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DISSELENETO DE *m*-TRIFLUORMETIL-DIFENILA MODULA AS ADAPTAÇÕES NEUROTÓXICAS E COMPORTAMENTAIS INDUZIDAS PELA RETIRADA DA MORFINA EM CAMUNDONGOS

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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**

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Santa Maria, RS
2019

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RESUMO

DISSELENETO DE *m*-TRIFLUORMETIL-DIFENILA MODULA AS ADAPTAÇÕES NEUROTÓXICAS E COMPORTAMENTAIS INDUZIDAS PELA RETIRADA DA MORFINA EM CAMUNDONGOS

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O uso contínuo da morfina é controverso em virtude do desenvolvimento da síndrome de abstinência, fenômeno envolvendo inúmeras adaptações neuronais, as quais resultam na manifestação dos sinais físicos e de um estado afetivo aversivo. Por outro lado, apesar do efeito do tipo antidepressivo exercido pelo disseleneto de *m*-trifluormetil-difenila (*m*-CF_3-PhSe)$_2$ estar associado à modulação dos receptores opioides em modelos animais, a administração repetida deste composto não induziu os efeitos adversos clássicos dos agonistas dos receptores μ, caracterizados pelo desenvolvimento da tolerância e dos sinais físicos de abstinência. Além disso, foi demonstrado que o (*m*-CF_3-PhSe)$_2$ preveniu as neuroadaptações e os sinais de recondicionamento em ratos expostos à anfetamina. Nesse contexto, a presente dissertação investigou os efeitos do (*m*-CF_3-PhSe)$_2$ sobre os sinais físicos e sobre o fenótipo do tipo depressivo durante a retirada da morfina, assim como os processos neuroadaptativos envolvidos no hipocampo de camundongos. Para a realização deste modelo experimental, foram utilizados camundongos Swiss machos, adultos (CEUA nº. 8756060317). Nos primeiros seis dias, os animais receberam doses crescentes de morfina (20 – 100 mg/kg), duas vezes ao dia, pela via subcutânea. A partir do sétimo dia, as injeções de morfina foram descontinuadas para a indução da síndrome de abstinência espontânea, sendo que, ao mesmo tempo, foi administrado o (*m*-CF_3-PhSe)$_2$ nos camundongos, em diferentes doses (5 e 10 mg/kg), uma vez ao dia, pela via intragástrica, durante os próximos três dias. No nono dia do protocolo experimental, transcorridos 30 minutos da última administração do composto, os animais foram submetidos aos testes comportamentais para avaliar a manifestação dos sinais físicos de abstinência e o fenótipo do tipo depressivo através dos testes da suspensão da cauda e do nado forçado, respectivamente. Posteriormente, o hipocampo foi removido para a realização dos ensaios *ex vivo*, incluindo os marcadores de estresse oxidativo, os níveis de proteínas relacionadas às defesas antioxidantes, às subunidades (2A e 2B) do receptor NMDA e às vias de sinalização do proBDNF e do *m*BDNF. Os resultados obtidos demonstraram que as adaptações hipocampais mediadas pelo desequilíbrio redox, pela redução nos níveis de receptores NMDA e pela estimulação da via pró-apoptótica do proBDNF/$p75^{NTR}$/JNK, sem afetar a via neurotrófica do *m*BDNF/TrkB/ERK/CREB podem estar relacionadas com o desenvolvimento dos sinais físicos e do fenótipo do tipo depressivo em camundongos abstinentes à morfina. Em contrapartida, o tratamento com (*m*-CF_3-PhSe)$_2$ em ambas as doses reverteu as adaptações comportamentais induzidas durante a abstinência à morfina em camundongos, porém a maior dose *per se* intensificou a manifestação de um dos parâmetros relacionados aos sinais físicos de abstinência. Além disso, o (*m*-CF_3-PhSe)$_2$ reestabeleceu o equilíbrio na sinalização redox e na plasticidade sináptica, ao inibir a sinalização do proBDNF, sem alterar a do *m*BDNF, assim como restaurou os níveis dos receptores NMDA no hipocampo de camundongos abstinentes à morfina. Em conclusão, o presente trabalho evidenciou que os efeitos neuroprotetores do (*m*-CF_3-PhSe)$_2$, mediados prioritariamente pela sua intrínseca propriedade antioxidante, modularam os eventos neurotóxicos hipocampais e assim, atenuaram a manifestação dos sinais físicos e do fenótipo do tipo depressivo em camundongos abstinentes à morfina.

Palavras-chave: Síndrome de abstinência à morfina. Fenótipo do tipo depressivo. Estresse oxidativo. Plasticidade sináptica. Selênio.

ABSTRACT

m-TRIFLUOROMETHYL-DIPHENYL DISELENIDE MODULATES THE NEUROTOXIC AND BEHAVIORAL ADAPTATIONS INDUCED BY MORPHINE WITHDRAWAL IN MICE

