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Vanessa Angonesi Zborowski

4,4'-DICLORO-DIFENIL DISSELENETO REVERTE O DÉFICIT DE MEMÓRIA INDUZIDO PELA CORTICOSTERONA EM CAMUNDONGOS

Santa Maria, RS
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Bioquímica Toxicológica**

Orientador: Prof. Dr. Gilson Zeni

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Aprovado em 29 de fevereiro de 2016:

Gilson Zeni, Dr. (UFSM)
(Presidente, Orientador)

Ana Cristina de Souza Guerra, Dr.ª (UFSC)

Maribel Antonello Rubin, Dr.ª (UFSM)

Santa Maria, RS
2016.

À minha família

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Roberto Shinyashiki

RESUMO

4,4'-DICLORO-DIFENIL DISSELENETO REVERTE O DÉFICIT DE MEMÓRIA INDUZIDO PELA CORTICOSTERONA EM CAMUNDONGOS

AUTOR: Vanessa Angonesi Zborowski
ORIENTADOR: Gilson Zeni

O estresse crônico ou mesmo uma única experiência traumática grave pode ter um impacto negativo na função cognitiva. Nesse sentido, os compostos orgânicos de selênio são moléculas que despertam grande interesse pelas diversas propriedades farmacológicas já reportadas, destacando-se entre elas os efeitos protetores sobre o prejuízo de memória em diferentes modelos experimentais. Assim, o presente estudo investigou o efeito da administração de p-cloro-difenil disseleneto $(p\text{-ClPhSe})_2$ em um modelo de prejuízo de memória relacionado com estresse induzido pela exposição à corticosterona em camundongos e os mecanismos envolvidos nesta ação. Para este fim foram utilizados camundongos adultos machos Swiss divididos em seis grupos: (I) óleo mineral e (II e III) $(p\text{-ClPhSe})_2$ nas doses de 1 ou 5 mg/kg, estes grupos receberam o veículo da corticosterona (1% etanol/H_20) na água de beber; grupo (IV) recebeu óleo mineral e (V e VI) receberam $(p\text{-ClPhSe})_2$ nas doses de 1 ou 5 mg/kg e corticosterona na água de beber. Os animais receberam costicosterona ou o seu veículo (1% etanol/H_20) na água de beber por quatro semanas. Na última semana de exposição à corticosterona, os animais foram tratados pela via intragástrica com $(p\text{-ClPhSe})_2$ ou óleo mineral (10 ml/kg) uma vez ao dia. Após, os animais realizaram os testes comportamentais tais como teste de reconhecimento do objeto (TRO), teste de localização do objeto (TLO), teste da esquiva passiva (TEP) para avaliar a memória. Avaliou-se também a atividade locomotora e exploratória dos animais. Para avaliar a toxicidade do composto, foram determinadas as atividades da aspartato aminotransferase (AST) e da alanina aminotransferase (ALT), e os níveis de ureia, colesterol total, triglicerídeos e da lipoproteína de alta densidade (HDL) no plasma dos camundongos. Além disso, amostras de córtex cerebral e de hipocampo foram obtidas para determinar as atividades das enzimas Na^+K^+ATPase, acetilcolinesterase (AChE) e a captação de glutamato. Os resultados demonstraram que o tratamento com o $(p\text{-ClPhSe})_2$ em ambas as doses reverteram o dano de memória no TRO, TLO e TEP induzido pela corticosterona em camundongos. Além disso, ambas as doses de $(p\text{-ClPhSe})_2$ reverteu o aumento da captação de glutamato em fatias de hipocampo de camundongos tratados com corticosterona. Em contrapartida, a captação de glutamato em fatias de córtex cerebral não foi alterada em camundongos expostos a corticosterona. A atividade da Na^+K^+ATPase e AChE não foram alteradas em hipocampo nem em córtex cerebral de camundongos tratados com corticosterona. Os parâmetros de toxicidade avaliados não foram alterados em camundongos tratados com $(p\text{-ClPhSe})_2$. Esse composto orgânico de selênio reverteu o prejuízo de memória relacionado com o estresse causado pela corticosterona e modulou a captação de glutamato em fatias de hipocampo de camundongos.

Palavras-chave: Selênio. Organoselênio. Memória. Glicocorticoides. Captação de Glutamato Cerebral.

ABSTRACT

4,4'-DICHLORO-DIPHENYL DISELENIDE REVERSES THE IMPAIRED MEMORY INDUCED BY CORTICOSTERONE IN MICE

AUTHOR: Vanessa Angonesi Zborowski

ADVISOR: Gilson Zeni

Chronic stress or even a single serious traumatic experience may have a negative impact on cognitive function. In this sense, organic selenium compounds are molecules that arouse great interest because they have various protective effects on memory deficits already reported in different experimental models. The present study investigated the effect of administration of p-chloro-diphenyl diselenide $(p\text{-}ClPhSe)_2$ in the memory impairment related to stress induced by exposure to corticosterone in mice and the action mechanisms involved. Male adult Swiss mice were divided into six groups: (I) mineral oil and (II and III) $(p\text{-}ClPhSe)_2$ at a dose of 1 or 5 mg/kg, these groups received the corticosterone vehicle (1% ethanol / H20) in drinking water; group (IV) received mineral oil and (V and VI) were given $(p\text{-}ClPhSe)_2$ at both doses and corticosterone in the drinking water. The animals received vehicle or corticosterone (1% ethanol/H20) by four weeks in drinking water. In the last week of corticosterone treatment once a day with $(p\text{-}ClPhSe)_2$ at a dose of 1 or 5mg/kg or mineral oil (10ml/kg) by the intragastric route. After that, behavioral tests such as object recognition test (ORT), object location test (OLT), step-down passive avoidance (SDPA) were perfomed. The locomotor and exploratory activities of mice were also evaluated. The toxicity of $(p\text{-}ClPhSe)_2$ was investigated by determining the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and the levels of urea, total cholesterol, triglycerides and high-density lipoprotein (HDL) in the plasma of mice. Samples of cerebral cortex and hippocampus were obteined to determine the activities of Na^+K^+ATPase and acetylcholinesterase (AChE) and glutamate uptake. The results demonstrated that the treatment with $(p\text{-}ClPhSe)_2$ at both doses was effective in reversing memory deficits in the ORT, OLT and SDPA induced by corticosterone in mice. In addition, both doses of $(p\text{-}ClPhSe)_2$ reversed the increase in glutamate uptake in hippocampal slices of mice treated with corticosterone. However, the glutamate uptake in cerebral cortical slices was not altered in mice exposed to corticosterone. The Na^+K^+ATPase and AChE activities were not changed in the hippocampus or cerebral cortex of mice treated with corticosterone. There were no signs of safety in $(p\text{-}ClPhSe)_2$-treated mice in the evaluated parameters. This organoselenium compound reversed the memory impairment associated with stress caused by corticosterone and modulated glutamate uptake in mice.

Keywords: Selenium. Organoselenium. Memory. Glucocorticoids. Cerebral glutamate uptake.

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MANUSCRITO

LISTA DE ABREVIATURAS

$(p\text{-}ClPhSe)_2$	4,4'-dicloro difenil disseleneto
$(PhSe)_2$	Difenil disseleneto
ACh	Acetilcolina
AChE	Acetilcolinesterase
AMPA	Alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico
AP-1	Ativação da proteína 1
BuChE	Butirilcolinesterase
ERGs	Elementos de resposta a glicocorticoides
HACT	Hormônio adrenocorticotrófico
HLC	Hormônio liberador de corticotrofina
HPA	Hipotálamo-pituitária-adrenal
iGluRs	Receptores de glutamato ionotrópicos
mAChRs	Receptores de acetilcolina muscarínicos
MCP	Memória de curto prazo
mGluRs	Receptores de glutamato metabotrópicos
MLP	Memória de longo prazo
nAChRs	Receptores de acetilcolina nicotínicos
NF-KB	Fator nuclear kappa-B
NMDA	Ácido N-metil-D-aspartato
OMS	Organização mundial da saúde
Pct70	Proteína de choque térmico 70
Pct90	Proteína de choque térmico 90
RGs	Receptores glicocorticoides
RMs	Receptores mineralocorticoides
SeCys	Selenocisteína
SeMet	Selenometionina
SNC	Sistema nervoso central

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1 INTRODUÇÃO

1.1 EIXO HIPOTÁLAMO-PITUITÁRIA-ADRENAL (HPA)

O eixo HPA é composto por sinais estimuladores e um ciclo de feedback negativo que regulam a sua atividade. Este eixo neuroendócrino é composto por três distintos locais anatômicos: o núcleo paraventricular do hipotálamo, a glândula pituitária e as glândulas adrenais (Figura 1)(CHROUSOS, 1995; CHROUSOS et al., 1985). O hormônio liberador de corticotrofina (HLC), através do sistema porta hipofisário, atinge o lobo anterior da glândula pituitária e se liga ao seu receptor de proteína G, que leva à produção e liberação do hormônio adrenocorticotrófico (HACT) para a circulação sistêmica (CALOGERO et al., 1988). O HACT após a ligação ao seu receptor de proteína G, nas células do córtex adrenal da zona fasciculada, induz a atividade de proteínas e a expressão dos genes implicados na via biossintética de glicocorticoides (cortisol em seres humanos, e corticosterona em roedores)(BORNSTEIN e CHROUSOS, 1999).

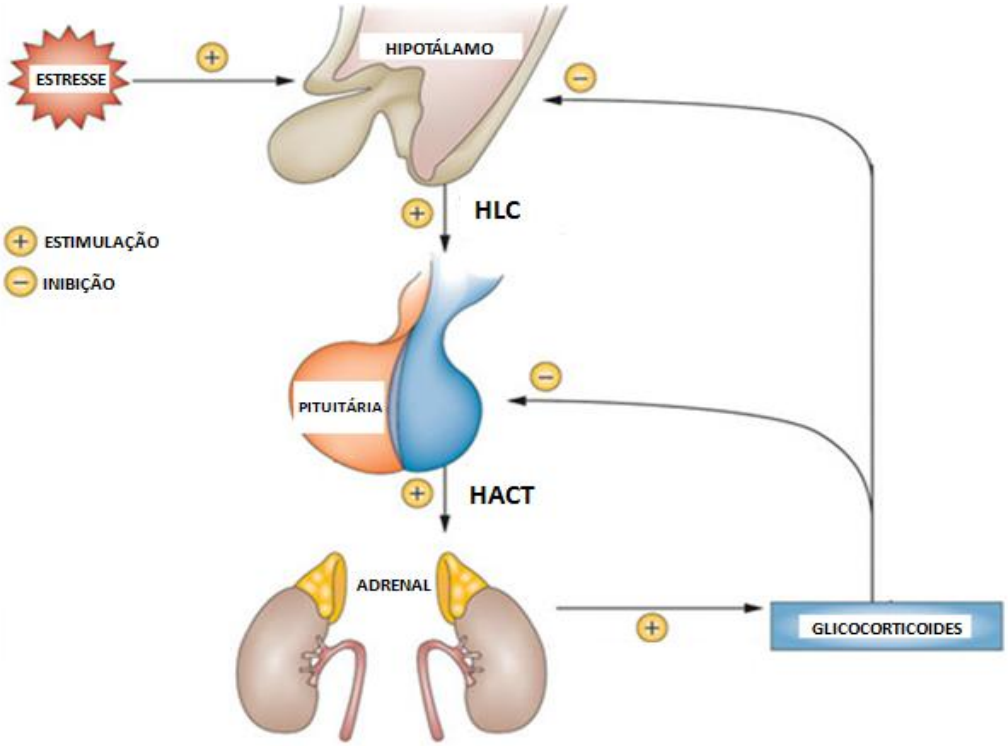

Figura 1. Diagrama esquemático do eixo hipotálamo-pituitária-adrenal (HPA). Adaptado de VITALE et al. (2013).

Os glicocorticoides são hormônios derivados de colesterol, que desempenham um papel fundamental na manutenção da homeostase relacionada ao estresse, além disso, estão envolvidos na manutenção do tônus cardiovascular adequado, na regulação do metabolismo como ações catabólicas no fígado, músculo e tecido adiposo, e exercem uma influência substancial na quantidade e qualidade da resposta inflamatória e imune (CHARMANDARI et al., 2005).

