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AÇÃO FARMACOLÓGICA DO 3-ALQUINIL SELENOFENO EM MODELOS DE CONVULSÃO EM RATOS JOVENS

TESE DE DOUTORADO

Ethel Antunes Wilhelm

Santa Maria, RS, Brasil
2012

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por

Ethel Antunes Wilhelm

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**.

Orientadora: Profª Drª Cristina Wayne Nogueira

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Universidade Federal de Santa Maria
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A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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como requisito parcial para obtenção do grau de
Doutor em Bioquímica Toxicológica

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Santa Maria, 02 de março de 2012

Mãe, Pai (in memorian), Mana e Juliano

pelo amor, exemplo, confiança e companheirismo,

dedico esta tese à vocês,

com todo o meu amor!!!

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(Fernando Pessoa)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
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AÇÃO FARMACOLÓGICA DO 3-ALQUINIL SELENOFENO EM MODELOS DE CONVULSÃO EM RATOS JOVENS

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LOCAL E DATA DA DEFESA: Santa Maria, 02 de março de 2012

As convulsões têm conseqüências importantes em termos de mortalidade e qualidade de vida da população afetada, sendo um fator de risco para o desenvolvimento de alterações cognitivas e anormalidades comportamentais. Tendo em vista as promissoras propriedades farmacológicas das moléculas contendo selênio, no **artigo 1** avaliamos a ação anticonvulsivante do (1-(2,5-difenilselenofeno-3-il)-3-metilpent-1-in-3-ol, que foi genericamente denominado de 3-alquinil selenofeno (3-ASP) frente as convulsões induzidas por pilocarpina (PC), pentilenotetrazole (PTZ) e cainato (KA) em ratos de 21 dias de vida. Os animais foram pré-tratados com 3-ASP (10, 25 ou 50 mg/kg; per oral, p.o.) ou veículo, 30 minutos antes da administração intraperitoneal (i.p.) de PC (400 mg/kg), PTZ (80 mg/kg) ou KA (45 mg/kg). Verificamos que o pré-tratamento com 3-ASP (50 mg/kg) aboliu as convulsões e a morte induzidas pela administração de PC. O 3-ASP (50 mg/kg) aumentou a latência para o primeiro episódio convulsivo, bem como, diminuiu a mortalidade e a incidência das convulsões causadas por PTZ e KA. Ainda no **artigo 1**, consideramos importante o estudo da ação antioxidante do 3-ASP (10, 25 e 50 mg/kg; p.o.) frente ao estresse oxidativo induzido pela PC (400 mg/kg, i.p.) em ratos de 21 dias de vida. Os resultados demonstraram que o pré-tratamento com 3-ASP mostrou-se eficaz na proteção contra a inibição da atividade cerebral da superóxido dismutase, diminuição dos níveis de ácido ascórbico, estimulação da atividade da catalase e aumento dos níveis de espécies reativas causadas pela PC. Adicionalmente, o 3-ASP protegeu contra a inibição da atividade da acetilcolinesterase e da Na^+,K^+-ATPase resultantes das convulsões induzidas pela PC. Em um segundo momento, o envolvimento dos sistemas glutamatérgico e GABAérgico na ação anticonvulsivante do 3-ASP foi verificado (**Artigos 1 e 2**). A combinação de doses sub-efetivas de 3-ASP (10 mg/kg, p.o.) e diazepam (agonista GABAérgico; 0,5 mg/kg, i.p.), 5S,10R (+)-5-metil-10,11-dihidro-5H-dibenzo [a,d] ciclohepteno -5,10- imina maleato (MK-801; antagonista não-competitivo do receptor NMDA; 0.1mg/kg, i.p.) ou 6,7-dinitroquinoxalina-2,3-diona (DNQX; antagonista de receptores não-NMDA; 5 mg/kg, i.p.) aumentou a latência para o primeiro episódio convulsivo, bem como diminuiu a incidência de convulsões induzidas pela PC. Por outro lado, a combinação de 3-ASP e 2-metil-6-feniletinil piridina hidroclorada (MPEP; antagonista do receptor glutamatérgico metabotrópico do tipo 5; 0,5 mg/kg, i.p.) não apresentou efeito protetor contra os episódios convulsivos. A administração oral de 3-ASP (50 mg/kg) causou uma inibição de 64% e 58% da captação de GABA no córtex e no hipocampo, respectivamente. Entretanto, nenhuma alteração na

captação de glutamato após a administração de 3-ASP (50 mg/kg) foi observada. Adicionalmente, no **artigo 2** investigamos a possível interação entre doses sub-efetivas de 3-ASP e inibidores da captação de GABA ou da GABA transaminase (GABA-T) frente às convulsões induzidas por PC em ratos de 21 dias de vida. Para isto, doses sub-efetivas de 3-ASP (10 mg/kg; p.o.) e ácido DL-2,4-diamino-*n*-butírico hidroclorado (DABA - um inibidor da captação de GABA; 2 mg/kg; i.p.) ou ácido aminooxiacético hemihidroclorado (AOAA – um inibidor da GABA-T; 10 mg/kg, i.p.) foram co-administrados em ratos de 21 dias de vida antes da administração de PC (400 mg/kg; i.p.). A presença de episódios convulsivos foi avaliada. Verificamos que o tratamento com o 3-ASP e DABA aboliu as convulsões induzidas por PC, corroborando com nossos resultados neuroquímicos. O mesmo foi observado quando foram administradas doses sub-efetivas de 3-ASP e AOAA. Por fim, no **artigo 3** investigamos o efeito do 3-ASP ou diazepam frente as convulsões, aumento da susceptibilidade ao desenvolvimento de convulsões e prejuízo na memória a longo prazo resultantes da convulsão febril induzida pela hipertermia. Os ratos de 21 dias de vida foram pré-tratados com 3-ASP (25, 50 ou 100 mg/kg; p.o), diazepam (1 ou 5 mg/kg; i.p.) ou veículo. Após o pré-tratamento, os animais foram expostos a uma temperatura de 41°C. Trinta dias após a exposição à hipertermia, avaliamos o aumento da susceptibilidade ao desenvolvimento de convulsões e prejuízo na memória a longo prazo. Verificamos que o pré-tratamento com 3-ASP ou diazepam não foi capaz de proteger contra o comportamento estereotipado, automatismos faciais e flexão corporal induzidos pela hipertermia. O efeito protetor do 3-ASP (100 mg/kg) contra o aumento da susceptibilidade ao desenvolvimento de convulsões e prejuízo na memória a longo prazo resultantes da convulsão febril induzida pela hipertermia foi verificado. O diazepam (1 ou 5 mg/kg) não protegeu contra o prejuízo na memória a longo prazo causado pela convulsão febril. Além disso, o tratamento com diazepam (1 mg/kg) nos animais mantidos a temperatura ambiente causou um significativo prejuízo cognitivo. Estes resultados sugerem que o 3-ASP apresenta ação anticonvulsivante em ratos de 21 dias de vida e que essa ação parece ser mediada pelos sistemas glutamatérgico e GABAérgico. Além disso, a ação anticonvulsivante do 3-ASP parece estar associada à sua atividade antioxidante. Por fim, o 3-ASP pode representar uma importante ferramenta para a proteção contra o aumento à susceptibilidade às convulsões e o prejuízo cognitivo resultantes das convulsões febris.

Palavras-chave: 3-alquinil selenofeno, selênio, anticonvulsivante, hipertermia, estresse oxidativo, ácido γ-aminobutírico, glutamato

ABSTRACT

Thesis of Doctor's Degree
Graduate Program in Biological Sciences: Toxicology Biochemical
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PHARMACOLOGICAL ACTION OF 3-ALKYNYL SELENOPHENE ON MODELS OF SEIZURES IN RAT PUPS

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DATE AND PLACE OF THE DEFENSE: Santa Maria, march 02, 2012

Seizures have important consequences in terms of mortality and quality of life of the affected population, being a risk factor for the development of cognitive and behavioral abnormalities. Considering the promising pharmacological properties of molecules containing selenium, in **article 1**, we evaluated the anticonvulsant action of 1-(2,5-diphenylselenophen-3-yl)-3-methylpent-1-yn-3-ol, generically called 3-alkynyl selenophene (3-ASP) against seizures induced by pilocarpine (PC), pentylenetetrazole (PTZ) and kainate (KA) in 21-days-old rats. Animals were pre-treated with 3-ASP (10, 25 or 50 mg/kg; per oral, p.o.) or vehicle, 30 minutes before of intraperitoneally administration of PC (400 mg/kg), PTZ (80 mg/kg) or KA (45 mg/kg). 3-ASP pre-treatment (50 mg/kg) abolished seizures and the death induced by PC administration. 3-ASP (50 mg/kg) increased the latency to the first convulsive episode, as well as decreased the mortality and incidence of seizures caused by PTZ and KA. In **article 1**, the antioxidant activity of 3-ASP (10, 25 or 50 mg/kg; p.o.) against the oxidative stress induced by PC (400 mg/kg, i.p.) in 21-days-old rats was evaluated. Our results demonstrated that 3-ASP pre-treatment was effective in protecting against the inhibition of cerebral activity of superoxide dismutase, decreased ascorbic acid levels, stimulation of catalase activity and increase of reactive species levels caused by PC. Additionally, 3-ASP protected against the inhibition of acetylcholinesterase and Na^+, K^+-ATPase activities resulting from convulsions induced by PC. The involvement of glutamatergic and GABAergic systems in the anticonvulsant action of 3-ASP was investigated (**Articles 1 and 2**). The combination of sub-effective doses of 3-ASP (10 mg/kg, p.o.) and diazepam (GABA agonist; 0,5 mg/kg, i.p.), 5S,10R-(+)-5-methyl-10,11-dihydro- 5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist; 0.1mg/kg, i.p.) or 6,7-dinitroquinoxaline-2,3-dione (DNQX, a non-NMDA receptor antagonist; 5 mg/kg, i.p.) was effective in increasing the latency to the first convulsive episode, as well as, in decreasing the incidence of convulsions induced by PC. On the other hand, the combination of 3-ASP and 2-methyl-6-phenylethynyl pyridine hydrochloride (MPEP, an antagonist of metabotropic glutamate receptor mGluR5; 0,5 mg/kg, i.p.) did not protect against convulsions. The oral administration of 3-ASP (50 mg/kg) caused an inhibition of 64% and 58% of GABA uptake in the cortex and hippocampus, respectively. However, no change in glutamate uptake after 3-ASP administration (50 mg/kg) was found. Additionally, in **article 2**, we investigated the possible interaction between sub-effective doses of 3-ASP and GABA uptake or GABA transaminase (GABA-T)

inhibitors against PC-induced seizures in 21-days-old rats. To this, sub-effective doses of 3-ASP (10 mg/kg; p.o.) and DL-2,4-diamino-*n*-butyric acid hydrochloride (DABA, an inhibitor of GABA uptake; 2 mg/kg; i.p.) or aminooxyacetic acid hemihydrochloride (AOAA; a GABA-T inhibitor; 10 mg/kg, i.p.) were co-administrated in 21-days-old rats before of PC administration (400 mg/kg). The treatment with 3-ASP and DABA abolished the PC-induced seizures. Similar results were found when sub-effective doses of 3-ASP and AOAA were administrated in 21-days-old rats. Finally, in the **article 3** we investigated the effect of 3-ASP or diazepam against convulsions, the increased susceptibility to the development of seizures and long term memory impairment resulting from febrile seizures induced by hyperthermia. 21-Days-old rats were pre-treated with 3-ASP (25, 50 or 100 mg/kg; p.o), diazepam (1 or 5 mg/kg; i.p.) or vehicle. After the treatment, animals were exposed to a stream of heated air to approximately 41°C. Thirty days after the exposure to hyperthermia, the susceptibility to the development of seizures and long term memory impairment were evaluated. We verified that the pre-treatment with 3-ASP or diazepam did not protect against stereotyped behavior, facial automatisms and body flexion induced by hyperthermia. The protective effect of 3-ASP (100 mg/kg) against the increased susceptibility to the development of seizures and long term memory impairment resulting from febrile seizures induced by hyperthermia was demonstrated. Diazepam (1 or 5 mg/kg) did not protect against long term memory impairment caused by febrile seizures. In addition, diazepam (1 mg/kg) treatment caused a significant cognitive impairment in animals kept at room temperature. These results suggest that 3-ASP had anticonvulsant action in 21-days-old rats and that this action appears to be mediated by glutamatergic and GABAergic systems. In addition, the anticonvulsant action of 3-ASP seems to be associated with its antioxidant activity. Finally, 3-ASP can represent an important tool to protect against increased susceptibility to seizures and cognitive impairment resulting from febrile seizures.

Key-words: 3-alkynyl selenophene, selenium, anticonvulsant, hiperthermia, oxidative stress, γ-aminobutyric acid, glutamate

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LISTA DE ABREVIATURAS

3-ASP - 3-alquinil selenofeno

AMPA - α-amino-3-hidroxi-5-metil-4-isoxazol-7 ácido propiônico

AOAA - ácido aminooxiacético hemihidroclorado

DABA - ácido DL-2,4-diamino-*n*-butírico hidroclorado

GABA - ácido γ-aminobutírico

GABA-T - GABA transaminase

GPx - glutationa peroxidase

GST - glutationa-S-transferase

KA - cainato

NMDA - N-metil-D-aspartato

SNC - sistema nervoso central

SOD - superóxido dismutase

SUMÁRIO

1 INTRODUÇÃO

A epilepsia é uma desordem neurológica caracterizada pelo aparecimento recorrente e imprevisível de convulsões espontâneas (Engel e Pedley, 2008). O comportamento convulsivo consiste em episódicas descargas anormais de um grupo de neurônios cerebrais, resultando em uma alteração da atividade cerebral que se caracteriza por manifestações motoras, sensitivas, sensoriais, psíquicas ou neurodegenerativas (Engelborghs e col., 2000). O tratamento farmacológico da epilepsia é ineficaz no controle das convulsões em aproximadamente 25% dos pacientes (Ben-Menachem e col., 2007), o que resulta em um alto custo social e financeiro para a sociedade.

As crises convulsivas são divididas em parciais e generalizadas. Na crise parcial há evidências clínicas e eletroencefalográficas que apontam o local de início do foco epiléptico, o qual se restringe a uma porção de um hemisfério cerebral. São classificadas também como simples, quando a consciência é preservada, ou complexas, quando ocorre a perda desta (Fisher e col., 2005; Anne e col., 2011). Na crise generalizada não há evidências do local de início do foco epilético e as descargas neuronais são bilaterais, ou seja, envolvem amplas áreas de ambos os hemisférios cerebrais. A consciência é quase sempre comprometida, e as manifestações motoras afetam os dois lados do corpo. As crises convulsivas acompanhadas de fenômenos motores são classificadas como: tônicas, quando o corpo fica rígido; clônicas, quando há contrações ritmadas seguidas de relaxamento em rápida sucessão; tônico-clônicas, se os dois sintomas estiverem presentes e mioclônicas, caso haja contrações não ritmadas de apenas um ou alguns grupos de músculos definidos. Quando não há fenômenos motores, as crises são denominadas atônicas (perda do tônus muscular, sem rigidez do corpo) ou de ausência (perda do contato com o meio) (Fisher e col., 2005; Anne e col., 2011).

Para entender as epilepsias, inúmeras abordagens têm sido feitas, tanto pelo estudo do tecido epiléptico humano, obtido após remoção cirúrgica do foco epiléptico, como através do estudo de tecidos cerebrais, provenientes de animais submetidos a diferentes modelos de epilepsia. A relevância de um modelo experimental é determinada pelo grau em que o modelo serve como testemunha do fenômeno natural. O *screening* farmacológico de drogas antiepilépticas e o

entendimento dos mecanismos envolvidos na epileptogênese baseiam-se em grande parte nos modelos animais de epilepsia (Mody e Schwartzkroin, 1997).

Os modelos experimentais de epilepsia são denominados agudos, quando o animal apresenta crises convulsivas somente durante a vigência do agente indutor. Estes modelos incluem a aplicação tópica ou injeção sistêmica de compostos que interferem no balanço neuroquímico responsável pela excitabilidade neuronal (Mody e Schwartzkroin, 1997). Os modelos mais comumente usados são: bloqueadores dos sistemas inibitórios de neurotransmissão (por exemplo antagonistas de receptores $GABA_A$); estimulantes dos sistemas excitatórios (por exemplo agonistas de receptores glutamatérgico e colinérgicos); estimulação elétrica direta ou indireta e exposição à hipertermia.

Os modelos de epilepsia são ditos crônicos quando as crises recorrem a intervalos variados de tempo, não sendo necessário o estímulo precipitante exógeno para desencadear cada crise. Estes modelos caracterizam-se por apresentar um fator casual conhecido, que induz o processo de epileptogênese, o qual, após determinada latência, culmina com crises epilépticas espontâneas (Naffah-Mazzacoratti, 1998). Os modelos mais utilizados são aqueles que mimetizam a epilepsia do lobo temporal, o tipo mais freqüente de epilepsia encontrado na população humana adulta. São exemplos: o modelo da pilocarpina (Turski e col., 1983), o modelo do ácido caínico (Bem-Ari, 1985) e o abransamento (*kindling*) (Goddard, 1967).

A incidência de convulsões é muito alta nos primeiros anos de vida, diminuindo na infância e adolescência (Grunewald, 2002). Crianças apresentam um alto risco de desenvolver convulsões, quando comparadas a adultos, e este risco é aumentado nos primeiros meses de vida. Durante o nascimento, os bebês podem sofrer insultos como trauma, hipóxia, isquemia, infecções perinatais, hemorragias intracraniais, distúrbios metabólicos e febre, o que pode resultar em convulsão (Holmes, 2005). Estudos utilizando modelos animais demonstram que o cérebro imaturo é mais vulnerável ao desenvolvimento de convulsões quando comparado ao cérebro adulto. Neurônios imaturos tendem a gerar periódicas descargas e estas facilitam a geração de oscilações patológicas (Khazipov e col., 2004).

No rato, a hiperplasia (aumento no número de células) neuronal prevalece na vida pré-natal, ocorrendo principalmente durante a última semana de gestação (Dobbing e Sands, 1971). A sinaptogênese no cérebro do rato ocorre principalmente

entre o 7º e 21º dia de vida pós-natal, podendo diferir de região a região (Davidson e Dobbing, 1966) (Figura 1). O aumento dos contatos sinápticos e a diferenciação destas conexões representam o começo do desenvolvimento químico e funcional do SNC. De acordo com Avishai-Eliner e col. (2002), a maturação cerebral de ratos de 21 dias de vida corresponde a crianças de 3-5 anos de idade, enquanto ratos recém nascidos correspondem a fetos humanos com 6 meses de gestação.

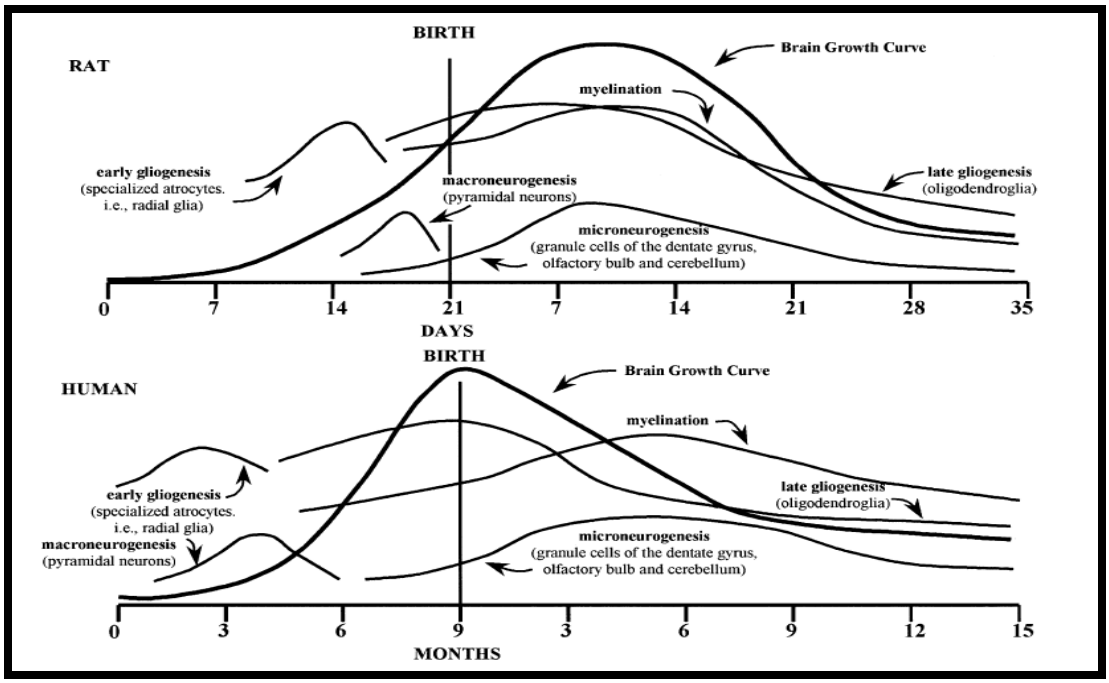


Figura 1 – Curva de Velocidade, comparando os índices relativos, duração e tempo do processo de desenvolvimento específico em cérebro de ratos e humanos. As curvas de crescimento cerebral (índices de mudança no peso cerebral) são sobrepostas em relação aos eventos desenvolvimentais no cérebro. Note a gênese precoce de astroglia e células piramidais em humanos, resultando na aquisição de aproximadamente 27% do peso cerebral adulto no tempo do nascimento, comparado à aproximadamente 12% do peso cerebral adulto visto em ratos ao nascimento. A curva de rápido crescimento cerebral em ratos é alterada para a direita, comparado aos humanos. **Fonte:** adaptado de Morgane e col., 2002.

A convulsão é um fator de risco para o desenvolvimento de alterações cognitivas e anormalidades comportamentais. Diversas evidências demonstram que as convulsões, em especial na infância, podem causar danos cerebrais que podem ser responsáveis por um declínio cognitivo e maior susceptibilidade ao desenvolvimento de epilepsia do lobo temporal na vida adulta (Cendes e col., 1993; Elger e col., 2004; Dubé e col., 2006).

