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DISSELENETO *m*-TRIFLUORMETIL DIFENILA ATENUA AS FASES DA SENSIBILIZAÇÃO MOTORA COMPORTAMENTAL INDUZIDAS POR MORFINA EM CAMUNDONGOS

Santa Maria, RS

2022

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Dissertação apresentada ao curso de Mestrado do Programa de pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de MESTRE EM BIOQUÍMICA TOXICOLÓGICA

Orientadora: Profª Dra. Cristina Wayne Nogueira

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Aprovada em 11 de fevereiro de 2022.

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Santa Maria, RS
2022

DEDICATÓRIA

Dedico o presente trabalho á minha família, e em especial ao serzinho que esta sendo gerada em meu ventre, benção da minha vida.

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RESUMO

DISSELENETO *m*-TRIFLUORMETIL DIFENILA ATENUA AS FASES DA SENSIBILIZAÇÃO MOTORA COMPORTAMENTAL INDUZIDAS POR MORFINA EM CAMUNDONGOS

AUTORA: Renata Fritzsche Rodrigues
ORIENTADORA: Prof.ª Dr.ª Cristina Wayne Nogueira

O disseleneto de *m*-trifluormetil-difenila (*m*-CF_3-$PhSe)_2$ possui múltiplos alvos, incluindo o sistema glutamatérgico e o opioide, além de possuir propriedades antioxidantes. A sensibilização locomotora comportamental, caracterizada pela hiperatividade, é um modelo utilizado em estudos relacionados à drogadição e dependência, e diversas vias contribuem com estes aspectos, como o sistema opioide e a modulação de receptores de dopamina e de glutamato. Além do mais, o desequilíbrio redox favorece esta condição. O objetivo deste trabalho foi investigar o efeito do (*m*-CF_3-$PhSe)_2$ nas fases de aquisição, retirada da morfina e na reexposição à droga na sensibilização locomotora comportamental induzida por morfina em camundongos. Para este estudo foram utilizados camundongos Swiss machos com 30 dias (CEUA 5302070619), estes foram tratados com salina ou morfina 10 mg/kg 2x ao dia durante 3 dias, permaneceram em abstinência nos próximos 5 dias e no nono dia receberam uma dose de salina ou morfina. A fim de avaliar o efeito do composto, o (*m*-CF_3-$PhSe)_2$ foi administrado durante a aquisição (primeiros 3 dias), na retirada da morfina ou na reexposição (nono dia), logo após a dose de morfina no nono dia, imediatamente a atividade locomotora dos animais foi avaliada. Determinamos marcadores de estresse oxidativo, além dos níveis proteicos dos receptores opioides, de dopamina e de glutamato em córtex cerebral. Os resultados mostraram que a reexposição à morfina aumentou o conteúdo dos receptores opioides (MOR, DOR e KOR) e dos receptores glutamatérgicos (NMDA 2A e 2B). Entretanto, diminuiu os níveis dos receptores de dopamina (D1 e D2), além de desencadear um desequilíbrio redox, visto que aumentou os níveis de espécies reativas e marcadores da peroxidação lipídica e alterou a atividade de enzimas antioxidantes. Conclui-se que o (*m*-CF_3-$PhSe)_2$ foi efetivo em atenuar a sensibilização locomotora induzida por morfina, além de proteger contra as alterações bioquímicas e moleculares causadas pela reexposição a morfina em camundongos.

Palavras-Chave: Atividade locomotora. Drogadição. Receptor dopaminérgico. Receptor glutamatérgico. Opioide. Espécies reativas.

ABSTRACT

m-Trifluormethyl-diphenyl diselenide attenuates all phases of morphine-induced behavioral locomotor sensitization in mice

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m-Trifluoromethyl-diphenyldiselenide (*m*-CF_3-PhSe)$_2$ has multiple targets, including the glutamatergic system and the opioid, in addition to having antioxidant properties. Behavioral locomotor sensitization, characterized by hyperactivity, is a drug addiction and dependence model used experimentally. Several pathways contribute to these aspects, such as the opioid system, dopamine and glutamate receptors modulation. Furthermore, redox imbalance favors this condition. This study aimed to investigate the effect of (m-CF_3-PhSe)$_2$ on the acquisition, morphine withdrawal, and drug re-exposure phases on morphine-induced behavioral locomotor sensitization in mice. This study used 30 day-old male Swiss mice (CEUA 5302070619). They were treated with saline or morphine 10 mg/kg twice a day for three days; in the next five days, they were kept abstinent, and received saline or morphine on the ninth day. To assess the compound effect on this protocol, (m-CF_3-PhSe)$_2$ was administered during acquisition (first 3 days), on morphine withdrawal or re-exposure (ninth day), soon after the morphine dose on the ninth day, the mice were challenged in the locomotor activity test. Markers of oxidative stress were determined, in addition to protein levels of opioid, dopamine, and glutamate receptors in the mouse cerebral cortex. The results showed that re-exposure to morphine increased the content of opioid (MOR, DOR and KOR) and glutamatergic receptors (NMDA 2A and 2B). However, it decreased the levels of dopamine receptors (D1 and D2), in addition to triggering a redox imbalance, as it increased the levels of reactive species and markers of lipid peroxidation and altered the activity of antioxidant enzymes. In conclusion, (*m*-CF_3-PhSe)$_2$ attenuated morphine-induced locomotor sensitization and protected against biochemical and molecular alterations caused by re-exposure to morphine in mice.

Keywords: Locomotor activity. Drug addiction. Dopaminergic receptor. Glutamatergic receptor.Opioid. Reactive species.

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(*m*-CF_3-PhSe)$_2$	Disseleneto *m*-trifluormetildifenila
4-HNE	4-Hidroxinonenal
AMPA	α-amino-3-hidroxi-5-ácido metil isoxazol-4-propiônico
AMPc	Adenosina monofosfato cíclico
CAT	Catalase
CEUA	Comitê de Ética no Uso de Animais
CONCEA	Conselho Nacional de Controle de Experimentação Animal
D1	Receptor de dopamina 1
D2	Receptor de dopamina 2
DA	Dopamina
DL_{50}	Dose letal em 50% dos animais
ER	Espécies reativas
GPx	Glutationa peroxidase
HO-1	Heme Oxigenase-1
Keap1	Kelch-like ECH–associated
NMDA 2A	Receptor N metil-D-aspartato subunidade 2ª
NMDA 2B	Receptor N metil-D-aspartato subunidade 2B
NMR	Ressonância magnética nuclear
NPSH	Tióis não proteicos
Nrf2	Nuclear factor erythroid 2-related factor
SNC	Sistema Nervoso Central
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico

SUMÁRIO

1 INTRODUÇÃO

1.1 SENSIBILIZAÇÃO MOTORA INDUZIDA POR MORFINA

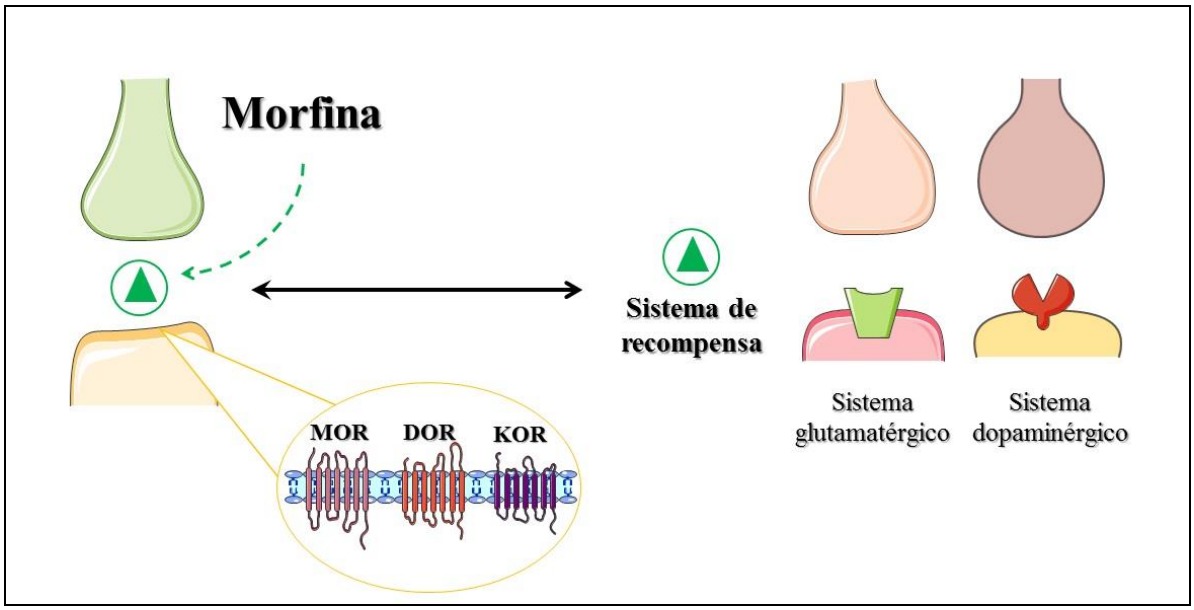
Os fármacos opioides são amplamente utilizados por serem potentes analgésicos (HERRERA et al., 2018), pois a dor é um fenômeno multidimensional complexo e provavelmente um dos sintomas mais frequentes apresentados na clínica (WORLD HEALTH ORGANIZATION, 2004). Nesta classe de fármacos, destaca-se a morfina, um alcaloide natural utilizado no tratamento de dor moderada a intensa, dor pós-operatória e tratamento paliativo do câncer. A morfina é um agonista completo, seus efeitos estão principalmente associados com a ativação do receptor MOR opioide e incluem analgesia, depressão respiratória, redução da motilidade gastrointestinal, náusea e sedação (GUTSTEIN e AKIL, 2005; STEIN et al., 1991).

O sistema opioide está envolvido em diversos processos fisiológicos, incluindo aqueles relacionados aos estímulos dolorosos, ao humor, à respiração e à recompensa (NOZAKI et al. 2014). Uma vez ativados, os receptores opioides desencadeiam uma série de eventos até causar a diminuição dos níveis de adenosina monofosfato cíclico (AMPc), inibição de canais de cálcio, e ativação de canais de potássio, o que diminui a excitabilidade neuronal e a liberação de neurotransmissores (AL-HASANI e BRUCHAS, 2011). No entanto, a dependência causada pelo uso prolongado de opioides limita o seu uso clínico e desencadeia um problema de saúde pública relacionado ao uso indevido destas drogas (MANCHIKANTI et al., 2006).