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Repeated use of morphine is controversial because it leads to the development of withdrawal syndrome, phenomenon involving a number of neuronal adaptations, which results on manifestations of physical signs and a state affective aversive. On the other hand, although the antidepressant-like effect exerted by m-trifluoromethyl-diphenyl diselenide (m-$CF_3PhSe)_2$ has been related to modulation of opioid receptors in animal models, repeated administration of this compound did not induce any characteristic undesirable effects of μ receptor agonists, characterized by tolerance and physical withdrawal signs. Furthermore, it has been shown that (m-$CF_3PhSe)_2$ prevented the neuroadaptations and the re-conditioning signs in rats exposed to amphetamine. In this context, the present study investigated the effect of (m-$CF_3PhSe)_2$ on the physical signs and on the depressive-like phenotype during morphine withdrawal, as well as the neuroadaptive processes involved in hippocampus of mice. The study was carried out using adult male Swiss mice (CEUA nº. 8756060317). In the first six days, the animals received escalating doses of morphine (20 – 100 mg/kg), twice a day, by the subcutaneous route. From the seventh day, the animals were treated with (m-$CF_3PhSe)_2$ at different doses (5 and 10 mg/kg), once a day intragastrically, over the next three days whereas morphine injections were discontinued to induce the spontaneous withdrawal syndrome. On the ninth day of the experimental protocol, 30 min after the last administration of (m-$CF_3PhSe)_2$, the animals performed the behavioral tests to assess the physical signs of withdrawal and the depressive-like phenotype on the tail suspension test and the forced swim test, respectively. After, the samples of hippocampus were collected for *ex vivo* analyses, including oxidative stress markers, protein levels related to antioxidant defenses, NMDA receptor subunits (2A and 2B) and the signaling pathways of the proBDNF and the *m*BDNF. The results demonstrated that hippocampal neuroadaptations mediated by the redox imbalance, the decrease on NMDA receptors levels and the stimulation of proBDNF/$p75^{NTR}$/JNK pro-apoptotic pathway without affecting *m*BDNF/TrkB/ERK/CREB neurotrophic signaling, may contribute to the development of physical signs and the depressive-like phenotype in morphine withdrawn-mice. In contrast, (m-$CF_3PhSe)_2$ treatment in both doses reversed the behavioral adaptations induced during morphine withdrawal in mice; however, the highest dose of this compound intensified one of the parameters related to physical withdrawal signs. Besides (m-$CF_3PhSe)_2$ reestablished the balance in redox signaling and in synaptic plasticity by inhibiting proBDNF signaling without altering that of *m*BDNF as well as restored the levels of NMDA receptor in hippocampus of morphine withdrawn-mice. In conclusion, the present study demonstrated that the neuroprotective effects of (m-$CF_3PhSe)_2$, mediated primarily by its antioxidant property, modulated the hippocampal neurotoxic events and, thus, attenuated the manifestation of physical signs and depressive-like phenotype in morphine withdrawn-mice.

Keywords: Morphine withdrawal syndrome. Depressive-like phenotype. Oxidative stress. Synaptic plasticity. Selenium.

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MANUSCRITO

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(*m*-CF_3-PhSe)$_2$	Disseleneto de *m*-trifluormetil difenila
AC	Adenilato ciclase
ALT	Alanina aminotransferase
AMPc	3’5’-Adenosina-monofosfatocíclico
AST	Aspartato animotransferase
ATP	Adenosina trifosfato
BDNF	Fator neurotrófico encefálico
CK	Creatina quinase
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
GSH	Glutationa reduzida
NMDA	N-metil-D-aspartato
Nrf2	Fator eritróide nuclear 2 relacionado ao fator 2
$p75^{NTR}$	Receptor neurotrófico p75
SNC	Sistema nervoso central
TrkB	Receptor de tirosina cinase B

SUMÁRIO

1 INTRODUÇÃO

1.1 FÁRMACOS OPIOIDES

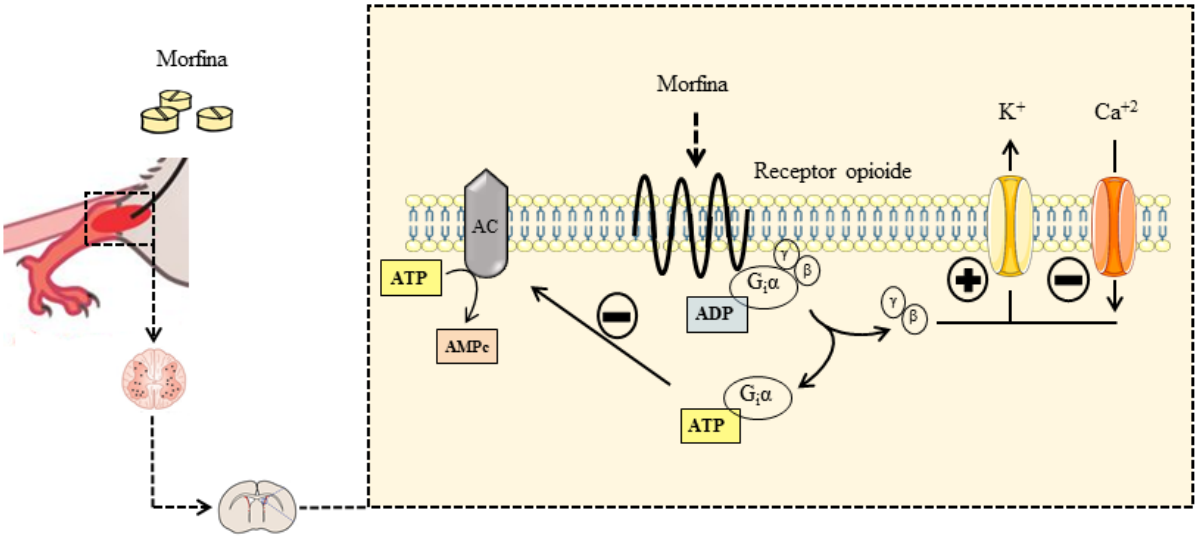
O ópio, principal substância extraída da planta *Papaver somniferum*, acompanha o desenvolvimento da civilização diante de uma perspectiva histórica, uma vez que este era amplamente utilizado pelos gregos para fins medicinais, assim como foi o protagonista de conflitos entre a Grã-Bretanha e a China em meados do século VII a.C. e XIX d.C., respectivamente. Inclusive nos dias atuais, os fármacos opioides ainda são prescritos como a principal intervenção farmacológica para o alívio da dor (CHERNY, 1996).

