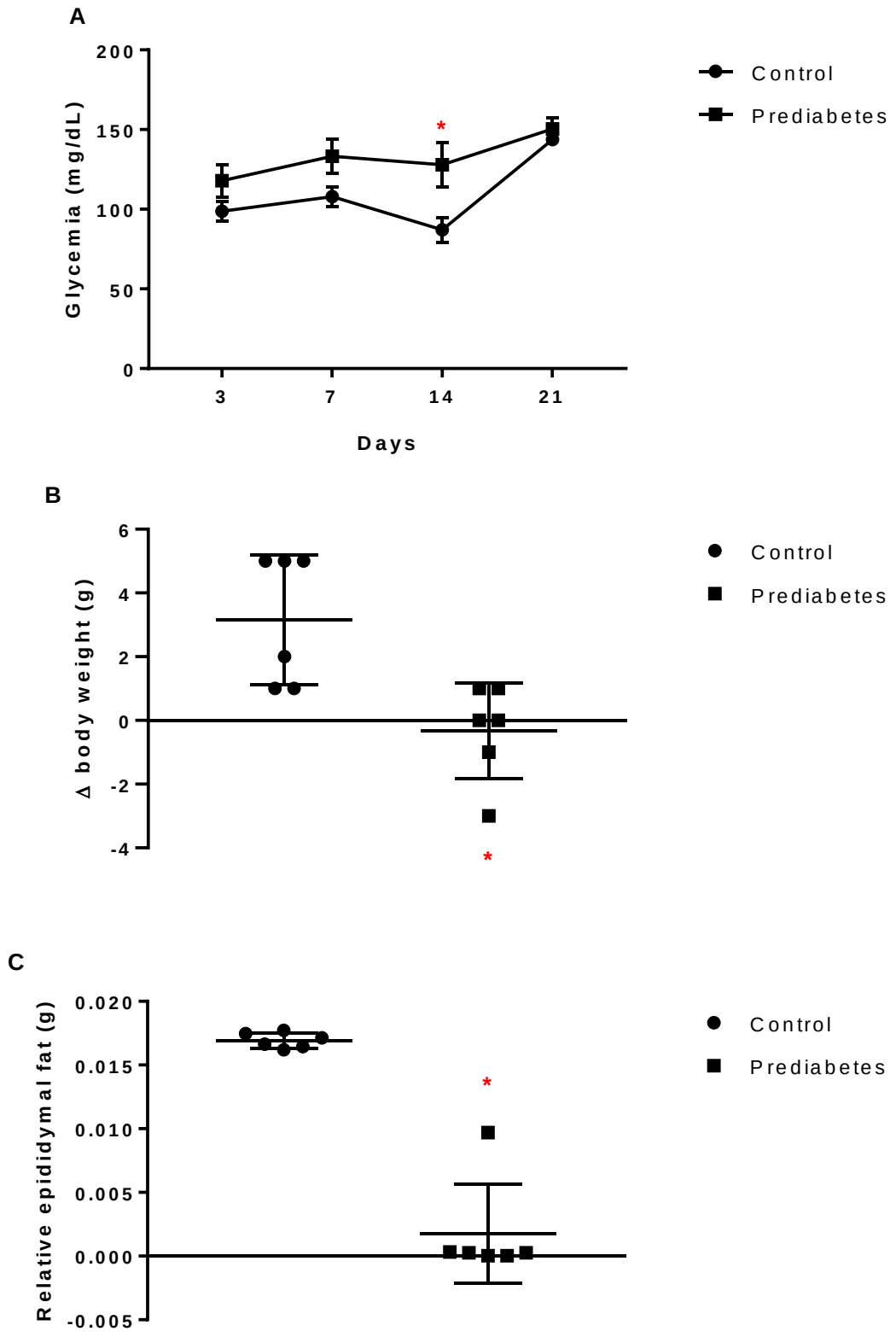

A
Glycemia (mg/dL)
200
150
100
50
0
3
7
14
21
Days
*
Control
Prediabetes
B
Δ body weight (g)
6
4
2
0
-2
-4
*
Control
Prediabetes
C
Relative epididymal fat (g)
0.020
0.015
0.010
0.005
0.000
-0.005
*
Control
Prediabetes

Figure 3

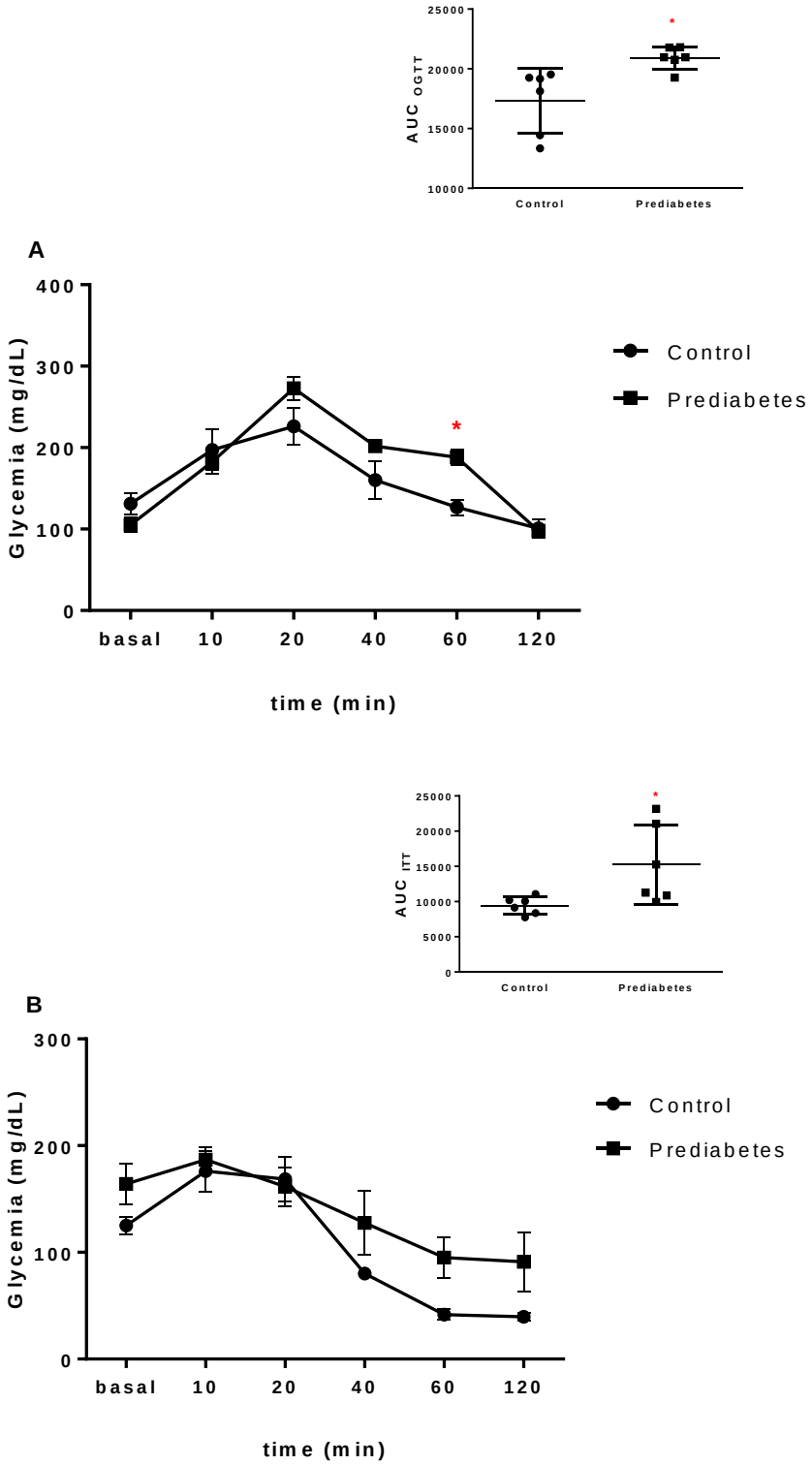

AUC OGTT
25000
20000
15000
10000
Control
Prediabetes
*
A
Glycemia (mg/dL)
400
300
200
100
0
basal
10
20
40
60
120
time (min)
Control
Prediabetes
*
AUC ITT
25000
20000
15000
10000
5000
0
Control
Prediabetes
*
B
Glycemia (mg/dL)
300
200
100
0
basal
10
20
40
60
120
time (min)
Control
Prediabetes

Figure 4

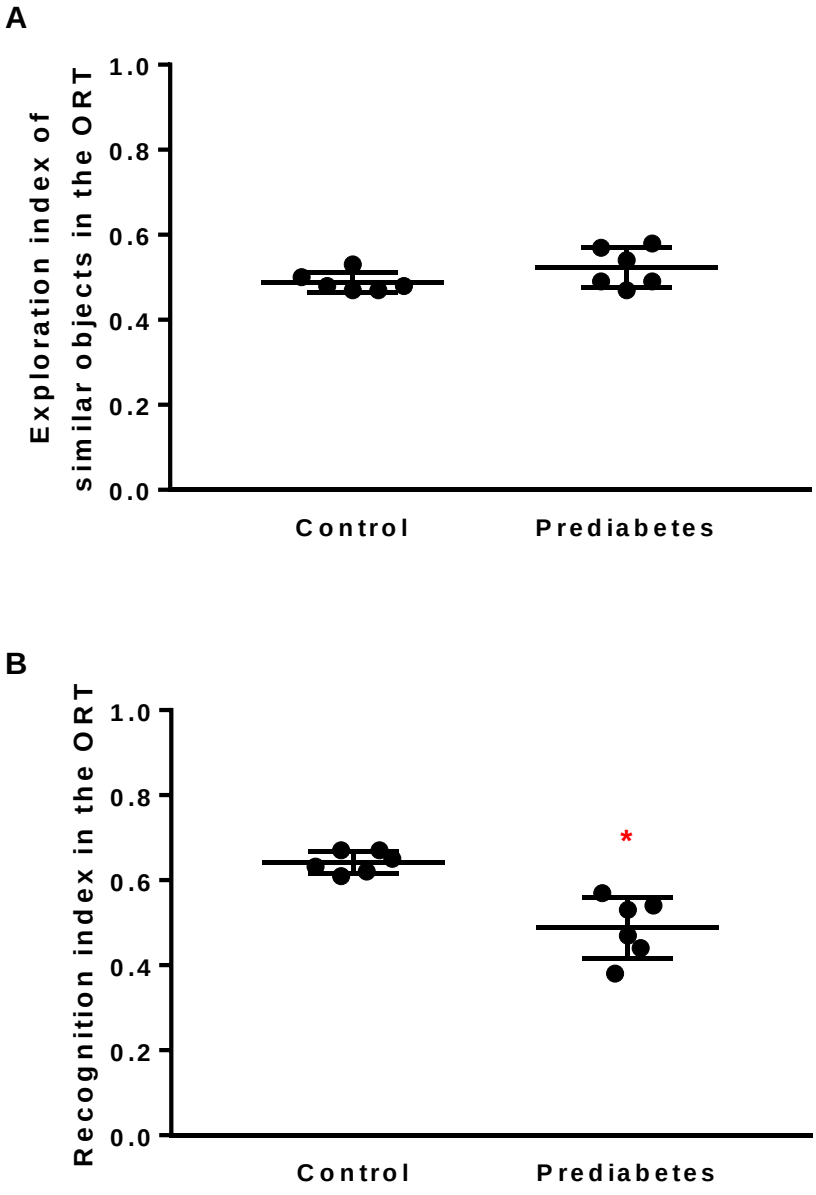

A
Exploration index of similar objects in the ORT
1.0
0.8
0.6
0.4
0.2
0.0
Control
Prediabetes
B
Recognition index in the ORT
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Prediabetes