A morfina ativa os receptores opioides na área tegmental ventral (ATV), aumenta os níveis de dopamina (DA) e consequentemente induz sensação de euforia durante o uso (HNASKO et al., 2005). A administração contínua de opioides, induz alterações adaptativas no sistema nervoso central (SNC), responsáveis pela tolerância, dependência física, sensibilização, desejo e recaída. A adição causada pela morfina envolve não apenas alterações contra adaptativas de peptídeos endógenos e receptores opioides, mas também mudanças em muitos outros sistemas de transmissores neuronais, tais como noradrenérgico, dopaminérgico ou glutamatérgico (SHALEV et al., 2002) (figura 1). O uso indevido de opioides é um problema global e crescente que afeta milhões somente nos Estados Unidos da América. Além dos riscos diretos à saúde, a dependência está associada a uma grave disfunção social (HAN et al., 2017).

O aumento progressivo da resposta motora à droga é característica principal da sensibilização locomotora comportamental (ROBINSON e BECKER, 1986), o qual se torna mais pronunciado quando há falta da droga, ou seja, após o período de retirada, este que é um fator importante na indução e manutenção da dependência da droga em organismos animais e humanos, além disso, a retirada contribui para a recaída do abuso de drogas (ROBINSON e BERRIDGE, 1993). A sensibilização comportamental a estimulantes tem sido o foco de muitos estudos, dada a relevância potencial de seus mecanismos subjacentes a vários estados psicopatológicos em seres humanos, desde a dependência de drogas até a esquizofrenia (KALIVAS e STEWART, 1991; KOOB e Le MOAL, 1997; ROBINSON e BECKER, 1986; SEGAL e SCHUCKIT, 1983). Esse fenômeno esta envolvido em certos aspectos da dependência, como o desejo por drogas e o comportamento compulsivo. Foi sugerido que o aumento da sensibilidade comportamental pode ser um mecanismo subjacente que aumenta o risco de desenvolver dependência de drogas. Em vista disso, a exposição repetida a um medicamento promove a reorganização neural, levando a um estado hipersensível nos circuitos de recompensa cerebral (ROBINSON e BERRIDGE, 1993, 2000, 2001).

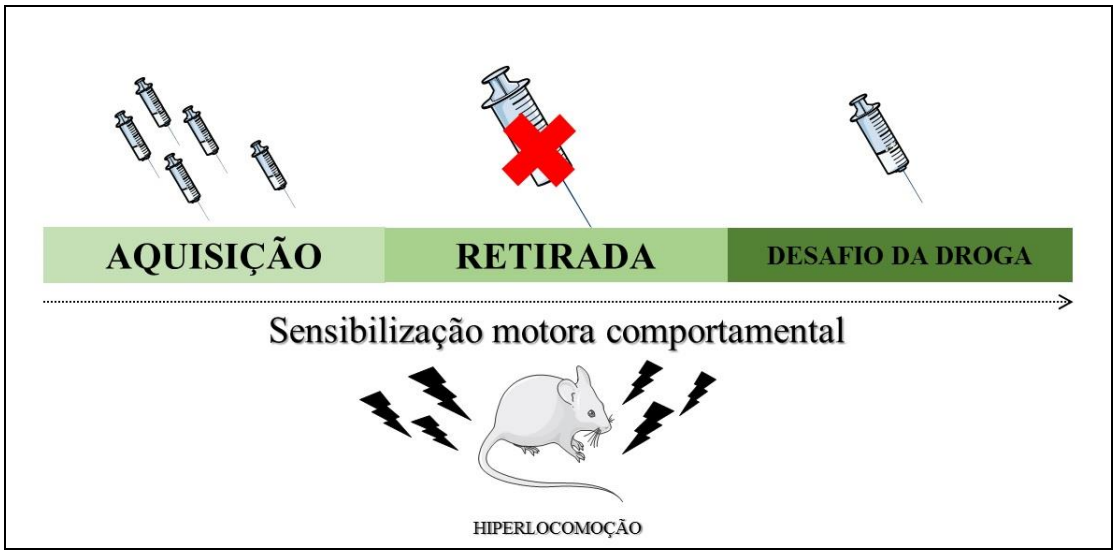
Figura 1 - Ativação do sistema opioide e do sistema de recompensa.



Fonte: (Arquivo pessoal do autor).

A sensibilização locomotora é um modelo animal amplamente utilizado em estudos sobre neurobiologia. A lógica deste modelo é que os efeitos subjetivos da droga de abuso aumentam com a exposição repetida à droga. A avaliação da atividade locomotora é uma ferramenta comum neste modelo, pois em roedores os efeitos recompensadores de uma droga de abuso estão diretamente relacionada à sensibilização aos seus efeitos locomotores estimulantes (VANDERSCHUREN e KALIVAS, 2000; VANDERSCHUREN e PIERCE, 2010). Para avaliar o perfil temporal, o modelo é dividido em três fases: (1) fase de indução, aquisição de sensibilização locomotora durante a exposição a medicamentos, (2) fase de desenvolvimento, período da retirada do fármaco e (3) fase de expressão, desafio da droga, para avaliar a persistência da sensibilização locomotora adquirida anteriormente (COELHOSO et al., 2013) (Figura 2).

Figura 2. Perfil temporal do protocolo de sensibilização motora comportamental.



Fonte: (Arquivo pessoal do autor)

Sabe-se que a ativação locomotora é uma adaptação comportamental induzida por fármacos opioides e a exposição a drogas aditivas. O receptor MOR opioidetem grande relevância na modulação deste comportamento, visto que microinjeções de agonistas deste receptor promoveram a sensibilização locomotora comportamental anteriormente(JOYCE e IVERSEN, 1979). Outros estudos envolvendo os receptores opioides destacam que em

camundongos *knockout* de receptor MOR mostraram uma diminuição na hiperlocomoção quando comparados com camundongos que possuíam este receptor(YOO et al. 2003).

Além do receptor MOR, o DOR (HEIDBREDER et al. 1996)e o KOR (SHIPPENBERG et al. 1996) também mostram envolvimento na sensibilização comportamental. Dados anteriores mostram que a presença de um antagonista do receptor DOR impede o desenvolvimento da sensibilização(SHIPPENBERG et al. 2009). Além disso, alguns resultados mostram que a ativação crônica do receptor KOR aumenta a atividade locomotora e também um comportamento compulsivo em animais (ESCOBAR et al. 2017; WEE e KOOB 2010).

1.2 SISTEMA DOPAMINÉRGICO E SISTEMA GLUTAMATÉRGICO

O circuito neuronal implicado neste fenômeno induzido por drogas está claramente envolvido com regiões cerebrais do sistema límbico (VANDERSCHUREN et al., 2000). Evidências convergentes indicam que a sensibilização comportamental depende da estimulação de receptores de aminoácidos excitatórios, como o glutamato e aspartato (KIM e VEZINA, 1999). O glutamato desempenha um papel importante na neurotransmissão no SNC, a transmissão rápida é mediada pelos receptores ionotrópicos de glutamato, como α-amino-3-hidroxi-5-ácido metil isoxazol-4-propiônico (AMPA) e N metil-D-aspartato (NMDA) (PARSONS et al., 1998; PIN e ACHER, 2002). Em relação aos mecanismos neuronais, há uma grande quantidade de dados que sugerem que a expressão da sensibilização comportamental de longo prazo depende da hipersensibilidade dos núcleos nervosos deDA (PIERCE e KALIVAS, 1997). Então, uma vez adquirida, é duradoura e tem uma relação temporal direta com alterações morfológicas e neuroquímicas na via mesolímbica e nos núcleos encefálicos que interagem com o sistema dopaminérgico (ROBINSON e KOLB, 1999; VANDERSCHUREN e PIERCE, 2010).

Muitos fatores podem influenciar a indução da sensibilização comportamental, está bem estabelecido que a DA desempenha um papel fundamental nos efeitos comportamentais das drogas de abuso, e o tratamento com morfina mostrou causar aumento na liberação de DAno corpo estriado por meio da ativação do receptor MOR(HYMAN et al. 2006). Muitos dados provam que todos os três receptores opióides (MOR, KOR e DOR) estão envolvidos na manutenção de DA no NAc (DI CHIARA e IMPERATO 1988). Algumas alterações neuroadaptativas foram observadas nos receptores de dopamina D1 e D2 em outros estudos,

uma diminuição dos níveis de expressão dos receptores de mRNA D1 e D2 foi observada no estriado de roedores sensibilizados com morfina, podendo ser causada por um aumento na concentração da DA estriatal, induzida pela dose de desafio de morfina (VANDERSCHUREN e KALIVAS 2000; LE MAREC et al. 2011; LISTOS et al. 2016).

Além do sistema dopaminérgico, o sistema glutamato é outro elemento-chave na dependência de drogas (KALIVAS et al. 2009). Os receptores de glutamato estão envolvidos em muitas atividades induzidas por drogas, como preferência condicionada de lugar (NARITA et al. 2000), dependência de opióides (BISAGA et al. 2001)e a hiperatividade locomotora condicionada pela morfina (BESPALOV e ZVARTAU 1996). Relatórios anteriores focados nas funções dos antagonistas do receptor NMDA mostraram-se eficazes para inibir a sensibilização locomotora (MENDEZ e TRUJILLO 2008), além disso há dados para provar que a regulação do NMDA subunidade 2B e os níveis totais de expressão de NMDA no NAc contribuem para a sensibilização locomotora comportamental induzida por morfina (BISAGA et al. 2001).

1.3 ESTRESSE OXIDATIVO ASSOCIADO A MORFINA

Há muitas evidências envolvendo o estresse oxidativo com o desenvolvimento dos efeitos adversos causados pelo tratamento com a morfina (CAI et al., 2016; JANG et al., 2017). A ativação de receptores opioides não gera somente um aumento nas espécies reativas, de oxigênio e nitrogênio, como também causa dano nas defesas antioxidantes, e desta forma induz um desequilíbrio redox celular (SKRABALOVA et al., 2013; MA et al., 2015). Recentemente, o papel do estresse oxidativo na ação da morfina tem recebido mais atenção. Um crescente corpo de evidências indicou que o estresse oxidativo está envolvido no desenvolvimento da dependência de várias drogas viciantes, incluindo morfina (CAI et al. 2016; ZENG et al. 2020).O estresse oxidativo altera o equilíbrio entre oxidantes e antioxidantes por acúmulo de ROS e/ou depleção de antioxidantes.Níveis aumentados de ROS podem alterar as estruturas de DNA e RNA e a expressão gênica (HABASHY et al. 2018).Em estudos anteriores, ratos que receberam morfina de forma sistêmica apresentaram um aumento no estresse oxidativo em regiões cerebrais como o córtex e hipocampo, juntamente com o comportamento de adição a morfina (FAMITAFRESHI et al., 2018). Mesmo com a administração subcutânea de morfina em um protocolo de tolerância e dependência foi possível notar um aumento significativo na peroxidação lipídica e uma diminuição nas defesas antioxidantes enzimáticas e não enzimáticas em regiões cerebrais de

camundongos (ABDEL-ZAHER et al., 2013a; ABDEL-ZAHER et al., 2013b). Anteriormente foi observado que a ativação de receptores opioides geram excesso de espécies reativas (SKRABALOVA et al. 2013) efoi visto tambem que os níveis de marcadores de estresse oxidativo estavam aumentados no córtex pré-frontal de ratos dependentes de morfina (FAMITAFRESHI e KARIMIAN 2018).Além disso, Abdel-Zaher e colaboradores relataram que os níveis de peróxido lipídico, o malondialdeído, aumentaram progressivamente no cérebro de camundongos tratados com morfina (ABDEL-ZAHER et al. 2013a). Em ratos, a injeção subcutânea de morfina também aumentou significativamente a peroxidação lipídica, além de diminuir a atividade da SOD (MOTAGHINEJAD et al. 2015).