Em geral a gênese da crise convulsiva envolve um desequilíbrio ocasionado por um aumento da transmissão excitatória e/ou diminuição da resposta inibitória (Mares e Kudová, 2008).

A neurotransmissão sináptica inibitória no sistema nervoso central (SNC) de mamíferos é mediada principalmente pelo ácido γ-aminobutírico (GABA). O GABA é sintetizado em 20 a 30% dos neurônios pelos chamados neurônios GABAérgicos e é indispensável para o controle de funções do SNC como a atividade locomotora, o aprendizado e o ritmo circadiano (Varju e col., 2001; Enna e Möhler, 2007). O GABA é armazenado em vesículas sinápticas e liberado para o meio extracelular de maneira dependente de cálcio, onde ativa seus receptores.

Os receptores GABAérgicos estão divididos em três classes, de acordo com propriedades farmacológicas, bioquímicas e eletrofisiológicas: $GABA_A$ e $GABA_C$ (receptores ionotrópicos) e $GABA_B$ (receptores metabotrópicos) (Olsen e De Lorey, 1999; Enna e Möhler, 2007). Os receptores $GABA_A$ e $GABA_C$ são canais iônicos que permitem a entrada de cloreto, provocando uma hiperpolarização localizada na membrana neuronal, o que dificulta o disparo do potencial de ação necessário para a liberação de neurotransmissores (Enna e Möhler, 2007). Portanto, a ação do GABA desencadeia a redução da excitabilidade neuronal. A inibição é um processo fundamental na atividade cerebral, e, portanto, a maior parte das células neuronais expressa estes receptores nas suas membranas celulares (Treiman, 2001). Os receptores $GABA_A$ apresentam sítios de ligação para GABA, benzodiazepínicos, barbitúricos, neuroesteróides, picrotoxina e etanol (Figura 2). Os receptores $GABA_B$ são receptores metabotrópicos (ligados a proteínas G), hiperpolarizam o neurônio aumentando a condutância do potássio e diminuindo o influxo de cálcio, tendo efeito inibitório lento. Os receptores $GABA_B$ presentes em terminais de axônios excitatórios e inibitórios, são ativados por baclofen e resistentes a drogas que modulam os receptores $GABA_A$ (Marshal e col., 1999; Enna e Möhler, 2007).

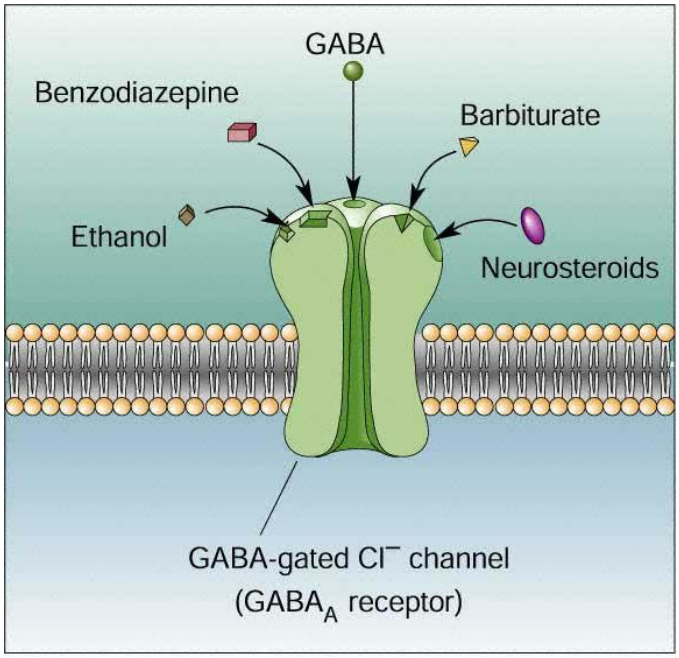


Figura 2 – Representação esquemática do receptor GABAérgico ionotrópico GABA$_A$. **Fonte:** http://img.medscape.com/pi/emed/ckb/neurology/1134815.png

A liberação pré-sináptica de GABA pode ser vesicular (dependente de cálcio e estimulada por altas concentrações de potássio) e não-vesicular (independente de cálcio e secundária à despolarização da membrana celular e influxo de sódio). A liberação não vesicular de GABA depende do transporte reverso deste (Treiman, 2001). Depois do GABA ser liberado dos terminais dos axônios e de agir sobre os receptores ionotrópicos e metabotrópicos, esta ação é terminada pela sua rápida recaptação via transportadores dependentes de íons sódio/cloreto, localizados nas membranas pré- e pós-sinápticas de neurônios e também em células gliais. A manutenção dos níveis normais dos neurotransmissores na fenda sináptica é uma condição indispensável para garantir funções cerebrais adequadas.

Após a recaptação, o GABA é catabolizado pela enzima GABA transaminase (GABA-T) a semialdeído succínico, que é convertido a ácido succínico pela ação da semialdeído ácido succínico desidrogenase, entrando, então, no ciclo de Krebs (Treiman, 2001; Enna e Möhler, 2007). O aumento da atividade da enzima GABA-T no cérebro diminui as concentrações de GABA e pode levar a convulsões, coma e óbito. A inibição desta enzima aumenta consideravelmente as concentrações sinápticas de GABA (Devaud, 2001).

O aminoácido L-glutamato é considerado o maior mediador de sinais excitatórios no SNC de mamíferos e está envolvido em uma variedade de processos

fisiológicos, tais como comunicação intracelular, crescimento e diferenciação, aprendizado e memória (Gereau e Swanson, 2008). Simultaneamente, o glutamato desempenha um importante papel na formação de redes neurais durante o desenvolvimento (Izquierdo, 2006). Conseqüentemente, um desequilíbrio na via glutamatérgica é um fator importante na gênese de muitas desordens neurológicas.

A síntese desse neurotransmissor ocorre nos terminais pré-sinápticos, predominantemente a partir da glutamina, a qual é sintetizada nas células gliais e transportada para os terminais nervosos, onde então é convertida em glutamato pela ação da enzima glutaminase. O glutamato ainda pode ser obtido a partir do α-cetoglutarato nas reações de transaminação e na aminação redutora, pela ação da enzima glutamato desidrogenase (Kvamme, 1998).

Quando liberado na fenda sináptica, as respostas fisiológicas ao glutamato ocorrem via ativação de receptores ionotrópicos e metabotrópicos, farmacologicamente e funcionalmente distintos, localizados nas membranas pré- e pós-sinápticas, bem como na membrana das células gliais (Gereau e Swanson, 2008) (Figura 3).

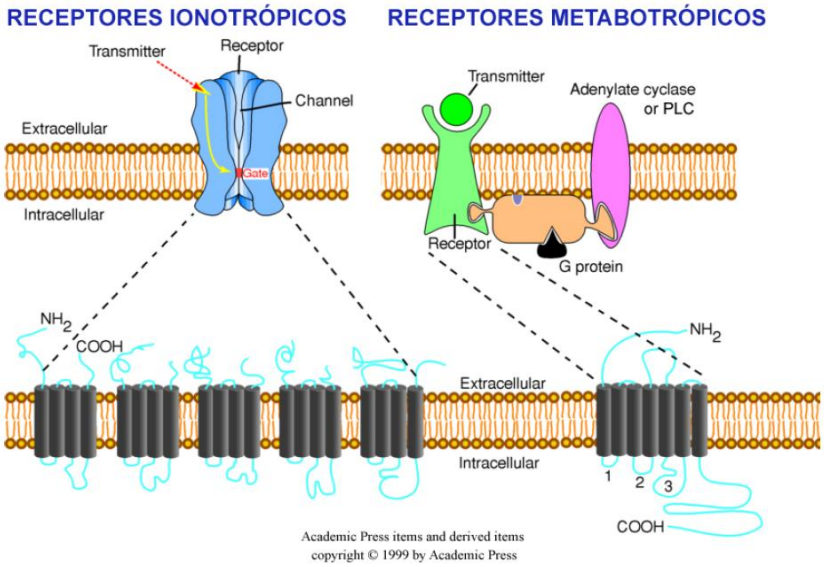


Figura 3. Receptores glutamatérgicos. Os receptores glutamatérgicos ionotrópicos (iGluRs) contêm um canal iônico cátion-específico e são subdivididos em três subtipos: a-amino-3-hidroxi-5-metil-4-isoxazolepropionato (AMPA), cainato e N-metil-D-aspartato (NMDA). Os receptores glutamatérgicos metabotrópicos (mGluRs) são acoplados ao sistema de transdução de sinal via adenilato ciclase ou fosfolipase C (PLC) e estão subdivididos em 8 subtipos (mGluR1-8). **Fonte:** Adaptado de Zigmond, Fundamental Neuroscience, Academic Press, 2003.

Os receptores ionotrópicos são canais iônicos que permeiam cátions através da membrana neuronal. Portanto, sua ativação provoca a despolarização da membrana sináptica e desencadeia uma resposta excitatória. Estes receptores são subdivididos em α-amino-3-hidroxi-5-metil-4-isoxazol-7 ácido propiônico (AMPA), cainato (KA) e N-metil-D-aspartato (NMDA), com base na sua sensibilidade a agonistas específicos (Cotmann e col., 1995).

Os receptores AMPA medeiam a neurotransmissão excitatória rápida e são canais com grande permeabilidade aos cátions monovalentes, sódio e potássio, e com baixa permeabilidade ao cálcio. Estes receptores possuem, ao menos, três sítios para ligantes: o sítio de união de glutamato (ou AMPA), um sítio de união que modula a desensibilização do receptor e outro que bloqueia o influxo de íons e que está localizado no interior do canal (Gereau e Swanson, 2008). Os receptores de KA diferem dos receptores AMPA por serem, além de permeáveis a íons sódio e potássio, relativamente permeáveis a íons cálcio (Ozawa e col., 1998; Gereau e Swanson, 2008). Esses receptores são encontrados em poucas áreas cerebrais, ao contrário dos receptores AMPA, que apresentam ampla distribuição no SNC. Além disso, a administração intracerebral ou parenteral de KA em ratos possui efeito convulsivo, e resulta num modelo de dano cerebral que se assemelha ao de pacientes com epilepsia lobo temporal (Kleinrok e col., 1995; Bortolatto e col., 2011).

Por sua vez, os receptores NMDA medeiam a transmissão sináptica excitatória lenta, são canais com grande permeabilidade ao cálcio e baixa permeabilidade ao sódio e potássio (Ozawa e col., 1998; Gereau e Swanson, 2008). O complexo do receptor NMDA apresenta diversos sítios para ligantes que regulam a abertura do canal iônico: um sítio para glutamato (ou NMDA), um para o co-agonista endógeno, glicina, um sítio no interior do canal para a união de bloqueadores [MK-801 e fenciclidina (PCP)], e sítios modulatórios, tais como: um sítio para o zinco (antagonista não-competitivo do receptor), outro para poliaminas, um sensível a modulação redox (modulado tanto por oxidantes quanto por redutores) e um sítio sensível a H^+ (Martin e col., 1995; Ozawa e col., 1998; Gereau e Swanson, 2008).

Além disso, o canal do receptor NMDA é bloqueado por magnésio de uma maneira dependente de voltagem, ou seja, nos neurônios em potencial de repouso, a ativação do receptor só ocorre se a membrana neuronal for despolarizada, o que

permite a saída de magnésio do interior do canal. O bloqueio dependente de voltagem do canal NMDA por magnésio pode ser visto como um mecanismo protetor intrínseco contra a entrada excessiva de cálcio na célula e a conseqüente toxicidade neuronal (Gereau e Swanson, 2008) (Figura 4).

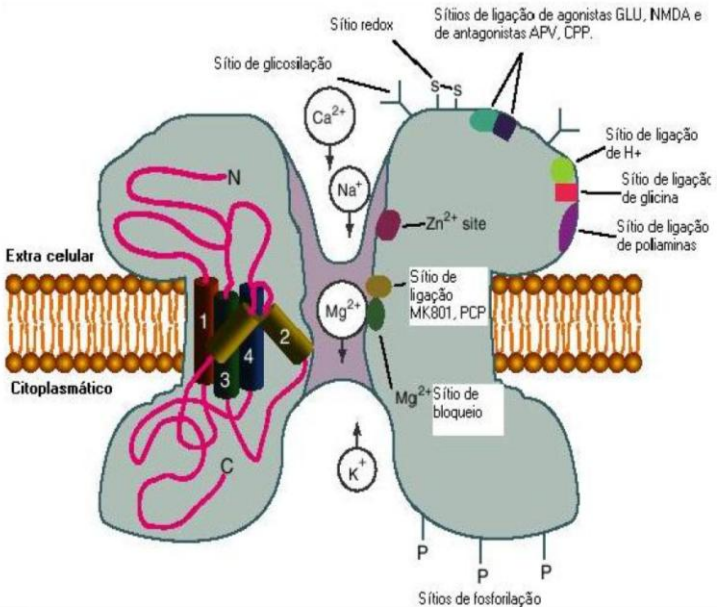


Figura 4. Representação esquemática do receptor NMDA. **Fonte:** Zigmond et al. (2003).

Diferentemente dos receptores ionotrópicos, os receptores glutamatérgicos metabotrópicos estão associados a sistemas de segundos mensageiros intracelulares. Estes receptores são acoplados a proteínas G (proteínas ligantes de nucleotídeos da guanina), as quais modulam a atividade de efetores intracelulares, tais como a adenilato ciclase e a fosfolipase C (Cotmann e col., 1995). Os receptores glutamatérgicos metabotrópicos, assim como o ionotrópico NMDA, estão envolvidos no processo de indução da plasticidade neuronal. Os receptores glutamatérgicos metabotrópicos encontram-se localizados nos terminais pré- e pós-sinápticos e nas células gliais, estes receptores também possuem papel importante na indução de convulsões e morte neuronal (Chapman, 2000).

A captação de glutamato é o principal mecanismo responsável pela manutenção dos níveis extracelulares de glutamato abaixo dos níveis tóxicos, sendo realizados por transportadores de glutamato presentes na membrana plasmática de neurônios e células gliais, principalmente em astrócitos (Danbolt, 2001). A captação do glutamato envolve dois sistemas de transporte: um carreador com alta afinidade e dependente de sódio, localizado nas membranas pré-sinápticas e gliais, e outro com

baixa afinidade e independente de sódio, localizado nas membranas das vesículas sinápticas. Devido à ação coordenada destes transportadores, o glutamato é armazenado nas vesículas, diminuindo sua concentração na fenda sináptica (Danbolt, 2001). Desta forma, a captação de moléculas de glutamato apresenta uma função vital na manutenção dos níveis de glutamato, a fim de evitar a ativação excessiva dos receptores glutamatérgicos e conseqüente excitotoxicidade. Estudos de Meldrum e colaboradores (1999) indicaram que alterações nos transportadores de glutamato podem desencadear um importante papel no processo epileptogênico. Além disso, a captação de glutamato tem importância vital no fornecimento de glutamato para síntese de GABA e glutationa (Danbolt, 2001).

Além das alterações nos sistemas neuroquímicos, outros mecanismos podem estar envolvidos no processo convulsivo. Um significante número de estudos sugere a associação entre a formação de espécies reativas e o desenvolvimento de episódios convulsivos (Waldbaum e Patel, 2010; Shin e col., 2011). O conceito formulado por Sies (1997) define estresse oxidativo como sendo um desequilíbrio entre a produção de agentes oxidantes e antioxidantes, em favor dos oxidantes, com potencial para ocasionar dano celular. As principais espécies reativas (oxidantes) vinculadas ao estresse oxidativo são: o radical ânion superóxido ($O_2^{\bullet -}$), radical hidroxil ($^{\cdot}OH$), peróxido de hidrogênio (H_2O_2), óxido nítrico (NO) e peroxinitrito ($ONOO^-$). Os seres vivos dispõem de mecanismos protetores para evitar o acúmulo destas espécies reativas, que incluem mecanismos enzimáticos e não enzimáticos. As principais enzimas antioxidantes são a superóxido dismutase (SOD), a catalase, a glutationa peroxidase (GPx) e a glutationa-S-transferase (GST). Essas enzimas evitam o acúmulo de $O_2^{\bullet -}$ e de H_2O_2, e a conseqüente produção de OH. As defesas não-enzimáticas incluem os antioxidantes lipofílicos (tocoferóis, carotenóides e bioflavonóides) e hidrofílicos (glutationa e ascorbato) (Halliwell e Gutteridge, 2007). As espécies reativas podem induzir um grande número de alterações nos constituintes celulares, incluindo inativação de enzimas, danos às bases nitrogenadas dos ácidos nucléicos e às proteínas, além de peroxidação dos lipídios de membrana (Halliwell e Gutteridge, 2007).

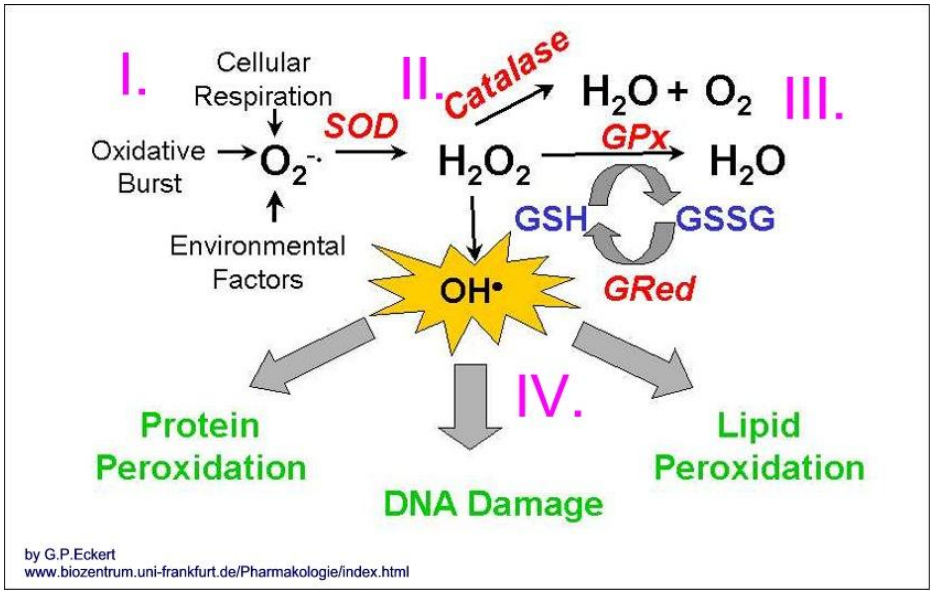


Figura 5: Representação esquemática dos mecanismos de defesa antioxidante enzimáticos e não enzimáticos. **Fonte:** www.biozentrum.uni-frankfurt.de/Pharmakologie/index.html.

Estudos bioquímicos têm mostrado que reações de oxidação podem ser importantes no desenvolvimento de patologias cerebrais e que estas estão associadas com um desequilíbrio da regulação redox no SNC (Halliwell e Gutteridge, 2007). De fato, o cérebro é particularmente mais susceptível ao estresse oxidativo, pois apresenta elevado consumo de O_2, baixa quantidade de defesas antioxidantes, altas concentrações de lipídios poliinsaturados (substratos para peroxidação lipídica) e concentrações elevadas de ferro (Sousa e col., 2003; Wajner e col., 2004; Halliwell e Gutteridge, 2007). Por outro lado, o estresse oxidativo também pode ocorrer em consequência de episódios convulsivos prolongados, sugerindo que este processo possa desempenhar um papel importante no dano cerebral induzido pela convulsão (Kaneko e col., 2002; Halliwell, 2006; Shin e col., 2011). Neste sentido, substâncias com ação antioxidante podem representar estratégias terapêuticas promissoras para o tratamento e/ou prevenção de doenças associadas ao estresse oxidativo.

O selênio, elemento traço essencial, apresenta um grande número de funções biológicas, sendo a mais importante a ação antioxidante. Sabe-se que esse elemento está presente como resíduo de selenocisteína no sítio ativo das enzimas glutationa peroxidase (Wingler e Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985) e selenoproteina P (Ursini e col., 1990), sendo que sua atividade redox é de fundamental importância para o sítio catalítico dessas enzimas (Wingler e Brigelius-Flohé, 1999).

Os efeitos neuroprotetores do selênio têm sido amplamente estudados, uma vez que esse elemento desempenha um importante papel para o cérebro. Trabalhos mostram que quando há depleção de selênio no organismo, o cérebro recebe uma oferta prioritária desse elemento com relação aos outros órgãos (Behne e col., 1988; Buckman e col., 1993; Whanger, 2001) e que quando a deficiência de selênio atinge também este órgão, a taxa de *turnover* dos neurotransmissores é alterada (Castano e col., 1997). Além disso, baixos níveis desse elemento no plasma estão associados com déficit cognitivo e pacientes com doenças neurodegenerativas, como Doença de Alzheimer, apresentaram menores concentrações cerebrais de selênio quando comparados a grupos com níveis normais de selênio (Corrigan e col., 1991). Estudos de Ramaekers (1994) e Weber e colaboradores (1991) demonstraram que a suplementação com selênio pode reduzir as convulsões epilépticas na infância. E ainda, em estudos pré-clínicos de dano cerebral, o selênio apresentou um efeito neuroprotetor (Crack e col., 2001; Zafar e col., 2003). Recentemente, Mahyar e colaboradores (2010) relataram a associação entre a deficiência de selênio e o desenvolvimento de convulsões febris.

Neste sentido, nas últimas décadas, os compostos orgânicos de selênio têm se destacado devido à descoberta de suas aplicações sintéticas, de suas diversificadas ações farmacológicas, além de sua menor toxicidade em relação às espécies inorgânicas (Parnham e Graf, 1991; Nogueira e col., 2004; Nogueira e Rocha, 2010).