Figure 5

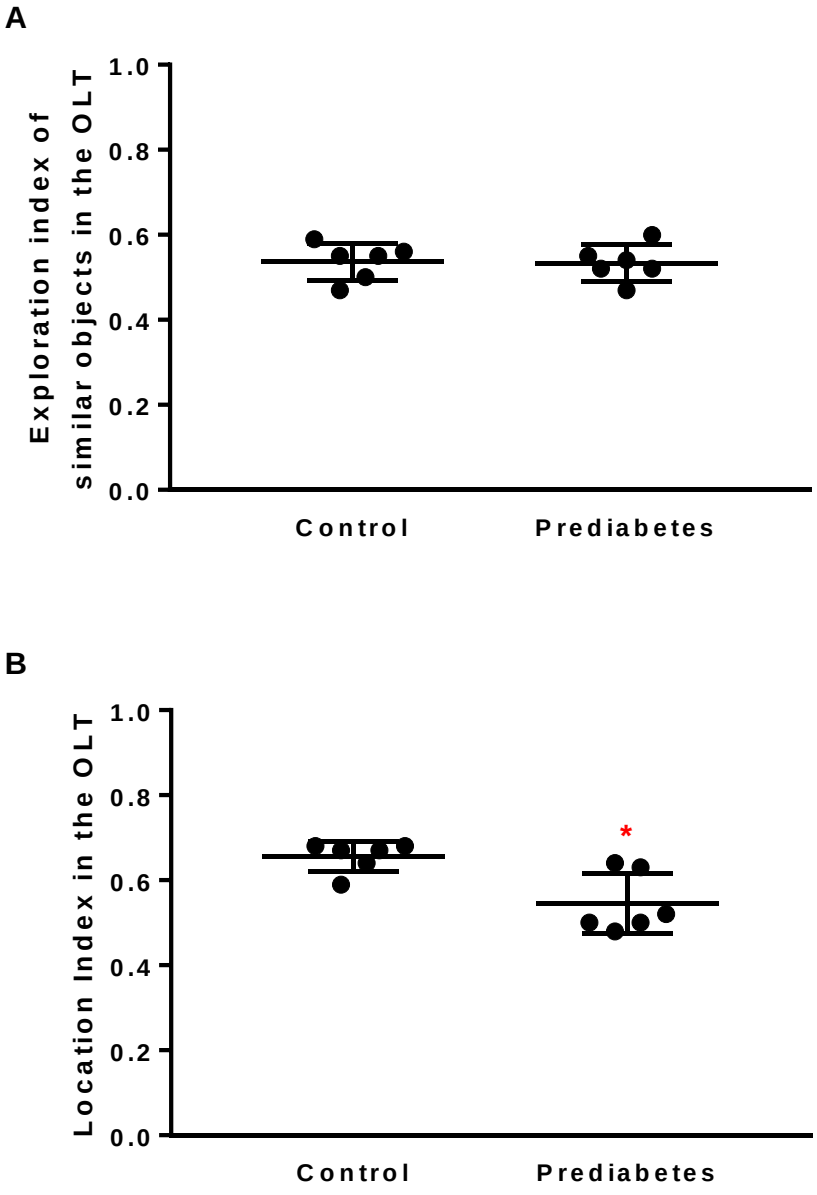

A
Exploration index of similar objects in the OLT
1.0
0.8
0.6
0.4
0.2
0.0
Control
Prediabetes
B
Location Index in the OLT
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Prediabetes

Figure 6

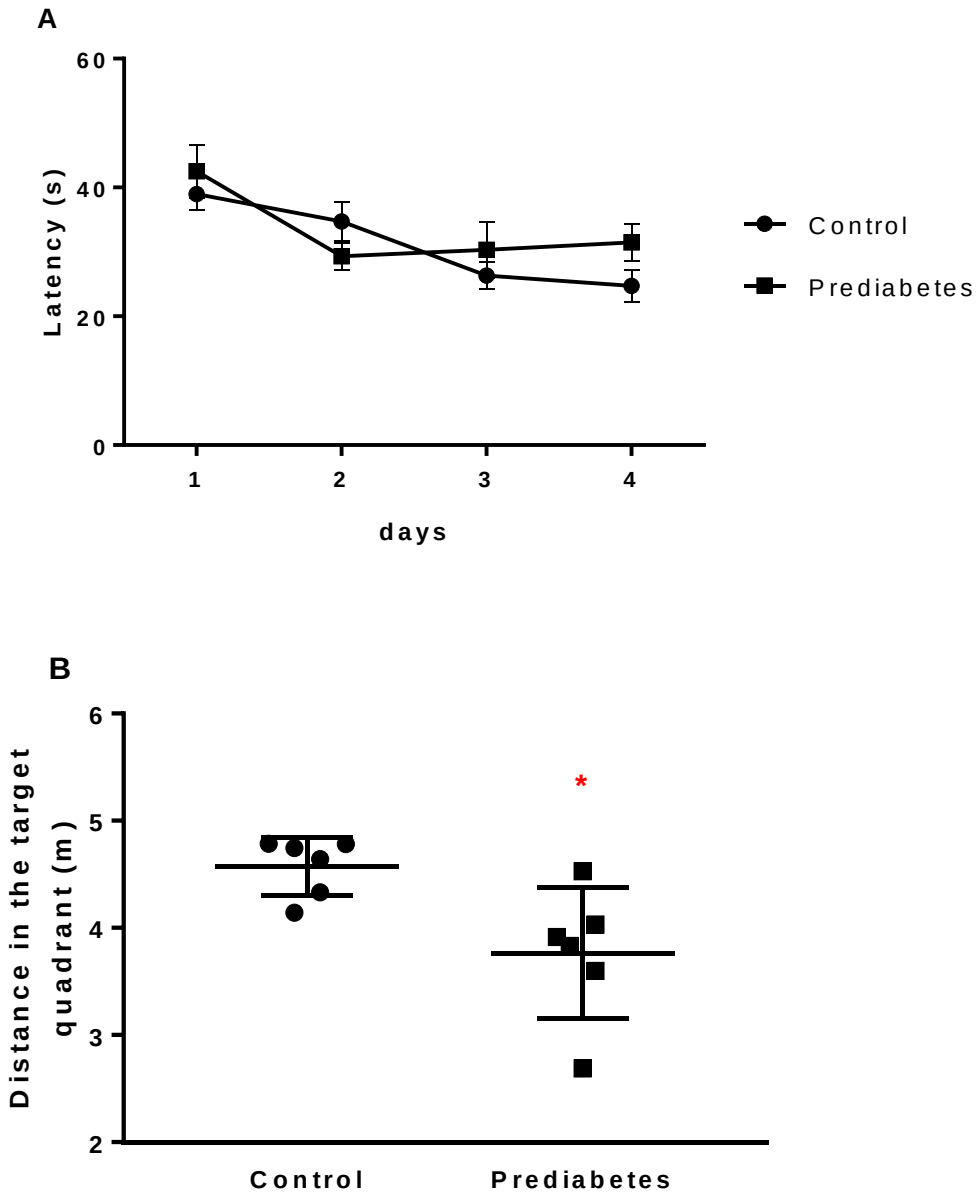

A
60
40
20
0
Latency (s)
1
2
3
4
days
Control
Prediabetes
B
6
5
4
3
2
Distance in the target quadrant (m)
*
Control
Prediabetes