Devido as suas propriedades lipofílicas, os glicocorticoides podem atravessar as membranas plasmáticas e ativar dois receptores intracelulares diferentes: receptores mineralocorticoides (RMs) e receptores glicocorticoides (RGs), também conhecidos, respectivamente, como receptor Tipo I e Tipo II (LU, N. Z. et al., 2006). Na ausência de glicocorticoides (no estado inativo), os RM e RG citoplasmáticos estão ligados a proteínas chaperonas complexas, incluindo proteínas de choque térmico pct70 e pct90 (GRAD e PICARD, 2007). A ligação do glicocorticoide ao seu receptor induz uma alteração conformacional, resultando na dissociação do complexo-receptor e resulta na sua translocação para o núcleo. No núcleo, ambos os MRs e GRs podem ligar-se a sequências específicas de 15 nucleotídeos do promotor de genes alvo, conhecidos como elementos de resposta a glicocorticoides (ERGs) e ativar diretamente a transcrição de genes alvo (ZALACHORAS et al., 2013). Os RMs e RGs também podem controlar respostas celulares rápidas por mecanismos que são independentes da translocação nuclear e regulação da expressão gênica, em vez disso ocorrer por meio de ações genômicas-independentes (GROENEWEG et al., 2011; PRAGER e JOHNSON, 2009), através de interações direta proteína-proteína com fatores de transcrição, incluindo ativação de proteína 1 (AP-1) e fator nuclear-kappaB (NF-kB) (RAY e PREFONTAINE, 1994).

Os efeitos biológicos intracelulares onipresentes de glicocorticoides são geralmente adaptativos; no entanto, a ativação inadequada ou excessiva do eixo HPA pode contribuir para o desenvolvimento de patologias (MCEWEN e STELLAR, 1993; MUNCK et al., 1984), e é considerado um fator de risco para doenças relacionadas ao estresse(STERNER e KALYNCHUK, 2010).

1.2 ESTRESSE

Nas últimas décadas, o termo estresse é usado para definir um estado de ameaça a homeostase. Em resposta a essa ameaça, os organismos desenvolveram um sistema altamente sofisticado que melhora as respostas neuroendócrinas centrais e periféricas. Se essas respostas adaptativas forem inadequadas, excessivas ou prolongadas, elas podem ter efeitos adversos graves na função vital fisiológica (CHARMANDARI et al., 2005; CHROUSOS, 2009).

A capacidade de defesa e adaptação de um organismo ao estresse depende da duração do estresse e da sensibilidade ao estressor, bem como da interação com as características individuais como: fatores genéticos, experiências prévias, memórias de situações e condições de saúde física e mental. As respostas ao estresse são resultantes de adequações do organismo e de mecanismos que controlam diversas funções e com isso, uma resposta inadequada a um estímulo estressor pode representar um risco de doença e/ou ameaças à saúde (CORTEZ e SILVA, 2007; TOFOLI et al., 2011).

O estresse crônico ou mesmo uma única experiência traumática grave pode ter um impacto negativo na função cognitiva e assim, levar ao desenvolvimento de diversas patologias (DE QUERVAIN et al., 2009). O efeito do estresse em funções cognitivas é amplamente dependente de características do estressor, como a intensidade, duração, cronicidade e previsibilidade do estresse que são características principais que afetam a cognição e a memória (LUPIEN et al., 2007). Os efeitos dos glicocorticoides na memória podem ser mediados através de seus receptores, ambos estão altamente expressos na região límbica, particularmente no hipocampo, que é uma estrutura com um papel essencial na memória (TASKER e HERMAN, 2011).

A corticosterona, o principal glicocorticoide liberado pela adrenal de roedores, exerce um papel crítico na regulação do eixo HPA e também na modulação de processos de aprendizado e memória (RICHARDSON et al., 2008). Achados experimentais têm demonstrado que o prejuízo na memória está correlacionado com o aumento dos níveis de glicocorticoides em modelos animais de estresse. Essa condição poderia ser mimetizada experimentalmente através da administração sistêmica de corticosterona ou de agonistas de receptores de glicocorticoides, que

desencadeiam um prejuízo no aprendizado e na recuperação de memória (ROOZENDAAL et al., 2004).

1.3 DOENÇAS RELACIONADAS AOS GLICOCORTICOIDES

1.3.1 Síndrome de Cushing

A Síndrome de Cushing é causada por exposição crônica ao excesso de cortisol (NIEMAN, 2015). Em aproximadamente 80% dos casos, a Síndrome de Cushing é consequência de uma hipersecreção de HACT, geralmente devido a um tumor da hipófise e raramente devido a um tumor extra secretório de HACT ou HLC. Nos outros 20% dos casos, a síndrome é consequência direta da superprodução autônoma pela glândula adrenal, por causa de tumores adrenocorticais unilaterais ou bilaterais, hiperplasia adrenal ou displasia (ARNALDI et al., 2003; NEWELL-PRICE et al., 2006; PIVONELLO et al., 2008). A apresentação clínica da Síndrome de Cushing é variável, em parte relacionada com a extensão e duração do excesso de cortisol. Quando o hipercortisolismo é intenso, os sinais e sintomas são inconfundíveis, em particular, fraqueza muscular, aumento da gordura abdominal, tronco e face, e aumento de estrias arroxeadas, sugerindo hipercortisolismo (NIEMAN, 2015). Devido ao fato de que os GCs tem uma distribuição pleiotrópica no sistema nervoso central (SNC), não é surpreendente que o excesso de GC crônico pode conduzir a alterações estruturais e funcionais no SNC (SONINO et al., 2010).

A gestão atual de pacientes com Síndrome de Cushing não pode ignorar os transtornos psiquiátricos e neurocognitivos, sua evolução durante a doença ativa e após a remissão da doença, e seu impacto na qualidade de vida (NIEMAN, 2015). A primeira descrição de distúrbios neuropsiquiátricos na Síndrome de Cushing ocorreu em 1932 por Harvey Cushing que destacou a presença de "distúrbios emocionais" como uma característica patológica da Síndrome de Cushing (CUSHING, 1969). Estes distúrbios incluem transtornos psiquiátricos, como depressão maior, mania e ansiedade, e distúrbios cognitivos, caracterizados principalmente por perturbações da memória e na concentração (SONINO e FAVA, 2001).

1.3.2 Doença de Addison

A Doença de Addison é uma condição autoimune rara decorrente de uma complexa interação de fatores genéticos, imunológicos e fatores ambientais, manifestando-se como insuficiência adrenocortical sintomática e necessitando de terapia de reposição de corticóide ao longo da vida (NAPIER e PEARCE, 2012). A primeira descrição de insuficiência adrenocortical foi em meados do século XIX, quando o Dr. Thomas Addison, um médico do Hospital de Guy, em Londres, descreveu sintomas de insuficiência adrenocortical e alterações nas glândulas supra-renais no momento da autópsia (ELLIS, 2009). Os sintomas de insuficiência adrenal incluem fadiga, náuseas, anorexia, tonturas posturais e cãibras musculares. Uma história de perda de peso, redução da força e desejo de sal também pode ser encontrada (NAPIER e PEARCE, 2012). Além disso, hiperpigmentação é um sinal específico de insuficiência adrenal primária, que afeta predominantemente áreas da pele submetidas a atrito, como os cotovelos, juntas, vincos palmares, lábios e mucosa bucal. A estimulação do receptor de melanocortina-1 na pele devido ao aumento do HACT circulante é uma das explicações para a hiperpigmentação (ARLT e ALLOLIO, 2003).

Como outras doenças auto-imunes, a Doença de Addison é mais frequente em mulheres e geralmente acomete indivíduos com idades entre 30 e 50 anos, embora possa afetar indivíduos de qualquer idade (KONG e JEFFCOATE, 1994). Essa doença era invariavelmente fatal até que na década de 1940 foram desenvolvidos os primeiros precursores do cortisol sintético usados para o tratamento da Doença de Addison (MITCHELL e PEARCE, 2012).

1.4 APRENDIZAGEM E MEMÓRIA

A aprendizagem é um processo pelo qual adquirimos conhecimento sobre o mundo, enquanto memória é um processo pelo qual o conhecimento é codificado, retido e, posteriormente, recuperado (KANDEL et al., 2000). A memória é o resultado de pelo menos três tipos de processamento distintos, mas relacionados entre si: aquisição, consolidação e evocação. A aquisição é um processo pelo qual novas informações aprendidas são tratadas e processadas por sistemas neurais específicos, logo após, tem-se a consolidação, que refere-se à fixação e

armazenamento de uma informação recém aprendida. Após, dá-se a evocação, um processo que permite a lembrança e o uso de informações retidas (KANDEL et al., 2000).

A formação de memórias é o resultado de mecanismos celulares e moleculares ativados em diferentes estruturas do cérebro. A habilidade de um animal para adaptar seu comportamento em resposta a estímulos ambientais depende da plasticidade estrutural e funcional de várias regiões cerebrais (GIOVANNINI et al., 2015).

Uma única experiência de aprendizagem inicia uma cascata de eventos, que pode conduzir para diferentes formas de memória como: a memória de curto prazo (MCP), que dura alguns minutos a horas, e de longo prazo (MLP) que dura dias, semanas, e durante toda vida (MCGAUGH, J. L., 1966). A memória de curto prazo tem uma capacidade limitada, a informação abordada está disponível apenas por um curto período de tempo. A memória de longo prazo é feita de uma rede neuronal funcionalmente sinérgica e tem também dois tipos: memória declarativa (explícita) de fatos e eventos, para pessoas, lugares e objetos e não declarativa (implícito) a memória para habilidades motoras e perceptivas (BORBELY et al., 2013). Alguns mecanismos celulares que estão na base do desenvolvimento da MCP se sobrepõem com as da MLP, mas existem outros mecanismos independentes (IZQUIERDO, IVÁN et al., 1998; IZQUIERDO, I. et al., 1999; IZQUIERDO, L. A. et al., 2002). Além disso, vários estudos relataram que certas quinases permitem um estado celular que beneficia a formação da MLP, sem influenciar na MCP (CAMMALLERI et al., 2003; DE CARVALHO MYSKIW et al., 2014; LU, Y. et al., 2011; MONCADA et al., 2011).

A memória não é uma função unitária, ela depende da atividade integrada de diversas estruturas cerebrais e sistemas de neurotransmissores e receptores, envolve vários mecanismos pós-sinápticos e vias de transdução de sinal (IZQUIERDO, IVÁN et al., 1998)

Os mecanismos de formação da memória por consequência de informações adquiridas incluem a formação de novas sinapses e a reformulação das sinapses já existentes, sendo este fenômeno chamado de plasticidade sináptica (GEINISMAN, 2000). Acredita-se que a modulação do tônus glutamatérgico seja um dos principais passos para a formação da memória (MCGAUGH, JAMES L e IZQUIERDO, 2000). Além disso, o sistema colinérgico desempenha um papel essencial nos processos

de aprendizado e memória (WINKLER et al., 1995). Dessa forma, sabe-se que tanto em animais quanto em humanos, o aprendizado e a memória podem ser modificados por drogas que afetam a função colinérgica central (YAMAZAKI et al., 2005). A memória é afetada quando as sinapses encarregadas de fazer ou evocar memórias encontram-se inibidas ou alteradas, como em condições de idade avançada, na qual ocorrem modificações neuronais fisiológicas e em muitas doenças que são acompanhadas de uma acelerada alteração neuronal (IZQUIERDO, IVÁN et al., 1998).

1.5 SISTEMA COLINÉRGICO

A via colinérgica consiste do neurotransmissor acetilcolina (ACh); da acetilcolinesterase (AChE), que hidrolisa ACh; da colina acetiltransferase, uma enzima que sintetiza a ACh; e dos receptores de ACh, os receptores nicotínicos (nAChR) e os receptores muscarínicos (mAChR) (ABREU-VILLACA et al., 2011). A ACh é um neurotransmissor necessário para o bom funcionamento da transmissão colinérgica que regula os processos de aprendizagem e memória (ISHRAT et al., 2009), e que também está envolvida na regulação da excitabilidade e plasticidade sináptica, além de participar de uma variedade de funções fisiológicas (LEVIN et al., 2006). A ACh liberada na fenda sináptica, desempenha sua função por intermédio de seus receptores ionotrópicos (nAChRs) e os receptores acoplado a proteína G (mAChRs), esses receptores de ACh modificam a atividade neuronal, através de múltiplas cascatas de sinalização (MOLAS e DIERSSEN, 2014).

