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PAPEL PROTETOR DO 2-FENILETINIL-BUTILTELÚRIO EM MODELOS DE DANO COGNITIVO EM CAMUNDONGOS E NA APOPTOSE EM CÉLULAS HUMANAS

TESE DE DOUTORADO

Ana Cristina Guerra de Souza

Santa Maria, RS, Brasil
2013

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Ana Cristina Guerra de Souza

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Orientadora: Profª Drª Cristina Wayne Nogueira

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Ana Cristina Guerra de Souza

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COMISSÃO EXAMINADORA:

Cristina Wayne Nogueira, Drª.
(Presidente/Orientadora)

Cláudia da Silva Funchal, Drª. (IPA)

Daiana da Silva de Ávila, Drª. (UNIPAMPA)

Maribel Antonello Rubin, Drª. (UFSM)

Michele Rechia Fighera, Drª. (UFSM)

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Aos meus queridos pais,
Flavio e Ilda,
dedico este trabalho
e todo o meu amor!

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Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.

Fernando Pessoa (Ricardo Reis)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

PAPEL PROTETOR DO 2-FENILETINIL-BUTILTELÚRIO EM MODELOS DE DANO COGNITIVO EM CAMUNDONGOS E NA APOPTOSE EM CÉLULAS HUMANAS

AUTORA: ANA CRISTINA GUERRA DE SOUZA
ORIENTADORA: CRISTINA WAYNE NOGUEIRA
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A memória inclui pelo menos três tipos de processamento relacionados entre si: aquisição, consolidação e evocação. A memória é afetada quando as sinapses encarregadas de fazer ou evocar memórias encontram-se alteradas. A doença de Alzheimer (DA) é a causa mais comum de demência. A DA é caracterizada por danos cognitivos, acúmulo de peptídeo β-amiloide (Aβ) e disfunção colinérgica. Além disso, o estresse oxidativo está associado à DA. Uma vez que ainda não há cura para a DA e as terapias atuais são apenas paliativas, torna-se importante a busca de novos compostos para melhorar danos cognitivos. Com o estudo dos efeitos biológicos de compostos de telúrio, muitas aplicações estão sendo descobertas. Neste sentido, o composto orgânico de telúrio 2-feniletinil-butiltelúrio (PEBT) apresenta efeito antioxidante. O objetivo deste estudo foi caracterizar o PEBT como uma alternativa promissora para a melhora e prevenção de danos cognitivos, usando modelos experimentais da DA em camundongos. Primeiramente, avaliou-se o efeito de uma única dose oral (p.o) de PEBT (10 mg/kg) na memória, utilizando a tarefa da esquiva inibitória. O tratamento com PEBT 1 h antes do treino, imediatamente após o treino ou 1 h antes do teste da esquiva inibitória aumentou a latência comparada com os animais controles, melhorando a aquisição, consolidação e evocação da memória, respectivamente. A captação de glutamato, mas não a liberação deste neurotrasmissor, foi inibida em córtex e hipocampo de camundongos após 1 h de tratamento com PEBT. Após 24 h, a inibição da captação de glutamato no córtex não foi mais evidenciada. A melhora da memória causada pelo PEBT parece ser mediada através da interação com os transportadores de glutamato. Além disso, o tratamento subcrônico com PEBT (1 mg/kg, p.o., por 10 dias) após a injeção de Aβ(25-35) (3 nmol/3 µl/*per site*, intracerebroventricular) reverteu o prejuízo no aprendizado e na memória causados por Aβ nas tarefas do labirinto aquático de Morris e na esquiva inibitória. O PEBT (10 mg/kg, p.o.), adminstrado 30 min antes da escopolamina (1 mg/kg, intraperitoneal), também protegeu do dano de memória causado por este agente anticolinérgico no labirinto aquático de Morris. Quando a escopolamina foi administrada 30 min antes do treino ou teste, ou imediatamente após o treino da esquiva inibitória houve dano na aquisição, evocação e consolidação da memória, respectivamente. O PEBT, administrado 30 min antes da escopolamina, protegeu do dano na consolidação e evocação da memória, mas não na aquisição. Não houve diferença nas atividades locomotora e exploratória dos animais tratados com o PEBT no teste do campo aberto. Finalmente, o efeito antiapoptótico do PEBT foi avaliado. Células do epitélio pigmentado da retina humana (linhagem ARPE-19) foram expostas ao estresse oxidativo induzido pelo fator de necrose tumoral-α (10 ng/ml) e H_2O_2 (600 µM). O PEBT (7.5 e 10 µM), quando pré-incubado por 1 h, protegeu contra a apoptose induzida pelo estresse oxidativo e este efeito permaneceu até 6 h após a indução. O PEBT (5 e 10 µM) inibiu a clivagem da poli(ADP-ribose) polimerase (PARP) induzida por estresse oxidativo e, também, restaurou a fosforilação da quinase regulada por sinal extracelular (ERK). O efeito protetor do PEBT contra o estresse oxidativo parece envolver a clivagem da PARP e a regulação da fosforilaçao de ERK, além de sua atividade antioxidante. Nesse sentido, os resultados apresentados nesta tese destacam o efeito do PEBT na melhora das três fases da memória, bem como em modelos de dano cognitivos em camundongos. Estes resultados parecem estar relacionados ao aumento do tônus glutamatérgico causado pelo PEBT e seu efeito antiapoptótico. Assim sendo, estes dados sugerem que o PEBT poderá, futuramente, ser considerado candidato para a prevenção de danos de memória, como aqueles observados na DA.

Palavras-chave: Telúrio. 2-Feniletinil-butiltelúrio. Sistema glutamatérgico. Memória. Doença de Alzheimer. Peptídeo β-amiloide. Escopolamina. Estresse Oxidativo. Apoptose

ABSTRACT

Thesis of Doctor's Degree
Federal University of Santa Maria, RS, Brazil

PROTECTIVE ROLE OF 2-PHENYLETHINYL-BUTYLTELLURIUM ON MODELS OF COGNITIVE DEFICITS IN MICE AND ON APOPTOSIS IN HUMAN CELLS

AUTHOR: ANA CRISTINA GUERRA DE SOUZA
ADVISOR: CRISTINA WAYNE NOGUEIRA
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Memory is considered to be a process that has several stages, including acquisition, consolidation and retrieval. Memory impairment occurs when important synapses are modified. Alzheimer's disease (DA) is the most common cause of dementia. DA is characterized by cognitive damage, accumulation of the pathogenic amyloid-β (Aβ) peptide, and cholinergic dysfunction. Moreover, oxidative stress is associated with DA. Therapies used for dementia are still palliative rather than curative. Consequently, new therapies are urgently required. Biological effects of tellurium compounds have been studied, leading to a set of interesting and promising applications. Accordingly, 2-phenylethinyl-butyltellurium (PEBT), an organotellurium compound, has been reported as antioxidant. The purpose of this study was to characterize PEBT as a promising alternative for memory improvement and prevention of cognitive deficits, using experimental models of DA in mice. Initially, the present study was conducted to evaluate the effect of a single oral administration (p.o.) of PEBT at a dose of 10 mg/kg on memory, employing the step-down inhibitory avoidance task. PEBT administered 1 h before training, immediately after training or 1 h before the test session of the step-down inhibitory avoidance task increased the step-down latency time in comparison to the control mice, improving acquisition, consolidation, and retrieval of memory, respectively. The glutamate uptake, but not glutamate release, by cerebral cortex and hippocampal slices of mice was inhibited after 1 h of treatment with PEBT. After 24 h of PEBT exposure, the inhibition of cerebral cortex glutamate uptake disappeared. The improvement of memory by PEBT seems most likely to be mediated through an interaction with the amino acid transporters of the glutamatergic system. Thereafter, a subchronic PEBT treatment (1 mg/kg, p.o., for 10 days) after injection of Aβ(25-35) (3 nmol/3 µl/per site, intracerebroventricular) reversed Aβ-induced learning and memory deficits in the Morris water maze and step-down inhibitory avoidance tasks. In addition, PEBT (10 mg/kg, p.o.), administered 30 min before scopolamine (1 mg/kg, intraperitoneal), ameliorated memory deficit induced by this amnesic agent in the Morris water maze. Further, scopolamine was given 30 min before training and test or immediately post-training of step-down inhibitory avoidance task, inducing damage on acquisition, retrieval, and consolidation of memory, respectively. PEBT, administered 30 min before scopolamine, improved consolidation and retrieval stages, but not acquisition. General locomotor and exploratory activities, evaluated in the open-field test, were similar in all mice. Finally, the antiapoptotic effect of PEBT was evaluated. Human retinal pigment epithelial cells (ARPE-19 cell line) were exposed to *in vitro* oxidative stress by 10 ng/ml tumor necrosis factor-α and 600 µM H_2O_2. One hour PEBT incubation at concentrations of 7.5 and 10 µM attenuated the apoptosis induced by oxidative stress. This effect lasted up to 6 hours after oxidative stress induction. PEBT (5 and 10 µM) inhibited oxidative stress-induced poly (ADP-ribose) polymerase (PARP) cleavage and restored extracellular-signal-related kinase (ERK) phosphorylation decreased by oxidative stress. The protective mechanism exerted by PEBT against oxidative stress may involve PARP cleavage, regulation of ERK pathway, as well as its known antioxidant properties. In conclusion, the finds of the present thesis point out the ameliorative effect of PEBT on memory stages (acquisition, consolidation and retrieval). Likewise, PEBT improved memory impairment in mice. These effects seem to be due to strengthen the physiological glutamatergic tonus by PEBT and the antiapoptotic effect of PEBT. Therefore, PEBT could be considered a candidate for the prevention of memory deficits such as those observed in DA.

Keywords: Tellurium. 2-Phenylethinyl-butyltellurium. Glutamatergic system. Memory. Alzheimer's disease. Amyloid-β peptide. Scopolamine. Oxidative stress. Apoptosis

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LISTA DE ABREVIATURAS

Aβ - peptídeo β-amiloide

Aβ1-40 - fragmento β-amiloide contendo 40 aminoácidos

Aβ1-42 - fragmento β-amiloide contendo 42 aminoácidos

Aβ25-35 - fragmento β-amiloide contendo 11 aminoácidos

ALT - alanina aminotransferase

AMPA - α-amino-3-hidróxi-5-metil-4-isoxazol-ácido propiônico

APP - proteína precursora amiloide

ARPE-12 - linhagem de células do epitélio pigmentado da retina

AST - asparato aminotransferase

BDNF - fator neurotrófico derivado do encéfalo

CAMK II - cálcio-calmodulina cinase tipo II

CREB - proteína ligante do elemento responsivo à adenosina 3',5'-monofosfato cíclico

COX-2 - ciclo-oxigenase-2

DA - doença de Alzheimer

EAAT - transportador de aminoácido excitatório

ERK - quinase regulada por sinal extracelular

iGluR - receptores ionotrópicos de glutamate

KA - ácido caínico

IL-1β - interleucina-1β

MAPK - proteínas cinases ativáveis por agentes mitógenos

mGluR - receptores metabotrópicos de glutamato

NMDA - N-metil-D-aspartato

PEBT - 2-feniletinil-butiltelúrio

PKA - proteína cinase dependente de adenosina 3',5'-monofosfato cíclico

PKC - proteína cinase dependente de cálcio

PKG - proteína cinase dependente de monofosfato cíclico de guanosina

PARP - poli(ADP-ribose) polimerase

TNF-α - Fator de necrose tumoral-α

SUMÁRIO

1 INTRODUÇÃO

1.1 Memória e aprendizado

Aprendizagem é o processo pelo qual nós adquirimos conhecimento sobre o mundo, enquanto memória é o processo pelo qual o conhecimento é codificado, retido e, posteriormente, recuperado (Kandel et al., 2003). A memória é um processo essencial para a experiência humana e pode ser caracterizada como a capacidade que um indivíduo tem de armazenar dados ou conhecimentos para, assim, modificar o próprio comportamento. Através da interação com o meio ambiente, adquirimos informações acerca do mundo e aprendemos a associar estímulos externos e respostas internas. A memória é, então, um registro das nossas percepções e um produto dos nossos sentidos (Baddeley, 1999).

As memórias podem ser classificadas de acordo com o tempo de permanência e conteúdo. Quanto ao conteúdo armazenado, as memórias declarativas são aquelas que registram informações, eventos ou conhecimentos adquiridos. A memória declarativa abrange a memória episódica, em que lembramos eventos específicos da memória do passado e a memória semântica que se refere à memória de conhecimentos gerais. Já as memórias procedurais são aquelas que tratam das aptidões tanto sensoriais quanto motoras, sendo ambas adquiridas de forma implícita e inconsciente (Izquierdo, 2002; Roberston et al., 2004).

Quanto ao tempo de permanência, podemos separá-las em memórias de curta duração ou de longa duração. As primeiras permanecem armazenadas por breves períodos, enquanto que as de longa duração podem se estender por dias, meses ou até mesmo anos. Apesar de partilharem diversos mecanismos, as memórias de curta e longa duração também possuem características próprias, as quais as constituem em dois processos relacionados, porém independentes (Izquierdo et al., 1998a, 1998b).

O hipocampo desempenha um papel fundamental na formação de memórias de curto e longo prazo (Izquierdo et al., 1998a). Diferentes áreas corticais aferentes e eferentes interagem com o hipocampo para regular a aquisição e o armazenamento de nova informação (Izquierdo e Medina, 1998). A formação de uma memória de longa duração envolve uma série de processos metabólicos no hipocampo e em outras estruturas cerebrais que compreendem diversas fases.As

memórias não são adquiridas imediatamente na sua forma final. É necessário um tempo no qual a memória vai sendo preparada para se tornar permanente. Durante os primeiros minutos e horas após a aquisição, as memórias estão suscetíveis a interferências, seja por outras memórias ou por drogas (McGaugh, 1966; 2000). A memória é o resultado de pelo menos três tipos de processamento distintos, mas relacionados entre si: aquisição, consolidação e evocação.

A aquisição ou aprendizagem refere-se aos processos pelos quais novas informações aprendidas são tratadas e processadas por sistemas neurais específicos, quando encontradas pela primeira vez. Em seguida, tem-se a consolidação, ou seja, a fixação e o armazenamento de uma informação recém adquirida ou aprendida. Refere-se àqueles processos que alteram a informação recém retida e ainda lábil, de modo a torná-la mais estável para a retenção em longo prazo. A consolidação envolve a expressão de genes e a síntese de novas proteínas, dando origem a alterações estruturais que mantêm a estabilidade da memória ao longo do tempo (Dudai, 2004).

À última etapa dá-se o nome de evocação ou lembrança, que se refere àqueles processos que permitem a lembrança e o uso das informações retidas. Embora algumas evocações de memória ocorram de forma espontânea, como resultados de uma flutuação na atividade neural, a evocação usualmente ocorre como resultado da integração entre a chegada da informação do meio ambiente com a rede de memórias já existentes. A evocação da memória leva à formação de novas memórias, combinadas com as memórias anteriores (Izquierdo e McGauch, 2000).

O aprendizado adquirido a partir de um novo estímulo pode ser alterado das mais variadas formas se uma ou mais das etapas de formação da memória for manipulada. A interferência pode ocorrer antes da exposição a uma nova experiência, ou nos momentos iniciais da aquisição. Para se estudar os efeitos de drogas especificamente sobre a consolidação, prefere-se a utilização após o treino (após a aquisição) (McGaugh, 1966; 2000). Já o processo de evocação pode ser interferido com tratamento aplicado antes do teste (Izquierdo et al., 1997).

A esquiva inibitória é o paradigma que melhor propicia a avaliação dos mecanismos envolvidos nas memórias de curta e longa duração. Por ser adquirido em uma única sessão de treino, o aprendizado da esquiva inibitória de uma única sessão permite isolar cada uma das fases do processamento da memória, aquisição, consolidação e evocação. Nesse sentido, paradigmas de múltiplas sessões apresentam problemas para a interpretação dos resultados, pois cada sessão envolve uma interação complexa entre as distintas etapas em que se subdivide o processamento e o armazenamento de nova informação (Izquierdo e Medina, 1997).

1.2 O sistema glutamatérgico e a formação da memória

O glutamato é o principal neurotransmissor excitatório no SNC e está envolvido em várias funções cerebrais, como aprendizado e memória, desenvolvimento e envelhecimento cerebral, comunicação celular e interação entre estruturas cerebrais (Ozawa et al., 1998; Danbolt, 2001; Segovia et al., 2001; Izquierdo et al., 2006; Tzingounis e Wadiche, 2007). Este aminoácido é sintetizado nos terminais pré-sinápticos, ficando estocado dentro de vesículas e sendo liberado na fenda sináptica após despolarização da célula, promovida por processos dependentes de cálcio (Hassel e Dingledine, 2006).

Após sua liberação sináptica, o glutamato exerce seus efeitos através de receptores específicos que são divididos em ionotrópicos e metabotrópicos. Os receptores ionotrópicos (iGluR) são canais que permitem a passagem de um cátion específico quando ativados por um agonista (Ozawa et al., 1998) e foram subdivididos em N-metil-D-aspartato (NMDA), α-amino-3-hidróxi-5-metil-4-isoxazol-ácido propiônico (AMPA) e ácido caínico (KA), de acordo com a sensibilidade a estes agonistas. Os receptores metabotrópicos (mGluR) pertencem a uma família de receptores que estão acoplados às proteínas ligantes de nucleotídeos da guanina (proteínas G), promovendo a modulação de efetores intracelulares que, por sua vez, ativam e/ou inibem diversos eventos de transdução de sinal (Ozawa et al., 1998; Kew e Kemp, 2005). O término da ação do glutamato se dá pela sua recaptação através de transportadores de glutamato na membrana plasmática. Para isso, existem cinco distintos transportadores de glutamato com alta afinidade e dependentes de sódio: transportador de glutamato-aspartato ou transportador de aminoácido excitatório 1 (EAAT1); transportador de glutamato-1 ou EAAT2; carreador de aminoácido excitatório 1 ou EAAT3; EAAT4; e EAAT5 (Danbolt, 2001; Gether et al., 2006) (Figura 1).

Os mecanismos de formação da memória por consequência de informações adquiridas incluem a formação de novas sinapses e a reformulação ou fortificação das sinapses já existentes, sendo este fenômeno chamado de plasticidade sináptica (Geinisman, 2000). Acredita-se que um aumento na liberação de neurotransmissores, principalmente o glutamato, seja o primeiro passo para a formação da memória (McGaugh, 2000; McGaugh e Izquierdo, 2000).

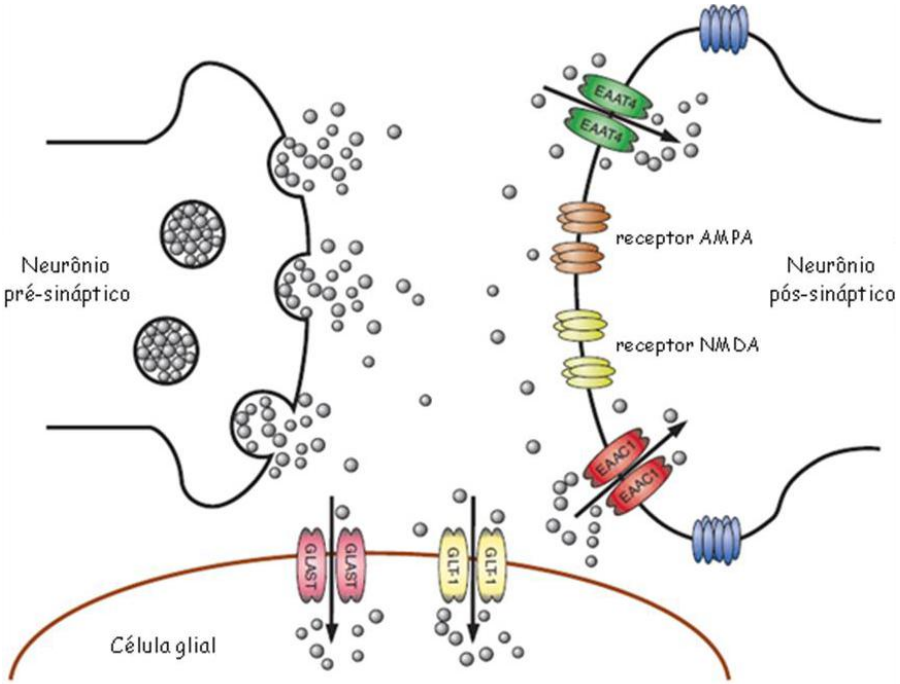

Figura 1: Representação da sinapse glutamatérgica mostrando a liberação de glutamato para a fenda sináptica, os receptores AMPA, NMDA e mGluR e os transportadores capturando o glutamato do meio extracelular (Adaptada de González e Robinson, 2004).

Uma vez liberado, o glutamato une-se ao receptor AMPA no neurônio pós-sináptico permitindo a entrada de íons sódio na célula, despolarizando-a. Como consequência da despolarização, o íon magnésio desobstrui o canal do receptor NMDA, que passa a responder ao glutamato, permitindo a entrada de íons cálcio na célula. Há, também, a ativação de mGluRs induzindo a liberação de íons cálcio das reservas intracelulares (Izquierdo e Medina, 1997; Abel e Lattal, 2001). O influxo de cálcio nas células origina uma cascata de sinalização com a ativação de várias proteínas-cinase, entre elas a proteína cinase dependente de cálcio (PKC) e cálcio-calmodulina cinase tipo II (CAMK II). A atividade dessas enzimas favorece a fosforilação de diversos tipos de receptores ao glutamato, ativando-os por várias horas (Izquierdo e McGaugh, 2000).

Simultaneamente à ativação do receptor NMDA, ocorre a ativação de receptores colinérgicos muscarínicos e de noradrenérgicos β. Também ocorre a ativação de outras proteínas cinases, como a proteína cinase dependente de GMPc (PKG), a proteína cinase dependente de AMPc (PKA) e as proteínas cinases ativáveis por agentes mitógenos (MAPK) que fosforilam uma série de outras proteínas. Ao fim da cascata, ocorre ativação da proteína nuclear CREB que participa de processos que levam à síntese de muitas proteínas (Dudai, 2004).

A PKG está envolvida na produção de mensageiros retrógrados, óxido nítrico, monóxido de carbono e fator de ativação plaquetário. Estas três substâncias aumentam a eficiência da sinapse através de mecanismos que promovem o aumento da liberação de glutamato (Dudai, 2004). Por sua vez, a ativação da PKC fosforila a proteína GAP-43 que está envolvida na mobilização do glutamato das vesículas sinápticas, fundamental para a formação da memória (Izquierdo e Medina,1997; Izquierdo et al., 2006). Dentre as famílias das MAPKs, a quinase regulada por sinal extracelular (ERK) é utilizada em todas as regiões cerebrais em que a plasticidade sináptica ocorre e a sua ativação é requerida para a formação de novas memórias (Cammarota et al., 2000). Outro fator importante para a formação da memória é a síntese do fator neurotrófico derivado do encéfalo (BDNF), uma neurotrofina relacionada com processos de plasticidade sináptica e memória, desenvolvimento cerebral, modulação das sinapses e arborização dendrítica. O BDNF, ao ativar seus receptores, pode ampliar a sinalização glutamatérgica por aumentar a fosforilação dos receptores NMDA e, desta maneira, contribuir para a formação da memória (Suen et al., 1997; Yamada K et al., 2002; Tyler e Pozzo-Miller, 2003).

1.3 Doença de Alzheimer

A memória é afetada quando as sinapses encarregadas de fazer ou evocar memórias encontram-se inibidas ou alteradas. Isso ocorre gradativamente na idade adulta, sendo um processo fisiológico que dificilmente gera um déficit funcional antes dos 80-85 anos de idade. Porém, muitas doenças são acompanhadas de uma aceleração da alteração neuronal fisiológica. Quando esse dano abrange funções superiores, tem-se a demência (Izquierdo, 2002).

O envelhecimento é o principal fator de risco para a demência, estima-se que apenas 6% dos casos de demência acometem pessoas com idade inferior a 65 anos (Kalaria et al., 2008). A demência é uma síndrome neurológica, geralmente crônica, caracterizada principalmente por uma progressiva e global perda da memória e da capacidade intelectual do individuo, de forma a interferir nas suas atividades sociais ou ocupacionais (Janca et al., 2006). A Doença de Alzheimer (DA) é a forma mais comum de demência entre a população idosa, correspondendo a 60-70% de todos os casos (Philipson et al., 2010). No mundo mais de 25 milhões de pessoas apresentaram

quadro clínico de demência, sendo a grande maioria devido à DA. Cerca de 5 milhões de novos casos ocorrem todo ano (Brookmeyer et al., 2007).

Segundo o censo realizado em 2010, a população atual de idosos acima de 60 anos no Brasil é de mais de 20 milhões e, as projeções mostram que em 2050 será de mais de 64 milhões (IBGE, 2011). Embora estes dados demonstrem um avanço na qualidade de vida, eles também alertam para a possibilidade de um significativo aumento no número de pessoas acometidas pela DA. No Brasil, a prevalência de demência na população com mais de 65 anos foi de 7,1%, sendo a DA responsável por 55% dos casos (Herrera et al., 2002). Nos EUA, estimativas de 2011 mostraram que 5,4 milhões de americanos possuem DA e outras demências, sendo que entre estes, 5,2 milhões possuem idade de 65 ou mais (Alzheimer's Association, 2011). Desta forma, se não houver avanços no tratamento, há previsão de aumento do número de casos nos EUA para 13,2 milhões em 2050 (Hebert et al., 2003), sendo estimado um alto custo para o cuidado dos pacientes, visto que em 2011 foram gastos cerca de 183 bilhões de dólares (Alzheimer's Association, 2011).

Além da idade, fatores genéticos e ambientais são apontados como fatores de risco para a DA. Além disso, a deficiência de fatores neurotróficos, defeitos mitocondriais, defeito no metabolismo energético e aumento na formação de espécies reativas são hipóteses que vem sendo estudadas para a DA (Hardy e Selkoe, 2002; Pratico, 2008). Em geral, os pacientes com alguma forma de demência evoluem para a morte em torno de três a dez anos após o diagnóstico (Brodaty et al., 2012). Infelizmente, ainda não há cura conhecida ou medidas preventivas eficazes para a maioria dos tipos de demência.

A DA foi caracterizada pelo neuropatologista alemão Dr. Alois Alzheimer em 1907 como uma doença neurológica progressiva das funções cognitivas, com perda de memória, alucinações, ilusões e comprometimento psicossocial (Alzheimer, 1907). Diferentemente do declínio das habilidades físicas e mentais decorrentes do envelhecimento, na DA o progressivo declínio intelectual é muito mais acentuado (Hardy e Selkoe, 2002; Stix, 2010). A DA é caracterizada por uma piora da memória. O paciente geralmente apresenta um início gradual da doença, e um progressivo e sequencial declínio nas funções cognitivas, comportamentais e motoras. Este declínio interfere com as atividades diárias do indivíduo e, consequentemente, com sua qualidade de vida (Alloul et al., 1998).