Os selenofenos, uma classe específica de compostos contendo selênio, também despertam interesse por apresentarem variadas ações biológicas. Estudos prévios já demonstraram que esses compostos apresentam propriedades antimicrobianas, anti-apoptóticas e antitumorais (Abdel-Hafez, 2005; Shiah e col., 2007; Juang e col., 2007; Rhoden e Zeni, 2011). Recentemente, Gai e colaboradores (2010) reportaram o perfil farmacológico do tipo antidepressivo do 3-(4-fluorofenilselenil)-2,5-difenilselenofeno em camundongos.

Estudos do nosso grupo de pesquisa têm demonstrado que o composto 3-alquinil selenofeno (1-(2,5-difenilselenofeno-3-il)-3-metilpent-1-in-3-ol; 3-ASP) (Figura 6), um composto heterocíclico contendo selênio em sua estrutura, possui atividade antioxidante. O 3-ASP apresenta ações anti-hiperalgésica e antinociceptiva em camundongos (Wilhelm et al., 2009A). O 3-ASP reduz a nocicepção causada pela administração intraplantar de formalina (primeira e segunda fase), glutamato,

bradicinina, acetato de forbol miristato (PMA), capsaicina e reduz a hiperalgesia mecânica induzida pelo Complemento Adjuvante de Freund (CFA) em camundongos. Este efeito antinociceptivo do 3-ASP não envolve mecanismos opioidérgicos (Wilhelm et al., 2009A). O 3-ASP administrado pela via oral (p.o.) nas doses de 1-50 mg/kg não apresenta toxicidade e não afeta a atividade locomotora em camundongos (Wilhelm et al., 2009b). Além disso, o 3-ASP apresenta efeito hepatoprotetor na injúria hepática aguda induzida por D-galactosamina/Lipopolissacarídeo, 2-nitropropano e tetracloreto de carbono em ratos (Wilhelm et al., 2009B, 2010).

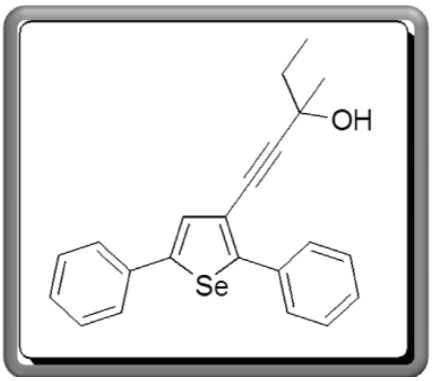


Figura 6. Estrutura química do composto 1-(2,5-difenilselenofeno-3-il)-3-metilpent-1-in-3-ol (3-ASP)

Desta forma, com base nas propriedades biológicas já descritas para os compostos orgânicos de selênio, em especial para os compostos selenofenos, o 3-ASP pode representar uma estratégia terapêutica bastante promissora para a prevenção da convulsão e das alterações causadas por episódios convulsivos.

2 OBJETIVOS

2.1 Objetivo Geral

Tendo em vista as promissoras ações farmacológicas dos compostos orgânicos de selênio, o objetivo geral do presente estudo foi investigar se o composto 3-alquinil selenofeno (3-ASP), uma molécula orgânica de selênio da classe dos selenofenos, apresenta ação anticonvulsivante em ratos jovens.

2.2 Objetivos Específicos

Considerando os aspectos já mencionados, os objetivos específicos deste trabalho compreenderam:

- Avaliar a ação neuroprotetora do 3-ASP em convulsões induzidas por pilocarpina (PC), pentilenotetrazole (PTZ) e KA em ratos jovens;
- Verificar o efeito do 3-ASP sobre o estresse oxidativo induzido por PC em ratos jovens através da determinação dos níveis espécies reativas, ácido ascórbico e atividade das enzimas catalase, glutationa peroxidase, glutationa-S-transferase e superóxido dismutase. Avaliar o efeito do 3-ASP e/ou PC sobre a atividade da Na^+, K^+-ATPase e acetilcolinesterase.
- Estudar a possível contribuição dos sistemas GABAérgico e glutamatérgico na ação anticonvulsivante do 3-ASP através da determinação da captação de GABA e glutamato e avaliações farmacológicas.
- Investigar o efeito do 3-ASP sobre as convulsões febris induzidas por hipertermia em ratos jovens;
- Avaliar a ação protetora do 3-ASP contra o prejuízo cognitivo e o aumento da susceptibilidade às convulsões causadas pela convulsão febril em ratos.

3 ARTIGOS CIENTÍFICOS

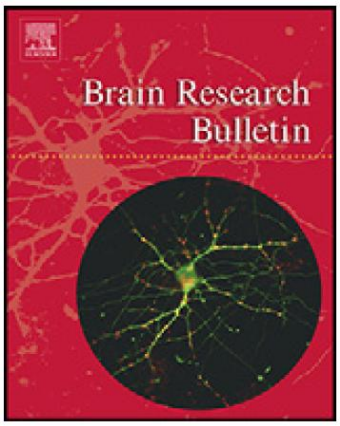
Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências dos artigos, encontram-se estruturados de acordo com as normas das respectivas revistas nas quais foram publicados.

3.1 Artigo 1

Efeitos anticonvulsivante e antioxidante do 3-alquinil selenofeno em ratos de 21-dias de idade no modelo de convulsões induzidas por pilocarpina

ANTICONVULSANT AND ANTIOXIDANT EFFECTS OF 3-ALKYNYL SELENOPHENE IN 21-DAY-OLD RATS ON PILOCARPINE MODEL OF SEIZURES

Ethel A. Wilhelm, Cristiano R. Jesse, Cristiani F. Bortolatto, CristinaW. Nogueira, Lucielli Savegnago



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Research report

Anticonvulsant and antioxidant effects of 3-alkynyl selenophene in 21-day-old rats on pilocarpine model of seizures

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ABSTRACT

This study investigated the anticonvulsant effect of 3-alkynyl selenophene (3-ASP) on pilocarpine (PC)-, pentylenetetrazole (PTZ)- and kainic acid (KA)-induced seizures and mortality in 21-day-old rats. Rats were pretreated by oral route (p.o.) with 3-ASP (10, 25 and 50 mg/kg) before intraperitoneal (i.p.) administration of PC (400 mg/kg), PTZ (80 mg/kg) or KA (45 mg/kg). 3-ASP increased the latency to the seizure onset on PTZ and KA models. At the dose of 50 mg/kg, 3-ASP avoided the death caused by PTZ and KA. 3-ASP (50 mg/kg) abolished seizures and death induced by PC in rats. To investigate the antioxidant effect of 3-ASP on rats exposed to PC, the activity of glutathione peroxidase (GPx), glutathione-S-transferase (GST), acetylcholinesterase (AChE), Na^+K^+ATPase, superoxide dismutase (SOD) and catalase (CAT) as well as the levels of reactive species (RS) and ascorbic acid (AA) were determined in brains of rats. 3-ASP protected against the increase in RS levels and CAT activity induced by PC in brains of rats. The decrease in the levels of AA and inhibition of Na^+K^+ATPase, SOD and AChE activities caused by PC were protected by 3-ASP. Subeffective doses of 3-ASP plus diazepam, 5S,10R-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) or 6,7-dinitroquinoxaline-2,3-dione (DNQX) increased the latency to the seizure onset induced by PC, suggesting the involvement of ionotropic glutamatergic and GABAergic receptors in anticonvulsant action of 3-ASP. The anticonvulsant and antioxidant effects of 3-ASP in 21-day-old rats on PC model were demonstrated.

1. Introduction

Prolonged seizures in the early developmental period can cause brain damage and lead to serious consequences later in life [5], which motivates the development of new animal models for early-life seizures [57]. The seizures can occur at any age, affecting at least 1–2% of the world population, they are far more common in children than adults [16]. The immature brain is more prone to seizures than the adult brain due to an imbalance between the development of excitation and inhibition [34,48]. This is manifested as differences in neuronal vulnerability, cellular and synaptic reorganization and regenerative processes. Collectively, recent studies suggest that the deleterious effects of seizures may not solely be a consequence of neuronal damage and loss per se, but could be due to the fact that seizures interfere with the highly regulated developmental processes in the immature brain [35].

The genesis of seizure involves a disturbance between inhibitory and excitatory neurotransmitter system of brain function [35]. Accordingly, an increase in excitatory amino acid transmission and a decrease in GABAergic inhibitory responses seem to be important for convulsion. In this context, γ-aminobutyric acid (GABA) is one of the main inhibitory transmitters in the central nervous system (CNS) [17]. GABA receptors are upregulated in response to seizures and GABA acts as amino acid excitatory on immature neurons due to high intracellular chloride levels. This ionophore complex is widely implicated in epilepsy [3]. The observation that many of the GABAergic synapses in the developing brain are excitatory, rather than inhibitory, might also increase the excitability of the immature brain and turn that more susceptible to convulsion [7]. GABA is excitatory during the first 2–3 weeks of life [28].

Glutamate is the main excitatory neurotransmitter in the mammalian CNS, involved in essential brain functions, such as neural development, learning and memory [47]. Altered excitatory amino acid neurotransmission, mediated primarily by glutamate, is a major cause of the imbalance of excitation and inhibition that contributes to hyperexcitability in the immature brain [49]. Glutamate

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exerts its toxicological effects by acting on both metabotrophic and ionotrophic receptors. These receptors reveal their potential role in a variety of central nervous system disorders such as epilepsy, pain, ischemia and neurodegenerative diseases [40]. Studies indicate that alterations in glutamate transporters can lead to epileptic phenotypes and that impairment in glutamate uptake may play an important role in epileptogenesis [52]. In this context, blockade of AMPA receptors can protect the brain from apoptotic and necrotic cell death by preventing neuronal excitotoxicity during pathophysiological activation of glutamatergic neurons [58]. In fact, 2,3-benzodiazepine AMPA antagonists can protect against seizures [58].

Evidence supports the hypothesis that experimental epilepsy is mediated by oxidative stress [6,13]. The activation of excitatory amino acid receptor can also trigger the formation of reactive oxygen species (ROS) [53]. The brain is a preferential target for the peroxidative process because it has a high content of polyunsaturated fatty acids [33]. This way, free radicals are neutralized by an elaborate antioxidant defense system consisting of enzymes such as catalase (CAT) and glutathione peroxidase (GPx) and non-enzymatic antioxidants such as ascorbic acid (AA). Thus, when ROS production is excessive, the intrinsic antioxidant scavenging capacity is overwhelmed resulting in the development of oxidative stress which can induce tissue injury and may activate apoptosis processes [61]. Many cerebral enzymes, such as Na^+, K^+ ATPase, are sensitive to situations associated with oxidative stress [10]. Accordingly, differences have been reported in free radical scavenging enzyme levels during the convulsive process [26].

The extent that prolonged seizure activity, i.e. status epilepticus (SE), and repeated, brief seizures affect neuronal structure and function in both the immature and mature brain has been the subject of increasing clinical and experimental research. In this context, the most widely used epilepsy models in immature rats are the administration of convulsant drugs such as kainic acid (KA), an agonist of KA type of glutamate receptors, and pilocarpine (PC), agonists of cholinergic muscarinic receptors [35].

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to design synthetic organoselenium compounds [4]. Accordingly, chalcogenophenes, a class of organochalcogen heterocycles containing a five-membered ring in the structure, have drawn the attention of researchers in view of their interesting biological activities [15,36]. In addition to their antioxidant action [42], chalcogenophenes were found to have antinociceptive and anti-inflammatory properties [42,67,30,66]. Among chalcogenophenes, selenophenes play an important role in organic synthesis [2] because of their excellent electrical properties and environmental stability.

Based on the considerations above, the objectives of the present study were to evaluate: (i) the anticonvulsant effect of 3-alkynyl selenophene (3-ASP) on PC-, pentylenetetrazole- and kainic acid-induced seizures and mortality in 21-day-old rats, (ii) the possible involvement of glutamatergic and GABAergic receptors in the anticonvulsant effect of 3-ASP in PC model, and (iii) the antioxidant effect of 3-ASP on oxidative stress induced by PC.

2. Materials and methods

2.1. Animals

Young male Wistar rats (40–50 g; 21-day-old) were obtained from a local breeding colony. Animals were housed in cages with free access to food and water. The animals were kept in separate animal rooms, on a 12-h light/12-h dark cycle, in an air-conditioned room (22 ± 2 °C). The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

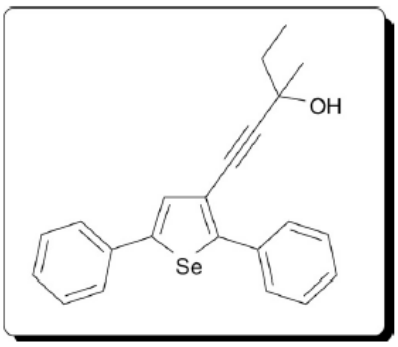

Fig. 1. Chemical structure of 3-ASP.

2.2. Drugs

Pilocarpine hydrochloride (PC), kainic acid (KA), pentylenetetrazole (PTZ), 5S,10R-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 2-methyl-6-phenylethynyl pyridine hydrochloride (MPEP) and diazepam were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

PC, PTZ, KA, MK-801 and MPEP were dissolved in 0.9% physiological saline. DNQX was dissolved in a minimum amount of dimethylsulfoxide (DMSO) and adjusted to the appropriate volume with 0.9% physiological saline. Diazepam is insoluble in saline or water, then it was dissolved in polyethylene glycol and adjusted to the appropriate volume with 0.9% physiological saline.

3-ASP (Fig. 1) was prepared in our laboratory according to the literature method [2]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that 3-ASP obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/HPLC. This drug was dissolved in canola oil.

2.3. In vivo experiments

2.3.1. Effect of 3-ASP on PC-, PTZ- and KA-induced seizures

The 21-day-old rats were treated orally by gavage with 3-ASP (10, 25 or 50 mg/kg.) or canola oil and 30 min after animals received PC (400 mg/kg, i.p.) [46], PTZ (80 mg/kg, i.p.) [63] or KA (45 mg/kg, i.p.) [45]. Animals treated with PC also received scopolamine hydrobromide (1 mg/kg, i.p.) 45 min before PC to avoid peripheral toxicity and diarrhea, masticatory and stereotyped movements [27].

Protocol 1 of 21-day-old rats treatment is given below:

- Group 1: canola oil + saline.
- Group 2: canola oil + PC or PTZ or KA.
- Group 3: 3-ASP (10 mg/kg, p.o.) + PC or PTZ or KA.
- Group 4: 3-ASP (25 mg/kg, p.o.) + PC or PTZ or KA.
- Group 5: 3-ASP (50 mg/kg, p.o.) + PC or PTZ or KA.

After the administration of convulsants, rats were observed by 1 h for behavioral changes. The latency for the onset of the tonic–clonic seizure episode was also recorded. Only animals with seizure activity were considered to calculate the latency to the onset of seizures. The latency to the death was observed in the cut-off of 60 min.

Since, studies have demonstrated that PC induced status epilepticus is followed by changes in the level of oxidative stress in several regions of the brain and reactive oxygen species (ROS) could be involved in the subsequent neuronal damage [27,18] and that 3-ASP abolished seizures in this model, the protective effect of 3-ASP against oxidative stress caused by PC was also investigated.

Subsequently to the seizure episode induced by PC, rats were decapitated. Animals which did not display seizure activity were considered protected and decapitated 1 h after the compound administration. The brains were immediately removed for determination of glutathione peroxidase (GPx), glutathione-S-transferase (GST), acetylcholinesterase (AChE), Na^+K^+ ATPase and catalase activities as well as reactive species (RS) and AA levels.

2.3.2. Effect of subeffective doses of 3-ASP plus diazepam, MK-801, DNQX or MPEP on PC-induced seizures

The 21-day-old rats were treated with diazepam, a GABAergic agonist (0.5 mg/kg, i.p.) [60], MK-801, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist (0.1 mg/kg, i.p.) [27], DNQX, a non-NMDA receptor antagonist (5 mg/kg, i.p.) [51] or MPEP, an antagonist of metabotropic glutamate receptor mGluR5 (0.5 mg/kg,

i.p.) [38] and 30 min after animals received 3-ASP (10 mg/kg, p.o.) or canola oil. PC (400 mg/kg, i.p.) [46] was injected 30 min after 3-ASP administration. All animals treated with PC received also scopolamine hydrobromide (1 mg/kg, i.p.) 45 min before the PC administration. No alteration was observed in animals that received DMSO, polyethylene glycol or saline when compared to canola oil (vehicles).

Protocol 2 of 21-day-old rats treatment is given below:

- Group 1: saline + canola oil + saline.
- Group 2: saline + canola oil + PC.
- Group 3: diazepam + canola oil + PC.
- Group 4: MK-801 + canola oil + PC.
- Group 5: DNQX + canola oil + PC.
- Group 6: MPEP + canola oil + PC.
- Group 7: diazepam + 3-ASP + PC.
- Group 8: MK-801 + 3-ASP + PC.
- Group 9: DNQX + 3-ASP + PC.
- Group 10: MPEP + 3-ASP + PC.

After the administration of PC, rats were observed as described the steps above in Section 2.3.1.

2.4. Ex vivo experiments

The samples of brain were homogenized in 50 mM Tris–HCl, pH 7.4 (1:5, w/v), and centrifuged at 2400 × g for 10 min to obtain the low-speed supernatant (S_1).

2.4.1. RS measurement

In order to verify the presence of oxidative imbalance induced by PC in brain from 21-day-old rats, RS levels were measured. To estimate the level of brain RS production, S_1 was diluted (1:10) in 50 mM Tris–HCl (pH 7.4) and incubated with 10 μl of dichlorofluorescein (DCF; 1 mM). The RS levels were determined by a spectrofluorimetric method, using 2′,7′-dichlorofluorescein diacetate (DCHF-DA) assay. The oxidation of DCHF-DA to fluorescent dichlorofluorescein is measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 15 min after the addition of DCHF-DA to the medium. The RS levels were expressed as AU.

2.4.2. GPx activity

GPx activity in S_1 of brain was assayed spectrophotometrically by the method of Wendel [64], through the glutathione/NADPH/glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. In this assay, the enzyme activity is measured indirectly by means of NADPH decay. H_2O_2 is decomposed, generating oxidized glutathione (GSSG) from reduced glutathione (GSH). GSSG is regenerated back to GSH by the glutathione reductase present in the assay media, at the expense of NADPH. The enzymatic activity was expressed in nmol NADPH/min/mg protein.

2.4.3. GST activity

GST activity in S_1 of brain was determined spectrophotometrically at 340 nm as described by Habig et al. [32]. An aliquot of 100 μl of S_1 was added in 0.1 M potassium phosphate buffer, pH 7.4. CDNB was used as substrate and GSH at 50 mM of the concentration. The enzymatic activity was expressed in nmol CDNB/min/mg protein.

2.4.4. CAT activity

CAT activity was assayed spectrophotometrically by the method of Aebi [1], which involves monitoring the disappearance of H_2O_2 in the S_1 presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 μl of the S_1 tissue and substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.5. The enzymatic activity was expressed in units (1 U decomposes 1 μmol of H_2O_2 per minute).

2.4.5. AA levels

AA levels determination was performed as described by Jacques-Silva et al. [37]. Proteins were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample at a final volume of 1 ml of the solution was incubated at 38 °C for 3 h then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of AA is related to tissue amount (μmol AA/g wet tissue).

2.4.6. SOD activity

Superoxide dismutase activity in S1 was assayed spectrophotometrically as described by Misra and Fridovich [44]. This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. The S1 was diluted 1:10 (v/v) for determination of SOD activity in the test day. Aliquots of supernatant were added in a Na_2CO_3 buffer 50 mM pH 10.3. Enzymatic reaction was started by adding of epinephrine. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. The enzymatic activity was expressed as Units (U)/mg protein.

2.4.7. Na^+, K^+ ATPase activity

Na^+, K^+ ATPase activity was determined in S_1 of brain. The reaction mixture for Na^+, K^+ ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 μl. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. Control samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The samples were incubated at 37 °C for 30 min, the incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. Na^+, K^+ ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was spectrofluorimetrically measured at 650 nm as described by Fiske and Subbarow [25] and Na^+, K^+ ATPase activity was expressed as nmolPi/mg protein/min.

2.4.8. AChE activity

Activity of AChE was carried out according to the method of Ellman et al. [21] using acetylthiocholine as substrate. The activity of AChE was spectrofluorimetrically measured at 412 nm and expressed as nmol/min/mg protein.

2.4.9. Protein quantification

Protein concentration was measured by the method of Bradford [11], using bovine serum albumin as the standard.

2.5. Statistic analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed using a one-way (effect of 3-ASP on PC-, PTZ- and KA-induced seizures and the effect of subeffective doses of 3-ASP plus diazepam, MK-801, DNQX or MPEP on the PC-induced seizures) or two-way (effect of 3-ASP on PC-induced oxidative stress) analysis of variance (ANOVA), followed by Newman–Keuls test when appropriate. Values of $p < 0.05$ were considered statistically significant. Seizure incidence was analyzed statistically by the χ^2 method and Fisher's Exact Test.

3. Results

3.1. Effect of 3-ASP on PC-, PTZ-, and KA-induced seizures

As shown in Table 1, 3-ASP (10 mg/kg) increased the latency (1.6 times) to the seizure onset induced by PC (400 mg/kg, i.p.) when compared to the PC group. 3-ASP (25 mg/kg) abolished the death induced by PC (400 mg/kg, i.p.), but did not alter the latency to the

Table 1
Effect of treatment with 3-ASP on pilocarpine-, pentylenetetrazole- and kainic acid-induced seizures and death in 21-day-old rats.