Evidencias experimentais demonstram que os inibidores da colinesterase melhoram a transmissão colinérgica diretamente através da inibição da enzima AChE, assim, permitindo um acúmulo de ACh na fenda sináptica (ANAND e SINGH, 2013). Sabe-se que a função prejudicada do sistema colinérgico tem sido relacionada com a Doença de Alzheimer, assim, uma estratégia farmacológica de tratamento foi criada para melhorar a função colinérgica pela utilização de inibidores da AChE (SCHLIEBS e ARENDT, 2006). O *Food and Drug Administration – USA(FDA)* aprovou quatro inibidores da acetilcolinesterase (tacrina, donepezil, rivastigmina e galantamina) para o tratamento de Doença de Alzheimer até o ano 2000 (CUMMINGS, 2000), porém esses fármacos são apenas paliativos na doença de Alzheimer.

1.6 SISTEMA GLUTAMATÉRGICO

O glutamato é o principal neurotransmissor excitatório no SNC (MILADINOVIC et al., 2015). Após a despolarização da membrana pré-sináptica, o glutamato é liberado para a fenda sináptica e liga-se a seus receptores ionotrópicos (iGluRs) ou metabotrópicos (mGluRs) na membrana pós-sináptica (Figura 2). Os iGluRs são canais iônicos dependentes de ligantes e incluem receptores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico (AMPA), cainato e ácido N-metil-D-aspartato tipos (NMDA) (LODGE, 2009). Enquanto que os receptores de AMPA e cainato medeiam principalmente influxo de sódio, os receptores de NMDA possuem uma condutividade elevada de cálcio (LEWERENZ e MAHER, 2015).

Em contraste com os outros iGluRs, a atividade dos receptores de NMDA é bloqueada por Mg^{+2} quando a membrana encontra-se no potencial de repouso, porém o canal iônico é prontamente desbloqueado por despolarização da membrana, que remove o Mg^{+2} do canal (VARGAS-CABALLERO e ROBINSON, 2004). O glutamato é amplamente encontrado intracelularmente e relativamente pouco (até um milhão de vezes menor) encontrado no meio extracelular. Isso estabelece um gradiente de concentração necessário para a transmissão sináptica rápida (NICHOLLS e ATTWELL, 1990).

Considerando que o glutamato tem um papel amplo nos processos centrais e periféricos, não é surpreendente que a liberação, absorção, metabolismo e sinalização desse neurotransmissor sejam constantemente regulados (MELDRUM, 2000). Os níveis de glutamato são regulados por transportadores de aminoácidos excitatórios (EAAT2), que removem o excesso de moléculas de glutamato do líquido extracelular, e estão predominantemente presentes em astrócitos (GREWER e RAUEN, 2005; TZINGOUNIS e WADICHE, 2007; VANDENBERG e RYAN, 2013). Os transportadores de glutamato são dependentes do íon sódio e necessitam, portanto da funcionalidade da enzima $Na^{+}K^{+}$ATPase para regular as concentrações intracelulares de sódio e potássio e conduzir a captação do neurotransmissor (ROSE et al., 2009). Portanto, alterações na atividade da $Na^{+}K^{+}$ATPase podem influenciar diretamente a sinalização do glutamato, a atividade neural e o comportamento animal. Os transportadores de glutamato mantêm baixas as concentrações de glutamato na fenda sináptica, protegendo contra a excitotoxicidade. O termo excitotoxicidade foi utilizado pela primeira vez por OLNEY (1986), e resulta da

ativação excessiva de iGluRs o que leva a uma perda característica de estruturas pós-sinápticas, incluindo dendritos e corpos celulares (CHOI, 1994).

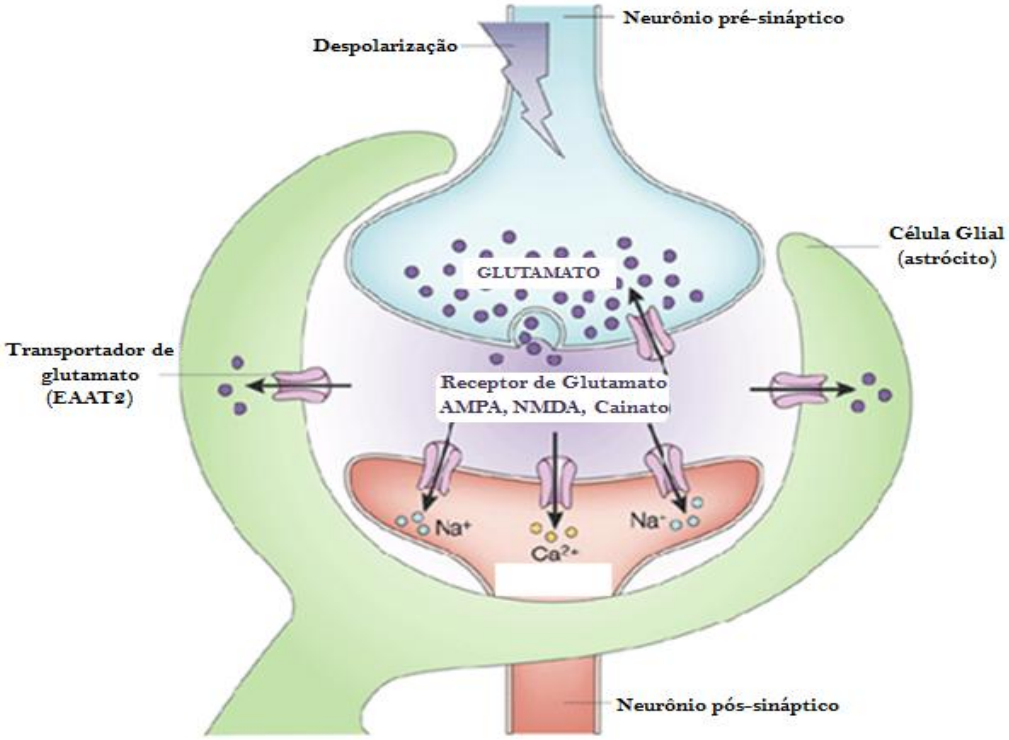

Figura 2. Ilustração esquemática da sinapse glutamatérgica. Adapatado de SYNTICHAKI e TAVERNARAKIS (2003).

1.7 COMPOSTOS ORGÂNICOS DE SELÊNIO

O Selênio (Se) é um micronutriente que foi descoberto em 1817 pelo médico e químico sueco Jöns Jacob Berzelius (COMASSETO, 2010). Antigamente, o selênio foi reconhecido como uma substância tóxica devido a casos de envenenamento na pecuária em solos ricos neste elemento (NOGUEIRA e ROCHA, 2011). Apesar deste início desfavorável, anos de estudo e experimentação esclareceram o papel essencial do elemento Se no organismo (RAYMAN, 2000). Assim, a história biológica do selênio foi marcada por um contraste entre seus efeitos benéficos e tóxicos (NOGUEIRA e ROCHA, 2011).

Durante a última década, um grande número de dados científicos tem revelado de forma conclusiva o papel crucial do Se na manutenção imuno-endócrina, metabólica, e na homeostase celular dos indivíduos (BROWN e ARTHUR, 2001; RAYMAN, 2009). Esse elemento é encontrado principalmente em

alimentos como a castanha-do-pará, alho, cebola, brócolis, cereais, ovos e carnes (DUMONT et al., 2006). A Organização Mundial de Saúde (OMS) recomenda uma ingestão diária de 34-35 µg para adultos (FAO/OMS, 2002), seja através da ingestão de alimentos comuns, de origem animal e vegetal, ou por suplementação (DUMONT et al., 2006; RAYMAN, 2008).

O Se está presente em alimentos principalmente na forma de selenocisteína (SeCys) e selenometionina (SeMet) (forma orgânica), que provavelmente são absorvidos no intestino delgado, enquanto o selenito e o selenato (formas inorgânicas) estão presentes apenas em quantidades mínimas em alguns gêneros alimentícios (THIRY et al., 2013). A eficiência da absorção de Se varia entre as diferentes espécies (SeMet > SeCys > selenato > selenito), indicando que a biodisponibilidade do Se é dependente da sua espécie (THIRY et al., 2013) e que compostos dietéticos de Se podem ser considerados pró-fármacos, cuja atividade é regulada pela via metabólica e pelo estado redox de células e órgãos (WEEKLEY e HARRIS, 2013).

Estudos demonstram que o elemento Se é efetivo em prevenir inúmeras condições neurodegenerativas (LOEF et al., 2011; XIONG et al., 2007). Tem-se o interesse pela química e bioquímica de compostos orgânicos contendo Se, tendo em vista que, estes compostos vêm demonstrando ter atividades biológicas promissoras (NOGUEIRA e ROCHA, 2010), as quais se incluem ação antioxidante (MIORELLI et al., 2008), antinociceptiva (SAVEGNAGO et al., 2007) e ansiolítica (BRUNING et al., 2009).

Nesse contexto, é relevante o fato do composto orgânico de selênio, disseleneto de difenila $(PhSe)_2$ (Figura 3) possuir propriedades de melhorar a performance cognitiva em roedores nos testes de reconhecimento do objeto (ROSA et al., 2003), labirinto em Y e labirinto aquático de Morris (SOUZA et al., 2010; STANGHERLIN et al., 2008). Uma modificação da estrutura do $(PhSe)_2$ como a adição do átomo de cloro na estrutura, resulta em um composto orgânico de selênio chamado 4,4'-dicloro-difenil disseleneto $(p\text{-ClPhSe})_2$ (Figura 4). Esse composto foi estudado com eficácia em modelos de dano cerebral em camundongos (PRIGOL et al., 2009), no déficit cognitivo em ratos velhos (BORTOLATTO et al., 2012) e também apresentou atividade do tipo anorexígena em ratos (BORTOLATTO et al., 2015). Dados do nosso grupo de pesquisa demonstram que a DL50 de uma administração aguda em camundongos de $(PhSe)_2$ é >312mg/kg e para $(p\text{-ClPhSe})_2$

é >381mg/kg (NOGUEIRA e ROCHA, 2010). Essa classe de compostos têm propriedades farmacológicas com doses pelo menos 50 vezes menor do que aquelas em que têm efeitos tóxicos, assim, podem ser considerados compostos que causam baixa toxicidade(SAVEGNAGO et al., 2009).

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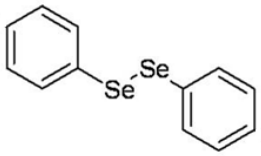

Figura 3. Estrutura química do composto disseleneto de difenila

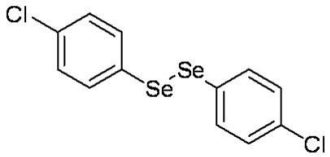

Figura 4. Estrutura química do composto p-clorodifenil disseleneto

De acordo com a ação promissora de melhorar a memória do (*p*-ClPhSe)$_2$ em ratos velhos, visamos a busca por compostos que minimizem o dano de memória, principalmente relacionadas ao estresse no qual atualmente não tem tratamento adequado.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Considerando os aspectos mencionados, o objetivo desse estudo foi avaliar a ação do composto 4,4'-dicloro-difenil disseleneto $(p\text{-ClPhSe})_2$ no déficit cognitivo induzido pela administração exógena de corticosterona em camundongos, bem como investigar os mecanismos pelos quais o $(p\text{-ClPhSe})_2$ age neste modelo.

2.2 OBJETIVOS ESPECÍFICOS

- Investigar se o tratamento com $(p\text{-ClPhSe})_2$ reverte alterações causadas pela corticosterona em testes comportamentais na avaliação da memória de camundongos, tais como teste do reconhecimento do objeto, teste de localização do objeto e esquiva passiva;

- Investigar se o tratamento com $(p\text{-ClPhSe})_2$ reverte alterações gerada pela corticosterona na atividade da bomba sódio/potássio ATPase (Na^+K^+ATPase) e da acetilcolinesterase (AChE) e na captação de glutamato em córtex cerebral e hipocampo de camundongos;

- Avaliar se o tratamento com $(p\text{-ClPhSe})_2$ altera parâmetros de toxicidade, na alanina aminostrasferase (ALT), aspartato aminotransferase (AST), ureia, colesterol total, triglicerídeos, lipoproteína de alta densidade (HDL), e as atividades locomotora e exploratória em camundongos.

3 Resultados

Os resultados que fazem parte dessa dissertação estão apresentados na forma de um manuscrito. Os itens introdução, materiais e métodos, resultados, discussão e referências bibliográficas do manuscrito estão dispostos de acordo com a recomendação do periódico científico no qual está submetido. O resultado apresentado no Apêndice A é complementar aos resultados do manuscrito e está dividido nas seguintes seções: Materiais e Métodos, Resultados e Discussão.