Entre as alterações histopatológicas encontradas por Alois Alzheimer destacam-se os emaranhados neurofibrilares e as placas senis (Figura 2). Os emaranhados neurofibrilares são formados pela deposição da proteína Tau que é uma proteína solúvel, altamente expressa no cérebro, que promove a junção e estabilização dos microtúbulos que, por sua vez, são responsáveis por modular a organização funcional do neurônio. Enquanto que as placas senis são formadas pelo acúmulo e agregação de peptídeo beta-amiloide (Aβ) gerado pelo processamento proteolítico da proteína precursora amiloide (APP) (Laferla et al., 2007; Stix, 2010).

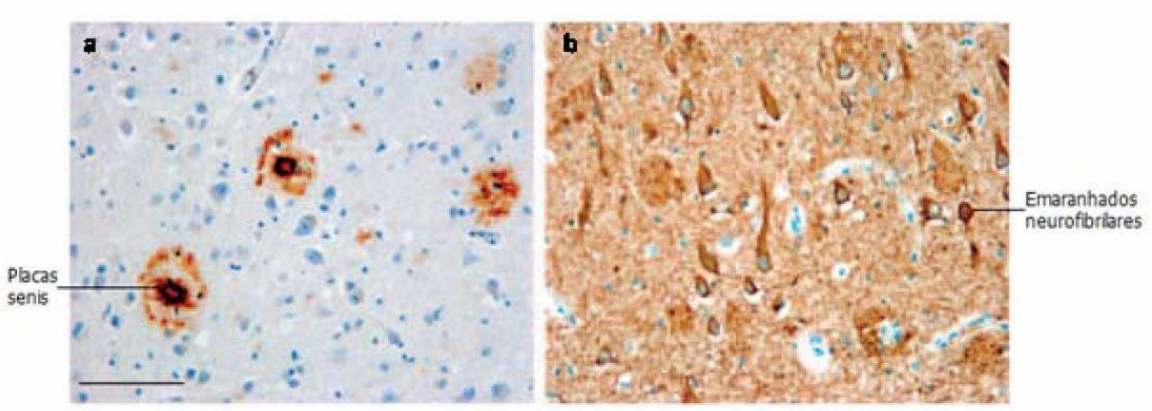

Figura 2: Alterações morfológicas características do tecido cerebral na DA. **a**) Placas senis constituídas por depósitos extracelulares do peptídeo Aβ. **b**) Emaranhados neurofibrilares intracelulares constituídos pela proteína tau hiperfosforilada. Escala 100 µm (Adaptada de Haass e Selkoe, 2007).

APP é uma glicoproteína transmembrana amplamente expressa na superfície celular de neurônios e glia. A APP pode ser processada por duas vias distintas: a via amiloidogênica e a via não-amiloidogênica (Figura 3) (Querfurth e Laferla, 2010). A via não-amiloidogênica, iniciada pela α-secretase, é a via predominante e nela não há produção do peptídeo Aβ. A α-secretase cliva a APP no domínio Aβ, liberando ao meio extracelular o fragmento solúvel sAPPα. O fragmento remanescente na membrana composto por 83 aminoácidos é clivado pela γ-secretase, liberando um pequeno fragmento hidrofóbico (p3) com funções ainda desconhecidas (Laferla et al., 2007). Por outro lado, na via amiloidogênica, um processo proteolítico anormal, a β-secretase cliva APP imediatamente antes do domínio Aβ, liberando o fragmento solúvel sAPPβ ao meio extracelular. O fragmento C-terminal amiloidogênico (C99) aderido à membrana é clivado pela γ-secretase, liberando ao meio extracelular o peptídeo Aβ composto de 38 a 43 aminoácidos (Haass e Selkoe, 2007; Querfurth e Laferla, 2010). O peptídeo contendo 40 aminoácidos (Aβ1-40) é a

forma predominantemente produzida e está fracamente associado à formação das placas senis. Já o peptídeo contendo 42 aminoácidos (Aβ1-42) é a forma mais responsável pela geração de placas senis, por ser mais hidrofóbico e mais propenso a formar fibrilas (Laferla et al., 2007).

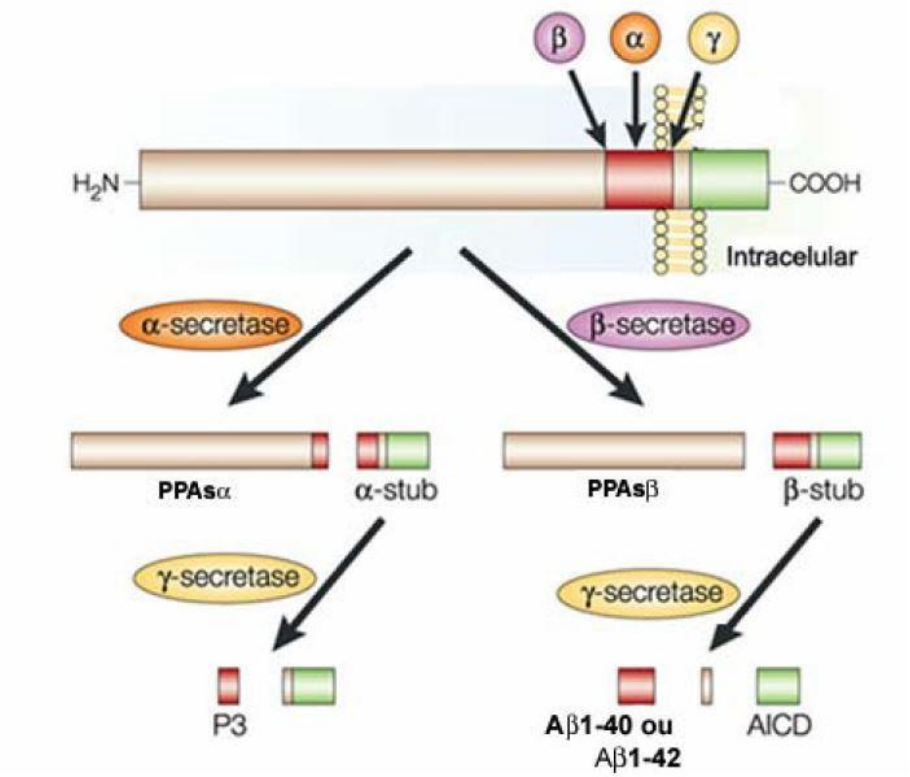

Figura 3: Processamento proteolítico da APP. O processamento não-amiloidogênico (via α-secretase) ocorre no domínio Aβ. O processamento amiloidogênico (via β-secretase e γ-secretase) gera o peptídeo Aβ (Adaptado de Laferla et al., 2007).

A cascata amiloide descrita, não só conduz à disfunção neuronal, como também pode estar envolvida no mecanismo que induz a formação dos emaranhados neurofibrilares que são inclusões filamentosas compostas pela Tau hiperfosforilada. Na DA, a Tau hiperfosforilada torna-se insolúvel, desprende-se dos microtúbulos e acumula-se intracelularmente na forma filamentosa (Querfurth e Laferla, 2010). Microtúbulos desestabilizados, devido à hiperfosforilaçao da Tau, levam à desestruturação do citoesqueleto, alterando o transporte neuronal e contribuindo para disfunção sináptica e neurodegeneração (Hernandez e Avila, 2007). Além disso, o aumento nos níveis de emaranhados neurofibrilares pode intensificar a resposta imunológica e conduzir ao estresse oxidativo (Lee et al., 2010).

Embora mecanismos diferentes pareçam estar envolvidos na patogênese da DA, muitas evidências demonstram que a neuroinflamação contribui para a sua progressão (Khandelwal et

al., 2011). Na DA, tem sido proposto que uma fagocitose ineficiente de Aβ pela microglia, a consequente ativação microglial e liberação de mediadores inflamatórios e fatores neurotóxicos contribuem para a progressão da DA (Akiyama et al., 2000). De acordo com isso, já foi monstrado um aumento nos níveis de diversos mediadores pró-inflamatórios no cérebro de pacientes com DA (Galimberti et al., 2003).

Muitas evidências correlacionam o estresse oxidativo e a disfunção mitocondrial como um fator central na etiologia da DA (Coskun et al., 2012). A toxicidade mitocondrial induzida pelo Aβ não está completamente elucidada, mas vários mecanismos estão envolvidos, tais como o aumento da permeabilidade das membranas mitocondriais, a perturbação da homeostase do cálcio e a alteração da fosforilação oxidativa com uma consequente produção excessiva de espécies reativas (Cummings, 2004; Coskun et al., 2012; Verri et al., 2012). O peptídeo Aβ pode causar estresse oxidativo, gerando espécies reativas e produtos da peroxidação lipídica que são capazes de danificar a mitocôndria, levando à morte celular por apoptose (Chauhan e Chauhan, 2006; Petrozzi et al., 2007). De acordo com isso, em cérebro de pacientes com DA, foi encontrada morte celular por apoptose (Stadelmann et al., 1999). As características da doença, bem como sua progressão podem ser explicadas pela ativação da cascata apoptótica induzida por Aβ (Behl, 2000). Nesse sentido, modelos para o estudo de apoptose são empregados utilizando uma linhagem de células do epitélio pigmentado da retina humana (linhagem ARPE-19). O estresse oxidativo pode induzir a morte celular por apoptose nas células ARPE-19, sendo uma ferramenta importante para a investigação de drogas com potencial farmacológico (Sparrow e Cai, 2001; Mukherjee et al., 2004).

O entendimento do mecanismo neurotóxico induzido pelo peptídeo Aβ, responsável pela neurodegeneração na DA é essencial para o desenvolvimento de agentes terapêuticos capazes de evitar, retardar e até retroceder a degeneração. Modelos *in vitro* e *in vivo* utilizando peptídeos Aβ sintéticos tem sido bastante empregados como uma alternativa eficaz de menor custo aos modelos de animais transgênicos. Dentre estes, os modelos animais de injeção intracerebral de fragmentos do peptídeo Aβ têm contribuído consideravelmente para avanços na compreensão dos mecanismos envolvidos na neurotoxicidade do Aβ e possuem um papel importante na avaliação de potenciais agentes terapêuticos (Van Dam e De Deyn, 2006). Entre esses fragmentos, o peptídeo sintético de 11 aminoácidos (Aβ25-35) representa a região biologicamente ativa do peptídeo Aβ1-42 (Pike et al., 1995) (Figura 4), exercendo toxicidade similar ao peptídeo

originado a partir do processamento anormal da APP, tais como danos no aprendizado e memória, apoptose neuronal, disfunção colinérgica e estresse oxidativo (Stepanichev et al., 2006; Tohda et al., 2003).

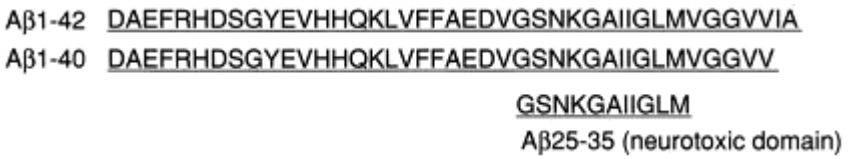

Figura 4: Peptídeo sintético de 11 aminoácidos Aβ25-35 representa a região biologicamente ativa do peptídeo Aβ1-42 (Adaptado de Yamada e Nabeshima, 2000).

1.4 Sistema colinérgico

O sistema colinérgico desempenha um papel essencial nos processos de aprendizado e memória (Winkler et al., 1995). Durante o envelhecimento, os neurônios colinérgicos sofrem uma moderada degeneração, resultando em uma hipofunção colinérgica, e esta tem sido relacionada com os declínios cognitivos progressivos no envelhecimento (Schliebs e Arendt, 2011). Além disso, a degeneração do sistema colinérgico é a mais dramática entre os sistemas de neurotransmissão afetados na DA (Racchi et al., 2004; Cummings et al., 2004).

A síntese do neurotransmissor acetilcolina ocorre nos terminais nervosos a partir de dois precursores, colina e acetil-coenzima A, pela ação da enzima colina acetiltransferase. Após sua formação, a acetilcolina é liberada na fenda sináptica, onde poderá interagir com dois tipos de receptores, muscarínicos e nicotínicos. Na fenda sináptica, a enzima acetilcolinesterase hidrolisa a acetilcolina em colina e acetato. A colina liberada é então reciclada após captação no neurônio colinérgico (Schliebs e Arendt, 2006).

O sistema colinérgico tem importante papel nos processos de formação da memória e há evidências, tanto em animais como em humanos, de que o aprendizado e a memória podem ser modificados por drogas que afetam a função colinérgica central (Yamazaki et al., 2005). A escopolamina, um alcaloide derivado da *Atropa belladonna* (Zhang et al., 2008), age como um antagonista colinérgico, não-seletivo, dos receptores muscarínicos. O bloqueio de receptores colinérgicos muscarínicos interfere no armazenamento de novas informações, sendo que o mecanismo envolvido neste processo se pronuncia pela supressão da atividade neural excitatória (Atri et al., 2004). O efeito amnésico característico da administração de escopolamina a torna

uma ferramenta farmacológica interessante para estudos de novas drogas associadas a patologias do SNC, principalmente aquelas relacionadas ao sistema colinérgico, uma vez que contempla o déficit cognitivo e a perda da memória característicos de demência, bem como mimetiza sintomas da DA (Deiana et al., 2009).

1.5 Telúrio

O elemento telúrio é um semimetal pertencente ao grupo 16 da tabela periódica, denominada família dos calcogênios. O telúrio foi descoberto em 1782 pelo químico Frans-Joseph Mueller Von Reichestein, que estava estudando um minério contendo ouro, e isolado, pela primeira vez, em 1798 por Klaproth (Cooper, 1971). Este elemento pode apresentar-se com diferentes estados de oxidação: Te^{+6} (telurato), Te^{+4} (telurito), Te^{0} (telúrio elementar) e Te^{+2} (telureto) (Scansetti, 1992). É encontrado com maior frequência na forma de teluretos de ouro, bismuto, chumbo e prata. No Brasil, a química de telúrio foi introduzida pelo Prof. Rheinboldt, o qual se dedicou ao estudo sistemático de compostos orgânicos contento telúrio e sua aplicabilidade como intermediários em síntese orgânica (Petragnani, 1995; Comasseto et al., 1997; Zeni et al., 2006).

O Te^{0} é utilizado como componente de ligas metálicas, sendo adicionado ao chumbo para aumentar a sua resistência mecânica, durabilidade e diminuir a ação corrosiva do ácido sulfúrico nos processos industriais (Petragnani, 1995; Taylor, 1996). Ele também é utilizado na produção industrial de vidro e aço, e como um aditivo antidetonante na gasolina (Fairhill, 1969). Além disso, é empregado no processo de síntese de explosivos, na vulcanização da borracha, em lubrificantes sólidos e na petroquímica (Taylor, 1996). Ademais, o telúrio inorgânico pode ser encontrado em soluções oxidantes para polir metais (Yarema e Curry, 2005).

Esse elemento vem sendo muito empregado na manufatura de semicondutores particulados, sistemas de energia fotovoltaica e outros componentes eletrônicos, como microchips e DVD regraváveis (Yamada N et al., 2002; Zhang e Swihart, 2007; Wang et al., 2008). Além disso, estudos tem recentemente demonstrado o uso de nanopartículas de telureto de cádmio fluorescente como biomarcadores para análise de imagem (Green et al., 2007; Ba et al., 2010).

O telúrio está presente na composição de organismos vegetais, particularmente em membros da Família *Alliaceae*, como o alho (Larner, 1995). Pequenas quantidades de telúrio foram identificadas em fluidos corporais de camundongos, tais como sangue e urina (Siddik e Newman, 1988; Newman et al., 1989). Além disso, em 1967, Schoroeder e colaboradores determinaram, utilizando espectrometria de absorção atômica, que os organismos humanos possuiriam aproximadamente 600 mg de telúrio, uma quantidade relativamente grande em comparação com outros elementos-traço como o ferro e o zinco. Apesar desta grande quantidade, nenhuma função fisiológica foi ainda atribuída ao telúrio (Rezanka e Sigler, 2008).

O primeiro composto orgânico de telúrio foi sintetizado por Friedrich Wöhler em 1840 (Wöhler, 1840). Apenas a partir de 1970, os compostos orgânicos de telúrio começaram a ser explorados, refletindo no crescimento exponencial de artigos publicados desde então (Klaman, 1990). Vários destes compostos, com diferentes características e estruturas químicas, vem sendo estudados quanto às suas propriedades fármaco-toxicológicas.

1.5.1 Toxicologia dos compostos orgânicos de telúrio

O aumento do uso industrial de produtos químicos provoca riscos ocupacionais e ambientais para a saúde humana, e cresce a preocupação em relação aos potenciais efeitos adversos desses compostos. O telúrio pode ser prontamente absorvido pelo organismo, através da dieta, principalmente na forma de compostos orgânicos, mas também ocorre a absorção de telúrio inorgânico na forma de teluritos e teluratos (Larner, 1995; Ogra et al., 2008). Casos de intoxicação ocupacional aguda por telúrio são raros, entretanto, quando ocorrem, os sintomas são: dores de cabeça, sonolência, náuseas, alteração da frequência cardíaca, bem como odor característico de alho na respiração e na urina (Müller et al., 1989; Taylor, 1996).

Foi demonstrado que compostos orgânicos de telúrio, como o ditelureto de difenila, podem causar toxicidade renal e hepática em roedores (Meotti et al., 2003), além de toxicidade pulmonar (Pinton et al., 2011). Adicionalmente, alterações hematológicas foram causadas por esses compostos (Borges et al., 2007; Schiar et al., 2009). Também foi observada teratogênese em fetos de ratos, com altas taxas de mortalidade pré- e pós-natal (Stangherlin et al., 2005). Além disso, a prole exposta ao telúrio orgânico através do leite materno apresenta mudanças neurocomportamentais (Stangherlin et al., 2006).

A neurotoxicidade dos compostos orgânicos de telúrio está bem descrita na literatura. Estudos tem demonstrado que estes compostos podem modificar a funcionalidade do sistema glutamatérgico em ratos adultos e em desenvolvimento (Nogueira et al., 2002; Souza et al., 2010). Também foi descrita alterações nas proteínas do citoesqueleto, danificando a estrutura celular (Funchal et al., 2006). Enzimas como esqualeno monooxigenase, importante para a formação de mielina, NA^+/K^+ATPase, importante para atividade neuronal e creatina quinase, enzima chave no metabolismo energético, tiveram suas atividades inibidas por compostos orgânicos de telúrio (Wagner-Recio et al., 1994; Laden e Porter, 2001; Borges et al., 2005; Funchal et al., 2011). São relatadas, ainda, prejuízo na memória de ratos jovens em modelo de reconhecimento de objeto novo (Stangherlin et al., 2009).

A toxicidade dos compostos orgânicos de telúrio deve-se principalmente pela oxidação de grupos tióis de moléculas biologicamente ativas, inativando enzimas ou diminuindo a concentração de glutationa (Blais et al., 1972; Deuticke et al., 1992). A oxidação da glutationa pode ser um dos principais fatores da toxicidade causada pelos compostos orgânicos de telúrio (Barbosa et al., 1998; Nogueira et al., 2003). Recentemente foi demonstrada a inibição dos complexos I e II da cadeia respiratória via oxidação de grupos tióis (Puntel et al., 2012), podendo contribuir para os efeitos pró-oxidantes destes compostos, bem como seus efeitos neurotóxicos.

A intensidade da toxicidade depende da estrutura do composto de telúrio, da dose administrada e do tipo de animal testado (Nogueira et al., 2004). Embora compostos orgânicos de telúrio mostraram-se bastante tóxicos, como previamente descrito, outros compostos contendo telúrio foram avaliados em relação à sua toxicidade sistêmica em animais, sendo identificadas as faixas de dose segura para testes farmacológicos (Nyska et al., 1989; Borges et al., 2008; Ávila et al., 2006; 2008). Considerando isto e as possíveis propriedades farmacológicas terapeuticamente relevantes relatadas na literatura, a avaliação de novos compostos orgânicos de telúrio torna-se importante (Cunha et al., 2009).

1.5.2 Farmacologia dos compostos orgânicos de telúrio

Em 1987, Sredni e colaboradores descreveram pela primeira vez uma atividade farmacológica para um composto orgânico de telúrio, ao demonstrarem as propriedades imunomoduladoras do composto codificado como AS101 (telurato de tricloro amônio-

dioxoetileno-O,O') (Figura 5) em camundongos, mediando efeitos antitumorais (Hayun et al., 2006). Estudos com leucócitos mononucleares isolados de pacientes com lúpus eritematoso sistêmico tiveram resultados interessantes para o uso desse composto no tratamento de patologias auto-imunes (Alcocer-Varela et al., 1989).

A propriedade quimioprotetora dos compostos orgânicos de telúrio, proveniente de seus efeitos citotóxicos, começou a ser explorada por diversos grupos de pesquisa. A indução de morte celular por apoptose em células tumorais (Sailer et al., 2003; 2004; Hayun et al., 2006), bem como a inibição da atividade cisteíno-proteásica da catepsina B (Cunha et al., 2005) os torna candidatos para drogas com atividade antimetastática. Utilizando células de leucemia promielocítica humana, Abondanza e colaboradores (2008) demonstraram que o composto orgânico de telúrio (IV) (RT-04) (Figura 5), que também inibe a ação da catepsina B, induz apoptose por aumentar atividade de caspases e reduzir a expressão de Bcl-2. Diferentemente, um organotelureto apresentou efeito antitumoral *in vitro* e *in vivo* em modelo de câncer de cólon por via independente de caspases. A morte por necrose das células tumorais foi observada por efeitos pró-oxidantes do composto (Coriat et al., 2011).

No contexto da terapia antitumoral, Engman e colaboradores (2003) sintetizaram uma série de compostos orgânicos de telúrio e observaram que alguns eram inibidores da tiorredoxina redutase (TRXr), a qual tem sua expressão gênica aumentada nas células tumorais (Grogan et al., 2000). Dessa forma, estes compostos inibem o crescimento de células cancerosas.

Estudos clínicos de fase II vem sendo realizados com o composto AS101 em pacientes com câncer (Sredni et al., 1995; 1996). Além disso, o AS101 foi eficaz em proteger células tronco da medula óssea dos efeitos tóxicos da quimioterapia (Guest e Uetrecht, 2001). Este composto afeta a produção de interleucinas, reduzindo os níveis plasmáticos das mesmas em um modelo de sepse induzida por lipopolissacarídeo em camundongos (Brodsky et al., 2007). Também, o pré- e pós-tratamento com AS101 em um modelo de isquemia em camundongos foi capaz de reduzir o edema e a região infartada, melhorando as funções neurológicas dos animais. Ademais, o AS101 foi capaz de proteger células neuronais do estresse oxidativo e do processo apoptótico e reduzir os níveis de cálcio intracelular aumentados por glutamato, caracterizando uma função neuroprotetora deste composto (Okun et al., 2007).

Teluretos vinílicos mostraram um efeito do tipo antidepressivo no teste de suspensão da cauda realizado em camundongos, sem alterar a locomoção destes animais (Okoronkwo et al.,

2009). AS101 e outro composto orgânico de telúrio, RF-07 (organotelurano) (Figura 5), possuem atividade antiepileptogênica, em parte pela capacidade de inibição da catálise mediada por caspases (Persike et al., 2008). O AS101 também protege os neurônios dopaminérgicos e melhora a função motora em um modelo animal de doença de Parkinson (Sredni et al., 2007). Outra atividade farmacológica do AS101 é a indução de crescimento de cabelo em camundongos e em adolescentes com alopecia (Sredni et al., 2004).

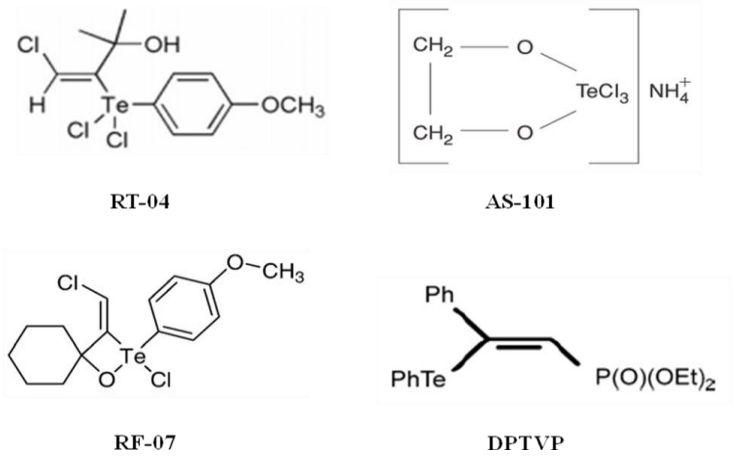

Figura 5: Estrutura química dos compostos RT-04, AS-101, RF-07 e DPTVP.

A atividade anti-helmíntica de compostos orgânicos contendo telúrio foi demonstrada em camundongos infectados com *Trichinella spiralis* (Ordyntseva et al., 1988). Soni e colaboradores (2005) mostraram a atividade antibacteriana de uma série de compostos orgânicos de telúrio. Estes compostos foram eficazes contra bactérias gram-positivas (*Baclilus subtilis*, *Staphylococcus aureus*) e gram-negativas (*Escherichia coli*, *Pseudomonas aeruginosa* e *Salmonella sp.*). Recentemente, o composto RF-07 foi capaz de destruir o parasita *Leishmania (Leishmania) chagasi in vitro* e *in vivo* em concentrações não tóxicas, podendo ser uma alternativa para o tratamento da leishmaniose visceral (Pimentel et al., 2012).

Além destas importantes propriedades, a atividade antioxidante de muitos compostos orgânicos de telúrio está bem descrita (Nogueira et al., 2004; Cunha et al., 2009). Estudos mostraram que DPTVP (diethyl 2-phenyl-2 tellurophenyl vinylphosphonate) (Figura 5), um telureto vinílico, possui efeito antioxidante *in vitro* e *in vivo* sem alterar o sistema glutamatérgico ou apresentar efeitos tóxicos significativos quando administrado subagudamente em camundongos (Ávila et al., 2006; 2007; 2008). Outros teluretos vinílicos também apresentaram

resultados bastante promissores, uma vez que possuem atividade antioxidante e baixa toxicidade (Savegnago et al., 2006; Borges et al., 2008). Importante atividade neuroprotetora foi atribuída ao DPTVP em um modelo de neurotoxicidade induzida por manganês, o que pode ser explicada pela atividade antioxidante deste composto (Ávila et al., 2010). Recentemente, um modelo experimental utilizando o nematoide *Caenorhabditis elegans* demonstrou o DPTVP como um agente antienvelhecimento, uma vez que DPTVP aumentou a sobrevivência e o tempo de vida dos vermes expostos ao manganês (Ávila et al., 2012).

Tem sido demonstrado que alguns compostos orgânicos de telúrio possuem capacidade de mimetizar a atividade da enzima glutationa peroxidase (Engman et al., 1992; Andersson et al., 1993; Kanda et al., 1999; Ren et al., 2001; You et al., 2003; Braga et al., 2009). Esses compostos orgânicos de telúrio têm sido sugeridos como catalisadores em potencial na redução de peróxidos na presença de tióis (Wirth, 1998; Mishra et al., 2006). Dessa forma, houve um considerável interesse no potencial antioxidante de compostos contra muitos agentes pró-oxidantes (Briviba et al.,1998; Jacob et al., 2000; Gay et al., 2010; Borges et al, 2008; Acker et al., 2009). Como por exemplo, um composto orgânico de telúrio foi capaz de prevenir os efeitos do peróxido de hidrogênio e do radical hidroxila *in vitro*, além de proteger dos efeitos lesivos de espécies reativas de oxigênio e de nitrogênio em hemácias, sinaptossomas de rato e em cultura de células hipocampais, o que permite uma possível aplicação dessa molécula no tratamento das doenças neurodegenerativas cuja patogênese envolva o estresse oxidativo (Kanski et al., 2001).