Figure 7

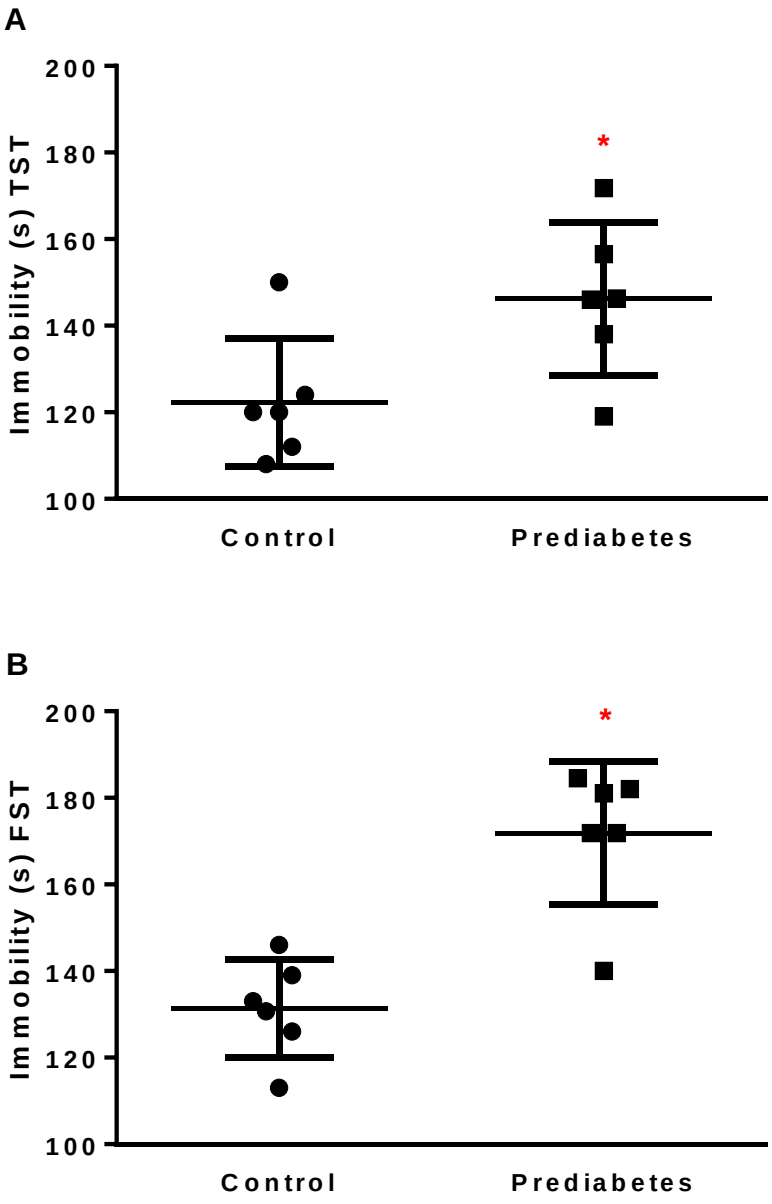

A
Immobility (s) TST
200
180
160
140
120
100
*
Control
Prediabetes
B
Immobility (s) FST
200
180
160
140
120
100
*
Control
Prediabetes

Figure 8

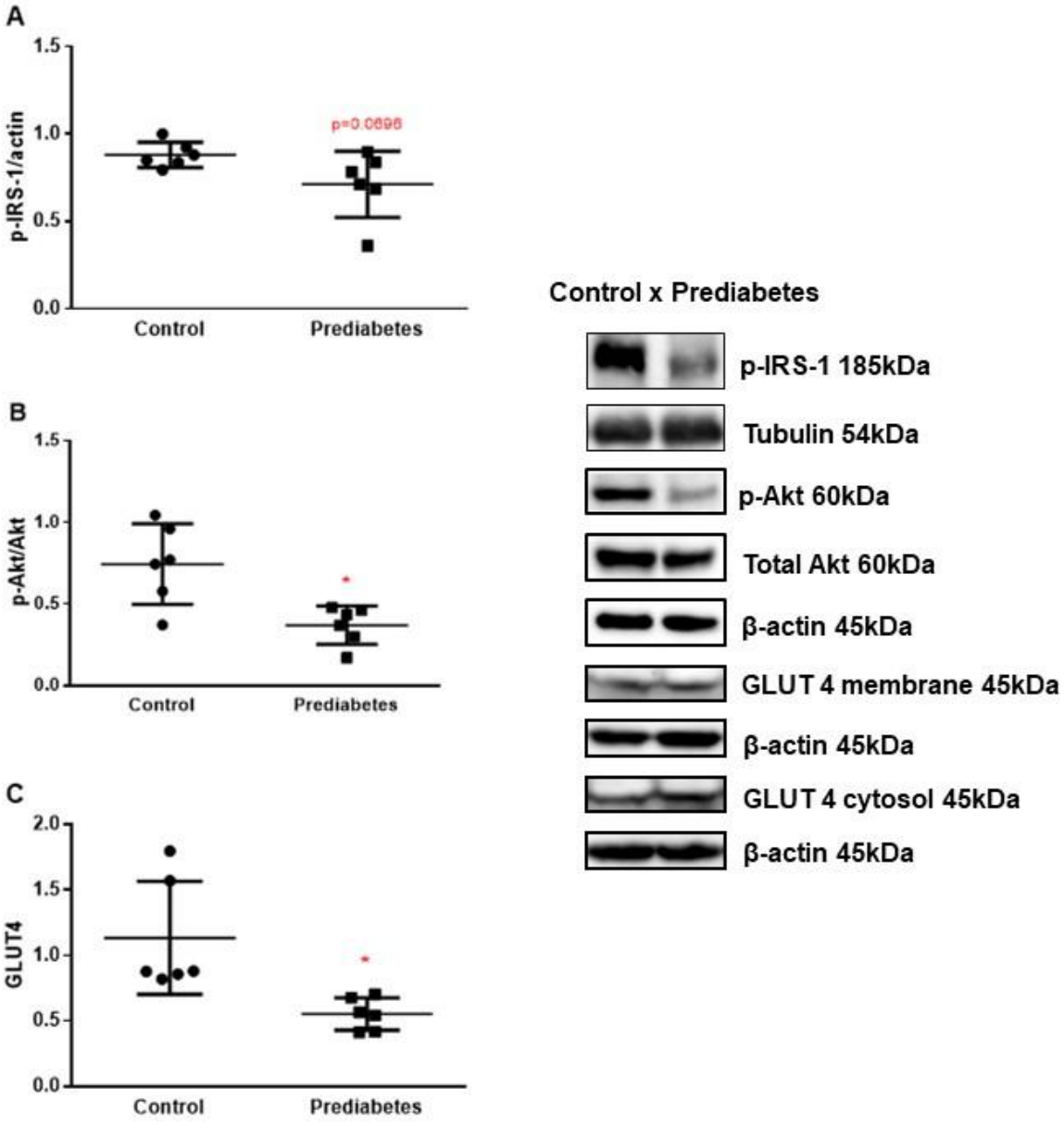
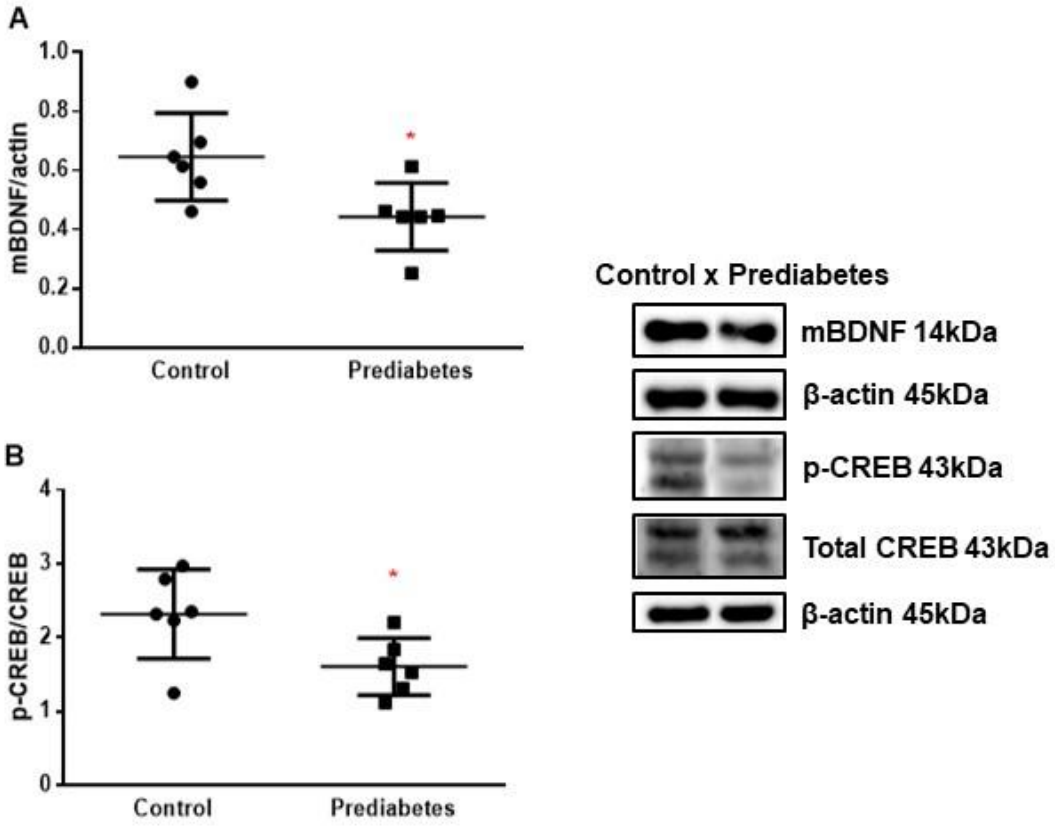

A
p-IRS-1/actin
1.5
1.0
0.5
0.0
p=0.0696
Control
Prediabetes
B
p-Akt/Akt
1.5
1.0
0.5
0.0
*
Control
Prediabetes
C
GLUT4
2.0
1.5
1.0
0.5
0.0
*
Control
Prediabetes
Control x Prediabetes
p-IRS-1 185kDa
Tubulin 54kDa
p-Akt 60kDa
Total Akt 60kDa
β-actin 45kDa
GLUT 4 membrane 45kDa
β-actin 45kDa
GLUT 4 cytosol 45kDa
β-actin 45kDa

Figure 9


A
mBDNF/actin
1.0
0.8
0.6
0.4
0.2
0.0
Control
Prediabetes
B
p-CREB/CREB
4
3
2
1
0
Control
Prediabetes
Control x Prediabetes
mBDNF 14kDa
β-actin 45kDa
p-CREB 43kDa
Total CREB 43kDa
β-actin 45kDa

Supplementary data

Memory impairment and depressive-like phenotype are accompanied by downregulation of hippocampal insulin and BDNF signaling pathways in prediabetic mice

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Supplementary Table 1 List of primary antibodies used by Western Blot assay.

Supplementary Table 2 Food and water intake, and diuresis of prediabetic mice.

Supplementary Table 3 Physical and chemical properties of urine in prediabetic mice.

Supplementary Table 4 Protein, glucose and ketones in urine of prediabetic mice.

Supplementary Table 5 Hepatic and renal markers of toxicity in prediabetic mice.

Supplementary Table 6 Locomotor and exploratory activities in prediabetic mice.

Table 1S List of primary antibodies used by Western Blot assay.