Groups	Seizures		Death	
	n/N^a	Latency (min)	n/N^b	Latency (min)
Control	0/9	ns	0/9	nl
3-ASP 10	0/9	ns	0/9	nl
3-ASP 25	0/9	ns	0/9	nl
3-ASP 50	0/9	ns	0/9	nl
PC	8/8	21.24 ± 4.71	5/8	41.76 ± 12.00
3-ASP 10 + PC	7/8	34.10 ± 17.00*	5/8	47.71 ± 10.00
3-ASP 25 + PC	2/8	21.00 ± 2.00	7/8	nl
3-ASP 50 + PC	0/8*	ns	0/8*	nl
PTZ	8/8	3.35 ± 0.85	3/8	26.56 ± 6.01
3-ASP 10 + PTZ	8/8	4.34 ± 1.93	3/8	22.14 ± 8.90
3-ASP 25 + PTZ	4/8	11.42 ± 4.52#	1/8	26.22 ± 0.00
3-ASP 50 + PTZ	1/8#	27.01 ± 0.00#	0/8#	nl
KA	9/9	19.86 ± 6.63	4/9	44.02 ± 4.91
3-ASP 10 + KA	9/9	28.08 ± 10.03&	4/9	46.86 ± 6.34
3-ASP 25 + KA	4/9	37.02 ± 8.19&	1/9	45.53 ± 0.00
3-ASP 50 + KA	2/9	40.65 ± 2.39&	0/9&	nl

Animals were treated intraperitoneally (i.p.) with 3-ASP (10–50 mg/kg) and 30 min after they received PC (400 mg/kg, i.p.), PTZ (80 mg/kg, i.p.) or KA (45 mg/kg, i.p.). *Abbreviations*: PC, pilocarpine; PTZ, pentylenetetrazole; KA, kainic acid; 3-ASP 10, 3-alkynyl selenophene 10 mg/kg; 3-ASP 25, 3-alkynyl selenophene 25 mg/kg; 3-ASP 50, 3-alkynyl selenophene 50 mg/kg. Results for the latency to the first seizure are expressed as means ± S.E.M.

[a] Number of animals which presented seizures/*N* of animals per group.

[b] Number of animals which died/*N* of animals per group (χ^2 method and Fischer's exact probability test).

* $p < 0.05$ as compared to the PC group.

\# $p < 0.05$ as compared to the PTZ group.

& $p < 0.05$ as compared to the KA group (ANOVA and Student–Newman–Keuls test).

onset of seizure. 3-ASP (50 mg/kg) prevented seizures and death induced by PC (400 mg/kg, i.p.) in 21-day-old rats (Table 1).

No alteration in the latency to the onset of seizure and death induced by PTZ (80 mg/kg, i.p.) in the animals treated with 3-ASP (10 mg/kg) was observed. Conversely, an increase in the latency (3.4 times) to the seizure onset induced by PTZ (80 mg/kg, i.p.) was observed in animals treated with 3-ASP (25 mg/kg). 3-ASP (50 mg/kg) abolished death and increased the latency (8.0 times) to the onset of seizure induced by PTZ (80 mg/kg, i.p.) (Table 1).

3-ASP (10 and 25 mg/kg) increased the latency (1.4 and 1.8 times, respectively) to the onset of seizure induced by KA (45 mg/kg, i.p.) but did not abolish death induced by KA. 3-ASP (50 mg/kg) abolished death and increased the latency (2.0 times) to the onset seizure induced by KA (45 mg/kg, i.p.) (Table 1).

3.2. Effect of subeffective doses of 3-ASP plus diazepam, MK-801, DNQX or MPEP on PC-induced seizures

Table 2 shows that treatment with diazepam (0.5 mg/kg, i.p.), MK-801 (0.1 mg/kg, i.p.), DNQX (5 mg/kg, i.p.) or MPEP (0.5 mg/kg, i.p.), 30 min before PC administration, neither alter the latency to the seizure onset nor abolish death in 21-day-old rats when compared to the PC-treated group.

As shown in Table 2, the administration of diazepam (0.5 mg/kg, i.p.) or MK-801 (0.1 mg/kg, i.p.) together with 3-ASP (10 mg/kg) increased the latency (1.6 and 2.0 times, respectively) to the seizure onset ($p < 0.05$). Treatment with MK-801 (0.1 mg/kg, i.p.) plus 3-ASP (10 mg/kg) abolished death induced by PC in rats. It was found an increase in the latency (1.72 times) to the seizure onset and in the latency to the death (1.4 times) when DNQX (5 mg/kg, i.p.) was administrated together with 3-ASP (10 mg/kg) (Table 2). The latency to the onset of seizure and the number of animals which died were not altered by administration of MPEP (0.5 mg/kg, i.p.) plus 3-ASP (10 mg/kg) compared to PC-treated group.

3.3. RS measurement

Two-way ANOVA of RS levels revealed a significant 3-ASP × PC interaction ($F_{3,63} = 3.88$; $p < 0.0131$). Post hoc comparisons showed that PC significantly increased RS levels in brain of 21-day-old rats when compared to the control group ($F_{1,63} = 127.53$; $p < 0.0001$). Treatment with 3-ASP at all doses protected against the increase in brain RS levels caused by PC in 21-day-old rats ($F_{1,63} = 10.34$; $p < 0.0001$) (Table 3). 3-ASP at all doses did not alter RS levels when compared to the control group (Table 3).

Table 2
Effect of the subeffective dose of 3-ASP plus diazepam, MK-801, DNQX or MPEP on the pilocarpine-induced seizures in 21-day-old rats.

Groups	Seizures		Death	
	n/N^a	Latency (min)	n/N^b	Latency (min)
Pilo	8/8	21.24 ± 4.71	5/8	41.76 ± 12.00
Dzp + PC	8/8	15.87 ± 4.71	6/8	52.12 ± 7.96
Dzp + 3-ASP 10 + PC	4/8	33.40 ± 7.46*	1/8	48.00 ± 0.00
MK801 + PC	9/9	21.56 ± 4.16	7/9	35.16 ± 12.43
MK801 + 3-ASP 10 + PC	2/9	43.35 ± 4.63*	0/9*	nl
DNQX + PC	7/7	20.37 ± 1.84	5/7	37.87 ± 3.21
DNQX + 3-ASP 10 + PC	3/9	38.69 ± 3.97*	1/9	58.17 ± 0.00*
Mpep + PC	8/8	24.04 ± 7.03	6/8	47.12 ± 7.10
Mpep + 3-ASP 10 + PC	7/8	31.90 ± 8.47	5/8	47.97 ± 6.57

The 21-day-old rats were treated with diazepam (0.5 mg/kg, i.p.), MK-801 (0.1 mg/kg, i.p.), DNQX (5 mg/kg, i.p.) or MPEP (0.5 mg/kg, i.p.) and 30 min after animals received 3-ASP (10 mg/kg, p.o.) or saline (0.9%, i.p.). *Abbreviations*: PC, pilocarpine; 3-ASP 10, 3-alkynyl selenophene 10 mg/kg. The PC (400 mg/kg, i.p.) was administrated 30 min after of the 3-ASP administration. Results for the latency to the first seizure are expressed as means ± S.E.M.

[a] Number of animals which presented seizures/N of animals per group.

[b] Number of animals which died/N of animals per group (χ^2 method and Fischer's exact probability test).

* $p < 0.05$ as compared to the PC group (ANOVA and Student–Newman–Keuls test).

Table 3
Effect of 3-ASP on RS, glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities in brain of 21-day-old rats after pilocarpine-induced seizures.

Groups	RS	GST	GPx
Control	14.60 ± 3.85	119.71 ± 31.22	256.25 ± 25.35
3-ASP 10	14.19 ± 3.26*	132.12 ± 30.58*	242.62 ± 28.32*
3-ASP 25	11.75 ± 3.83*	139.50 ± 19.97*	265.75 ± 31.45*
3-ASP 50	11.11 ± 3.55*	132.50 ± 29.27*	271.12 ± 53.98*
PC	30.69 ± 6.02#	217.50 ± 26.28#	163.87 ± 25.82#
3-ASP 10 + PC	21.82 ± 3.46*,#	206.00 ± 27.79#	173.40 ± 27.31#
3-ASP 25 + PC	21.06 ± 4.33*,#	200.90 ± 31.93#	177.50 ± 39.12#
3-ASP 50 + PC	20.74 ± 2.65*,#	205.36 ± 34.06#	190.45 ± 53.09#

The results are expressed as mean ± S.E.M., $n = 8–12$ rats/group. The RS levels were expressed as AU means arbitrary units, GST activity as nmol CDNB/mim/mg protein and GPx activity as nmol NADPH/min/mg protein. *Abbreviations*: PC, pilocarpine; 3-ASP 10, 3-alkynyl selenophene 10 mg/kg; 3-ASP 25, 3-alkynyl selenophene 25 mg/kg; 3-ASP 50, 3-alkynyl selenophene 50 mg/kg.

\# $p < 0.05$ compared to the control group.

* $p < 0.05$ compared to the PC group, by ANOVA followed by Student–Newman–Keuls.

3.4. GPx activity

A significant main effect of PC ($F_{1,63} = 82.71$; $p < 0.0001$) was observed. Post hoc comparisons revealed that PC decreased GPx activity. The decrease in GPx activity induced by PC (400 mg/kg, i.p.) was not altered in brain of 21-day-old rats treated with 3-ASP when compared to the control group (Table 3).

GPx activity remained unaltered in the brain of rats which received 3-ASP at the doses of 10, 25 and 50 mg/kg when compared to the control group (Table 3).

3.5. GST activity

A significant main effect of PC ($F_{1,63} = 115.76$; $p < 0.0001$) was observed. Post hoc comparisons revealed that PC increased GST activity. As shown in Table 3, the increase in GST activity induced by PC (400 mg/kg, i.p.) was not altered in brain of 21-day-old rats treated with 3-ASP when compared to the control group.

3-ASP at all doses did not alter GST activity when compared to the control group (Table 3).

3.6. CAT activity

Two-way ANOVA of CAT activity revealed a significant 3-ASP × PC interaction ($F_{3,63} = 3.96$; $p < 0.0004$). Post hoc comparisons showed that the treatment of animals with PC (400 mg/kg, i.p.) produced an increase in CAT activity in brain of 21-day-old rats in comparison to the control group ($F_{1,63} = 11.34$; $p < 0.0013$) (Table 4).

3-ASP at the dose of 10 mg/kg decreased CAT activity when compared to the PC group. Rats treated with 3-ASP (25 and 50 mg/kg) presented CAT activity similar to the activity found in control rats ($F_{3,63} = 5.17$; $p < 0.0029$) (Table 4).

CAT activity remained unaltered in the brain of rats which received 3-ASP, at all doses, when compared to the control group (Table 4).

3.7. AA levels

Two-way ANOVA of AA levels revealed a significant 3-ASP × PC interaction ($F_{3,63} = 3.42$; $p < 0.0225$). Post hoc comparisons showed

that the treatment of animals with PC significantly decreased AA levels in brain of 21-day-old rats when compared to the control group ($F_{1,63}$ = 17.43; p < 0.0001). Treatment with 3-ASP (25 and 50 mg/kg) protected against the decrease in AA levels induced by PC in 21-day-old rats when compared to the PC group (Table 4).

3-ASP (10, 25 and 50 mg/kg) did not alter AA levels when compared to the control group (Table 4).

3.8. SOD activity

Two-way ANOVA of SOD activity revealed a significant 3-ASP × PC interaction ($F_{3,63}$ = 2.98; p < 0.0502). Post hoc comparisons showed that the treatment of animals with PC (400 mg/kg, i.p.) produced an decrease in SOD activity in brain of 21-day-old rats in comparison to the control group ($F_{1,63}$ = 11.40; p < 0.0038) (Table 4). Treatment with 3-ASP (50 mg/kg) protected the decrease in SOD activity induced by PC in 21-day-old rats when compared to the PC group (Table 4).

3-ASP (10, 25 and 50 mg/kg) did not alter SOD activity when compared to the control group ($F_{3,63}$ = 2.32; p < 0.1141) (Table 4).

3.9. Na^+, K^+ ATPase activity

A significant main effect of PC ($F_{1,63}$ = 16.94; p < 0.0001) was observed. Post hoc comparisons revealed that PC decreased Na^+, K^+ ATPase activity. As shown in Table 4, 3-ASP at all doses protected against the inhibition of AChE activity induced by PC when compared to the PC group (Table 5).

3-ASP at all doses did not alter AChE activity when compared to the control group (Table 5).

3.10. AChE activity

A significant main effect of PC ($F_{1,63}$ = 14.06; p < 0.0004) was observed. Post hoc comparisons revealed that PC decreased AChE activity. Table 5 shows that 3-ASP (50 mg/kg) protected against the inhibition of AChE activity caused by PC.

3-ASP at all doses did not alter AChE activity when compared to the control group (Table 5).

4. Discussion

The results of the present study demonstrate that 3-ASP has anticonvulsant action in 21-day-old rats in the PC model; protects against biochemical alterations caused by PC and that its anticonvulsant effect can be attributed, at least in part, to the involvement of ionotropic glutamatergic and GABAergic receptors This study also reveals that 3-ASP increases the latency to the onset of seizure induced by PTZ and KA in 21-day-old rats. This conclusion derives from the following results; the administration of 3-ASP: (i) at the dose of 50 mg/kg abolished seizures and the death induced by PC (400 mg/kg, i.p.); (ii) together with diazepam, a GABAergic agonist (0.5 mg/kg, i.p.), MK-801, a non-competitive NMDA receptor antagonist (0.1 mg/kg, i.p.) or DNQX, a non-NMDA receptor antagonist (5 mg/kg, i.p.), an increase in the latency to the seizure onset and in the latency to the death induced by PC; (iii) together with MPEP, an antagonist of metabotropic glutamate receptor mGluR5 (0.5 mg/kg, i.p.), did not alter the latency to the onset seizure and the number of animals who died in the PC model; (iv) at all doses protected against the increase in RS levels and CAT activity and the decrease in AA levels in brain of 21-day-old rats caused by PC; (v) protected against the decrease of Na^+, K^+ ATPase, SOD and AChE activities induced by PC, and (vi) increased the latency to the onset of seizure induced by PTZ (80 mg/kg, i.p.) and KA (45 mg/kg, i.p.).

The use of animal seizure models is essential in the discovery and development of new drugs for the treatment of epileptic seizures. PTZ is a convulsant chemical agent used in experimental models for induction of seizures [19]. Experiments revealed that a single administration of PTZ produced benzodiazepine-binding decrease, suggesting that PTZ-induced chemical seizure may be associated with significant changes of the GABAergic system [50]. Similarly, the PC animal model of epilepsy is a common experimental tool in epilepsy research. Many microdialysis studies demonstrated significant alterations of glutamate and GABA concentrations in PC-induced seizure [14]. In addition, the KA treated rat is an experimental model of temporal lobe epilepsy [9]. KA is thought to exert its action, at least in part, by augmenting glutamate release through pre-synaptic receptors [23]. Furthermore, marked decrease in glutamate decarboxylase activity, catalyzing the formation of GABA, has been found in several brain regions such as amygdala, piriform cortex, lateral septum and the hippocampus [55]. 3-ASP, at the dose of 50 mg/kg, abolished seizures and death induced by PC in 21-day-old rats, demonstrating its anticonvulsive effect in this model. Conversely, 3-ASP increased the latency to the seizure onset and at the dose of 50 mg/kg abolished death induced by PTZ and KA. These results suggest that the anticonvulsant effect is dependent of the seizure model used. In this context, there is evidence of possible involvement of the glutamatergic and GABAergic systems in the anticonvulsant effect observed in PC model.

Blockade of glutamatergic receptors can protect the brain from apoptotic and necrotic cell death by preventing neuronal excitotoxicity during pathophysiological activation of glutamatergic neurons.

Table 4
Effect of 3-ASP on AA levels, catalase (CAT) and superoxide dismutase (SOD) activities in brain of 21-day-old rats after pilocarpine-induced seizures.

Groups	AA	CAT	SOD
Control	719.63 ± 26.40	9.37 ± 2.53	28.83 ± 3.00
3-ASP 10	698.91 ± 37.12*	10.08 ± 2.61*	26.50 ± 2.07
3-ASP 25	691.96 ± 48.01*	10.03 ± 2.35*	26.02 ± 1.98
3-ASP 50	704.18 ± 28.90*	10.05 ± 2.85*	27.04 ± 0.69
PC	551.59 ± 65.83#	17.54 ± 3.11#	21.48 ± 1.03#
3-ASP 10 + PC	581.91 ± 84.02#	12.06 ± 4.03*,#	19.58 ± 1.20#
3-ASP 25 + PC	674.31 ± 52.76*	10.98 ± 3.07*	23.46 ± 1.21
3-ASP 50 + PC	679.90 ± 50.18*	8.45 ± 1.90*	28.15 ± 1.74*

The results are expressed as mean ± S.E.M., n = 8–12 rats/group. The AA levels were expressed as µmol AA/g wet tissue, CAT activity as units (1 U decomposes 1 µmol of H_2O_2 per minute) and SOD activity as units (U)/mg protein. *Abbreviations*: PC, pilocarpine; 3-ASP 10, 3-alkynyl selenophene 10 mg/kg; 3-ASP 25, 3-alkynyl selenophene 25 mg/kg; 3-ASP 50, 3-alkynyl selenophene 50 mg/kg.

# p < 0.05 compared to the control group.

* p < 0.05 compared to the PC group, by ANOVA followed by Student–Newman–Keuls.

Table 5
Effect of 3-ASP on Na^+K^+ATPase and acetylcholinesterase (AChE) activities in brain of 21-day-old rats after pilocarpine-induced seizures.

Groups	Na^+K^+ATPase	AChe
Control	9.16 ± 3.72	4.05 ± 1.04
3-ASP 10	8.23 ± 2.94*	3.54 ± 0.72*
3-ASP 25	8.54 ± 3.07*	3.74 ± 0.97*
3-ASP 50	8.07 ± 2.01*	3.76 ± 1.98*
PC	3.65 ± 1.53#	1.72 ± 0.73#
3-ASP 10 + PC	6.62 ± 2.63*	2.46 ± 1.33#
3-ASP 25 + PC	6.39 ± 2.59*	2.75 ± 1.61
3-ASP 50 + PC	7.03 ± 2.18*	3.56 ± 0.89*

The results are expressed as mean ± S.E.M., n = 8–12 rats/group. The Na^+K^+ATPase and AChE activities were expressed as nmolPi/mg protein/min and nmol/min/mg prot, respectively. *Abbreviations*: PC, pilocarpine; 3-ASP 10, 3-alkynyl selenophene 10 mg/kg; 3-ASP 25, 3-alkynyl selenophene 25 mg/kg; 3-ASP 50, 3-alkynyl selenophene 50 mg/kg.

# p < 0.05 compared to the control group.

* p < 0.05 compared to the PC group, by ANOVA followed by Student–Newman–Keuls.

Animal experiments provided evidence for the potential usefulness of non-competitive AMPA antagonists in the treatment of human ischemic and neurodegenerative disorders including stroke, multiple sclerosis, Parkinson's disease, periventricular leukomalacia and motoneuron disease. 2,3-Benzodiazepine AMPA antagonists can protect against seizures, decrease levodopa-induced dyskinesia in animal models of Parkinson's disease demonstrating their utility for the treatment of a variety of CNS disorders [58].

Additionally, several studies have showed that brain endothelial cells act as a signal transducer or amplifier, especially, under pathological conditions, such as epileptic seizure. Further analysis of the interactions among neurons, astrocytes, and endothelial cells may provide a better understanding of the processes of neuropathological disorders as well as facilitating the development of new treatments [59].

In order to investigate the involvement of glutamatergic and GABAergic systems in the anticonvulsant effect of 3-ASP, we administrated a subeffective dose of 3-ASP (10 mg/kg) together with subeffective doses of diazepam, MK-801, DNQX or MPEP. Experimental evidence suggests that the ionotropic glutamatergic receptor is involved in the anticonvulsant effect of 3-ASP. In fact, the administration of 3-ASP together with MK-801 or DNQX increased the latency to the seizure onset and the latency to the death induced by PC. Conversely, the administration of 3-ASP together with MPEP did not alter the latency to the onset seizure and the number of animals who died, excluding the involvement of glutamatergic metabotropic receptor in this event. The fact that 3-ASP plus diazepam, at subeffective doses, increased the latency to the onset of seizure and the latency to the death induced by PC in 21-day-old rats can be attributed, at least in part, to the involvement of GABAergic receptor. However, more studies are necessary to elucidate the complete mechanism involved in the protective effect of 3-ASP against seizures induced by PC.

Since studies have demonstrated that PC induced status epilepticus is followed by changes in the level of oxidative stress in several regions of the brain and ROS could be involved in the subsequent neuronal damage [18,29], the PC model was used to investigate the protective effect of 3-ASP against alterations caused by oxidative stress induced by PC.

The relationship between seizures and ROS is well known as the epileptiform activity causes excessive free radical production of ROS, a factor believed to be involved in the mechanism leading to cell death and neurodegeneration [20,55]. We showed that RS formation in brain was increased after seizure episodes in 21-day-old rats, confirming the involvement of free radical in PC-induced brain injury. Indeed, 3-ASP treatment decreased RS levels in brain of 21-day-old rats, thus suggesting that this compound acts positively on free radicals caused by the PC administration.

In this study we observed a decrease in GPx activity in animals that received treatment with PC. The decrease of GPx activity has a toxic effect even in normoxia and it indicates that basal oxygen radical production can be damaging for the cell and should be controlled [43]. Besides, our research group demonstrated for the first time the stimulation of GST activity in the brain of young rats treated with PC [38]. In this study, we confirmed this increase. We believed that the stimulation of GST activity observed may be associated with an increase of ROS levels. In this way, authors have reported that GST is an antioxidant defense and serves to protect the tissues against oxidative stress [39]. In addition, 3-ASP was not effective in protecting the alteration in GPx and GST activities. We believe that 3-ASP does not exhibit its protective effects by acting on the redox system of GSH.

Under oxidative stress, protective factors such as CAT, SOD and AA are activated in the defense against oxidative injury. AA protects the brain against injury resulting from ischemia and excitatory amino acid toxicity [41,56]. Here, we demonstrated a decrease in AA levels caused by PC-induced seizures. It suggests that this decrease may be associated with an increase of ROS levels. 3-ASP protected this decrease, demonstrating its protector effect against oxidative injury caused by PC-induced seizures. The seizures alter oxidative stress by activation of free radicals scavenging enzymes such as CAT, indicating its neuroprotective effect [24]. Furthermore, in this study we confirmed that the treatment with PC (400 mg/kg, i.p.) produced the stimulation of CAT activity in brain of 21-day-old rats as demonstrated by us in a previous study [38].