Numerosos sistemas antioxidantes endógenos estão em vigor para garantir que os níveis de ROS seja adequadamente reduzido, incluindo a via de sinalização Nrf2-Keap1, o Nrf2 é um fator de transcrição que se liga a Elementos de Resposta Antioxidante na região promotora de genes envolvidos na regulação redox (HAYES e DINKOVA-KOSTOVA 2014) (Figura 3). Especificamente, a redução do Nrf2 e o aumento do estresse oxidativo contribuem para o estado patogênico. Por sua vez, isso permite que o superóxido, os radicais hidroxila e o peróxido de hidrogênio se acumulem nas células, portanto, um declínio na função do Nrf2 é um componente crítico de vários processos (SCHMIDLIN et al. 2019).

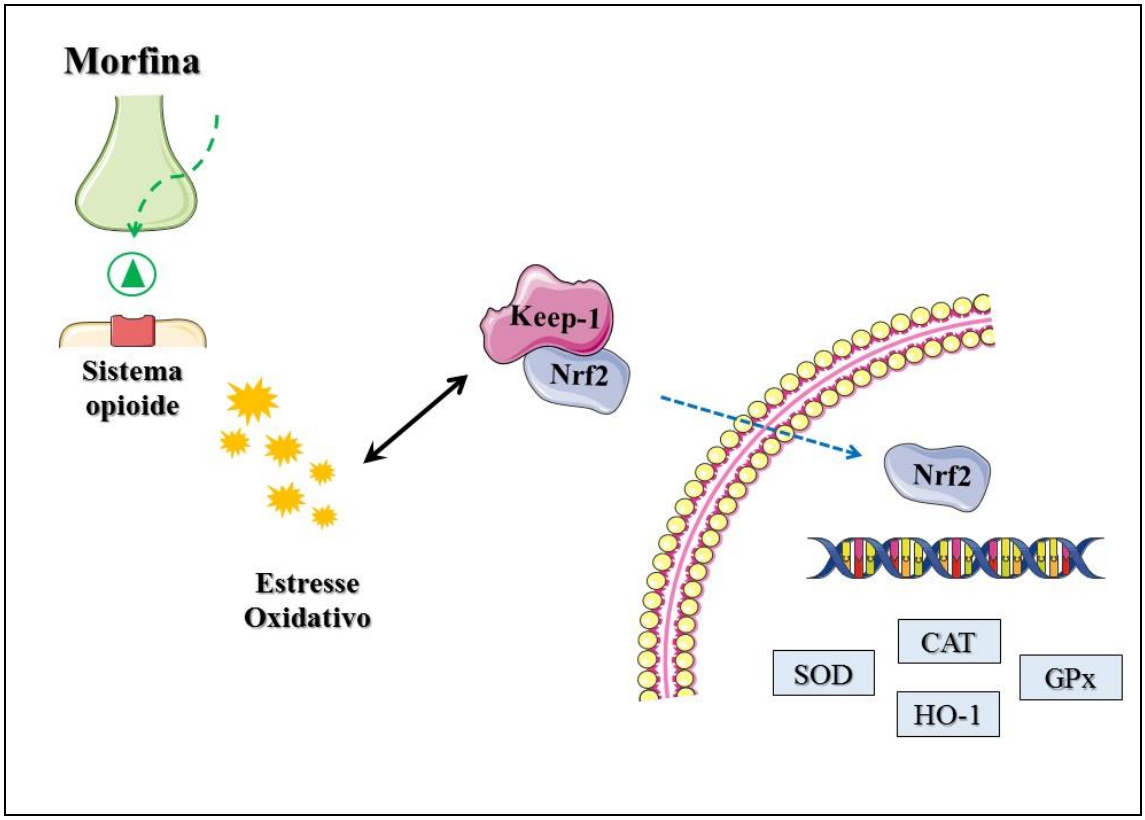
Em um estudo *in vitro*, foi visto que a exposição celular a drogas aditivas causam um aumento significativo nos níveis de ROS nas células através da inibição da expressão de Nrf2 nuclear (LIANG et al. 2020), outros autores tambem ressaltam a relação entre o aumento do dano oxidativo e a regulação negativa de Nrf2 (BELLEZZA et al. 2018), apoiando ainda mais o papel do Nrf2 como um regulador crucial do sistema antioxidante celular.Assim sendo, acreditasse que compostos que sejam efetivos em atenuar o quadro de estresse oxidativo possam auxiliar na proteção do desenvolvimento da sensibilização motora, e consequentemente da dependência.

1.4 DISSELENETO *m*-TRIFLUORMETIL DIFENILA

Neste contexto, o selênio é um micronutriente essencial conhecido, principalmente, por sua importância na manutenção de funções biológicas em organismos vivos (FLOHE et al., 1973; ROTRUCK et al. 1973). Além disso, na sua forma de selenocisteína, faz parte da constituição das selenoproteínas as quais desempenham funções fisiológicas importantes, principalmente, antioxidante (MOCCHEGIANI et al., 2014). O selênio é encontrado no organismo constituindo macromoléculas de diferentes tipos, como por exemplo, a

glutationaperoxidase e a tioredoxinaredutase, as quais são conhecidas pelo seu papel na proteção contra peroxidação lipídica e danos celulares oxidativos (BRABOSA et al. 2017; TINGGI 2008). Sabe-se que o selênio é importante para o SNC não somente pelo seu efeito antioxidante mas também por manter o estado redox celular, melhorar a dinâmica mitocondrial,e por participar na regulação dos canais de Ca^{2+} e na modulação da neurogênese (PAPP et al., 2007).

Figura 3 - Estresse oxidativo e transcrição do Nrf2.



Fonte: (Arquivo pessoal do autor).

Os compostos orgânicos de selênio apresentam importantes propriedades farmacológicas já reportadas (NOGUEIRA et al., 2004), e nesse contexto, a inserção de grupamentos CF_3 na posição meta do diaril disseleneto deu origem ao disseleneto *m*-trifluormetildifenila (*m*-CF_3-PhSe)$_2$, composto que destacamos neste estudos, pois os efeitos antinociceptivo (BRUNING et al., 2011), ansiolítico e antidepressivo (BRUNING et al., 2011), além de propriedades antioxidantes (BRUNING et al., 2015), tem sido descritas. Embora, não existam estudos

demonstrando a relação estrutura atividade do composto (*m*-CF_3-PhSe)$_2$, a presença do grupamento trifluorometila possivelmente contribua para seus efeitos farmacológicos duradouros e ampla distribuição (BRUNING et al., 2014). Por se tratar de um composto lipofílico, atravessa a barreira hematoencefálica e deste modo exerce seu efeito farmacológico no SNC. O (*m*-CF3-PhSe)$_2$ atua sobre diferentes sistemas de neurotransmissão, tais como serotoninérgico, opioide e glutamatérgico. No entanto, diferentemente da morfina, este composto não induz sinais de abstinência e tolerância em camundongos (BRUNING et al., 2015; ROSA et al., 2017). Além disto, este composto orgânico de selênio foi efetivo contra as alterações comportamentais induzidas pela droga de abuso anfetamina (SEGAT et al., 2016).

O (*m*-CF3-PhSe)$_2$ administrado de forma aguda, pela via oral em camundongos apresentou uma DL50, ou seja, dose letal a 50% dos animais tratados, correspondente a 278 mg/kg, e foram observadas alterações bioquímicas em marcadores de toxicidade renal e hepática (SAVEGNAGO et al., 2009). Quanto ao tratamento repetido, o composto quando administrado em doses de até 50 mg/kg pela via intragástrica (i.g.), durante oito dias, não causou toxicidade sistêmica (ROSA et al., 2017) evidenciando-se que o uso contínuo do (*m*-CF_3-PhSe)$_2$ seja relativamente seguro. Assim, estes estudos sugerem uma reduzida toxicidade do (*m*-CF_3-PhSe)$_2$, em roedores.

Desta forma, parte-se da necessidade de desenvolver compostos que atenuem ou bloqueiem efeitos adversos induzidos pela morfina, como o desenvolvimento da sensibilização motora comportamental. Ao analisar os mecanismos envolvidos no desenvolvimento da sensibilização motora comportamental induzida pela morfina e as propriedades do composto (*m*-CF_3-PhSe)$_2$, é possível que o tratamento com o (*m*-CF_3-PhSe)$_2$ durante as 3 fases seja efetivo em proteger do desenvolvimento da sensibilização motora comportamental induzida por morfina, através da modulação de receptores alterados pela morfina, além de atenuar o estresse oxidativo induzido pela morfina no SNC.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar o efeito do (*m*-CF_3-PhSe)$_2$ nas fases de aquisição, retirada da morfina e no desafio da re-exposição à droga em um modelo de sensibilização motora comportamental induzida por morfina, afim de propor uma intervenção terapêutica que auxilie contra o desenvolvimento da dependência a morfina.

2.2 OBJETIVOS ESPECÍFICOS

a) Explorar o efeito protetor do (*m*-CF_3-PhSe)$_2$ administrado durante as fases de aquisição, retirada e de reexposição no desenvolvimento da sensibilização motora em parâmetros comportamentais;
b) Determinar a contribuição do sistema opioide, dopaminérgico e glutamatérgico na ação do (*m*-CF_3-PhSe)$_2$ em um modelo de sensibilização motora comportamental induzida por morfina em camundongos;
c) Investigar o efeito do (*m*-CF_3-PhSe)$_2$ no estresse oxidativo em um modelo de sensibilização motora comportamental induzida por morfina em camundongos;

3 DESENVOLVIMENTO

O desenvolvimento da presente dissertação está apresentado na forma de artigo aceito na revista *Journal of Trace Elements in Medicine and Biology*. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio artigo conforme formatação e estrutura exigida pela revista.