Antibody name	Molecular weight (kDa)	Type	Company	Dilution
β-Actin	45	mouse	Cell Signaling Technology	1:5000
pAkt	60	rabbit	Cell Signaling Technology	1:1000
Akt	60	rabbit	Cell Signaling Technology	1:1000
BDNF	14	rabbit	Abcam	1:1000
pCREB	43	rabbit	Cell Signaling Technology	1:1000
CREB	43	rabbit	Cell Signaling Technology	1:1000
GLUT4	45	rabbit	Abcam	1:1000
pIRS-1	185	rabbit	Cusabio	1:1000
Tubulin	54	mouse	Abcam	1:5000

Akt (Protein kinase B); BDNF (Brain-derived neurotrophic factor); CREB (cAMP response element binding); GLUT4 (glucose transporter 4); IRS-1 (Insulin receptor substrate 1).

Table 2S Food and water intake and diuresis of prediabetic mice.

	Control	Prediabetes
Food intake (g)	0.83 ± 0.16	0.83 ± 0.47
Water intake (g)	0.83 ± 0.40	1.33 ± 0.42
Diuresis (ml)	0.78 ± 0.19	1.08 ± 0.23

Data are reported as mean ± S.E.M of 6 animals/group. Data were analyzed by an unpaired Student's test.

Table 3S Physical and chemical properties of urine in prediabetic mice.

	Control	Prediabetes
Leucocytes (WBC/µL)	-	-
Urobilinogen (mg/dL)	-	-
Bilirrubin (mg/dL)	-	-
Erythrocytes (RBC/µL)	-	-
Nitrite	-	-
pH	6.75 ± 0.25	5.91 ± 0.41
Density	1015 ± 1.82	1015 ± 1.82

Data are reported as mean ± S.E.M of 6 animals/group. Data were analyzed by an unpaired Student's test.

Table 4S Protein, glucose and ketones in urine of prediabetic mice.

Parameters	Results	Control	Prediabetes
Protein	Negative	**6/6**	**5/6**
	+		**1/6**
	++		
	+++		
Glucose	Negative	**6/6**	**4/6**
	+		**2/6**
	++		
	+++		
Ketones	Negative	**6/6**	**5/6**
	+		**1/6**
	++		
	+++		

Representative numbers of 6 animals/group. The statistical analysis was not performed.

Table 5S Hepatic and renal markers of toxicity in prediabetic mice.

	Control	Prediabetes
AST (U/L)	75.5 ± 1.1	90.6 ± 5.1*
ALT (U/L)	60.5 ± 2.7	68.0 ± 5.1
Urea (mg/dL)	24.5 ± 0.2	21.8 ± 1.6

Data are reported as mean ± S.E.M of 6 animals/group. *$p<0.05$ when compared with the control group. Data were analyzed by an unpaired Student's test.

Table 6S Locomotor and exploratory activities in prediabetic mice.

	Control	Prediabetes
Crossing	605.8 ± 16.2	555.0 ± 45.0
Distance (mm)	10217.8 ± 294.8	9131.5 ± 554.4

Data are reported as mean ± S.E.M of 6 animals/group. Data were analyzed by an unpaired Student's test.

4 DISCUSSÃO

No Diabetes tipo 1 vários fatores genéticos e ambientais podem resultar na perda progressiva de massa e/ou função das células beta do pâncreas, que se manifesta clinicamente com a hiperglicemia (AAD, 2019). Quando a hiperglicemia se estabelece, embora as taxas de progressão das complicações possam diferir, os pacientes começam a desenvolver complicações crônicas. Assim, a caracterização da fisiopatologia do diabetes, exigirá, no futuro, um melhor entendimento sobre os diferentes caminhos das complicações do diabetes (SKYLER et al., 2017).

Nesse sentido, evidências sugerem que o diabetes está associado a um desempenho reduzido em vários domínios da função cognitiva, sendo que o tempo do diabetes não-controlado e a falta do controle glicêmico, podem ter um impacto no tipo e na gravidade do comprometimento cognitivo dos pacientes (ZILLIOX et al., 2016). Além do déficit de memória, existe uma relação importante do diabetes com a depressão, assim tem sido relatado que as taxas de prevalência de depressão podem ser até três vezes maiores em pacientes com diabetes em comparação com a população em geral (ROY; LLOYD, 2012). Nessa perspectiva, elucidar os vínculos entre o déficit de memória e a depressão no diabetes torna-se importante, uma vez que as bases moleculares destas doenças podem ser convergentes. Um aspecto importante seria o entendimento das origens comuns, a fim de melhorar os tratamentos farmacológicos de forma ampla, não apenas em um único alvo específico da doença.

Nesse contexto, os compostos orgânicos de selênio têm chamado a atenção devido às propriedades farmacológicas reportadas na literatura, principalmente, aquelas relacionadas aos efeitos benéficos do tipo neuroendócrinos e neuropsicológicos (BORTOLATTO et al., 2017; CECHELLA et al., 2018; GAI et al., 2014; ROSA et al., 2016). Além disso, sabe-se que uma deficiência em Se no organismo pode levar a predisposição para o desenvolvimento de doenças, tais como o câncer, as doenças cardiovasculares e o diabetes (WANG et al., 2016). O interesse pelo composto orgânico de selênio, (*p*-ClPhSe)$_2$ surgiu devido aos efeitos biológicos apresentados no estudo de BORTOLATTO et al. (2012), em que este composto protegeu contra o fenótipo do tipo depressivo e o prejuízo cognitivo em ratos machos velhos.

Com o objetivo de continuar as descobertas acerca dos efeitos promissores do (*p*-ClPhSe)$_2$ relacionados ao efeito do tipo antidepressivo e no déficit de memória, os experimentos dessa tese avaliaram as propriedades farmacológicas do (*p*-ClPhSe)$_2$ na comorbidade déficit de memória/depressão em um modelo de diabetes induzida por ETZ em camundongos Swiss, bem como buscou-se elucidar possíveis alvos de ação do composto em

estudo. Inicialmente, os experimentos do **artigo 1** foram realizados com o intuito de avaliar a ação do (*p*-ClPhSe)$_2$ contra o prejuízo de memória induzido por diabetes em camundongos e o papel da via de sinalização do BDNF/TrkB no hipocampo desses animais. Enquanto o **artigo 2** teve como foco avaliar os efeitos do tipo antidepressivo do (*p*-ClPhSe)$_2$ nos animais diabéticos assim como investigar a contribuição da via de sinalização Keap1/Nrf2/HO-1 no córtex cerebral dos camundongos diabéticos induzidos com ETZ. Além disso, o **manuscrito 1** foi desenvolvido simultaneamente aos artigos citados anteriormente e teve como objetivo avaliar se os animais pré-diabéticos demonstravam prejuízos de memória e o fenótipo do tipo depressivo, assim como investigar as vias de sinalização AMPK/GLUT4 e Akt/CREB/BDNF no hipocampo dos camundongos pré-diabéticos.

Para o **artigo 1**, primeiramente foi realizado um estudo piloto em que foram testadas diferentes doses de ETZ para induzir o diabetes em camundongos Swiss. Visto que, de acordo com a literatura, a dose de ETZ necessária para induzir diabetes varia entre 100-300 mg/kg para camundongos (DEEDS et al., 2011) e de 40-60 mg/kg para ratos (SZKUDELSKI, 2001), quando administrada em uma única injeção pela via intraperitoneal. Portanto, após o experimento piloto, a dose escolhida de ETZ para induzir hiperglicemia em camundongos Swiss foi de 200 mg/kg de ETZ administrada pela via intraperitoneal. Vale destacar que, mesmo utilizando essa dose na qual a maioria dos animais tornam-se hiperglicêmicos, alguns camundongos não tiveram a glicemia maior que 200 mg/dl, parâmetro utilizado para caracterizar o diabetes. Esses animais, que não apresentaram glicemia superior a 200 mg/dl, foram denominados primeiramente como animais “resilientes a hiperglicemia” e não foram descartados do estudo, entretanto, foram conduzidos como grupo “resilientes” visto que não se encaixavam nem no grupo diabetes e nem no grupo controle. Os resultados obtidos com esses animais deram origem ao **manuscrito 1** desta tese.

Após a confirmação da diabetes (**artigo 1**) também foram verificados outros parâmetros que são característicos desta doença, tais como a hemoglobina glicada, a perda de peso, a poliúria, a polidipsia e a polifagia, bem como análises físico-químicas da urina. Segundo a *Associação Americana de Diabetes* de 2019, as pessoas com diabetes tipo 1 geralmente apresentam os sintomas característicos de poliúria, polidipsia e polifagia e ainda aproximadamente um terço apresentam cetoacidose diabética (DABELEA et al., 2014). Os indícios do início do diabetes tipo 1 podem ser variáveis e não apresentar todos os sintomas clássicos. No entanto, no **artigo 1** foi observado alteração em todos os parâmetros característicos da diabetes citados anteriormente.

Nesse contexto, para avaliar os efeitos farmacológicos do (*p*-ClPhSe)$_2$ sobre os parâmetros clássicos da diabetes, foram testadas primeiramente as doses de 1 e 5 mg/kg do composto. Após 7 dias de tratamento com (*p*-ClPhSe)$_2$ com ambas as doses, o composto apresentou efeitos significativos contra a hiperglicemia. Além disso, observou-se que a dose de 5 mg/kg foi suficiente para reverter a hiperglicemia dos camundongos diabéticos. Nesse sentido, sabe-se que alguns compostos orgânicos de selênio apresentam o efeito anti-hiperglicêmico podendo estar relacionado com propriedades insulino-miméticas desses compostos (COSTA et al., 2012; QUINES et al., 2017b). De fato, foi demonstrado por QUINES et al. (2017b) que o (*p*-ClPhSe)$_2$ apresentou ação insulino-mimética *in vitro* assim como reverteu a disfunção metabólica de carboidratos hepáticos induzidas por alto consumo de frutose em ratos Wistar. Além disso, recentemente este mesmo autor, utilizando o modelo *C.elegans*, revelou que (*p*-ClPhSe)$_2$ pode reduzir os níveis de glicose e triglicerídeos modulando a sinalização do tipo insulina/IGF-1 de maneira dependente de AGE-1/DAF-16. Esses achados sugerem que o tratamento com (*p*-ClPhSe)$_2$ apresenta efeitos farmacológicos atuando no metabolismo da glicose (QUINES et al., 2018a).