Studying the seizures model induced by PC administration, we found a decrease in SOD activity in brain of 21-day-old rats after seizure induction in accordance with Bellissimo and collaborators [8]. In addition, we can suggest that the lipid peroxidation could be dependent of the decrease of SOD activity. SOD activity can also be inhibited by high amount of H_2O_2, released during the O_2^- dismutation [54]. Consequently, the excess of H_2O_2 produced could be responsible for the oxidative damage found in the brain of these animals.

PC-induced seizures produce several changes in parameters related to release and/or synthesis of brain neurotransmitters (monoamines and amino acids) of adult rats [12]. Turski and collaborators [62] demonstrated that the reduction in the metabolism of ACh in brain is essential for installation of seizures and for propagation and establishment of SE induced by PC. In this study we observed that 3-ASP protected against inhibition of AChE activity caused by PC in the brain of rats.

Na^+, K^+ ATPase is responsible for the maintenance of ionic gradient necessary for neuronal excitability [22]. The activity of this enzyme is decreased in cerebral ischemia [65], epilepsy [31]. Accordingly, we observed that 3-ASP protected against the decrease in Na^+, K^+ ATPase activity caused by PC.

Collectively, the results of the present study demonstrate that 3-ASP abolished seizures and the death induced by PC in 21-day-old rats. These effects were associated with antioxidant property of 3-ASP against the increase in RS levels and CAT activity and the decrease in AA levels and Na^+, K^+ ATPase and AChE activities in brain caused by PC. We suggest that the anticonvulsant action of 3-ASP can be attributed, at least in part, to the involvement of ionotropic glutamatergic and GABAergic systems. Thus, this study further confirms the anticonvulsant activity of 3-ASP in the PC model and the drug ability in reducing the oxidative stress in this model, although, more studies are necessary to elucidate the complete mechanism involved in these effects.

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3.2 Artigo 2

Envolvimento dos sistemas GABAérgico e glutamatérgico na atividade anticonvulsivante do 3-aquinil selenofeno em ratos de 21 dias de vida.

INVOLVEMENT OF GABAERGIC AND GLUTAMATERGIC SYSTEMS IN THE ANTICONVULSANT ACTIVITY OF 3-ALKYNYL SELENOPHENE IN 21-DAYS-OLD RATS

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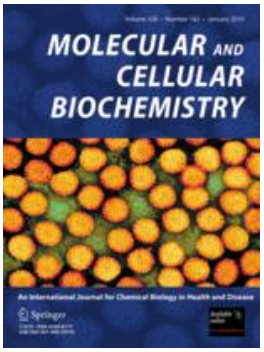

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Involvement of GABAergic and glutamatergic systems in the anticonvulsant activity of 3-alkynyl selenophene in 21-days-old rats

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Abstract

In this study, we investigated the role of GABAergic and glutamatergic systems in the anticonvulsant action of 3-alkynyl selenophene (3-ASP) in a pilocarpine (PC) model of seizures. To this purpose, 21-days-old rats were administered with an anticonvulsant dose of 3-ASP (50 mg/kg, per oral, p.o.) and [^{3}H]*γ*-aminobutyric acid (GABA) and [^{3}H]glutamate uptake were carried out in slices of cerebral cortex and hippocampus. [^{3}H]GABA uptake was decreased in cerebral cortex (64%) and hippocampus (58%) slices of 21-days-old rats treated with 3-ASP. By contrast, no alteration was observed in [^{3}H]glutamate uptake in cerebral cortex and hippocampus slices of 21-days-old rats that received 3-ASP. Considering that drugs that increase synaptic GABA levels, by inhibiting its uptake or catabolism, are effective anticonvulsants, we further investigated the possible interaction between sub-effective doses of 3-ASP and GABA uptake or GABA transaminase (GABA-T) inhibitors in PC-induced seizures in 21-days-old rats. For this end, sub-effective doses of 3-ASP (10 mg/kg, p.o.) and DL-2,4-diamino-*n*-butyric acid hydrochloride (DABA, an inhibitor of GABA uptake – 2 mg/kg, intraperitoneally; i.p.) or aminooxyacetic acid hemihydrochloride (AOAA; a GABA-T inhibitor – 10 mg/kg, i.p.) were co-administrated to 21-days-old rats before PC (400 mg/kg; i.p.) treatment and the appearance of seizures was recorded. Results demonstrated that treatment with AOAA and 3-ASP or DABA and 3-ASP significantly abolished the number of convulsing animals induced by PC. The present study indicates that 3-ASP reduced [^{3}H]GABA uptake, suggesting that its anticonvulsant action is related to an increase in inhibitory tonus.

Keywords: selenium; selenophene; anticonvulsant; *γ*-aminobutyric acid; glutamate; pilocarpine.

1 Introduction

Epilepsy affects 1-2% of human worldwide and has a peak incidence in the first year of life [1]. The genesis of seizure involves a disturbance between inhibitory and excitatory neurotransmitter system of brain function. The balanced activity of the inhibitory and excitatory neurotransmitter system in the brain is of essential importance for the normal brain function, and any disturbance in this genuine balance can lead to seizure activity [2]. Nearly all epileptic seizures are characterized by predominance of excitation over inhibition either simultaneously in many brain structures (primary generalized convulsive seizures) or in a part of the brain (partial, focal seizures) [2].

In the mammalian central nervous system (CNS), glutamate is the main excitatory neurotransmitter, being essential for normal brain functions [3]. However, overstimulation of the glutamatergic system, which occurs when extracellular glutamate levels increase over the physiological range, is involved in many acute and chronic brain diseases (excitotoxicity) such as epilepsy [4]. In addition, *γ*-aminobutyric acid (GABA) is recognized as the principal inhibitory neurotransmitter in the cerebral cortex [5]. Potentiation of GABAergic inhibition is the main mechanism of action of many antiepileptic drugs [6, 7]. Consequently, virtually all receptors, metabolic enzymes and transporters involved in GABAergic or glutamatergic neurotransmission can be considered as valid targets when designing new CNS-active drugs.

The enhancement of the GABAergic signal transduction can be effected either by direct receptor agonism or allosteric modulation of the GABA receptors, as it is accomplished by benzodiazepines or barbiturates [8]. On the other hand, the GABAergic neurotransmission can be enhanced by increasing the concentration of

GABA in the synaptic cleft. This may be achieved by inhibition of enzymatic GABA degradation or by blocking specific high affinity GABA transport proteins responsible for the removal of synaptic GABA [8]. These GABA transporters are located in the cell membranes of the pre-synaptic nerve terminals and also in those of glial cells. In fact, it has been suggested that the reduction of GABA levels in the synaptic cleft increases predisposition to seizures, indicating that GABA modulates seizure susceptibility [9].

Additionally, glutamate homeostasis in the brain is maintained by its well balanced release, uptake, and metabolism. The removal of glutamate from the synaptic cleft is the major mechanism for modulating glutamate actions and maintaining extracellular glutamate concentration below the neurotoxic levels. Glutamate uptake processes involve two transport systems located at distinct cellular levels: a high affinity Na^+-dependent carriers located at the cell membranes of neural and glial cells [10], and a low affinity Na^+-independent carrier located at the membrane of synaptic vesicles. Inhibition of glutamate uptake contributes for an increase in extracellular glutamate concentration, which ultimately leads to over stimulation of the glutamatergic system [11]. Over stimulation of the glutamatergic system may promote a process known as excitotoxicity, leading to cell death [3].

Previous reports have reported that 3-alkynyl selenophene (3-ASP) exhibits different pharmacological properties. 3-ASP is a hepatoprotective, antinociceptive, anti-allodynic and antioxidant drug [12-14]. Recently, we demonstrated the anticonvulsant activity of this organoselenium compound. These effects were associated with its antioxidant property on oxidative stress induced by pilocarpine (PC) administration [13]. By using pharmacological tools, our results suggested the

involvement of ionotropic glutamatergic and GABAergic receptors in the anticonvulsant action of 3-ASP [13].

In this study, we intend to investigate the contribution of [^{3}H]GABA and [^{3}H]glutamate uptake in the anticonvulsant activity of 3-ASP in a PC model of seizures. Considering that drugs that increase GABA synaptic levels, by inhibiting its uptake or catabolism, are effective anticonvulsants, we also investigated the possible interactions between 3-ASP and a GABA uptake inhibitor or 3-ASP and a GABA transaminase (GABA-T) inhibitor, using PC-induced seizures in 21-days-old rats.

2 Materials and methods

2.1 Animals

Male Wistar rats (40–50 g; 21-day-old) were obtained from a local breeding colony. Animals were housed in cages with free access to food and water, they were kept in a separate animal room, on a 12-h light/12-h dark cycle (with lights on at 7 a.m.), in an air-conditioned room (22 ± 2 ◦C). The research was submitted and approved by the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.2 Drugs

L-[^{3}H]*γ*-aminobutyric acid (GABA) (specific activity 40 Ci/mmol) and L-[^{3}H]glutamate (specific activity 50 Ci/mmol) were purchased from Amersham International, UK. Choline chloride was purchased from Sigma Chemical CO (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from

standard commercial suppliers. 3-ASP was prepared in our laboratory according to the literature method [15]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that 3-ASP obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by Gas Chromatography–Mass Spectrometry (GC/MS). This drug was dissolved in canola oil.

2.3 Uptake assay

[^{3}H]GABA and L-[^{3}H]glutamate uptake assays were carried out in slices of cortex and hippocampus of 21-days-old rats according to the method described by Schweigert et al. [16]. Animals were divided into two groups: control (canola oil, 1 ml/kg, p.o.) and 3-ASP (50 mg/kg, p.o., an anticonvulsant dose). Animals were decapitated after 30 minutes (min) of drug or vehicle administration; brains were immediately removed and submerged in 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. Cortex and hippocampus were dissected and coronal slices (0.4 mm) were obtained using a Mc Illwain tissue chopper. Slices were transferred to multiwell dishes and washed with 1.0 ml HBSS. After 10 min of pre incubation, the uptake assay was performed by adding 13.3 µM (hippocampus) and 6.6 µM (cortex) L-[^{3}H]glutamate or 16.6 ηM (hippocampus) and 8.3 ηM (cortex) [^{3}H]GABA in 300 µl HBSS at 37 °C. Incubation was terminated after 5 min (hippocampus) or 7 min (cortex) by three ice-cold washes with 1 ml HBSS immediately followed by the addition of 0.5 M NaOH, which was kept overnight. 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. Both uptakes were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). Results were expressed as pmol of L-[^{3}H]glutamate or [^{3}H]GABA uptake/mg protein.min^{-1}.

2.4 Protein determination

Protein concentration was measured by the method of Bradford [17], using bovine serum albumin (1 mg/ml) as a standard.

2.5 Behavioral tests

Different doses (2 to 16 mg/kg; intraperitoneally; i.p.) of DL-2,4-diamino-*n*-butyric acid hydrochloride (DABA, an inhibitor of GABA uptake) were tested against seizures induced by PC (400 mg/kg; i.p.) to obtain a sub-effective dose. Based on the results obtained, sub-effective doses of 3-ASP (10 mg/kg; p.o.) [10] and DABA (2 mg/kg, i.p.) were co-administrated to 21-days-old rats 30 min prior to the PC injection [13, 18] and the appearance of tonic-clonic seizures was recorded.

Different doses (10 to 20 mg/kg, i.p.) of aminooxyacetic acid hemihydrochloride (AOAA; a GABA-T inhibitor) were tested to obtain a sub-effective dose. Subsequently, sub-effective doses of 3-ASP (10 mg/kg, p.o.) [13] and AOAA (10 mg/kg, i.p.) were co-administrated to 21-days-old rats. Treatment times for 3-ASP and AOAA prior to the PC injection were 30 and 20 min, respectively [13, 18]. The appearance of tonic-clonic seizures was recorded.

After the administration of PC, 21-days-old rats were observed by 1 h for behavioral changes (tremors, stereotyped movements - increased activity of biting, scratching, wet-dog shakes; loss of muscle tone, clonic and tonic movements). The latency for the onset of the tonic–clonic seizure episode was recorded. Only animals with seizure activity were considered to calculate the latency to the onset of seizures.

2.6 Statistical analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed using a non-paired t-test. Values of $p < 0.05$ were considered statistically significant. Seizure incidence was statistically analyzed using the χ^2 method and Fisher's exact test.

3 Results

3.1 [^{3}H]*GABA and* [^{3}H]*Glutamate uptake*

[^{3}H]GABA uptake was decreased in cerebral cortex (64%) and hippocampus (58%) slices of 21-days-old rats treated with 3-ASP (50 mg/kg) when compared to the control group (Fig. 1A).

No alteration was observed in [^{3}H]glutamate uptake in both cortex and hippocampus of 21-days-old rats treated with 3-ASP (50 mg/kg) (Fig. 1B).

3.2 Behavioral tests

The number of convulsing animals resulting of PC administration was not altered by 3-ASP pre-treatment (10 mg/kg). 3-ASP (10 mg/kg) increased the latency to the first convulsive episode induced by PC (Table 1).

Pre-treatment with DABA, at the dose of 2 mg/kg, did not reduce the number of convulsing animals in the PC model and did not alter the behavioral seizure when compared to animals treated with PC (Table 1). DABA administered at doses of 8 and 16 mg/kg, decrease the number of animals that had seizures, but did not alter the behavioral seizure when compared to animals treated with PC (Table 1). No alteration was observed in the latency to the onset of seizures when compared to PC group. Co-treatment with DABA (2 mg/kg, i.p.) and 3-ASP (10 mg/kg, p.o.) completely abolished the appearance of seizures induced by PC (Table 1).

AOAA, at the dose of 10 mg/kg, was not effective in protecting against seizures induced by PC (Table 2). AOAA, at doses of 15 and 20 mg/kg, decreased the number of convulsing animals induced by PC. Pre-treatment with AOAA did not alter the behavioral seizure when compared to PC group. The latency to the onset of seizures was not altered when compared to PC group (Table 1). Co-treatment with sub-effective doses of AOAA and 3-ASP abolished seizures induced by PC in 21-days-old rats (Table 1).

4 Discussion

GABAergic function in the CNS could be potentiated with GABA receptor agonists [19] or inhibitors of GABA catabolism [20]. Besides, GABA function is potentiated by inhibition of GABA uptake from the synaptic cleft [21]. In this context, we demonstrated that 3-ASP inhibited [^{3}H]GABA uptake in cerebral cortex and hippocampal slices of 21-days-old rats, suggesting that its anticonvulsant action in the PC model of seizures could be associated to an increase in GABA levels in the synaptic cleft and consequently potentiation of inhibitory tonus. Similarly, Prigol et al. [22] have reported that *m*-trifluoromethyl-diphenyl diselenide, an organoselenium

compound with potential anticonvulsant, inhibited the [^{3}H]GABA uptake in cerebral cortex slices of mice.

Altered excitatory amino acid neurotransmission, mediated primarily by glutamate, is a major cause of the imbalance of excitation and inhibition that contributes to hiperexcitability in the immature brain [23]. Recently, we showed the involvement of glutamatergic receptors in the anticonvulsant action of 3-ASP [13]. Our experimental evidence suggests that the ionotropic glutamatergic receptor is involved in the anticonvulsant effect of 3-ASP. Conversely, we exclude the involvement of glutamatergic metabotropic receptor in this event. In this sense, in the present study we evaluated the possible involvement of [^{3}H]glutamate uptake in the anticonvulsant action of 3-ASP. Glutamate uptake is a vital step for glutamatergic neurotransmission. Uptake is one of the mechanisms by which glutamate is removed from the synaptic cleft, and its inhibition contributes for an increase in extracellular glutamate concentrations, which ultimately leads to over stimulation of the glutamatergic system [24]. Here, no alteration on [^{3}H]glutamate uptake in slices of cerebral cortex and hippocampus of animals treated with 3-ASP was observed. The results of the present study indicate that [^{3}H]glutamate uptake is not directly involved in the anticonvulsant action of 3-ASP in the PC model of seizures.

The elaboration of an efficacious mode of treatment for patients with drug-resistant epilepsy is now one of the main challenges facing clinicians and scientists [25, 26]. Among them, the combination of conventional and novel antiepileptic drugs has been included as the most efficacious, offering considerable protection against seizures with a minor tendency to produce side effects [27]. Considering the present results and that drugs that increase synaptic GABA levels by inhibiting uptake or

GABA catabolism are effective anticonvulsants, we investigated if the combination of sub-effective doses of 3-ASP and inhibitors of GABA uptake or GABA-T are effective against seizures induced by PC in 21-days-old rats.

GABA-T is a mitochondrial enzyme, which degrades GABA into succinic semialdehyde [28, 29]. GABA-T decreases the level of GABA in the brain and also increases the level of L-glutamate, therefore; producing the excitation of neurons by dual mechanisms [28]. The inhibition of enzyme GABA-T increases the GABA concentration in the brain, then, decreasing the susceptibility to convulsions and epileptic conditions, as shown by some reports [30, 31]. AOAA is a potent inhibitor of GABA-T [29]. Our results demonstrated that the association of sub-effective doses of AOAA and 3-ASP abolished seizures induced by PC in 21-days-old rats. In fact, it has been reported [6] that molecules with GABA-T inhibitory property exhibit significant protection and play a central role in the management of epilepsy.

In addition, the combination of sub-effective doses of 3-ASP and DABA (an inhibitor of GABA uptake) was effective in protecting against seizures induced by PC in 21-days-old rats. An inhibition of GABA uptake could represent higher GABA levels in the synaptic cleft, favoring the inhibitory system. New anticonvulsants, namely vigabatrin, tiagabine, gabapentin and topiramate, with a mechanism of action considered to be primarily via an effect on GABA, were licensed [5].

Although a number of classical GABAergic analogues are useful as pharmacological tools in epilepsy researches, they were shown to be inefficient in therapy due to their low permeability at the blood–brain barrier [32]. The high lipophilicity of 3-ASP and its subsequent possible ability to cross the blood-brain barrier could add to explain its anticonvulsant activity. In this context, it has been demonstrated that diphenyl diselenide, another organoselenium compound, is a

highly lipophylic compound and therefore exhibits a concentration–time profile characterized by an early peak concentration and rapid distribution from blood to the CNS, where it exerts its pharmacological and toxicological effects [33-35].

5 Conclusion

In conclusion, we reported that 3-ASP reduced [^{3}H]GABA uptake, suggesting that its anticonvulsant action is related to an increase in inhibitory tonus. In addition, our results indicate that [^{3}H]glutamate uptake was not involved in 3-ASP anticonvulsant action in 21-days-old rats. However, more studies are necessary to elucidate other mechanisms related to the anticonvulsant action of 3-ASP.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Acknowledgments

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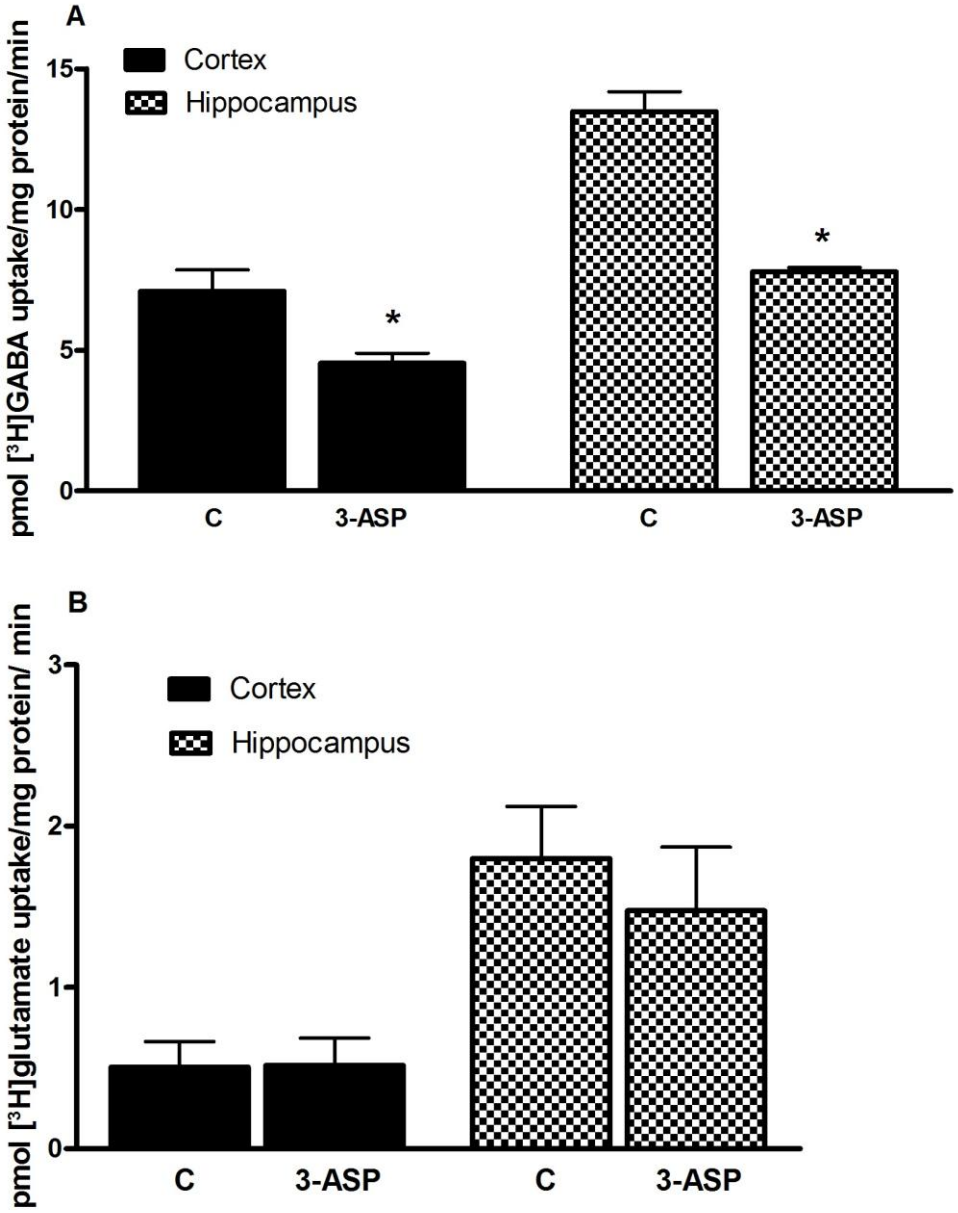
Legends

Figure 1. Effect of a single oral dose of 3-ASP on [^{3}H]GABA uptake (**panel A**) and [^{3}H]Glutamate uptake (**panel B**). [^{3}H]GABA and [^{3}H]Glutamate uptakes were carried out in slices of cortex and hippocampus of 21-days-old rats treated per oral route with 3-ASP at the dose of 50 mg/kg. *Abbreviation:* C = control; 3-ASP = 3-alkynyl selenophene. Data are expressed as means ± S.E.M. * $p < 0.05$ compared to the control group.