Nesse sentido, foi demonstrado que teluroacetilenos, uma classe de compostos orgânicos de telúrio, possuem atividade antioxidante *in vitro* avaliada em cérebro de ratos. Estes compostos reduzem a peroxidação lipídica e a oxidação de proteínas induzidas por estresse oxidativo. Estes compostos inibem a atividade da enzima sulfidrílica delta-aminolevulinato desidratase *in vitro*, um parâmetro de toxicidade, entretanto esta inibição ocorre em concentrações maiores àquelas necessárias para que apresentem atividade antioxidante (Souza et al., 2009). Além do mais, o composto 2-feniletinil-butiltelúrio (PEBT) (Figura 6), um composto teluroacetileno, protegeu contra o dano oxidativo causado por nitroprussiato de sódio em cérebro de camundongos, sugerindo um efeito antioxidante *in vivo* deste composto. Embora este composto não apresente atividade mimética da glutationa peroxidase, o PEBT foi capaz de aumentar *per se* a atividade desta enzima em cérebro de camundongos. O aumento na atividade desta enzima antioxidante

somada ao efeito scavenger de radicais exercido pelo PEBT pode explicar o efeito antioxidante deste composto *in vivo* (Souza et al., 2009).

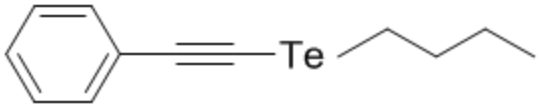

Figura 6: Estrutura química do 2-feniletinil-butiltelúrio (PEBT).

Dada às limitadas medidas terapêuticas efetivas para o dano de memória apresentado pelos pacientes, torna-se importante a busca de novos compostos (Brunbech e Sabers, 2002). Uma das principais tendências das investigações é a pesquisa de novas drogas com propriedades neuroprotetoras (Elinos-Calderón et al., 2009; Wildburger et al., 2009).

2 OBJETIVOS

2.1 Objetivo geral

Tendo em vista as atividades farmacológicas dos compostos orgânicos de telúrio e a busca de um novo agente promissor a ser empregado no tratamento de danos cognitivos, o objetivo desta tese foi avaliar o efeito do PEBT sobre a memória em camundongos e em modelos de dano cognitivo nestes animais, bem como o efeito deste composto na apoptose em células ARPE-19.

2.2 Objetivos específicos

a) Avaliar o efeito do PEBT na aquisição, consolidação e evocação da memória na tarefa da esquiva inibitória em camundongos;
b) Determinar o efeito do PEBT sobre o sistema glutamatérgico através da captação e liberação de glutamato em córtex cerebral e hipocampo de camundongos;
c) Investigar o efeito do PEBT no modelo de dano cognitivo induzido por peptídeo β-amilóide(25-35) em camundongos através das tarefas do labirinto aquático de Morris e esquiva inibitória;
d) Verificar o efeito do PEBT frente ao prejuízo na aquisição, consolidação e evocação da memória causado por escopolamina utilizando as tarefas do labirinto aquático de Morris e esquiva inibitória em camundongos;
e) Investigar o efeito antiapoptótico do PEBT em cultura de células ARPE-19;
f) Determinar possíveis mecanismos envolvidos na ação antiapoptótica do PEBT em cultura de células ARPE-19.

3 RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de dois artigos e dois manuscritos científicos. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências dos artigos encontram-se estruturados de acordo com as normas das respectivas revistas nas quais foram publicados ou submetidos para publicação.

3.1 Artigo I

2-Phenylethynyl-butyltellurium improves memory in mice

Ana Cristina Guerra Souza, Carmine Inês Acker, Bibiana Mozzaquatro Gai, José Sebastião dos Santos Neto, Cristina Wayne Nogueira

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2-Phenylethynyl-butyltellurium improves memory in mice

Ana Cristina Guerra Souza, Carmine Inês Acker, Bibiana Mozzaquatro Gai, José Sebastião dos Santos Neto, Cristina Wayne Nogueira *

Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, SM, RS, CEP 97105-900 Santa Maria, Brazil

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ABSTRACT

The present study was conducted to evaluate the effect of 2-phenylethynyl-butyltellurium (PEBT), an organotellurium compound, at doses of 5 and 10 mg/kg on memory, employing the step-down inhibitory avoidance task in mice. Moreover, the involvement of glutamate uptake and release in cerebral cortex and hippocampus of mice was investigated. A single oral administration (p.o.) of PEBT at the dose of 10 mg/kg 1 h before training (acquisition), immediately after training (consolidation) or 1 h before the test session (retrieval) of the step-down inhibitory avoidance task increased the step-through latency time in comparison to the control mice. In the open-field test, no significant differences in the number of crossings and rearings were observed among groups. The [^{3}H]glutamate uptake by cerebral cortex and hippocampal slices of mice was significantly inhibited after 1 h of treatment with PEBT. After 24 h of PEBT exposure, only the hippocampal [^{3}H]glutamate uptake was inhibited. The [^{3}H]glutamate release by cerebral cortex and hippocampal synaptosomes of mice was not altered. These results suggest that PEBT improved memory stages (acquisition, consolidation and retrieval) in the step-down inhibitory avoidance task in mice. The improvement of memory by PEBT seems most likely to be mediated through an interaction with the amino acid transporters of the glutamatergic system.

1. Introduction

The biological effects of inorganic and organic tellurium compounds have been studied, leading to a set of interesting and promising applications (Cunha et al., 2009). For instance, pre-administration of an organotellurane avoided the establishment of the *status epilepticus* in rats (Persike et al., 2008). Besides, tellurides are promising antitumoral drugs and their chemoprotective effects can be related to their cytotoxic properties and to their ability to inhibit important enzymes necessary for the tumor growth (Engman et al., 2000; Cunha et al., 2005). Additionally, Ávila et al. (2010) demonstrated the neuroprotective activity of a vinylic telluride compound against Mn-induced neurotoxicity.

Organotellurium compounds have been also reported as antioxidants in several models of oxidative stress (Briviba et al., 1998; Jacob et al., 2000), especially in brain (Ávila et al., 2008). Recently, our research group showed the antioxidant effect of telluroacetylenes on rat brain homogenate *in vitro* (Souza et al., 2009). Moreover, 2-phenyletinil-butyltellurium (PEBT) (Fig. 1), a telluroacetylene compound, protected against oxidative damage caused by sodium nitroprusside in mouse brain, suggesting an antioxidant effect *in vivo* of this compound (Souza et al., 2009).

Glutamate has a pivotal role in neuroplasticity, learning and memory processes (Flood et al., 1990; Izquierdo and Medina, 1997; Castellano et al., 2001; Whitlock et al., 2006). The central nervous system strictly regulates the fine balance between glutamate release and uptake. When glutamate is released in the synaptic cleft, it is uptaked by specific high affinity Na^+-dependent amino acid transporters, which are mainly present in glial cells, and metabolized by the glutamine pathway, transported as glutamine to the neurons and stored as glutamate now in the vesicles of pre-synaptic neuron to be released again (Fykse and Fonnum, 1996; Danbolt, 2001; Sheldon and Robinson, 2007). In that way, facilitated glutamate transmission leads to consequent increase in learning (Lhullier et al., 2004; Mameli et al., 2005).

In view of the pharmacological properties of organotellurium compounds, the present study evaluated the effect of PEBT on the three stages of memory, acquisition, consolidation and retrieval, employing the step-down inhibitory avoidance task in mice. Moreover, the involvement of glutamate uptake and release in the improvement of memory caused by PEBT were investigated.

2. Materials and methods

2.1. Chemicals

PEBT was prepared according to the literature method (Comasseto et al., 1996). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed

* Corresponding author. Tel.: +55 55 3220 8140; fax: +55 55 3220 8978.
E-mail address: criswn@quimica.ufsm.br (C.W. Nogueira).

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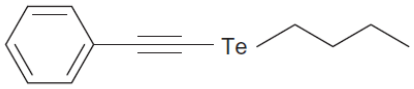

Fig. 1. Chemical structure of 2-phenylethynyl-butyltellurium (PEBT).

that PEBT synthesized exhibited analytical and spectroscopic data in full agreement with its assigned structure. PEBT was diluted in canola oil. L-[^{3}H]glutamate (specific activity 30 Ci/mmol) was purchased from Amersham International, UK. All other chemicals were obtained of the analytical grade and from standard commercial suppliers.

2.2. Animals

The experiments were conducted using male adult Swiss mice (25–35 g) from our own breeding colony. Animals were maintained at 22 ± 2 °C with free access to water and food (Guabi, RS, Brazil), under a 12:12 h light/dark cycle (with lights on at 7:00 a.m.). Mice were acclimatized to the laboratory for at least 1 h before testing. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

2.3. Behavioral tasks

2.3.1. Step-down inhibitory avoidance

Non-spatial long-term memory was investigated using a step-down inhibitory avoidance task according to the method of Sakaguchi et al. (2006), with some modifications. Each mouse was placed on the platform, and the latency to step-down (four paws on the grid) was automatically recorded in training and test sessions. In the training session, upon stepping down, the mouse received a 0.5 mA scrambled foot shock for 2 s. Test sessions were performed 24 h later, with the same procedure except that no shock was administered after stepping down; an upper cutoff time of 300 s was set. Six to eight animals were used per group. PEBT at the doses of 5 or 10 mg/kg orally (p.o.) (Souza et al., 2009), or vehicle (canola oil 10 ml/kg, p.o.) were given 1 h before training (acquisition), immediately post-training (consolidation), or 1 h before test (retrieval). The oral route dominates contemporary drug therapy and is considered to be safe, efficient and easily accessible with minimal discomfort compared to other routes of administration (Lennernäs, 2007).

2.3.2. Open-field

Spontaneous locomotor activity was measured in the open-field test (Walsh and Cummins, 1976). The open-field was made of plywood and surrounded by walls 30 cm in height. The floor of the open-field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3). Each animal was placed individually at the center of the apparatus and observed for 4 min to record the locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of time rearing on the hind limbs). Six to eight animals were used per group. The locomotor and exploratory activities were evaluated after the test session of the step-down inhibitory avoidance task.

2.4. Ex vivo assay

In order to investigate the possible mechanisms involved in the effect of PEBT on memory, glutamate uptake and release assays were carried out 1 h (training) or 24 h (test of memory) after oral administration of PEBT (10 mg/kg).

2.4.1. [^{3}H] Glutamate uptake

Glutamate uptake was performed according to Thomazi et al. (2004). One and 24 h after oral administration of PEBT, mice were killed by cervical dislocation and the brains were immediately removed. Slices (0.4 mm) were obtained by transversally cuts of cortex and hippocampus using a McIlwain chopper. Experiments were made in triplicates. Slices were pre-incubated for 15 min at 37 °C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 1.11 glucose, adjusted to pH 7.2. Then, 0.66 and 0.33 μCi ml^{-1} L-[^{3}H]glutamate for hippocampus and cortex, respectively, in a 100 μM final concentration of glutamate were added. Incubation was stopped after 5 or 7 min for hippocampus and cortex, respectively, with three ice-cold washes of 1 ml HBSS, immediately followed by the addition of 0.5 N NaOH, which was then kept overnight. An aliquot of 10 μl was removed to protein determination. Unspecific uptake was measured using the same protocol described above, with differences in the temperature (4 °C) 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 pmol [^{3}H]glutamate uptake/mg protein min^{-1}.

2.4.2. Synaptosomal preparation

Synaptosomal preparations were obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C, as previously described (Dunkley et al., 1986) with few modifications. Briefly, homogenates (10%, w/v) from cortex and hippocampus were made in 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 6.25 mM dithiotreitol (DDT) (pH 7.4), and centrifuged at 800*g* for 10 min. The supernatants containing synaptosomes were subjected to 23%, 15%, 7% and 3% Percoll solution density gradient centrifugation at 24,000*g* for 10 min. The synaptosomal fractions were isolated, suspended and homogenized in buffered HBSS containing low K^+ (pH 7.4), containing in mM: 133 NaCl, 2.4 KCl, 1.2 KH_2PO_4, 1.09 $MgSO_4$, 27.7 HEPES, 1.2 glucose and 0.001 $CaCl_2$ and centrifuged at 21,000*g* for 15 min. The supernatant was removed and the pellet gently resuspended in HBSS buffer.

2.4.3. Synaptosomal [^{3}H] glutamate release

Determination of [^{3}H]glutamate release was accomplished as described by Migues et al. (1999). Prior to the release assay, synaptosomal preparations from cortex and hippocampus of mice were loaded with labeled [^{3}H]glutamate for 15 min at 37 °C. Incubation was performed in a non-depolarizing medium (low potassium), containing, in mM: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 12, $CaCl_2$ 1.0 in the presence of 0.5 μM of glutamate (0.1 μCi [^{3}H]glutamate). Aliquots of labeled synaptosomal preparations were centrifuged at 16,000*g* for 1 min. Supernatants were discarded and the pellets were washed four times in the medium by centrifugation at 16,000*g* for 1 min (at 4 °C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in the same buffer and incubated for 1 min at 37 °C. Incubation was terminated by immediate centrifugation (16,000*g* for 1 min). Radioactivity present in supernatants and pellets was separately determined. The [^{3}H]glutamate release was calculated as the percentage of total amount of radiolabel glutamate present at the start of the incubation period in preloaded synaptosomes.

2.4.4. Protein determination

Protein concentration was measured according to Bradford (1976), using bovine serum albumin (1 mg/ml) as the standard.

2.5. Statistical analysis

Step-through latencies are expressed as median and interquartile range, since these data demonstrated a non parametric distribution. Statistical analysis of step-down inhibitory avoidance task was carried out by Kruskal–Wallis test. All other results are presented as means ± S.E.M. The statistical significant difference between groups of the open-field test was calculated by means of one-way analysis of variance (ANOVA) followed by Duncan's test when appropriate. Statistical analysis of glutamate uptake and release was carried out by Student's *t*-test. *P* values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

3. Results

3.1. Behavioral tasks

3.1.1. Effect of PEBT on the step-down inhibitory avoidance task

Fig. 2 shows the effect of PEBT on the step-down inhibitory avoidance task in mice. During the training session in the step-down inhibitory avoidance task, there was no difference in the step-through latency time among groups. Oral administration of PEBT, at the dose of 10 mg/kg, 1 h before the training (acquisition) (Fig. 2a) and immediately after the training session (consolidation) (Fig. 2b) to mice increased the step-through latency in comparison to the control group. The dose of 10 mg/kg of PEBT administrated 1 h before the test session (retrieval) increased the step-through latency time in comparison to the control group (Fig. 2c). The lowest dose of PEBT (5 mg/kg) did not alter the step-through latency time in the three stages of memory (Fig. 2a–c).

3.1.2. Effect of PEBT on the open-field test

Locomotor and exploratory activities evaluated after the test session of the step-down inhibitory avoidance task are shown in Fig. 3. Administration of PEBT at both doses pre-training (Fig. 3a), immediately post-training (Fig. 3b) and before test (Fig. 3c) did not alter the number of crossings and rearings in the open-field test in mice.

3.2. Ex vivo assay

3.2.1. Effect of PEBT on the [^{3}H]glutamate uptake

Fig. 4 shows the effect of PEBT (10 mg/kg, p.o.) on the [^{3}H]glutamate uptake by cerebral cortex and hippocampal slices of mice. One hour after PEBT administration, the [^{3}H]glutamate uptake in cerebral cortex and hippocampus was significantly inhibited around of 61% and 37%, respectively (Fig. 4a and b, respectively). After 24 h of PEBT administration, the hippocampal [^{3}H]glutamate uptake remained significantly inhibited around of 51% (Fig. 4d). The effect of PEBT on cerebral cortex [^{3}H]glutamate uptake disappeared after 24 h administration (Fig. 4c).

3.2.2. Effect of PEBT on the [^{3}H]glutamate release

Fig. 5 shows the effect of PEBT (10 mg/kg, p.o.) on the [^{3}H]glutamate release by cerebral cortex and hippocampal synaptosomes of mice. At 1 and 24 h after PEBT administration, the [^{3}H]glutamate release was not altered in comparison to the control group.

4. Discussion

In this study, we demonstrated that PEBT, a telluroacetylene compound, induced memory improvement when administered to mice before training (effect on memory acquisition), immediately after training (effect on memory consolidation) and before test (effect on memory retrieval) of step-down inhibitory avoidance task. Moreover, the inhibition of [^{3}H]glutamate uptake was proven to be involved in the PEBT improvement of memory.

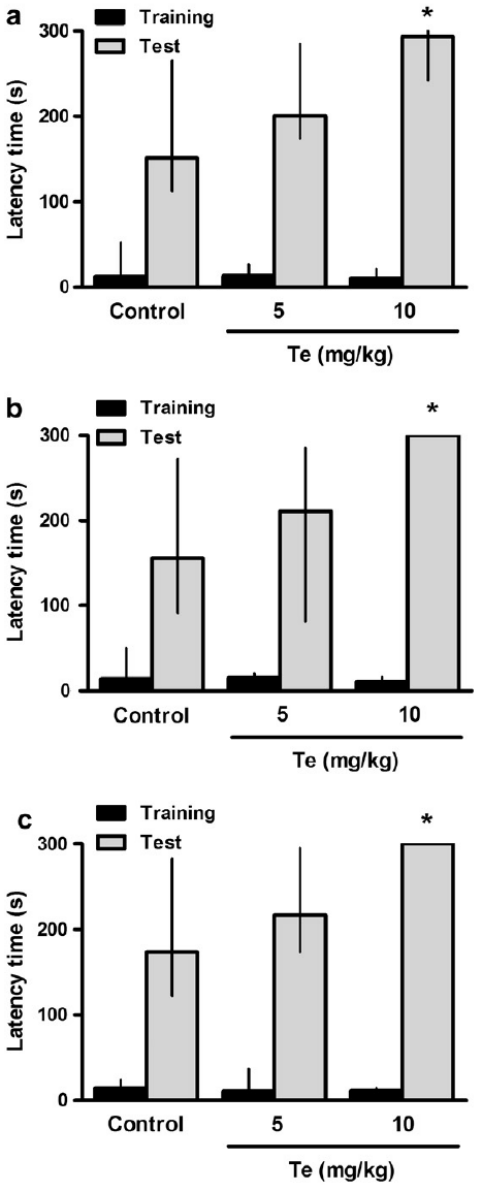

Fig. 2. Effect of PEBT on the step-down inhibitory avoidance task in mice. PEBT at the dose of 5 or 10 mg/kg was administered, p.o., 1 h before training session (acquisition) (a), immediately post-training (consolidation) (b) and 1 h before test (retrieval) (c). Each column represents median with interquartile range from 6 to 8 animals per group. Data were analyzed by Kruskal–Wallis test. (∗) $P<0.05$ when compared to the control group. *Abbreviation*: Te – PEBT.

Memory is often considered to be a process that has several stages, including acquisition, consolidation and retrieval (Abel and Lattal, 2001). Memory acquisition occurs as the animal learns an association between a context and a shock. During consolidation, which can last from minutes to hours, this memory is moved from a labile to a more fixed state. During retrieval, the animal is returned to the conditioning context, where memory for the context-shock association is assessed (Abel and Lattal, 2001).

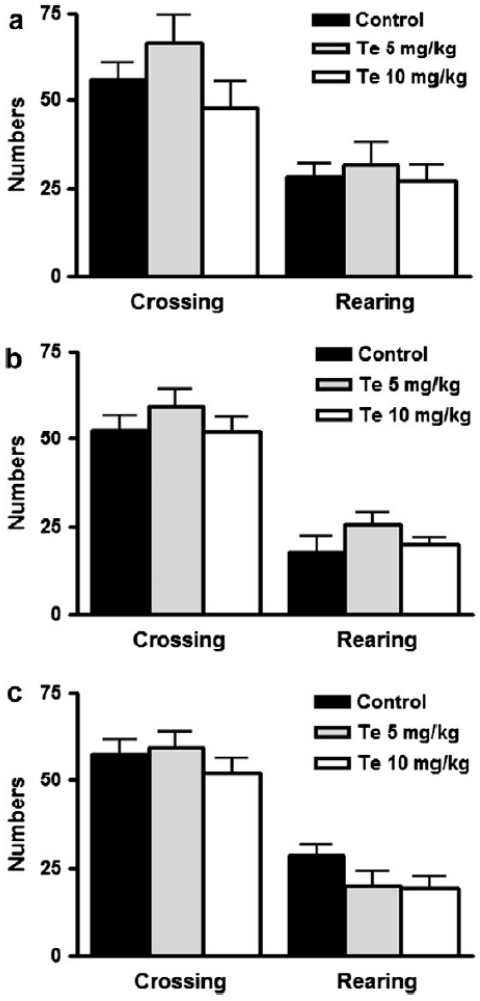

Fig. 3. Effect of PEBT on the open-field test in mice. Locomotor and exploratory activities were evaluated after the test session of the step-down inhibitory avoidance task. Administration of PEBT at doses of 5 and 10 mg/kg (p.o.) pre-training (a), post-training (b) and pre-test (c). Each column represents mean ± S.E.M. from 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Duncan's test when appropriate. *Abbreviation*: Te – PEBT.

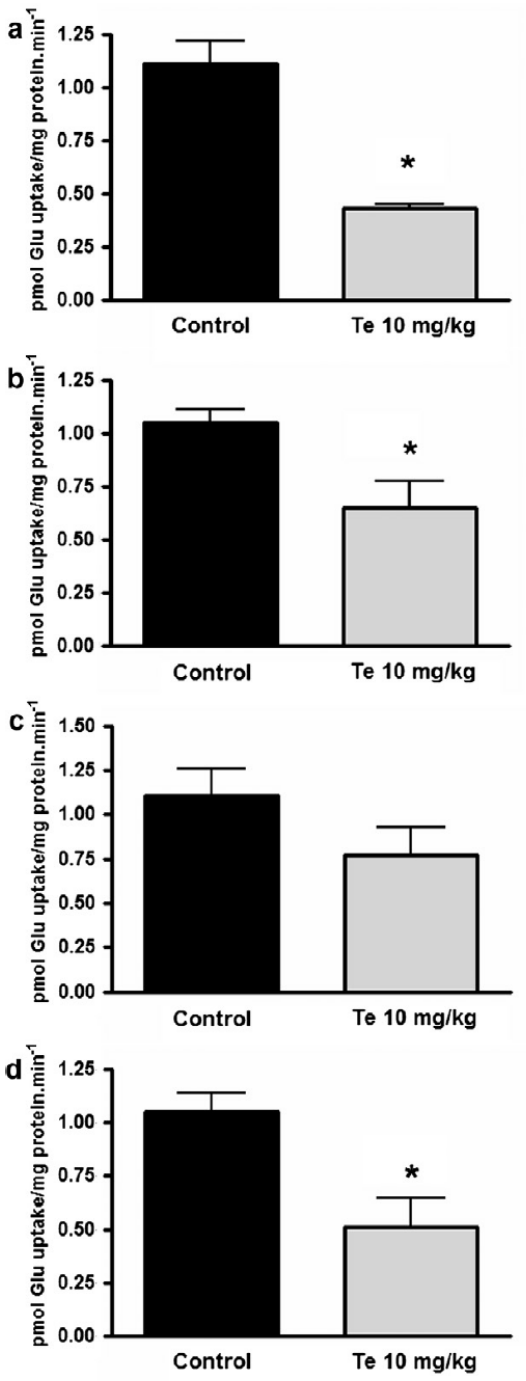

Fig. 4. Effect of PEBT on the [^{3}H]glutamate uptake by cerebral cortex and hippocampal slices of mice. Cerebral cortex (a) and hippocampal (b) [^{3}H]glutamate uptake 1 h after administration of PEBT at the dose of 10 mg/kg (p.o.). Cerebral cortex (c) and hippocampal (d) [^{3}H]glutamate uptake 24 h after administration of PEBT at the dose of 10 mg/kg (p.o.). Each column represents mean ± S.E.M. from experiments performed in triplicates. Statistical analysis was performed by Student's *t*-test. (*) $P < 0.05$ when compared to the control group. *Abbreviation*: Te – PEBT.

The results of the present investigation showed that a single administration of PEBT (10 mg/kg, p.o.), 1 h before training of step-down inhibitory avoidance task, increased the step-through latency. In other words, PEBT improved the acquisition of memory in mice. Furthermore, the effect of post-training administration of PEBT on the consolidation process was evaluated. In memory studies, where drugs are administered after, not before training, the drug's effects can be attributed to influences in the consolidation of memory, a process which takes place immediately after the training experience and lasts for few hours [for a review see (McGaugh, 1989; Castellano et al., 2001)]. PEBT (10 mg/kg, p.o.) administered immediately after training enhanced memory consolidation due to the increase in the step-through latency. Pre-test administration of drugs may affect retrieval process which implies the reactivation of memories and variety of factors can modify retrieval at the time of testing (McGaugh, 2000). In the present study, pre-test administration of PEBT (10 mg/kg, p.o.) improved retrieval of memory in the step-down inhibitory avoidance task. By contrast, 5 mg/kg dose of PEBT did not improve acquisition, consolidation or retrieval. Moreover, it is important to mention that PEBT did not cause impairment in the locomotor activity and exploratory behavior of mice assessed by the open-field test.

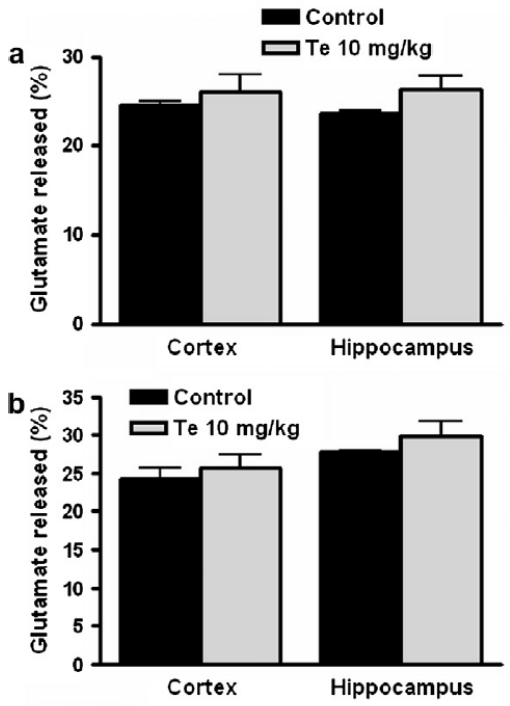

Fig. 5. Effect of PEBT on the [^{3}H]glutamate release by cerebral cortex and hippocampal synaptosomes of mice. Cerebral cortex and hippocampal synaptosomes (a) [^{3}H]glutamate release 1 h after administration of PEBT at the dose of 10 mg/kg (p.o.). Cerebral cortex and hippocampal synaptosomes (b) [^{3}H]glutamate release 24 h after administration of PEBT at the dose of 10 mg/kg (p.o.). Each column represents mean ± S.E.M. from experiments performed in triplicates. Statistical analysis was performed by Student's *t*-test. *Abbreviation*: Te – PEBT.