Devido as suas similaridades no que tange à estrutura química, os agonistas opioides exógenos mimetizam a ação antinociceptiva fisiológica dos peptídeos endógenos opioides ao interagirem com os distintos receptores opioides μ, κ e δ, proteínas transmembrana acopladas à proteína G inibitória, os quais promovem a dissociação entre as subunidades Gα e Gβγ quando ativados e consequentemente, modulam diversas vias efetoras intracelulares (CHILDERS e SNYDER, 1978; RAFFA, 2014). Dentro desse contexto, uma via de transdução de sinal clássica e possivelmente a mais estudada pelos cientistas evidencia que a estimulação dos receptores opioides modula os canais iônicos de potássio (K^+) e de cálcio (Ca^{+2}) em diversas estruturas relacionadas ao sistema nervoso central (SNC), inclusive no hipocampo, no *locus coeruleus* e na área tegmental ventral (AL-HASANI e BRUCHAS, 2011; PERGOLIZZI et al., 2017).

Conforme demonstrado na Figura 1, a ativação e abertura dos canais de condutância retificadores de K^+ prolongam o limiar de transmissão da dor através da hiperpolarização celular e da inibição da atividade neuronal tônica (THOMPSON et al., 2015). Por outro lado, o influxo deficitário do Ca^{+2} para dentro da célula devido a inibição das correntes voltagem dependentes deste íon altera o próprio funcionamento das vesículas, a liberação de neurotransmissores excitatórios, assim como inibe a atividade da enzima adenilato ciclase (AC) responsável pela conversão da molécula de adenosina trifosfato (ATP) em 3’5’-adenosina-monofosfato-cíclico (AMPc) (ZHANG et al., 2005). Diante de tantas evidências, os pesquisadores sugerem que os canais iônicos de K^+ e Ca^{+2} são substratos altamente conservados e modulados pelos receptores opioides (AL-HASANI e BRUCHAS, 2011).

Além disso, todos os receptores opioides encontram-se abundantemente dispostos nas principais regiões relacionadas à condução de um estímulo nocivo, incluindo em áreas periféricas, nos gânglios da raiz dorsal, na medula espinhal e no encéfalo. Assim, os agonistas opioides endógenos e exógenos podem inibir a sinalização de um estímulo nocivo através de uma modulação a nível supra espinhal, assim como através das vias ascendentes e descendentes. Por tais motivos, os fármacos opioides perduram por séculos como os mais potentes e eficazes para o manejo da dor sob diversas condições (AL-HASANI e BRUCHAS, 2011; PERGOLIZZI et al., 2017).

Figura 1 – Vias de sinalização envolvidas na ação antinociceptiva de fármacos opioides.



Esquema simplificado do mecanismo de ação dos fármacos opioides sobre os seus respectivos receptores localizados na superfície celular. Estes receptores, através da cascata da proteína Gi, modulam os canais iônicos de K^+ e de Ca^{+2}, assim como inibem a atividade da AC.

1.2 A CRISE EPIDÊMICA GLOBAL DE OPIOIDES

Em 1995, a instituição “American Pain Society” declarou que o grau de percepção da dor representaria o quinto sinal vital avaliado durante a anamnese em consultas médicas, juntamente com a aferição da pressão arterial, da temperatura, da frequência cardíaca e respiratória (MORONE e WEINER, 2013). Diante desta nova medida implementada, a crescente corrente ideológica pela busca de tratamentos mais efetivos para o controle da dor,

aliado à flexibilização de políticas governamentais em relação à utilização de narcóticos culminou no decreto da epidemia dos fármacos opioides (MANCHIKANTI et al., 2012).

Ao longo dos últimos anos, houve um aumento acentuado no uso abusivo de heroína e de fentanil ilicitamente fabricados, assim como na prescrição indiscriminada de medicamentos opioides ao redor do mundo, particularmente nos Estados Unidos (OSTLING et al., 2018). Apesar das medidas públicas adotadas pelo governo norte americano refletirem em uma redução substancial na prescrição de opioides, a intoxicação não intencional por opiáceos persiste como a principal causa de mortes acidentais em adultos entre 25 e 64 anos (DUNN et al., 2018). Nesse contexto, as estatísticas apontam que tanto o consumo geral de opioides, quanto o número de fornecimento de prescrição elevaram-se à níveis alarmantes ao decorrer dos anos 2000. Tais dados embasaram as evidências de que no ano de 2015 cerca de 63% da incidência de mortes envolvendo overdose de drogas de abuso foram provocadas por opioides, sendo que aproximadamente metade destas mortes estavam diretamente relacionadas com a prescrição abusiva destes medicamentos (GUY et al., 2017; JONES, M. R. et al., 2018).

Embora essa epidemia tenha se expandido progressivamente pela maioria dos países desenvolvidos, o acesso às prescrições de fármacos opioides ainda é extensivamente restrito e limitado nas nações de baixa ou média renda (SEYA et al., 2011). No que se refere ao Brasil, o consumo médio de opioides é relativamente menor quando comparado aos países de alta renda. Entretanto, este panorama vem sendo gradualmente alterado neste país e nas demais regiões da América Latina nos últimos anos de acordo com a necessidade de tratamentos mais eficazes e agressivos para o tratamento da dor crônica em pacientes não oncológicos. Diante disso, um estudo recente realizado no Brasil revelou que o número de prescrições de opioides subiu de 8,28 para 44,25 receitas distribuídas para 1000 pessoas, ou seja, houve um aumento de 465% na venda de fármacos opioides registrados nas farmácias durante um período de seis anos, sendo que a oxicodona e a codeína são os líderes em venda (KRAWCZYK et al., 2018).