Disseleneto difenila substituido com *m*-CF3 atenua todas as fases da sensibilização locomotora comportamental induzida por morfina em camundongos.

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m-CF_3-substituted diphenyl diselenide attenuates all phases of morphine-induced behavioral locomotor sensitization in mice

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ABSTRACT

Background: Behavioral sensitization, thought to underlie some aspects of drug dependence, is typically measured as increased locomotion in response to repeated administration of a drug. The study aimed to investigate the (*m*-CF3-PhSe)$_2$ effects on the acquisition, withdrawal, and re-exposure phases of morphine-induced behavioral locomotor sensitization.

Methods: Swiss male mice were treated with saline or morphine at 10 mg/kg twice a day for 3 days; those of the morphine group were kept in the morphine withdrawal period (5 days). On day 9, mice were re-exposed to morphine. (*m*-CF3-PhSe)$_2$ (10 mg/kg) or vehicle was administered at all phases of morphine protocol, and mice performed locomotor activity test. Oxidative stress markers and the levels of opioid, dopamine, and glutamate receptors were determined in samples of the cerebral cortex. (*m*-CF3-PhSe)$_2$ administered at all phases of protocol attenuated morphine-induced locomotor sensitization.

Results: Mice exposed to morphine showed reduced weight gain and increased locomotor activity, but (*m*-CF_3-PhSe)$_2$ treatment attenuates the weight gain and behavioral hyperlocomotion effects. (*m*-CF3-PhSe)$_2$, independent of the administration phase, modulated the increase of opioidergic (MOR, DOR, KOR) and glutamatergic (NMDA 2A and 2B) protein contents and attenuated redox imbalance in the cerebral cortex of mice exposed to morphine. However, (m-CF3-PhSe)$_2$ did not modulate cortical protein levels of dopaminergic (D1 and D2) receptors in the acquisition phase of morphine-induced locomotor sensitization protocol.

Conclusion: (*m*-CF_3-PhSe)$_2$ was effective against the behavioral and molecular alterations caused by morphine at all phases of locomotor sensitization.

1. Introduction

Morphine is a valuable drug in clinical practice because of its analgesic efficacy and potency [1]; however, its chronic use has several adverse effects, such as tolerance and dependence. Morphine use is limited because of its addictive properties, acting on opioid receptors in the ventral tegmental area (VTA), increasing dopamine release, and inducing euphoria for the user [2]. An intermittent administration of that drug leads to development of behavioral sensitization, defined as an enhanced systemic, mainly motor stimulation, response to the same dose of morphine or any other addictive substance [3,4].

Exposure to opiates induces locomotor sensitization, commonly manifesting in behavioral experiments by the increased locomotor activity of rodents, promoting a greater desire for the drug or craving. It plays a critical role in the development of addiction, especially in the high rate of relapse among the drug addicts even after very long periods of abstinence [5,6].

Behavioral sensitization includes three distinct phases: induction (acquisition), development (withdrawal), and expression (re-exposure). Induction refers to the immediate neurophysiological consequences associated with the drug administration, the first contact with morphine. Development refers to a protracted alteration in gene expression and neuroplasticity within the reward circuit when the drug is withdrawn. The expression is the behavioral reflection of the long-term consequences of the initial drug-induced neuroplastic changes within the reward circuitry, pronounced by the challenge dose: the re-exposure to the drug [7].

Although the mechanisms behind opioid-induced behavioral sensitization are not yet fully understood, the influence of various neurotransmitters, including dopamine [8] and glutamate [9], has been

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experimentally demonstrated.

The neuronal circuit involved in this drug-induced phenomenon is related to brain regions in the limbic system, such as the VTA and the nucleus accumbens (NAc). These structures have connections with the cortex, a part of a mesocortical circuit [10], which controls brain functions related to limbic neurotransmission, so impairment in the cortical area could lead to changes in this circuit [7].

Addictive drugs play a central role in dopamine-increased neurotransmission in mesocortical limbic brain, such as cerebral cortex, which is related to rewarding, motivation, and around [11,12], thus cerebral cortex is the structure critical in the development of sensitization.

Increasing evidence indicates that systemic morphine administration increased cerebral cortical and hippocampal oxidative stress along with the addiction to morphine behavior in rats [13], leading us to hypothesize that antioxidant compounds could protect against the behavioral locomotor sensitization development.

Considering the search for new therapeutic approaches to mitigate opioid-induced adverse effects, such as dependence, we emphasize *m*-trifluoromethyl-substituted diphenyl diselenide (m-$CF_3PhSe)_2$ that has some unique properties [14]. (m-$CF_3PhSe)_2$ modulates the opioidergic [15–17] and glutamatergic systems [18], and reduces re-conditioning symptoms induced by amphetamine in rats [19]. However, different from morphine, the well-known antioxidant (m-$CF_3PhSe)_2$ lacks tolerance and withdrawal symptoms in mice [18,20].

The present study aimed to evaluate the (m-$CF_3PhSe)_2$ effects on different phases of morphine-induced behavioral locomotor sensitization in mice.

2. Materials and methods

2.1. Animals

Adult male Swiss-Webster mice (30-day-old, 30–35 g) were maintained in a temperature-controlled room (22 ± 2 °C, 33 % humidity) on a 12 h dark 12 h light cycle with lights turned on at 7:00 a.m. The animals were allocated in polypropylene cages (five mice/cage), which were continuously sanitized for mice welfare. They had free access to a commercial diet (GUABI, RS, Brazil) and filtered water. The experiments were performed following the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil (registered under the number 5302070619) and affiliated to the Council for Control of Animal Experiments (CONCEA). The procedures followed what recommends the NIH Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines (record number 10684). All efforts were made to reduce both suffering and the number of animals to a minimum.

2.2. Chemicals

The compound (m-$CF_3PhSe)_2$ was synthesized according to the methodology previously described by Paulmier [21] and characterized by Nuclear Magnetic Resonance (NMR). The compound (Fig. 1) was dissolved in canola oil and administered to mice by the intragastric (i.g.) route.

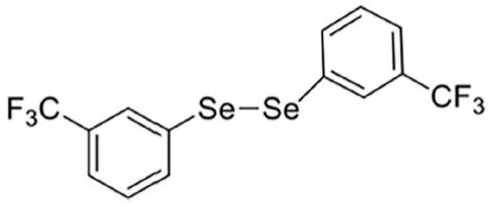

Fig. 1. Chemical structure of (m-$CF_3PhSe)_2$.

Morphine was bought from MCW medical and hospital products (Santa Cruz do Sul, RS, Brazil) under the reference drug Dimorf (morphine sulfate, 10 mg/kg) and dissolved in a physiological solution for the subcutaneous (s.c.) administration in mice. All drugs were administered in a constant volume of 10 mg/kg body weight.

2.3. Experimental design

The experimental design timeline illustrated in Fig. 2 depicts that the behavioral motor sensitization protocol lasted 9 days. Mice received (s.c.) saline solution 0.9 % or morphine at a dose of 10 mg/kg, whereas (m-$CF_3PhSe)_2$ was administered (i.g.) at the dose of 10 mg/kg. The selected dose and time of (m-$CF_3PhSe)_2$ treatments were based on our previously published reports [15,22,23]. Forty-five mice were separated into five groups (9 mice/group):

I – Control group (Fig. 2A) - mice received saline solution 0.9 % (s.c) twice a day on days 1, 2, 3, and 9.

* We did not run the (m-$CF_3PhSe)_2$ *per se* group because a pilot experiment revealed that mice in this group performed similar to those in the control group (supplementary material).

II – Morphine group (Fig. 2A) - mice received morphine twice a day on days 1, 2, and 3 and were kept in the morphine withdrawal period for 5 days. On day 9, mice were re-exposed to morphine [24].

III – (m-$CF_3PhSe)_2$ group in the acquisition phase (Fig. 2B) - 10 min after (m-$CF_3PhSe)_2$ administration, mice received morphine, twice a day, on days 1, 2, and 3 and were kept in the morphine withdrawal period for 5 days. On day 9, mice were re-exposed to morphine.

IV – (m-$CF_3PhSe)_2$ group in the withdrawal phase (Fig. 2C) - mice received morphine twice a day on days 1, 2, and 3. Mice were treated once a day with (m-$CF_3PhSe)_2$ for the morphine withdrawal period (5days). On day 9, mice were re-exposed to morphine.

V – (m-$CF_3PhSe)_2$ group in the re-exposure phase (Fig. 2D) - mice received morphine twice a day on days 1, 2, and 3 and were kept in the morphine withdrawal period for 5 days. On day 9, mice were treated once a day with (m-$CF_3PhSe)_2$ 30 min before morphine re-exposure.

2.4. In vivo

2.4.1. Body weight profile

The body weight of mice was daily recorded. At the end of the protocol, the individual body weight gain (g) was calculated by the difference between the baseline body weight, obtained before the beginning of treatment, and the body weight at the end of treatment.

2.4.2. Locomotor activity

Mice (9 per group) were individually evaluated in a transparent acrylic apparatus (50 cm × 48 cm × 50 cm) connected to a monitor with photocell beams, containing 16 infrared sensors for the automatic recording of animal position and the general locomotor activity (Insight, Ribeirao Preto, SP, Brazil). Each mouse was placed in the center of the box and allowed to explore freely for 1 h [24]. The number of crossings, speed, and total distance traveled were recorded.

2.5. Ex vivo assays

After the behavioral tests, mice were euthanized, and the cerebral cortex was immediately collected on a cold plate and stored at -80 °C until further analysis. The experimental number of samples was randomly reduced to 6 for all assays, excepting for the western blot assay, in which 5 animals per group were used.

2.5.1. Sample preparations

2.5.1.1. Low-speed supernatant. The samples were homogenized in 50 mM Tris HCl pH 7.4, 1:5 (v/w), and centrifuged at 2,500 × g for 10 min.

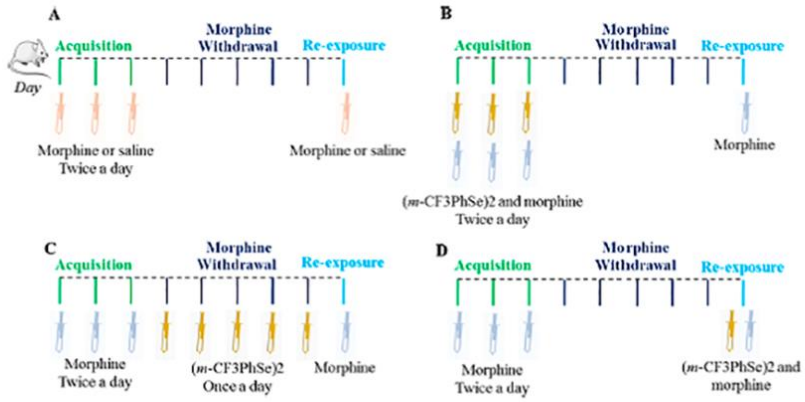

Fig. 2. Timeline of the experimental design. The animals received morphine at a dose of 10 mg/kg or saline (s.c) twice a day (days 1, 2, and 3) and on day 9 (A). In the acquisition phase (B), during the morphine withdrawal (C), or before the morphine re-exposure (D) mice received $(m\text{-}CF_3\text{-}PhSe)_2$ at a dose of 10 mg/kg (i.g).