Based upon PEBT effect on cognitive enhancement in mice and considering the facilitatory effect of the glutamatergic system on the memory of various tasks, we investigated the possible involvement of glutamatergic neurotransmission in the PEBT action. The amino acid glutamate, the main excitatory neurotransmitter in the mammalian brain, is known to play important roles in several physiological processes, such as cognition and neural plasticity of synaptic connections (Meldrum, 2000; Mattson, 2008). Our results demonstrated that PEBT at the dose of 10 mg/kg inhibited [^{3}H]glutamate uptake, but not [^{3}H]glutamate release, in cerebral cortex and hippocampus of mice. Accordingly, diphenyl diselenide and diphenyl ditelluride, organochalcogen compounds, did not alter [^{3}H]glutamate release by rat brain synaptosomes *in vivo* (Nogueira et al., 2002). Therefore, the [^{3}H]glutamate uptake seems to be related, at least in part, to the mechanisms by which PEBT induces cognitive enhancement in the step-down inhibitory avoidance task in mice. These findings are consistent with those reported by different research groups (Daisley et al., 1998; Lhullier et al., 2004; Mameli et al., 2005), in which facilitated glutamate transmission with consequent increase in learning has been demonstrated. It is important to point out that an excessive increase of glutamate concentration in the synaptic cleft may produce neurotoxic effects associated with an over stimulation of the glutamatergic system, a process known as excitotoxicity, leading to cell death. An unbalanced increase or decrease in the glutamatergic system is highly neurotoxic. In fact, a fine tuning of glutamatergic system functioning is essential for proper brain functioning (Ozawa et al., 1998; Mattson, 2008).

Similar to PEBT, diphenyl diselenide and diphenyl ditelluride are able to inhibit [^{3}H]glutamate uptake (Souza et al., 2010). These compounds oxidize sulfhydryl groups of glutamate transporter proteins, disrupting the glutamatergic system (Moretto et al., 2007). The redox modulation of glutamate transporter proteins has been demonstrated by using agents that oxidize thiol groups, such as 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) and dithiol chelating agents. In fact, DTNB and dithiol chelating agents inhibit the glutamate uptake (Trotti et al., 1996, 1997; Nogueira et al., 2001). Moreover, ebselen, another organochalcogen compound, selectively modulates the redox site of the NMDA receptor by oxidizing thiol groups of the receptor *in vitro* (Herin et al., 2001) and the peripheral glutamatergic system (Meotti et al., 2009).

Studies of our research group demonstrated that PEBT inhibited *in vitro* δ-aminolevulinate dehydratase (ALA-D) activity, a sulfhydryl-containing enzyme, in rat brain homogenate. In this study, dithiothreitol restored δ-ALA-D activity (Souza et al., 2009). Since the mechanism involved in δ-ALA-D inhibition caused by PEBT is related to their ability to oxidize sulfhydryl groups, it is possible that PEBT inhibits [^{3}H]glutamate uptake by oxidation of SH– groups of glutamate transporter proteins. The specific high affinity Na^+-dependent amino acid transporters contain reactive –SH groups in their structure that are modulated by their redox status (Trotti et al., 1999). From these results it is possible to hypothesize that PEBT alters the redox modulation of reactive amino acids in glutamate transporter proteins. It is important to highlight that the oxidation of sulfhydryl groups of glutamate transporter proteins was spontaneously recovered since cerebral cortex [^{3}H]glutamate uptake inhibition disappeared after 24 h of administration.

5. Conclusion

In conclusion, the present study established, for the first time, that PEBT administration to mice caused cognitive enhancement in the three evaluated memory phases (acquisition, consolidation and retrieval) in the step-down inhibitory avoidance task. Based upon data from the literature pointing to a modulatory effect of glutamate on behavioral performances, results presented here could indicate that by decreasing glutamate uptake, PEBT could strengthen the physiological glutamatergic tonus, consequently improving memory on the step-down inhibitory avoidance task.

Acknowledgments

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3.2 Artigo II

2-Phenylethynyl-Butyltellurium Attenuates Amyloid-b Peptide(25–35)-Induced Learning and Memory Impairments in Mice

Ana Cristina Guerra Souza, Marcel Henrique Marcondes Sari, Simone Pinton, Cristiane Luchese, José Sebastião Santos Neto, Cristina Wayne Nogueira

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2-Phenylethynyl-Butyltellurium Attenuates Amyloid-β Peptide(25–35)-Induced Learning and Memory Impairments in Mice

Ana Cristina Guerra Souza,[1] Marcel Henrique Marcondes Sari,[1] Simone Pinton,[1] Cristiane Luchese,[2] José Sebastião Santos Neto,[1] and Cristina Wayne Nogueira[1]*

[1]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, Brasil
[2]Mestrado em Nanociências, Centro de Ciências Tecnológicas, Centro Universitário Franciscano, Santa Maria, Brazil

Our previous study demonstrated that 2-phenylethynyl-butyltellurium (PEBT), an organotellurium compound, enhances memory in mice. In this study, the effects of PEBT on cognitive impairment induced by $A\beta_{25–35}$ were assessed by Morris water maze and step-down inhibitory avoidance tasks. Mice received a single intracerebroventricular injection of $A\beta_{25–35}$ (3 nmol/3 μl/per site) and a daily oral administration of PEBT (1 mg/kg, for 10 days). PEBT significantly improved Aβ-induced learning deficits on the training session in the Morris water maze. At the probe trial session, PEBT significantly decreased the escape latency and increased the number of crossings in the platform local compared with the Aβ-treated group. PEBT significantly improved Aβ-induced memory impairment in the step-down inhibitory avoidance task. General locomotor activity was similar in all groups. This study showed that PEBT ameliorated the impairments of spatial and nonspatial long-term memory evaluated on Morris water maze and step-down inhibitory avoidance tasks, respectively. The results suggest that PEBT could be considered a candidate for the prevention of memory deficits such as those observed in Alzheimer's disease.

Key words: tellurium; organotellurium; memory; amyloid-$\beta_{25–35}$; Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia among the elderly (Hebert et al., 2003). Clinically AD is characterized by progressive loss of memory and cognition, and pathologically it is characterized by senile plaques and neurofibrillary tangles in the brain (Blennow et al., 2006). The senile plaques are made up mainly of amyloid-β (Aβ) peptide, a 39–42-amino-acid peptide formed by the proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases (Selkoe, 2004). The most abundant Aβ species in the brain of AD is $A\beta_{1–40}$, which can be truncated into a more toxic fragment, $A\beta_{25–35}$ (Kubo et al., 2002).

The drugs currently used for treating AD patients are mostly palliative, but a search for drugs that can delay or even reverse the neurodegenerative process is actively underway (Blennow et al., 2006; Mount and Downtown, 2006). The cerebral microinjection of Aβ causes amnesia and is considered a suitable animal model for testing new protective strategies eventually relevant to manage the early phases of AD (Harkany et al., 1999).

In the search for neuroprotective agents, organochalcogen compounds have attracted our attention. One important reason for this is that some studies already suggest that organoselenium compounds have a neuroprotective effect in a model of sporadic dementia of Alzheimer's type (Pinton et al., 2010, 2011) and against cognitive impairment induced by scopolamine (Souza et al., 2010) in mice. The other reason is that organotellurium compounds exert anticonvulsant effects associated with the inhibition of caspases (Persike et al., 2008), enhance neuronal survival, and improve functional outcome in an ischemic stroke model in mice (Okun et al., 2007). Moreover, a vinylic telluride compound has neuroprotective activity against Mn-induced neurotoxicity (Ávila et al., 2010).

Recently, our research group showed that 2-phenylethynyl-butyltellurium (PEBT; Fig. 1), a telluroacetylene compound, caused cognitive enhancement in the three evaluated memory phases (acquisition, consolidation, and retrieval) on the mouse step-down inhibitory avoidance task. The improvement of memory by PEBT seems to be mediated by decreasing glutamate uptake (Souza et al., 2012). In addition, this compound has an antioxidant effect in vitro and in vivo (Souza et al., 2009).

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*Correspondence to: Cristina Wayne Nogueira, Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brasil. E-mail: criswn@quimica.ufsm.br

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In light of the above observations, the objective of the present study was to assess the effect of PEBT on memory deficit induced by administration of aggregated $A\beta_{25–35}$ in mice as the first step to evaluate its potential value for the treatment of AD. To attain this end, the Morris water maze and step-down inhibitory avoidance tasks were used.

MATERIALS AND METHODS

Chemicals

PEBT was prepared according to the method of Comasseto et al. (1996). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed that the PEBT synthesized exhibited analytical and spectroscopic data in full agreement with its assigned structure. PEBT was diluted in canola oil. The Aβ peptide (fragment 25–35) was obtained from Sigma (St. Louis, MO). Aβ peptide was dissolved in double-distilled water and aggregated by incubation at 37°C for 4 days before use. All other chemicals were of analytical grade and from standard commercial suppliers.

Animals

The experiments were conducted using male adult Swiss mice (25–35 g) from our own breeding colony. Animals were maintained at 22°C ± 2°C with free access to water and food (Guabi, Rio do Sul, Brazil), under a 12:12-hr light/dark cycle (with lights on at 7:00 AM). Mice were acclimated to the laboratory for at least 1 hr before testing. Animals were used in accordance with the guidelines published in the NIH *Guide for the care and use of laboratory animals* and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and also according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

Experimental Protocol

Mice were divided into four groups of eight to 10 animals each. Mice belonging to groups 2 and 4 received PEBT (1 mg/kg, oral, dissolved in canola oil) once per day during the experiment (9 days). Animals of groups 3 and 4 received $A\beta_{25–35}$ aggregated form (3 nmol/3 µl/per site, intracerebroventricularly, dissolved in double-distilled water). Group 1 received only vehicles. The dosage of $A\beta_{25–35}$ was based on the work of H.H. Wang et al. (2001). Intracerebroventricular injections were given as described by Haley and McCormick (1957) and modified by Laursen and Belknap (1986), with the bregma fissure as a reference point. Morris water maze, step-down inhibitory avoidance, and open-field tasks were performed on the fifth, eighth, and ninth days after $A\beta_{25–35}$ injection, respectively (Fig. 2).

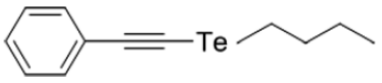

Fig. 1. Chemical structure of 2-phenylethynyl-butyltellurium (PEBT).

Morris Water Maze

The Morris water maze consisted of a pool (120 cm diameter, 50 cm height) made of black plastic and filled with water (22°C ± 2°C) to a height of 30 cm. The water's height was sufficient to avoid floor walking rather than swimming for the adult mice (1984). Black plastic beads were evenly spread over the water surface to camouflage the escape platform (8 cm diameter), which was made of black plastic and covered with a wire mesh grid to ensure a firm grip. The pool was placed in a room with several extra-maze visual cues, such as counters, posters, a dangling wire, and a pole. For the acquisition phase, mice were placed next to and facing the wall successively in north (N), south (S), east (E), and west (W) positions. The escape platform was hidden 1 cm below water level in the middle of the northwest (NW) quadrant. Behaviors were recorded and analyzed using the ANY-maze video tracking system (Stoelting Co., Wood Dale IL). The experimenter was hidden from the view of the animals but was able to follow their swimming trajectories on a video monitor, on which the pool had earlier been separated into four equally spaced quadrants, and the platform location was designated. The latency to reach the platform location was measured in four trial sessions during 3 days. The latencies were calculated as mean of total time spent in four trials of each day. The mice remained on the platform for at least 40 sec after each trial. Whenever the mice failed to reach the escape platform within the 1-min cutoff period, they were retrieved from the pool and placed on it for 40 sec. After the swim, the mice were kept dry in a plastic holding cage filled with paper towels. Twenty-four hours after the acquisition phase, a probe trial was conducted. The probe trial was conducted by removing the platform and placing the mouse next to the S side and facing the N side. The number of crossings over the former platform position and the latency to find the platform were measured for a single 1-min trial. The speed (m/sec) and distance traveled (m) were also recorded.

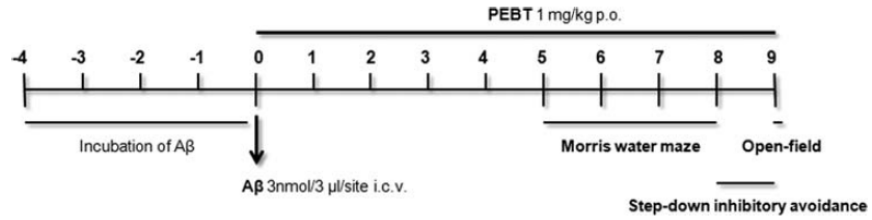

Fig. 2. Experimental schedule.

Step-Down Inhibitory Avoidance

Nonspatial long-term memory was investigated by using a step-down inhibitory avoidance task according to the method of Sakaguchi et al. (2006), with some modifications. Each mouse was placed on the platform, and the latency to step-down (four paws on the grid) was automatically recorded in training and test sessions. In the training session, upon stepping down, the mouse received a 0.5-mA scrambled foot shock for 2 sec. Test sessions were performed 24 hr later, with the same procedure except that no shock was administered after stepping down; an upper cutoff time of 300 sec was set.

Open Field

Spontaneous locomotor activity and exploratory behavior were measured in the open-field test (Walsh and Cummins, 1976). The open field was made of plywood and surrounded by walls 30 cm in height. The floor of the open field, 45 cm in length and 45 cm in width, was divided by masking tape markers into nine squares (three rows of three). Each animal was placed individually at the center of the apparatus and observed for 4 min to record the locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of time rearing on the hind limbs).

Statistical Analysis

Data are expressed as mean ± SEM. Spatial learning training data from Morris water maze was analyzed using repeated-measures ANOVA. Data from all other tasks were analyzed by two-way ANOVA followed by Duncan's test, when appropriate. Main effects are presented only when the higher second order interaction was nonsignificant. $P < 0.05$ was considered significant.

RESULTS

Morris Water Maze

Two-way repeated-measures ANOVA of the latency to reach the platform in the 3 days of spatial learning training demonstrated a significant main effect of day ($F_{2,64} = 33.99$, $P < 0.05$). Post hoc comparisons demonstrated that there were statistically significant differences among groups only at the third day. Compared with control animals, mice belonging to the Aβ-treated group showed less ability to find the platform and learn its location at day 3 of the acquisition phase. This effect was significantly protected by PEBT treatment (Fig. 3A).

In the probe trial, the two-way ANOVA of the latency to reach the platform position yielded a significant PEBT × Aβ interaction ($F_{1,32} = 7.408$, $P < 0.05$). Post hoc comparisons demonstrated that Aβ-treated group presented an increased latency to find the platform position compared with the control group. The PEBT treatment effectively reversed the increase in the latency to reach the platform position induced by Aβ (Figs. 3B, 4A–D).

The two-way ANOVA of the number of crossings over the platform position yielded a significant PEBT × Aβ interaction ($F_{1,32} = 7.093$, $P < 0.05$). Post hoc comparisons showed that Aβ-treated mice decreased the number of crossings of the platform location compared with the control group. PEBT significantly attenuated the effects of Aβ on the number of crossings (Fig. 3C).

There were no group differences for Morris water maze performance as measured by the average swim speed ($F_{1,32} = 0.00$, not significant [NS]) and the total distance traveled ($F_{1,32} = 0.01$, NS) during the probe trial (Table I).

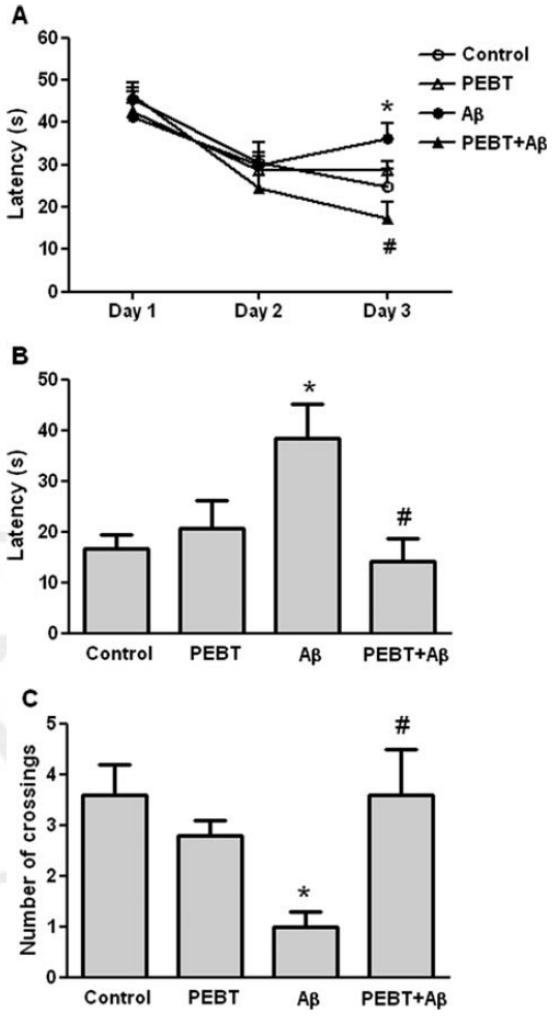

Fig. 3. Effect of PEBT on $A\beta_{25-35}$-induced memory impairment in the Morris water maze task in mice. **A:** Average time to locate the platform during the training sessions. Time to locate the platform area (**B**) and number of crossings of the platform location (**C**) during the probe trial session. Each column represents mean ± SEM from eight to 10 animals per group. Statistical analysis was performed by using repeated-measures ANOVA (data of the training sessions) and by two-way ANOVA followed by Duncan's test when appropriate. $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the Aβ group.

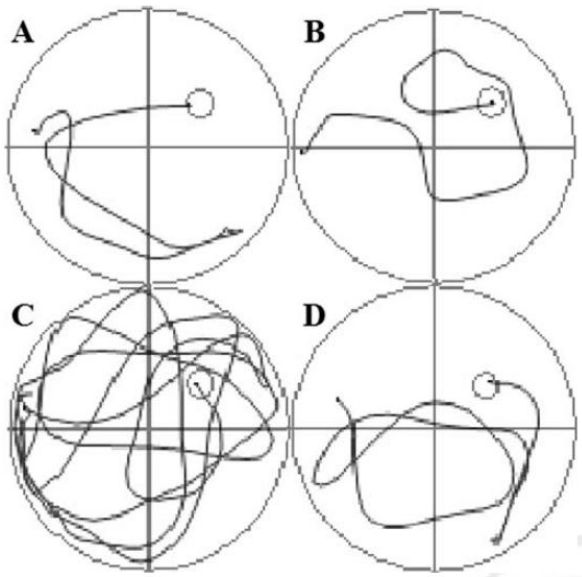

Fig. 4. Representation of latency to reach platform location. Swimming paths during the probe trial session in the water Morris maze. **A:** Control. **B:** PEBT. **C:** Aβ. **D:** PEBT + Aβ.

TABLE I. Effect of PEBT and $A\beta_{25-35}$ on Locomotor Parameters Evaluated in the Morris Water Maze Task[a]

	Average speed (m/sec)	Total distance travelled (m)
Control	0.197 ± 0.010	11.8 ± 0.7
PEBT	0.204 ± 0.010	12.1 ± 0.6
Aβ	0.195 ± 0.009	11.6 ± 0.5
PEBT + Aβ	0.201 ± 0.007	12.0 ± 0.4

Data are means ± SEM from eight to 10 animals per group.

Step-Down Inhibitory Avoidance

During the training session in the step-down inhibitory avoidance task, there was no difference in the step-down latency among groups ($F_{1,34} = 0.00$, NS). In the test session, the two-way ANOVA of step-down latency revealed a significant PEBT × Aβ interaction ($F_{1,34} = 0.46$, $P < 0.05$). Post hoc comparisons showed that Aβ-treated mice decreased the step-down latency in comparison with the control group. PEBT significantly prevented the latency decrease in mice exposed to Aβ. Moreover, the PEBT treatment increased the step-down latency in comparison with the control group (Fig. 5).

Open Field

Locomotor and exploratory activities measured in the open-field test did not differ significantly among groups (Table II). The two-way ANOVA of the number of crossings ($F_{1,34} = 0.81$, NS) and rearings ($F_{1,34} = 1.62$, NS) revealed no significant differences.

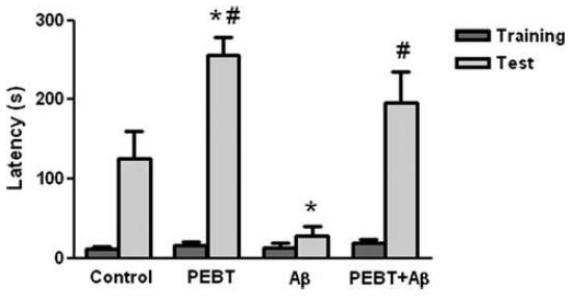

Fig. 5. Effect of PEBT on $A\beta_{25-35}$-induced memory impairment in the step-down inhibitory avoidance task in mice. Each column represents mean ± SEM from eight to 10 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test when appropriate. $^{\star}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the Aβ group.

TABLE II. Effect of PEBT and $A\beta_{25-35}$ on Locomotor Parameters Evaluated in the Open-Field Task[a]

	Number of crossings	Number of rearings
Control	63.6 ± 8.4	26.6 ± 4.9
PEBT	58.7 ± 5.4	38.3 ± 4.0
Aβ	56.3 ± 6.4	33.2 ± 4.1
PEBT + Aβ	62.9 ± 5.7	34.3 ± 3.8

Data are means ± SEM from eight to 10 animals per group.

DISCUSSION

Providing a therapeutic strategy to reduce the progressive cognitive decline in AD would improve patients' well-being. In this study, we have provided an alternative to prevent cognitive impairment induced by Aβ. The subchronic administration of PEBT, a telluroacetylene compound, attenuated $A\beta_{25-35}$-induced learning and memory deficits in mice evaluated on Morris water maze and step-down inhibitory avoidance tasks.

Aβ species play a prominent role in the pathogenesis of AD, a pathology that disrupts memory performance (Hardy and Selkoe, 2002; Klein et al., 2004). This Aβ-induced cognitive disruption may result primarily from a synaptic dysfunction, which then spreads to include a pattern of neuronal death (Coleman et al., 2004). Reported evidence suggests that the fraction 25–35 of Aβ is the functional neurotoxic domain (Pike et al., 1995). In this view, cognitive deficits induced by a single i.c.v. administration of $A\beta_{25-35}$ have been documented in mice (X.Y. Wang et al., 2001; Um et al., 2006; Dall'Igna et al., 2007).

It is important to note that AD is associated with a variety of hippocampus-dependent memory deficits, such as impairment in long-term memory formation (Bach et al., 1999). The Morris water maze is usually accepted as an indicator of spatial learning and reference memory, which reflects long-term memory (Morris, 1984; D'Hooge and De Deyn, 2001). On the third day of the

acquisition phase in the Morris water maze test, the Aβ-treated group presented an increased latency to find the platform location, showing the impairment on learning. PEBT significantly shortened the escape latency prolonged by Aβ treatment during the training. In the probe trial, an impairment of long-term memory was observed in the Aβ-treated group, as shown by the elevated latency to reach the platform location and the decreased number of crossings in this place. The results of the present investigation showed that a subchronic administration of PEBT ameliorated the performance of mice in both parameters evaluated with the Morris water maze. Because the swimming speed and total distance traveled during the probe trial were not significantly different among groups, the performance impairment seen in the Aβ group is not due to any perceptual deficits, such as vision or motor deficits.

The ameliorative effect of PEBT on nonspatial long-term memory was investigated in the step-down inhibitory avoidance task. Aβ-induced memory impairment was verified by decreasing the step-down latency, whereas PEBT prevented this impairment of nonspatial memory. In addition, the PEBT treatment increased the step-down latency in comparison with the control group. This result is in accordance with our previous study demonstrating the enhancement of memory with a single dose of PEBT on the mouse step-down inhibitory avoidance task (Souza et al., 2012).

The open field is commonly used to analyze animal behavior based on natural conflict between exploration and aversion against open areas in a novel environment. The total number of grid squares traversed in the field normally serves as an index of locomotion activity, whereas the sum of rearings and grid squares traversed reflect exploratory activity (Schmitt and Hiemke, 1998). There were no significant differences in the number of crossings and rearings for any experimental group in the open-field test, so the pharmacological effect of PEBT is independent of the influence of motor and exploratory disturbances in mice.

In a previous study, we demonstrated that PEBT was effective for improving acquisition, consolidation, and retrieval of memory on the step-down inhibitory avoidance task in mice (Souza et al., 2012). In the same study, PEBT inhibited [^{3}H]glutamate uptake but not [^{3}H]glutamate release in cerebral cortex and hippocampus of mice. In addition, other organochalcogen compounds have been reported as memory enhancers (Rosa et al., 2003; Stangherlin et al., 2008). In this context, diphenyl diselenide $(PhSe)_2$ ameliorates scopolamine-induced memory impairment in mice (Souza et al., 2010). Moreover, a substituted analog of $(PhSe)_2$, improves memory in mice in the model of sporadic dementia of Alzheimer's type (SDAT) induced by i.c.v. injection of streptozotocin. This compound exerts its neuroprotective effect in the SDAT model by inhibiting acetylcholinesterase activity and its antioxidant properties (Pinton et al., 2010, 2011).

Accumulating evidence suggests that oxidative stress is involved in the mechanism of Aβ-induced neurotoxicity (Zhu et al., 2004). Although the mechanisms underlying the preventive effect of PEBT against Aβ are unclear, we can suggest that PEBT's antioxidant properties, demonstrated in vitro and in vivo (Souza et al., 2009), contribute to its ameliorative effect on Aβ-induced learning and memory impairment in mice. Accordingly, antioxidants, such as vitamin E, *Ginkgo biloba*, and ferulic acid, have been used in attempts to treat or prevent AD (Sano et al., 1997; Christen, 2000; Yan et al., 2001).

Data reported here demonstrate that PEBT improves long-term memory deficits induced by $A\beta_{25-35}$, a model of AD, in mice. Although additional investigations are necessary to determine the neurochemical mechanisms involved in the effect of PEBT on cognition, this compound may have potential for preventing and/or delaying progression of learning and memory impairments in AD.

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The authors declare that there are no conflicts of interest.