Figures

Figure 1



Table

Table 1. Effect of sub-effective doses of 3-ASP and DABA or AOAA against PC-induced seizures in rats.

Groups	n/N[a]	Latency[b]
Control	0/8 *	ns
PC	8/8	13.76 ± 2.38
PC + 3-ASP	7/8	35.00 ± 15.00*
DABA 2 + PC	8/8	12.50 ± 2.77
DABA 8 + PC	5/8	23.20 ± 7.55
DABA 16 + PC	5/8	11.40 ± 1.50
DABA 2 + 3-ASP + PC	0/7 *	ns
AOAA 10 + PC	8/8	19.63 ± 4.43
AOAA 15 + PC	5/8	23.30 ± 2.58
AOAA 20 + PC	3/8	12.67 ± 4.70
AOAA 10 + 3-ASP + PC	0/7*	ns

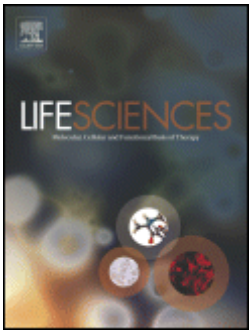
[a] Number of animals which presented seizures/ number of animals per group; [b] Latency to first seizure episode, expressed in minutes. Different doses of DABA (2 to 16 mg/kg; i.p.) or AOAA (10 to 20 mg/kg; i.p.) were tested against seizures induced by PC (400 mg/kg; i.p.) to obtain a sub-effective dose. 3-ASP was administered at the dose of 10 mg/kg (p.o). *Abbreviations: ns* = no seizures; *AOAA* = aminooxyacetic acid hemihydrochloride; *DABA* = DL-2,4-diamino-*n*-butyric acid hydrochloride; PC= pilocarpine; 3-ASP = 3-alkynyl selenophene . *$p < 0.05$ as compared to the PC group.

3.3 Artigo 3

Convulsões hipertérmicas aumentam a resposta ao pentilenotetrazol e induzem disfunção cognitiva: efeito protetor do 3-alquinil selenofeno

HYPERTHERMIC SEIZURES ENHANCE RESPONSIVENESS TO PENTYLENETETRAZOLE AND INDUCE COGNITIVE DYSFUNCTION: PROTECTIVE EFFECT OF 3-ALKYNYL SELENOPHENE

Ethel A. Wilhelm, Ana Cristina G. Souza, Bibiana M. Gai, Pietro M. Chagas, Juliano A. Roehrs, Cristina W. Nogueira



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Hyperthermic seizures enhance responsiveness to pentylenetetrazole and induce cognitive dysfunction: protective effect of 3-alkynyl selenophene

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Abstract

Aims: In this study we investigated the effect of pre-treatment with 3-alkynyl selenophene (3-ASP) against the increase in responsiveness to pentylenetetrazole [PTZ seizure threshold] and cognitive dysfunction induced by experimental febrile seizures (FS). The effects of 3-ASP were compared to those of diazepam (DZP).

Main methods: Young rats, at postnatal day 21, developed seizures after exposure to a stream of heated air to approximately 41°C. A non-spatial long-term memory and PTZ seizure threshold were determined 30 days after FS. The behavioural seizures were stereotyped followed by facial automatisms, often followed by body flexion. Young rats were pre-treated with 3-ASP (50 and 100 mg/kg; per oral route), DZP (1 and 5 mg/kg; intraperitoneally) or vehicle.

Key findings: 3-ASP and DZP pre-treatments were not effective in protecting against seizures induced by FS. 3-ASP pre-treatment protected against the increase in responsiveness to PTZ and cognitive dysfunction induced by FS. DZP pre-treatment was effective in protecting against the increase in responsiveness to PTZ, but not, against the impaired memory induced by FS.

Significance: 3-ASP pre-treatment protected against impairment of memory performance in step-down passive avoidance task and the increase in the susceptibility to seizures caused by FS early in life of rats.

Keywords: hyperthermia; 3-alkynyl selenophene; selenium; diazepam; memory.

Introduction

Fever is the most common manifestation of the innate immune response to invading pathogens, which could promote deleterious effects, particulary in the neonatal period (Ellis et al. 2005). Febrile seizures (FS) are the most common seizure disorder in childhood, occurring in 2-5% of children (Sadleir and Scheffer 2008).

In humans, retrospective analyses have considered FS as a risk factor for the development of temporal lobe epilepsy (TLE) (Cendes et al. 1993). Actually, it is not proven that FS causes TLE. FS can be a symptom of other factors that lead to the epileptogenic process. In addition, epidemiological clinical studies suggest that children with prolonged FS are at risk for long-term cognitive disturbances (Chang et al. 2001). FS in themselves might affect normal neuronal function within the hippocampal circuit (Baram and Shinnar 2001).

Although FS is the most common form of seizures in children, the pathophysiology is still not fully understood (Shinnar 2006). Risk factors for FS include genetic factors (Shinnar 2006), micronutrient deficiency (e.g., iron, zinc) (Burhanoglu et al. 1996; Daoud and Batieha 2002), and immunologic reactions (Heida et al. 2009). Selenium (Se) is an important micronutrient that has antioxidant effects in cells, especially in brain cells (Anderson 2004). Weber et al. (1991) reported that plasma Se and blood glutathione peroxidase activity were severely reduced in children with intractable seizures. Recently, Mahyar et al. (2010) showed that the serum Se level of children with simple FS was significantly lower than that of febrile children without seizure. It seems that there is a relationship between serum Se deficiency and FS.

In this context, organoselenium compounds may be promising for the treatment and/or prevention of FS and their effects. In fact, organoselenium compounds have been widely studied, demonstrating several pharmacological activities, particularly

the anticonvulsant activity (Nogueira et al. 2004). Previous studies have reported that 3-alkynyl selenophene (3-ASP), an organoselenium compound, exhibits different pharmacological properties (Wilhelm et al. 2009a, 2009b, 2009c). 3-ASP has anticonvulsant and antioxidant effects on a pilocarpine model of seizures in 21-days-old rats (Wilhelm et al. 2009a). 3-ASP has protective effects in the model of hepatotoxicity induced by D-galactosamine and lipopolysaccharide (Wilhelm et al. 2009b), carbon-tetrachloride and 2-nitropropane in rats (Wilhelm et al. 2010) and exerts antinociceptive and anti-allodynic (Wilhelm et al. 2009c) activities.

Under this point of view, the objectives of this study were: i) to investigate the effect of 3-ASP pre-treatment against the increase in responsiveness to PTZ and cognitive dysfunction induced by experimental FS in young rats; ii) to evaluate the effects of 3-ASP in comparison to those of diazepam, an antiepileptic drug.

Materials and methods

Animals

Young male Wistar rats (40-50 g; 21-day-old) were obtained from a local breeding colony. Animals were housed in cages with free access to food and water. Animals were kept in a separate animal room, on a 12-h light/12-h dark cycle (with lights on at 7:00 a.m.), in an air-conditioned room (22 ± 2 °C) and were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (018/2011).

Drugs

Pentylenetetrazole (PTZ) and diazepam (DZP) were purchased from Sigma (St. Louis, MO, USA). PTZ was dissolved in 0.9% physiological saline. DZP is insoluble in

saline or water and then it was dissolved in polyethylene glycol and adjusted to the appropriate volume with 0.9% physiological saline. 3-ASP (figure 1) was prepared in our laboratory according to the literature method (Alves et al. 2008). Analyses of the ^{1}H NMR and ^{13}C NMR spectra showed that 3-ASP obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/MS. This drug was dissolved in canola oil.

Experimental FS

To investigate the effects of FS, we employed a well established model of experimental FS with some modifications (Auvin et al. 2009; Dubé et al. 2000, 2006). In the model used in this study, young rats at postnatal day 21 (P21) developed seizures after exposure to a stream of heated air to approximately 41°C for 30 min. P21 was chosen based on a previous study performed by us (Wilhelm et al. 2009a), which reveals that 3-ASP is an anticonvulsant agent for 21-days-old rats in the pilocarpine model. The seizures occur at the age where hippocampal development is equivalent to that of young human (Baram et al. 1997). The rectal temperature and the behaviour of the young rats were continuously monitored. The rectal temperature was noted at baseline, during the induction of hyperthermia, at the onset of seizure behaviour and at the time when the maximal temperature was reached. Animals were exposed to temperature of 40 ± 1.5 ºC for 30 min. The young rats were then removed and placed on a cool surface until they regained posture and their core temperature returned to baseline. Behavioural seizures induced by 30 minutes of hyperthermia consisted of sudden cessation of activity accompanied by facial automatisms, often followed by body flexion (stage 1) (Racine 1972). The

experimental FS in young rats at P21 was similar to that of described by Dubé et al. (2000, 2006), using rats at P11-P16.

Design of the study

At P21, young rats were divided in 10 groups (n= 7-8): Animals of the **control group** received vehicle (1 ml/kg) and remained at room temperature. Rats of the **FS group** received vehicle (1 ml/kg) and were exposed to hyperthermia. Animals of **3-ASP groups** were pre-treated with 3-ASP at the dose of 50 mg/kg or 100 mg/kg (per oral; p.o.). After 30 min, animals were separated in two groups: one that was kept at room temperature and another that was exposed to hyperthermia. Animals of **DZP groups** were pre-treated with DZP at the dose of 1 mg/kg or 5 mg/kg (intraperitoneally; i.p.). After 1 hour, animals were separated in two groups: one that was kept at room temperature and another that was exposed to hyperthermia.

The doses of 3-ASP and DZP were chosen based on previous studies (Mishra et al. 2010; Wilhelm et al. 2009a). Young rats were exposed to hyperthermia by 30 min. A non-spatial long-term memory, PTZ seizure threshold and the locomotory activity were determined 30 days after experimental FS.

Locomotor activity: Open field test (OFT)

In order to control for possible sensorimotor effects induced by experimental FS or the drug treatments, behaviour during exploration of an open field was evaluated 30 days after experimental FS induced by hyperthermia. The open field was a 40×45 cm arena surrounded by 50 cm high walls, made of plywood with a frontal glass wall. The floor of the arena was divided into 9 (3x3) equal squares by black lines. Animals

were placed in the rear left corner and left to explore the field freely for 4 min. Line crossings and rearings were counted (Walsh and Cummins 1976).

Cognitive dysfunction: Step-down passive avoidance task

To investigate the cognitive impairment induced by experimental FS, the non-spatial long-term memory of rats was investigated using a step-down passive avoidance task according to the method of Sakaguchi et al. (2006) with modifications in the intensity of electric shock and in the exposure time. During the training session, each rat was placed on the platform. When it stepped down and placed its four paws on the grid floor, an electric shock (0.5 mA) was delivered for 2 s. In method used by Sakaguchi et al. (2006), mice were submitted to the intensity of electric shock of 0.3 mA and the electric shock was delivered for 9s. In the present study, we used rats, then, a pilot study was performed and modifications were necessary. The intensity of electric shock was changed to 0.5 mA and the delivered was for 2s. In addition, the animals of the hyperthermia group had greater difficulty in learning at the training day, thus, a higher intensity of electric shock was necessary. The retention test was performed 24 h after training in a similar manner. Each rat was placed again on the platform and the step-down transfer latency time was recorded. Step-down passive avoidance task was made 30 days after experimental FS.

Responsiveness to PTZ

To investigate the possible increase in susceptibility to seizures induced by experimental FS, a PTZ seizure threshold was determined 30 days after experimental FS. PTZ was administered i.p. at the dose of 60 mg/kg, a

subconvulsive dose (based on a pilot study). The animals were observed during 30 min after PTZ administration in Plexiglas cages.

The convulsive behaviour was evaluated according to the following rating scale described by Racine (1972): 0 = no reaction; 1 = stereotype mouthing, eye blinking and/or mild facial clonus; 2 = head nodding and/or severe facial clonus; 3 = myoclonic jerks in the forelimbs; 4 = clonic convulsions in the forelimbs with rearing and 5 = generalized clonic convulsions associated with loss of balance, which were expressed as Racine's score.

Statistical analysis

The normality of data was analyzed using a D'Agostino and Pearson omnibus normality test. Data with normal distribution (crossings, rearings, latency, temperature and duration of seizures) were evaluated using a one- or two-way analysis of variance (ANOVA) (Pre-treatment x Experimental FS), followed by Duncan's Multiple Range Test when appropriate. Main effects were presented only when the second order interaction was non-significant. All data of experiments were expressed as means $\pm$ S.E.M. Seizure incidence was statistically analyzed using the χ^2 method and Fisher's exact test. Data without normal distribution were evaluated using non parametric tests. Data of pre-treatment (Step-down passive avoidance task and PTZ seizure threshold) were analyzed using a Scheirer – Ray – Hare test (an extension of Kruskal–Wallis test) followed by Dunns post-hoc test. These data were expressed as median with interquartile range. Values of $p<0.05$ were considered statistically significant.

Results

Experimental FS

The behaviour seizures in the experimental FS were stereotyped accompanied by facial automatisms, often followed by body flexion (stage 1; Racine, 1972). None of controls had any behavioural seizures noted. 3-ASP pre-treatment, at the dose of 50 or 100 mg/kg, was not effective in protecting against the experimental FS. Similary, DZP (1 and 5 mg/kg) did not protect animals against seizures induced by hyperthermia. Animals were exposed to temperature of 40 ± 1.5 ºC for 30 min. The duration of seizure episode was similar between groups (figure 2B). The temperature profile through the hyperthermia episode is demonstrated in figure 2. Pre-treatment with 3-ASP and DZP did not alter hyperthermic temperature (figure 2A). Mortality was not observed in animals exposed to FS. No alteration was observed in the latency to the onset of FS (figure 2A). To obtain these experimental evidences were used 7-8 animals/group.

Locomotory activity: OFT

The spontaneous locomotor activity measured in the OFT did not differ significantly among groups. No significant difference was found in the number of crossings and rearings between groups ($p > 0.05$) (table 1). As demonstrated in table 1, we used 7-8 animals/group to obtain these experimental evidences.

Cognitive dysfunction: Step-down passive avoidance task

The latency for rats to step down of the platform in the training session is shown in figures 3A and B. No difference in the latency for rats to step down of the platform in the training session was observed among groups. In the test session, the latency to

step down of the platform was decreased for rats exposed to hyperthermia when compared to those from the control group ($p<0.05$) (figures 3A and B).

At both doses, 3-ASP pre-treatment was effective in protecting against the impairment of memory induced by experimental FS ($p<0.05$). The latency of rats pre-treated with 3-ASP to step down the platform was similar to that of control animals (figure 3A). 3-ASP per se, at both doses, did not alter the latency to step down of the platform (figure 3A).

As demonstrated in figure 3B, DZP pre-treatment, at the doses of 1 and 5 mg/kg, did not protect against the impairment of memory induced by FS. The latency of rats pre-treated with DZP to step down the platform was similar to that of the FS group (figure 3B). An impairment of memory was observed in animals treated with DZP (1 mg/kg) and kept at room temperature. The latency of rats pre-treated with DZP (1 mg/kg) and kept at room temperature to step down the platform was decreased when compared to the control group ($p<0.05$) (figure 3B). To obtain these experimental evidences were used 7-8 animals/group.

Responsiveness to PTZ

FS in young rats resulted in a decrease in the PTZ seizure threshold assessed in adult rats ($p<0.05$) (figures 4A and B). 3-ASP pre-treatment (100 mg/kg) increased the PTZ seizure threshold when compared to the FS group ($p<0.05$) (figure 4A).

As demonstrated in figure 4B, DZP pre-treatment, only at the dose of 5 mg/kg, was effective in protecting against the decrease in PTZ seizure threshold induced by experimental FS ($p<0.05$).

3-ASP or DZP treatment reduced the number of convulsing animals when compared to hyperthermia group in the PTZ model. No alteration was found in latency to

generalized clonic convulsions induced by PTZ in animals pre-treated with 3-ASP or DZP when compared to the hyperthermia group (table 1). As demonstrated in table 1, we used 7-8 animals/group to obtain these experimental evidences.

Discussion

The present study demonstrated that the experimental FS in young rats, induced by exposure to hyperthermia, caused a memory impairment and increased the responsiveness to PTZ. Using the FS model, we came to the following two findings: first, 3-ASP pre-treatment protected against the cognitive dysfunction in step-down passive avoidance task and the increase long-term in the responsiveness to PTZ induced by experimental FS. Second, DZP pre-treatment was effective in protecting against the increase in responsiveness to PTZ, but not, against the impairment of memory in step-down passive avoidance task induced by experimental FS.

In accordance with studies performed by Dubé and collaborators (2006, 2009) the electrographic seizures induced by hyperthermia in rat pups was accompanied by behavioural seizures characterized by sudden cessation of activity, facial automatisms and body flexion (Stage 1, Racine 1972). Although the age of animals used by us was different from that of Dubé et al. (2006, 2009), the rat behaviour observed in the present study was similar to those described. FS occur in 3-5% of children between the age of 6 months and 5 years (Berg and Shinnar 1996). According to Avishai-Eliner et al. (2002) the brain maturity of 21-days-old rats corresponds to that of 3 to 5-year-old children. Additionally, Sobaniec-Łotowska and Łotowska (2011) demonstrated the neuroprotective of topiramate using an experimental model of febrile seizures in 22 to 30-days-old rats.

Epilepsy, though not cured, is usually controlled with available medications. Apart from the epilepsy disease itself producing cognitive impairment, the conventional anticonvulsant drugs can also produce cognitive deficits (Herranz 2007). With regard to behavior, the animals that were pre-treated with 3-ASP or DZP were not protected against stereotyped behaviour, facial automatisms, and body flexion induced by hyperthermia exposure. However, we can not rule out any protection by these two drugs because both pre-treatments (3-ASP and DZP) exerted protective effects against dysfunctions caused by experimental FS. The difficulties in identifying the possible protective effects of 3-ASP and DZP may have been hampered by the fact that the behaviours in experimental FS were modest when compared to chemical models of seizures. A limitation of the present study is that animals were not monitored for spontaneous recurrent seizures. Thus, further studies are necessary to better understand the mechanisms involved in the protective effects of 3-ASP and DZP. Results about efficacy of benzodiazepines are controversial. According to Uhari et al. (1995) benzodiazepines do not act at the level of the triggering mechanisms of FS and; furthermore, the available evidence indicates that benzodiazepines do not protect against FS or recurrent FS when applied at a dose of 0.2 mg/kg. On the other hand, Knudsen (1996, 2000) reported that acute anticonvulsive treatment with benzodiazepines is effective in aborting FS.

A closer inspection of the results further revealed that the experimental FS in young rats induced a cognitive impairment, demonstrated in the step-down passive avoidance task. In this task, the animal learns to avoid an adjoining chamber where shock was previously delivered. Hence, the animal suppresses its natural tendency to enter the confined spaces. Thus, this task assesses the ability of animals to retain and recall information about the environment as well as the foot shock (Rodriquiz and

Wetsel 2006). In this study, animals exposed to hyperthermia showed a decreased latency to step down the platform as compared to those that were kept at room temperature, demonstrating that these animals had a cognitive deficit, which is in accordance with the study performed by Yang et al. (2009).

Interestingly, 3-ASP pre-treatment protected against the impairment of memory in step-down passive avoidance task induced by experimental FS.The relevance of this result is supported by the fact that the FS have been considered as a risk factor for the development of TLE (Cendes et al. 1993) and for long-term cognitive disturbances (Chang et al. 2001). By contrast, DZP, a clinically used antiepileptic drug, did not protect against impairment of memory observed using step-down passive avoidance task in animals exposed to experimental FS and caused a cognitive impairment in animals that were kept at room temperature. Antiepileptic drug treatment may last a lifetime in epileptic patients and during treatment with antiepileptic drugs, a variety of side effects may occur. One of the important side effects of the conventional antiepileptic drugs is the cognitive impairment (Herranz 2007). In fact, phenobarbitone, phenytoin and valproate have been shown to have harmful effects on the immature brain, sometimes with very intense impairment of cognitive development (Herranz 2007). Similarly, a significant deterioration of cognitive function after treatment with carbamazepine has been observed (Wesnes et al. 2009). Based on these considerations, in this study we demonstrate the protective effect of 3-ASP and highlight its positive effect when compared to the antiepileptic drug used in the clinical practice, in special DZP.