3.1 MANUSCRITO

p-Chloro-diphenyl diselenide reverses memory impairment-related to stress caused by corticosterone and modulates hippocampal [3H]glutamate uptake in mice

Vanessa A. Zborowski, Marcel H. M. Sari, Suélen O. Heck, Eluza C. Stangherlin, José S. S. Neto, Cristina W. Nogueira, Gilson Zeni*

Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, CEP 97105-900, RS, Brasil

*Correspondence should be sent to:

Gilson Zeni

Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, CEP 97105-900, RS, Brasil.

Phone: 55-55-3220-8140

FAX: 55-55-3220-8978

E-mail: gzeni@ufsm.br

ABSTRACT

Chronic stress or chronically high levels of glucocorticoids can result in memory impairment. This study aimed to investigate if 4,4'-dichloro-diphenyl diselenide $(p\text{-ClPhSe})_2$ reverses memory impairment-related to stress caused by corticosterone administration in mice and its possible mechanism of action. Swiss mice received corticosterone (20 µg/ml) in their drinking water during four weeks. In the last week, the animals were treated with $(p\text{-ClPhSe})_2$ (1 or 5 mg/kg) by the intragastric route (i.g.) once a day for 7 days. The cognitive performance of mice was assessed through the object recognition test (ORT), the object location test (OLT) and the step-down passive avoidance test (SDPA), some of predictive tests of memory. Biochemical parameters were determined and locomotor activity of mouse was performed to gain insight in $(p\text{-ClPhSe})_2$ toxicity. The findings demonstrated that treatment with $(p\text{-ClPhSe})_2$ in both doses was effective in reversing memory deficits in the ORT, the OLT and the SDPA caused by corticosterone exposure in mice. Treatment with $(p\text{-ClPhSe})_2$ at both doses reversed the increase in the $[^3H]$ glutamate uptake by hippocampal slices of mice treated with corticosterone. By contrast, $[^3H]$ glutamate uptake by brain cortical slices was not altered in mice exposed to corticosterone. The Na^+K^+ATPase activity was not altered in hippocampus and cerebral cortices of mice treated with corticosterone. There was no sign of toxicity in mice treated with $(p\text{-ClPhSe})_2$. This organoselenium compound reversed memory impairment-related to stress caused by corticosterone and modulated hippocampal $[^3H]$ glutamate uptake in mice.

Keywords: selenium, organoselenium, memory, glucocorticoids, glutamate uptake.

1 Introduction

Stress is characterized by physiological changes that occur in response to novel or threatening stimuli. These changes comprise a cascade of neuroendocrine events mediated by stress systems such as the hypothalamic–pituitary–adrenal (HPA) axis. Its activation triggered the release of hypothalamic corticotropin-releasing hormone (CRH) which in turn releases pituitary adrenocorticotropin-releasing hormone (ACTH), culminating in the secretion of adrenal glucocorticoids (cortisol in humans and corticosterone in rodents) into the circulatory system [1].

Experimental and clinical data have shown a hypersecretion of glucocorticoids in neurodegenerative diseases such as Alzheimer's disease [2]. Corticosterone, the major glucocorticoid in rodents, plays a critical role in the regulation of the HPA axis and in manifold effects on health, emotion, and cognition [3, 4]. It has been reported impairment in the memory function and its interconnection with increased glucocorticoid levels in animal models of stress. This condition could be experimentally mimicked through systemic administration of corticosterone or glucocorticoid receptor agonists which trigger an inhibitory influence on learning and memory retrieval [5].

Selenium is an essential trace element for growth, metabolism, development, immune function and antioxidant defense system in rodents and in human beings as well [6, 7]. This element is involved in the maintence of physiologic functions especially due to its in selenium containing proteins known as selenoproteins [8] provides protection from free-radical-induced cell damage [9, 10], modulates the function of the thyroid gland and has been shown potential antiviral effects [11, 12]. An inverse relationship between Se status and the incidence of various diseases has been observed in epidemiological studies [10, 13]. Abnormal levels of Se were found in the plasma of patients with impaired cognitive functions and neurological

disorders [14]. In addition, it is important to remark that diminished selenium concentrations in the brain affect its normal function and can cause neuronal loss and metabolic disturbances [15]. One of the most studied organoselenium compounds is diphenyl diselenide, $(PhSe)_2$, a simple synthetic compound which exhibits numerous biological actions. It has been reported that this compound is a potent anti-inflammatory, antioxidant, antidepressant- and anxiolytic-like agent in different animal models [16, 17]. In addition, evidence has been found to support the idea that $(PhSe)_2$ improves memory and learning in mice [18] and enhances acquisition and retention of spatial memory of rats in the water maze and T-maze tests [19]. In this way, 4,4'-dichloro-diphenyl diselenide (p-ClPhSe)$_2$, a derivative compound of $(PhSe)_2$, has been reported to have antidepressant-like activity in the rat forced swimming test[20], anorexigenic [21] and memory enhancer actions in the object location test in aged rats [20].

Regarding the promising memory enhancer actions of (p-ClPhSe)$_2$ in old rats [20] and the search for drugs that minimize memory impairment induced by glicocorticoids, the aim of the present study was to investigate whether (p-ClPhSe)$_2$ reverses memory impairment-related to stress caused by corticosterone administration in mice and its possible mechanism of action.

2 Experimental Procedure

2.1 Animals

The study was conducted using adult (2 months) male Swiss mice (25-35g), conventional, which were obtained from a local breeding colony from The Central Animal Facility that provides a centre for the maintenance, by skilled and experienced personnel, of quality animals in a controlled environment of the Federal University of Santa Maria. The animails were maintained at 22 ± 2 0C under a 12:12h light/dark cycle, with lights turned on

at 7:00 a.m. Commercial diet (Guaiba, RS, Brazil) and water are sterilized and were supplied ad libitum.

The experiment was performed using a randomized schedule on separate groups of animals that 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 (nº 6997050115). 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 Drugs

The compound (*p*-ClPhSe)$_2$ (Fig. 1) was prepared and characterized by the method previously described to Paulmier[22] and accurately evaluated before its use. Analysis of the 1H NMR and 13CNMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. (*p*-ClPhSe)$_2$ chemical purity (99.9%) was determined by GC-MS. Corticosterone was obtained from Sigma (St. Louis, MO, USA) and all the other chemicals were of analytical grade and obtained from standard commercial suppliers.

(*p*-ClPhSe)$_2$ and corticosterone were dissolved in mineral oil and in water with ethanol (1% EtOH/H_2O), respectively. The organoselenium compound was administered to mice in a constant volume of 10 ml/kg of body weight and appropriate vehicle-treated groups were simultaneously assessed as well.

2.3 Experimental design

The experimental design of this study is depicted in figure 1. The animals were randomly assigned in six different groups (n = 10 mice/group) as following:

- Group I: vehicle (1% EtOH/H_2O) + mineral oil

- Group II: corticosterone + mineral oil
- Groups III and IV: vehicle (1% EtOH/H_2O) + (*p*-ClPhSe)$_2$ (1 or 5 mg/kg/day)
- Groups V and VI: corticosterone + (*p*-ClPhSe)$_2$ (1 or 5 mg/kg/day)

The animals received corticosterone or vehicle (1% EtOH/H_2O) in drinking water, during four weeks. The concentration of corticosterone in the drinking water (20 µg/ml) was adjusted based on the vehicle consumption [23].

On the last week of corticosterone exposure, the animals received intragastrically (i.g) (*p*-ClPhSe)$_2$ (1 or 5 mg/kg) or mineral oil (10 ml/kg) one time a day. The doses of (*p*-ClPhSe)$_2$ and time of treatment were chosen based on a previous study conducted in our research group [24].

Twenty-four hours after treatment with (*p*-ClPhSe)$_2$, animals were challenged in activity chamber (AC) to evaluate locomotor and exploratory activities and on subsequent days the object recognition test (ORT), the object location test (OLT) and the step-down passive avoidance (SDPA) test were carried out as shown in figure 2.

Immediately after the behavioral tests, the animals were slightly anesthetized with ketamine-xylazine for blood collection by heart puncture with anticoagulant heparin. The blood samples were centrifuged to obtain the plasma that was quickly frozen and used to determine the parameters of toxicity.

Because some behavioral tests are aversive to the animals, such as SDPA test, and this could cause neurochemical and endocrine changes during and after the behavior [25], in another set of experiments, in which the animals were not submitted to the behavioral tests, the same treatment protocol was carried out; the brains were removed and hippocampi and

total cortices were excised to determine: [^{3}H]glutamate uptake (n = 4 mice/group) and $Na^{+}K^{+}$ATPase activity (n = 8 mice/group).

2.4 Behavioral tests

Before object recognition tests (ORT), the animals were habituated in an open-field apparatus without objects during 5 min. This equipment was made of plywood and surrounded by walls of 30 cm height, the floor had 45 cm length and 45 cm width. This procedure was useful to familiarize the mice with the arena as a context habituation trial for memory tests.

The exploration time in objects was defined as sniffing or touching with the nose and/or forepaws. The objects were positioned 10 cm from the walls of apparatus. Periods in which the mice moved around, climbed over or sat on the objects were not recorded. The animals were allowed to explore the objects for 5 min, and during this period the total time that mice spent exploring each of two objects was recorded and then the mouse was brought back to its home cage.

The results were expressed as exploratory preference according to the following formula: [time spent in the novel object /(time spent in the familiar object + time spent in the novel object)] for the ORT. Data on OLT were expressed as: [time spent in the novel location/(time spent in the familiar location + time spent in the novel location)].

Moreover, the objects and the field were cleaned with EtOH/H_2O solution after each trial to prevent olfactory cues.

2.4.1 Object Recognition Test (ORT)

The ORT was performed as described to Rosa et al. [18], 24h after mice habituation. The training was conducted by placing one mouse into the field, where two identical objects

(objects A1 and A2; duple Lego colorful toy) were positioned in two adjacent corners, 10 cm from the walls.

After ninety minutes of training, the test session was performed for evaluation of memory in ORT. For this, the animals were put back into the same place that was used in the training session but one of the two objects was replaced by another object (object B) with similar textures, color and size, but with distinctive shapes. Animals were allowed to explore the objects during 5 min and the time spent was recorded as described in section 2.4.

2.4.2 Object Location Test (OLT)

This test was performed in the same apparatus used in the ORT. The training was conducted by placing one mouse into the field where two objects (objects A1 and C; duple Lego colorful toy) were positioned in two adjacent corners, 10 cm from the walls [26] with some modifications. Thus, 2 h later the session training, object C was moved to a location that was diagonally opposite to object A1, and the animals were allowed to explore the objects during 5 min and the evaluation of the exploration was done as described in section 2.4.

2.4.3 Step-down Passive Avoidance (SDPA)

The non-spatial long-term memory was investigated through the step-down passive inhibitory avoidance task according to Sakagushi et al.[27] with some modifications. The apparatus consisted of a single box in which the floor was made of a metal grid connected to a shock scrambler. It has also a safe platform, where each animal was placed before starting the experiment which consists in training and 24 h later a test session was carried out. The animal was set in the platform and when it stepped down with its four paws on the grid floor an electric shock (0.5 mA) was delivered for 2 s. The test sessions were performed 24 h later following the same procedure but no shock was given after stepping down. The latency to

step-down was automatically recorded in training and test sessions and besides that, an upper cut-off time of 300s was set.

2.4.4 Activity chamber (AC)

The AC was performed to verify the treatment effect with corticosterone and/or $(p\text{-}ClPhSe)_2$ on the animals' locomotion. The apparatus consists in a clear acrylic chamber (500 x 480 x 500 mm) equipped with 16 infrared sensors for the automatic recording of horizontal activity (Model EP149, Insight Instruments Ltda, São Paulo, BR). General locomotor activity and the mouse's position in the chamber are detected by breaks of the photocell beams which are recorded by the software. Animals were placed in the center of the apparatus and allowed to freely explore it during 4 min. In this time, the number of crossings (number of segments crossed with the four paws) and rearings (number of times reared on the hind limbs), average velocity (mm/s) and total distance traveled (dm) were recorded.