After centrifugation, the portion S1 was collected and used to determine the levels of thiobarbituric acid reactive substances (TBARS), reactive species (RS), and non-protein thiol (NPSH). S1 was also used to determine the activities of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD).

2.5.1.2. Western blot. The samples were homogenized in HEPES buffer solution pH 7.4 (1 mM added with cocktail of inhibitors, Sigma-Aldrich Company, St. Louis, Missouri, United States). The sample protein concentration (2 $\mu g.\mu L^{-1}$) was obtained by dilution in Laemmli buffer 4x solution (500 mM Tris/HCl pH 6.8; glycerol, 10 % sodium dodecyl sulphate, and 2% bromophenol blue) [25], and 2-β-mercaptoethanol.

2.5.2. Oxidative markers

2.5.2.1. TBARS levels. The naturally occurring lipid peroxidation is an important biomarker of oxidative stress and is determined in a spectrophotometer, as oxidized lipids react with thiobarbituric acid and then forms a chromophore [26]. An S1 aliquot was incubated at 95 °C for 2 h with a solution containing 0.8 % thiobarbituric acid (TBA), acetic acid buffer pH 3.4, and 8.1 % sodium dodecyl sulfate (SDS). After the incubation time, the samples were read in a spectrophotometer at 532 nm and the results were expressed in the nmol equivalent of malondialdehyde (MDA)/mg protein.

2.5.2.2. RS levels. A fluorescence method previously described by Loetchutinat et al. (2005) was used to determine RS. An S1 aliquot was incubated in 10 mM Tris/HCl pH 7.4 for 1 h at 37 °C in the presence of 1 mM 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) in the dark. After that, dichlorofluorescein was detected from the oxidation of DCFH-DA to reveal the levels of RS. The fluorescence emission intensity was recorded at 525 nm, with an excitation of 480 nm. The levels of RS were expressed in fluorescence Unit (UF)/mg of protein.

2.5.3. Antioxidant defenses

2.5.3.1. CAT activity. The CAT activity was measured following the method of Aebi (1984). Briefly, an aliquot of S1 was incubated with 50 mM potassium phosphate buffer pH 7.0, and H_2O_2 was added to start the reaction. The absorbance was measured for 2 min, every 15 s, at 240 nm and 37 °C by a UV microplate reader. The results were expressed in activity (K)/μg protein.

2.5.3.2. GPx activity. The GPx activity depends on the conversion of reduced glutathione to oxidized glutathione. A system containing 0.5 M potassium phosphate buffer with 5 mM ethylenediaminetetraacetic acid (EDTA) pH 7.0, 10 mM azide, 10 mM glutathione, 1.6 mM NADPH, and 2.5 U/mL glutathione reductase (GR) and an S1 aliquot were incubated in a UV microplate. The reaction started with 4 mM H_2O_2 added to this system [27]. The absorbance was measured for 3 min, every 15 s, at 340 nm and 37 °C. The activity was expressed in nmol NADPH/min/mg of protein.

2.5.3.3. SOD activity. The enzyme activity was carried out as described by Misra and Fridovich (1972). The principle of this assay is based on the ability of SOD to inhibit adrenaline auto-oxidation to adrenochrome. For this, aliquots of S1 were added in 50 mM sodium carbonate buffer. The reaction was started when epinephrine was added to the medium. The enzymatic reaction was measured in a VIS-microplate reader at 480 nm. The results were expressed in U/mg of protein.

2.5.4. Western blot

The samples (20 μL of protein/well) and the marker protein (Bio-Rad, São Paulo, Brazil) were separated electrophoretically in an SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane using Transfer-Blot® Turbo ™ Transfer System (1.0 mA; 45 min, Bio-Rad).

Membranes were blocked with a 3% bovine albumin solution for 1 h and incubated overnight at 4 °C with the primary antibodies anti-nuclear factor (erythroid-derived 2)-like 2 (Nrf2), anti-Kelch-like ECH -associated protein 1 (Keap-1), anti-4-hydroxinonenal (4-HNE), anti-hemeoxygenase (HO-1), anti-D1 receptor, anti-D2 receptor, anti-opioid mu receptor (MOR), anti-opioid kappa receptor (KOR), anti-opioid delta receptor (DOR), and anti-*N*-methyl D-Aspartate (NMDA 2A and 2B). B-actin and B-tubulin were used as constitutive proteins (Table 1).

After incubation with the primary antibodies, the membranes were washed and then incubated with the respective secondary antibodies conjugated with peroxidase for 1 h at room temperature. For the protein detection, a chemiluminescence kit (Amersham, São Paulo, Brazil) was used and the signals were captured with Amersham Imager 600 (GE health care life sciences). The bands were quantified using the Image J software (NIH, Bethesda, Maryland, USA). The results were expressed as a percentage of control.

2.5.5. Protein determination

The protein concentration was determined by the Bradford method [28] using bovine serum albumin (1 mg/mL) as a standard or the

Table 1
List of primary antibodies and their properties.

Antibody	*Molecular Weight*	*Type*	*Company*
4-HNE	70 kDa	Mouse	Abcam
D1	74 kDa	Mouse	Santa Cruz Biotechnology
D2	51 kDa	Mouse	Santa Cruz Biotechnology
DOR	58 kDa	Rabbit	Santa Cruz Biotechnology
HO-1	34 kDa	Mouse	Abcam
Keap-1	69 kDa	Goat	Santa Cruz Biotechnology
KOR	43 kDa	Rabbit	Santa Cruz Biotechnology
MOR	55 kDa	Goat	Santa Cruz Biotechnology
NMDA 2A	180 kDa	Rabbit	Cell Signaling Technology
NMDA 2B	190 kDa	Rabbit	Cell Signaling Technology
Nrf2	61 kDa	Mouse	Santa Cruz Biotechnology
β-actin	45 kDa	Mouse	Cell Signaling Technology
β-tubulin	54 kDa	Mouse	Abcam

4-HNE: 4-Hydroxinonenal; D1: dopaminergic repector; D2: domainergic receptor; DOR: δ opioid receptor; HO-1: Heme Oxygenase; Keap-1: Kelch-like ECH –associated; KOR: κ opioid receptor; protein 1 MOR: μ opioid receptor; NMDA2A: N-methyl D-Aspartate 2A; NMDA 2B: N-methyl D-Aspartate 2B; Nrf2: Nuclear factor (erythroid-derived 2)-like 2.

bicinchoninic acid solution [29].

2.6. Statistical analyses

Descriptive statistic data were expressed as the mean (s) ± SEM. All analyses were performed using the GraphPad software (GraphPad Software, San Diego, CA, USA). Data normality was estimated using the Shapiro-Wilk normality tests. All experimental data were analyzed by the One-way ANOVA, followed by the Bonferroni test. Although all experimental groups were compared among them, only the effects of morphine (morphine x control) and those of (m-CF_3-PhSe)$_2$ in the acquisition, withdrawal, and re-exposure phases ((m-CF_3-PhSe)$_2$ x morphine group) were considered. Differences among groups were considered statistically significant when probability values were less than 0.05 ($P < 0.05$).

3. Results

3.1. (m-CF_3-PhSe)$_2$ treatment attenuates behavioral hyperlocomotion in the three phases of locomotor sensitization induced by morphine

The results illustrated in Fig. 3 show the weight profile during the experimental protocol (A) and the weight gain (B) at the end of the protocol. The one-way ANOVA analyses indicated that morphine reduced the mouse weight gain [$F_{(4,40)} = 6.06$, $P = 0.004$] compared to the control group. (m-CF_3-PhSe)$_2$ treatment in the acquisition ($P < 0.01$) and the morphine withdrawal ($P < 0.001$) phases effectively attenuated the reduction in the weight gain in mice exposed to morphine.

Fig. 4 depicts the effects of morphine on distance traveled (mm) (A), number of crossings (B), and speed (mm/s) (C) of mice. The one-way ANOVA analyses showed a significant difference for morphine group on distance traveled (mm) [$F_{(4,40)} = 27.64$, $P = 0.000$], number of crossings [$F_{(4,40)} = 21.13$, $P = 0.000$], and speed (mm/s) [$F_{(4,40)} = 24.31$, $P = 0.000$] compared to the control group. Treatment with (m-CF_3-PhSe)$_2$ in the acquisition, the morphine withdrawal, and the re-exposure phases ($P < 0.001$) attenuated the parameters of locomotor activity in mice exposed to morphine.

3.2. (m-CF_3-PhSe)$_2$ treatment modulates opioid receptor contents increased by morphine in the mouse cerebral cortex

Morphine increased the MOR [$F_{(4,20)} = 7.45$, $P = 0.0032$], DOR [$F_{(4,20)} = 19.19$, $P = 0.000$], and KOR [$F_{(4,20)} = 17.20$, $P = 0.028$] protein contents in the cerebral cortex of mice (Fig. 5A-C) compared to the control group, and treatment with (m-CF_3-PhSe)$_2$ at the three phases was effective against this effect ($P < 0.05$).

3.3. (m-CF_3-PhSe)$_2$ treatment modulates protein contents of dopaminergic and glutamatergic receptors altered by morphine in the mouse cerebral cortex

Fig. 6 shows that morphine modulated D1 (A), D2 (B), NMDA 2A (C), and NMDA 2B (D) protein contents in the cerebral cortex of mice. The one-way ANOVA analyses indicated a decrease in the protein levels of cortical D1 [$F_{(4,20)} = 9.20$, $P = 0.036$] and D2 [$F_{(4,20)} = 6.47$, $P = 0.017$] in the morphine group when compared to the control group. (m-CF_3-PhSe)$_2$ treatment in the morphine withdrawal and re-exposure phases reversed the decrease in dopaminergic receptors ($P < 0.05$) of morphine exposed mice.

The protein levels of NMDA 2A [$F_{(4,20)} = 13.94$, $P = 0.001$] and NMDA 2B [$F_{(4,20)} = 6.83$, $P = 0.003$] were increased in the cerebral cortex of mice exposed to morphine and (m-CF_3-PhSe)$_2$ treatment, at all phases, was effective in attenuating the increase in these proteins ($P < 0.05$).