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3.3 Manuscrito I

2-Phenylethynyl-butyltellurium enhances learning and memory impaired by scopolamine in mice

Ana Cristina G Souza, César A Bruning, Carmine I Acker, José S S Neto, Cristina W Nogueira

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2-Phenylethynyl-butyltellurium enhances learning and memory impaired by scopolamine in mice

Ana Cristina G Souza, César A Bruning, Carmine I Acker, José S S Neto, Cristina W Nogueira*

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

Short Title: PEBT improves memory impairment in mice

*Correspondence should be sent to:

Cristina Wayne Nogueira

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

Phone: 55-55-3220-8140

FAX: 55-55-3220-8978

E-mail:criswn@quimica.ufsm.br

The authors declare that there are no conflicts of interest

Abstract

Taking into account the memory enhancement property of 2-phenylethynyl-butyltellurium (PEBT) and the constant search for drugs that improve cognitive performance, the present study was designed to investigate the PEBT effect on cognitive impairment induced by scopolamine in mice. PEBT (10 mg/kg, gavage) was administered to mice one hour before the probe trial of the Morris water maze task. Memory impairment was induced by scopolamine (1 mg/kg, intraperitoneal) 30 min before the probe trial. PEBT significantly ameliorated scopolamine-induced long-term memory deficit, as indicated by decrease in escape latency and increase in the number of crossings in the platform location when compared with the amnesic mice. In order to evaluate the effect of PEBT on different phases of memory (acquisition, consolidation, and retrieval) impaired by scopolamine, the step-down inhibitory avoidance task was used. Scopolamine was given 30 min before training (acquisition), test (retrieval) or immediately post-training (consolidation). PEBT, administered 30 min before scopolamine, increased step-down latency in memory-impaired mice, improving consolidation and retrieval stages, but not acquisition. No significant alterations in locomotor and exploratory behaviors were found in animals treated with PEBT and/or scopolamine. PEBT improved memory deficits during consolidation and retrieval induced by scopolamine.

Keywords: Tellurium; Organotellurium; Scopolamine; Memory; Morris Water Maze; inhibitory avoidance

1 Introduction

During the last decade a considerable interest in the study of tellurium compounds as pharmacological agents has emerged. Organic and inorganic tellurium compounds have shown antioxidant, antimicrobial, antihelmintic, immunomodulatory, and antitumoral properties (Wieslander et al., 1998; Urig and Becker, 2006; Cunha et al., 2009; Ba et al., 2010; Sredni, 2012; Tiekink, 2012). Lately, the vinylic telluride protection against manganese-induced aging and toxicity was demonstrated (Ávila et al., 2012). Furthermore, organotellurium compounds have demonstrated anticonvulsant effects, enhancement of neuronal survival, and improvement of functional outcome in an ischemic stroke model in mice (Okun et al., 2007; Persike et al., 2008).

2-Phenylethynyl-butyltellurium (PEBT) (Figure 1) is a telluroacetylene compound that has antioxidant properties *in vitro* and *in vivo* in brains of rodents (Souza et al., 2009). Recently, our research group showed that PEBT enhances acquisition, consolidation, and retrieval of memory in the step-down inhibitory avoidance task in mice. The improvement of memory by PEBT seems to be mediated by decreasing glutamate uptake (Souza et al., 2012). In addition, this compound improves long-term memory deficits induced by amyloid beta peptide (25-35), a model of Alzheimer disease, in mice (Souza et al., 2013).

Cholinergic neuromodulation is known to influence multiple cognitive processes, including memory and attention (Klinkenberg et al., 2010; Graef et al., 2011). Cholinergic agonists can facilitate memory, whereas cholinergic antagonists can impair memory (Mattson, 2004). In this way, blockade of the cholinergic muscarinic receptors by the antagonist scopolamine results in impaired performance on learning and memory tasks (Rusted and Warburton, 1988; Klinkenberg and Blokland, 2011). For this reason, scopolamine has been used

to model cognitive deficits associated with aging and dementia, such as Alzheimer disease (Ebert and Kirch, 1998; Azizi et al., 2012; Gupta and Gupta, 2012).

On the basis of the above considerations, the aim of the present study was to investigate if PEBT ameliorates long-term memory deficits induced by scopolamine in the mouse Morris water maze task. Moreover, the PEBT effect was evaluated on different phases of memory (acquisition, consolidation, and retrieval) in amnesic mice using the step-down inhibitory avoidance task.

2 Materials and methods

2.1 Chemicals

PEBT was prepared according to the literature method (Comasseto et al., 1996). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed that synthesized PEBT exhibited analytical and spectroscopic data in full agreement with its assigned structure. PEBT was diluted in canola oil. (–) Scopolamine hydrobromide was purchased from the Sigma Chemical Co. (St Louis, Missouri, USA). Scopolamine was dissolved in saline (0.9% NaCl).

2.2 Animals

The behavioral experiments were conducted using male adult Swiss mice (25–35 g) from our own breeding colony. Animals were maintained at 22 ± 2 ^{0}C with free access to water and food (Guabi, RS, Brazil), under a 12:12 h light/dark cycle (with lights on at 7:00 a.m.). Mice were acclimatized to the laboratory for at least 1 hour before testing. Animals were used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (CEUA 041/2012).

2.3 Morris water maze

The Morris water maze consisted of a pool (120 cm diameter, 50 cm height) made of black plastic and filled with water (22 ± 2 ^{0}C) to a height of 30 cm. The water's height was sufficient to avoid floor walking rather than swimming for the adult mice (Morris, 1984). Black plastic beads were evenly spread over the water surface to camouflage the escape platform (8 cm diameter), which was made of black plastic and covered with a wire mesh grid to ensure a firm grip as the mice climbed onto the platform. The pool was placed in a room with several extra-maze visual cues, such as counters, posters, a dangling wire, and a pole. For the acquisition phase, mice were placed next to and facing the wall successively in north (N), south (S), east (E), and west (W) positions. The escape platform was hidden 1 cm below water level in the middle of the northwest (NW) quadrant. Behaviors were recorded and analyzed using the ANY-maze^{TM} video tracking system (Stoelting Co., Wood Dale IL, USA). The experimenter was hidden from the view of the animals but was able to follow their swimming trajectories on a video monitor, on which the pool was earlier separated into four equally spaced quadrants and the platform location was designated. The latency to reach the platform location was measured in four trial sessions during 3 days. The latencies were calculated as mean of total time spent in four trials of each day. The mice remained on the platform for at least 40s after each trial. Whenever the mice failed to reach the escape platform within the 1 min cut-off period, they were retrieved from the pool and placed on it for 40s. After the swim, the mice were kept dry in a plastic holding cage filled with paper towels. A probe trial was conducted twenty-four hours after the acquisition phase by removing the platform and placing the mouse next to the S side facing the walls, in front of the N side. The number of crossings over the former platform position and the latency to find platform location were measured for a single 1-min trial. The speed (m/s) and distance traveled (m) were

also recorded. During the acquisition phase, mice were not treated, constituting a total of 38 animals. Mice were divided into four groups of eight to nine animals each: 1-Control; 2-PEBT; 3-Scop; 4-PEBT+Scop. One hour before the probe trial, mice belonging to groups 2 and 4 were treated with PEBT (10 mg/kg, gavage). Thirty min later, memory impairment was induced by administration of scopolamine (1 mg/kg, intraperitoneal) in animals of groups 3 and 4. Group 1 received only vehicles (canola oil 10 ml/kg, gavage and saline 5 ml/kg, intraperitoneal).

2.4 Step-down inhibitory avoidance

Non-spatial long-term memory was investigated using a step-down inhibitory avoidance task according to the method of Sakaguchi et al. (2006), with some modifications. Each mouse was placed on the platform, and the latency to step-down with four paws on the grid was automatically recorded in training and test sessions. In the training session, upon stepping down, the mouse received a 0.5 mA scrambled foot shock for 2 seconds. Test sessions were performed 24 hours later with the same procedure, except that no shock was administered after stepping down. An upper cutoff time of 300 seconds was set. Mice were divided into four groups of seven to nine animals each: 1-Control; 2-PEBT; 3-Scop; 4-PEBT+Scop. Animals of groups 3 and 4 received scopolamine (1 mg/kg, intraperitoneal) 30 min before training (acquisition), test (retrieval) or immediately post-training (consolidation). Mice belonging to groups 2 and 4 were treated with PEBT (10 mg/kg, gavage) 30 min before scopolamine (Figure 2). Group 1 received only vehicles (canola oil 10 ml/kg, gavage and saline 5 ml/kg, intraperitoneal).

2.5 Open-Field

Spontaneous locomotor activity and exploratory behavior were measured in the open-field test (Walsh and Cummins, 1976). The open-field was made of plywood and surrounded by walls 30 cm in height. The floor of the open-field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3). Each animal was placed individually at the center of the apparatus and observed for 4 min to record the locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of time rearing on the hind limbs). The locomotor and exploratory activities were evaluated after the test session of the step-down inhibitory avoidance task. Seven to nine animals were used per group.

2.6 Statistical Analysis

All data present normal distribution and are expressed as mean ± S.E.M. For the training session in the Morris water maze, the data were analyzed using repeated measure analysis of variance (ANOVA). Data from all other tasks were analyzed by two-way ANOVA (PEBT X Scopolamine) followed by the Duncan's test, when appropriate. Main effects are presented only when the higher second order interaction was non-significant. *P* values less than 0.05 ($P<0.05$) were considered as indicative of significance.

3 Results

3.1 Morris water maze

All untreated mice showed a general decrease in overall latency to find the platform location throughout the training session (days 1 to 3) (Figure 3A). In the probe trial, the two-way ANOVA of the latency to achieve the platform position yielded a significant PEBT X

Scopolamine interaction [$F_{1,30}$ = 5.74, $p<0.05$]. *Post hoc* comparisons demonstrated that scopolamine-treated mice showed an increased latency to find the platform location compared to the control group. The PEBT treatment significantly reversed the increase in the latency to reach the platform position induced by scopolamine (Figure 3B). The two-way ANOVA of the number of crossings over the platform location revealed a significant PEBT X Scopolamine interaction [$F_{1,30}$ = 4.34, $p<0.05$]. *Post hoc* comparisons showed that scopolamine administration decreased the number of crossings on the platform location when compared with the control mice. PEBT significantly attenuated the effects of scopolamine on the number of crossings (Figure 3C). The two-way ANOVA of average swim speed [$F_{1,30}$ = 0.00, not significant (NS)] and total distance traveled [$F_{1,30}$ = 0.18, NS] did not demonstrate significant difference among groups (Table 1).

3.2 Step-down inhibitory avoidance

During the training session in the step-down inhibitory avoidance task, there was no difference in the step-down latency among groups (data not shown). Figure 4 shows the effects of PEBT on scopolamine-induced amnesia in the test session of step-down inhibitory avoidance task in mice. For memory acquisition phase, the two-way ANOVA of step-down latency revealed a significant PEBT X Scopolamine interaction [$F_{1,29}$ = 5.37, $p<0.05$] (Figure 4A). The pre-training injection of scopolamine reduced the step-down latency in comparison to the control group. PEBT, administered 30 min before scopolamine, did not protect against this reduction in the latency.

For consolidation phase, the two-way ANOVA of step-down latency yielded a significant PEBT X Scopolamine interaction [$F_{1,25}$ = 4.53, $p<0.05$] (Figure 4B). Scopolamine administered

immediately post-training decreased the step-down latency, which was protected by the pre-administration of PEBT.

For retrieval phase, the two-way ANOVA of step-down latency revealed significant main effects of PEBT [$F_{1,27} = 26.36$, $p<0.05$] and scopolamine [$F_{1,27} = 11.04$, $p<0.05$] (Figure 4C). The reduction in the step-down latency induced by the pre-test scopolamine administration was protected by PEBT treatment. Also, the PEBT treatment *per se* increased the step-down latency in comparison to the control group in consolidation and retrieval of memory.

3.3 Open-field

Locomotor and exploratory activities assessed in the open-field test did not differ significantly among groups (Table 2). The two-way ANOVA of the number of crossings [$F_{1,30} = 0.49$, NS (pre-training); $F_{1,26} = 1.36$, NS (post-training); $F_{1,27} = 0.33$, NS (pre-test)] and rearings [$F_{1,30} = 0.51$, NS (pre-training); $F_{1,26} = 1.06$, NS (post-training); $F_{1,27} = 1.85$, NS (pre-test)] revealed no significant difference.

4 Discussion

Since manipulation of cholinergic activity considerably influences cognitive performance (Blokland, 1996), scopolamine, a cholinergic muscarinic antagonist, was used as a memory and learning impairing agent (Klinkenberg and Blokland, 2010) to investigate the effect of PEBT on memory. The PEBT treatment was effective against the long-term memory impairment evaluated in the Morris water maze task. In addition, the acute administration of PEBT improved the

consolidation and retrieval phases but not acquisition of memory impaired by scopolamine in the step-down inhibitory avoidance task in mice.

The Morris water maze learning task is used to assess hippocampal-dependent spatial learning ability. Escape latency reductions from day to day reflect learning with respect to long-term memory (Morris, 1894). During the training session of the Morris water maze task, all untreated mice learned the platform location by decreasing in overall latency to find the platform place. In the probe trial session, the prolongation in escape latency induced by scopolamine was significantly decreased by PEBT treatment. Besides, PEBT protected against the reduction in the number of crossings in the place where the platform was located. Therefore, PEBT was effective in improving impaired spatial long-term memory induced by scopolamine in the mouse Morris water maze task.

Our research group showed that PEBT caused cognitive enhancement in the three evaluated memory phases (acquisition, consolidation, and retrieval) on the mouse step-down inhibitory avoidance task (Souza et al., 2012). In accordance, our current results corroborate with the previous ones, since the pre-training and pre-test administration of PEBT alone increased the step-down latency compared to the control mice. However, the enhancement of memory consolidation was not seen by PEBT, probably due to the difference in the treatment protocol or also by considering the numerous technical issues for getting the step down test to work reproducible. Step-down inhibitory avoidance involves learning not to step down from a platform in order to avoid a mild footshock. It is usually acquired in one single trial, which makes it ideal for studying processes initiated by training uncontaminated by prior or further trials, rehearsals, or retrievals (Gold, 1986; Izquierdo, 1989). The chief finding of this study is that PEBT reversed scopolamine-induced amnesia for long-term memory in two memory phases, consolidation and

retrieval, in the step-down inhibitory avoidance task. The PEBT treatment was effective against the impairment of memory consolidation induced by scopolamine, which was injected immediately post-training to mice. Moreover, PEBT protected against the decrease of step-down latency induced by scopolamine pre-test administration, showing an improvement of memory retrieval. However, in the acquisition stage, the pre-training administration of PEBT was absolutely ineffective in memory performance impaired by scopolamine. It may be explained by considering that two compounds are injected before a demanding acquisition phase where the injections may alert the mice and induce anxiety and interfere with their attention. Thus, PEBT improves memory consolidation and retrieval not only in control mice (Souza et al., 2012) but also in scopolamine-induced memory impaired mice.

It is substantial to remark that all treatments neither cause impairment in the number of crossings and rearings assessed by the open-field task nor in swimming speed and total distance traveled evaluated by the Morris water maze task in mice. Scopolamine has been found to increase or decrease locomotor activity, or even no effect at all. Thus, the role of scopolamine in locomotor behavior appears to be dependent on various experimental factors, such as strain, sensitivity of the test, drug doses, etc. Besides, the anxiety could be present as an underlying effect on locomotor behavior (Klinkenberg and Blokland, 2010).

The cholinergic system is involved in many physiological processes, including synaptic plasticity and learning and memory (Power et al., 2003; Weinberger, 2006). The inhibitory avoidance response is a cholinergic-dependent learning task, undermined by pre- or post-training administration of muscarinic antagonists (McGaugh and Izquierdo 2000). As seen in this study, the systemic administration of scopolamine impairs acquisition, consolidation, and retrieval in the step-down inhibitory avoidance protocol (da Silva et al., 2009). The role of acetylcholine in

learning and memory processes has been put forward (Hasselmo and McGaughy, 2004; Hasselmo, 2006). However, the role of this neurotransmitter is complex, since it is mainly involved in attentional processes (Blokland, 1995; Sarter and Bruno, 1997). The cholinergic lesion contributes to the severity of the cognitive and behavioral deficits, especially in the areas of memory and attention. A profound loss of cortical cholinergic innervation is a nearly invariant feature of advanced Alzheimer's disease (Beatty et al., 1986; Mesulam et al., 2004). Nevertheless, cholinergic lesion is unlikely to be a major determinant of the clinical symptoms or of the neuropathological lesions (Mesulam et al., 2004), and multiple neurotransmitter systems are involved in Alzheimer's disease (Gsell et al., 2004).

In addition to cholinergic system, it has long been recognized that the glutamatergic system has a pivotal role in neuroplasticity, learning, and memory processes (Castellano et al., 2001; Mattson, 2008). The interaction of these two systems is important for memory (Pakpour et al., 2010), and acetylcholine may function to facilitate glutamate activity by coordinating states of acquisition and recall in the cortex and hippocampus (Aigner, 1995). Our previous study indicates that by decreasing glutamate uptake, PEBT could strengthen the physiological glutamatergic tonus (Souza et al., 2012). Consequently, PEBT could improve the impairment in consolidation and retrieval of memory caused by scopolamine, which decreased the cholinergic function in the step-down inhibitory avoidance task. Further studies should be necessary to elucidate the absence of protection by PEBT in the scopolamine-induced memory acquisition impairment as well as other mechanisms involved in the improvement of memory by PEBT.

Taken together, the results of the present study reveal that the PEBT treatment ameliorated spatial long-term memory in the Morris water maze, and consolidation and retrieval of memory impaired by scopolamine in the step-down inhibitory avoidance in mice. The PEBT

cognitive-enhancing activity might offer a useful therapeutic choice to alleviate memory impairments, especially those related to the cholinergic dysfunction.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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Table 1: Effect of PEBT and scopolamine on locomotor parameters evaluated in the mouse Morris water maze task.

	Average speed (m/s)	**Total distance travelled (m)**
Control	0.211 ± 0.010	13.78 ± 0.8
PEBT	0.206 ± 0.007	12.33 ± 0.4
Scopolamine	0.228 ± 0.005	14.28 ± 0.4
PEBT+ Scopolamine	0.223 ± 0.012	13.35 ± 0.7

Data are reported as means ± S.E.M from 8 to 9 animals per group. Statistical analysis was performed by two-way ANOVA

Table 2: Effect of PEBT and scopolamine on the number of crossings and rearings evaluated in the mouse open-field task.

	Pre-training		**Post-training**		**Pre-test**	
	Crossings	Rearings	Crossings	Rearings	Crossings	Rearings
Control	58.7 ± 8	31.9 ± 3	54.4 ± 6	28.7 ± 3	54.9 ± 6	26.5 ± 2
PEBT	47.8 ± 5	26.3 ± 3	54.6 ± 2	27.8 ± 4	57.3 ± 6	19.4 ± 4
Scopolamine	53.9 ± 5	28.8 ± 3	53.14 ± 5	32.0 ± 3	58.1 ± 14	14.5 ± 5
PEBT+Scopolamine	56.0 ± 7	33.0 ± 5	61.4 ± 4	35.0 ± 4	70.8 ± 7	19.5 ± 6

Data are reported as means ± S.E.M from 7 to 9 animals per group. Statistical analysis was performed by two-way ANOVA.

Figure legends

Figure 1: Chemical structure of 2-phenylethynyl-butyltellurium (PEBT).

Figure 2: Experimental schedule for step-down inhibitory avoidance task. Experimental protocol to investigate the effects of PEBT in acquisition (A), consolidation (B) and retrieval (C) of memory in a model of scopolamine-induced memory impairment in mice.

Figure 3: Effect of PEBT (10 mg/kg, gavage) on scopolamine (1 mg/kg, i.p.)-induced memory impairment in the Morris water maze task in mice. (A) Average time to find the platform during the training sessions. (B) Time to locate the platform area, and (C) number of crossings of the platform location during the probe trial session. Each column represents mean ± S.E.M. from 9 to 10 animals per group. Statistical analysis was performed using one-way ANOVA (data of the training sessions) and by two-way ANOVA followed by the Duncan's test when appropriate. (a, b, c) $P<0.05$ when compared to the other groups. (*) $P<0.05$ when compared to the control group. (#) $P<0.05$ when compared to the scopolamine group. *Abbreviation: SCOP – scopolamine.*

Figure 4: Effect of PEBT (10 mg/kg, gavage) on scopolamine (1 mg/kg, i.p.)-induced memory deficit in the step-down inhibitory avoidance task in mice. Test session of (A) pre-training (acquisition), (B) post-training (consolidation), and (C) pre-test (retrieval) treatments. Each column represents mean ± S.E.M. from 7 to 9 animals per group. Statistical analysis was performed by two-way ANOVA followed by the Duncan's test when appropriate. (*) $P<0.05$ when compared to the control group. (#) $P<0.05$ when compared to the scopolamine group. *Abbreviation: SCOP – scopolamine.*

Figures

Figure 1

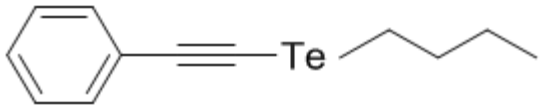


Figure 2

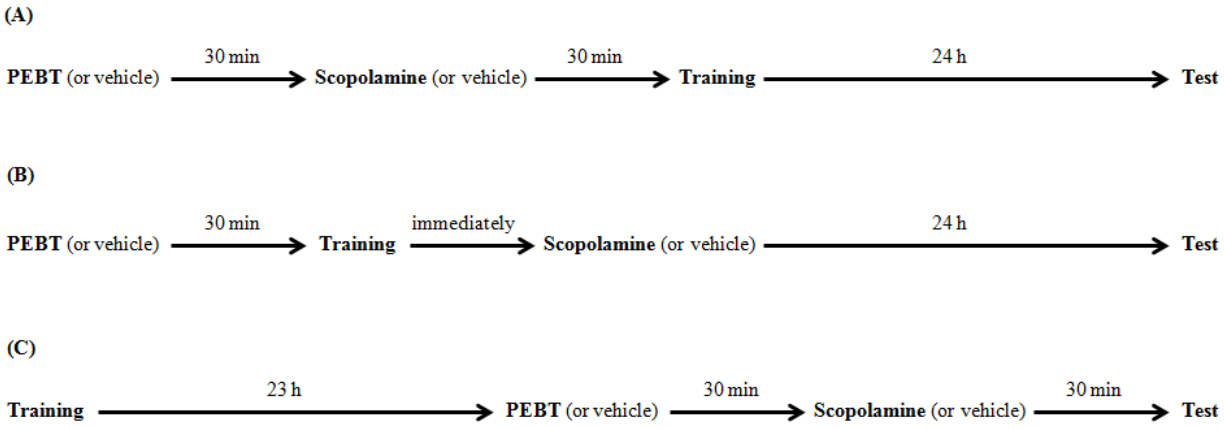


Figure 3A

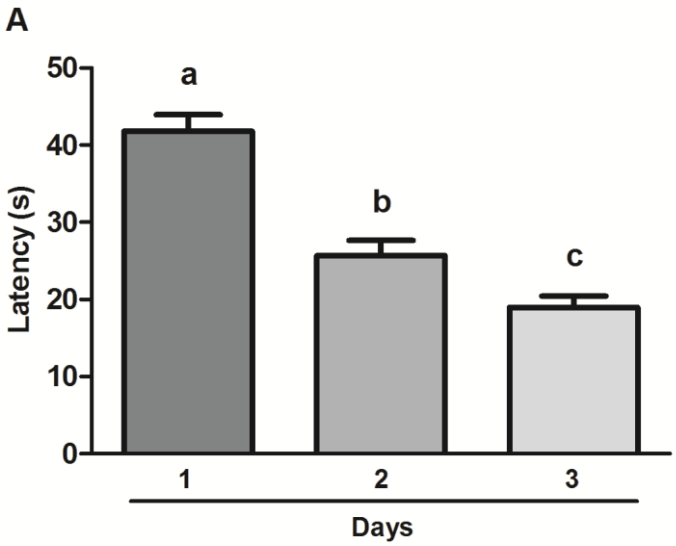


Figure 3B

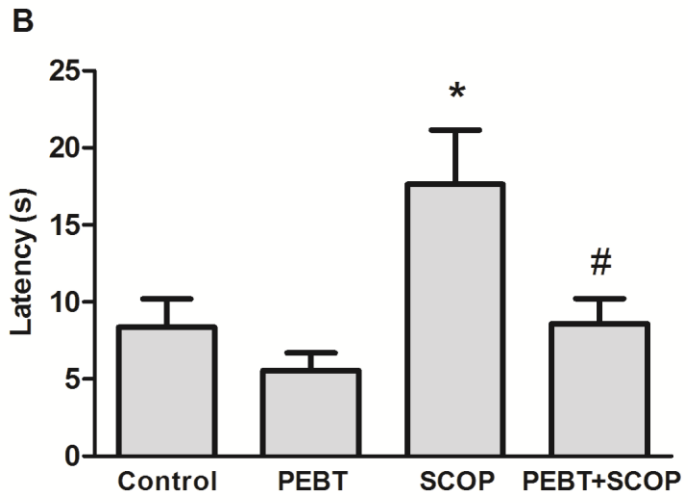


Figure 3C

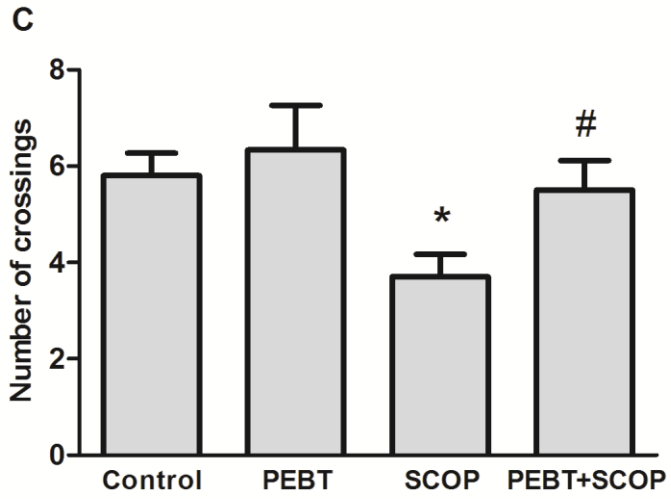


Figure 4A

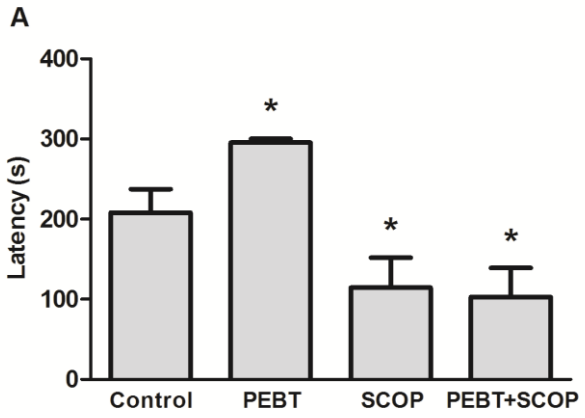


Figure 4B

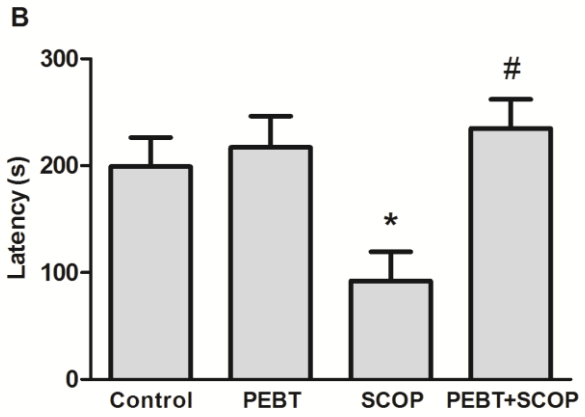
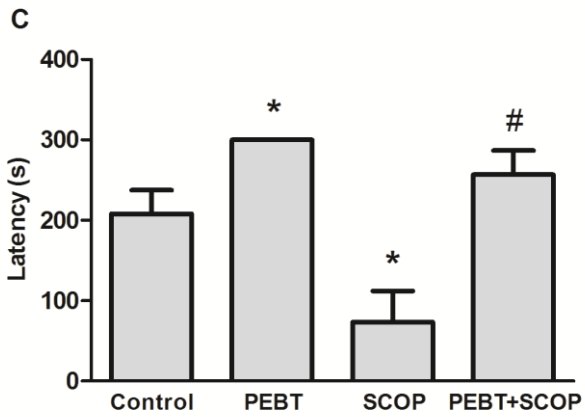


Figure 4C



3.4 Manuscrito II

Protective effect of 2-phenylethinyl-butyltellurium against oxidative stress in human retinal pigment epithelium

Ana Cristina G Souza, Pranab K Mukherjee, Veronica B Balaszczuk, José S S Neto, Cristina W Nogueira, Nicolas G Bazan

Protective effect of 2-phenylethinyl-butyltellurium against oxidative stress in human retinal pigment epithelium

Ana Cristina G Souza[1], Pranab K Mukherjee[2], Veronica B Balaszczuk[2], José S S Neto[1], Cristina W Nogueira[1], Nicolas G Bazan[2]*

[1]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, Brazil

[2]Neuroscience Center of Excellence and Department of Ophthalmology, Louisiana State University Health Sciences Center School of Medicine, New Orleans, LA 70112

*Correspondence should be sent to:

Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, 2020 Gravier St., Suite D, New Orleans, LA 70112, USA

Tel.: 504-599-0831

E-mail: nbazan@lsuhsc.edu

Abstract

2-Phenylethinyl-butyltellurium (PEBT) is an organotellurium compound with antioxidant properties. Oxidative stress-induced retinal pigment epithelial (RPE) cell death is involved in the pathogenesis of ocular diseases. In the present study, human RPE cells (ARPE-19 cell line) were exposed to in vitro oxidative stress by 10 ng/ml tumor necrosis factor-α and 600 μM H_2O_2. We observed an increase of apoptosis in cells exposed to oxidative stress, which could be significantly attenuated by 1 hour PEBT treatment at concentrations of 7.5 and 10 μM. This effect was observed even 6 hours after oxidative stress induction. PEBT at concentrations of 5 and 10 μM inhibited oxidative stress-induced poly (ADP-ribose) polymerase (PARP) cleavage. Likewise, PEBT at both concentrations restored extracellular-signal-related kinase (ERK) phosphorylation decreased by oxidative stress. c-Jun N-terminal kinase and p38 phosphorylation were not altered by PEBT and/or oxidative stress. Interleukin-1 β-stimulated cyclooxygenase-2 (COX-2) gene promoter and oxidative stress-induced COX-2 expression were not inhibited by PEBT. The protective mechanism exerted by PEBT against oxidative stress may involve PARP cleavage, regulation of ERK pathway, as well as its known antioxidant properties. In summary, these results showed, for the first time, that PEBT is an antiapoptotic compound in ARPE-19 cells and may have a potential therapeutic role in oxidative stress-related retinal diseases.