2.5 Ex vivo assays

2.5.1 L-[^{3}H]glutamate uptake assay

L-[^{3}H]glutamate uptake assay was carried out in slices of total cortex and hippocampus of mice according to the method described by Thomazi et al.[28]. After separation of the tissue (cortex and hippocampus) coronal slices (0.4 mm) were obtained using a Mc Illwain tissue chopper and slices were submerged in Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO4, 4.17 NaHCO3, 5.36 KCl, 0.44 KH_2PO4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, and 1.11 glucose, adjusted to pH 7.2. After 15 min of pre-incubation, the uptake assay was performed by adding 13.3 µM (hippocampus) and 6.6 µM (cortex) L-[^{3}H]glutamate in 300 µl HBSS at 37ºC. Incubation was finished after 5 min (hippocampus) or 7 min (cortex) by three ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 M NaOH, which was kept overnight. Unspecific uptake was

measured using the same protocol described above, with differences in temperature (4ºC) and medium composition (choline chloride instead of sodium chloride). Na^+-dependent uptake was considered as the difference between the total uptake and the unspecific uptake. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). Results were expressed as nmol of L-[^{3}H] glutamate uptake/mg protein/min.

2.5.2 Na^+K^+ATPase activity

For this assay, it was used a reaction mixture containing 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl (pH 7.5), at a final volume of 500 µl. Control samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. The samples were incubated at 37 oC for 30 min. The incubation was stopped by adding trichloroacetic acid solution (10%) with 10 mM $HgCl_2$. Na^+K^+ATPase activity was calculated by the difference between the two assays. The release of phosphorus was measured by the method of Fiske and Subbarow[29]. Enzyme activity was expressed as nmol Pi formed/mg protein/min.

2.5.3 Toxicity

In order to investigate any possible (*p*-ClPhSe)$_2$ toxic effect caused by its administration in mice, the plasma samples of animals were obtained to evaluate biochemical markers of acute hepatic damage, aspartate (AST) and alanine aminotransferase (ALT) enzyme activities, which were expressed as U/l. The renal function was analyzed by determining plasma urea levels, being expressed as mg/dl. Total cholesterol, high density lipoprotein (HDL) and triglycerides levels were expressed as mg/dl. All these parameters were determined by enzymatic colorimetric methods using commercial kits (Labtest Diagnostica, MG, Brazil) according to the manufacturer's instructions. Standard controls were

run before each determination and the values obtained for the different biochemical parameters were always in agreement with the expected ranges.

2.6 Protein determination

Protein concentration was measured according to Bradford[30], using bovine serum albumin (1mg/ml) as the standard sample.

2.7 Statistical Analysis

Data were expressed as mean (s) ± S.E.M. of the mean (s) of n observations, representing the number of animals used in the experimental groups. The normal distribution was tested according to the normality of D'Agostino and statistically evaluated by analysis of variance of Two-way ANOVA followed by the Newman-Keuls test. The statistical program GraphPad Prism software and Statistica were used. Probability values less than 0.05 ($p < 0.05$) were considered to be statistically significant.

3 Results

3.1 Behavioral tests

3.1.1 (*p*-ClPhSe)$_2$ effect on the mouse ORT

(*p*-ClPhSe)$_2$ reversed the recognition index in the ORT impaired in mice treated with corticosterone. The two-way ANOVA of the recognition index data in the memory test revealed a significant corticosterone and (*p*-ClPhSe)$_2$ interaction [$F(2,54) = 5.55$, $p < 0.05$]. Post-hoc comparisons showed that the recognition index for the memory of corticosterone-treated mice was significantly lower than that of the vehicle-treated control mice and both doses of (*p*-ClPhSe)$_2$ significantly reversed this reduction (Fig 3). Besides, Table 1 shows values mean in seconds of exploration by mice in each object in ORT.

3.1.2 (*p*-ClPhSe)$_2$ effect on the mouse OLT

(*p*-ClPhSe)$_2$ reversed spatial memory impaired by corticosterone in mice. The two-way ANOVA of the recognition index in OLT revealed a significant corticosterone and (*p*-ClPhSe)$_2$ interaction [$F(2,54) = 12,06$, $p < 0.05$]. Post-hoc comparisons showed that the exploratory preference of corticosterone-treated mice was reduced in comparison to the one of vehicle-treated control mice. Furthermore, both doses of (*p*-ClPhSe)$_2$ were effective against spatial memory impairment caused by corticosterone (Fig. 4).

3.1.3 (*p*-ClPhSe)$_2$ effect on the mouse SDPA

Fig. 5 shows the effect of corticosterone exposure and/or (*p*-ClPhSe)$_2$ in the step-through latency. There was no difference among groups during the training session in the step-down passive avoidance test ($p > 0.05$).

The (*p*-ClPhSe)$_2$ reversed the reduction in the step-down latency caused by the treatment with corticosterone in mice. The two-way ANOVA of step-down latency revealed a significant corticosterone and (*p*-ClPhSe)$_2$ interaction [$F(2,54) = 16,36$, $p < 0.05$] in the test session. Post hoc comparisons indicated that corticosterone-treated mice decreased the step-through latency when compared with that of the control group and both doses of (*p*-ClPhSe)$_2$ were effective in reducing the memory impairment induced by corticosterone.

3.1.4 (*p*-ClPhSe)$_2$ effect on the mouse locomotor activity

Neither corticosterone nor (*p*-ClPhSe)$_2$ altered the parameters of locomotor and exploratory activities in mice. Two-way ANOVA of locomotor and exploratory activities parameters (crossing, rearing, average velocity and total distance traveled) evaluated in the activity chamber were not significantly altered in all experimental groups ($p > 0.05$) (Table 2).

3.2 Ex vivo assays

3.2.1 (*p*-ClPhSe)$_2$ effect on [^{3}H] glutamate uptake

Fig. 4 shows the effect of exposure to corticosterone and/or treatment with (*p*-ClPhSe)$_2$ on the [^{3}H] glutamate uptake of total cortical and hippocampal slices of mice. Two-way ANOVA of [^{3}H] uptake glutamate in the total cortex did not show a significant corticosterone and (*p*-ClPhSe)$_2$ interaction [$F(2,18) = 0,15$, $p > 0.05$] (Fig 6A).

The (*p*-ClPhSe)$_2$ reversed the increase in the [^{3}H] glutamate uptake in the hippocampus caused by treatment with corticosterone in mice. Two-way ANOVA of [^{3}H] glutamate uptake in the hippocampus revealed a significant corticosterone and (*p*-ClPhSe)$_2$ interaction [$F(2,18) = 7,37$, $p < 0.05$]. Post hoc comparisons showed that corticosterone-treated mice increased the [^{3}H] glutamate uptake when compared to that of the control group. Moreover, (*p*-ClPhSe)$_2$ was effective, at both doses, against the increase of [^{3}H] glutamate uptake induced by corticosterone (Fig. 6B).

3.2.2 (*p*-ClPhSe)$_2$ effect on Na^+K^+ATPase activity

The Na^+K^+ATPase activity measured in both cerebral structures did not significantly differ among groups. Two-way ANOVA of Na^+K^+ATPase activity data revealed no significant corticosterone and (*p*-ClPhSe)$_2$ interaction in total cortex [$F(2,42) = 0,25$, $p > 0,05$] and hippocampus [$F(2,42) = 0,20$, $p > 0,05$] (Fig. 7A and B, respectively).

3.2.3 (*p*-ClPhSe)$_2$ effect on toxicity

The (*p*-ClPhSe)$_2$ did not cause toxicity in mice. Two-way ANOVA of ALT and AST activities as well as urea, cholesterol, triglycerides and HDL levels indicated no significant corticosterone and (*p*-ClPhSe)$_2$ interaction ($p > 0.05$)(Table 3).

4 Discussion

The present study revealed that (*p*-ClPhSe)$_2$ reversed memory impairment-related to stress caused by corticosterone administration in mice which was demonstrated in the ORT, OLT and SDPA. Furthermore, our results also showed that repeated treatment with (*p*-ClPhSe)$_2$ during seven days neither trigger impairment on locomotor and exploratory activities nor caused overt signs of toxicity in mice. This study showed that (*p*-ClPhSe)$_2$, at both doses tested, had memory enhancer effects and restored glutamate uptake in the hippocampus of mice exposed to corticosterone.

It has been reported that chronic stress makes deleterious impacts on learning and memory process [31] and, an increase in corticosterone levels could be a plausible explanation for cognition impairment [32]. According to the experimental protocol employed in the present study, memory impairment was caused by the addition of corticosterone in the drinking water, a model of chronic stress [33].

The object recognition test used in this study is a type of non-aversive and non-spatial memory [34], which requires no external motivation, reward or punishment for the animals, and the exploration of the novel object reflects the use of learning and recognition memory [35]. In our study, animals treated with corticosterone showed a memory deficit characterized by a lower discrimination index compared to that of control mice in the ORT. It is well documented that chronic elevation of circulating corticosterone concentrations, systemic administration of corticosterone or glucocorticoid receptor agonists exerts an inhibitory influence on learning and memory [36, 37]. Accordingly, in this study the administration of both doses of (*p*-ClPhSe)$_2$ reversed memory impairment induced by corticosterone in the ORT. Differently to data Bortolatto et al.[20] in which demonstrate that (*p*-ClPhSe)$_2$ had no effect on short-term ORT in aged rats, however, memory enhancer action of organoselenium

compounds in the object recognition test has been reported by us, in these studies a diet supplemented with $(PhSe)_2$, a parent compound of $(p\text{-}ClPhSe)_2$, improved memory in old [38] and middle-age rats [39].

Object location memory suggests that discrimination of the displaced object reflects spatial memory formation [40], the OLT has a spatial component which is not present in ORT. Our findings showed that corticosterone caused memory impairment in the OLT being demonstrated by a reduction in the exploratory preference of a novel object location. This memory deficit was reversed by the treatment with both doses of $(p\text{-}ClPhSe)_2$, reinforcing the memory enhancer effects of this organoselenium compound in mice. Our data corroborated with findings reported in the literature using the novel object location paradigm, in which $(p\text{-}ClPhSe)_2$ protected against cognitive impairment caused by aging in male rats [20]. It is possible that 5 min habituation be less effective than 3-5 daily exposures to the object chamber, along with daily mild handling to allow for behavioral or endocrine habituation. Therefore, we acknowledge a single 5-minute habituation to the object recognition chamber 24h prior to tests as a limitation of this study.

A trial passive avoidance paradigm represents a model of associative learning where the effects of stressors or stress hormones can be studied [41]. The memory impairment induced by corticosterone was also demonstrated in this study by the SDPA test and both doses of $(p\text{-}ClPhSe)_2$ were also effective in improving memory. In a model of memory impairment induced by streptozotocin in mice, $(MeOPhSe)_2$, a diaryl diselenide substituted with the methoxyl group into the aromatic ring, had memory enhancing effects when evaluated in the step-down-type passive-avoidance test [42].

It has long been recognized that the glutamatergic system plays a pivotal role in neuroplasticity [43] as well as cognition, learning and memory processes [44]. In this study

both doses of (*p*-ClPhSe)$_2$ were effective in modulating glutamate uptake increased in hippocampus of mice exposed to corticosterone. Our findings are in agreement with Fontella et al.[45] who showed an increase in the glutamate uptake in the hippocampus of animals after chronic restraint stress. Organoselenium compounds have been reported to modulate glutamate neurotransmission; protecting neuronal cells from injury induced by glutamate [46, 47] and inhibiting glutamate transport [48]. The inhibitory effect of organoselenium compounds on glutamate uptake has been demonstrated to be related to the oxidation of critical protein –SH groups [49-51].

Considering the well established involvement of glutamatergic neurotransmission in learning and memory processes [52], the decrease of glutamate levels in the synaptic cleft triggers a disruption in its neurotransmission and could result in cognitive and memory impairment. Our findings suggest that the treatment with (*p*-ClPhSe)$_2$ restored the glutamate homeostasis by modulating the hippocampal glutamate uptake and reversed memory impairment in ORT, OLT and SDPA caused by corticosterone exposure to mice. Although object location memory is hippocampal dependent, object recognition memory is heavily dependent on cortical function and step down avoidance is also not heavily reliant on hippocampal function. However, the biochemical measures were only found to be significant in the hippocampus, and not in cerebral cortex. One possible explanation for this question would be the fact that we used total cerebral cortex tissue instead of using specific regions of cortex for the glutamate uptake, which could have dissipated the effect. This fact is assumed by us as one limitation of this study.

The underlying neural mechanisms by which stress exerts its effects on learning and memory in the brain are still not completely clear and little is known about a way through which the stress-induced changes are accomplished. Although, N-methyl-D-aspartate

receptors seem to be involved, changes in glutamate transmission and calcium influx, affecting the potential for synaptic plasticity [53] also appear to be associated.

Considering what was mentioned before, it is possible that the corticosterone effects on the cerebral cortex might be different than those in the hippocampus. It is likely that some prefrontal cortex neurons respond phasically, wherein a separate population of neurons responds to a stressful stimulus with a sustained increase that persists in the absence of stimulus, furthermore, some neurons rapidly become less responsive to the same stressor [54].