3.4. (m-CF_3-PhSe)$_2$ treatment attenuates redox imbalance induced by morphine in the mouse cerebral cortex

The one-way ANOVA analyses showed a significant difference in the levels of RS [$F_{(4,25)} = 11.31$, $P = 0.028$], TBARS [$F_{(4,25)} = 8.19$, $P = 0.001$], and 4-HNE [$F_{(4,20)} = 23.08$, $P = 0.000$], which were increased in

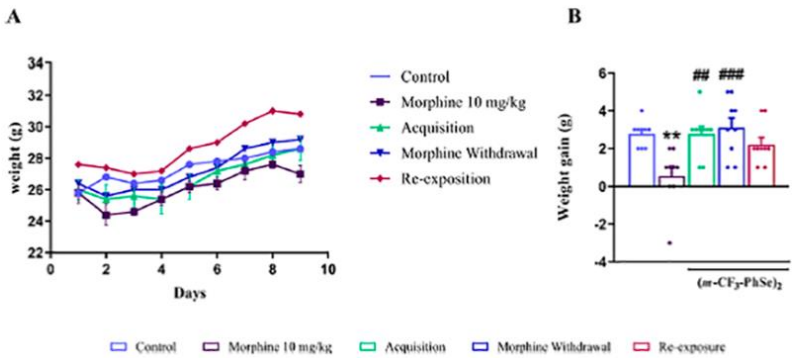

Fig. 3. The mouse weight profile during the experimental protocol (A) and the weight gain at the end of protocol (B). Data are expressed as mean ± S.E.M of 9 animals per group. Asterisk denotes significance levels when compared to the control group: (**) $P < 0.01$. Hashtag denotes significance levels when compared to the morphine-treated group: (##) $P < 0.01$ and (###) $P < 0.001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

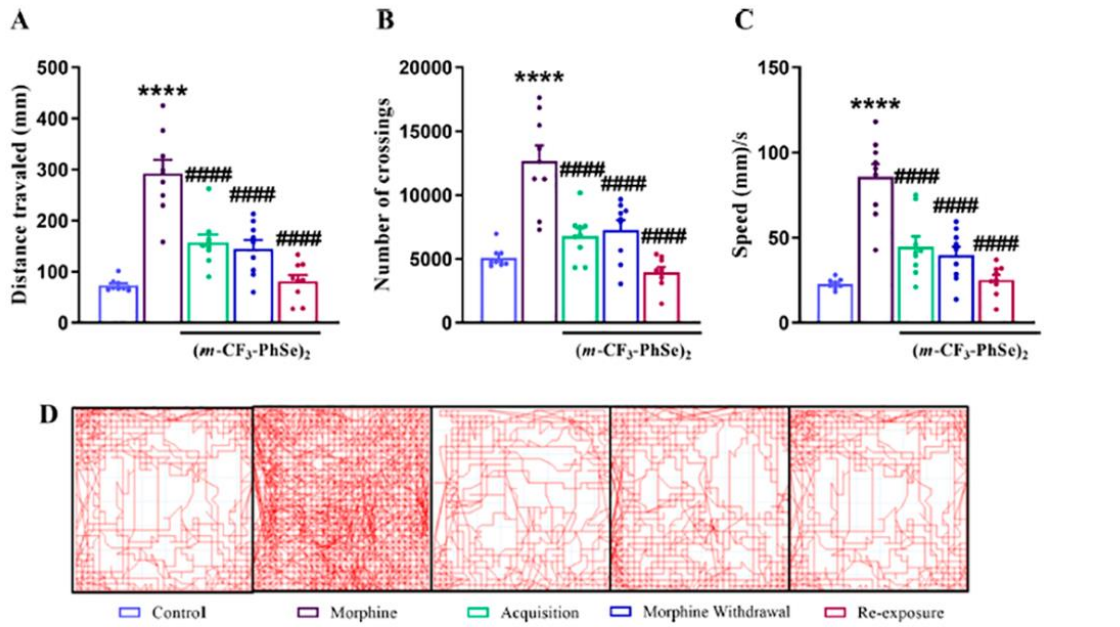

Fig. 4. Effects of (m-CF_3-PhSe)$_2$ (10 mg/kg) on the parameters of mouse locomotor activity, distance traveled (mm) (A), number of crossings (B), and speed (mm/s) (C). Data are expressed as mean ± S.E.M of 9 animals per group. Asterisk denotes significance levels when compared to the control group: (****) $P < 0.0001$. Hashtag denotes significance levels when compared to the morphine-treated group: (####) $P < 0.0001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

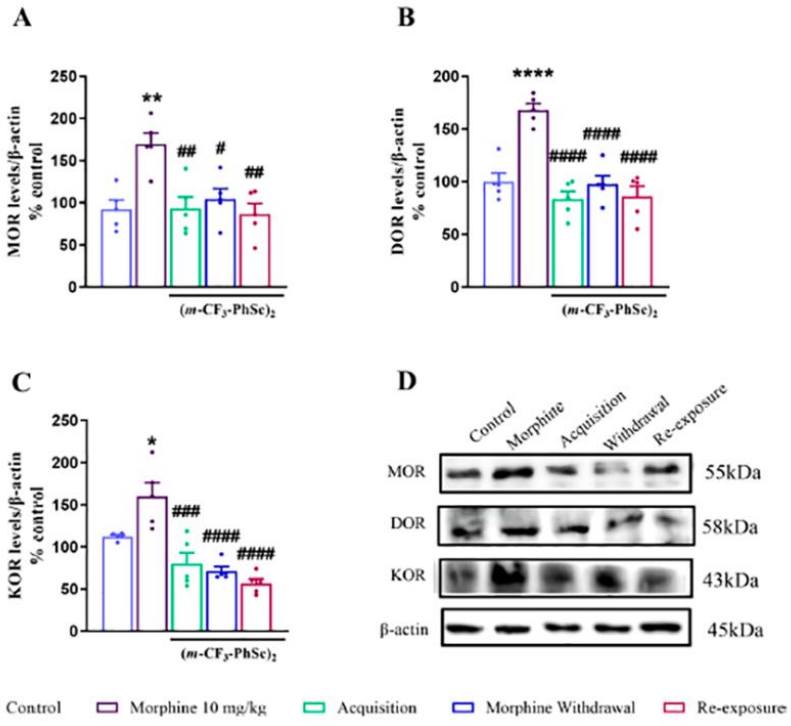

Fig. 5. Effects of (m-CF_3-PhSe)$_2$ (10 mg/kg) on MOR(A), DOR (B), and KOR (C) protein levels and their representative blot bands (D). Data are expressed as mean ± S.E.M of 5 animals per group. Asterisk denotes significance levels when compared to the control group: (*) $P < 0.05$, (**) $P < 0.01$, and (****) $P < 0.0001$. Hashtag denotes significance levels when compared to the morphine-treated group: (#) $P < 0.05$, (##) $P < 0.01$, (###) $P < 0.001$, and (####) $P < 0.0001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

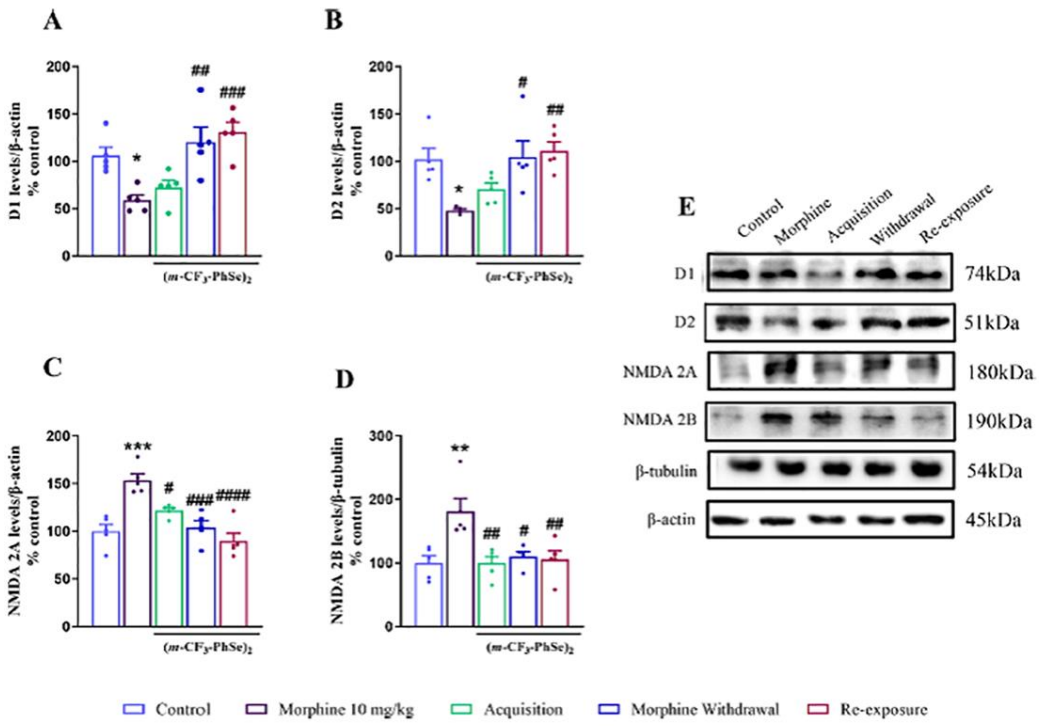

Fig. 6. Effects of $(m\text{-}CF_3\text{-}PhSe)_2$ (10 mg/kg) on cerebral cortical D1 (A), D2 (B), NMDA 2A (C), and NMDA 2B (D) receptor contents. Representative blot bands (E). Data are expressed as mean ± S.E.M of 5 animals per group. Asterisk denotes significance levels when compared to the control group: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$. Hashtag denotes significance levels when compared to the morphine-treated group: (#) $P < 0.05$, (##) $P < 0.01$, (###) $P < 0.001$, and (####) $P < 0.0001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

the cerebral cortex of mice exposed to morphine compared to the control group (Fig. 7A-C). $(m\text{-}CF_3\text{-}PhSe)_2$ treatment in the acquisition, morphine withdrawal, and re-exposure phases reversed the increase in the levels of RS, TBARS, and 4-HNE protein ($P < 0.05$) in the cerebral cortex of mice exposed to morphine.

The results illustrated in Fig. 8 show the CAT activity (A), GPx activity (B), and SOD activity (C) in the cerebral cortex of mice. The statistical analyses indicated a decrease in the SOD activity [$F_{(4,25)} = 4.93$, $P = 0.020$] but an increase in the CAT [$F_{(4,25)} = 8.05$, $P = 0.000$] and GPx activities [$F_{(4,25)} = 4.71$, $P = 0.010$] in the cerebral cortex of mice exposed to morphine.