Keywords: Tellurium; Organotellurium; Retinal Pigment Epithelial; Oxidative Stress; Apoptosis

1 Introduction

The retinal pigment epithelium (RPE) cells perform a wide variety of critical functions during the retina embryonic development as well as throughout adult life to maintain normal vision (Grunwald, 2009). The RPE, located between the light sensitive outer segments of the photoreceptors and the blood supply of the choroid, is required for light absorption, epithelial transport, retinoid recycling, and removal of molecular components that shed from the photoreceptor in order to have continued vision (Strauss, 2005). The functions and location of this tissue, coupled with the fact that RPE cells are non-mitotic, have contributed to the theory that the RPE accumulates oxidative damage throughout the life. Such oxidative damage produces tissue dysfunction over time, predisposing the development of age-related macular degeneration (AMD), which is the leading cause of blindness in the elderly all around the world (Beatty et al., 2000; Cai et al., 2000).

Aging predisposes cells to oxidative stress, a condition where the amount of intracellular oxidizing agents becomes increased with a concurrent decline in the defensive systems of the cells (Cannizzo et al., 2011). In the RPE, H_2O_2 is produced during daily phagocytosis of shed photoreceptor outer segments (Stinson et al., 1991; Miceli et al., 1994) and is generated as a consequence of light irradiation of the pigment melanin (Sarna et al., 2003). The phagocytosis-generated H_2O_2 causes mitochondrial dysfunction and damage mitochondrial DNA in human RPE cells (Ballinger et al., 1999). Oxidative stress is one of the most common apoptosis inducing factors in several organs, from the intestinal and cardiovascular systems to neuronal cells (Ferencz et al. 2002; Vaudry et al. 2002; Racz et al. 2007). Moreover, it has been shown that also in pigment epithelial cell death, contributes in the pathogenesis of retinal diseases (Beatty et al., 2000; Kalariya et al. 2008; Kook et al. 2008).

The research of novel and potent antioxidants plays an important role in targeting therapeutic interventions in several diseases. In this way, studies have reported the antioxidant activity of several organotellurium compounds (Cunha et al., 2009; Ba et al., 2010). Since these sort of compounds may mimic glutathione peroxidase activity (Engman et al., 1994), there has been a considerable interest in organotellurium compounds as potential antioxidants against several prooxidant agents (Briviba et al.,1998; Jacob et al., 2000; Ren et al., 2001; Borges et al, 2008; Gay et al., 2010; Avila et al., 2012). Telluroacetylenes, a class of organotellurium compounds, exhibit antioxidant effect on rat brain homogenate in vitro (Souza et al., 2009). Moreover, 2-phenylethinyl-butyltellurium (PEBT) (Figure 1), a telluroacetylene compound, protects against oxidative damage caused by sodium nitroprusside in mouse brain, suggesting an antioxidant effect of this compound in vivo (Souza et al., 2009). This makes PEBT a promising candidate for the use in the developing antioxidant therapies.

Considering the above mentioned and given that RPE is frequently exposed to high oxidative stress, the present study was designed to explore whether apoptosis triggered by oxidative stress can be attenuated by PEBT in ARPE-19 cells, a human RPE cell line.

2 Materials and methods

2.1 Chemicals

PEBT was prepared according to the literature method (Comasseto et al., 1996). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed that PEBT synthesized exhibited analytical and spectroscopic data in full agreement with its assigned structure. PEBT was diluted in dimethyl sulfoxide. Media and additives were purchased from Invitrogen, and FBS was purchased from Tissue Culture Biologicals Inc. (Seal Beach, CA). Tumor necrosis factor-alpha (TNF-α),

Interleukin-1 beta (IL-1β), and Hoechst 33342 were purchased from Sigma (St. Louis, MO). A construct that carried an 830-bp cyclooxygenase-2 (COX-2) promoter DNA fragment, which drives luciferase expression, was used to study modulation of proinflammatory gene expression. FuGENE 6 Transfection Reagent was obtained from Promega. COX-2, poly (ADP-ribose) polymerase (PARP), extracellular-signal-related kinase (ERK), p38, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). c-Jun N-terminal kinase (JNK) antibody was obtained from Cell Signaling Technology (Danvers, MA). Buffers and supplies for Western blot and Immunocytochemistry were obtained from Invitrogen, Bio-Rad, and Sigma.

2.2 ARPE-19 Cell Culture

ARPE-19 cells (a human RPE cell line) were obtained from American Type Culture Collection (Manassas, VA) and used at passages 25-35 in all experiments. ARPE-19 cells were plated and grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 ^{0}C, 5% CO_2, 99% relative humidity for 72 hours (Philp et al., 2003).

2.3 Exposure of ARPE-19 Cells to Oxidative Stress

After 72 h growth, ARPE-19 cells were serum-starved for 8 hours in DMEM/F12 medium containing 0.5% FBS. The serum-starved cells were pretreated with PEBT (1.0, 2.5, 5.0, 7.5, and 10 μM). Oxidative stress was induced 1 hour after PEBT exposure by treatment with TNF-α (10

ng/ml) and H_2O_2 (600 µM) (Mukherjee et al., 2004) for 14 hours to detect Hoechst-positive apoptotic cells or for 6 hours for Western blot and Immunocytochemistry analyses (Figure 2). A post-treatment with PEBT (10 µM) was performed at 2, 4, 6, and 8 hours after oxidative stress to detect Hoechst-positive apoptotic cells. An experiment without oxidative stress induction was also carried out to evaluate the survival of ARPE-19 cells treated only with PEBT (5, 10, and 50 µM) for 15 hours.

2.4 Hoechst Staining

Hoechst staining was performed in 6-well plates. ARPE-19 cells were fixed with methanol for 15 min, washed with 1X phosphate-buffered saline (PBS), and then loaded with 2 µM of Hoechst in 1X PBS for 15 min at room temperature before imaging using a Nikon DIAPHOT 200 microscope under UV fluorescence. Images were recorded by a Hamamatsu Color Chilled 3CCD camera and Photoshop 5.0 software (Adobe Systems, Mountain View, CA).

2.5 Transfection Assay

ARPE-19 cells growing in 6-well plates for 24 hours were transfected with 5 µg of a human COX-2-luciferase construct (830 bp) by FuGENE 6. Forty-eight hours later, ARPE-19 cells were serum starved for 8 hours and pretreated with PEBT (5 and 10 µM). One hour later, cells were treated with IL-1β (10 ng/ml) for 6 hours (Mukherjee et al., 2004). Cell homogenates were made and luciferase assay was performed.

2.6 Luciferase Activity Assay

Cells were mixed with 2X luciferase assay buffer [Analytical Luminescence Laboratory (ALL) buffer, 0.2 M K2PO4/2 mM dithiothreitol/30 mM MgSO4/10 mM ATP]. The reaction was initiated by 1 mM luciferin and the relative light units were determined by using an ALL luminometer recording over a 20 sec interval. The luciferase assays were performed on triplicate and normalized for protein content with the Bio-Rad protein assay kit.

2.7 Western Blot Analysis

ARPE-19 cells growing in 6-well plates were exposed to conditions previously described in 2.3 section. The protein content was estimated (Bio-Rad) and the samples were boiled for 3 min with 2X Laemmli buffer containing 100 mM of dithiothreitol. Equal amounts of cell extract protein (10–20 µg) were loaded onto and separated by a NuPAGE precast gel. The gel was run in an Invitrogen XCell running system using MOPS buffer. The transference took place in the iBLOT system following the manufacturer's instructions. Membranes were incubated with primary antibodies overnight at 4 °C, washed, and incubated for 1 hour with the secondary antibody at room temperature. The membranes were developed using ECL (GE Healthcare) following the manufacturer's instructions. For the standardization of the immunoblotting, GAPDH monoclonal antibody was used.

2.8 Immunocytochemistry

ARPE-19 cells were cultured in 4-well chamber slides stimulated according to the experimental design (2.3 section), and fixed with methanol for 15 minutes at –20°C. Blocking was performed with 5% BSA and 0.3% Triton for 2 hours at room temperature. Cells were incubated with antibody against phosphorylated ERK in 1% goat serum in PBS overnight at 4°C.

Secondary antibody conjugated with Alexa Fluor 488 was used for 2 hours at room temperature. Finally, the nuclei were stained with Hoechst as above described. Slides were mounted in Vectashield fluorescent medium (Vector Laboratories, Burlingame, CA) and analyzed using Axioplan 2 deconvolution microscope. Images were obtained and processed using SlideBook 4.2 and 5.0 software (Intelligent Imaging Innovations Inc., Denver, CO). Cellular counting was made using the Image J program (Java-based image-processing program. National Institutes of Health) with a magnification of 10× by clicking on immuno-positive cells. The counting was carried out by a treatment blind investigator.

2.9 Statistical Analysis

The results are presented as mean ± S.E.M.. Data were analyzed using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls test when appropriate. P values less than 0.05 ($P<0.05$) were considered as indicative of significance.

3 Results

3.1 PEBT protects ARPE-19 cells from oxidative stress-induced apoptosis

Figure 3 shows the PEBT effect against oxidative stress-induced apoptosis in ARPE-19 cells. Oxidative stress induced by 10 ng/ml TNF-α and 600 μM H_2O_2 led to a marked increase in Hoechst-positive cells. PEBT at concentrations of 7.5 and 10 μM, incubated 1 hour before oxidative stress, attenuated the damage by decreasing the Hoechst-positive cells (Figures 3A and B). The presence of 10 μM PEBT, even 6 hours after TNF-α/ H_2O_2, ensured protection (Figures 3C and D). ARPE-19 cells when treated only with PEBT (5, 10, and 50 μM) maintained the survival and the number of cells the same of those of the control group (Figure 4).

3.2 Oxidative stress-induced PARP cleavage is attenuated by PEBT

Western blot analysis of PARP cleavage confirmed the antiapoptotic effect of PEBT (Figure 5). Pretreatment of PEBT at concentrations of 5 and 10 μM reduced oxidative stress-induced PARP cleavage to control values. PEBT at a concentration of 10 μM without the presence of TNF-α/H_2O_2 did not change the PARP cleavage.

3.3 IL-1β- and oxidative stress-mediated COX-2 expression are not inhibited by PEBT

TNF-α/H_2O_2-induced oxidative stress increased COX-2 expression in ARPE-19 cells analyzed by Western blot (Figure 6A). Transfected cells with the human COX-2 promoter containing a luciferase reporter gene showed a prominent increase in COX-2 promoter expression induced by IL-1β (Figure 6B). PEBT, at both concentrations, was not effective in counteract the proinflammatory COX-2 gene and protein expression. PEBT at 10 μM without the presence of TNF-α/H_2O_2 or IL-1β did not change COX-2 expression.

3.4 PEBT restores ERK phosphorylation decreased by oxidative stress

Oxidative stress significantly decreased ERK phosphorylation, while PEBT completely restored this parameter evaluated by Western blot (Figure 7F). Subsequent experiments with immunocytochemistry analysis indicated that TNF-α/H_2O_2 treated cells showed faint phospho-ERK staining, confirming the inhibition of ERK by oxidative stress. PEBT 10 μM effectively increased phospho-ERK staining to control levels (Figures 7A-E). Together with western blot analysis, the immunocytochemistry experiment showed the PEBT protection against the

oxidative stress-induced inhibition of ERK phosphorylation in ARPE-19 cells. PEBT alone did not alter phospho-ERK expression. JNK and p38 phosphorylation were also investigated by Western blot and no differences were founded (Figures 8A and 8B).

4 Discussion

The present study was conducted to investigate for the first time the PEBT effect on oxidative stress-triggered apoptosis in human pigment epithelial cells (ARPE-19 cell line). The results indicated that PEBT prevented ARPE-19 cells from apoptosis caused by H_2O_2/TNF-α-induced oxidative stress. The mechanism of PEBT induced cytoprotection was probably through the ERK pathway and the inhibition of PARP cleavage.

The ARPE-19 cells used in this study are widely utilized as a model of human RPE that exhibit lower variability than primary RPE cultures and posses RPE-like properties, including polarization, tight junction formation, phagocytosis of rod outer segments and immunologic responses (Dunn et al., 1996; Fukuoka and Medof, 2001; Chowers et al., 2004).

The retina, which is exposed to both sunlight and very high levels of oxygen, is exceptionally rich in polyunsaturated fatty acids, which makes it a favorable environment for the generation of reactive oxygen species (Beatty et al., 2000). Therefore, accumulated oxidative damage in the largely non-mitotic RPE monolayer is likely to cause tissue dysfunction that may contribute to the pathogenesis of retinal diseases (Delcourt et al., 1999; Beatty et al., 2000; Cai et al., 2000). Oxidative stress-induced apoptosis is a major contributor to cell death, and it has been shown to play a role also in pigment epithelial cell degeneration (Kalariya et al. 2008; Kook et al. 2008; Antonelli et al. 2012; Kalonia et al. 2012). The present study has shown that PEBT protects ARPE-19 cell line against oxidative stress-induced apoptotic cell death. This result provided

evidence by the decrease in Hoechst-positive cells which were markedly increased by the combination of TNF-α and H_2O_2. Not only the PEBT pre-treatment was effective but also the post-treatment protected against the H_2O_2/TNF-α-induced apoptosis in ARPE-19 cells. The PEBT activity lasted until six hours after the induction of oxidative stress, displaying a wide window of cytoprotection. Besides, it is important to mention that PEBT did not alter the survival of ARPE-19 cells.

Once oxidative stress can trigger apoptosis, the cleavage of PARP was evaluated to gain some insight into the protective mechanism of PEBT in ARPE cells. In response to oxidative stress in those cells, apoptotic pathway is turned on, resulting in caspase activation (Mukherjee et al., 2004). Caspase, in turn, inactivates PARP by cleavage, inducing to cell death. The PARP cleavage has been regarded as an evidence of caspase activation and has been widely used as a hallmark of cell apoptosis (Boulares et al., 1999; Hassa et la., 2006). In this study, we found an increase in PARP cleavage cells exposed to oxidative stress. In the presence of PEBT, the level of PARP cleavage was restored suggesting an anti-apoptotic activity of this compound. Accordingly, AS101, an organotellurium compound, rescues neurons from apoptosis in a Parkinson's disease model, in great part due to inhibition of apoptotic processes exerted by caspases (Sredni et al., 2007). Moreover, AS101 also mediates antiapoptotic effects in this fulminant hepatic failure model by decreasing PARP cleavage and enhancing non-cleaved fragment formation, which mediates DNA repair (Brodsky et al., 2009).

Oxidative stress and inflammation are known to be associated with RPE apoptosis in AMD (Dunaief et al., 2002; Liang and Godley, 2003; Jiang et al., 2008; Augustin and Kirchhof, 2009). COX-2, another major factor in cell damage, is an inducible enzyme that catalyzes the synthesis of prostaglandins and is involved in oxidative stress and cell function. COX-2 is

actively regulated in the RPE (Ershov and Bazan, 1999). Transfect ARPE-19 showed a prominent increase in COX-2 promoter expression induced by IL-1β. Furthermore, oxidative stress caused enhanced COX-2 expression. PEBT neither counteracted the cytokine COX-2 gene induction nor inhibited the oxidative stress-triggered high COX-2 expression in ARPE-19 cell line. Thus, due to a lack of defense against proinflammatory agents, this parameter might not be accountable for the protective effect of PEBT in oxidative stress-induced apoptosis.

MAPKs (mitogen-activated protein kinases) are important regulatory proteins through which various extracellular signals are transduced into intracellular events. MAPKs, which include ERK, JNK, and p38 subfamilies, also control cell survival (Waskiewicz and Cooper, 1995; Robinson and Cobb, 1997). By and large, activation of ERK has been linked to cell survival, whereas JNK and p38 are linked to induction of apoptosis (Xia et al., 1995). The current results showed that both JNK and p38 phosphorylation remained unchanged by oxidative stress and/or PEBT treatments. In contrast, treatment of cells with H_2O_2/TNF-α inhibited ERK by decreasing ERK phosphorylation, which was completely prevented by PEBT. This data goes along with previous studies that suggested that over activation of ERK may participate in the defense signaling against oxidative stress damage in cells (Glotin et al., 2006). The immuncytochemistry analysis corroborates with the western blot one, since PEBT increased the faint phospho-ERK staining induced by oxidative stress. Likewise, the organotellurium AS101 prevents apoptosis and induces cell differentiation and survival by activating the ERK pathway (Makarovsky et al., 2003). Our findings support a protective role of PEBT in ARPE-19 cells exposed to a H_2O_2/TNF-α challenge through the activation of ERK inhibited by oxidative stress. Accordingly, the ERK mediated survival signal has been suggested to protect ARPE-19 against

oxidative stress-induced damage, while the prevention of ERK phosphorylation reduces this protective effect (German et al., 2006; Tsao et al., 2006; Dong et al., 2011).

Although the exact mechanism by which PEBT exerts protective effects against oxidative stress in ARPE-19 cells requires to be further elucidated, our results point out the involvement of the ERK signaling pathway, since this compound restores the ERK phosphorilation, as well as the reduction of PARP cleavage, confirming the antiapoptotic effect of PEBT. In addition, the antioxidant properties of PEBT may contribute to its ameliorative effect of oxidative stress-induced apoptosis. Previous studies afford this statement, once PEBT restores lipid peroxidation, protein oxidation induced by sodium nitroprusside, and shows radical scavenging property *in vitro* at concentrations equal or lower than 10 µM (Souza et al., 2009). In other words, PEBT exhibited antioxidant activity at the same concentrations in which the antiapoptotic effect was observed in ARPE-19 cells.

Taken together, our results indicate that PEBT has antiapoptotic effects in oxidative stress-induced cell death in retinal pigment epithelial cells. The protective mechanism exerted by PEBT may involve the regulation of ERK pathway and inhibition of PARP cleavage associated with its antioxidant properties. Hence, this compound may be a useful pharmacological tool capable of preventing oxidative stress cell death in human ocular diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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Figure legends

Figure 1: Chemical structure of 2-phenylethynyl-butyltellurium (PEBT).

Figure 2: Experimental schedule.

Figure 3: TNF-α/H_2O_2-induced oxidative stress in ARPE-19 cells is inhibited by PEBT. After plating, cells were serum starved and exposed to 10 ng/ml TNF-α/600 μM H_2O_2. Apoptosis was analyzed after 14 hours. PEBT was added 1 hour before (**A** and **B**) or at different times after oxidative stress (**C** and **D**). The figures **A** and **C** illustrate the appearance of Hoechst-positive cells and the figures **B** and **D** demonstrate the percentage of apoptosis upon exposure to TNF-α/ H_2O_2 and the effect of PEBT. Data are reported as mean ± S.E.M. * $p<0.05$ as compared to the OS group (black bar) (one way ANOVA/Newman-Keuls). *Abbreviation: OS – oxidative stress.*

Figure 4: Effect of PEBT in ARPE-19 cells. After plating, cells were serum starved. PEBT was incubated at different concentrations. Hoechst staining was analyzed after 15 h. (**A**) Illustrates the Hoechst positive cells upon exposure to PEBT. (**B**) Shows the number of cells per field. (**C**) Represents the percentage of survival cells. Data are reported as mean ± S.E.M. (one way ANOVA/Newman-Keuls).

Figure 5: Oxidative stress-induced PARP cleavage is attenuated by PEBT. After plating, cells were serum starved and exposed to 10 ng/ml TNF-α/600 μM H_2O_2 for 6 hours. PEBT was

incubated one hour before H_2O_2/TNFα exposure. Data are reported as mean ± S.E.M. *p<0.05 as compared to control group (white bar). #p<0.05 as compared to OS group (black bar) (one way ANOVA/Newman-Keuls). *Abbreviation: OS – oxidative stress.*

Figure 6: IL-1β- and oxidative stress-mediated COX-2 expression are not inhibited by PEBT. (**A**) After plating, cells were serum starved and exposed to 10 ng/ml TNF-α/600 μM H_2O_2 for 6 hours. PEBT was incubated one hour before H_2O_2/TNFα exposure. (**B**) ARPE-19 cells were transfected with human COX-2 (830 bp) promoter-luciferase construct. After plating, cells were serum starved and exposed to 10 ng/ml IL-1β for 6 hours. PEBT was added one hour before IL-1β exposure. Data are reported as mean ± S.E.M. *p<0.05 as compared to control group (white bar) (one way ANOVA/Newman-Keuls).