The glutamatergic homeostasis is not related only with the glutamatergic neurons but also the astrocytes integrity are indispensable for such system [55]. It has been reported that glutamate uptake from the extracellular space occurs mainly via the glial glutamate transporters where a glutamate molecule is accompanied by three Na^+ and one H^+ in exchange for one K^+ [56]. Efficient uptake of glutamate into astrocytes via the glutamate/Na^+ cotransporters depends on the transmembrane Na^+gradient generated by Na^+K^+ATPase [57]. It is known that the released of glutamate in the synaptic cleft need to be rapidly cleared from the extracellular space to avoid detrimental effects of glutamate receptors inappropriate activation [58]. In this context, the Na^+K^+ATPase activity in slices of cerebral cortex and hippocampus of mice was not altered by corticosterone in the experimental protocol used in this study. Considering our results, it is possible to suggest that the exposure to corticosterone in the mice interferes in the glutamate uptake and Na^+K^+ATPase activity is not involved in this effect.

Although the toxicity parameters were evaluated in a different time course than the one used for the behavioral experiments in this study, no signals of toxicity related to hepatic and renal functions were immediately found after the end of treatment with (*p*-ClPhSe)$_2$ in rats [59] supporting the findings of toxicity obtained in this study. Moreover, our previous

study indicates that modifications in chemical structures of disubstituted diaryl diselenides, such as the introduction of chloro into the aromatic ring of diphenyl diselenide, did not introduce toxicity. In fact, calculated LD50 values for a single oral application of (*p*-ClPhSe)$_2$ in mice were estimated to be >381 mg/kg, which is similar to that of obtained for diphenyl diselenide, a parent compound. Oral administration of (*p*-ClPhSe)$_2$ was accompanied by a decrease in body weight gain, which was parallel to the decrease in food and water intake [24].

The present study showed that memory functions were negatively affected by corticosterone, possibly due to a disruption of the glutamate homeostasis. Confirming our assumptions, the treatment with both doses of (*p*-ClPhSe)$_2$ reversed memory impairment triggered by corticosterone exposure and also regulate the glutamate uptake in the hippocampus which could explain its memory enhancer effects.

Acknowledgments

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Captions

Fig. 1 Schematic representation of the experimental design. Corticosterone solution or vehicle was given to mice during 4 weeks. In the last week, mice received a daily i.g. administration of (*p*-ClPhSe)$_2$ (1 or 5 mg/kg) represented by the chemical structure. Behavioral tests started at 24h after the last dose of (*p*-ClPhSe)$_2$ and were performed in consecutive days as illustrated in this figure, activity chamber (AC), object recognition test (ORT), object location test (OLT) and step-down passive avoidance test (SDPA). *Ex vivo* assays were determined at 96h after the last dose of (*p*-ClPhSe)$_2$.

Fig. 2 Schematic representation of the behavioral design. Behavioral tests started at 24h after the last dose of (*p*-ClPhSe)$_2$ and were performed in consecutive days as illustrated in this figure. Activity chamber (AC) and habituation were carried out 24h after treatment with (*p*-ClPhSe)$_2$, the object recognition training and memory in ORT were performed 48h after treatment with (*p*-ClPhSe)$_2$, the training and object location test (OLT) and step-down passive avoidance (SDPA) training were carried out 72h after the last dose of (*p*-ClPhSe)$_2$. The SDPA test was performed 96h after the treatment with (*p*-ClPhSe)$_2$.

Fig 3 (*p*-ClPhSe)$_2$ effect (1 and 5 mg/kg, i.g.) on the recognition index of corticosterone-treated mice in the object recognition test. Each column represents the mean ± SEM to ten animals per group. *$p<0.05$ when compared with the control group and #$p<0.05$ when compared with the corticosterone group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test.

Fig. 4 (*p*-ClPhSe)$_2$ effect (1 and 5 mg/kg, i.g.) on the location index of corticosterone treated mice in the object location test. Each column represents the mean ± SEM to ten animals per group. * $p<0.05$ when compared to the control group and #$p<0.05$ when compared with the corticosterone group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test.

Fig. 5 (*p*-ClPhSe)$_2$ effect (1 and 5 mg/kg, i.g.) on corticosterone-induced memory impairment in the step-down inhibitory avoidance test in mice. Each column represents the mean ± SEM to ten animals per group. * $p<0.05$ when compared with the control group and # $p<0.05$ when

compared with the corticosterone group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test.

Fig. 6 (*p*-ClPhSe)$_2$ effect (1 and 5 mg/kg, i.g.) on [^{3}H] glutamate uptake of cortical **(A)** and hippocampal **(B)** slices in mice treated with corticosterone. Each column represents the mean ± SEM to four animals per group. *p<0.05 when compared with the control group and #p<0.05 when compared with the corticosterone group. Data were analyzed through two-way ANOVA followed by the Newman–Keuls test.

Fig. 7 (*p*-ClPhSe)$_2$ effect (1 and 5 mg/kg, i.g.) on Na^+K^+ATPase activity of cerebral total cortex **(A)** and hippocampus **(B)** of corticosterone treated mice. Each column represents the mean ± SEM. Data were analyzed through two-way ANOVA.

Figures

Figure 1

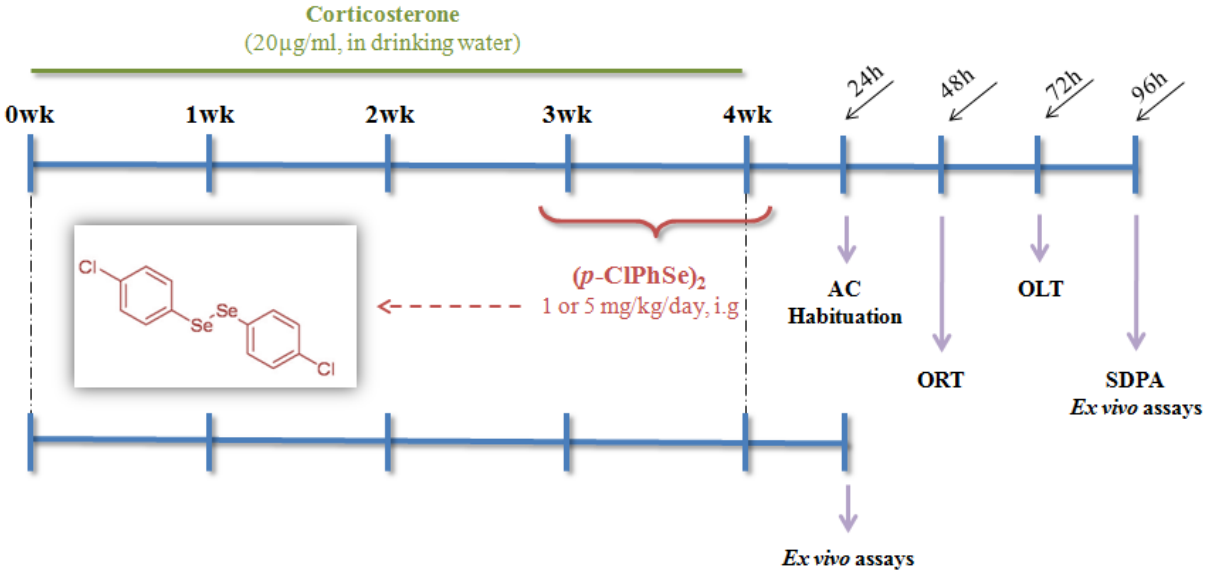


Figure 2

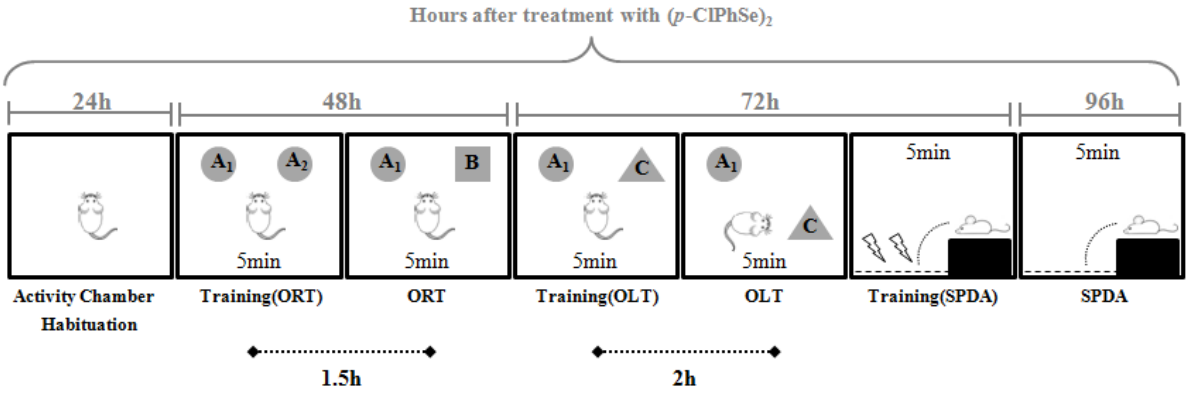

Hours after treatment with (p-ClPhSe)2
24h
48h
72h
96h
A1
A2
B
C
5min
Activity Chamber Habituation
Training(ORT)
ORT
Training(OLT)
OLT
Training(SPDA)
SPDA
1.5h
2h

Figure 3

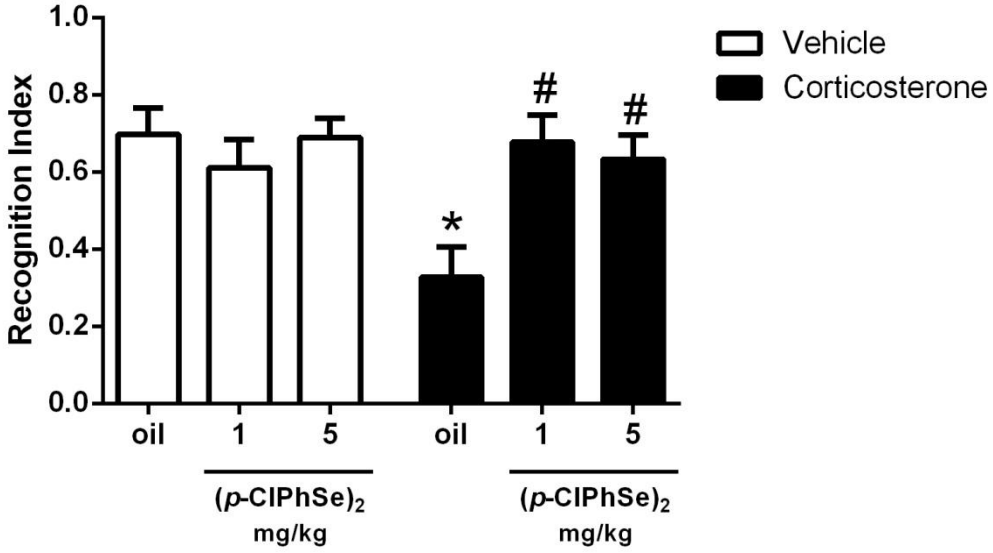

Recognition Index
1.0
0.8
0.6
0.4
0.2
0.0
oil
1
5
oil
1
5
*
#
#
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg
Vehicle
Corticosterone

Figure 4

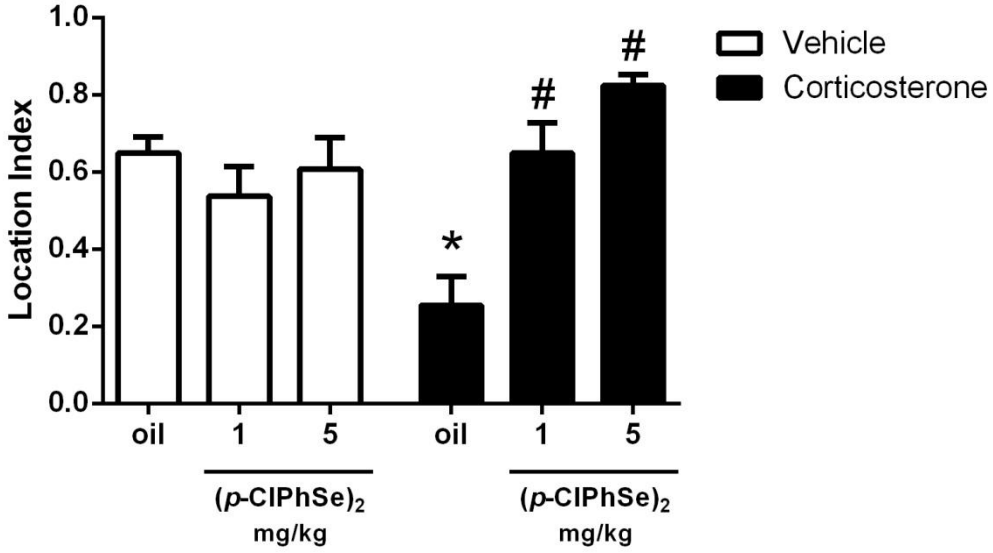

Location Index
1.0
0.8
0.6
0.4
0.2
0.0
oil
1
5
oil
1
5
*
#
#
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg
Vehicle
Corticosterone

