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AVALIAÇÃO DO EFEITO DO DISSELENETO DE DIFENILA NA NOCICEPÇÃO INDUZIDA PELA ADMINISTRAÇÃO NEONATAL DE GLUTAMATO MONOSSÓDICO EM RATOS

DISSERTAÇÃO DE MESTRADO

Suzan Gonçalves Rosa

Santa Maria, RS, Brasil
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AVALIAÇÃO *DO EFEITO DO DISSELENETO DE DIFENILA NA NOCICEPÇÃO INDUZIDA PELA ADMINISTRAÇÃO NEONATAL DE GLUTAMATO MONOSSÓDICO EM RATOS*

Suzan Gonçalves Rosa

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

Orientadora: Prof.ª Dr.ª Cristina Wayne Nogueira

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Universidade Federal de Santa Maria
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elaborada por
Suzan Gonçalves Rosa

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

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Dedico esta dissertação aos meus pais Geraldo e Ana e meus irmãos Sandrigo e Ricardo, por todo apoio, incentivo e amor incondicional!

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"O sucesso nasce do querer, da determinação e da persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis."

José de Alencar

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

AVALIAÇÃO DO EFEITO DO DISSELENETO DE DIFENILA NA NOCICEPÇÃO INDUZIDA PELA ADMINISTRAÇÃO NEONATAL DE GLUTAMATO MONOSSÓDICO EM RATOS

AUTOR: SUZAN GONÇALVES ROSA
ORIENTADORA: CRISTINA WAYNE NOGUEIRA
Data e Local da Defesa: Santa Maria, 9 de setembro de 2014

O Glutamato monossódico (GMS) tem sido alvo de investigação devido aos seus efeitos toxicológicos. A administração neonatal de GMS em animais pode influenciar na organização morfológica e eletrofisiológica do cérebro, levando a distúrbios de comportamento na idade adulta, incluindo o aumento da sensibilidade à dor. O presente estudo teve como objetivo pesquisar o mecanismo pelo qual o GMS induz nocicepção e avaliar o efeito do disseleneto de difenila $(PhSe)_2$, um composto orgânico de selênio com propriedades farmacológicas já documentadas, na nocicepção induzida por GMS. Ratos Wistar recém-nascidos foram tratados com dez injeções subcutâneas de GMS na dose de 4,0 g/kg ou salina, uma vez por dia. Aos 60 dias de vida, os ratos receberam $(PhSe)_2$ (1mg /kg) ou o veículo (óleo de canola), por via intragástrica, uma vez ao dia, durante 7 dias. Testes comportamentais (atividade locomotora, teste da placa quente, imersão da cauda e alodinia mecânica) foram realizados após trinta minutos do último tratamento com $(PhSe)_2$. Além disso, ensaios *ex vivo* foram realizados para determinar a atividade das enzimas Na^+, K^+-ATPase e da Ca^{2+}-ATPase, os níveis de citocinas e a captação de glutamato em hipocampo. Os resultados demonstraram um aumento na nocicepção induzida por GMS no teste da placa quente e no teste de alodinia mecânica, porém não no teste de imersão da cauda. O $(PhSe)_2$ diminuiu todos os comportamentos nociceptivos induzidos pelo GMS. O GMS estimulou a atividade da Na^+, K^+-ATPase e da Ca^{2+}-ATPase e induziu o aumento dos níveis de citocinas pró-inflamatórias, bem como a diminuição da citocina anti-inflamatória, IL-10, e da captação de glutamato no hipocampo de ratos. O tratamento com $(PhSe)_2$ protegeu contra estas alterações. Estes resultados demonstraram mecanismos de ação envolvidos na nocicepção induzida pelo GMS e a ação antinociceptiva do $(PhSe)_2$ após injeções neonatais de GMS em ratos através da diminuição da excitotoxicidade e neuroinflamação hipocampal associada à administração de GMS em ratos.

Palavras-chave: Gluatamato monossódico; excitotoxicidade; nocicepção; selênio; disseleneto de difenila; ATPases

ABSTRACT

Dissertation of Master's Degree
Postgraduate Programme in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

EVALUATION OF DIPHENYL DISELENIDE EFFECT IN THE NOCICEPTION INDUCED BY NEONATAL ADMINISTRATION OF MONOSODIUM GLUTAMATE IN RATS

AUTHOR: SUZAN GONÇALVES ROSA
ADVISOR: CRISTINA WAYNE NOGUEIRA
Date and Place of the Defense: Santa Maria, September 9, 2014.

Monosodium glutamate (MSG) has been the target of research due to its toxicological effects. Neonatal administration of MSG in animals can affect the morphological and electrophysiological organization of the brain, leading to behavioral disorders in adulthood, including increased pain sensitivity. The present study aimed to investigate the mechanism of action by which MSG induces nociception and the effect of diphenyl diselenide $(PhSe)_2$, an organoselenium compound with pharmacological properties already documented, on nociception induced by MSG. Newborn Wistar rats were treated with ten subcutaneous injections of MSG at a dose of 4.0 g/kg or saline, once a day. At the 60th day of life, rats received daily $(PhSe)_2$ (1 mg/kg) or vehicle (canola oil) by intragastric route for 7 days. The behavioral tests (locomotor activity, hot plate, tail-immersion and mechanical allodynia) were carried out. In addition, hippocampal ex vivo assays were performed to determine Na+, K+-ATPase and Ca2+-ATPase activities, cytokines levels and [3H] glutamate uptake. The results demonstrated that MSG increased nociception in the hot plate test, but not in the tail immersion test, and in the mechanical allodynia stimulated by Von-Frey Hair. $(PhSe)_2$ decreased all nociceptive behaviors induced by MSG. MSG increased hippocampal Na+,K+-ATPase and Ca2+-ATPase activities and pro-inflammatory cytokines levels as well as decreased the anti-inflammatory cytokine (IL-10) and the [3H]glutamate uptake in hippocampi of rats. $(PhSe)_2$ treatment protected against these alterations. These results demonstrated the mechanisms of action involved in nociception induced by MSG and the antinociceptive action of (PhSe)2 after neonatal injections of MSG in rats through the decrease hippocampal excitotoxicity and neuroinflammation associated to the administration of MSG in rats.

Key words: Monosodium glutamate; excitotoxicity; nociception; selenium; diphenyl diselenide, ATPases.

LISTA DE FIGURAS

INTRODUÇÃO

MANUSCRITO

LISTA DE ABREVIATURAS

AMPA- alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico

BHE- Barreira hematoencefálica

CFA- Adjunvante completo de Freund

IL-1β- Interleucina 1β

IL-6- Interleucina 6

IL-18- Interleucina 18

IP_3- Inositol trifosfato

GMS- Glutamato monossódico

NMDA- N-metil D-Aspartato

OMS- Organização mundial da saúde

PIP_2- Fosfatidilinositol bifosfato

SNC- Sistema nervoso central

TNFα- Fator de necrose tumoral alfa

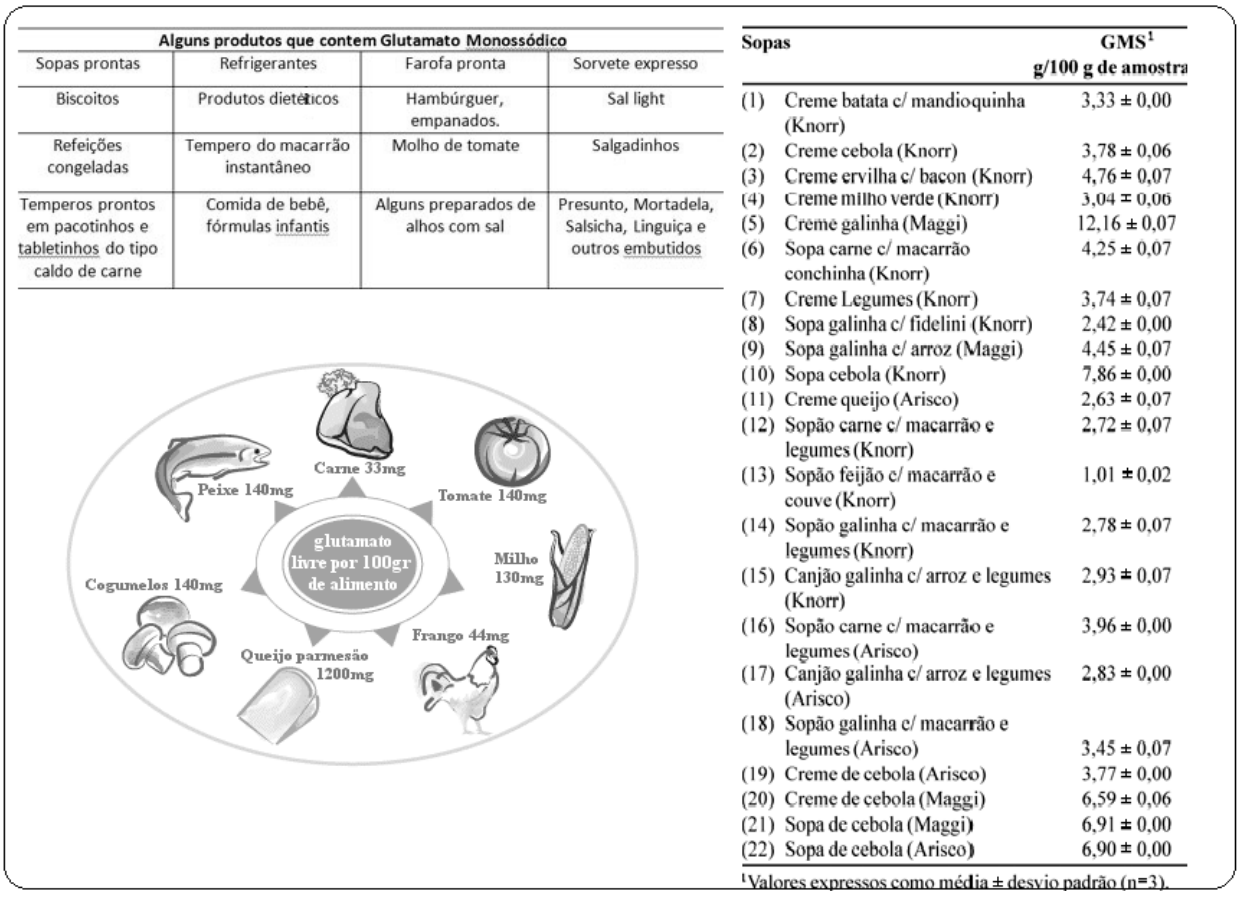
VGCCs- Canais de cálcio voltagem-dependentes

SUMÁRIO

1. INTRODUÇÃO

1.1 Glutamato monossódico

O glutamato monossódico (GMS), um aminoácido excitatório derivado do ácido glutâmico, é um dos aminoácidos não essenciais mais abundantes na natureza. O GMS é largamente utilizado como realçador de sabor em aditivos alimentares (Figura 1), portanto, a preocupação com a segurança na sua utilização tem aumentado nos últimos tempos. A toxicidade do glutamato monossódico tornou-se uma área de investigação em animais e seres humanos desde que estudos demonstraram que o GMS induz necrose neuronal aguda (OLNEY e SHARPE, 1969) que pode resultar em distúrbios metabólicos e comportamentais graves em animais e humanos (DINIZ et al., 2004; INSAWANG et al., 2012; ROTIMI et al., 2012).