Após a caracterização do modelo de diabetes, com o intuito de avaliar comportamentos relacionados a processos de memória dos animais, realizaram-se testes preditivos de memória como o TRO, TLO e o teste LAM. As tarefas com objetos, ORT e OLT, têm sido amplamente utilizadas no estudo dos mecanismos neurobiológicos subjacentes à formação da memória (MURAI et al., 2007). Ambos os testes envolvem o mesmo manuseio dos animais, habituação e treinamento com dois objetos, sendo que a principal diferença ocorre no dia do teste, em que no ORT, um objeto é substituído por um novo e no OLT, um objeto é movido para um novo local. Esses testes não requerem reforços, ou seja, é puramente baseado na preferência inata dos roedores em explorar “o novo” (LEGER et al., 2013). Além disso, o TRO foi desenvolvido para testar a memória não espacial em roedores e o TLO acessa um tipo de memória espacial, sem a necessidade de reforçadores convencionais, como alimento ou imersão em água (ENNACEUR; DELACOUR, 1988). Quando combinados, esses dois testes podem permitir abordar questões experimentais envolvendo diferentes regiões cerebrais e alvos moleculares (VOGEL-CIERNIA; WOOD, 2014).

A fim de corroborar as análises realizadas com testes de objetos, buscou-se avaliar a memória utilizando um teste com reforço, através do teste do LAM. Esse teste é preconizado para avaliar especificamente o aprendizado e a memória espacial de longa duração (D'HOOGE; DE DEYN, 2001). Dessa forma, o teste do LAM tornou-se uma das ferramentas

laboratoriais mais utilizadas na neurociência comportamental para acessar memórias espaciais (BARNHART et al., 2015). Para realização desse teste comportamental, o aparato foi construído em uma sala reservada seguindo as especificações dimensionais da literatura (MORRIS, 1984). Esse aparato consiste em uma grande piscina circular cheia de água na qual uma pequena plataforma de fuga permanece submersa (TOMAS PEREIRA; BURWELL, 2015). Pistas visuais foram confeccionadas e adicionadas em torno do aparato para que o animal se localize, espacialmente, durante as séries de treinamentos, e aprendam a encontrar a plataforma e "fugir" da água (TOMAS PEREIRA; BURWELL, 2015; VORHEES; WILLIAMS, 2006). A aprendizagem espacial e o desempenho no teste do LAM parecem depender de diferentes regiões do cérebro que constituem uma rede neural integrada (VORHEES; WILLIAMS, 2006). Entretanto, está bem estabelecido que a integridade do hipocampo é essencial para os processos de memória espacial (BARNHART et al., 2015; PEARCE et al., 1998).

Nesse sentido, no **artigo 1** foi possível observar que os animais diabéticos apresentaram prejuízo de memória nos testes comportamentais realizados, enquanto o tratamento com o (*p*-ClPhSe)$_2$ reverte este prejuízo, quando avaliado nos três diferentes testes comportamentais, os quais avaliam diferentes tipos de memória. Vale ressaltar que o tratamento com o (*p*-ClPhSe)$_2$ foi efetivo tanto na memória espacial (TLO e LAM), como na memória não espacial (TRO). Além disso, foi possível perceber que a dose de 1 mg/kg do (*p*-ClPhSe)$_2$ não foi efetiva contra o prejuízo de memória no TLO, por isso as análises posteriores do estudo foram realizadas apenas com a dose de 5 mg/kg do composto, que foi efetiva em reverter o dano de memória em todos os testes comportamentais avaliados no **artigo 1**. Corroborando com esses resultados, um estudo prévio demonstrou que o tratamento com (*p*-ClPhSe)$_2$ na dose de 5 mg/kg durante o mesmo período de administração utilizado no artigo 1, foi efetivo contra o comprometimento de memória induzido por estresse em camundongos Swiss (ZBOROWSKI et al., 2016), reforçando o potencial efeito farmacológico apresentado pelo tratamento com (*p*-ClPhSe)$_2$ frente à prejuízos de memória.

Em seguida, para fins comparativos, surgiu a ideia do tratamento com uma droga de uso clínico, um controle positivo, para isso foi escolhida a metformina. Esse medicamento é amplamente utilizado na clínica há muitos anos devido a sua ação anti-hiperglicêmica, que é consequência da redução da produção de glicose devido à inibição da gliconeogênese hepática (SHAW et al., 2005) e, em menor grau, aumento da captação de glicose mediada por insulina no músculo esquelético (MCINTYRE et al., 1991). Dessa forma, com objetivo de verificar uma redução da hiperglicemia, assim como foi observado após o tratamento com

composto, foi escolhido à dose de 200 mg/kg de metformina, que é o equivalente a 1400 mg/kg administrada em seres humanos (OLIVEIRA et al., 2016; TERPSTRA, 2001). Os camundongos diabéticos receberam metformina duas vezes ao dia, intragastricamente, do dia 3 ao dia 21 de protocolo. Interessantemente, o tratamento com a metformina foi efetivo em reverter o aumento dos parâmetros metabólicos como a poliúria, a polidipsia e a polifagia causados pela diabetes, no entanto, não foi possível verificar a redução da hiperglicemia nos camundongos após o tratamento com a metformina. Dessa forma, como o objetivo proposto para comparar a metformina com (*p*-ClPhSe)$_2$ não teve êxito, optou-se por encerrar os estudos em relação ao controle positivo, e seguir com as análises somente com o tratamento com (*p*-ClPhSe)$_2$.

Após avaliação comportamental, buscando investigar os mecanismos pelos quais o (*p*-ClPhSe)$_2$ reverteu os danos de memória causados pelo diabetes, o **artigo 1** explorou a contribuição das proteínas relacionadas com a via de sinalização do BDNF/TrkB na região do hipocampo cerebral dos animais. Para a avaliação dessas proteínas, foi proposta a realização da técnica de Western Blot. Durante essa época foi necessário realizar uma repadronização da técnica desenvolvida no nosso laboratório, devido a algumas modificações dos tampões, principalmente, o de homogeneização de tecido que era utilizada naquele momento. Após alguns ensaios, chegou-se a uma repadronização da técnica. A partir disso, as amostras de hipocampo dos camundongos submetidos ao protocolo experimental foram homogeneizadas para análise do conteúdo de proteínas da via de sinalização pretendida.

Sabe-se que as interações sinérgicas entre a atividade neuronal e a plasticidade sináptica reguladas pelo BDNF fazem dele um regulador fundamental para os mecanismos moleculares subjacentes à plasticidade sináptica e aos processos de memória (CHEN et al., 2017). Nesse sentido, no **artigo 1** foi observado que animais diabéticos apresentaram a redução dos níveis de BDNF maduro bem como de seu receptor TrkB, agregado a um aumento do precursor do BDNF. Em contrapartida, foi possível observar um aumento nos níveis de BDNF maduro e do TrkB bem como a redução dos níveis do seu precursor no hipocampo de camundongos tratados com o (p-ClPhSe)$_2$ quando comparados aos animais diabéticos. Corroborando com esses resultados, alguns compostos da mesma classe do (*p*-ClPhSe)$_2$ apresentaram efeitos semelhantes, aumentando os níveis de BDNF no hipocampo de ratos adultos (ROSA et al., 2016). Além disso, também foi proposto um papel neuroprotetor desses compostos através da modulação da sinalização da via BDNF/TrkB em um modelo de doença de Parkinson (SAMPAIO et al., 2017).

Para ampliar o conhecimento em relação à modulação da via de sinalização do BDNF/TrkB neste estudo, foi proposto mensurar proteínas *downstream* da via que, em conjunto, estão diretamente envolvidas com a aprendizagem e a memória. Baseado nisso, ainda no **artigo 1** avaliou-se o efeito do tratamento com o (*p*-ClPhSe)$_2$ no conteúdo da Akt, ERK e CREB, proteínas *downstream* da via BDNF/TrkB. Após a quantificação do conteúdo dessas proteínas, foi possível observar que os animais diabéticos apresentaram redução na fosforilação de ERK e CREB no hipocampo, sem alterar a fosforilação de Akt, ao passo que o tratamento com (*p*-ClPhSe)$_2$ foi efetivo em reverter as alterações nos níveis das proteínas alteradas nos animais com diabetes. De fato, acredita-se que o BDNF seja o alvo transcricional mais conhecido da proteína de ligação ao elemento de resposta ao AMPc (CREB), emergindo como um importante modulador sináptico (JIANG et al., 2016). Nesse cenário, ERK e CREB são proteínas reconhecidas como pontos críticos de convergência nas vias de sinalização que regulam a transcrição de genes para a aprendizagem e a memória (DAVIS et al., 2000). Os dados obtidos nesta tese corroboram os da literatura, nos quais ZHONG e colaboradores demonstraram um prejuízo de memória e a redução dos níveis da proteína CREB em hipocampo de ratos diabéticos. Isso sugere que o dano de memória induzido pela diabetes está associado ao prejuízo na sinalização da proteína CREB (HAN et al., 2012; ZHONG et al., 2016).

No **artigo 1**, realizou-se as análises de coloração de FJC que mostraram um aumento de células positivas para FJC no hipocampo, especificamente na região do giro dentado, de animais diabéticos e que o tratamento com (*p*-ClPhSe)$_2$ foi efetivo em reverter este efeito, indicando a ação neuroprotetora do composto. Neste sentido, BIESSELS e REIJMER demonstraram que a hiperglicemia persistente pode contribuir para a redução do volume do hipocampo e aumentar o número de células positivas para FJC em ratos diabéticos, o que é um indício de morte neuronal (BIESSELS; REIJMER, 2014). Estes dados são consistentes com estudos realizados em culturas de células PC12 (modelo de cultura neuronal), que mostram uma diminuição na viabilidade das células induzida por altos níveis de glicose (AFRAZI et al., 2014; AMINZADEH et al., 2014; LIU et al., 2013; YANG et al., 2017; YANG et al., 2014). Estudos com humanos também reportam a associação entre hiperglicemia e a redução do hipocampo, tanto na seção transversal quanto longitudinal em crianças e jovens adultos (MARZELLI et al., 2014; MUSEN et al., 2006). Entretanto, esse dado levanta algumas questões em relação aos possíveis mecanismos que possam estar envolvidos na ação do (*p*-ClPhSe)$_2$, em razão de outros pesquisadores terem mostrado o

envolvimento do estresse oxidativo, da inflamação e das vias apoptóticas na neurodegeneração em regiões do hipocampo na diabetes (YANG et al., 2014).