Figure 5

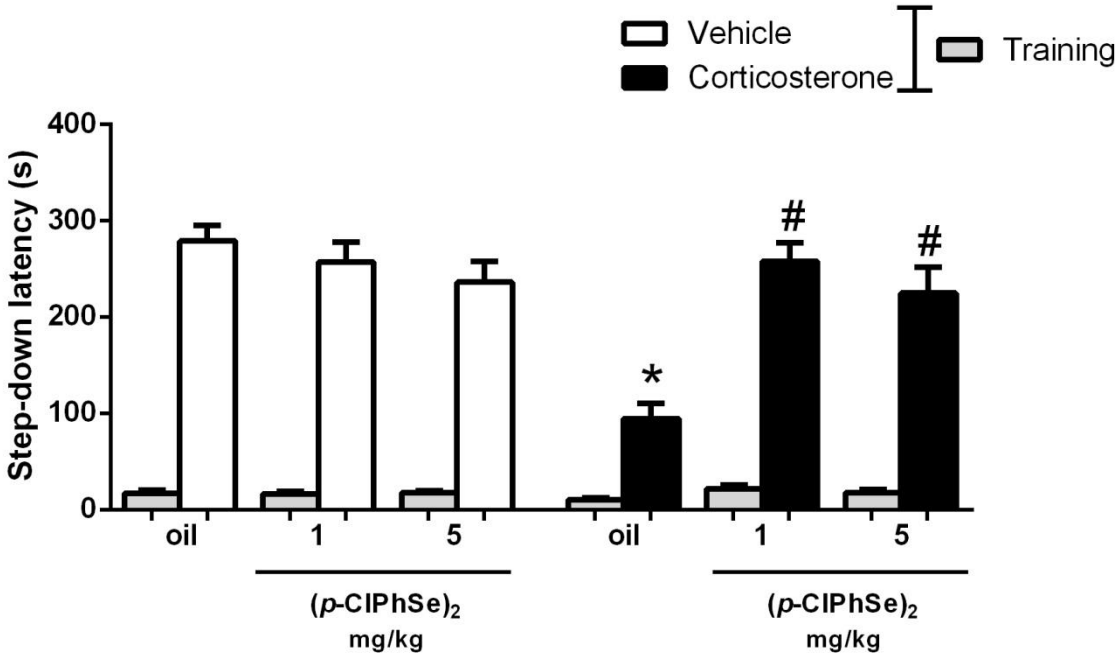

Vehicle
Corticosterone
Training
Step-down latency (s)
400
300
200
100
0
*
#
#
oil
1
5
oil
1
5
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg

Figure 6

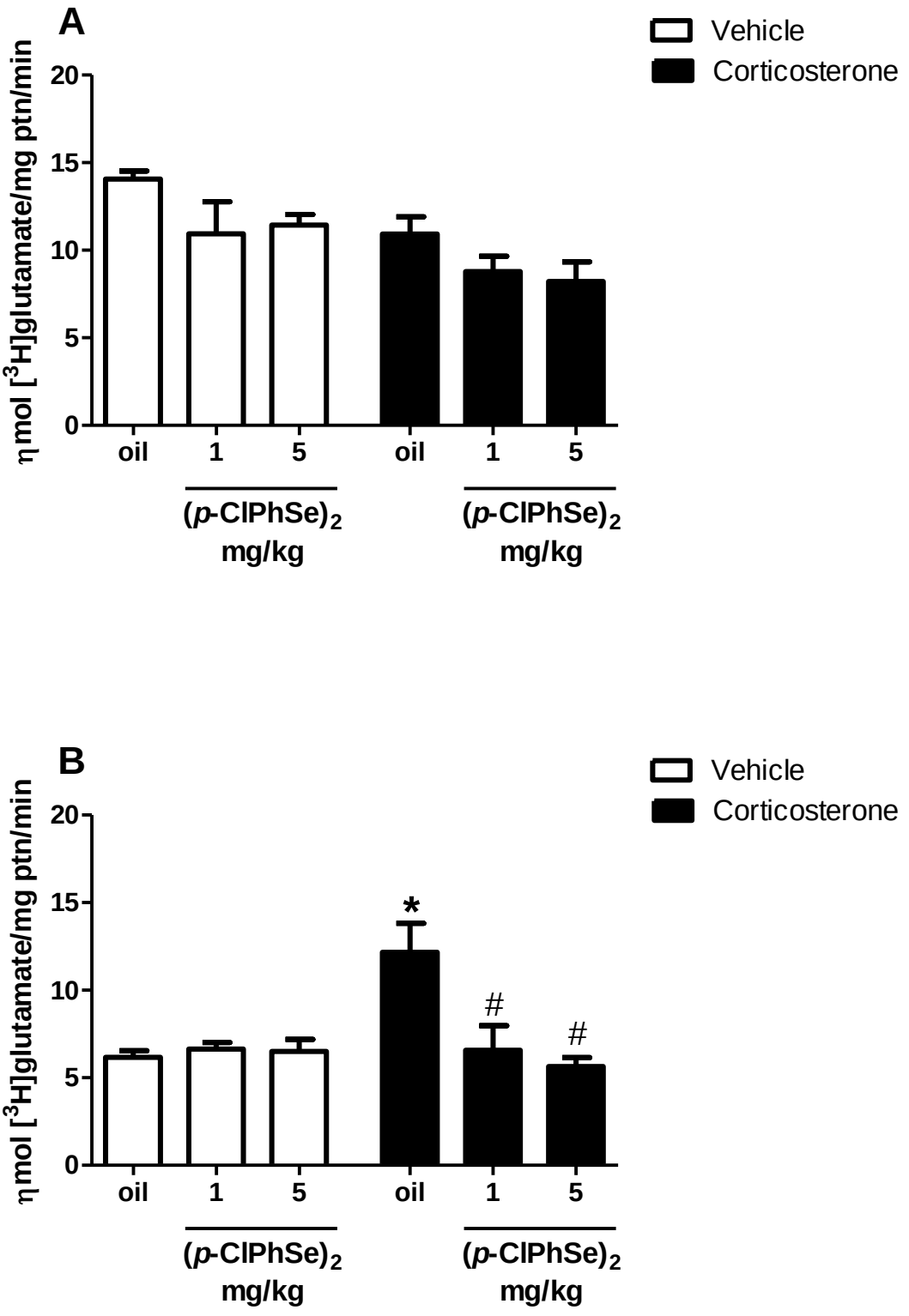
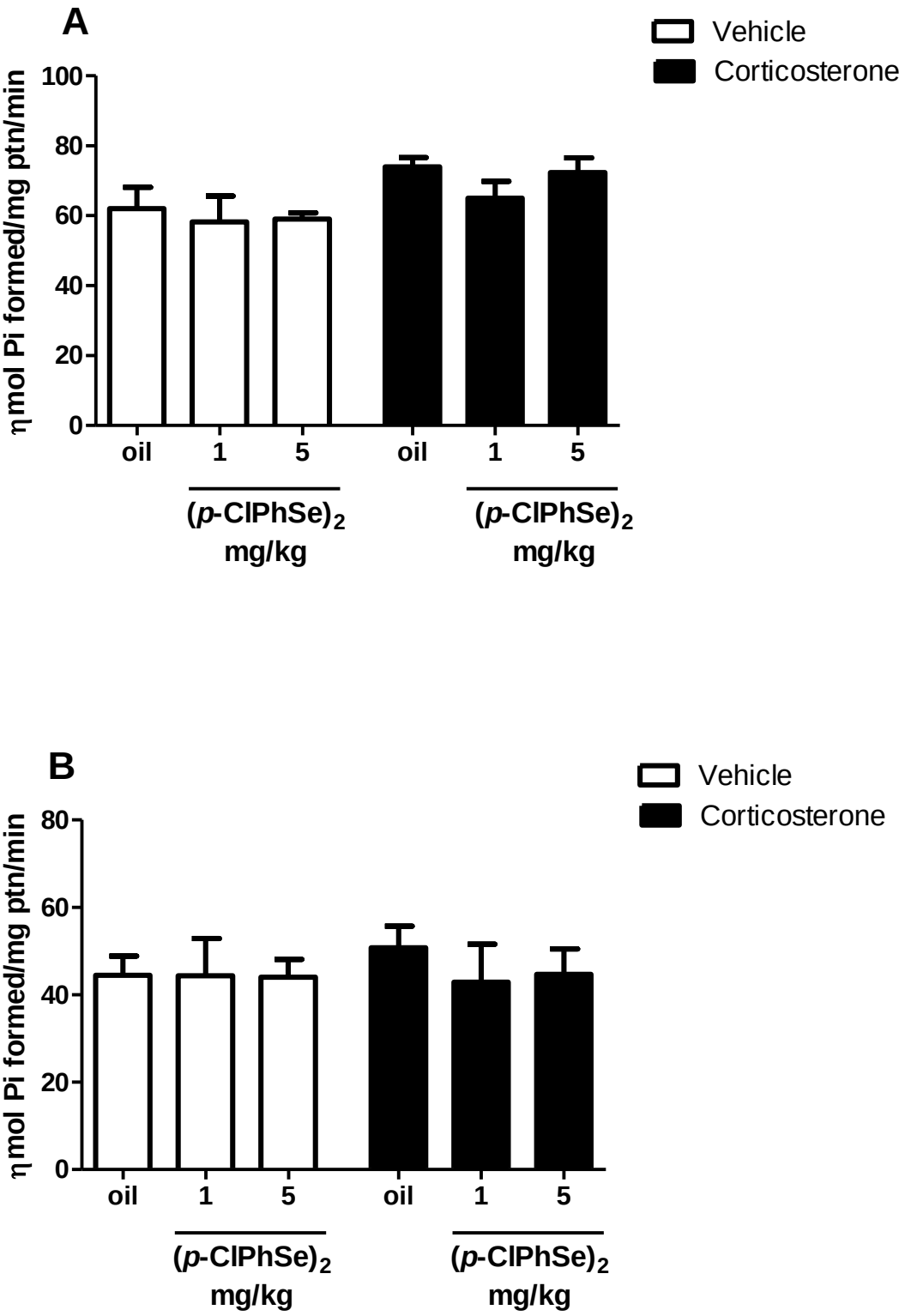

A
Vehicle
Corticosterone
ηmol [3H]glutamate/mg ptn/min
20
15
10
5
0
oil
1
5
oil
1
5
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg
B
Vehicle
Corticosterone
ηmol [3H]glutamate/mg ptn/min
20
15
10
5
0
*
#
#
oil
1
5
oil
1
5
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg

Figure 7


A
Vehicle
Corticosterone
ηmol Pi formed/mg ptn/min
100
80
60
40
20
0
oil
1
5
oil
1
5
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg
B
Vehicle
Corticosterone
ηmol Pi formed/mg ptn/min
80
60
40
20
0
oil
1
5
oil
1
5
(p-ClPhSe)2
mg/kg
(p-ClPhSe)2
mg/kg

Tables

Table 1. Effect of corticosterone and (*p*-ClPhSe)$_2$.on the time of exploration (s) in each object in the mice object recognition test.

Vehicle						**Corticosterone**					
oil		**1mg/kg**		**5mg/kg**		**oil**		**1mg/kg**		**5mg/kg**	
A1	B	A1	B	A1	B	A1	B	A1	B	A1	B
5.0 ± 1.6	7.0 ±1.9	4.8 ± 1.3	9.1 ± 2.5	6.4 ± 2.1	9.8 ± 1.6	4.2 ± 0.9	2.9 ± 0.8	5. 3 ± 1.5	8.9 ± 1.7	6.7 ± 2.3	8.8 ± 1.8

Values are expressed as the mean ± S.E.M.

Table 2. Locomotor parameters of mice evaluated in the activity chamber after exposure to corticosterone and treatment with (*p*-ClPhSe)$_2$.