Alguns produtos que contem Glutamato Monossódico			
Sopas prontas	Refrigerantes	Farofa pronta	Sorvete expresso
Biscoitos	Produtos dietéticos	Hambúrguer, empanados.	Sal light
Refeições congeladas	Tempero do macarrão instantâneo	Molho de tomate	Salgadinhos
Temperos prontos em pacotinhos e tabletinhos do tipo caldo de carne	Comida de bebê, fórmulas infantis	Alguns preparados de alhos com sal	Presunto, Mortadela, Salsicha, Linguiça e outros embutidos

Sopas		GMS[1] g/100 g de amostra
(1)	Creme batata c/ mandioquinha (Knorr)	3,33 ± 0,00
(2)	Creme cebola (Knorr)	3,78 ± 0,06
(3)	Creme ervilha c/ bacon (Knorr)	4,76 ± 0,07
(4)	Creme milho verde (Knorr)	3,04 ± 0,06
(5)	Creme galinha (Maggi)	12,16 ± 0,07
(6)	Sopa carne c/ macarrão conchinha (Knorr)	4,25 ± 0,07
(7)	Creme Legumes (Knorr)	3,74 ± 0,07
(8)	Sopa galinha c/ fidelini (Knorr)	2,42 ± 0,00
(9)	Sopa galinha c/ arroz (Maggi)	4,45 ± 0,07
(10)	Sopa cebola (Knorr)	7,86 ± 0,00
(11)	Creme queijo (Arisco)	2,63 ± 0,07
(12)	Sopão carne c/ macarrão e legumes (Knorr)	2,72 ± 0,07
(13)	Sopão feijão c/ macarrão e couve (Knorr)	1,01 ± 0,02
(14)	Sopão galinha c/ macarrão e legumes (Knorr)	2,78 ± 0,07
(15)	Canjão galinha c/ arroz e legumes (Knorr)	2,93 ± 0,07
(16)	Sopão carne c/ macarrão e legumes (Arisco)	3,96 ± 0,00
(17)	Canjão galinha c/ arroz e legumes (Arisco)	2,83 ± 0,00
(18)	Sopão galinha c/ macarrão e legumes (Arisco)	3,45 ± 0,07
(19)	Creme de cebola (Arisco)	3,77 ± 0,00
(20)	Creme de cebola (Maggi)	6,59 ± 0,06
(21)	Sopa de cebola (Maggi)	6,91 ± 0,00
(22)	Sopa de cebola (Arisco)	6,90 ± 0,00

[1]Valores expressos como média ± desvio padrão (n=3).

Figura 1- Produtos que contêm GMS. Adaptado de International Glutamate information service.

O sistema nervoso central é um importante alvo das ações do GMS, particularmente no período neonatal, momento em que a barreira hemato-encefálica não esta totalmente formada e o cérebro ainda esta em desenvolvimento. A fase de maturação do sistema nervoso de roedores ocorre principalmente durante o período de lactação, período correspondente ao primeiro ano de vida em humanos (MORGANE et al., 1978). Assim, o estudo dos efeitos do GMS tornou-se de grande importância, uma vez que uma parte crescente da população humana consome o glutamato monossódico como um condimento alimentar em produtos industrializados, principalmente crianças, as quais se tornam susceptíveis ao desenvolvimento de obesidade e aos efeitos neurotóxicos do GMS, quando adultos (BEYREUTHER et al., 2007).

Em modelos animais, o tratamento subcutâneo com GMS 4 mg/kg no período neonatal, induz a destruição de corpos celulares neuronais localizados no sistema nervoso central. Além disso, a administração do GMS causa degeneração da retina, do nervo óptico e de regiões do córtex cerebral (CHAPARRO-HUERTA et al., 2002; TAMAS et al., 2004), sendo que as principais lesões induzidas por GMS ocorrem em neurônios do núcleo arqueado do hipotálamo (XU et al., 2007). Os déficits funcionais resultantes destas lesões têm sido utilizados por diferentes autores para explicar as respostas neuroendócrinas, como o acúmulo de tecido adiposo, intolerância à glicose, hiperinsulinemia e resistência à insulina, (BALBO et al., 2007; NARDELLI et al., 2011) encontradas nesses animais. A administração neonatal do GMS também leva a degeneração de células piramidais CA1 do hipocampo, o que é associado com deficiência de aprendizagem (ISHIKAWA et al., 1997b), hiperexcitabilidade, alterações do comportamento motor (LOPEZ-PEREZ et al., 2010), atraso no desenvolvimento de reflexos neurológicos e habilidades de coordenação (KISS et al., 2007; KISS et al., 2005). Além disso, alguns estudos têm demonstrado que o GMS induz estresse oxidativo e hepatotoxicidade em ratos (ONYEMA et al., 2006) bem como produz alteração no perfil lipídico, com elevação de espécies reativas de oxigênio (ROS), redução da atividade de enzimas antioxidantes, aumento da infiltração de macrófagos e da expressão de TNF-α e IL-6 em tecido adiposo, bem como aumento dos níveis plasmáticos de TNF-α e IL-6 (FURUYA et al., 2010).

Mudanças no limiar nociceptivo e alterações nas respostas analgésicas observados em animais na idade adulta também são associadas ao tratamento com

GMS no período neonatal (BADILLOMARTINEZ et al., 1984; VANDENBUUSE et al., 1985).

Um aumento agudo nas concentrações de glutamato intracerebroventricular e em hipocampo é observado após a administração de GMS (LOPEZ-PEREZ et al., 2010). Altas concentrações de glutamato cerebral durante o desenvolvimento do sistema nervoso podem levar a super ativação de receptores glutamatérgicos induzindo morte celular por apoptose excitotóxica ou necrose, dependendo da intensidade da ativação do receptor (JOHNSTON, 2005). Acredita-se, portanto, que a excitotoxicidade induzida por GMS é resultado de um aumento do influxo de cálcio através da estimulação excessiva de receptores glutamatérgicos N-metil-D-aspartato (NMDA) (CHAPARRO-HUERTA et al., 2002)

1.2 Excitoxicidade

Excitotoxicidade, proposta inicialmente por OLNEY e SHARPE (1969), é um processo patológico responsável por vários distúrbios neurológicos, provocado pela ativação excessiva ou prolongada dos receptores de aminoácidos excitatórios (DONG et al., 2009). O principal neurotransmissor excitatório no cérebro é o glutamato, o qual inicia rapidamente a transmissão sináptica (YU et al., 2008) e desempenha um importante papel na plasticidade sináptica, aprendizagem, memória e outras funções cognitivas. No entanto, esse neurotransmissor pode causar grandes danos quando encontrado em elevadas concentrações no tecido cerebral (OKUBO et al., 2010). Portanto, os mecanismos que mantêm as concentrações extracelulares de glutamato baixas são essenciais para as funções cerebrais (YU et al., 2008). Sob condições fisiológicas de transmissão sináptica, o canal do receptor glutamatérgico NMDA é fechado e só ativado durante breves períodos de tempo para permitir que os íons Ca^{2+} e outros cátions se movam para dentro da célula para exercer suas funções fisiológicas (SPANDOU et al., 2007).

Porém, em condições patológicas, estímulos despolarizantes, como o aumento do K^+ extracelular, fazem com que o glutamato, produzido a partir da glutamina por ação da enzima glutaminase, seja liberado do terminal pré-sináptico para a fenda sináptica e torne-se, então, livre para se ligar com os canais dos receptores ionotrópicos (AMPA e NMDA) e / ou com receptores metabotrópicos no neurónio pós-

sináptico. A ligação com os receptores AMPA permite a troca de Na^+ e K^+, enquanto a ligação do glutamato com receptores NMDA permite influxo de Ca^{2+} no neurônio pós-sináptico. Além disso, a interação do glutamato com os receptores metabotrópicos provoca a hidrólise do fosfatidilinositol-4,5-bifosfato (PIP_2) produzindo o inositol-1,4,5-trifosfato (IP_3), que, em seguida, estimula a liberação de Ca^{2+} armazenado no retículo endoplasmático liso (SER). Esses eventos levam a um excesso de Ca^{2+} dentro da célula nervosa, o qual estimula o início de vias de sinalização intracelular dependentes de Ca^{2+} (CHEN e LIPTON, 2006), desencadeando processos catabólicos que resultam em lesão neuronal (NDOUNTSE e CHAN, 2009) e que caracterizam a excitotoxicidade (Figura 2).

Os mecanismos responsáveis pelo aumento dos níveis de glutamato extracelular incluem maior liberação e menor captação de glutamato (GILGUN-SHERKI et al., 2002a). Além disso, a homeostase do glutamato extracelular no sistema nervoso central é regulada pela atividade de transportadores de aminoácidos excitatórios (EAATs). Os transportadores de glutamato são dependentes do íon sódio e necessitam, portanto da funcionalidade da enzima Na^+, K^+ - ATPase para regular as concentrações intracelulares de sódio e potássio e conduzir a captação do neurotransmissor (ROSE et al., 2009b). Existem evidências de que os transportadores de glutamato e Na^+, K^+ - ATPase são parte dos mesmos complexos macromoleculares e operam juntamente para regular a neurotransmissão glutamatérgica nos astrócitos e neurônios (ROSE et al., 2009b; ZHANG et al., 2009). Portanto, alterações na atividade da Na^+, K^+ - ATPase podem influenciar diretamente a sinalização do glutamato, a atividade neural e o comportamento animal.