$(m\text{-}CF_3\text{-}PhSe)_2$ treatment in the three phases of locomotor sensitization was effective against decreasing SOD activity in the cerebral cortex of mice exposed to morphine (Fig. 7C, $P < 0.05$).

$(m\text{-}CF_3\text{-}PhSe)_2$ administered before the re-exposure to morphine effectively modulated the GPx activity (Fig. 8B, $P < 0.05$). In the acquisition and morphine withdrawal phases, $(m\text{-}CF_3\text{-}PhSe)_2$ treatment ($P < 0.05$) reversed the CAT activity increased in the cerebral cortex of mice exposed to morphine (Fig. 8A).

The one-way ANOVA analyses of Nrf2 [$F_{(4,20)} = 6.40$, $P = 0.007$] and Keap-1 levels [$F_{(4,20)} = 28.36$, $P = 0.04$] revealed a decrease in these protein levels of morphine exposed mice when compared to those in the control group (Fig. 9A-B). $(m\text{-}CF_3\text{-}PhSe)_2$ administration in the three phases of locomotor sensitization was effective against the decrease in Keap-1 levels ($P < 0.05$). Regarding Nrf2 levels, $(m\text{-}CF_3\text{-}PhSe)_2$ administered in the morphine withdrawal and before the re-exposure to morphine led to an increase in the cerebral cortical protein levels of Nrf2 when compared to the morphine-treated mice ($P < 0.05$).

Fig. 9C shows that morphine administration increased the protein levels of HO-1 [$F_{(4,20)} = 14.57$, $P = 0.009$] in the cerebral cortex of mice and $(m\text{-}CF_3\text{-}PhSe)_2$ treatment, in the three phases, was effective against this increase ($P < 0.01$).

4. Discussion

The main findings of this study indicate the effectiveness of $(m\text{-}CF_3\text{-}PhSe)_2$ administered at acquisition, withdrawal, and re-exposure phases against behavioral locomotor sensitization induced by morphine. This compound administered at all phases modulated the protein levels of opioidergic (MOR, DOR, and KOR) and glutamatergic (NMDA 2A and 2B) receptors and redox imbalance in the cerebral cortex of morphine-exposed mice. Further, $(m\text{-}CF_3\text{-}PhSe)_2$ administered at withdrawal and re-exposure phases also modulated the cortical protein levels of dopaminergic (D1 and D2) receptors in morphine-exposed mice.

Morphine administration can induce neurochemical effects, including alterations in the protein contents and neurotransmission adaptations in the brain, which would result in behavioral response expressed by increased locomotor activity of animals [5,30]. There is some evidence suggesting that locomotor sensitization of rodents mimics the central nervous system changes that occur in the human dependence [31,32]; therefore, this animal model has been used to study mechanisms related to addiction.

The memorable somatic symptom of withdrawal period is the difficulty in weight gain induced by morphine, apparent from one day after discontinuation of repeated opioid administration to rats [33] and mice [34]. In agreement with the previously published data, the findings of this study demonstrate that mice exposed to morphine showed reduced weight gain.

The present results show that $(m\text{-}CF_3\text{-}PhSe)_2$ administration at all phases of the morphine sensitization protocol was effective against the

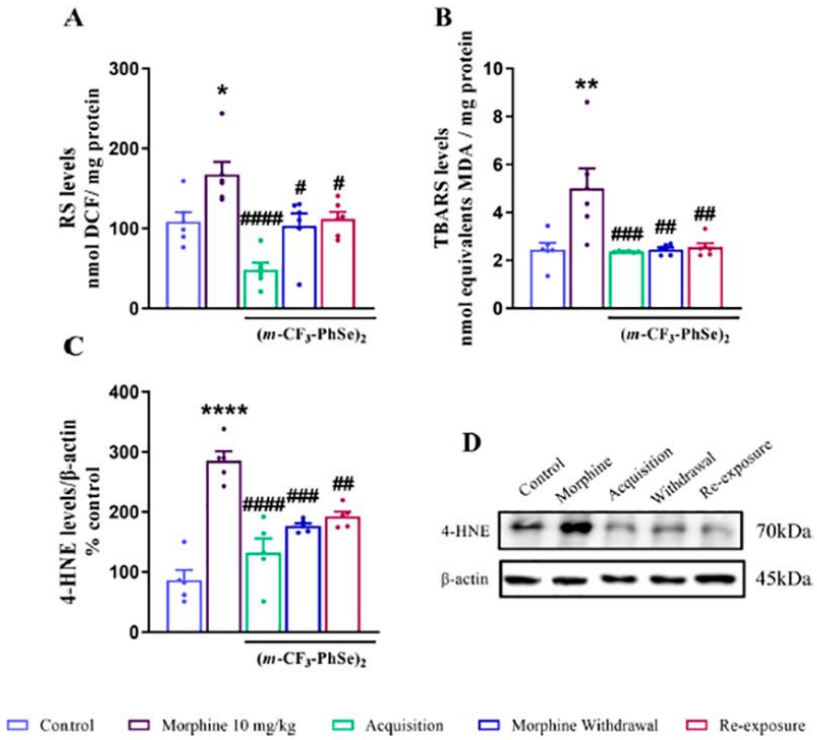

Fig. 7. Effects of $(m\text{-}CF_3\text{-}PhSe)_2$ (10 mg.kg^{-1}) on the levels of RS (A), TBARS (B), 4-HNE (C), and 4-HNE representative blot bands (D). Data are expressed as mean ± S.E.M of 6 animals per group for RS, and TBARS levels, and 5 per group for the western Blot assay in 4-HNE levels. Asterisk denotes significance levels when compared to the control group: (*) $P < 0.05$, (**) $P < 0.01$, and (****) $P < 0.0001$. Hashtag denotes significance levels when compared to the morphine-treated group: (#) $P < 0.05$, (##) $P < 0.01$, (###) $P < 0.001$, and (####) $P < 0.0001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

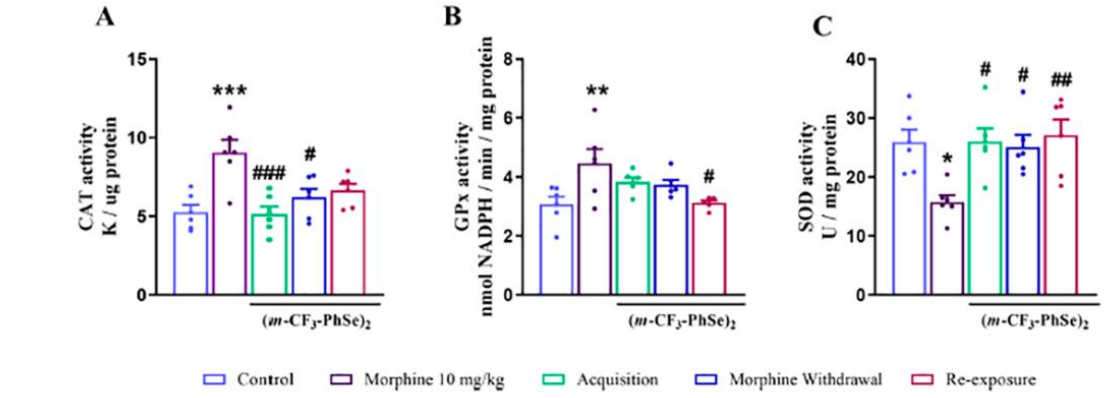

Fig. 8. Effects of $(m\text{-}CF_3\text{-}PhSe)_2$ (10 mg/kg) on the CAT (A), GPx (B), and SOD activities (C). Data are expressed as mean ± S.E.M of 6 animals per group. Asterisk denotes significance levels when compared to the control group: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$. Hashtag denotes significance levels when compared to the morphine-treated group: (#) $P < 0.05$, (##) $P < 0.01$, and (###) $P < 0.001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

increase in cerebral cortical MOR, DOR, and KOR protein contents and mouse hyperlocomotion. Some recent findings support the idea that the modulation of opioid receptors contributes to the $(m\text{-}CF_3\text{-}PhSe)_2$ antidepressant-like effect on lifestyle-induced depression mouse model [35].

Most of the literature converges to the view that locomotor sensitization is a behavioral adaptation induced by addictive drugs, in which different neurotransmitter systems play a role [36–38]. Therefore, MOR modulates morphine-induced locomotion [39] and MOR-knockout mice exhibit reduced locomotor activity compared to wild type ones [40].

It has also been reported that a DOR antagonist prevents morphine-induced sensitization [41] and the involvement of this opioid receptor in drug sensitization induced by the abuse drug, cocaine [42].

It is recognized that abused drugs activate the κ-opioid system [43]; the KOR activation increases locomotor sensitization and has an important role in driving compulsive drug intake [43,44]. Morphine, via the MOR activation, increases dopamine release in the VTA [45,46], which affects the response to abused drugs [47].

The results reveal that $(m\text{-}CF_3\text{-}PhSe)_2$ administered at the withdrawal and the re-exposure phases of the morphine protocol was

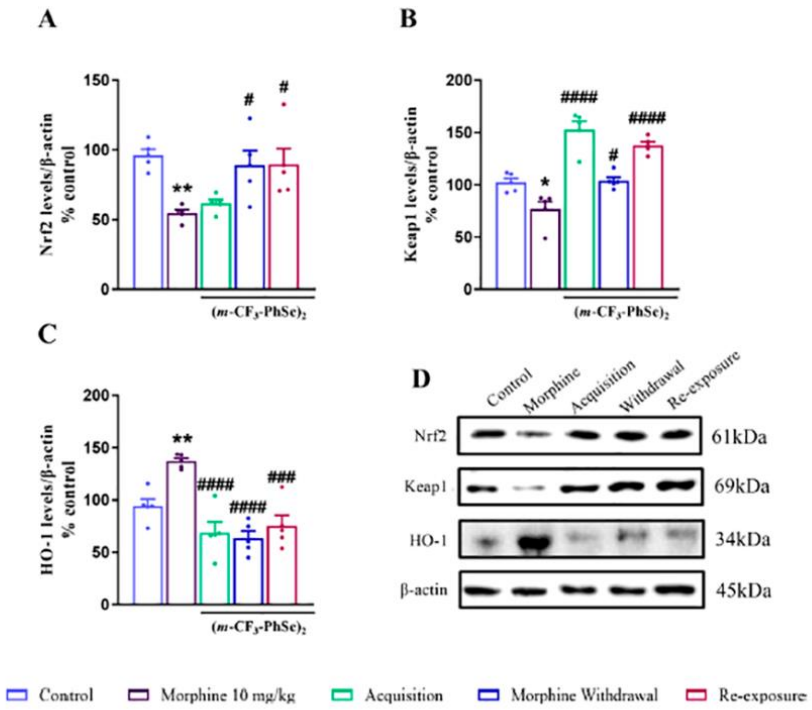

Fig. 9. Effects of $(m\text{-}CF_3\text{-}PhSe)_2$(10 mg/kg) on cerebral cortical Nrf2 (A), Keap-1 (B), and HO-1 (C) protein contents. Representative blot bands (D). Data are expressed as mean ± S.E.M of 5 animals per group. Asterisk denotes significance levels when compared to the control group: (*) $P < 0.05$ and (**) $P < 0.01$. Hashtag denotes significance levels when compared to the morphine-treated group: (#) $P < 0.05$, (###)$P < 0.001$, and (####)$P < 0.0001$. The results were analyzed by one-way ANOVA followed by Bonferroni's test.

effective to restore the levels of D1 and D2 receptors in the cerebral cortex of mice. It has been proposed that during morphine dependence, dopamine and morphine exert opposite effects, and the withdrawal phase has been associated with a down-regulation of postsynaptic dopamine D1 and D2 receptors [48].