Paradoxalmente, nenhum estudo realizado até o momento comprovou a eficácia do tratamento a longo prazo com opioides na redução da intensidade da dor crônica. No entanto, já é bem descrito na literatura que a exposição crônica a tais fármacos, como a morfina e seus derivados, induz inúmeras alterações adaptativas e alostáticas no SNC desencadeando um estado dependente da droga no indivíduo (EVANS e CAHILL, 2016). Somando-se a isso, estudos epidemiológicos sugerem uma relação direta entre o aumento na prevalência de morbidades e a crise epidêmica de opioides, ou seja, sugere-se que os crescentes casos de

transtornos psiquiátricos, de hiperalgesia e de incapacidade estejam associadas ao uso abusivo de opioides (JONES, M. R. et al., 2018). Paralelamente a isso, as prescrições indiscriminadas de medicamentos opioides sobrecarregaram os sistemas hospitalares tanto de forma direta, através do aumento na admissão de pacientes nessas instituições para o tratamento de desintoxicações e do transtorno do uso de opioides, quanto indireta por meio da propagação de doenças infecciosas dentro da comunidade devido ao uso compartilhado de seringas, incluindo a hepatite C e o vírus HIV (OSTLING et al., 2018; WEJNERT et al., 2016).

Nesse contexto, as overdoses, os crimes e o transtorno devido ao uso de substâncias (TUS) são decorrentes das prescrições abusivas de opioides e geram um ônus econômico equivalente a bilhões de dólares por ano para os Estados Unidos (FLORENCE et al., 2016). Ademais, sugere-se que a síndrome de abstinência seja um dos principais fatores responsáveis para a recidiva pela busca de drogas lícitas e ilícitas (SPANAGEL e WEISS, 1999). Portanto, a crise epidêmica dos opioides ainda é considerada um dos problemas de saúde pública mais agravantes em países desenvolvidos e em desenvolvimento sob uma perspectiva social, econômica e política.

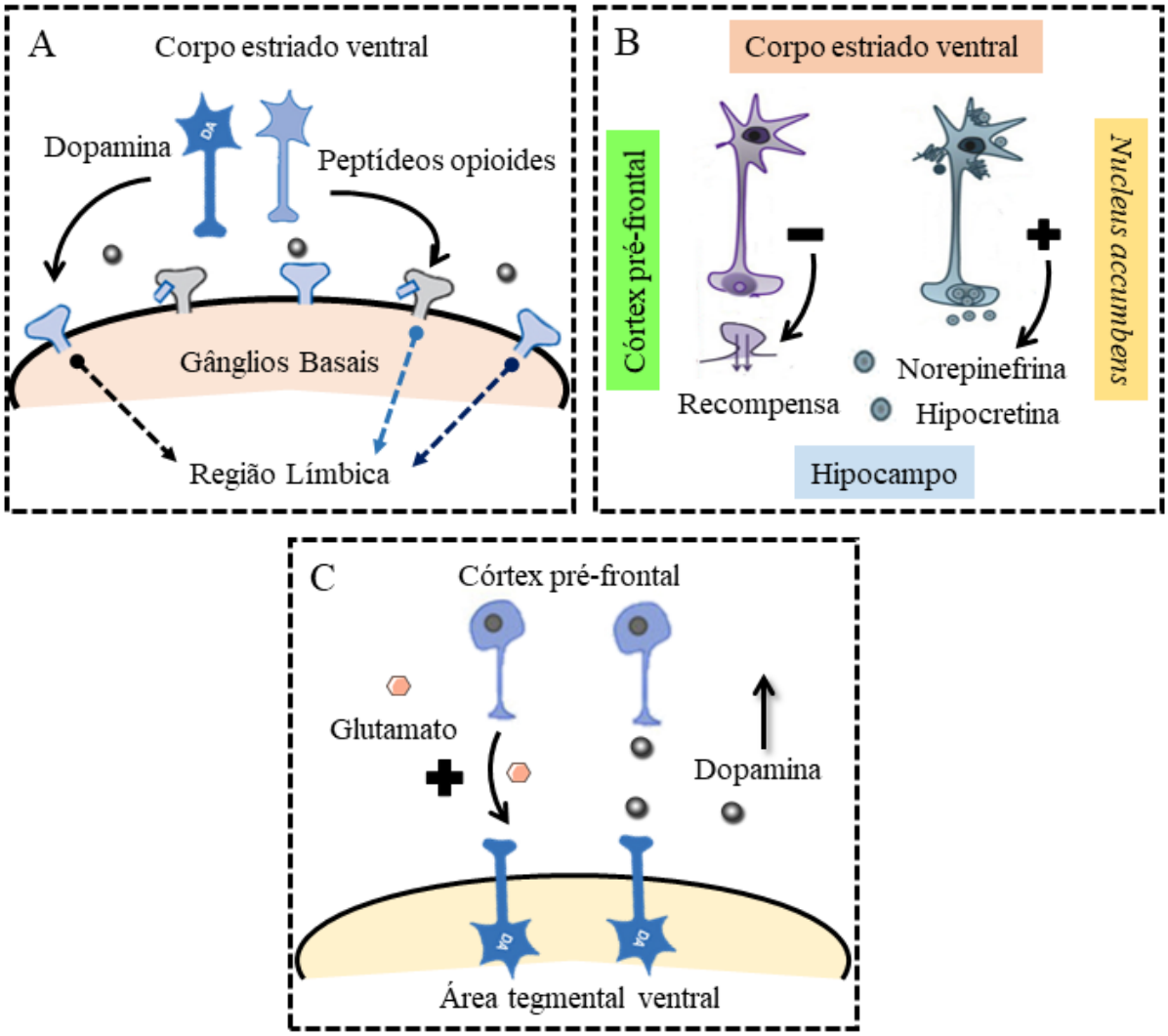
1.3 TRANSTORNO DEVIDO AO USO DE SUBSTÂNCIAS