Além disso, íons Ca^{2+} são mediadores chave de danos excitotóxicos. Sob condições fisiológicas estes íons ativam enzimas as quais influenciam uma ampla variedade de componentes celulares que regulam processos celulares (NICHOLLS e CHALMERS, 2004), incluindo o crescimento e diferenciação celular e atividade sináptica. Porém, em eventos excitotóxicos a liberação sináptica excessiva de glutamato pode levar à desregulação da homeostase do Ca^{2+} e consequentes danos neuronais. Portanto, existem mecanismos homeostáticos para manter as concentrações intracelulares de Ca^{2+} baixas e espacialmente e temporalmente localizadas (LIPTON, 2008). Assim, além da Na^+, K^+ - ATPase, a enzima Ca^{2+} - ATPase também desempenha um papel importante nos eventos de excitotoxicidade,

uma vez que regula a homeostase do Ca^{2+}, o qual desempenha um papel primordial na excitotoxicidade induzida por glutamato (TYMIANSKI et al., 1993b)

Além do envolvimento dos receptores glutamatérgicos com a excitotoxicidade e danos ao sistema nervoso central (SNC), estudos têm demonstrado que a super ativação destes receptores, especialmente do receptor do NMDA esta envolvida no desenvolvimento de sensibilidade a dor inflamatória (FUNDYTUS, 2001; LI e NEUGEBAUER, 2004; PETRENKO et al., 2003)

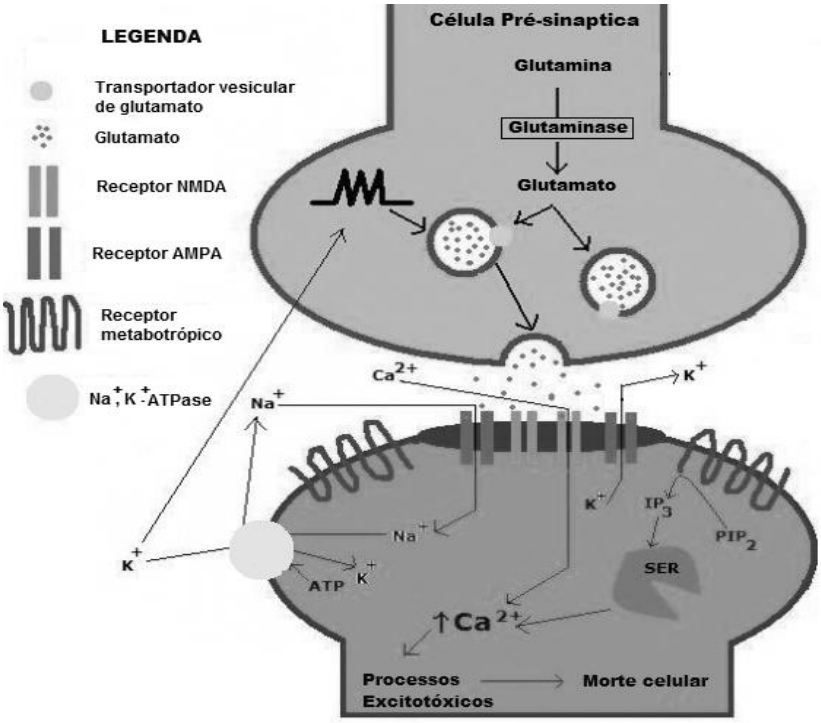


Figura 2- Sinapse glutamatérgica e excitotoxicidade. Adaptado de (MESSING, 2006)

1.3 Dor

A dor aguda é decorrente da ativação térmica, mecânica ou química de subconjuntos de neurônios aferentes primários (nociceptores) que transmitem a informação de dor para a medula espinhal, de onde é retransmitida a níveis supra-espinhais. Assim, o processo nociceptivo engloba desde eventos de detecção do estímulo, que pode ter diferentes origens, até a condução deste ao SNC, onde esse estímulo é processado e respondido (BASBAUM et al., 2009a). Além disso, os nociceptores aferentes podem ser modulados de forma que eles não só sinalizam a dor aguda, mas também contribuem para condições de dor persistente, a qual está

associada à produção e liberação de múltiplos mediadores inflamatórios, incluindo neurotransmissores e neuromoduladores (GRIFFIS et al., 2006).

Diversos fatores estão associados com a gênese e a transmissão do estímulo nociceptivo, entre eles, os neurotransmissores excitatórios, principalmente o glutamato (JULIUS e BASBAUM, 2001) óxido nítrico, peptídeos, como a substância P (HARRISON e GEPPETTI, 2001), bradicinina (CALIXTO et al., 2000), prótons, prostaglandinas e leucotrienos (FERREIRA, 1972). Além disso, alguns mediadores podem ativar direta ou indiretamente canais iônicos voltagem-dependente e cascatas de proteínas quinase, levando a alterações na permeabilidade da membrana o que favorece a transmissão de impulsos elétricos ao longo das fibras (MILLAN, 1999; PARADA et al., 2003; WOOLF e SALTER, 2000). Assim estes agem em conjunto, não só para manter a atividade dos nociceptores aferentes primários e sustentar a dor, mas também para aumentar a sensibilidade nociceptiva de tal forma que estímulos inócuos produzam dor (JULIUS e BASBAUM, 2001).

A nocicepção envolve diferentes estruturas através da interação entre várias vias anatômicas e neurofisiológicas. Estudos têm demonstrado que a formação do hipocampo pode estar envolvida no surgimento da nocicepção (KLAMT e PRADO, 1991; MCKENNA e MELZACK, 1992), bem como a intensidade de estímulos nociceptivos esta positivamente relacionada com a amplitude do potencial excitatório pós-sináptico em células piramidais CA1 do hipocampo (WEI et al., 2000). Além disso, esta estrutura do cérebro é vulnerável a elevadas concentrações de GMS no início da vida (BEAS-ZARATE et al., 2001), quando estímulos nocivos podem alterar a atividade neuronal no hipocampo (LORENZ et al., 2008).

Existem vários relatos de que o glutamato provoca hiperalgesia através da excitação direta de fibras aferentes terminais (ANDERSON e SWANSON, 2000; GEGELASHVILI et al., 2000). Além disso, a sinalização glutamatérgica pode definir o início, duração e intensidade dos estímulos nocivos da periferia. Após a comunicação sináptica, há um aumento na capacidade de resposta dos neurônios a estímulos subsequentes, conhecido como sensibilização central, processo pelo qual um estado de hiperexcitabilidade do SNC leva a um maior processamento de mensagens nociceptivas (WOOLF, 1983).

Na dor aguda, a liberação de glutamato de terminais nociceptores centrais gera correntes excitatórias pós-sinápticas em outros neurônios do corno dorsal. Isto ocorre principalmente por meio da ativação de receptores de glutamato ionotrópicos pós-

sinápticos (AMPA e Cainato). A soma das correntes excitatórias geradas no neurônio pós-sináptico resulta na transmissão da mensagem de dor para neurónios de ordem superior. Nestas condições, os receptores de glutamato NMDA não estão ativados, porém, em situações mais crônicas, a ativação de fibras C e nociceptores leva a ativação de canais iônicos voltagem-dependentes, VGCCs. Os VGCCs estão associados com as proteínas vesiculares Ca^{2+}-dependentes as quais podem levar ao influxo de Ca^{2+}, despolarização de neurônios pós-sinápticos e um consequente aumento na liberação de neurotransmissores, como o glutamato, que pode ativar receptores NMDA.

O aumento do influxo de cálcio pode fortalecer as conexões sinápticas entre os nociceptores e neurônios de transmissão de dor e ativar uma série de cascatas de sinalização que irão aumentar a excitabilidade neuronal e facilitar a transmissão de mensagens de dor para o cérebro (LATREMOLIERE e WOOLF, 2009) (Figura 3). A dor pode resultar de duas condições diferentes: aumento da capacidade de resposta de neurônios de transmissão da dor na medula espinhal (sensibilização central), ou redução dos limites de ativação de nociceptores (sensibilização periférica). Na sensibilização central, a dor é produzida pela atividade de fibras sensoriais primárias não-nociceptivas, enquanto a sensibilização periférica é produzida quando nociceptores terminais ficam expostos a produtos de dano tecidual e inflamação.

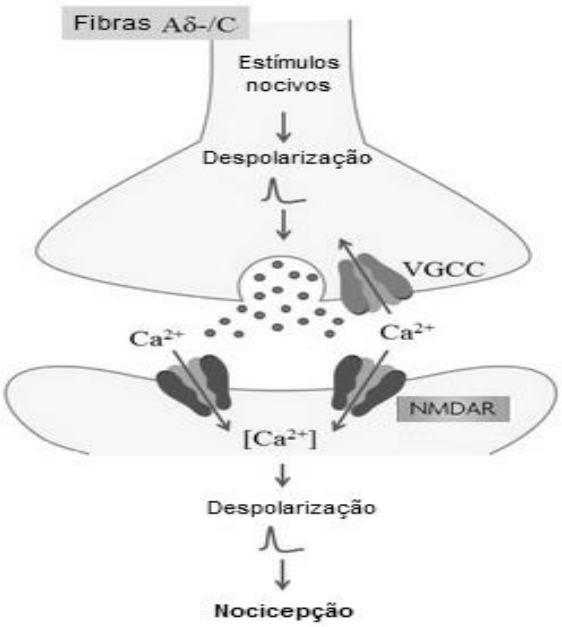


Figura 3 – Nocicepção mediada por receptor glutamatérgico NMDA. Adaptado de (SCHMIDTKO et al., 2010)

1.4 Inflamação

A inflamação é uma reação fisiológica complexa e mediada por diferentes eventos moleculares e celulares. Apesar de possuir caráter essencialmente protetor na eliminação do agente agressor, em alguns casos o processo inflamatório pode tornar-se muito intenso prejudicando o próprio organismo (LIBBY, 2007; MOALEM e TRACEY, 2006). Estímulos inflamatórios desencadeiam a síntese e a liberação de substâncias capazes de causar alterações morfológicas e bioquímicas no local da lesão e nos tecidos adjacentes. Essas modificações instalam-se gradualmente, moduladas por fatores celulares específicos e que também caracterizam a fase do processo inflamatório (PAUL, 1998).

Dor e inflamação estão associadas com várias condições fisiopatológicas. Lesões teciduais podem iniciar respostas inflamatórias acompanhadas pela dor. As células danificadas liberam o conteúdo intracelular e ativam células do sistema imunológico levando a produção de mediadores inflamatórios (JULIUS e BASBAUM, 2001). Embora os sinais de dor sejam processados no SNC, mediadores inflamatórios e citocinas produzidas a partir de células imunes ativadas também podem estimular nociceptores terminais no tecido periférico e aumentar a sensibilidade à dor (JULIUS e BASBAUM, 2001). Estes mediadores agem diretamente sobre nociceptores terminais para ativar e produzir dor ou para sensibilizar terminais periféricos, levando à hipersensibilidade a estímulos subsequentes (REN e DUBNER, 2010).