Figure 7: PEBT restores ERK phosphorylation decreased by oxidative stress. After plating, cells were serum starved and exposed to 10 ng/ml TNF-α/600 μM H_2O_2 for 6 hours. PEBT was incubated one hour before H_2O_2/TNFα exposure. (**A**, **B**, **C** and **D**) Represent immunocytochemistry staining of phospho-ERK (green). Nuclei were visualized using Hoechst (blue) Scale bar: 50 μm. (**E**) Shows quantitative analysis of phospho-ERK immunopositive cells. (**F**) Represents phospho-ERK expression by immunoblotting. Data are reported as mean ± S.E.M. *p<0.05 as compared to control group (white bar). #p<0.05 as compared to OS group (black bar) (one way ANOVA/Newman-Keuls). *Abbreviation: OS – oxidative stress.*

Figure 8: JNK and p38 phosphorylation are not modified after incubation with PEBT and in ARPE-19 cells oxidative stress. After plating, cells were serum starved and exposed to 10 ng/ml TNF-α/600 μM H_2O_2 for 6 hours. PEBT was incubated one hour before H_2O_2/TNFα exposure. (**A**) Represents JNK and (**B**) shows p38 expression. Data are reported as mean ± S.E.M. (one way ANOVA/Newman-Keuls). *Abbreviation: OS – oxidative stress.*

Figure 1

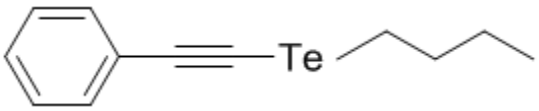


Figure 2

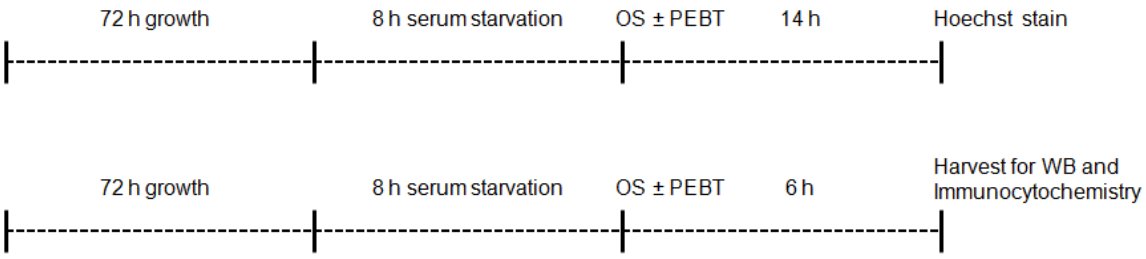
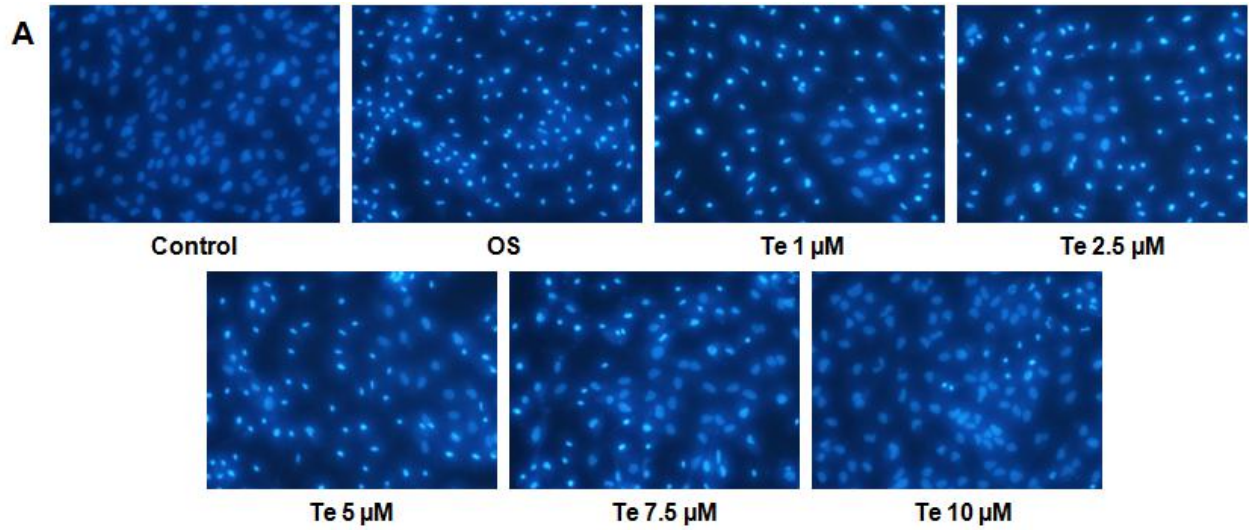


Figure 3A



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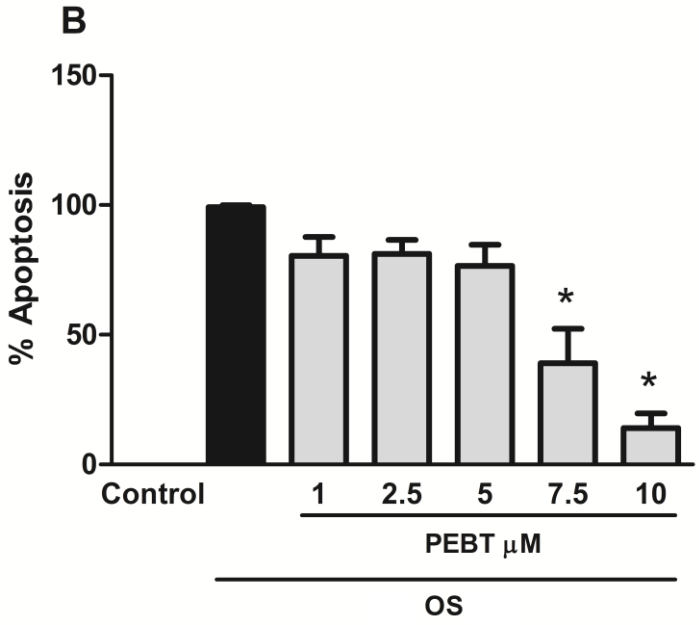
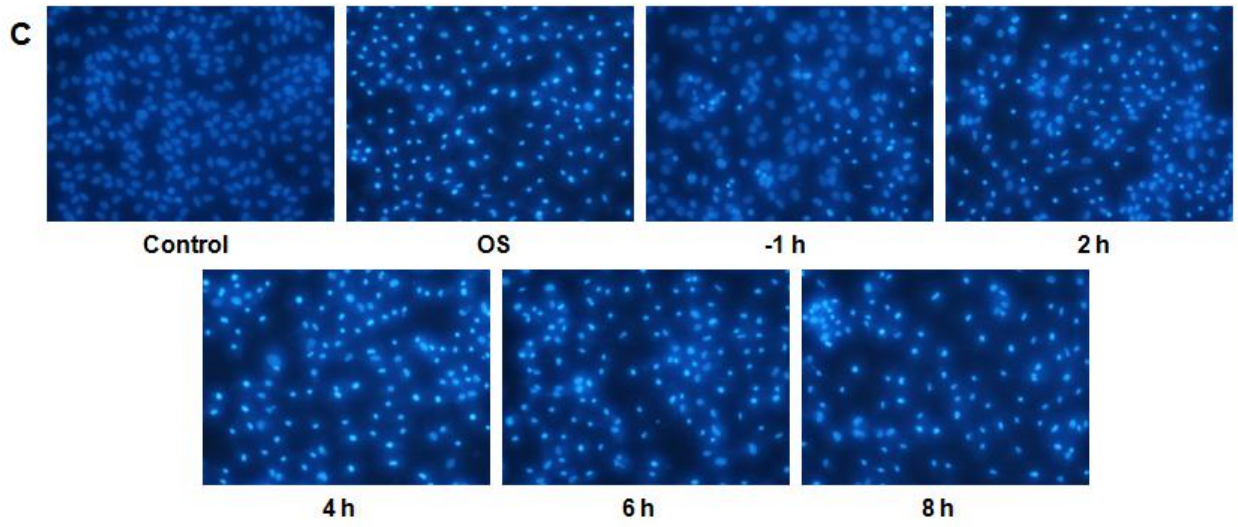


Figure 3C



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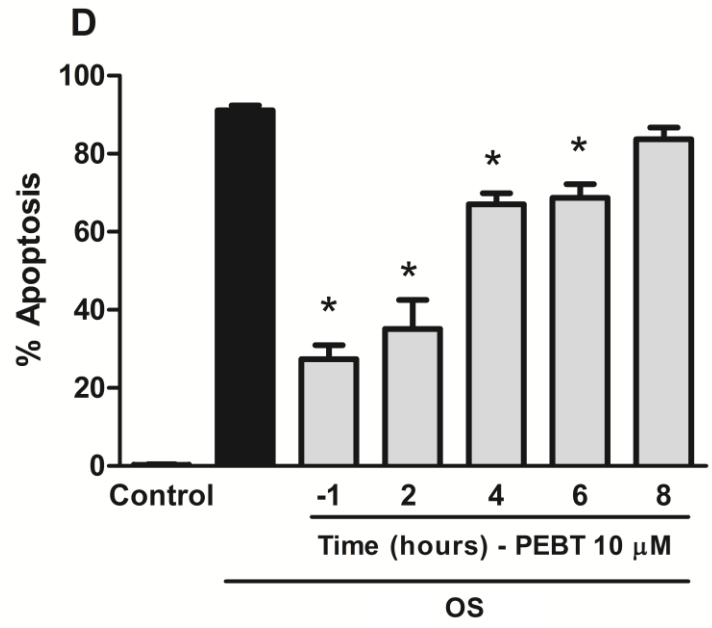

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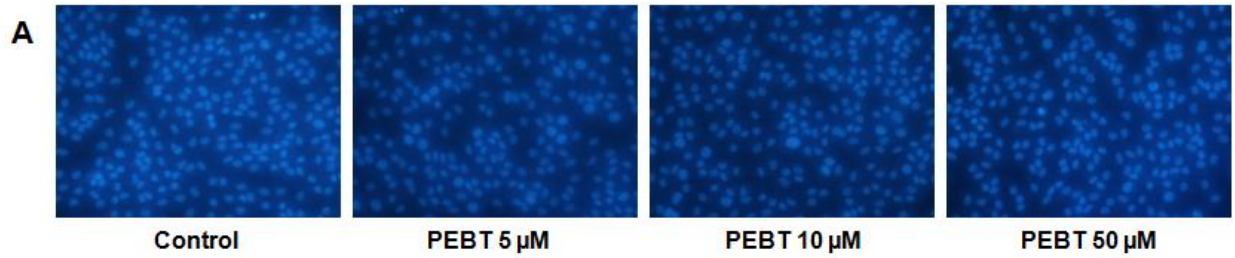

Figure 4B

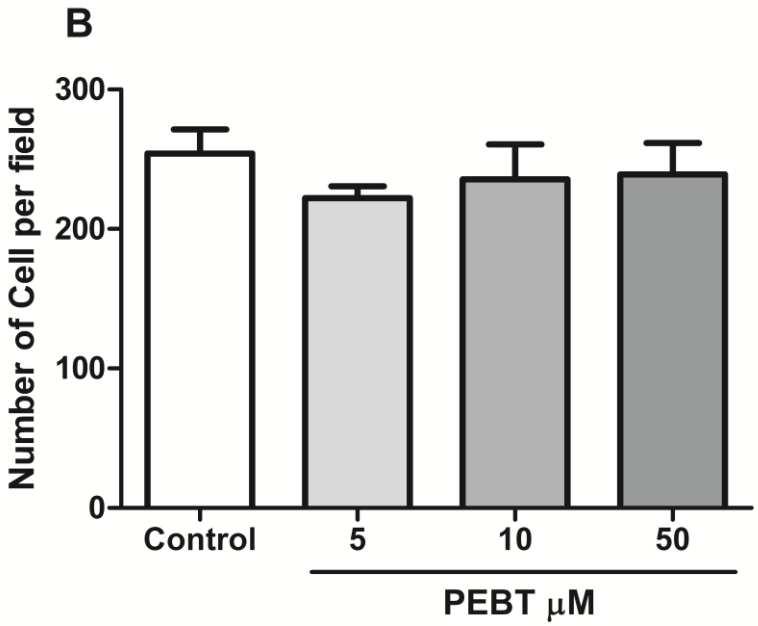

Figure 4C

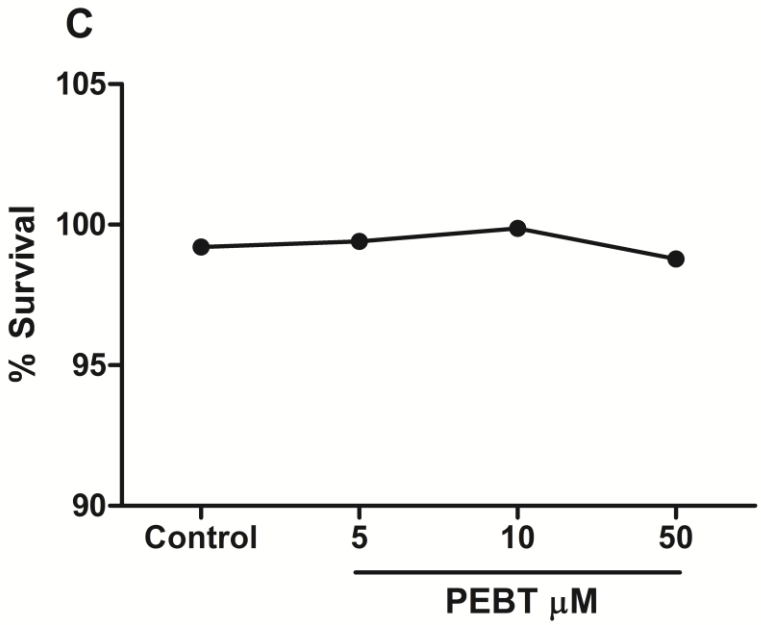

Figure 5

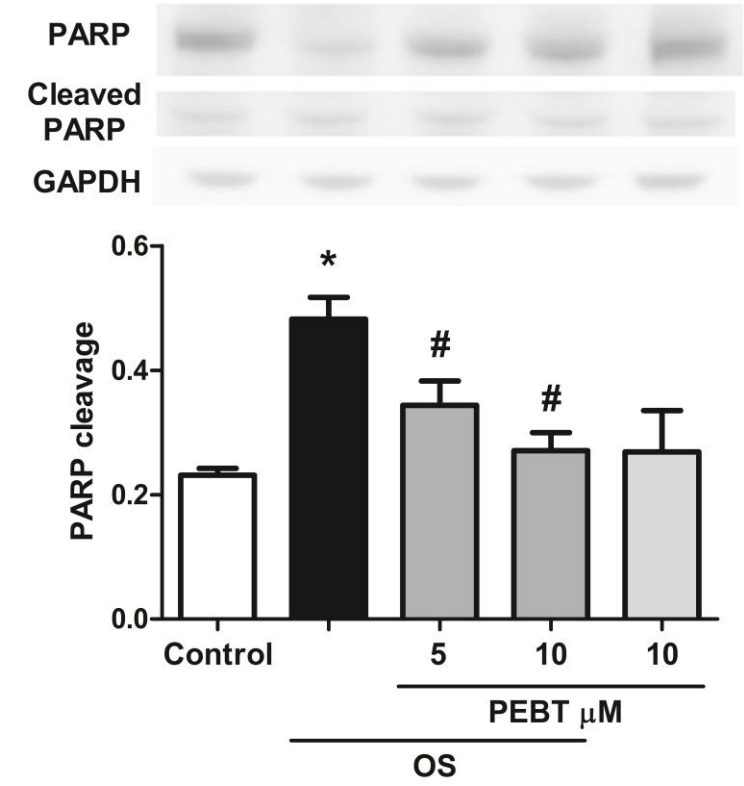
PARP
Cleaved PARP
GAPDH
PARP cleavage
0.6
0.4
0.2
0.0
*
#
#
Control
5
10
10
PEBT μM
OS

Figure 6A

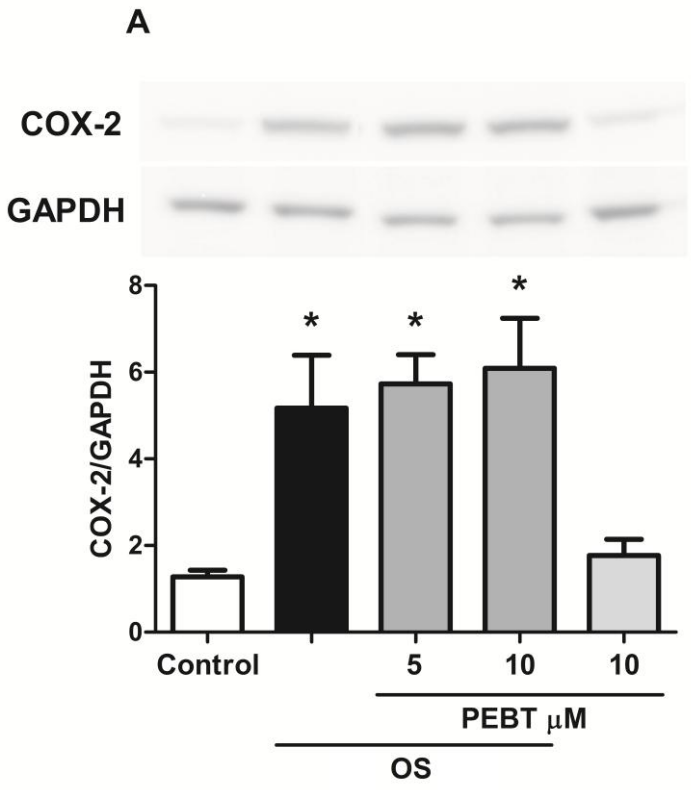

Figure 6B

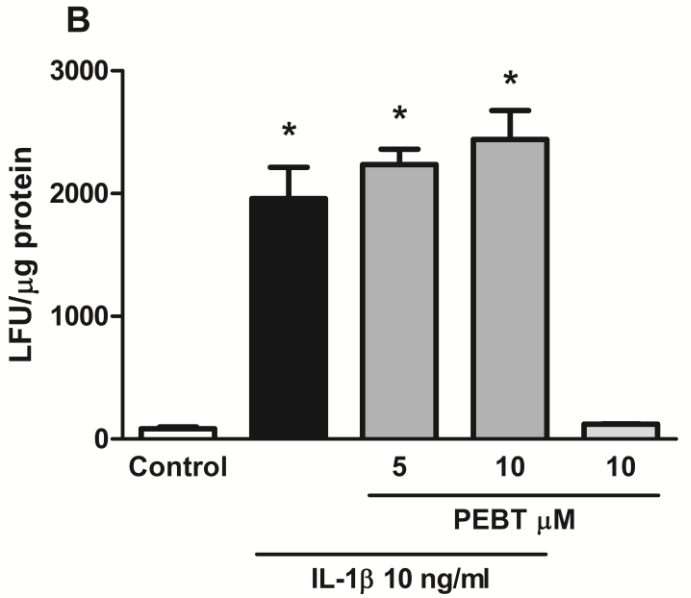

Figure 7A,B,C,D

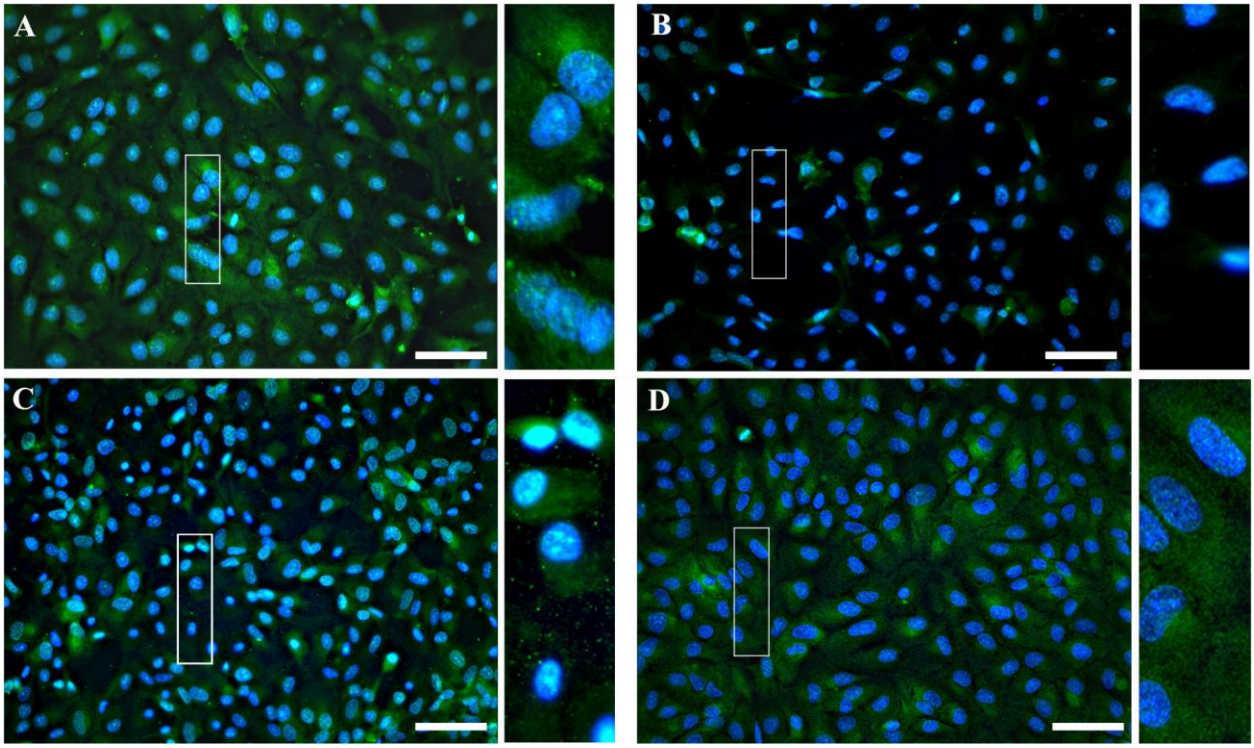

Figure 7E

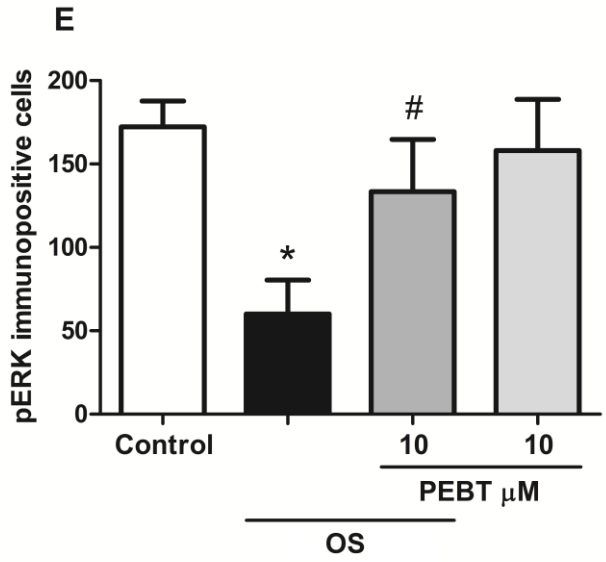

Figure 7F

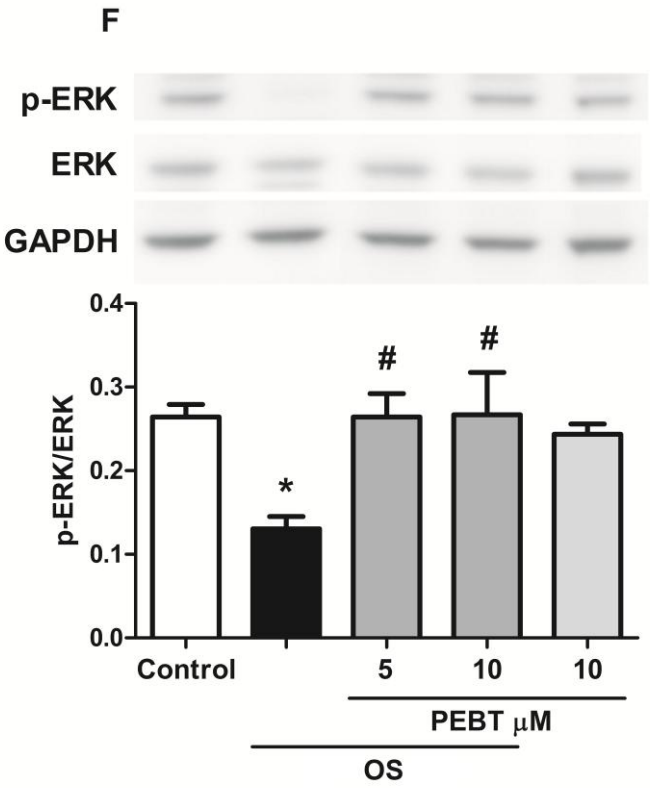
F
p-ERK
ERK
GAPDH
p-ERK/ERK
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Figure 8A

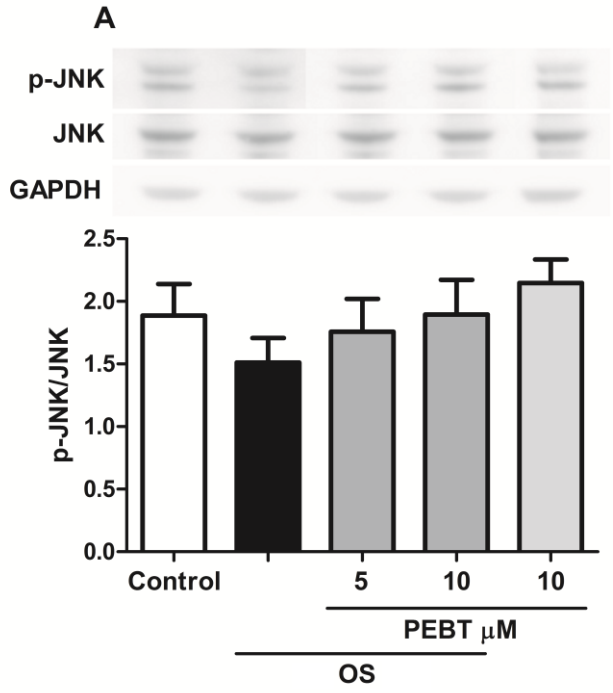
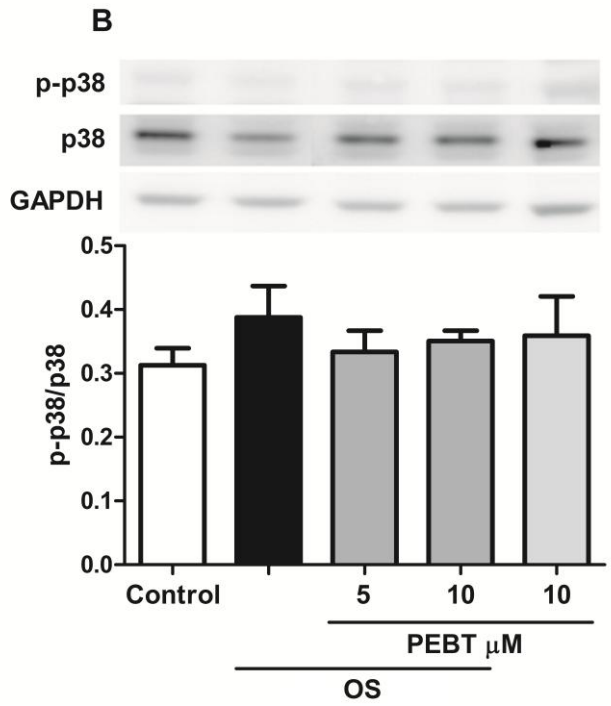


Figure 8B



4 DISCUSSÃO

Existem três etapas definidas no processamento da memória: aquisição, consolidação e evocação (Izquierdo, 2002). Durante a aquisição ocorre a integraçao entre estímulos. Nesta estapa o traço mnemônico é muito intenso, portanto experiências recentes são recordadas com mais nitidez. Das memórias adquiridas nem todas serão preservadas. A consolidação consiste no processo de filtração e na posterior fixação do traço mnemônico necessário para a posterior evocação (McGaugh, 2000; Abel e Lattal, 2001). Uma memória só pode ser medida quando for evocada ou expressa, assim a melhor maneira de estudar e avaliar o armazenamento da memória se dá através da evocação, quando observamos a mudança de comportamento do animal devido ao processo de memorização. A memória pode ser modulada por fatores externos ao seu processo de formação (Izquierdo, 2002).

De modo geral, os resultados aqui apresentados mostram que uma única adminstração de PEBT é capaz de melhorar as três etapas da memória estudadas nesta tese, aquisição, consolidação e evocação, na tarefa da esquiva inibitória (**Artigos I, II e Manuscrito I**). Observou-se que a facilitação da transmissão glutamatérgica pode estar envolvida no efeito do PEBT sobre a memória (**Artigo I**). Mais além, verificou-se que o PEBT melhorou o desempenho da memória em modelos de dano cognitivo induzidos por Aβ(25-35) e escopolamina em camundongos (**Artigo II e Manuscrito I**). Posteriormente, a atividade antiapoptótica do PEBT foi demonstrada *in vitro* (**Manuscrito II**).

Os resultados obtidos no **Artigo I** revelam que uma única dose de PEBT 1 hora antes do treino, imediatamente após o treino ou 1 hora antes do teste da esquiva inibitória é capaz de melhorar a aquisição, consolidação e evocação da memória, respectivamente. Estes resultados foram evidenciados devido ao aumento na latência em que os camundongos tratados com PEBT descem na grade em comparação com os animais tratados apenas com o veículo. Os achados do **Manuscrito I** confirmam a melhora da memória causada pelo PEBT nas fases de aquisição e evocação avaliadas na esquiva inibitória. Entretanto, não houve diferença na consolidação da memória, provavelmente devido a mudanças no tempo de tratamento. Surpreendentemente, 10

dias de tratamento com PEBT, em dose menor à anteriormente usada, também foi eficaz em melhorar a memória no teste da esquiva inibitória em camundongos (**Artigo II**).