Dessa forma no **artigo 1**, demonstrou-se que o tratamento com o (*p*-ClPhSe)$_2$ na dose de 5 mg/kg administrado no regime de 7 dias consecutivos, mesmo sem reverter todos os parâmetros metabólicos característicos do fenótipo diabético dos animais, apresentou efeitos positivos na modulação da via hipocampal do BDNF/TrkB e neuroproteção, contribuindo para os efeitos promissores do composto contra o dano de memória apresentado pelos animais com diabetes.

Somando-se a isso, o referido modelo de diabetes mostra uma variedade de alterações no SNC, as quais se assemelham a depressão em humanos, entre elas algumas modificações celulares como a alteração dos níveis de neurotransmissores, na função do eixo HPA, no sistema imunológico, no estresse oxidativo, na perda neuronal e o prejuízo da plasticidade sináptica contribuindo para o quadro de depressão associada ao diabetes (BEAUQUIS et al., 2010; MARITIM et al., 2003; PATEL et al., 2018; PRABHAKAR et al., 2015). Portanto, agregado a esses fatos o modelo do diabetes tornou-se atrativo para o estudo da comorbidade memória/depressão sob os efeitos do (*p*-ClPhSe)$_2$ tanto em relação aos efeitos comportamentais quanto em alterações bioquímicas. Dessa forma, no **artigo 2** objetivou-se investigar o efeito antidepressivo do (*p*-ClPhSe)$_2$ bem como os alvos de ação do composto, utilizando-se o mesmo modelo de diabetes e a dose eqüipotente do composto, que foi efetiva contra o prejuízo de memória, utilizados no **artigo 1**.

No que se refere aos testes comportamentais relacionados a depressão, o TNF é um dos modelos comportamentais mais comumente usados para avaliar o comportamento do tipo depressivo. Esse teste envolve a pontuação do comportamento ativo (natação e escalada) ou passivo (imobilidade) quando os roedores são forçados a nadar em um cilindro do qual não há escapatória. A redução do tempo imóvel é interpretada como um efeito antidepressivo, desde que não aumente a atividade locomotora geral do animal (SLATTERY; CRYAN, 2012). Nesse sentido, o TNF é uma das ferramentas padrão para o *screening* de novos antidepressivos em modelos animais (PORSOLT et al., 1979). Dessa forma, no **artigo 2**, quando realizado o TNF com os animais diabéticos foi possível observar um aumento no tempo de imobilidade, indicando um quadro do tipo depressivo nos animais. Em contrapartida, quando os camundongos diabéticos foram tratados com o (*p*-ClPhSe)$_2$ observou-se o efeito do tipo antidepressivo exercido pelo tratamento, ou seja, reduziu o tempo de imobilidade dos animais diabéticos, mostrando o efeito farmacológico do (*p*-ClPhSe)$_2$ frente ao fenótipo do tipo depressivo em animais diabéticos. Corroborando com

estes dados, o efeito do tipo antidepressivo do (*p*-ClPhSe)$_2$ tem sido reportado, pelo nosso grupo de pesquisa, em diferentes modelos experimentais (BORTOLATTO et al., 2012; HECK et al., 2019).

Ainda no **artigo 2** utilizou-se outro paradigma comportamental, o TSC, para avaliar os efeitos do tratamento com o composto orgânico de selênio em animais diabéticos. Esse teste também é útil na triagem de possíveis medicamentos antidepressivos. Visto que, nesse teste o camundongo é suspenso por sua cauda com uma fita adesiva, em uma posição que não possa escapar ou agarrar-se a superfícies próximas (CAN et al., 2012) e durante seis minutos o tempo imóvel é quantificado. Quando os camundongos diabéticos realizaram o TSC ocorreu um aumento no tempo de imobilidade, mostrando um comportamento do tipo depressivo também nesse teste. Ainda, para confirmar a ação do tipo antidepressiva do composto, o tratamento com (*p*-ClPhSe)$_2$ diminuiu o tempo de imobilidade dos animais diabéticos no TSC.

Em conjunto, os resultados do **artigo 2** demonstraram que o tratamento com (*p*-ClPhSe)$_2$ na dose de 5 mg/kg administrado por 7 dias foi efetivo contra o comportamento do tipo depressivo induzido pelo diabetes em camundongos, tanto no TNF quanto no TSC. A partir desses resultados comportamentais, buscou-se compreender o mecanismo pelo qual o (*p*-ClPhSe)$_2$ poderia estar desempenhando a ação do tipo antidepressiva.

Os dados atuais da literatura indicam que a fisiopatologia da depressão pode estar distribuída em muitas regiões e circuitos cerebrais. Portanto, no **artigo 2** explorou-se a região do córtex cerebral, visto que essa região parece ter uma atividade diminuída em pacientes gravemente deprimidos, o que coincide com a tomada de decisão prejudicada e a maior propensão a agir negativamente, resultando em comportamento suicida (DESMYTER et al., 2011; KIMBRELL et al., 2002; RIGUCCI et al., 2010). De fato, alterações no eixo HPA foram descritas em pacientes deprimidos (DEAN; KESHAVAN, 2017), entre elas estão à hipersecreção de hormônio liberador da corticotrofina do hipotálamo, prejuízo no *feedback* negativo do eixo, glândulas suprarrenais aumentadas, hipercortisolemia e diminuição da supressão do cortisol em resposta à dexametasona (PRUESSNER et al., 2003). Além disso, o córtex pré-frontal medial, o hipocampo e a amígdala são áreas cerebrais alteradas pelo aumento de glicocorticoides (DEAN; KESHAVAN, 2017). Dessa forma, no **artigo 2** avaliou-se alguns parâmetros do eixo HPA no córtex cerebral, a fim de elucidar o possível envolvimento dos glicocorticoides no fenótipo depressivo em camundongos diabéticos. Visto que a associação biológica entre depressão e diabetes é atribuída a um eixo HPA desregulado e hiperativo (BADESCU et al., 2016).

Os resultados encontrados no **artigo 2** demonstram que ocorreu uma diminuição no conteúdo de receptores glicocorticoides no córtex cerebral, adicionado ao aumento do peso relativo da glândula adrenal dos camundongos diabéticos. Entretanto, foi possível observar que o tratamento com o (*p*-ClPhSe)$_2$ não reverteu essas alterações causada pelo diabetes. Isso não significa, necessariamente, que o composto não possa estar envolvido com outros parâmetros do eixo HPA não avaliados no **artigo 2**, porém suspeitamos que (*p*-ClPhSe)$_2$ não esteja envolvido nesse alvo da fisiopatologia da depressão associado ao diabetes.

Outro objetivo proposto para o **artigo 2** foi avaliar a ação do (*p*-ClPhSe)$_2$ no perfil redox no córtex cerebral dos animais diabéticos. Sabe-se que o cérebro é particularmente vulnerável a danos oxidativos devido à alta utilização de oxigênio e subsequente geração de subprodutos de radicais livres, com modestas defesas antioxidantes (COBLEY et al., 2018). O estresse oxidativo é definido como um desequilíbrio persistente entre a oxidação e os antioxidantes, que leva ao dano das macromoléculas celulares (LIU et al., 2015). O dano oxidativo tem uma contribuição importante nas pesquisas relacionadas a doenças cardiovasculares, diabetes, câncer e doença de Alzheimer (VALKO et al., 2007). Além disso, existem estudos sugerindo que o estresse oxidativo pode estar aumentado em vários transtornos psiquiátricos, incluindo a depressão (PANDYA et al., 2013). Dessa forma, acredita-se que a inclusão de um tratamento com antioxidantes na depressão pode ser uma opção auxiliar junto a tratamentos convencionais quando ocorre simultaneamente ao diabetes.

Os resultados apresentados no **artigo 2** evidenciaram um desequilíbrio da homeostase redox no córtex cerebral dos animais diabéticos, visto pelo aumento do dano oxidativo (TBARS e ROS) e a redução de uma das defesas antioxidante enzimática da célula - SOD, o que pode contribuir para o quadro do tipo depressivo apresentado por esses animais. Em contrapartida, o tratamento com o (*p*-ClPhSe)$_2$ modulou o estado redox e combateu o estresse oxidativo causado pelo diabetes no córtex cerebral. De fato, nas últimas décadas tem sido mostrado o potencial efeito antioxidante dos compostos orgânicos de selênio (BORTOLATTO et al., 2012; LUCHESE et al., 2009; PINTON et al., 2011; PRIGOL et al., 2009). Corroborando com os resultados obtidos no **artigo 2**, as propriedades antioxidantes do (*p*-ClPhSe)$_2$ foram recentemente demonstradas no córtex pré-frontal de camundongos expostos a dexametasona (HECK et al., 2019).

Levando em consideração que o estresse oxidativo esteja associado a ambas as doenças, diabetes e depressão, é possível prever que o estresse oxidativo desencadeie uma sucessão de eventos moleculares que podem potencializar mutuamente estas doenças. Nesse

sentido, reconhecendo a importância da relação entre o estresse oxidativo e o diabetes, foi intensificado a investigação no que tange ao comprometimento do sistema diabético em responder ao aumento da carga oxidativa. A célula sob condições de estresse oxidativo é regulada, principalmente, a nível transcricional, pela via do Nrf2/Keap1 (DAVID et al., 2017), que regula a expressão de mais de 100 genes e funções relacionadas ao estresse oxidativo e à sobrevivência celular. Dentre estes genes e funções destacam-se proteínas antioxidantes, enzimas de desintoxicação de fases I e II (como as hemeoxigenases), homeostase da glutationa, bem como a expressão de vários fatores de crescimento e fatores de transcrição (KENSLER et al., 2007).

Considerando esses fatos, os resultados do **artigo 2** ressaltam a contribuição da via de sinalização do Nrf2/Keap1/OH-1 no córtex cerebral de animais diabéticos com fenótipo depressivo. Ficou constatado que camundongos diabéticos apresentam redução nos níveis das proteínas Nrf2, Keap1 e OH-1, demonstrando uma *dowmregulation* na via de sinalização do Nrf2/Keap1/OH-1. Em compensação, os níveis dessas proteínas alteradas nos animais diabéticos e depressivos foram normalizados após o tratamento com (*p*-ClPhSe)$_2$, indicando a contribuição dessa sinalização celular no combate do estresse oxidativo e, consequentemente, promovendo um efeito do tipo antidepressivo nos camundongos diabéticos.