As citocinas inflamatórias representam elementos-chave na comunicação entre as células do sistema imunológico, regulando respostas teciduais à infecção, inflamação e estresse. Quando encontradas no SNC, as citocinas podem ter duas origens: os órgãos imunes periféricos e/ou o próprio SNC. Apesar da existência da barreira hematoencefálica (BHE), citocinas produzidas perifericamente podem entrar no SNC por difusão passiva nos órgãos circunventriculares, por transporte ativo através da BHE (BANKS, 2006; BANKS et al., 2001; MAIER, 2003), ou ainda podem ser sintetizadas por células imunitárias residentes ou periféricas que invadem o SNC (RANSOHOFF e ENGELHARDT, 2012). Além disso, macrófagos, monócitos e linfócitos perivasculares podem ser recrutados dentro do SNC para ampliar a resposta inflamatória (RANSOHOFF et al., 2003).

Concentrações excessivas de glutamato extracelular agem sobre os receptores glutamatérgicos microgliais e podem contribuir para a propagação dos processos

inflamatórios (POCOCK e KETTENMANN, 2007), assim como, mediadores inflamatórios podem aumentar a liberação de glutamato, podendo causar um círculo vicioso (Figura 4). Além disso, citocinas como o TNF-α podem potenciar a excitotoxicidade mediada por glutamato por dois mecanismos complementares: indiretamente, pela inibição do transporte de glutamato, e diretamente, através do aumento na expressão de receptores de glutamato ionotrópicos localizados nas sinapses. A IL-1β (MANDOLESI et al., 2013) e o TNF-α (CARMEN et al., 2009; TOLOSA et al., 2011) também estão envolvidos na redução na expressão de transportadores de glutamato durante a neuroinflamação (TAKAKI et al., 2012). Neste sentido, já foi demonstrado que subunidades NR1 de receptores glutamatérgicos NMDA são ativadas durante inflamação periférica (PENG et al., 2011), sugerindo um papel do sistema glutamatérgico na inflamação.

Assim, a regulação dos níveis de glutamato não é apenas crucial no SNC, mas também a nível periférico, para evitar perturbações na transmissão sensorial. Dessa forma, a nocicepção pode estar sujeita a múltiplos níveis de controle bioquímico e farmacológico, envolvendo uma diversidade de tipos de células e mediadores solúveis (JULIUS e BASBAUM, 2001). Tendo isto em vista, substâncias capazes de neutralizar vias de sinalização nociceptiva, tanto a nível periférico quanto central podem ser promissoras no controle da dor.

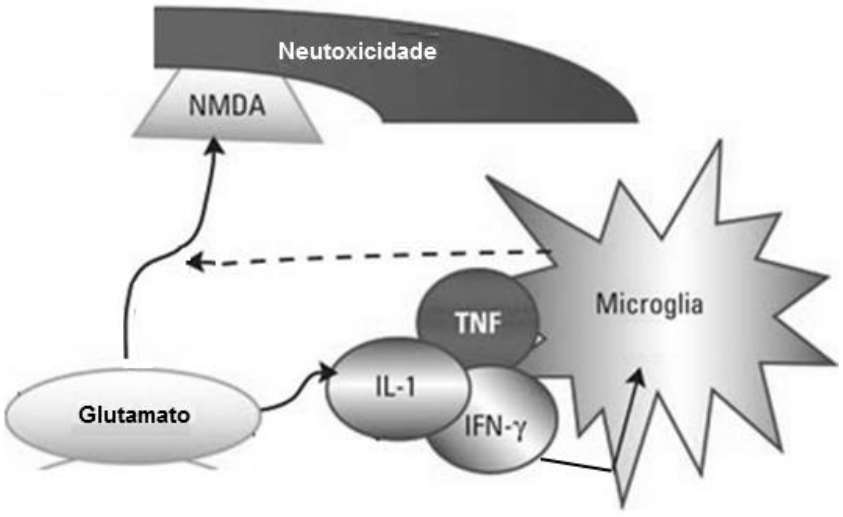


Figura 4- Interação bidirecional entre mediadores inflamatórios e neurotoxicidade glutamatérgica. Adaptado de MCNALLY et al. (2008)

1.5 Compostos orgânicos de Selênio

O selênio (Se) é um elemento químico não metálico da família dos calcogênios da tabela periódica o qual foi descoberto pelo químico sueco Jöns Jacob Berzelius em 1817 (COMASSETO, 2010). Este elemento traço é nutricionalmente essencial para mamíferos, com papéis fisiológicos como, por exemplo, como componente estrutural de enzimas antioxidantes (RAYMAN, 2000). Portanto, a Organização Mundial de Saúde (OMS) recomenda uma ingestão diária de 34-35 μg para adultos (FAO/OMS, 2002), seja através da ingestão de alimentos comuns, de origem animal e vegetal, ou por suplementação (DUMONT et al., 2006; RAYMAN, 2008). Além disso, a biodisponibilidade do selênio varia de acordo com a fonte e estado nutricional do indivíduo, sendo significativamente maior para as formas orgânicas de selênio (KIM e MAHAN, 2001; YOUNG et al., 1982)

Compostos orgânicos de selênio tem sido bastante estudados, uma vez que trabalhos já demonstraram as diversas atividades farmacológicas que eles apresentam (NOGUEIRA et al., 2004b). Além das suas propriedades antioxidantes (BORTOLATTO et al., 2013b; MEOTTI et al., 2004; MULLER et al., 1984), os compostos de selênio possuem atividade neuroprotetora (PORCIUNCULA et al., 2001; ROSSATO et al., 2002), anti-hipertensiva, anticâncer, antiviral, imunossupressora, antimicrobiana, ansiolítica, antidepressiva e anti-inflamatória (BRUNING et al., 2009; NOGUEIRA et al., 2003; SAVEGNAGO et al., 2008; ZASSO et al., 2005b). As propriedades antinociceptivas desses compostos também estão descritas e tem sido amplamente estudadas demonstrando que compostos orgânicos de selênio podem ser drogas relevantes para a gestão da dor.

Estudos anteriores realizados em nosso laboratório demonstraram que o composto orgânico de selênio, disseleneto de difenila $(PhSe)_2$ (Figura 5), um disseleneto de diarila de síntese simples, possui ampla distribuição nos tecidos (PRIGOL et al., 2012a) e produz efeitos antinociceptivos, dependentes da dose, em modelos químicos e térmicos de nocicepção em roedores (DA ROCHA et al., 2013b; NOGUEIRA et al., 2003; ZASSO et al., 2005b). Além disso, já foi relatado que o $(PhSe)_2$ reduz a alodínia causada por injeção intraplantar de adjuvante completo de Freund (CFA), bem como a alodinia induzida pela constrição parcial do nervo ciático, além de atenuar a hiperalgesia aguda induzida pelo glutamato, bradicinina e prostaglandina E2 em ratos (JESSE et al., 2008; SAVEGNAGO et al., 2007a).

Os mecanismos envolvidos na ação antinociceptiva deste composto incluem interação com receptores serotoninérgicos, glutamatérgicos e vias nitrérgicas, bem como sistemas peptidérgicos e vanilóide (SAVEGNAGO et al., 2007c; ZASSO et al., 2005b). Assim, compostos orgânicos de selênio, como o $(PhSe)_2$, são potenciais fontes de novas substâncias químicas relevantes para o tratamento da dor e outras aplicações terapêuticas. Sob este ponto de vista, considerando o uso crescente e precoce de GMS na dieta da população e a necessidade de busca por drogas capazes de atenuar os danos resultantes do uso deste aditivo alimentar, o estudo do efeito antinociceptivo do $(PhSe)_2$ na dor induzida por GMS torna-se de grande importância.

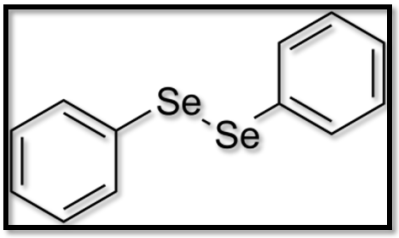


Figura 5– Estrutura química do composto $(PhSe)_2$

2. OBJETIVOS

2.1 Objetivo geral

Considerando os aspectos mencionados, o objetivo desse estudo foi avaliar a ação do composto disseleneto de difenila $(PhSe)_2$ na nocicepção induzida por glutamato monossódico administrado (MSG) no período neonatal em ratos, bem como investigar os mecanismos pelos quais o $(PhSe)_2$ age neste modelo.

2.2 Objetivos específicos

- Avaliar o efeito antinociceptivo do tratamento com $(PhSe)_2$ na hiperalgesia térmica e alodínia mecânica induzida por GMS, em ratos;
- Avaliar o envolvimento das enzimas Na^+ K^+- ATPase e Ca^{2+}- ATPase na nocicepção induzida por GMS e na ação antinociceptiva do $(PhSe)_{2;}$
- Pesquisar se a nocicepção induzida por GMS está acompanhada de um quadro inflamatório, e avaliar o possível efeito antiinflamatório do $(PhSe)_2$ neste modelo;
- Investigar o envolvimento do sistema glutamatérgico na nocicepção induzida por GMS e na ação antinociceptiva do $(PhSe)_{2;}$

3. Resultados

Os resultados que fazem parte dessa dissertação estão apresentados na forma de um manuscrito. Os itens materiais e métodos, resultados, discussão e referências bibliográficas do manuscrito estão dispostos de acordo com a recomendação do periódico científico no qual está submetido.

3.1 Manuscrito

Antinociceptive action of diphenyl diselenide in the nociception induced by neonatal administration of monosodium glutamate in rats

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Abstract

Monosodium glutamate (MSG) is a neuroexcitatory amino acid commonly used as flavoring of foods and its neonatal administration in animals leading to behavioral and physiological disorders in adulthood, including increased pain sensitivity. However, little is known about the mechanism of action by which MSG induces nociception. This study evaluated the effect of diphenyl diselenide $(PhSe)_{2,}$ an organoselenium compound with pharmacological properties already documented, on nociception induced by MSG. Newborn Wistar rats received ten subcutaneous injections of MSG at a dose of 4.0 g/kg or saline, once a day. At the 60^{th} day of life, rats were treated daily with $(PhSe)_2$ (1 mg/kg) or vehicle (canola oil) by intragastric route for 7 days. The behavioral tests (locomotor activity, hot plate, tail-immersion and mechanical allodynia) were carried out. In addition, hippocampal ex vivo assays were performed to determine Na^+, K^+-ATPase and Ca^{2+}-ATPase activities, cytokines levels and [^{3}H]glutamate uptake. The results demonstrated that MSG increased nociception in the hot plate test, but not in the tail immersion test, and in the mechanical allodynia stimulated by Von-Frey Hair. $(PhSe)_2$ decreased all nociceptive behaviors induced by MSG. MSG increased hippocampal Na^+,K^+-ATPase and Ca^{2+}-ATPase activities and pro-inflammatory cytokines levels. MSG decreased the anti-inflammatory cytokine and the [^{3}H]glutamate uptake in hippocampi of rats and $(PhSe)_2$ protected against these alterations. The results indicated some mechanisms of action that contribute to nociception induced by MSG in rats. This study also demonstrated that $(PhSe)_2$ protected against MSG- induced nociception.