	Vehicle			**Corticosterone**		
	Oil	**1mg/kg**	**5mg/kg**	**Oil**	**1mg/kg**	**5mg/kg**
Crossings	325 ± 46.1	343.1 ± 34.2	332.5 ± 17.7	289 ± 31.1	318.4 ± 39.3	291.0 ± 42.4
Rearings	10.3 ± 1.9	12.2 ± 1.7	10.4 ± 1.2	10.6 ± 1.1	9.8 ± 2.1	10.7 ± 1.9
Velocity (mm/s)	24.9 ± 2.5	24.3 ± 2.5	24.4 ± 1.4	21.4 ± 2.6	23.8 ± 2.7	21.0 ± 2.6
Distance (dm)	52.2 ± 6.5	52.9 ± 6.8	55.4 ± 3.5	45.8 ± 4.9	50.0 ± 6.1	47.1 ± 6.7

Values are expressed as the mean ± S.E.M . Data were analysed through a two-way analysis of variance (ANOVA).

Table 3. Biomarkers of hepatic and renal toxicity after exposure to corticosterone and treatment with (*p*-ClPhSe)$_2$.

	Vehicle			Corticosterone		
	Oil	**1mg/kg**	**5mg/kg**	**Oil**	**1mg/kg**	**5mg/kg**
ALT	39.5 ± 3.3	32.2 ± 3.6	30.5 ± 3.6	46.8 ± 4.0	30.3 ± 3.6	31.8 ± 4.0
AST	136.0 ± 8.3	157.6 ± 13.9	170.1 ± 11.5	184.2 ± 15.0	148.7 ± 17.3	158.2 ± 7.1
Urea	49.3 ± 3.1	43.5 ± 4.0	42.5 ± 3.2	43.4 ± 2.4	45.7 ± 2.7	47.4 ± 3.5
Cholesterol	83.3 ± 2.3	83.2 ± 2.2	75.5 ± 5.0	89.8 ± 4.5	77.9 ± 4.6	77.5 ± 4.0
HDL	74.1 ± 4.4	61.9 ± 3.6	61.4 ± 5.3	69.1 ± 4.6	61.8 ± 5.1	59.6 ± 3.1
Triglycerides	94.4 ± 5.7	120.1 ± 7.3	127 ± 6.4	100.0 ± 6.7	124.7 ± 12.6	96.9 ± 9.4

Values are expressed as the mean ± S.E.M. Data were analysed through a two-way analysis of variance (ANOVA).

4 CONCLUSÃO

Os resultados apresentados nesta dissertação permitem concluir que:

O $(p\text{-ClPhSe})_2$ não apresenta sinais de toxicidade neste protocolo de tratamento, visto que não houve alteração nos parâmetros bioquímicos e nem na atividade locomotora e exploratória dos animais;

O $(p\text{-ClPhSe})_2$ é efetivo em reverter o prejuízo cognitivo em animais induzidos com corticosterona, avaliados nos testes comportamentais utilizados neste protocolo de estudo;

O tratamento com $(p\text{-ClPhSe})_2$ e/ou corticosterona não alteraram a atividade das enzimas Na^+K^+ATPase e AChE em córtex cerebral e hipocampo de camundongos;

O $(p\text{-ClPhSe})_2$ foi efetivo em reverter o aumento da captação de glutamato induzido pela corticosterona em hipocampo de camundongos, em contrapartida, o tratamento com $(p\text{-ClPhSe})_2$ e/ou corticosterona não alteraram a captação de glutamato no córtex cerebral dos animais.

Em conjunto os dados deste estudo mostram um déficit de memória causado pela administração exógena de corticosterona, e que o $(p\text{-ClPhSe})_2$ em ambas as doses foi efetivo em reverter esse prejuízo cognitivo dos animais. Além disso, a ação do $(p\text{-ClPhSe})_2$ esta envolvida com a modulação da homeostase glutamatérgica. Entretanto, mais estudos são necessários para melhor elucidar o mecanismo exato pelo qual o composto age, bem como sugerir novos alvos da ação nootrópica do $(p\text{-ClPhSe})_2$.

6 PERSPECTIVAS

A seguir, as perspectivas para trabalhos futuros são:

Avaliar o possível efeito do $(p\text{-ClPhSe})_2$ envolvido em outros parâmetros do sistema colinérgico e glutamatérgico, visando melhor esclarecer o mecanismo pelo qual o composto age.

Avaliar o envolvimento de algumas proteínas e neurotrofinas que fazem parte do processo de aprendizagem e memória, e assim, descobrir se o composto estaria envolvido em alguma via de sinalização de proteínas.

Realizar outros protocolos que englobam o eixo HPA e a memória, para elucidar a via exata pela qual o $(p\text{-ClPhSe})_2$ exerce seu papel nootrópico.

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APÊNDICE A

Resultado complementar

O objetivo para realização desta técnica foi para avaliar um possível envolvimento em um dos parâmetros envolvidos na via colinérgica.

Materiais e Métodos

Atividade da AChE

O protocolo experimental de administração da corticosterona e do composto $(p\text{-ClPhSe})_2$ foi desenvolvido conforme o que está descrito no manuscrito (Figura 1). A dosagem da atividade da AChE ocorreu no mesmo dia da dosagem da atividade da Na^+K^+ATPase. Amostras de cortex e hipocampo foram homogeneizadas em tampão de sacarose 0.25 M e centrifugadas em 2555g por 10 min. A atividade da AChE foi realizada de acordo com o método de ELLMAN et al. (1961) usando acetilcolina como substrato. A atividade da AChE foi mensurada espectrofotometricamente no comprimento de onda de 412 nm e expressa por µmol AcSCh/h/mg proteína.

Resultado

Efeito do $(p\text{-ClPhSe})_2$ na atividade da AChE

A atividade da AChE dosada em ambas estruturas cerebrais não foram significativamente diferentes entre os grupos. ANOVA de duas vias da atividade da AChE para o córtex [$F(2,24) = 0,32$, $p > 0,05$] e hipocampo [$F(2,24) = 0,38$, $p > 0,05$] (Fig. 1A e B, respectivamente), revela que não houve interação significativa entre corticosterona e $(p\text{-ClPhSe})_2$.

Figura 1

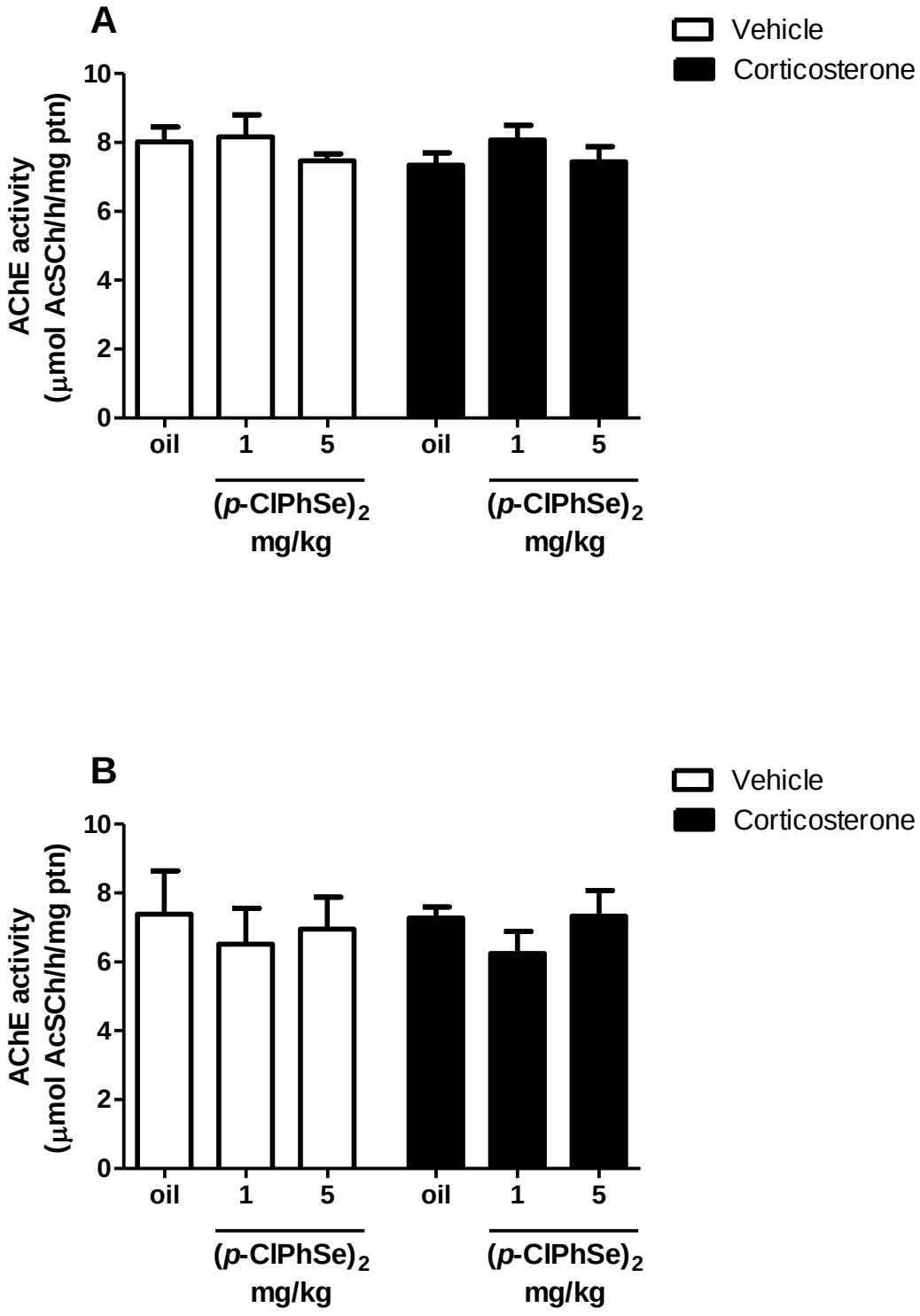

Figura 1. Efeito do (p-ClPhSe)$_2$ (1 e 5 mg/kg, ig) na atividade da acetilcolinesterase de córtex **(A)** e de hipocampo **(B)** cerebral de camundongos tratados com corticosterona (20µg/ml). Cada coluna representa a média ± SEM de quatro ou cinco animais por grupo. Os dados foram analisados usando ANOVA de duas vias.

Discussão

Existe uma relação bem estabelecida entre o sistema colinérgico e os processos cognitivos. A ACh é um neurotransmissor necessário para o bom funcionamento da transmissão colinérgica que regula os processos de aprendizagem e memória (ISHRAT et al., 2009). Esse neurotransmissor é sintetizado pela enzima colina acetiltransferase, que catalisa a acetilação de colina com acetil-CoA. A ACh é acumulada em vesículas sinápticas através de um transportador vesicular de ACh, que é controlado pelo gradiente de prótons transvesicular ligado a uma enzima ATPase (PARSONS, 2000). A liberação de ACh na fenda sináptica é desencadeada por despolarização induzida pelo fluxo de Ca^{2+} em que ocorre a ligação da ACh a seus receptores (WEVERS, 2011). A hidrólise da ACh ocorre em milissegundos pela AChE ou butirilcolinesterase (BuChE), que gera ácido acético e colina pronto para recaptação e re-síntese.

Vários neurotransmissores, vias de transdução de sinal e os fatores neurotróficos têm sido implicados nos efeitos do stress no cérebro (PAVLIDES et al., 2002). Portanto, uma estratégia para o tratamento de prejuízos cognitivos derivados do estresse, foi desenvolvida para modular a função colinérgica através da inibição da atividade da AChE (PORCEL e MONTALBAN, 2006).

Neste sentido, investigou-se a atividade da AChE em diferentes estruturas cerebrais, tais como o córtex e o hipocampo, áreas responsáveis pela função cognitiva, visto que a indução pela corticosterona causa déficit de memória. Os resultados demonstram que nenhum grupo experimental alterou a atividade da AChE. No entanto, como mencionado anteriormente, sabe-se que a via colinérgica é composta por: neurotransmissor; a enzima de hidrolisa a ACh (AChE); colina acetiltransferase, uma enzima que sintetiza ACh; e os receptores de acetilcolina, (nAChR) e (mAChR) (ABREU-VILLACA et al., 2011) e, portanto, este resultado da atividade da AChE, não exclui um mecanismo de ação do $(p\text{-ClPhSe})_2$ no sistema colinérgico, ao passo que a determinação de um único parâmetro é insuficiente para excluir o envolvimento desse sistema.