Nesse contexto, como o estresse oxidativo é considerado um fenômeno citotóxico, pode-se dizer que existe uma contribuição do estresse oxidativo em processos neurodegenerativos (HSIEH; YANG, 2013). Para apoiar esse argumento, estudos desenvolvidos com pacientes que sofrem de depressão apresentaram alterações volumétricas na amígdala, hipocampo e córtex pré-frontal (CAMPBELL; MACQUEEN, 2006). Como o estresse oxidativo pode modificar estrutural e funcionalmente proteínas e enzimas, e alterar a composição das membranas, causando peroxidação lipídica, este pode ser uma das causas subjacentes de morte celular neuronal e glial na depressão (MARIA MICHEL et al., 2012). Tendo em vista esses dados, foi proposto avaliar no **artigo 2** processos neurodegenerativos, utilizando-se a coloração de FJC no córtex dos animais diabéticos. Logo, foi visto que os camundongos diabéticos apresentaram aumento das células positivas para FJC no córtex cerebral, enquanto o tratamento com o (*p*-ClPhSe)$_2$ reverteu a expressão aumentada deste marcador. Esse dado corrobora com a literatura que reporta um aumento nas células positivas para FJC no córtex, hipotálamo e hipocampo de ratos diabéticos (WANG et al., 2014). Embora sejam necessários mais estudos para elucidar de que maneira acontece a neurodegeneração cortical, os resultados mencionados aqui sugerem que o estresse oxidativo pode estar contribuindo para a redução das células neuronais nesse modelo.

No decorrer dos experimentos dos **artigos1** e **2**, paralelamente, realizou-se as análises que estão contidas no **manuscrito 1**, visto que até aquele momento não estava claro para nós em qual dos grupos experimentais os animais “resilientes” se enquadrariam. Um fato paralelo a execução deste experimento, foi o fechamento do biotério de experimentação do PPGBTox, local em que os animais em experimentação permaneciam no prédio 19. A partir disso, foi necessária uma reorganização de todos os alunos, e um espaço em laboratórios vizinhos foi cedido para que os experimentos não cessassem por completo. Tendo em vista a redução da demanda e a disponibilidade de alocação dos animais, e ainda prezando pelas diretrizes dos 3R’s de utilização animal, surgiu a ideia de investigar os animais “resilientes”, que teoricamente, não pertenciam a nenhum grupo experimental dos relatados até aqui.

Em um primeiro momento, os animais que estão no **manuscrito 1** realizaram testes comportamentais relacionados a memória, assim como no **artigo 1**, e testes associados com o fenótipo do tipo depressivo, assim como no **artigo 2**, os testes transcorreram concomitantemente aos demais grupos experimentais dos artigos 1 e 2. Com estes resultados foi possível observar que os animais administrados com a ETZ, que não apresentavam hiperglicemia, apresentaram prejuízo de de diferentes tipos de memória, quando avaliados nos testes de TRO, OLT e LAM, bem como o fenótipo do tipo depressivo, vistos pelos testes do TNF e TSC, quando comparados ao grupo controle. Então se concluiu que, esses animais, de fato, não eram correspondentes aos animais do grupo controle. A partir desse momento, o foco do estudo foi investigar e elucidar porque esses animais apresentaram prejuízo de memória e fenótipo do tipo depressivo, apesar de não apresentarem características metabólicas semelhantes aos animais diabéticos.

Interessantemente, descobriu-se que esses animais (**manuscrito 1**) apresentavam perda de peso, perda relativa de gordura epididimal, um discreto aumento de glicemia (84 - 183 mg/dl) e um aumento da área sobre a curva nos testes de tolerância a glicose e a insulina quando comparados aos animais do grupo controle. A partir dessas análises, esses animais administrados com a ETZ que não apresentavam glicemia >200 mg/dl foram denominados como **pré-diabéticos**, visto que os critérios para o diagnóstico de pré-diabetes incluem o aumento da glicemia de jejum, prejuízo na tolerância à glicose e o aumento de HbA1c, podendo ou não apresentar resistência à insulina. Somando-se a isso, ainda no **manuscrito 1**, foi avaliado também o consumo de água, comida, diurese e as propriedades físico-químicas da urina dos animais pré-diabéticos, entretanto, esses parâmetros não foram diferentes do grupo controle. Mesmo sabendo que os estudos, geralmente, utilizam doses menores de ETZ para

induzir pré-diabetes (CHEN et al., 2018; VATANDOUST et al., 2018), nossa hipótese é que esses animais estariam em um estágio pré-diabético.

Nesse contexto, os resultados comportamentais observados pelo grupo pré-diabético no **manuscrito 1**, estão de acordo com estudos da literatura, os quais mostram que roedores pré-diabéticos demonstraram uma associação do prejuízo de memória à redução da microestrutura do hipocampo (SOARES et al., 2013). Além disso, pacientes pré-diabéticos apresentam risco aumentado de declínio cognitivo, quando comparados com uma população normoglicêmica (J et al., 2009; YAFFE et al., 2004). Ainda, animais pré-diabéticos apresentam propensão a ter um fenótipo do tipo depressivo (GUO et al., 2018), como observado no **manuscrito 1**, e estudos têm proposto que pacientes com sintomas depressivos e pré-diabetes tem um risco aumentado de progressão ao diabetes comparados com aqueles com apenas um desses fatores de risco (GRAHAM et al., 2017).

Com base nos efeitos apresentados nos testes comportamentais nos animais com pré-diabetes, foi proposto investigar a via de sinalização da insulina no hipocampo desses animais. Essas análises foram sugeridas, visto que a ligação da insulina ao receptor inicia uma cascata complexa de sinalização intracelular que sustenta uma variedade de funções neurais (KULLMANN et al., 2016), como aprendizado e memória (BIESSELS; REAGAN, 2015). Além disso, a resistência à insulina (RI) cerebral pode provocar vários efeitos biológicos abrangendo o envelhecimento e o funcionamento cognitivo (KULLMANN et al., 2016). Sabe-se que os receptores de insulina estão amplamente distribuídos nas regiões cerebrais e a presença de RI no hipocampo sugere seu envolvimento funcional no aprendizado e memória (DOU et al., 2005). As cascatas moleculares da via da insulina são compostas por um grande número de moléculas de sinalização, entre as diversas vias de sinalização, o IRS-1/PI-3K/Akt e a proteína contendo SH2 e colágeno (Shc)/MAPK no cérebro foram sugeridas para processos de aprendizado e memória (ZHAO et al., 2004).

Sabendo dessas informações, avaliou-se que seria relevante a quantificação dos níveis dessas proteínas em animais pré-diabéticos. De particular importância, os resultados encontrados no **manuscrito 1** demonstraram, pela primeira vez, que os camundongos pré-diabéticos apresentaram redução na sinalização IRS-1/Akt/GLUT4 hipocampal quando comparados aos do grupo controle. A redução dos níveis de receptores de insulina no hipocampo de ratos pré-diabéticos que consumiram uma dieta rica em sacarose por 9 semanas foi associado a um dano de memória espacial de curto e longo prazo (SOARES et al., 2013). Ainda, é relatado que jovens com níveis de glicemia de jejum e teste de tolerância a glicose alterada apresentam sintomas depressivos, bem como no mesmo estudo foi observado uma

prejudicada sensibilidade à insulina no hipotálamo de pacientes *post mortem* que apresentavam depressão (LI et al., 2016). Essas evidências nos permitem propor a contribuição da sinalização dessas proteínas IRS-1/Akt/GLUT4 no prejuízo de memória e no fenótipo do tipo depressivo em camundongos pré-diabéticos.

Como os experimentos realizados no **artigo 1** e no **manuscrito 1** ocorreram paralelamente, e demonstrou-se que os animais diabéticos apresentaram uma redução na cascata de sinalização do BDNF, foi proposto avaliar, então, o que estaria acontecendo naqueles animais no estágio pré-diabético. Então, para expandir o conhecimento acerca dos mecanismos envolvidos na patogenia do pré-diabetes, foi objetivado no **manuscrito 1** investigar outras vias proteicas que reforçariam a ideia de prejuízo de memória/depressão em animais pré-diabéticos. Nesse cenário, tem sido evidenciado em estudos que os níveis de BDNF cerebral são fundamentais no que tange aos processos neuropsicológicos, como a disfunção cognitiva e as doenças psiquiátricas (ZHONG et al., 2016). Tendo em vista esse conhecimento, o próximo passo do **manuscrito 1** fora analisar as vias de sinalização responsáveis pela síntese dessa neurotrofina, posto que o BDNF desempenha uma função essencial na sobrevivência e na plasticidade neuronal (KOWIANSKI et al., 2018). Com base na literatura, considera-se que uma das vias de sinalização responsável por ativar fatores de transcrição, que regulam a expressão de genes das neutrofinas é o CREB (KOWIANSKI et al., 2018).

Dessa forma, no **manuscrito 1** foi observado que camundongos pré-diabéticos tinham uma diminuição nos níveis das proteínas mBDNF e da razão pCREB/CREB na região do hipocampo cerebral. Esses achados sugerem que a diminuição do fator de transcrição, reduz a produção de neurotrofina cerebral. Além disso, outra proteína envolvida com a ativação de fatores de transcrição é a proteína Akt (LIM et al., 2008; MIZUNO et al., 2003). Evidências indicam que a Akt fosforila o CREB e, assim, induz a transcrição de BDNF, promovendo seus efeitos, como a plasticidade neuronal (DONG et al., 2018; MAYR; MONTMINY, 2001). Levando isso em consideração, da mesma forma que as outras proteínas analisadas, a razão pAkt/Akt se mostrou diminuída no hipocampo de animais pré-diabéticos. Com isso, pode-se sugerir que a modulação da via de sinalização do BDNF/Akt/CREB contribui para os efeitos de dano de memória e do fenótipo do tipo depressivo apresentados pelos animais pré-diabéticos. Estudos revelam que a modulação do BDNF/CREB leva a maior suscetibilidade à depressão em roedores (ADVANI et al., 2009; HOSHAW et al., 2005), assim como a disfunção da via do BDNF/Akt/CREB tem sido associada ao comprometimento da memória,

e ao aumento da suscetibilidade a transtornos neuropsiquiátricos, como os transtornos afetivos em animais (CAO et al., 2019; MLYNIEC et al., 2014; WANG et al., 2017).