Atualmente, a junta médica aderiu à terminologia “transtorno devido ao uso de substâncias” não só por ser mais abrangente, mas também por não estar associada a uma conotação pejorativa uma vez que engloba os termos “abuso”, “dependência” e “vício às drogas” em uma única definição. Normalmente, o uso dessas substâncias, como o álcool, os alucinógenos e os opioides, começa de forma ocasional e recreativa, e rapidamente progride para o uso abusivo, necessitando de quantidades cada vez maiores e mais frequentes para obter o efeito almejado. Nesse contexto, o TUS é definido como uma doença crônica e recorrente, geralmente acompanhada por prejuízos neurológicos, tais como déficit na memória, na motivação e na percepção, desinibição comportamental, instabilidade emocional, anedonia e depressão (PAU et al., 2002).

De modo geral, sugere-se que o uso repetido ou a retirada dessas substâncias, incluindo a morfina, induzem persistentes adaptações nos neurocircuitos cerebrais que desencadeiam efeitos psicoativos desejados ou indesejados, respectivamente. Estes estímulos, positivos e negativos, favorecem a formação de um estado de dependência à substância.

(CUNHA-OLIVEIRA et al., 2008; EVANS e CAHILL, 2016; HYMAN et al., 2006). A partir desse pressuposto, o TUS é amplamente caracterizado pelo desenvolvimento de um comportamento compulsivo e incontrolável pela busca e ingestão da substância, assim como o aparecimento de um estado emocional negativo durante períodos de restrição ao acesso da mesma. Assim, conforme ilustrado na Figura 2, o TUS é descrito como um ciclo envolvendo três estágios principais: a intoxicação, a abstinência e a antecipação (KOOB, 2015; KOOB e VOLKOW, 2016).

Figura 2 - As bases neurobiológicas dos três estágios envolvendo o transtorno devido ao uso de substâncias.



Representação esquemática dos principais neurotransmissores e dos demais componentes celulares presentes na região límbica responsáveis pelo estágio de intoxicação (A), pelo estágio de abstinência (B) e pelo estágio de antecipação (C).

1.3.1 Estágio de intoxicação

O estágio de intoxicação corresponde a fase inicial do desenvolvimento de um comportamento compulsivo em resposta às substâncias. Em resumo, essa fase envolve o aumento na liberação do neurotransmissor dopamina e de peptídeos endógenos opioides no corpo estriado ventral durante a exposição repetida as drogas. A ativação dessa área recruta os gânglios da base, que além de projetarem suas conexões para outros neurocircuitos, estão intimamente relacionados com o aprendizado processual direcionado para a formação de um comportamento de rotina ou de um hábito (Figura 2A) (KOOB e VOLKOW, 2016; MITCHELL et al., 2012; VOLKOW et al., 2007).

1.3.2 Estágio de abstinência

O estágio de abstinência é particularmente caracterizado pela manifestação de sinais aversivos físicos e motivacionais. Brevemente, sugere-se que a restrição ao acesso às substâncias modula negativamente o sistema de recompensa, assim como ativa os sistemas cerebrais relacionados ao estresse na região mesocorticolímbica. Nesse contexto, diversos estudos demonstraram o papel dos agentes moduladores do estresse, como o fator liberador de corticotrofina, a norepinefrina e a hipocretina, no desenvolvimento de um comportamento estressante e até mesmo aversivo (CARLEZON et al., 2000; KOOB e LE MOAL, 2001). A combinação destes dois processos neuroadaptativos pode evocar uma motivação poderosa pelo desejo, compulsão e busca pela substância (Figura 2B) (SOLECKI et al., 2019).

Devido à relevância desta fase para o desenvolvimento do TUS e a emergente crise epidêmica dos opioides, os processos neuroadaptativos e comportamentais envolvidos na síndrome de abstinência induzida por fármacos opioides estão sendo extensivamente estudados em modelos animais (GOELDNER et al., 2011; ZANOS et al., 2016). Notavelmente, embora a morfina seja considerada um dos mais potentes analgésicos opioides prescritos na clínica para o manejo da dor sob diversas condições, a intrínseca propriedade de reforço associada aos agonistas do receptor μ induzem tolerância e sinais graves de abstinência, restringindo o uso a longo prazo deste fármaco (HUTCHINSON et al., 2011; KIM et al., 2016; LERESCHE et al., 2015).