Key words: Monosodium glutamate; excitotoxicity; nociception; selenium; diphenyl diselenide, ATPases.

Introduction

The human diet has changed greatly during the past decades and with this the introduction of industrialized foods in diet of children widely increased, since, processed foods are more palatable by the use of food additives to preserve flavor and enhance taste. One of the most commonly consumed food additives is monosodium glutamate (MSG), a neuroexcitatory amino acid used as a flavoring agent (MCCABE e ROLLS, 2007).

Earlier studies have demonstrated that MSG has some adverse effects in humans and experimental animals. In rodents, the administration of high doses of MSG during early stages of brain development induces destruction of sites in the hypothalamus (ABE et al., 1990), which provokes neuroendocrine abnormalities. These abnormalities can result in animal functional and behavioral disorders in adulthood, including obesity, hyperexcitability, impairment of memory, anxiogenic, depressive-like behaviors, pain-sensitivity and changes in analgesic responses (BADILLO-MARTINEZ et al., 1984; COLLISON et al., 2010; LOPEZ-PEREZ et al., 2010; QUINES et al., 2014; VAN DEN BUUSE et al., 1985).

Even though little is known about the mechanism of action by which MSG induces nociception, studies have demonstrated that MSG neonatal exposure leads to degeneration in hippocampal CA1 pyramidal cells (BEAS-ZARATE et al., 2002; ISHIKAWA et al., 1997a). In addition, previous data indicate that the hippocampal formation is involved in emergence of nociception (KLAMT e PRADO, 1991; MCKENNA e MELZACK, 1992) as well as the intensity of nociceptive stimulation is positively related to amplitude of excitatory postsynaptic potentials in hippocampal CA1 pyramidal cells (WEI et al., 2000). Thus, the study deals with the involvement of hippocampus in nociception induced by MSG and compounds effective in blocking nociceptive signaling pathways implicated in this process are of great importance.

Organoselenium compounds have roused interest due to their biological and pharmacological activities (NOGUEIRA et al., 2004a). Besides to their antioxidant properties (BORTOLATTO et al., 2013a; BRUNING et al., 2012), these compounds have neuroprotective (ABDEL-HAFEZ e ABDEL-WAHAB, 2008), anti-inflammatory and antinociceptive properties (CHAGAS et al., 2014; MARCONDES SARI et al., 2014). Of particular importance, diphenyl diselenide $(PhSe)_2$, an organoselenium compound, has been documented as promising pharmacological agent in a number of experimental

models, which showed that this compound has antinociceptive and anti-inflammatory properties (DA ROCHA et al., 2013a; LUCHESE et al., 2012). The mechanism of action by which $(PhSe)_2$ elicits antinociceptive action involves the modulation of serotonergic, nitrergic and glutamatergic systems (SAVEGNAGO et al., 2007b; ZASSO et al., 2005a). Regarding the pharmacokinetic properties, $(PhSe)_2$ shows a wide tissue distribution profile (PRIGOL et al., 2012b)

Based on the above considerations, the present study was designed to investigate the effect of $(PhSe)_2$ in nociception induced by MSG in rats. The possible mechanisms related to the $(PhSe)_2$ antinociceptive action were also investigated.

Materials and Methods

Animals

Newborn Wistar rats from our own breeding colony were used. The animals were kept on a 12 h light/dark cycle with lights on at 7:00 a.m., at room temperature (22 ± 1^0C) with free access to water and food. The experiments were performed according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Drugs

Diphenyl diselenide $(PhSe)_2$ (Fig. 1) was prepared in our laboratory according to the method described by (PAULMIER, 1986) and the chemical purity (99.9%) was determined by gas chromatography–mass spectrometry (GC/MS). Analysis of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra showed analytical and spectroscopic data in full agreement with its assigned structure. $(PhSe)_2$ was diluted in canola oil.

Monosodium glutamate (MSG) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Experimental design

Newborn Wistar rats received ten subcutaneous injections of MSG at a dose of 4.0 g/kg or saline (0.9%) in a similar volume (1 ml/kg), once a day starting at day one postnatal (KLINGBERG et al., 1987). Pups were weaned at the 21st day of life. At the 60th day of life, the female rats were divided into four groups. MSG and control groups were treated with $(PhSe)_2$ (1 mg/kg) or vehicle (canola oil, 1ml/kg) by the intragastric (i.g.) route once a day, for 7 days. Thirty minutes after last treatment, the animals were submitted to locomotor activity monitor and nociceptive tests: hot plate, tail-immersion and mechanical allodynia tests. After the behavioral tests, rats were killed by cervical dislocation and hippocampi, a target structure for neonatal MSG action (BEAS-ZARATE et al., 2002), were quickly removed for *ex vivo* assays.

Behavioral tests

Spontaneous locomotor activity

With the purpose of excluding sedative or motor abnormality, spontaneous locomotor activity of rats was performed in the locomotor activity monitor. The locomotor activity monitor is a Plexiglas box (45 × 45 × 45 cm) surrounded by a frame consisting of 32 photocells mounted on opposite walls (16 L × 16 W, spaced 2 cm apart) that continuously tracks the animal's movement. Animals were placed in the center of the apparatus and allowed to freely explore the arena during 4 min. Motor activity was monitored with the Insight® Monitor Activity System and the rat position in the chamber are detected by breaks of the photocell beams. Number of crossings and rearings, average velocity (mm/s) and total distance traveled (dm) were recorded.

Hot plate test

The hot-plate test was carried out according to the method described previously (WOOLFE e MACDONALD, 1944). In this test, the animals were placed in a glass cylinder on a heated metal plate maintained at 55 ± 1 ^{0}C. The latency of nociceptive responses such as licking or shaking one of the paws or jumping was recorded as the

reaction time. In order to avoid damage to the paws of the animals, the time standing on the plate was limited to 60 s.

Tail-immersion test

The tail-immersion test was conducted as described previously (JANSSEN et al., 1963). The test was performed by immersing the lower 3.5 cm of the tail into a cup freshly filled with water from a large constant-temperature (55 ºC) bath until the typical tail withdrawal response was observed. A 7 s cut-off was imposed.

Mechanical Allodynia test

The mechanical allodynia was measured as described before (BORTALANZA et al., 2002). Rats were further acclimatized in individual clear Plexiglas boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paw. The withdrawal response frequency of the left hind paw was measured following 10 applications (duration of 1-2 s each) of 2 g von Frey hairs (VFH; Stoelting, Chicago, IL, USA).

***Ex vivo* assays**

Tissue Preparation

The hippocampus samples of all animals were homogenized in 50 mM Tris/HCl at pH 7.4. The homogenate was centrifuged at 2.500 g for 10 min at 4 ^{0}C to yield a low-speed supernatant fraction (S1). Freshly prepared S1 was used for the determination of Na^{+}, K^{+}-ATPase and Ca^{2+}- ATPase activities and for the measurement of cytokine levels.

Na^{+}, K^{+}-ATPase activity

The Na^{+}, K^{+}-ATPase activity was measured according to (WYSE et al., 2000). An aliquot of S1 was added to the reaction mixture for Na^{+}, K^{+}-ATPase activity assay containing 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 for 30 min at 37 ^{0}C, and the incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. The Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of FISKE e SUBBAROW (1925). The enzymatic activity was expressed as nmol Pi/min/mg protein.

Ca^{2+}- ATPase activity

The Ca^{2+}- ATPase activity was measured as previously described by ROHN et al. (1993) with minor modifications (TREVISAN et al., 2009). Briefly, the assay medium consisted of 30 mM Tris-HCl buffer (pH 7.4), 0.1 mM EGTA, 3 mM $MgCl_2$ and 100 µg of protein in the presence or absence of 0.4 mM $CaCl_2$, in a final volume of 200 µl. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. After incubation for 60 min at 37 ^{0}C, the reaction was stopped by adding trichloroacetic acid solution (50% TCA). The released inorganic phosphate (Pi) was measured by the method of FISKE e SUBBAROW (1925) . The Ca^{2+}- ATPase activity was calculated by subtracting the activity measured in the presence of Ca^{2+} from that determined in the absence of Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA). The enzymatic activity was expressed as nmol Pi/min/mg protein.

Determination of cytokines

The levels of IL-1, IL-6, TNFα, INFγ and IL-10 in the hippocampus was measured using commercial ELISA kits for rat as described by the manufacturer (eBIOSCIENCE®, San Diego, USA). The results are expressed in pg/mg of protein.

[^{3}H]Glutamate uptake assay

The [^{3}H] glutamate uptake assay was carried out in slices (0.4 mm) of hippocampus obtained using a McIllwain chopper. The glutamate uptake was performed according to the method described by THOMAZI et al. (2004). The slices were transferred to multiwell dishes and washed with 1.0 ml Hank's buffered salt solution

(HBSS). After 10 min of pre-incubation, the uptake assay was performed by adding 13.3 μM [^{3}H] glutamate in 300 μl HBSS at 37 ^{0}C. Incubation was terminated after 5 min 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 ^{0}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. Results were expressed as pmol of [^{3}H] glutamate uptake /mg protein/min.

Protein determination

The protein concentration was measured by the Coomassie blue method according to BRADFORD (1976) using bovine serum albumin (1 mg/ml) as standard.

Statistical Analysis

The normality of data was analyzed using a D'Agostino and Pearson omnibus normality test. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Newman–Keuls test. All data of experiments were expressed as means ± SEM. Probability *p* values less than 0.05 ($p < 0.05$) were considered as statistically significant.

Results

Behavioral tests

Spontaneous locomotor activity

Neither the injection of MSG nor $(PhSe)_2$ treatment altered the number of crossings and rearings, velocity and total distance traveled ($p > 0.05$) by rats in the locomotor activity monitor (Table 1).