Using the PTZ seizure threshold, we found that the experimental FS led to increased long-term in the responsiveness to PTZ 30 days after the hyperthermia exposure. Seizures early in life can profoundly and permanently change the hippocampal circuit

in a pro-epileptogenic direction (Dubé et al. 2000). In this sense, it has been demonstrated that the hyperthermic seizure-induced alterations of limbic excitability may require transient structural injury, but are mainly due to functional changes in the expression of genes coding for specific receptors and channels, leading to altered functional properties of hippocampal neurons (Brewster et al. 2005). Our findings showed that pre-treatment with 3-ASP and DZP protected against the increase in responsiveness to PTZ induced by experimental FS. Based on this result we suggest that 3-ASP is a promissory compound to the prevention or treatment of impairment of memory and increase in long-term in the responsiveness to PTZ caused by experimental FS. Moreover, the protective effects of 3-ASP can be attributed, at least in part, to its antioxidant activity and modulation of GABAergic system (Wilhelm et al. 2009a, 2012). In fact, Günes et al. (2009) demonstrated that FS may cause significant oxidative stress, and these changes in oxidant status may be a step along the way to cell damage subsequent to FS. Pathogenesis mechanisms of FS may be associated with increased excitatory amino acids, such as glutamate, and decreased inhibitory amino acids, such as γ-aminobutyric acid. Nitric oxide and carbon monoxide are also suggested to play roles in neurotoxicity caused by the excitatory amino acids during FS (Yang and Qin 2004). Thus, more studies are necessary to elucidate the complete mechanism involved in the protective effects of 3-ASP and DZP.

Conclusions

Taken together these data indicate that: I) the experimental FS early in life caused an impairment of memory performance later in adulthood in step-down passive avoidance task, which was accompanied by an increase in the susceptibility to

seizures (responsiveness to PTZ); II) 3-ASP protected against impairments induced by experimental FS and did not cause cognitive deficits when evaluated in step-down passive avoidance task. However, further studies are necessary to better understand the mechanisms involved in these beneficial effects.

Acknowledgements

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Conflict of Interest statement

There are no conflicts of interest.

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Legends

Figure 1 - Chemical structure of 3-ASP.

Figure 2 - Effect of 3-ASP or DZP pre-treatment on latency to the onset of FS and on temperature measured using rectal probes at the time of hyperthermic seizure induction (A) and on the FS duration. Animals were pre-treated with 3-ASP (50 mg/kg or 100 mg/kg, p.o.) or DZP (1 mg/kg or 5 mg/kg, i.p.) and were exposed to hyperthermia (7-8 animals/group). The results are expressed as mean ± S.E.M. Data were analyzed by using a one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test. (*) $p < 0.05$ as compared to the vehicle (at room temperature).

Figure 3 - Effect of 3-ASP (A) or DZP (B) pre-treatment on the step-down passive avoidance task, 30 days after experimental FS. Animals were pre-treated with 3-ASP (50 mg/kg or 100 mg/kg, p.o.) or DZP (1 mg/kg or 5 mg/kg, i.p.) and were exposed to hyperthermia (7-8 animals/group). Data were analyzed by using the non parametric Sheirer-Ray-Hare test and expressed as median with interquartile range. ($^{\#}$) $p < 0.05$ as compared to the vehicle (at room temperature); (*) $p < 0.05$ as compared to the hyperthermia group.

Figure 4 - Effect of 3-ASP (A) or DZP (B) pre-treatment on PTZ seizure thresholds, 30 days after experimental FS. Animals were pre-treated with 3-ASP (50 mg/kg or 100 mg/kg, p.o.) or DZP (1 mg/kg or 5 mg/kg, i.p.) and were exposed to hyperthermia (7-8 animals/group). Data were analyzed by using the non parametric Sheirer-Ray-Hare test and expressed as median with interquartile range. ($^{\#}$) $p < 0.05$ as compared to the vehicle (at room temperature); (*) $p < 0.05$ as compared to the hyperthermia group.

Figure 1

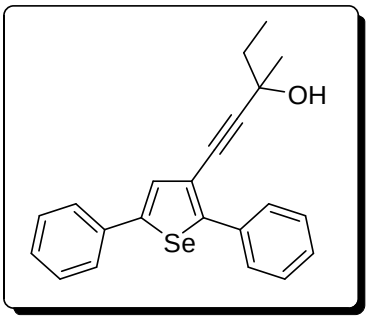

Figure 2

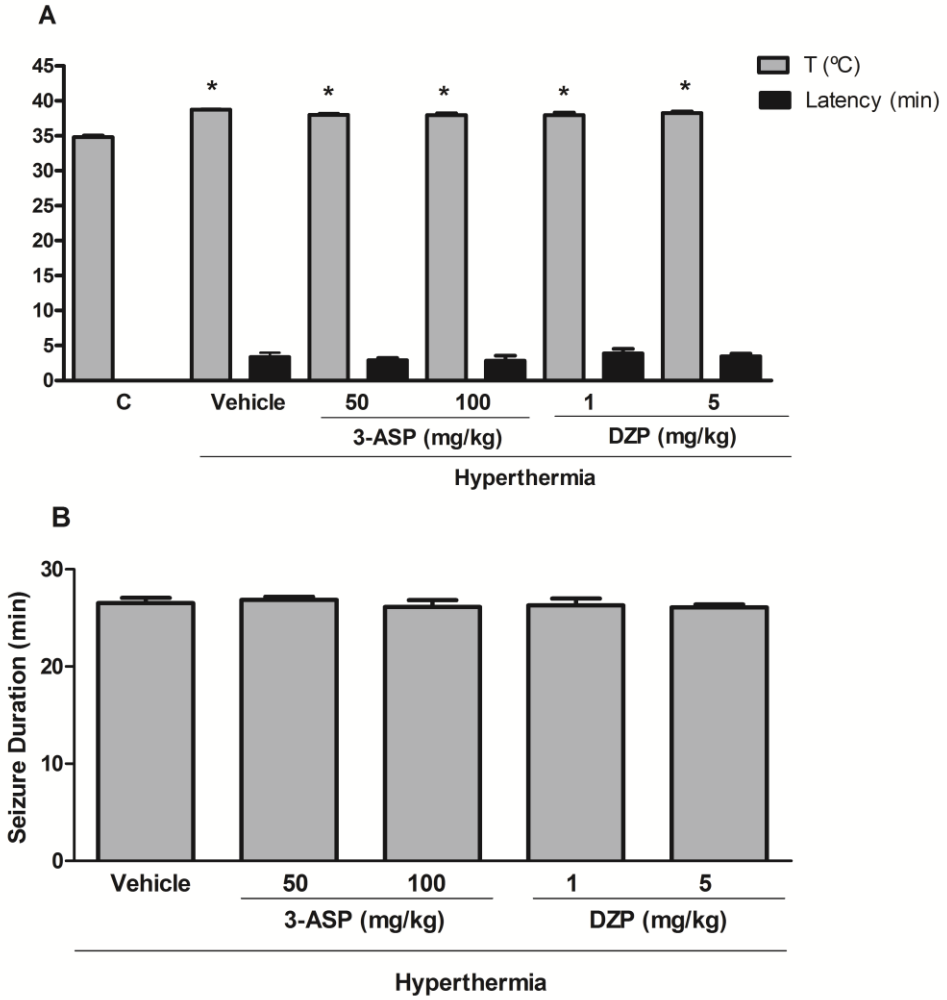
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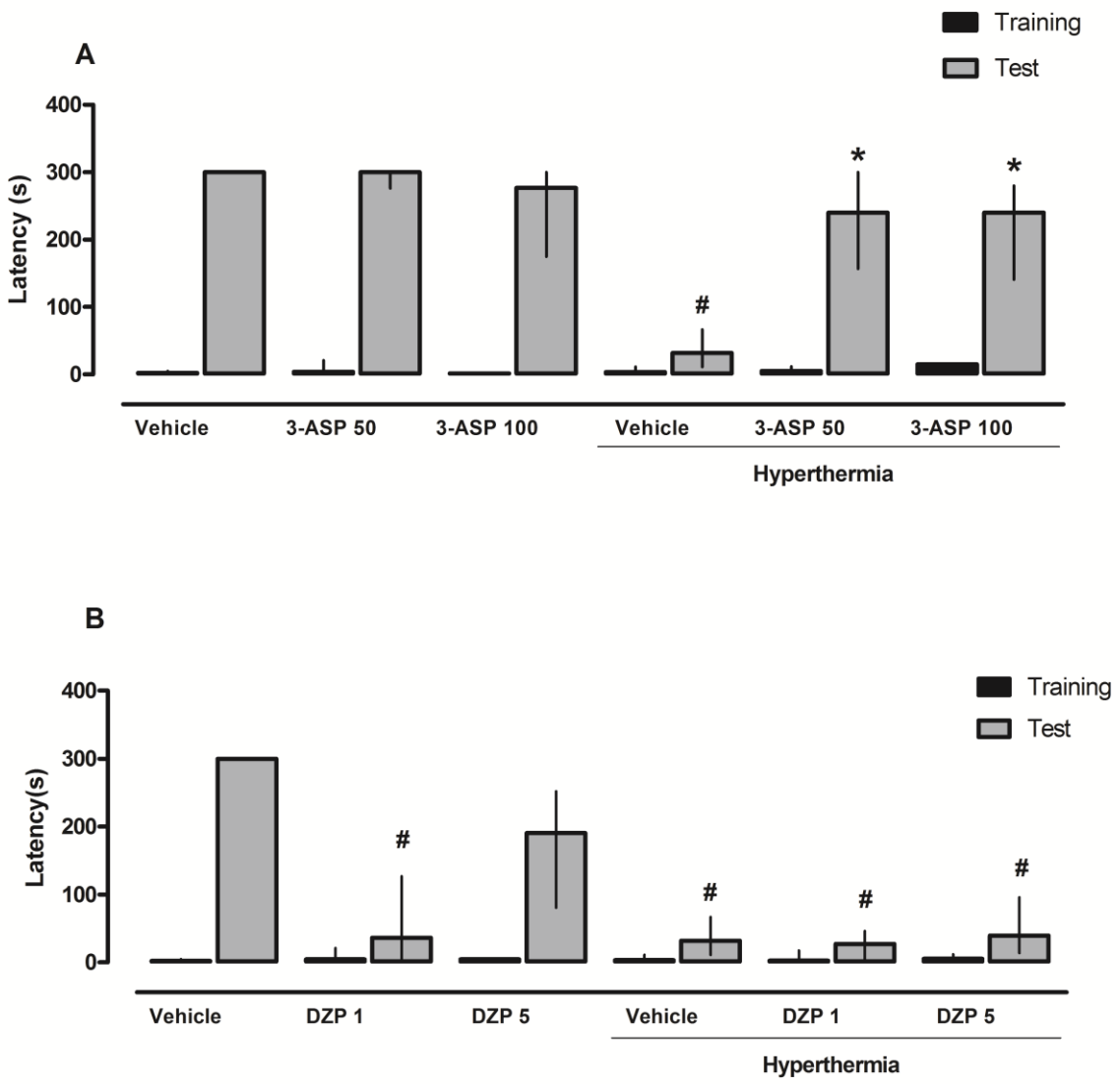
Figure 3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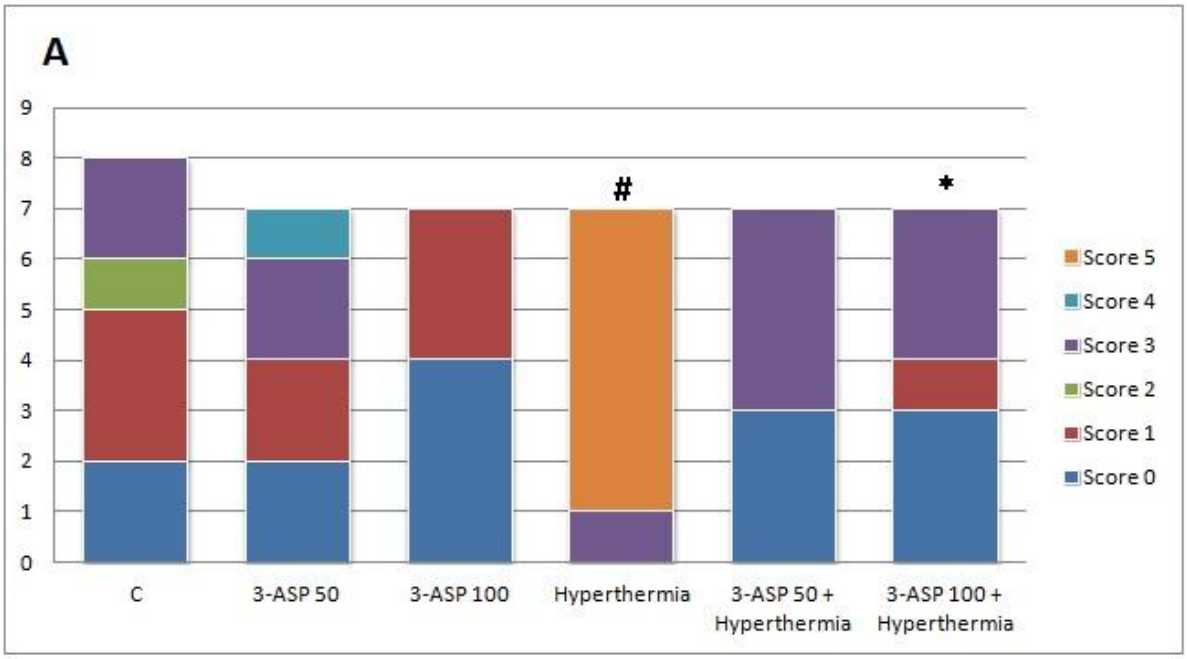
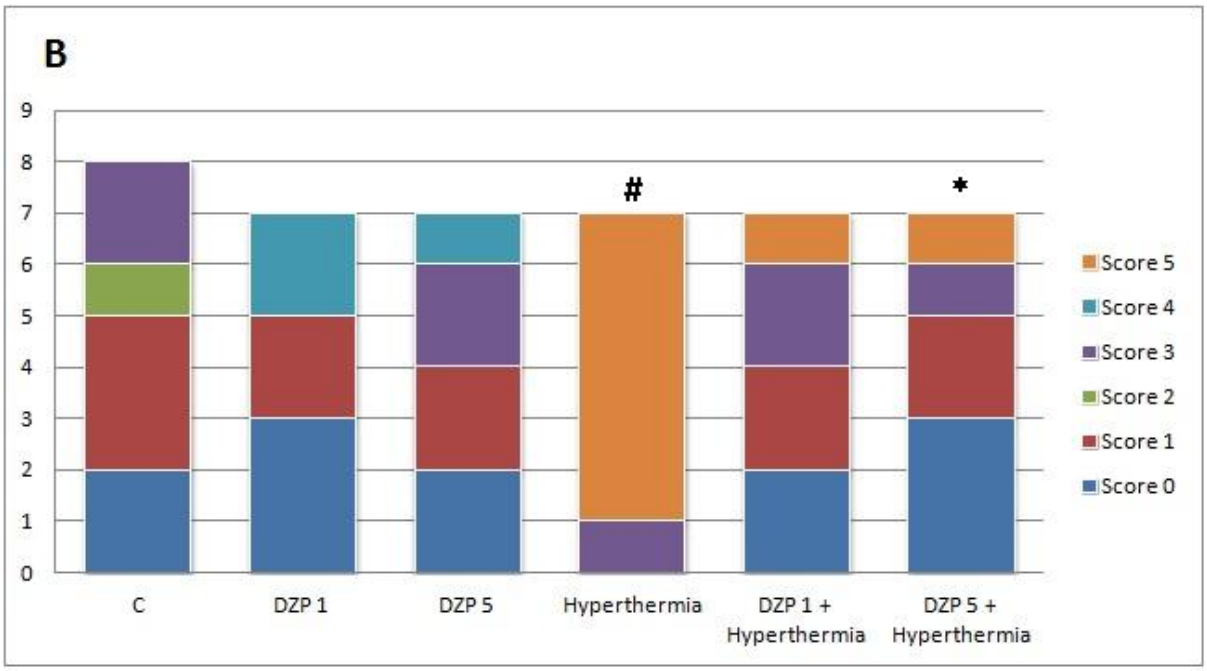
3-ASP 100
Hyperthermia
B
Training
Test
Latency(s)
400
300
200
100
0
#
#
#
#
Vehicle
DZP 1
DZP 5
Vehicle
DZP 1
DZP 5
Hyperthermia

Figure 4


A
9
8
7
6
5
4
3
2
1
0
#
*
C
3-ASP 50
3-ASP 100
Hyperthermia
3-ASP 50 + Hyperthermia
3-ASP 100 + Hyperthermia
Score 5
Score 4
Score 3
Score 2
Score 1
Score 0


B
9
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*
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DZP 1
DZP 5
Hyperthermia
DZP 1 + Hyperthermia
DZP 5 + Hyperthermia
Score 5
Score 4
Score 3
Score 2
Score 1
Score 0

Table 1. Effect of pre-treatment with 3-ASP or DZP on latency to PTZ-induced generalized clonic convulsions and locomotor activity 30 days after experimental FS.

Groups	Latency to PTZ-induced generalized clonic convulsions (min)	n/N[a]	Crossings	Rearings
Control	ns	0/8	37.63 ± 2.41	19.63 ± 1.70
Hyperthermia (H)	1.34 ± 0.21	7/8	31.88 ± 3.07	22.29 ± 3.83
Pre-treatment 3-ASP (mg/kg)				
50	ns	0/7	41.67 ± 2.66	20.67 ± 1.17
100	ns	0/7	45.50 ± 1.65	20.33 ± 1.02
50 + H	ns	0/7	52.00 ± 1.83	22.67 ± 1.22
100 + H	ns	0/7	48.00 ± 2.09	23.33 ± 2.42
Pre-treatment DZP (mg/kg)				
1	ns	0/7	47.17 ± 1.97	21.17 ± 2.44
5	ns	0/7	44.33 ± 2.12	20.50 ± 1.38
1 + H	3.00 ± 0.00	1/7	40.25 ± 1.23	20.88 ± 1.07
5 + H	1.35 ± 0.00	1/7	37.38 ± 1.47	19.75 ± 0.90

[a] Number of animals which presented seizures/ number of animals per group. *ns* = no seizures. The results are expressed as mean ± S.E.M. Data were analyzed by using a two-way analysis of variance (ANOVA). Seizure incidence was statistically analyzed using the χ^2 method and Fisher's exact test.

4 DISCUSSÃO

O interesse por compostos naturais ou sintéticos que possam prevenir, reverter ou retardar o desenvolvimento de diversas patologias tem crescido consideravelmente na comunidade científica nas últimas duas décadas. Neste contexto, muitos trabalhos têm demonstrado o efeito biológico e o potencial farmacológico de diferentes moléculas pertencentes à classe dos selenofenos (Abdel-Hafez, 2005; Xiao e Parkin, 2006; Juang e col., 2007; Shiah e col., 2007; Wilhelm e col., 2009a,b, 2010; Gay e col. 2010). Os selenofenos são intermediários sintéticos muito utilizados em química orgânica, consistindo numa ferramenta sintética bastante útil (Rhoden e Zeni, 2011). Assim, este trabalho demonstrou, pela primeira vez, que o composto (1-(2,5-difenilselenofeno-3-il)-3-metilpent-1-in-3-ol, que foi genericamente denominado de 3-alquinil selenofeno (3-ASP), apresentou ação anticonvulsivante em ratos jovens. O conjunto de resultados (**Artigos 1 e 2**) sugere o envolvimento dos sistemas GABAérgico e glutamatérgico neste efeito. Além disso, a ação anticonvulsivante do 3-ASP parece estar associada à sua atividade antioxidante. Por fim, através dos resultados reportados no **artigo 3**, verificamos a ação protetora do 3-ASP contra o aumento da susceptibilidade à convulsões e prejuízo na memória a longo prazo resultantes das convulsões induzidas por hipertermia em ratos jovens.

Considerando que o cérebro imaturo é mais vulnerável ao desenvolvimento de convulsões quando comparado ao cérebro adulto, e que neurônios imaturos tendem a gerar periódicas descargas e essas facilitam a geração de oscilações patológicas (Khazipov et al., 2004), avaliamos importante a investigação da ação anticonvulsivante do 3-ASP em ratos jovens (21 dias de vida).

Baseado nas considerações acima, na primeira fase deste estudo (**Artigo 1**), avaliamos a ação anticonvulsivante do 3-ASP em modelos animais de convulsão induzidas por PC, PTZ e KA em ratos de 21 dias de vida. Nossos resultados demonstraram o potencial anticonvulsivante do 3-ASP (50 mg/kg, *per oral*) no modelo de convulsões agudas induzidas por PC. Nos demais modelos estudados, o composto não aboliu as convulsões, no entanto, aumentou a latência para o primeiro episódio convulsivo, bem como, diminuiu a mortalidade e a incidência das convulsões. Os compostos orgânicos de selênio apresentam importante natureza

lipofílica (Nogueira e Rocha, 2010), o que nos leva a inferir que o cérebro é um dos tecidos-alvo da ação destes compostos. Assim, nossos resultados são consistentes com a idéia de que o 3-ASP pode modular os processos cerebrais. Entretanto, no **artigo 3**, o pré-tratamento com 3-ASP (50 e 100 mg/kg, *per oral*) não protege contra o comportamento estereotipado, automatismos faciais e flexão corporal (estágio 1, conforme escala de Racine, 1972) induzidos pela hipertermia. Contudo, nós não podemos excluir por completo uma possível ação protetora do 3-ASP, uma vez que este protegeu contra as disfunções resultantes das convulsões induzidas pela hipertermia, e uma análise eletroencefalográfica não foi realizada. Considerando os resultados obtidos podemos sugerir que a ação anticonvulsivante do 3-ASP é dependente do modelo convulsivo utilizado.