Os modelos pré-clínicos envolvendo o uso indiscriminado destas substâncias são excelentes ferramentas para o estudo dos mecanismos da toxicodependência, tanto para prevenir quanto para tratar essa condição e as comorbidades associadas a ela (LYNCH et al., 2010). Nessa perspectiva, modelos experimentais de síndrome de abstinência em roedores foram previamente caracterizados, e mimetizam os sintomas físicos e emocionais aversivos induzidos por opiáceos durante a fase de desintoxicação em humanos (PELES et al., 2007; VEILLEUX et al., 2010). Além disso, as condições que restabelecem a procura por drogas em animais de laboratório são semelhantes àquelas que desencadeiam a recaída em humanos demonstrando, portanto, a validade preditiva dos modelos animais para o estudo do TUS (LYNCH et al., 2010; SANCHIS-SEGURA e SPANAGEL, 2006).

1.3.2.1 Adaptações comportamentais envolvidas no estágio de abstinência

A síndrome de abstinência, caracterizada por diversos sintomas autonômicos e psicológicos, emerge durante períodos de restrição à exposição da morfina ou outras drogas. A manifestação dos sinais autonômicos ocorre nas primeiras horas de abstinência, incluindo diarreias, bocejos, perda de apetite, dores abdominais e náuseas (KIRBY et al., 1990; SPANAGEL e WEISS, 1999). À medida que os sinais físicos desaparecem, os sintomas psicológicos aversivos gradualmente se intensificam ao longo dos próximos dias de abstinência (GOELDNER et al., 2011). Nesse âmbito, estudos epidemiológicos prévios demonstraram uma forte relação entre o TUS e os transtornos de humor, tais como ansiedade, depressão e disforia (GRANT et al., 2004; WEN et al., 2017).

Historicamente, o uso indiscriminado de opioides aumenta as taxas de incidência do transtorno depressivo, sendo essa a comorbidade mais comum no ramo da psiquiatria (GRELLA et al., 2009). Em roedores, os indicadores da síndrome de abstinência física como o tremor da pata, o ranger dos dentes e o “comportamento saltitante” podem ser quantificados até uma semana após a retirada da morfina (WEI et al., 1973), enquanto que o comportamento do tipo depressivo emerge a partir do terceiro dia de abstinência e perdura por até quatro semanas ou mais (ANRAKU et al., 2001; JIA et al., 2013). Apesar de ser considerado um dos fatores determinantes para a recaída (BREWER et al., 1998), o comportamento do tipo depressivo é relativamente negligenciado em modelos pré-clínicos de síndrome de abstinência.

1.3.2.2 Mecanismos neuroadaptativos envolvidos no estágio de abstinência

O hipocampo, como parte do sistema límbico, tem sido identificado como um importante substrato neural nos processos adaptativos subjacentes à síndrome de abstinência induzido por opioides (HYMAN et al., 2006). Responsável pelo aprendizado espacial e pela formação da memória (MORRIS et al., 2003), esta estrutura encefálica possui conexões eferentes com as demais regiões pertencentes ao sistema límbico e, portanto, se torna crucial para disseminar as informações associadas as drogas (JONES, S. e BONCI, 2005; NESTLER, 2002). Mais especificamente, foi demonstrado que a administração de naloxona no hipocampo dorsal precipitou os sinais físicos de abstinência em camundongos tratados com morfina evidenciando, assim, o envolvimento direto do hipocampo na síndrome de abstinência (TREMBLAY e CHARTON, 1981).

Enquanto que o estágio da intoxicação envolve as propriedades reforçadoras positivas das drogas, na abstinência, os processos opostos denominados de reforço negativo são estimulados e ambos contribuem para a recaída ao uso da droga (KOOB, 2015). Nesse contexto, as constantes adaptações moleculares e celulares hipocampais podem estar relacionadas com o aparecimento dos sinais aversivos afetivos e hedônicos durante o estágio da abstinência (KOOB e LE MOAL, 2001). Inúmeros estudos descrevem o papel fundamental do estresse oxidativo no desenvolvimento de diversas doenças, incluindo o transtorno depressivo e a síndrome de abstinência (ABDEL-ZAHER et al., 2013; SALEHPOUR et al., 2018). Diversos modelos experimentais estabeleceram que o aumento nos níveis de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) em cérebro de roedores está associado ao aparecimento dos sinais físicos induzidos pela abstinência a morfina (PINELLI et al., 2009; SKRABALOVA et al., 2013).

Além disso, foi evidenciado que, diante de um ambiente oxidativo, o fator eritróide nuclear 2 relacionado ao fator 2 (Nrf2) pode ser ativado para codificar os genes de enzimas envolvidas na síntese e na conjugação da glutationa (GSH) e de enzimas antioxidantes nas regiões límbicas de camundongos abstinentes à morfina (YUN et al., 2015). Assim, o estresse oxidativo induz abrangentes impactos sobre a função neuronal, sendo que um destes envolve modificações nas estruturas e nas funções das proteínas, inclusive nos receptores de N-metil-D-aspartato (NMDA) (CHOI et al., 2001). Estudos sugerem que as adaptações comportamentais induzidas pela abstinência à morfina podem ser explicadas pelas alterações na homeostasia do no sistema glutamatérgico, através do aumento do estresse oxidativo

(ABDEL-ZAHER et al., 2013; ALEKSEENKO et al., 2009). Nesse contexto, alterações nos níveis basais e na liberação pré-sináptica de glutamato, assim como na expressão e funcionalidade dos receptores NMDA no sistema límbico parecem estar envolvidas durante o estágio da abstinência (NODA e NABESHIMA, 2004; SIGGINS et al., 2003).