Hot-plate test

The two-way ANOVA of hot-plate data revealed a significant MSG × $(PhSe)_2$ interaction ($F_{1,27}$=23.2061, p<0.001). The administration of MSG to rats decreased the response latency to thermal stimuli as compared to the control group (p<0.001). $(PhSe)_2$ caused a significant blocked of thermal hyperalgesia induced by MSG in rats (p<0.001) (Fig. 2A)

Tail immersion test

The results of Fig. 2B show a significant main effect of $(PhSe)_2$ in the tail immersion test ($F_{1,25}$=14.0488, p<0.001). Although the subcutaneous injection of MSG did not change the tail-immersion response latency, the animals exposed to MSG and treated with $(PhSe)_2$ had an increase in the tail withdrawal response compared to the control group (p<0.01).

Mechanical allodynia test

The two-way ANOVA of mechanical allodynia data demonstrated a significant MSG × $(PhSe)_2$ interaction ($F_{1,24}$= 14.0713, p<0.001). Post hoc analyses indicated a significant increase in the mechanical sensitivity on the left hind paws of rats administered with MSG when compared to those of the control (p<0.001). Treatment with $(PhSe)_2$ was effective to decrease the mechanical allodynia induced by MSG injection (p<0.001) (Fig. 3)

***Ex vivo* assays**

Na^+, K^+-ATPase activity

Two-way ANOVA of hippocampal Na^+, K^+-ATPase activity revealed a significant MSG X $(PhSe)_2$ interaction ($F_{1,16}$=5.440, p<0.05). Post hoc comparisons demonstrated an increase in Na^+, K^+-ATPase activity induce by MSG administration when compared to with the control group (p<0.05). The $(PhSe)_2$ treatment totally

protected against alterations in Na^+, K^+-ATPase activity induced by MSG injections (p<0.01). (Fig. 4)

Ca^{2+}-ATPase activity

The two-way ANOVA of Ca^{2+}-ATPase activity data revealed a significant MSG X $(PhSe)_2$ interaction ($F_{1,18}$= 5.009, p<0.05) (Fig.5). Post hoc analysis showed a significant stimulation of Ca^{2+}+-ATPase activity in hippocampi of rats exposed to MSG (p<0.05) when compared to that of the control group. $(PhSe)_2$ treatment was effective against the increase in Ca^{2+}-ATPase activity caused by MSG (p<0.05).

Determination of cytokines

The two-way ANOVA of the pro-inflammatory cytokine levels demonstrated a significant MSG X $(PhSe)_2$ interaction in IL-1β, IL-6, TNFα and INFγ levels ($F_{1,12}$=9.584, p<0.01; $F_{1,11}$=7.780, p<0.05; $F_{1,12}$=5.475, p<0.05; $F_{1,12}$=10.622, p<0.01, respectively). Post hoc comparisons revealed an increase at all the pro-inflammatory cytokines levels in hippocampus of animals that received MSG (p<0.001) when compared to the control. The treatment with $(PhSe)_2$ was effective in protecting against the increased in cytokines levels induce by MSG (p<0.01). In addition, two-way ANOVA demonstrated a main effect of MSG in IL-10 levels ($F_{1,12}$= 7.411, p<0.05). The MSG administrations significantly decreased IL-10 levels (p<0.05) when compared to the control group. The treatment with $(PhSe)_2$ resulted in a tendency (but not statistically significant) to increase cytokine anti-inflammatory (IL-10) levels in rats MSG-treated (p=0.07) (Fig.6)

[^{3}H]Glutamate uptake assay

The Two-way ANOVA of these data revealed a significant MSG X $(PhSe)_2$ interaction ($F_{1,8}$=35.6137, p<0.001). Post hoc analyses indicated a significant decrease in [^{3}H] glutamate uptake by MSG and $(PhSe)_2$ groups when compared to the control group (p<0.05). The $(PhSe)_2$ treatment was effective to protect against the decrease in [^{3}H] glutamate uptake levels in hippocampus slices induced by MSG group (p<0.01) (Fig.7)

Discussion

In the present study, we investigated the effect of $(PhSe)_2$ in nociception induced by neonatal administration of MSG in rats using a behavioral tests battery. The mechanisms of action by which $(PhSe)_2$ attenuated MSG-induced nociception were evaluated in this study. Our results demonstrated that the MSG administration to rats reduced the thermal withdrawal latency in the hot plate test, but not in the tail immersion test, and increased the response frequency of VFH stimulation.

The tail-immersion and hot-plate tests have been widely used as experimental models to measure nociception, especially for the screening of analgesic drugs (DEWEY et al., 1969). Tail-immersion is regarded as a spinal reflex, but the mechanism of response could also involve higher brain structures (JENSEN e YAKSH, 1986), while the hot-plate test produces two kinds of behavioral response, which are paw licking and jumping. Both of these are considered to be supraspinally integrated responses (CHAPMAN et al., 1985). In addition, mechanical hypersensitivity is stimulated by traditional methods such as Von-Frey hairs. Mechanical allodynia is defined as a pain sensation generated by physiological stimulation of low-threshold sensitive and occurs due to changes of central processing of impulse activity in primary afferent neurons which feed into central nociceptive pathways (LAMBERT et al., 2009). Considering that MSG treatment altered mechanical allodynia and the withdrawal latency in the hot plate, but did not change the response latency in tail immersion test, we can suppose that nociception induced by MSG involves supraspinal reflexes rather than spinal ones.

The hippocampus contributes to several major functions of the brain, including learning and memory, energy-intake regulation, reward related mechanisms and pain (DAVIDSON et al., 2007; ERFANPARAST et al., 2010; KARAMI et al., 2002; KENNEY e GOULD, 2008; KHANNA et al., 2004). This brain structure is vulnerable to exposure to high concentrations of MSG early in life (BEAS-ZARATE et al., 2001), when excessive activation of glutamate receptors induces both cell death and changes in the cytoarchitecture of the surviving pyramidal neurons of the hippocampal CA1 field (BEAS-ZARATE et al., 2002). In this study, we investigated the possible alterations caused by MSG in hippocampus since this brain structure plays an important role in nociception (CECCARELLI et al., 1999; SCHNEIDER et al., 2001; WEI et al., 2000) and hippocampal neuronal activity can be modulated by noxious stimuli (LORENZ et al., 2008).

Glutamate is a critical neurotransmitter for excitatory synaptic transmission and for the generation and maintenance of pain hypersensitivity via activation of glutamate receptors (BASBAUM et al., 2009b). The mechanisms responsible for the elevation of extracellular glutamate levels include enhanced release of glutamate and the reduction of glutamate uptake (GILGUN-SHERKI et al., 2002b). Previous findings demonstrated that the neurotoxic effects of MSG treatment early in life induce nociceptive alterations of these animals in adulthood (BADILLO-MARTINEZ et al., 1984; VAN DEN BUUSE et al., 1985). Our results for the first time showed a decrease in [^{3}H]glutamate uptake in hippocampi of rats administered with MSG in the early life, which could account for the accumulation of glutamate in the synaptic cleft and consequently cause excitotoxicity. Assuming this hypothesis, the increase of glutamate in the synaptic cleft could be related to the decrease of nociception threshold caused by MSG in rats. In addition, $(PhSe)_2$ treatment protected against the decrease of [^{3}H]glutamate uptake caused by MSG, suggesting that regulation of glutamate uptake contributes to antinociceptive action of this compound. Accordingly, an interaction with the glutamatergic system is one of mechanisms of action by which $(PhSe)_2$ has antinociceptive action in acute models of nociception (SAVEGNAGO et al., 2007d).

The increase of extracellular glutamate involves sustained elevations of intracellular calcium levels through glutamate transporters, leading to an imbalance of sodium ions across plasma membranes (GILGUN-SHERKI et al., 2002b). Glutamate transporters are sodium-dependent proteins that putatively rely indirectly on Na^+, K^+-ATPase to generate ion gradients that drive transmitter uptake (ROSE et al., 2009a). This enzyme is present at high concentrations in the brain and other nervous tissue, where it plays several roles in the maintenance of the electrochemical gradient across the plasma membrane underlying resting and action potentials besides modulate the neurotransmitter release and uptake (STAHL e HARRIS, 1986). Previous studies have demonstrated that glutamate transporters and Na^+, K^+- ATPase are part of the same macromolecular complexes and operate as a functional unit to regulate glutamatergic neurotransmission in astrocytes and neurons (ROSE et al., 2009a; ZHANG et al., 2009). As a consequence, alterations in Na^+, K^+- ATPase activity directly affects neurotransmitter signaling, neural activity and animal behavior.

In addition to Na^+, K^+- ATPase activity be closely related to neuronal excitotoxicity through its function in maintaining the ionic equilibrium cell, Ca^{2+}- ATPase plays an important role also in excitotoxic events. Intracellular Ca^{2+} signaling is

fundamental in neuronal functioning and disruption of Ca^{2+} homeostasis plays a primary role in the glutamate evoked excitotoxicity (TYMIANSKI et al., 1993a). In this study, neonatal MSG treatment stimulated the activities of Na^{+}, K^{+}- ATPase and Ca^{2+} ATPase. These data suggest a cellular ionic imbalance that corroborates with the decrease of glutamate uptake induced by MSG, resulting in an excitotoxic event. The stimulation of both ATPases caused by MSG may compensate the decrease of glutamate uptake. Moreover, $(PhSe)_2$ protected against the stimulation of Na^{+}, K^{+}- ATPase and Ca^{2+} ATPase activities. This result suggests that $(PhSe)_2$ antinociceptive action may be related to the cellular ionic concentration maintenance, protecting against excitotoxicity caused by MSG.

Additionally, the current study demonstrated that MSG increased pro-inflammatory cytokine levels such as TNF-α, IL-1β, IL-6 and INF-γ and decreased IL-10 levels, an anti-inflammatory cytokine. Cytokines are barely detectable in the CNS under physiological conditions, but they become rapidly upregulated by pathological events like neuroinflammation and excitotoxicity (MINAMI et al., 1991). Studies indicate that pro-inflammatory cytokines, particularly IL-1β, increase neuronal excitability (BERNARDINO et al., 2005; VEZZANI et al., 1999). Considering that IL-1β can also inhibit glutamate uptake in astrocytes (HU et al., 2000) it is plausible to propose that the increase of pro-inflammatory cytokines results in elevated extracellular glutamate levels. Moreover, TNF-α, IL-1β, IL-6 and INF-γ can also release pro-inflammatory mediators such as: nitric oxide, bradykinin, histamine and/or substance P at the site of the inflammatory process (MORIOKA et al., 2002; O'SHAUGHNESSY et al., 2006), contributing to the emergence and maintenance of pain.