On the other hand, dopamine and glutamate interaction has been related to the development of behavioral plasticity in response to addictive drugs [49,50]. Locomotor sensitization has been characterized by neuroadaptive changes that involve dopaminergic and glutamatergic interconnections between the limbic system and cerebral cortex [51].

The glutamatergic system has been reported as a key element in drug addiction [52,53]; glutamate receptors are involved in many drug-induced activities, such as conditioned place preference [54], opioid dependence [55], and locomotor hyperactivity conditioned by morphine [56]. Previous reports focusing on the role of NMDA receptor antagonists demonstrated that they inhibit morphine-induced locomotor sensitization [9]. In agreement with these findings, the present study reveals that morphine-induced locomotor sensitization was accompanied by an increase in the NMDA 2A and 2B subunit levels.

Regarding the $(m\text{-}CF_3\text{-}PhSe)_2$ effects, this compound has been reported to induce neuroprotective adaptations, modulating glutamatergic receptors, in a morphine withdrawal model [57]. Our data demonstrate the effectiveness of $(m\text{-}CF_3\text{-}PhSe)_2$ in modulating cerebral cortical NMDA 2A and 2B levels increased in morphine-induced locomotor sensitization protocol.

A growing body of evidence has indicated that oxidative stress is involved in the development of drug addiction, including morphine [58,59]; therefore, a well-known antioxidant property of $(m\text{-}CF_3\text{-}PhSe)_2$ [60–62] was reproduced in the morphine-induced locomotor sensitization protocol.

Different from the effects on withdrawal and re-exposure phases, $(m\text{-}CF_3\text{-}PhSe)_2$ was not effective in modulating the content of D1 and D2 receptors in the acquisition phase of locomotor sensitization protocol. One plausible explanation for this result is that the D1 and D2 modulation occurs after a period of morphine abstinence. In other words, the modulation of dopaminergic receptors is associated with withdrawal symptoms and occurs with the challenge dose of morphine following the hyperdopaminergic condition leading to the saturation of the dopamine receptors [48,63]. However, even though the compound did not restore the levels of D1 and D2, it was effective against the locomotor behavior induced by morphine [64]. Moreover, $(m\text{-}CF_3\text{-}PhSe)_2$ has been reported as an effective agent to alleviate symptoms of the morphine-induced withdrawal syndrome in mice [57].

5. Conclusion

$(m\text{-}CF_3\text{-}PhSe)_2$ attenuated all phases of morphine-induced behavioral locomotor sensitization in mice. This organoselenium compound, administered at all phases, modulated the protein levels of opioidergic (MOR, DOR, and KOR) and glutamatergic (NMDA 2A and 2B) receptors and redox imbalance in the cerebral cortex of morphine-exposed mice. $(m\text{-}CF_3\text{-}PhSe)_2$ did not modulate cortical protein levels of dopaminergic (D1 and D2) receptors in the acquisition phase of morphine-induced locomotor sensitization protocol.

CRediT authorship contribution statement

Renata F. Rodrigues and Bruna C.W. Fulco carried out in the experimental protocol. Cristina W. Nogueira conceived Funding acquisition, Investigation, Resources and Supervision of all steps of this study. Renata F. Rodrigues and Cristina W. Nogueira analyzed data and wrote the Manuscript- original draft. The authors declare that all data were generated in-house and that no paper mill was used. All authors read and approved the manuscript in its present form

Author contribution

RFR and BCWF carried out in the experimental protocol. CWN conceived Funding acquisition, Investigation, Resources and Supervision of all steps of this study. RFR and CWN analyzed data and wrote the Manuscript- original draft. The authors declare that all data were generated in-house and that no paper mill was used. All authors read and approved the manuscript in its present form

Declaration of Competing Interest

The manuscript and the data reported here have not been published previosly and they are not under consideration for publication elsewhere. All listed authors have contributed significantly to the manuscript and consent to their names on the manuscript. There is no conflict of interest in the conduct. All other Authors have read the manuscript and have agreed to submit it in its current from for consideration for publication in the Journal.

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4 CONCLUSÃO

O tratamento com (*m*-CF_3-PhSe)$_2$ atenuou as alterações comportamentais e moleculares causadas pela morfina em todas as fases do desenvolvimento da sensibilização locomotora comportamental em camundongos. Os resultados demonstraram que os receptores opioides, D1, D2, NMDA 2A e 2B, estresse oxidativo e a via de sinalização Nrf2/Keap/HO-1 estão associados ao desenvolvimento de sensibilização locomotora comportamental induzida por morfina em camundongos.

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ANEXO A – CARTA DE APROVAÇÃO DO PROJETO DE PESQUISA PELA COMISSÃO DE ÉTICANO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE SANTA MARIA

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

CERTIFICADO

Certificamos que a proposta intitulada "Investigação da sensibilidade motora cruzada entre o disseleneto m-trifluormetildifenila (m-CF3-PhSe)2 e a morfina, e da ação do composto na recaída a morfina induzida por estresse em camundongos.", protocolada sob o CEUA nº 5302070619 (ID 002645), sob a responsabilidade de **Cristina Wayne Nogueira** *e equipe; Cristina Wayne Nogueira; Suzan Gonçalves Rosa; Carolina Cristóvão Martins; Franciele Martini* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 20/08/2019.

We certify that the proposal "Investigation of cross-motor sensitivity between m-trifluoromethyl-diphenyl diselenide (m-CF3-PhSe)2 and morphine, and the action of the compound on relapse to stress-induced morphine in mice.", utilizing 128 Heterogenics mice (128 males), protocol number CEUA 5302070619 (ID 002645), under the responsibility of **Cristina Wayne Nogueira** *and team; Cristina Wayne Nogueira; Suzan Gonçalves Rosa; Carolina Cristóvão Martins; Franciele Martini* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 08/20/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2019 a 08/2020 Área: Bioquímica E Biologia Molecular

Origem:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	25 a 25 dias	N:	128
Linhagem:	Swiss			Peso:	20 a 25 g		

Local do experimento: Sala 2424- Prédio 18 e Sala 3209- Prédio 19. Durante todo o curto período em que os animais estarão em nossa sala de experimentação laboratorial, a limpeza e a troca das palhas de cada uma das caixas serão efetuadas por um funcionário, que está devidamente treinado para realizar estes procedimentos, causando o mínimo possível de desconforto para os animais experimentais. O transporte dos animais ao local adequado para as atividades experimentais seguirá os seguintes cuidados: I- O transporte será realizado, preferencialmente, antes das 10h da manhã. Evitando o transporte nos horários de pico de temperatura e tráfego intenso; II- Os animais serão levados diretamente até seu destino final, a fim de minimizar o estresse a que serão submetidos neste momento; III - Os animais serão acondicionados em caixas apropriadas para o transporte, as quais permitam que se movimentem confortavelmente e proporcionem travamento adequado para impedir fugas, garantindo a segurança destes, do usuário e do meio ambiente; IV- As caixas de transporte serão previamente higienizadas e preparadas com cama apropriada, bem como estarão devidamente identificadas. V- Por questões de segurança a caixa de transporte estará sempre coberta com material que permita ventilação e impeça que os animais possam ser observados durante o percurso; VI- Antes do transporte os bebedouros serão retirados para evitar vazamentos.

Santa Maria, 10 de janeiro de 2022

Dra. Patrícia Bräunig
Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Profa. Dra. Vania Lucia Loro
Vice-Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

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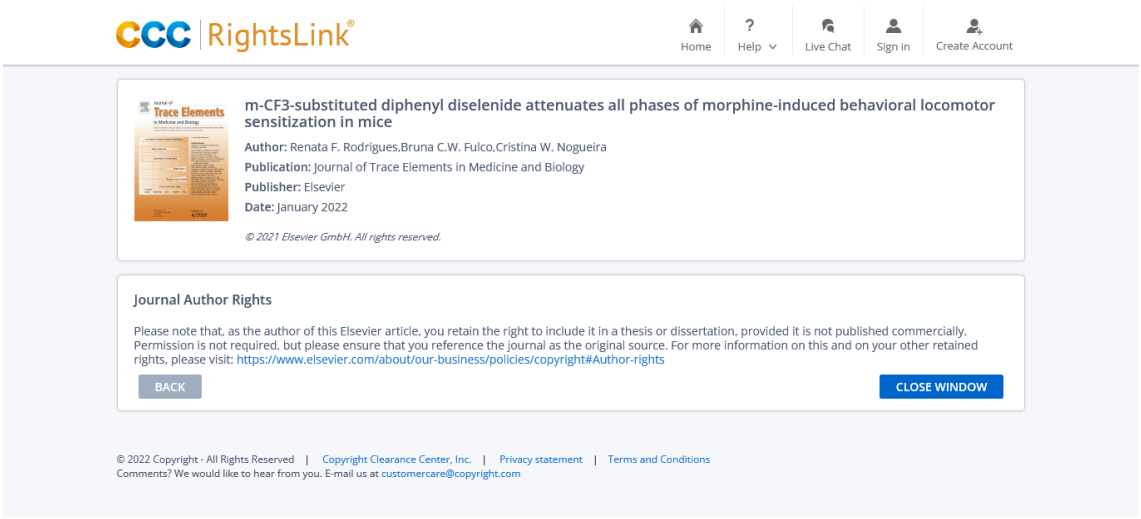
Horário de atendimento: 8h30 - 11h30 e 13h30 - 16h30 : e-mail: ceua.ufsm@gmail.com
CEUA N 5302070619

ANEXO B – AUTORIZAÇÃO DA REVISTA



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m-CF3-substituted diphenyl diselenide attenuates all phases of morphine-induced behavioral locomotor sensitization in mice

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