Além disso, está bem estabelecido o papel crítico dos receptores NMDA sobre a plasticidade sináptica (KAUER e MALENKA, 2007). Nesse âmbito, os sinais aversivos autonômicos e emocionais parecem estar associados às alterações na neuroplasticidade durante a exposição repetida ou a retirada da morfina, mediado pelo fator neurotrófico encefálico (BDNF) (PEREGUD et al., 2016; RUSSO et al., 2009). Diversos estudos demonstraram que a sinalização do proBDNF, através do seu receptor neurotrófico p75 ($p75^{NTR}$), promove a simplificação da árvore dendrítica e diminui a densidade das espinhas no hipocampo, uma vez que pode ativar vias relacionadas com a apoptose (YANG et al., 2014). Por outro lado, ao ser convertido em *m*BDNF, ativa o receptor de tirosina cinase B (TrkB) viabilizando a sobrevivência celular por meio do aumento na formação e na diferenciação das espinhas dendríticas e sinapses (HIESTER et al., 2013). Em modelos animais, sugere-se que a abstinência a morfina modula negativamente a plasticidade sináptica e a sobrevivência neuronal (FAN et al., 2003), o que pode ser atribuído aos efeitos neurotóxicos do pro-BDNF (BACHIS et al., 2017).

1.3.3 Estágio de antecipação

O estágio de antecipação é representado pelos processos neuroadaptativos que contribuem com o desejo e a recaída às drogas (TIFFANY et al., 2000). Através de modelos experimentais aplicados em roedores, foi evidenciado que as projeções glutamatérgicas, transmitidas a partir do córtex pré-frontal até os neurônios dopaminérgicos mesocorticais localizados na área tegmental ventral, desempenham um controle excitatório sobre as células dopaminérgicas em relação aos disparos e à liberação de dopamina no córtex pré-frontal (KOOB e VOLKOW, 2016; VANDERSCHUREN et al., 2005). Diante disso, foi postulado que a exacerbada atividade do sistema glutamatérgico sobre os neurônios dopaminérgicos via circuitos do córtex pré-frontal, desregula o funcionamento do controle executivo. Ou seja, esta fase está associada com os prejuízos na manutenção da informação espacial e com a

dificuldade na tomada de decisões cujas adaptações podem contribuir com o desejo e a recaída às drogas de abuso (Figura 2C) (KOOB, 2015).

1.4 SELÊNIO

O elemento químico selênio (Se), pertencente ao grupo dos calcogênios, é considerado um micronutriente essencial devido a sua relevância em diversas funções fisiológicas nos mamíferos, tais como nas defesas antioxidantes, na fertilidade, no metabolismo do hormônio tireoide e na resposta imunológica (SCHOMBURG, 2011; YOUN et al., 2008). Nesse contexto, as múltiplas e complexas propriedades biológicas do Se se manifestam nos seres humanos através da sua incorporação em 25 diferentes tipos de selenoproteínas amplamente distribuídas nos tecidos (OGAWA-WONG et al., 2016).

Além da importância do Se no que abrange os aspectos fisiológicos, diversos estudos o apontam como um dos elementos químicos vitais para o funcionamento do SNC (BURK e HILL, 2009). Diante disso, em situações de reduzida ingesta de Se, o SNC recebe oferta prioritária deste micronutriente em relação aos demais tecidos (BUCKMAN et al., 1993). Nesse âmbito, foi investigado o papel do elemento Se em patologias degenerativas envolvidas no SNC e evidenciou-se um efeito neuroprotetor. Os mecanismos pelo qual este micronutriente exerce neuroproteção são atribuídos a sua habilidade de estimular a biossíntese de selenoproteínas antioxidativas e de modular o influxo de Ca^{+2} através do canal iônico (MCKENZIE et al., 2002; NAZIROGLU, 2009; UGUZ e NAZIROGLU, 2012).

Pelo menos nos últimos 40 anos, estudos envolvendo o metabolismo, a toxicologia e a nutrição de Se e de seus compostos, tanto na forma inorgânica como na orgânica, proporcionaram uma melhor compreensão sobre as suas múltiplas funções biológicas e sobre o seu mecanismo único de incorporação em proteínas (SOLOVYEV, 2015). Nesse contexto, por aproximadamente duas décadas, os derivados orgânicos do elemento Se vêm sendo amplamente estudados em virtude da sua maior disponibilidade e menor toxicidade em comparação a forma inorgânica (NOGUEIRA e ROCHA, 2011; NOGUEIRA et al., 2004). Portanto, os compostos orgânicos de Se tornaram-se alvos de pesquisa em nosso grupo.