Our results demonstrated that the administration of $(PhSe)_2$ had an anti-inflammatory action, characterized by the reduction of pro-inflammatory cytokine levels (TNF-α, IL-1β, IL-6 and INF-γ) and a tendency (but not statistically significant) to increase the anti-inflammatory cytokine (IL-10) in rats treated with MSG. This is in agreement with previous studies of our research group that demonstrated a protection of $(PhSe)_2$ against the increase of pro-inflammatory cytokines in other experimental model (LUCHESE et al., 2012). Other important consideration is that the effect of $(PhSe)_2$ in decreasing IL-1 β levels induced by MSG can be one of the mechanisms by which this compound normalized the glutamate uptake in rats treated with MSG. This hypothesis can be supported by the study of HU et al. (2000) that demonstrated a link between IL-1β levels and the inhibition of glutamate uptake. These findings suggest that

the anti-inflammatory action of $(PhSe)_2$ contributes to its antinociceptive property induced by the neonatal administration of MSG in rats.

In conclusion, the results of the present study demonstrated that the administration of MSG induced alterations in [^{3}H] glutamate uptake, Na^+, K^+-ATPase and Ca^{2+}- ATPase activities, and in the levels of cytokines in hippocampus. These results contribute to clarify the mechanisms of action involved in nociception induced by MSG, suggesting targets for treatment of this worrying pathology since the use of MSG as a food additive is wide spreading all over the world. In addition, this study demonstrated the antinociceptive action of $(PhSe)_2$ after neonatal injections of MSG in rats. $(PhSe)_2$ was effective against excitotoxicity and neuroinflammation associated to the administration of MSG in rats, indicating a new role of $(PhSe)_2$ as an antinociceptive compound. Considering the results demonstrated in this study the concern about the consumption of MSG should increase. Nevertheless, further studies are needed to better understand the toxicological mechanisms by which MSG-induced behavioral alterations in rats.

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Conflict of interest

The authors declare they have no conflicts of interest to disclose.

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Figure Captions

Fig. 1. Chemical structure of diphenyl diselenide $(PhSe)_2$.

Fig.2. Effect of subcutaneous MSG-injection and intragastric treatment with $(PhSe)_2$ on the response latency to thermal stimuli in the hot plate test (A) and the tail immersion test (B). Data are reported as the means±SEM for seven to nine animals per group. **p<0.01, ***p<0.001 when compared to the control; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 when compared to the MSG group (two-way ANOVA followed by Newman–Keuls test).

Fig.3. Effect of subcutaneous MSG-injection and intragastric treatment with $(PhSe)_2$ on the percent response to mechanical stimulation in the left hind paw with 2g of von Frey hair filaments. Data are reported as the means ± SEM for six to eight animals per group. ***p<0.001 when compared to the control group; $^{\#\#\#}$p<0.001 when compared to the MSG group (two-way ANOVA followed by Newman–Keuls test).

Fig.4. Effect of subcutaneous MSG-injection and intragastric treatment with $(PhSe)_2$ on Na^+, K^+-ATPase activity in rat hippocampal homogenates. Results are expressed as nanomole Pi per milligram of protein per minute. Data are reported as the means ± SEM for four to six animals per group. *p<0.05 when compared to the control group; $^{\#\#}$p<0.01 when compared to the MSG group (two-way ANOVA followed by Newman–Keuls test).

Fig.5. Effect of subcutaneous MSG-injection and intragastric treatment with $(PhSe)_2$ on Ca^{2+}-ATPase activity in rat hippocampal homogenates. Results are expressed as nanomole Pi per milligram of protein per minute. Data are reported as the means ± SEM for five to six animals per group. *p<0.05 when compared to the control group; $^{\#}$p<0.05 when compared to the MSG group (two-way ANOVA followed by Newman–Keuls test).

Fig.6. Effect of subcutaneous MSG-injection and intragastric treatment with $(PhSe)_2$ on pro-inflammatory cytokines IL-1β (A) ,IL-6 (B), TNFα (C) and INFγ (D) and anti-inflammatory cytokines (IL-10) levels in rat hippocampal homogenates. Data are reported as the means ± SEM for four animals per group. *p<0.05 ***p<0.001 when

compared to the control group; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ when compared to the MSG group (two-way ANOVA followed by Newman–Keuls test).

Fig.7. Effect of subcutaneous MSG-injection and intragastric treatment with $(PhSe)_2$ on [3^{H}]glutamate uptake levels in hippocampus slices of rats. Data are reported as the means ± SEM for three animals per group. *p<0.05 when compared to the control group; $^{\#\#}p<0.01$ when compared to the MSG group (two-way ANOVA followed by Newman–Keuls test).

Figure 1.

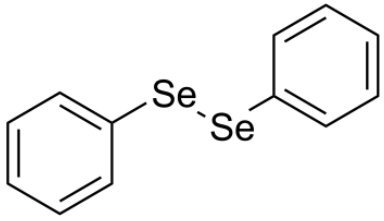


Figure 2.

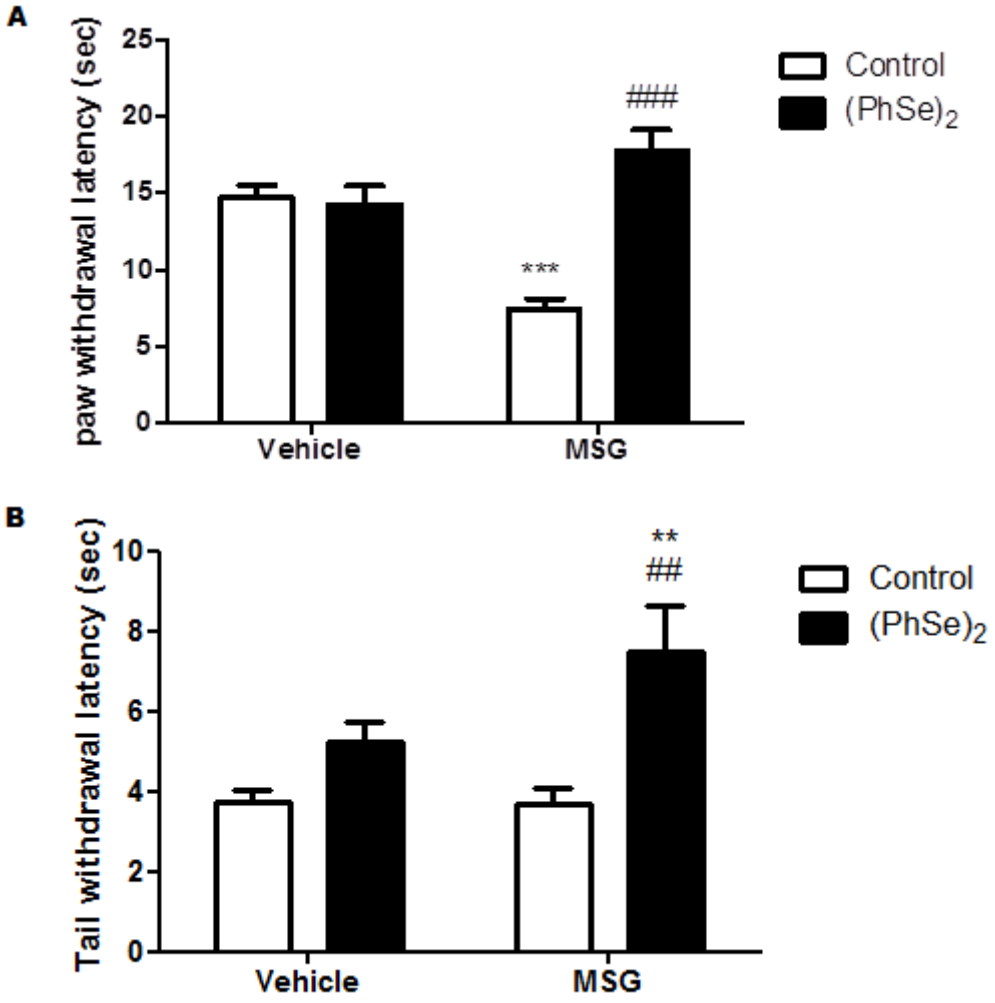

A
paw withdrawal latency (sec)
25
20
15
10
5
0
***
###
Vehicle
MSG
Control
(PhSe)2
B
Tail withdrawal latency (sec)
10
8
6
4
2
0
**
##
Vehicle
MSG
Control
(PhSe)2

Figure 3.

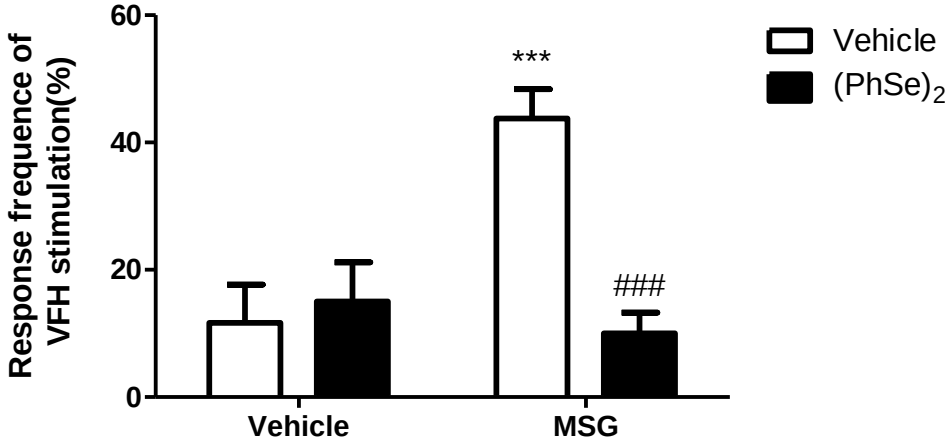

Response frequence of VFH stimulation(%)
60
40
20
0
***
###
Vehicle
MSG
Vehicle
(PhSe)2

Figure 4.