A ação convulsiva do PTZ ocorre através do bloqueio do canal de cloreto do complexo do receptor $GABA_A$, inibindo, portanto, canais ativados por GABA (Macdonald e Barker, 1978). Estudos de união específica de radioligantes sugerem que o PTZ age sobre os sítios benzodiazepínico e picrotoxínico do complexo do receptor $GABA_A$ (Rehavi e col., 1982; Ramanjaneyulu e Ticku, 1984). O KA, potente agonista do receptor de glutamato, ao ser administrado, promove a ativação de receptores glutamatérgicos ionotrópicos (AMPA/KA), principalmente expressos na região límbica (predominantemente no hipocampo e na amígdala) (Löscher e Schimdt, 1988). Em geral, as convulsões induzidas pela PC parecem depender da ativação de receptores muscarínicos, como também da participação de outros sistemas de neurotransmissão: dopaminérgico, serotoninérgico, GABAérgico e glutamatérgico (Freitas, 2011). Por fim, os mecanismos envolvidos no desenvolvimento das convulsões febris induzidas pela hipertermia são bastante complexos e ainda existem poucas evidências a respeito. De acordo com Reid e colaboradores (2009), as convulsões febris envolvem fatores genéticos, diminuição da inibição mediada pelo receptor $GABA_A$ - através da diminuição da liberação de GABA dos terminais pré-sinápticos e da diminuição da funcionalidade do receptor $GABA_A$ pós-sináptico. Uma diminuição da amplitude e da duração da corrente pós-sináptica inibitória e um aumento da captação de GABA estão associados à hipertermia (Qu e col., 2007). Estudos experimentais sugerem que a hipertermia causa um aumento na taxa de respiração, o que por sua vez leva a uma alcalose respiratória e esta a um aumento da excitabilidade neuronal (Reid e col., 2009). Entretanto, ainda não se pode afirmar se este mecanismo também está associado

ao desenvolvimento de convulsões febris em humanos. Considerando os diversos eventos envolvidos nos modelos animais estudados, tornou-se importante uma análise mais detalhada dos mecanismos envolvidos na ação anticonvulsivante do 3-ASP.

A função GABAérgica no SNC pode ser potencializada por inibidores da captação de GABA, agonistas GABAérgicos e inibidores do seu catabolismo. No **artigo 1**, nós demonstramos o envolvimento do sistema GABAérgico, uma vez que, a combinação de doses sub-efetivas de 3-ASP e diazepam (agonista GABAérgico) foi eficaz em aumentar a latência para o primeiro episódio convulsivo, bem como diminuir a incidência de convulsões induzidas pela PC. Corroborando com estes resultados, o 3-ASP apresentou ação protetora contra as convulsões induzidas por PTZ (antagonista do receptor $GABA_A$). Para ampliar estas evidências, no **artigo 2**, avaliamos o efeito do 3-ASP sobre a captação de GABA, em córtex e hipocampo de ratos de 21 dias de vida. Surpreendentemente, a administração oral de 3-ASP (50 mg/kg) causou uma inibição de 64% e 58% da captação de GABA no córtex e no hipocampo, respectivamente. Desta forma, a ação anticonvulsivante do 3-ASP está associada a um aumento dos níveis de GABA na fenda sináptica e conseqüentemente à potenciação do tônus inibitório. Considerando que as drogas que aumentam os níveis sinápticos de GABA, por inibir sua captação ou catabolismo, são anticonvulsivantes efetivos, investigamos, através de ferramentas farmacológicas, a possível interação entre doses sub-efetivas de 3-ASP e inibidores da captação de GABA ou da GABA transaminase (GABA-T) frente às convulsões induzidas por PC em ratos de 21 dias de vida. Como demonstrado no **artigo 2**, o tratamento com o 3-ASP (10 mg/kg) e o ácido DL-2,4-diamino-*n*-butírico hidroclorado (DABA; 2 mg/kg - um inibidor da captação de GABA) aboliu as convulsões induzidas por PC, corroborando com os resultados obtidos com ferramentas neuroquímicas. O mesmo foi observado quando foram administradas doses sub-efetivas de 3-ASP e do ácido aminooxiacético hemihidroclorado (AOAA; 10 mg/kg – um inibidor da GABA-T). Baseado nas considerações acima, nós podemos sugerir que a ação anticonvulsivante do 3-ASP está associada a um aumento do tônus inibitório pós-sináptico. Este conjunto de resultados pode justificar, pelo menos em parte, a ação protetora do 3-ASP contra as convulsões induzidas tanto por PTZ quanto por PC, a qual também envolve o sistema GABAérgico em sua gênese. Neste contexto, é importante destacar que as convulsões febris estão associadas à diminuição da

funcionalidade do receptor $GABA_A$ pós-sináptico (Reid e col., 2009), o que pode estar relacionado com a falta de efeito do 3-ASP e do diazepam (anticonvulsivante clássico) frente às convulsões causadas pela hipertermia (**artigo 3**).

Particularmente, um desequilíbrio em qualquer um dos sistemas de neurotransmissão excitatória ou inibitória no SNC pode estar implicado no desenvolvimento de processos convulsivos (Meldrum, 1995). De acordo com Raol e col. (2001), o aumento da neurotransmissão glutamatérgica está associado aos efeitos excitotóxicos das convulsões, além da neurodegeneração. Efetivamente, as principais vias excitatórias do SNC utilizam o glutamato como neurotransmissor (Meldrum e col., 1999). Através de ferramentas farmacológicas, no **artigo 1** nós avaliamos o envolvimento do sistema glutamatérgico na ação anticonvulsivante do 3-ASP. Para isso, a combinação de doses sub-efetivas de 3-ASP e MK-801 (antagonista não-competitivo do receptor NMDA), DNQX (antagonista de receptores não-NMDA) ou MPEP (antagonista do receptor glutamatérgico metabotrópico do tipo 5, mGluR5) foi avaliada frente às convulsões induzidas por PC. Observamos que a combinação do 3-ASP com os antagonistas glutamatérgicos ionotrópicos foi eficaz em aumentar a latência para o primeiro episódio convulsivo, bem como diminuir a incidência de convulsões e a morte induzidas pela PC. Por outro lado, a combinação de 3-ASP e do antagonista mGluR5 não apresentou efeito protetor contra os episódios convulsivos. Adicionalmente, avaliamos o efeito do 3-ASP sobre a captação de glutamato em córtex e hipocampo de ratos de 21 dias de vida. Entretanto, nenhuma alteração na captação de glutamato após a administração de 3-ASP (50 mg/kg) foi observada (**artigo 2**). Considerando os resultados obtidos, nós sugerimos o possível envolvimento dos receptores glutamatérgicos ionotrópicos na ação anticonvulsivante do 3-ASP e descartamos o envolvimento do receptor glutamatérgico metabotrópico do tipo 5, bem como seu efeito sobre a captação de glutamato. Os resultados aqui demonstrados justificam parte da ação anticonvulsivante do 3-ASP frente às convulsões induzidas por KA (agonista glutamatérgico) e PC.

A injúria cerebral resultante das crises convulsivas é um processo dinâmico que compreende múltiplos fatores que contribuem para a morte neuronal. Estes podem envolver fatores genéticos, excitotoxicidade, disfunção mitocondrial, níveis de citocinas alterados e o estresse oxidativo (Ferriero, 2005). A atividade convulsiva, a nível celular, dá início a um significativo influxo de cálcio através de canais

dependentes de voltagem e canais iônicos dependentes de NMDA (Van Den Pol e col. 1996). Elevadas concentrações intracelulares de cálcio podem induzir a formação de espécies reativas (Shin e col., 2011). O estresse oxidativo e a produção de espécies reativas têm sido descritos tanto como uma causa quanto uma conseqüência das crises convulsivas (Jesberger e Richardson, 1991; Kunz, 2002; Patel, 2004; Ashrafi e col., 2007; Shin e col., 2011). Desta forma, consideramos importante o estudo da ação antioxidante do 3-ASP frente ao estresse oxidativo induzido pela PC em ratos de 21 dias de vida. No **artigo 1**, nós verificamos que a formação de espécies reativas foi aumentada após os episódios convulsivos, confirmando o envolvimento de dano oxidativo na injúria cerebral induzida pela PC. Além disso, o pré-tratamento com 3-ASP mostrou-se efetivo contra a formação destas espécies reativas.

A produção de espécies reativas de oxigênio, de nitrogênio, entre outras espécies reativas, é parte integrante do metabolismo humano e é observada em diversas condições fisiológicas. As espécies reativas têm importante função biológica, como na fagocitose, fenômeno em que essas espécies são produzidas para eliminar o agente agressor. Por outro lado, quando sua produção é exacerbada, o organismo dispõe de um eficiente sistema antioxidante que consegue controlar e restabelecer o equilíbrio. O estresse oxidativo resulta do desequilíbrio entre o sistema pró e antioxidante, com predomínio dos oxidantes, com dano conseqüente.

Neste estudo, observamos uma inibição da atividade da selenoenzima GPx nos animais expostos à PC. A atividade enzimática da GPx é um dos meios de controle do organismo dos níveis de H_2O_2 e hidroperóxidos lipídicos, oriundos do ataque de espécies radicalares. Desta forma, uma inibição da atividade da GPx representa uma diminuição do controle dos níveis destas espécies oxidativas, o que pode ser responsável, pelo menos em parte, pelo aumento da produção de espécies reativas observada neste estudo. Nosso resultado corrobora com o estudo realizado por Weber e col. (1991) que sugere uma correlação entre a deficiência na atividade da GPx e as convulsões infantis. Adicionalmente, verificamos uma estimulação da atividade da GST nos animais expostos à PC. Nós acreditamos que a estimulação da atividade da GST representa um mecanismo compensatório ao aumento das espécies reativas induzido pelas convulsões causadas pela PC. De fato, diversos autores reportaram que a GST, além de ser uma enzima de fase II importante na

detoxificação de xenobióticos, é uma defesa antioxidante (Cnubben e col., 2001; Luchese e col., 2009). Como demonstrado no **artigo 1**, o pré-tratamento com o 3-ASP não protegeu contra estas alterações na atividade das enzimas GPx e GST. Nós acreditamos que o 3-ASP não exibe sua ação anticonvulsivante por atuar na modulação destas enzimas do ciclo redox da glutationa.

Em adição à GPx e GST, outros sistemas de defesa antioxidante operam em conjunto, dentre estes destacam-se a SOD, a catalase e o ácido ascórbico. A SOD, presente na quase totalidade dos organismos eucarióticos, catalisa a dismutação do $O_2^{\bullet -}$ em H_2O_2 (McCord e Fridovich, 1969). O H_2O_2 por sua vez pode ser degradado pela ação da catalase, resultando em água e oxigênio molecular (O_2) (Farber, 1990). O ácido ascórbico mostra-se eficiente contra as espécies reativas e desta forma age na proteção de biomembranas contra a peroxidação (Rose, 1987). No **artigo1**, observamos que o pré-tratamento com 3-ASP mostrou-se eficaz na proteção contra a inibição da atividade cerebral da SOD, diminuição dos níveis de ácido ascórbico e estimulação da atividade da catalase causados pela exposição à PC. Estes resultados confirmam o seu potencial antioxidante frente ao estresse oxidativo causado pelas convulsões induzidas por PC. Baseado nos resultados obtidos, nós sugerimos que o 3-ASP pode estar agindo como antioxidante, neutralizando as espécies reativas oriundas do processo convulsivo e/ou modulando as defesas antioxidantes, ou ainda estar agindo como anticonvulsivante, e desta forma diminuindo a formação destas espécies oxidativas.

No protocolo experimental utilizando PC como agente convulsivante, foi observada uma inibição da atividade da Na^+,K^+-ATPase cerebral (**Artigo 1**). Essa importante enzima reguladora do potencial de membrana é responsável pelo transporte ativo dos íons sódio e potássio no SNC (Doucet, 1988). A inativação da Na^+,K^+-ATPase leva a uma despolarização parcial da membrana, seguida de uma entrada excessiva de cálcio para dentro das células neuronais, o que resulta em eventos tóxicos, tais como a excitotoxicidade (Beal et al., 1993). Além disso, a inibição da atividade dessa enzima está relacionada com o aumento da liberação de neurotransmissores excitatórios (Vizi e Vyskocil, 1979). Sendo assim, a inibição da atividade desta enzima parece ser um dos mecanismos relacionados à excitotoxicidade causada pela PC. De acordo com vários autores, a inibição da atividade da Na^+, K^+- ATPase se deve à sua sensibilidade ao dano oxidativo, uma vez que esta é uma enzima sulfidrílica de membrana (Jamme e col., 1995; Morel e

col., 1998). Nossos resultados sugerem o envolvimento da modulação da atividade Na^+, K^+- ATPase na ação anticonvulsivante do 3-ASP, entretanto não podemos afirmar se o 3-ASP está modulando diretamente a enzima ou está agindo de forma indireta impedindo sua inibição.

Uma vez que a neurotransmissão colinérgica está implicada na instalação das crises convulsivas induzidas pela PC, no **artigo 1** avaliamos a atividade da acetilcolinesterase. De fato, Turski e col. (1987) reportaram que a redução no metabolismo cerebral da acetilcolina é essencial para a instalação e propagação das convulsões induzidas pela PC. O pré-tratamento com 3-ASP foi eficaz contra a inibição da atividade da acetilcolinesterase resultante da exposição à PC. Este resultado corrobora com o estudo realizado por Freitas e col (2006), que demonstra a inibição da atividade da acetilcolinesterase no córtex, hipocampo e estriado de ratos expostos à PC. As observações deste estudo sugerem que a modulação da atividade da acetilcolinesterase (direta ou indiretamente) contribui para a ação anticonvulsivante do 3-ASP.

Após verificarmos os efeitos protetores do 3-ASP contra as convulsões causadas por agentes químicos, avaliamos o efeito deste composto frente as convulsões febris em ratos. As convulsões febris afetam de 2 a 5% das crianças entre as idades de 6 meses e 6 anos (Sadleir e Scheffer, 2008). Estudos em humanos têm considerado a convulsão febril infantil como um fator de risco para o desenvolvimento da epilepsia do lobo temporal quando adultos (Cendes e col., 1993). Adicionalmente, estudos epidemiológicos sugerem que crianças que tiveram convulsão febril prolongada estão propensas a apresentar prejuízos cognitivos (Chang e col., 2001). De fato, no **artigo 3** verificamos que a convulsão febril induzida pela hipertermia em ratos de 21 dias de vida causou um aumento da susceptibilidade ao desenvolvimento de convulsões e prejuízo na memória a longo prazo. Outro resultado importante demonstrado neste trabalho foi a ação protetora do 3-ASP contra estes danos resultantes da convulsão febril induzida pela hipertermia. Em comparação com o diazepam, droga anticonvulsivante clássica, o 3-ASP mostrou-se mais eficaz, uma vez que o diazepam não foi capaz de proteger contra o prejuízo na memória a longo prazo causado pela convulsão febril. Adicionalmente, verificamos que o tratamento com diazepam nos animais mantidos a temperatura ambiente causou prejuízo cognitivo, o que corrobora com outros estudos que demonstram que drogas anticonvulsivantes convencionais apresentam

diversos efeitos colaterais, sendo o mais importante o prejuízo cognitivo (Herranz, 2007; Wesnes e col., 2009). A ação protetora do 3-ASP pode ser atribuída, em parte, a sua ação antioxidante. De acordo com Güneş e col (2009) as convulsões febris podem causar estresse oxidativo, e estas mudanças no estado oxidativo podem estar associadas ao subseqüente dano celular verificado após a convulsão febril. Além disso, os mecanismos envolvidos na patogênese destas convulsões envolvem os sistemas glutamatérgico e GABAérgico. Assim, podemos sugerir que a ação protetora do 3-ASP pode envolver a modulação destes sistemas. Por fim, mais estudos são necessários para melhor elucidar os mecanismos envolvidos nestas ações protetoras efetuadas pelo pré-tratamento com o 3-ASP.

Além das considerações apresentadas neste estudo, é importante ressaltar que os compostos orgânicos de selênio modulam circuitos neuronais. A exposição aguda ao ebselen inibe a liberação de [^{3}H]glutamato em sinaptossoma de cérebro de rato (Nogueira e col., 2002). Prigol e col. (2009) demonstraram que o disseleneto de difenila di-*m*-trifluormetil atenua as convulsões induzidas por PTZ em camundongos por inibir a captação de GABA em fatias de córtex cerebral. Adicionalmente, alguns receptores e canais iônicos, como o receptor NMDA, canais de potássio dependentes de voltagem e receptor $GABA_A$ são conhecidos por ser sensíveis à modulação redox (Ruppersberg e col., 1991). Neste contexto, Nogueira e Rocha (2010) sugerem que a modulação redox de moléculas específicas de alto peso molecular contendo grupamentos tióis podem contribuir para os efeitos farmacológicos de organocalcogênios.

Nosso conjunto de resultados demonstra que o 3-ASP inibe a captação de GABA e modula os receptores inibitórios GABAérgicos. Além disso, verificou-se que o 3-ASP é capaz de modular a atividade das enzimas Na^+,K^+,-ATPase e acetilcolinesterase. Através de ferramentas farmacológicas, podemos sugerir que a modulação de receptores glutamatérgicos ionotrópicos está envolvida na ação anticonvulsivante do 3-ASP. Uma diminuição do estresse oxidativo resultante das crises convulsivas também foi observado após o pré-tratamento com o 3-ASP. Uma representação esquemática das vias de sinalização envolvidas nas crises convulsivas e os possíveis alvos do 3-ASP estão demonstrados na **figura 2**.

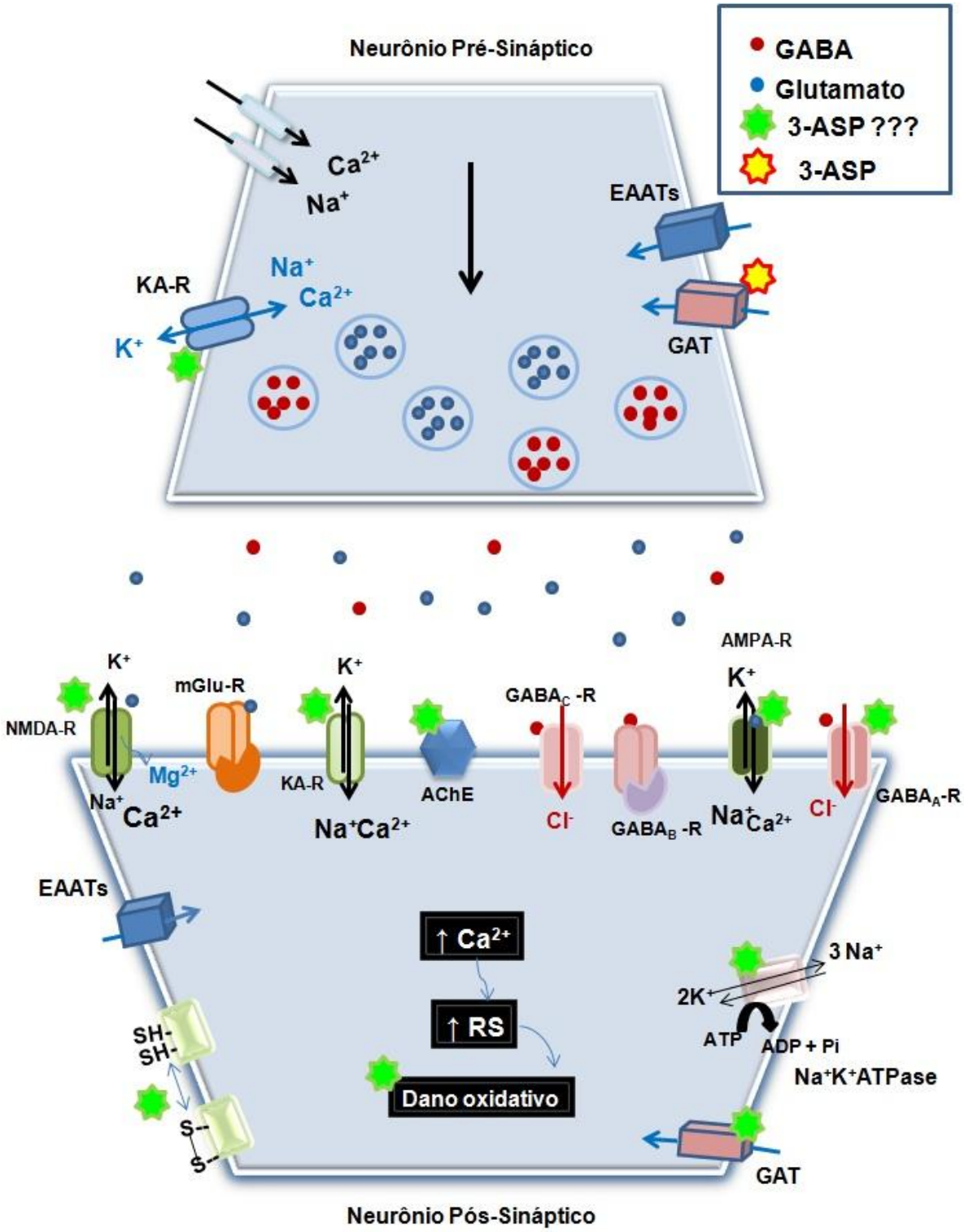

Figura 7 – Representação esquemática de possíveis alvos de ação do 3-ASP que poderiam contribuir para seu potencial anticonvulsivante. Abreviações: NMDA-R → receptor NMDA; KA-R → receptor KA; AMPA-R → receptor AMPA; EAATS → transportadores de aminoácidos excitatórios; GAT→ transportadores de GABA; $GABA_A$-R → receptor $GABA_A$; $GABA_B$-R → receptor $GABA_B$; $GABA_C$-R → receptor $GABA_C$; RS→ espécies reativas; AChE → acetilcolinesterase.

5 CONCLUSÕES

De acordo com os resultados apresentados nesta tese, podemos concluir e/ou inferir o seguinte:

- O 3-ASP apresentou ação anticonvulsivante nos modelos de PC, KA e PTZ em ratos de 21 dias de vida (**Artigo 1**).

- Baseado nos resultados reportados no **artigo 1**, a ação anticonvulsivante do 3-ASP parece estar associada à sua atividade antioxidante, uma vez que o 3-ASP mostrou-se eficaz na proteção contra o estresse oxidativo induzido pelas convulsões resultantes da exposição a PC.

- O conjunto de resultados (**Artigos 1 e 2**) sugere o envolvimento dos sistemas GABAérgico e glutamatérgico nesta ação.

- O pré-tratamento 3-ASP apresenta ação protetora contra o aumento da susceptibilidade às convulsões e prejuízo na memória a longo prazo resultantes das convulsões induzidas por hipertermia em ratos 21 dias de vida (**Artigo 3**).

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