Com base nos resultados que foram mencionados até aqui, seria relevante utilizar em animais pré-diabéticos uma ferramenta farmacológica que interviesse sobre as vias de sinalização IRS-1/Akt/GLUT4 e/ou do BDNF/CREB hipocampal. Como mencionado no **artigo 1**, o tratamento com o (*p*-ClPhSe)$_2$ apresentou efeitos moduladores na via do BDNF em camundongos diabéticos. Dessa forma seria interessante, em um futuro próximo, avaliar o efeito do tratamento com o (*p*-ClPhSe)$_2$ no protocolo de pré-diabetes.

Após a discussão em torno dos resultados obtidos, é importante notar que nessa tese foi utilizado um modelo animal que mimetiza a etiologia do (pré)diabetes e que replica suas complicações. Além disso, os resultados apresentados aqui também demonstram que o (*p*-ClPhSe)$_2$ é um composto que apresenta efeitos farmacológicos no prejuízo de memória e ação do tipo antidepressiva em camundongos diabéticos, sendo que interage em diferentes vias de sinalização neuroquímica e em diferentes regiões cerebrais. Nesse sentido, pode-se inferir que o (*p*-ClPhSe)$_2$ tem ação em múltiplos alvos, nos quais pode desempenhar suas ações farmacológicas.

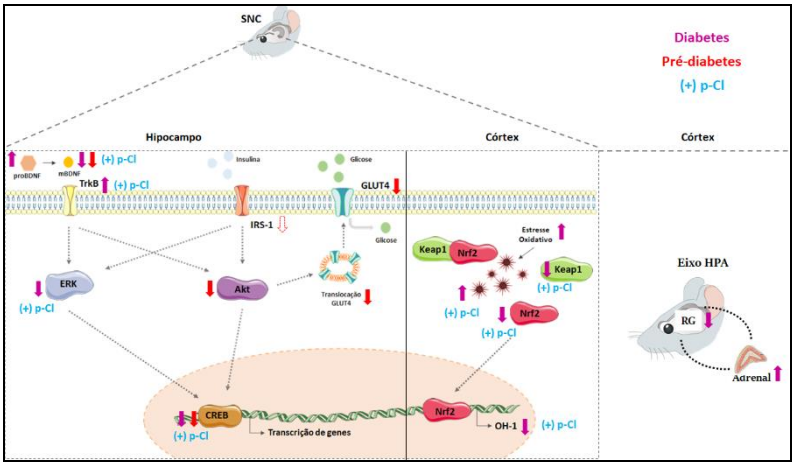
Entendendo que o (pré)diabetes é uma doença complexa que apresenta vários mecanismos etiopatológicos diferentes que ainda precisam ser adequadamente caracterizados, propõe-se uma estratégia de multialvos para o controle mais eficaz e rápido dos sintomas centrais e comórbidos das doenças. Recentemente, uma abordagem moderna muito promissora sugere o design de ligantes direcionados para múltiplos alvos, com base no paradigma "uma molécula, múltiplos alvos", adotado especificamente para o tratamento de distúrbios com mecanismos patológicos complexos (DAS; BASU, 2017; MILLAN, 2006). Assim, destaca-se a importância de pesquisas relacionadas às patologias heterogêneas bem como de compostos que tenham efeitos sobre diferentes alvos, como o composto orgânico de selênio (*p*-ClPhSe)$_2$.

Finalmente, esta tese contribui para o esclarecimento de algumas vias de proteínas no SNC que estão envolvidas com fenótipos comportamentais de memória e depressão em modelos tanto de pré-diabetes como de diabetes em camundongos, reforçando a importância da pesquisa básica, que busca revelar os mecanismos e talvez, no futuro, apontar tratamentos para doenças que afetam um grande número de indivíduos ao redor do mundo.

5 CONCLUSÃO

Os resultados apresentados nesses estudos indicam que o (*p*-ClPhSe)$_2$ (I) apresentou efeito contra o dano em diferentes tipos de memória induzido pelo diabetes em camundongos, modulando uma via importante para os processos de aprendizagem e memória, como a do BDNF/TrkB no hipocampo, (II) demonstrou efeito do tipo antidepressivo em um modelo de diabetes, protegendo contra o estresse oxidativo no córtex cerebral dos camundongos diabéticos, que foi associado a modulação do sistema Keap1/Nrf2. Agregado a esses resultados, foi descoberto que animais pré-diabéticos apresentaram (III) prejuízo em diferentes tipos de memória e no fenótipo do tipo depressivo, possivelmente, (IV) através do prejuízo na sinalização das vias IRS-1/Akt/GLUT4 e BDNF/CREB no hipocampo dos camundongos pré-diabéticos (Figura 7). A díade depressão-prejuízo de memória pode apresentar muitos mecanismos patofisiológicos e uma molécula multialvo seria uma alternativa interessante para tratar essa comorbidade do diabetes. Considerando os efeitos apresentados pelo (*p*-ClPhSe)$_2$, esse composto orgânico de selênio poderia ser considerado, no futuro, como uma opção terapêutica associada a medicamentos já utilizados para o diabetes, com o intuito de tratar as complicações da doença.

Figura 7. Esquema geral mostrando os efeitos no SNC após administração de ETZ periférica. Flechas **roxas** indicam efeitos apresentados nos camundongos **diabéticos** e flechas **vermelhas** indicam efeitos apresentados nos camundongos **pré-diabéticos**. Ainda, **(+) *p*-Cl** em **azul** representa os efeitos do (*p*-ClPhSe)$_2$ na modulação positiva frente ao dano nos camundongos diabéticos. (+) p-Cl = **(*p*-ClPhSe)$_2$**



SNC: Sistema nervoso central; BDNF: fator neurotrófico derivado do encéfalo; TrkB: receptor de tirosina quinase; ERK: quinase regulada por sinal extracelular; Akt: proteína quinase B; CREB: proteína de ligação ao elemento de resposta ao cAMP; IRS-1: substrato do receptor de insulina-1; GLUT4: transportador de glicose 4; Keap1: proteína 1 associada à ECH tipo Kelch; Nrf2: fator nuclear eritroide 2-relacionado ao fator 2; OH-1: hemeoxigenase-1; HPA: eixo hipotálamo-pituitária-adrenal; RG: receptor de glicocorticoide.

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6 ANEXOS

Anexo A - Carta de aprovação do projeto de pesquisa pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (UFSM)

Universidade Federal de Santa Maria 1960

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "AVALIAÇÃO DO EFEITO TERAPÊUTICO DO COMPOSTO 4-4'-DICLORO-DIFENIL-DISELENETO FRENTE A PREJUÍZOS COGNITIVOS INDUZIDOS POR ESTREPTOZOTOCINA EM CAMUNDONGOS SWISS", protocolada sob o CEUA nº 2964150317 (ID 001457), sob a responsabilidade de **Cristina Wayne Nogueira** *e equipe; Vanessa Angonesi Zborowski; Suelen Osorio Heck; Nicolas Klummer Bastos* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 07/06/2017.

We certify that the proposal "Therapeutic effect of 4,4'-Dichloro-diphenyl disselenide against memory impairment induced by streptozotocin in mice", utilizing 390 Heterogenics mice (390 males), protocol number CEUA 2964150317 (ID 001457), under the responsibility of **Cristina Wayne Nogueira** *and team; Vanessa Angonesi Zborowski; Suelen Osorio Heck; Nicolas Klummer Bastos* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 06/07/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 05/2017 a 10/2018 Área: Bioquímica E Biologia Molecular

Origem:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	50 a 60 dias	N:	390
Linhagem:	Swiss			Peso:	25 a 35 g		

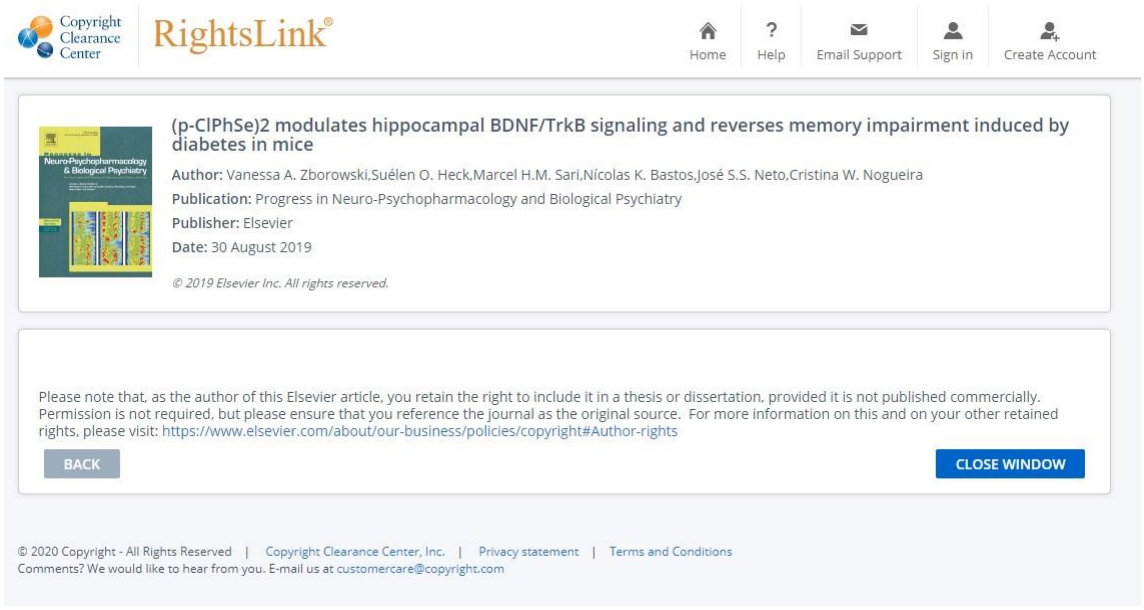
Local do experimento: Experimentos serão realizados no prédio 18 e 19, salas de laboratório.

Santa Maria, 06 de janeiro de 2020

Profa. Dra. Patrícia Severo do Nascimento
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Anexo B - Autorização para reprodução do artigo científico intitulado: *(p-ClPhSe)$_2$ modulates hippocampal BDNF/TrkB signaling and reverses memory impairment induced by diabetes in mice*



Anexo C - Autorização para reprodução do artigo científico intitulado: *Keap1/Nrf2/HO-1 signaling pathway contributes to p-chlorodiphenyl diselenide antidepressant-like action in diabetic mice*

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