A formação da memória envolve fenômenos plásticos, como o aumento da produção e liberação do neurotransmissor glutamato (Dudai, 2004; Izquierdo, 2002). Dessa maneira, foi estudada uma possível interação entre o PEBT e o sistema glutamatérgico através da captação e liberação de glutamato em córtex e hipocampo de camundongos tratados com PEBT por 1 ou 24 horas. Os resultados mostraram que a liberação de glutamato não foi alterada em ambas as estruturas estudadas após 1 ou 24 horas da adminstração de PEBT. Por outro lado, o PEBT inibiu a captação de glutamato tanto em córtex quanto em hipocampo, aumentando o tônus glutamatérgico. Então, a inibição da captação de glutamato parece ser, pelo menos em parte, um dos mecanimos pelo qual o PEBT melhora a memória em camundongos. De acordo com esta hipótese, foi mostrado que um aumento na transmissão glutamatérgica é capaz de melhorar a memória (Daisley et al., 1998; Lhullier et al., 2004; Mameli et al., 2005). Os transportadores de glutamato são susceptíveis às variações do estado redox de suas proteínas (Trotti et al., 1998). Levando em consideração que o PEBT pode oxidar grupos tiólicos (Souza et al., 2009), é possível supor que o PEBT inibe os transportadores de glutamato via oxidação de grupos tiólicos presentes nos mesmos. Esta oxidação de grupos tiólicos parece ser espontaneamente recuperada, visto que, após 24 horas de tratamento com o PEBT, a inibição da captação de glutamato no córtex cerebral voltou aos níveis do controle (**Artigo I**).

É importante destacar que os tratamentos com o PEBT utilizados neste estudo foram avaliados quanto a uma possível toxicidade. Testes de campo aberto foram realizados para detectar alterações motoras ou exploratórias que poderiam afetar o desempenho dos animais tratados com o PEBT nos testes aqui avaliados. Os resultados mostraram que não houve alteração no teste do campo aberto, descartando interferências locomotoras sobre o aprendizado e a memória dos animais tratados com o PEBT. Igualmente, não houve alterações nos animais tratados com Aβ e escopolamina, comprovando que os danos de memória não são devidos a alterações locomotoras (**Artigos I, II e Manuscrito I**). Reforçando essa ideia, a distância percorrida e a velocidade de natação de todos os animais tratados não foram alteradas na tarefa do labirinto aquático de Morris (**Artigo II e Manuscrito I**). Também não foram observadas diferenças entre animais tratados, de forma aguda ou subcrônica, com o PEBT e o veículo nos

parâmetros de toxicidade hepática (AST e ALT) ou renal (ureia) estimados neste trabalho (**Anexo I**).

Visto que o número de pessoas com demência está previsto para dobrar a cada 20 anos e a prevalência de DA quase duplica a cada 5 anos após os 65 anos de idade (Brookmeyer et al., 2007), e dada a escassez de medidas terapêuticas efetivas para o déficit de memória, evidencia-se a importância da busca de novos compostos. Então, a melhora da memória em camundongos causada pelo PEBT leva ao estudo deste composto em modelos de dano de memória em uma tentativa de buscar uma ferramenta farmacológica capaz de evitar a insidiosa perda de memória característica de doenças neurodegenerativas.

Os déficits cognitivos relacionados à DA estão principalmente associados à degeneração de neurônios colinérgicos, resultando em déficits da neurotransmissão colinérgica (Ubhi e Masliah, 2012). Sendo a escopolamina um antagonista muscarínico, esta droga é utilizada em modelos de estudo da amnésia em que se objetiva a procura de compostos que tenham capacidade de reverter os danos causados pela hipofunção colinérgica causada pela sua administração. No **Manuscrito I**, constatou-se que, quando os camundongos receberam uma única dose de PEBT antes da adminstração intraperitoneal de escopolamina, houve uma melhora significativa no desempenho dos camundongos no teste do labirinto aquático de Morris. Enquanto os animais tratados com a escopolamina apresentaram aumento na latência para achar o local da plataforma e diminuição nos cruzamentos sobre este local no dia do teste, o PEBT protegeu contra o dano na memória espacial causado pela escopolamina.

Os resultados do **Manuscrito I** mostraram, ainda, que a escopolamina, quando adminstrada antes do treino, imediatamente após o treino ou antes do teste da esquiva inibitória, prejudicou a aquisição, a consolidação e a evocação da memória, respectivamente. Embora o pré-tratamento com o PEBT não tenha sido eficaz em prevenir o dano na aquisição da memória no paradigma avaliado, o PEBT preveniu significativamente o dano na consolidação e evocação da memória causado pela escopolamina. Assim, o PEBT melhora a consolidação e evocação da memória, não apenas em condições fisiológicas, isto é, na ausência de dano (**Artigo I**), como também melhora estas fases da memória na presença de um prejuízo colinérgico.

Na DA, em que o sistema colinérgico é um dos principais alvos da neurodegeneração característica da patologia, o uso de inibidores da enzima acetilcolinesterase é uma das principais estratégias no manejo dos sintomas (Castellani et al., 2010). Pode-se observar que o PEBT não

modifica a atividade da acetilcolinesterase em córtex e hipocampo de camundongos tratados por 1 ou 24 horas com este composto. Assim, a atividade da acetilcolinesterase não está envolvida nos mecanismos de proteção do PEBT frente ao dano causado pela escopolamina (**Anexo II**).

Sabe-se que a acetilcolina está envolvida em muitas funções cognitivas, inclusive o aprendizado e a memória. Neurônios colinérgicos frequentemente inervam os terminais pré-sinápticos que modulam a atividade de vários sistemas no encéfalo. No hipocampo, por exemplo, a liberação pré-sináptica de acetilcolina pode regular a atividade de neurônios glutamatérgicos e GABAérgicos. Consequentemente, perturbações do sistema colinérgico podem levar a disfunções em outros sistemas de neurotransmissores e nas funções por eles desempenhadas (Gold, 2003; Miranda et al., 2003). Então, a interação dos sistemas glutamatérgico e colinérgico é importante para a memória (Pakpour et al., 2010). Por inibir a captação de glutamato (**Artigo I**), o PEBT pode aumentar o tônus glutamatérgico e, assim, melhorar o dano colinérgico causado pela escopolamina na consolidação e evocação da memória (**Manuscrito I**).

O peptídeo Aβ é o principal componente das placas senis e o maior contribuinte para a patogênese da DA, resultando em severa perda neuronal e disfunção sináptica (Selkoe, 2002; Newman et al., 2007). Tem sido proposto que o peptídeo Aβ(25-35) representa a região biologicamente ativa do Aβ(1-42) (Pike et al., 1995), sendo amplamente usado em experimentos tanto *in vitro* quanto *in vivo* como um modelo da DA (Misiti et al., 2005; Kosuge et al., 2006; Nassif et al., 2007). Neste contexto, o modelo de dano de memória causado pela adminstração intracerebroventricular do peptídeo Aβ(25-35) foi usado no **Artigo II**.

Utilizando-se o paradigma do labirinto aquático de Morris, que avalia a memória espacial de longo-prazo, observou-se um prejuízo no aprendizado no terceiro dia de treino no grupo tratado com Aβ. O tratamento diário com o PEBT foi capaz de proteger contra esse dano. No dia do teste, em que a plataforma submersa é retirada, os camundongos tratados com Aβ apresentaram um aumento na latência para achar o local da plataforma, bem como uma diminuição nos cruzamentos sobre este local, caracterizando o dano na memória. Esse dano foi completamente protegido pelo PEBT. A memória não espacial de longo prazo também foi avaliada. Utilizando-se o paradigma da esquiva inibitória, verificou-se que o PEBT restaurou completamente o dano na memória causado pela injeção intracerebroventricular de Aβ, de modo a aumentar a latência para descer na grade, que foi diminuída pelo Aβ. Assim, o tratamento

subcrônico com o PEBT foi capaz de melhorar o aprendizado e a memória dos animais tratados com Aβ avaliados no labirinto aquático de Morris e na esquiva inibitória (**Artigo II**).

Muitas evidências sugerem que o estresse oxidativo está envolvido no mecanismo de neurotoxicidade induzida por Aβ (Zhu et al., 2004). Foi verificado que o estresse oxidativo está aumentado em cérebro de pacientes com DA (Varadarajan et al., 2000; Butterfield et al., 2007a). Baseado nisto, antioxidantes como a vitamina E, *Ginkgo biloba* e o ácido ferúlico tem sido usados como uma tentativa de prevenir danos na DA (Sano et al., 1997; Christen, 2000; Yan et al., 2001). Embora parâmetros de estresse oxidativo não tenham sido avaliados no modelo de DA causado por injeção intracerebroventricular do peptídeo Aβ(25-35) (**Artigo II**), pode-se sugerir que a atividade antioxidante exercida pelo PEBT *in vitro* e *in vivo*, previamente descrita (Souza et al., 2009), pode contribuir para o efeito protetor deste composto no dano de aprendizado e memória causado por Aβ. O PEBT aumenta *per se* a atividade da enzima antioxidante glutationa peroxidase, protege contra a peroxidação lipídica e a oxidação de proteínas induzidas por estresse oxidativo, além de apresentar a atividade *scavenger* de radicais livres *in vitro* (Souza et al., 2009). Tal efeito antixidante do PEBT pode ter sido responsável, também, por proteger células do epitélio pigmentado da retina contra os efeitos citotóxicos induzidos pelo estresse oxidativo gerado por H_2O_2/TNF-α (**Manuscrito II**).

Estudos realizados em vários modelos celulares indicam que a morte celular induzida pelo Aβ é um processo apoptótico (Yankner et al., 1989; Cotman e Su, 1996). De acordo, o peptídeo Aβ pode causar estresse oxidativo, gerando radicais livres e produtos da peroxidação lipídica responsáveis pela neurodegeneração na DA (Butterfield et al., 2007b). Deste modo, tornou-se importante a investigação do efeito antiapoptótico do PEBT. Para isso, utilizou-se um modelo de estresse oxidativo para induzir apoptose em células do epitélio pigmentado da retina (linhagem ARPE-19). A ARPE-19 é uma linhagem celular de crescimento rápido que mantém as mesmas propriedades das células do epitélio pigmentado da retina humana (Dunn et al., 1996). Células ARPE-19 têm sido utilizadas no estudo de crescimento celular, diferenciação e apoptose (Janssen et al., 2000; Alizadeh et al., 2001; Sparrow e Cai, 2001). Os resultados do **Manuscrito II** apresentam o efeito antiapoptótico do PEBT em células ARPE-19. Estes resultados mostram que o PEBT reduziu a morte celular por apoptose induzida pelo estresse oxidativo gerado pela combinação de H_2O_2 e TNF-α. Tanto a pré- quanto a pós-incubação de PEBT protegeu contra a indução de apoptose. A ação antiapoptótica do PEBT foi verificada em até seis horas após a

indução do estresse oxidativo, evidenciando que o PEBT apresenta uma janela ampla de citoproteção. Visto que o PEBT protege as células ARPE-19 contra a morte por apoptose, pode-se predizer que este composto irá igualmente proteger os neurônios, uma vez que as células do epitélio pigmentado da retina têm origem neuroectodérmica semelhantemente às células gliais e neuronais (Bharti et al., 2011). Além disso, estudos mostraram que as células do epitélio pigmentado da retina podem ser diferenciadas em neurônios (Amemiya et al., 2004; Chen et al., 2006).

A clivagem da poli(ADP-ribose) polimerase (PARP) foi avaliada a fim de investigar os mecanismos pelos quais o PEBT poderia estar exercendo seu efeito protetor frente ao dano causado pelo estresse oxidativo nas células ARPE-19. A PARP está envolvida em vários processos celulares de reparação do DNA (Schreiber et al., 2006) e é alvo de caspases, as quais clivam a PARP inibindo sua atividade. Ao passo que o estresse oxidativo ativa caspases (Mukherjee et al., 2004), a clivagem da PARP tem sido utilizada como um marcador de morte celular por apoptose (Decker and Muller, 2002; Hassa et al., 2006). Neste trabalho, o estresse oxidativo induziu clivagem da PARP, a qual foi protegida pela incubação com o PEBT, confirmando seu efeito antiapoptótico. Estudos têm mostrado que a exposição de células ao Aβ apresentam ativação de caspase e, consequentemente, clivagem da PARP (Kim et al., 2007; Oh et al., 2011). Dessa forma, pode-se sugerir um efeito antiapoptótico do PEBT via clivagem da PARP em um modelo de neurotoxicidade induzido por Aβ.

Nas últimas décadas, o papel da COX-2 e prostaglandinas nas doenças cerebrais vem sendo extensivamente estudado. A expressão aumentada da COX-2 tem sido associada com a neurotoxicidade (Minghetti, 2007). Acredita-se que a COX-2 seja de grande importância nas respostas inflamatórias (O'banion, 1999). Os resultados mostraram que as células transfectadas com o promotor do gene da COX-2 tiveram a expressão desse gene aumentada devido à presença de IL-1β. Da mesma maneira, a exposição de células ao estresse oxidativo causou um aumento na expressão da enzima COX-2. O pré-tratamento com o PEBT não foi capaz de proteger contra a indução da expressão do gene e da enzima COX-2. Assim, o efeito antiapoptótico do PEBT em células ARPE-19 não pode ser explicado pela inibição do evento inflamatório induzido pelo estresse oxidativo (**Manuscrito II**). Em várias condições patológicas, como na doença de Alzheimer, ocorre o aumento da expressão da enzima COX-2 (Pasinetti, 1998; Minghetti et al., 1999; Mirjany et al., 2002). Assim, parece improvável que o PEBT possa melhorar o dano de

memória causado pelo Aβ via inibição de eventos inflamatórios. Apesar disso, mais estudos devem ser realizados para esclarecer a atuação do PEBT na cascata inflamatória.

A ativação da ERK está associada à sobrevivência e diferenciação neuronal (Qui e Green, 1992; Karmarkar et al., 2011). A ativação da ERK pode participar na defesa contra o dano celular induzido pelo estresse oxidativo (Glotin et al., 2006). Neste estudo, verificou-se que o estresse oxidativo induzido por H_2O_2/TNF-α diminuiu a fosforilação da ERK, inibindo-a. O PEBT restaurou significativamente a fosforilação da ERK aos níveis do controle, sendo um dos mecanismos pelos quais este composto apresenta efeito antiapoptótico.

Baseado nos resultados apresentados nos **Artigos I** e **II** e nos **Manuscritos I** e **II** é possível admitir que o PEBT possui propriedades favoráveis à melhora e à conservação do aprendizado e da memória em camundongos. Essas propriedades podem ser atribuídas, pelo menos em parte, ao aumento no tônus glutamatérgico (**Artigo I**), à inibição da morte celular por apoptose (**Manuscrito II**) e, ainda, às propriedades antioxidantes *in vitro* e *in vivo* (Souza et al., 2009) exercidas por este composto (Figura 7). Assim sendo, o PEBT possui efeitos promissores, podendo, futuramente, ser uma alternativa para o tratamento de danos cognitivos.

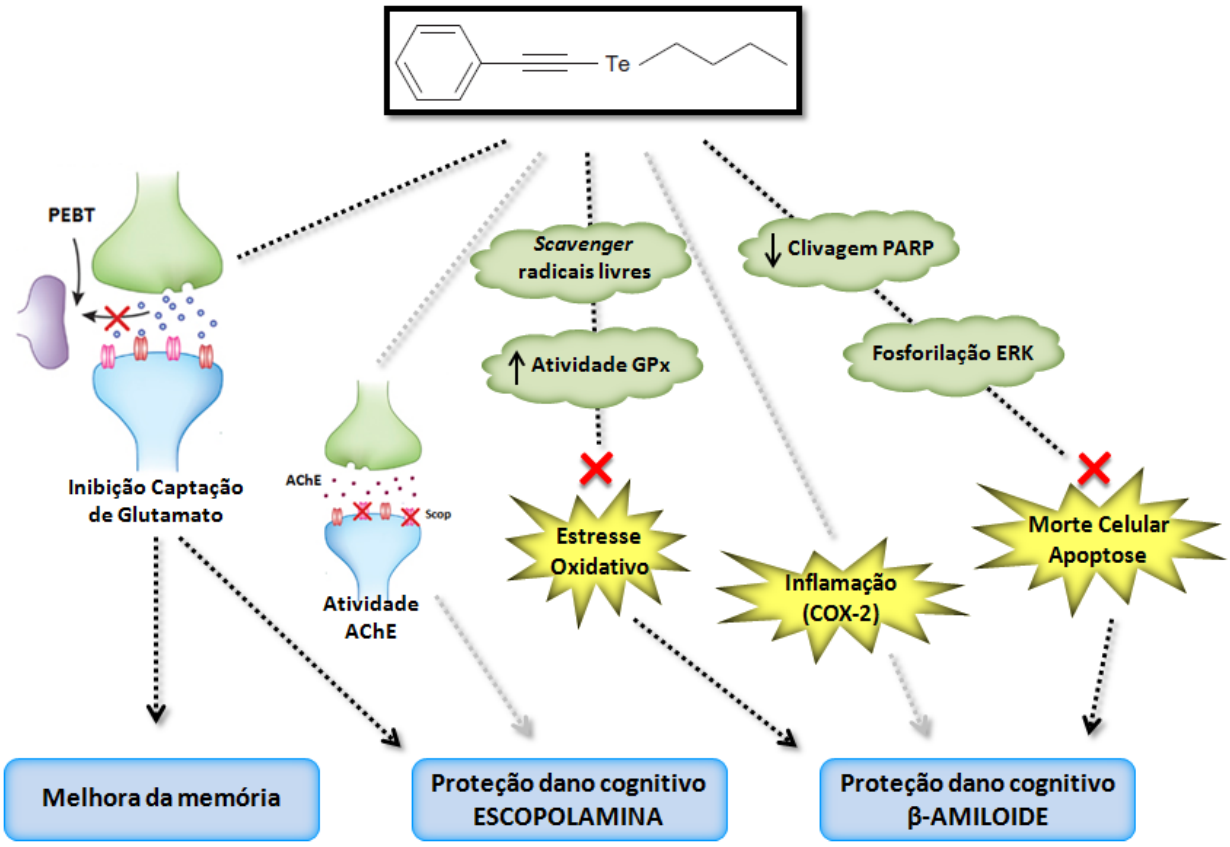


Figura 7. Esquema geral dos efeitos do PEBT apresentados nessa tese. O PEBT parece melhorar a memória e o dano cognitivo causado pela escopolamina por inibir a captação de glutamato. Os efeitos antiapoptótico e antioxidante do PEBT parecem proteger contra o dano causado pelo Aβ. O PEBT não altera a atividade da acetilcolinesterase e não age contra a inflamação. Linhas em preto: ações do PEBT sugeridas nessa tese. Linhas em cinza: vias que o PEBT não altera. Abreviaturas: Scop: escopolamina. AChE: acetilcolinesterase. GPx: glutationa peroxidase.

5 CONCLUSÕES

Baseado nos resultados apresentados nesta tese, pode-se concluir que:

a) O PEBT melhorou as três fases da memória aqui estudadas, aquisição, consolidação e evocação, na tarefa da esquiva inibitória em camundongos;
b) Um dos mecanismos envolvidos na melhora da memória causada pelo PEBT pode ser explicado pela diminuição da captação de glutamato, em que o tônus glutamatérgico é aumentado;
c) O tratamento subcrônico com o PEBT melhorou o desempenho de camundongos com prejuízo de memória causado pelo Aβ(25-35) nas tarefas do labirinto aquático de Morris e esquiva inibitória;
d) O PEBT protegeu contra o dano na memória induzido pela escopolamina no paradigma do labirinto aquático de Morris, além de proteger contra o dano na consolidação e evocação causado por este agente anticolinérgico na tarefa da esquiva inibitória;
e) O PEBT impediu a morte celular por apoptose induzida por estresse oxidativo *in vitro*, provavelmente por inibir a clivagem da PARP e restaurar a atividade da ERK.

Com este estudo, pode-se concluir que o PEBT possui propriedades de melhorar a memória tanto em condições fisiológicas quanto na presença de dano cognitivo. Por esta razão, o PEBT poderá, no futuro, ser considerado candidato para a prevenção de danos de memória, como aqueles observados na DA.

6 PERSPECTIVAS

Tendo em vista os efeitos promissores na memória apresentados pelo PEBT, as perspectivas para futuros trabalhos são:

a) Investigar o efeito do PEBT na persistência da memória em camundongos;
b) Avaliar as vias envolvidas na melhora da memória excercida pelo PEBT;
c) Estudar o efeito do PEBT na expressão dos receptores glutamatérgicos;
d) Investigar a ação do PEBT no sistema colinérgico;
e) Avaliar o efeito de PEBT contra a citotoxicidade induzida por Aβ em células neuronais;
f) Verificar se o PEBT interfere na produção de Aβ, inibindo a via amiloidogênica;
g) Investigar outros mecanismos envolvidos na ação antiapoptótica do PEBT;
h) Estudar a toxicidade e a farmacocinética do PEBT.

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ANEXOS

Anexo 1

Resultados da avaliação de parâmetros séricos de toxicidade hepática, alanina aminotransferase (ALT) e aspartato aminotransferase (AST), e toxicidade renal, ureia. Os camundongos foram tratados com PEBT na dose de 10 mg/kg (p.o.) por 24 horas ou na dose de 1 mg/kg por 10 dias. As atividades da ALT e AST, bem como os níveis de ureia não foram alterados pelo tratamento com o PEBT.

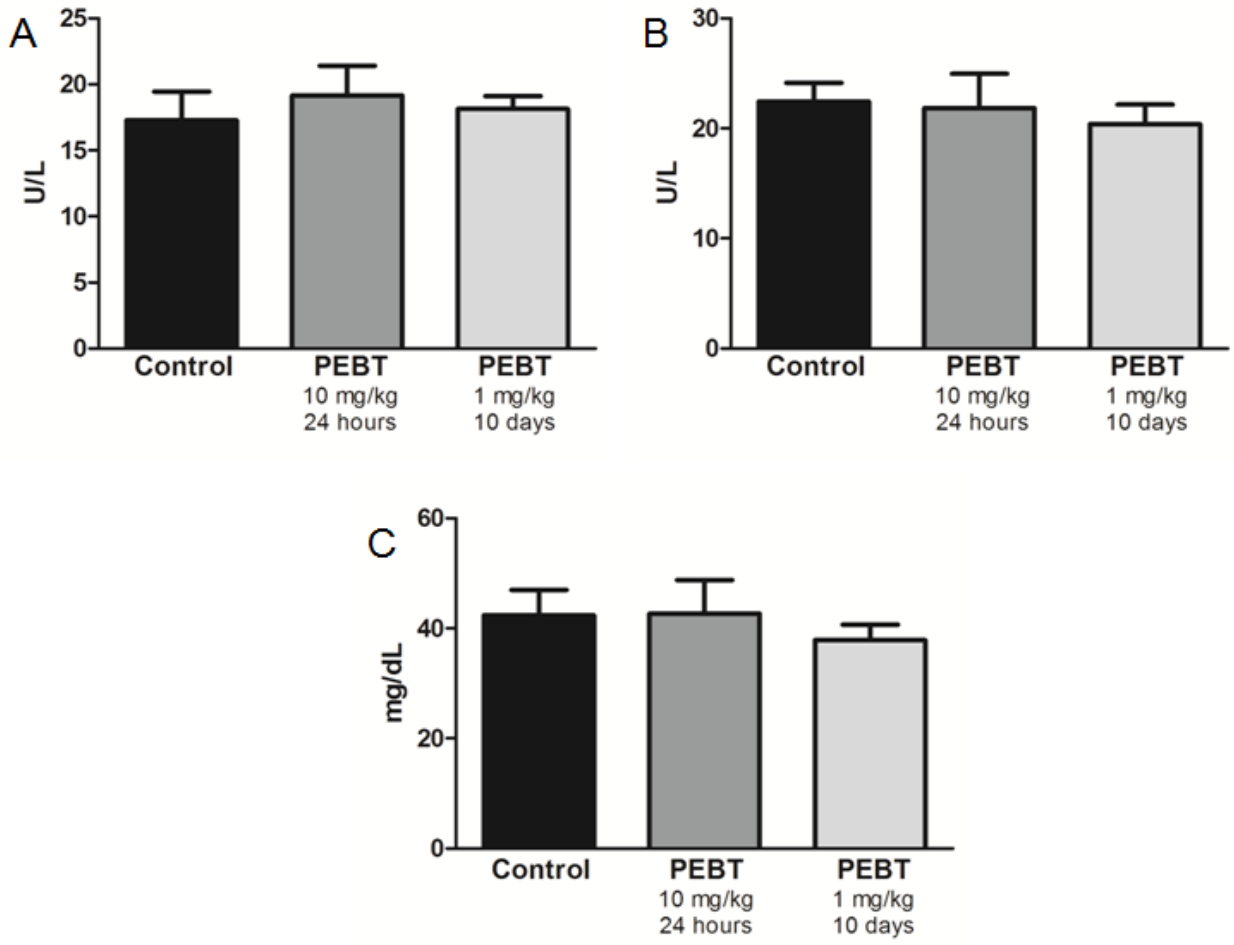

Figura 1. Efeito do tratamento agudo ou subcrônico com PEBT sobre a atividade da ALT (A), AST (B) e níveis de ureia (C) em camundongos. O PEBT foi adminstrado v.o. na dose de 10 mg/kg por 24 horas ou na dose de 1 mg/kg por 10 dias. Os dados estão expressos como média ± EP e foram avaliados usando ANOVA de uma via. Os valores de $p<0,05$ foram considerados significativos.

Anexo 2

Resultados do efeito de PEBT na atividade da acetilcolinesterase em córtex e hipocampo de camundongos. Os animais foram tratados com PEBT na dose de 10 mg/kg (p.o.) por 1 ou 24 horas. A atividade da acetilcolinesterase não foi alterada pelo tratamento com PEBT.

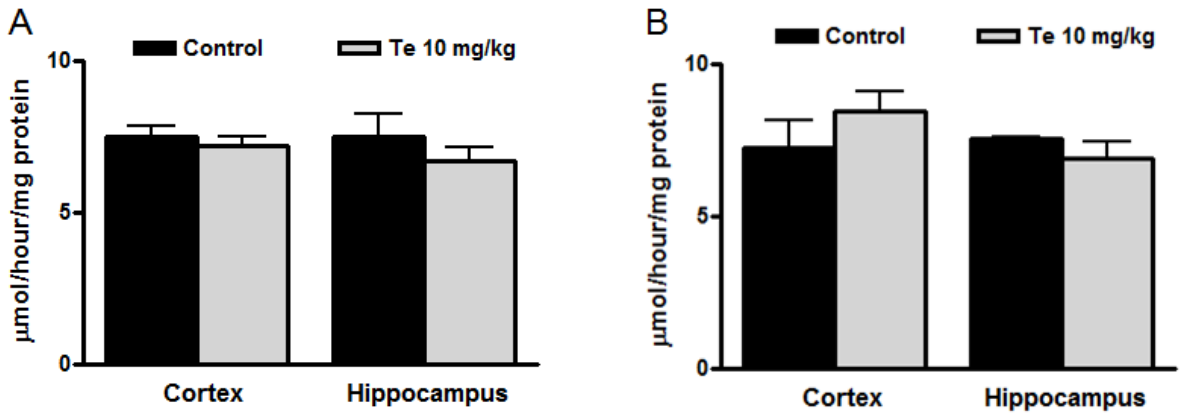

Figura 2. Efeito de PEBT (10 mg/kg, v.o.) por 1 (A) ou 24 (B) horas na atividade da acetilcolinesterase em córtex e hipocampo de camundongos. Os dados estão expressos como média ± EP e foram avaliados usando ANOVA de uma via. Os valores de $p<0,05$ foram considerados significativos.