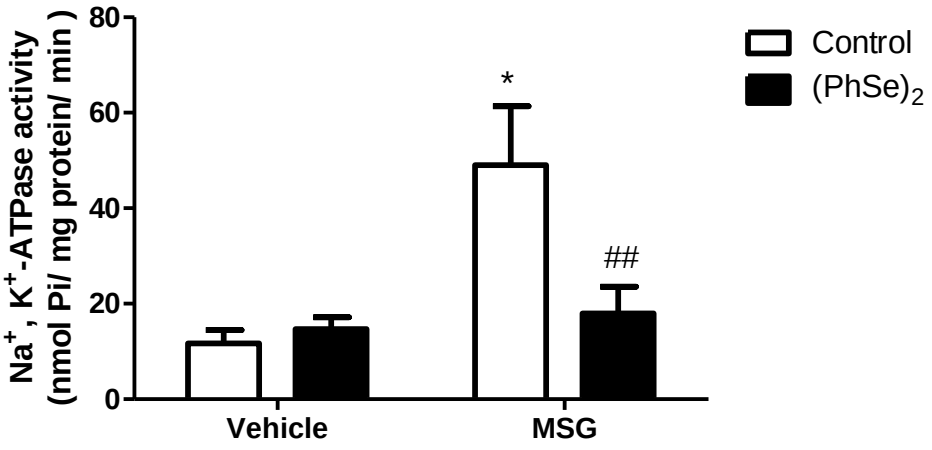

Na+, K+-ATPase activity
(nmol Pi/ mg protein/ min)
80
60
40
20
0
*
##
Vehicle
MSG
Control
(PhSe)2

Figure 5.

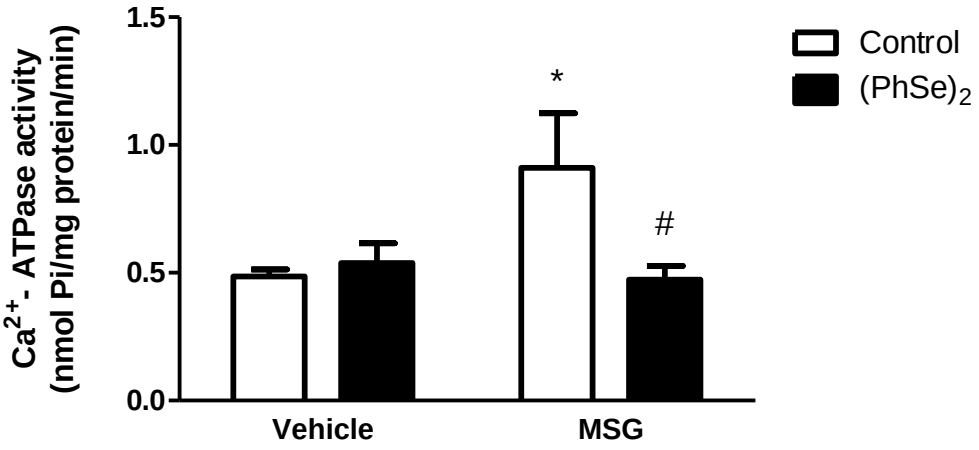

Ca2+- ATPase activity
(nmol Pi/mg protein/min)
1.5
1.0
0.5
0.0
*
#
Vehicle
MSG
Control
(PhSe)2

Figure 6.

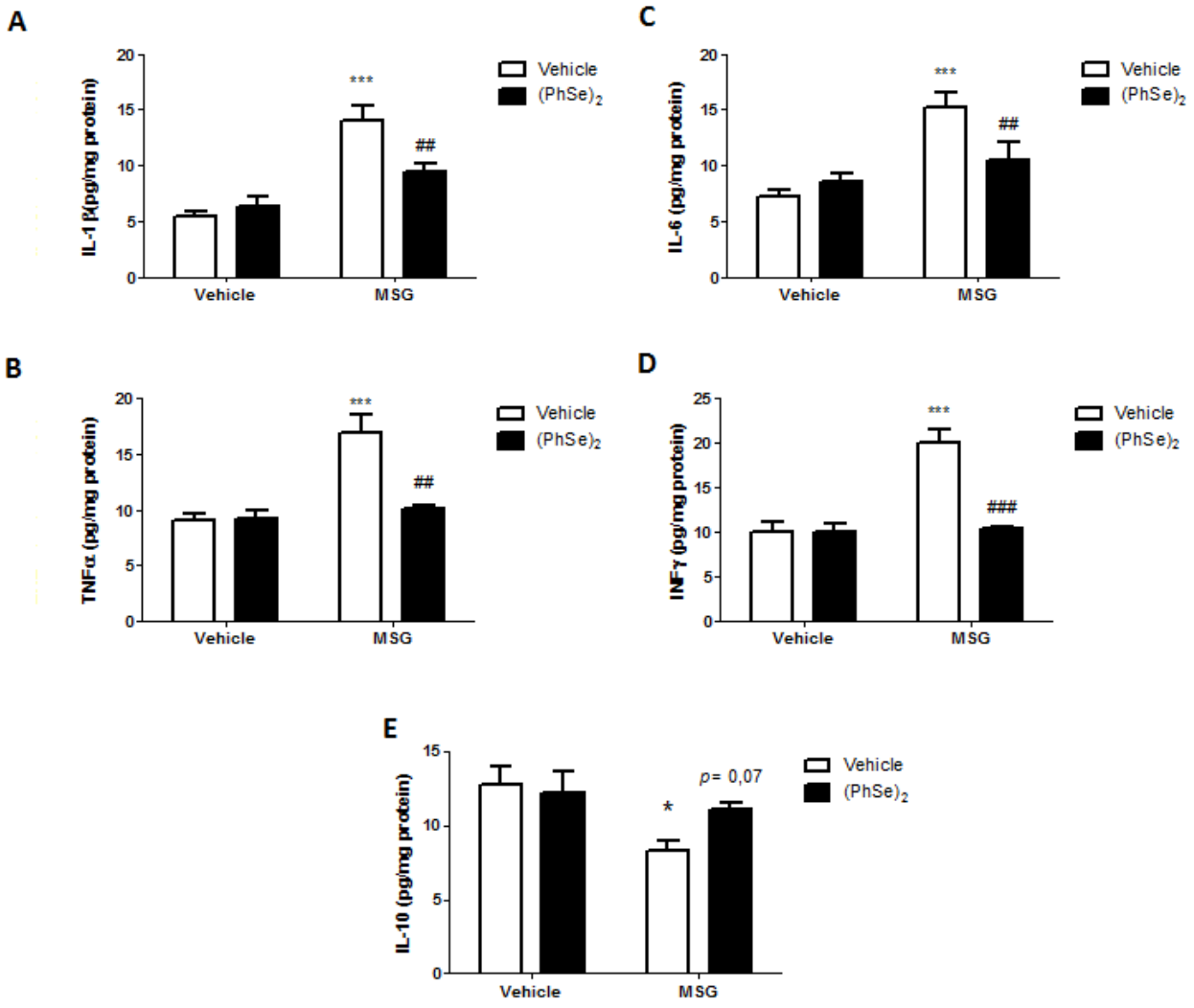
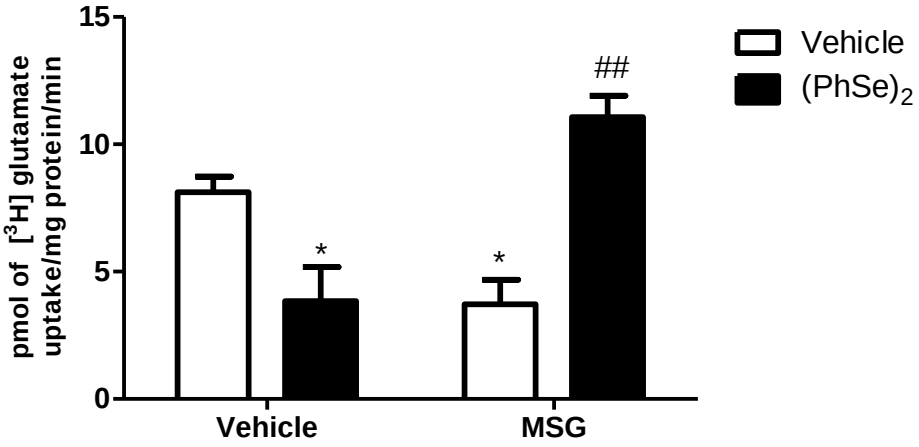

A
IL-1 β(pg/mg protein)
20
15
10
5
0
***
##
Vehicle
MSG
Vehicle
(PhSe)2
C
IL-6 (pg/mg protein)
20
15
10
5
0
***
##
Vehicle
MSG
Vehicle
(PhSe)2
B
TNFα (pg/mg protein)
20
15
10
5
0
***
##
Vehicle
MSG
Vehicle
(PhSe)2
D
INF-γ (pg/mg protein)
25
20
15
10
5
0
***
###
Vehicle
MSG
Vehicle
(PhSe)2
E
IL-10 (pg/mg protein)
15
10
5
0
*
p= 0,07
Vehicle
MSG
Vehicle
(PhSe)2

Figure 7.


15
10
5
0
pmol of [3H] glutamate uptake/mg protein/min
*
*
##
Vehicle
MSG
Vehicle
(PhSe)2

4. CONCLUSÃO

Os resultados apresentados nesta dissertação nos permitem concluir que:

✓ Administração neonatal de GMS induz hiperalgesia e alodínia em ratos aos 60 dias de vida a qual é revertida pelo $(PhSe)_2$;

✓ O GMS induz alterações na atividade das enzimas Na^+, K^+-ATPase e Ca^{2+}-ATPase e o tratamento com$(PhSe)_2$ reverte essas alterações;

✓ A nocicepção induzida por GMS esta relacionada com o aumento de respostas inflamatórias. O $(PhSe)_2$ reverte a inflamação induzida por GMS em hipocampo de ratos;

✓ A administração neonatal de GMS leva a diminuição da captação de glutamato em hipocampo e o $(PhSe)_2$ reverte esta alteração;

✓ Em conjunto os dados deste estudo sugerem que a nocicepção induzida por GMS envolve eventos excitotóxicos e inflamatórios e a ação do $(PhSe)_2$ no modelo estudado esta relacionada com sua ação neuroprotetora e anti-inflamatória;

Em conjunto os resultados desta dissertação contribuem para esclarecer os mecanismos de ação envolvidos na nocicepção induzida pelo MSG, sugerindo que a nocicepção induzida por GMS está relacionado com a excitotoxicidade e inflamação causados por este aditivo alimentar. Além disso, este estudo demonstrou a ação antinociceptiva $(PhSe)_2$ após injeções neonatais de MSG em ratos, a qual parece estar relacionado com sei efeito neuuprotetor e anti-inflamatório.Embora mais estudos sejam necessários para melhor elucidar os mecanismos toxicológicos pelos quais o GMS induz alterações comportamentais em ratos, os dados deste estudo contribuem para a compreensão dos mecanismos envolvidos na nocicepção induzida por GMS, bem como sugerem novos alvos da ação nociceptiva do composto $(PhSe)_2$.

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