1.4.1 Compostos orgânicos de selênio: Disseleneto de *m*-trifluormetil difenila

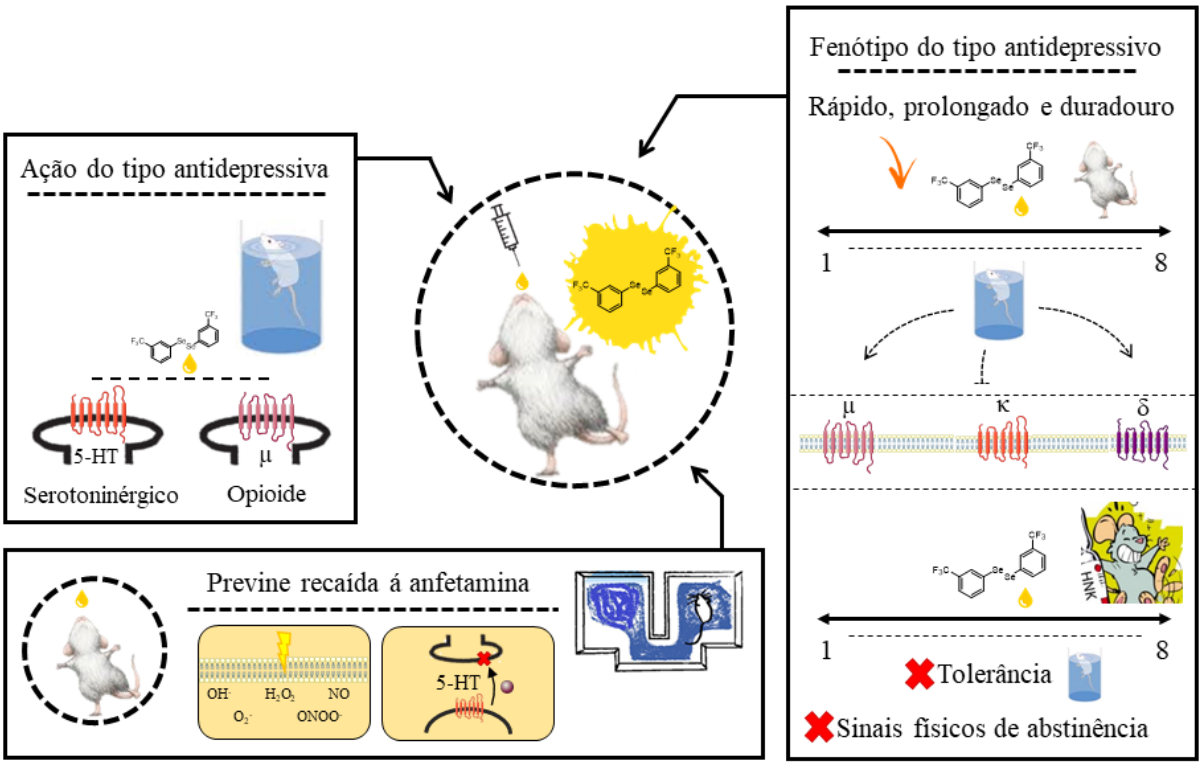
O disseleneto de *m*-trifluormetil difenila (*m*-CF_3-PhSe)$_2$, pertencente a classe dos organocalcogênios, tem sido amplamente descrito na literatura em virtude dos seus promissores efeitos em modelos experimentais de transtornos neuropsiquiátricos (BRUNING et al., 2015a; BRUNING et al., 2015b; SEGAT et al., 2016). Particularmente, a ação do tipo antidepressiva do (*m*-CF_3-PhSe)$_2$ foi comprovada em diferentes modelos animais relacionados aos transtornos de humor (ROSA et al., 2018a; ROSA et al., 2018b).

Assim como os seus análogos estruturais, a elevada lipofilicidade do (*m*-CF_3-PhSe)$_2$ permite que ele atravesse facilmente a barreira hematoencefálica e interaja com diferentes sistemas de neurotransmissores e cascatas intracelulares dentro do SNC (BRUNING et al., 2009; MACHADO et al., 2006). Nesse contexto, o envolvimento dos sistemas serotoninérgico e opioide parece estar relacionado com o efeito do tipo antidepressivo exercido pelo (*m*-CF_3-PhSe)$_2$ em camundongos (BRUNING et al., 2011). Estudos desenvolvidos recentemente demonstraram que a administração aguda do (*m*-CF_3-PhSe)$_2$ em roedores resulta em um fenótipo do tipo antidepressivo de início rápido e prolongado, enquanto que, ao longo de oito dias de tratamento repetido com este composto em diferentes doses (5, 10 e 25 mg/kg), os animais ainda apresentavam um comportamento do tipo antidepressivo. Especificamente, as bases farmacológicas sugerem que o (*m*-CF_3-PhSe)$_2$ modula o sistema opioide através da ativação dos receptores μ e δ e do bloqueio do receptor κ, contribuindo com o efeito do tipo antidepressivo característico desse composto (ROSA et al., 2017).

Diferentemente dos fármacos opioides clássicos, a administração repetida do (*m*-CF_3-PhSe)$_2$, sob as mesmas condições de doses e de duração de tratamento descritas anteriormente, não induziu o desenvolvimento da tolerância e da dependência os quais são caracterizados pela perda do efeito antidepressivo ao longo do tempo e pelos sinais físicos de abstinência, respectivamente (ROSA et al., 2017). Diante disso, tanto os efeitos modulatórios parciais sobre cada subtipo de receptor opioide, como as propriedades antioxidante e anti-inflamatória inerentes do (*m*-CF_3-PhSe)$_2$ parecem estar relacionadas com a ausência das manifestações comportamentais características da tolerância e da síndrome de abstinência em camundongos (ROSA et al., 2017). Somando-se a isso, um estudo prévio demonstrou que, através da modulação na homeostasia redox e no sistema serotoninérgico no córtex pré-frontal, o (*m*-CF_3-PhSe)$_2$ preveniu os sinais de recondicionamento em ratos expostos à anfetamina (SEGAT et al., 2016). Tais evidências indicam a relevância do efeito antioxidante deste composto nos processos adaptativos induzidos pelas drogas.

No que se refere a toxicidade dos disselenetos de diarila, foi demonstrado que a introdução de diferentes substituintes, retiradores ou doadores de elétrons, no grupamento arila do disseleneto de difenila não conferiu nenhuma toxicidade adicional (NOGUEIRA e ROCHA, 2011). Em conformidade, a avaliação da atividade de enzimas específicas, como a aspartato aminotransferase (AST), a alanina aminotransferase (ALT) e a creatina quinase (CK), assim como os níveis de ureia no plasma de camundongos mediante administração repetida do (*m*-CF_3-PhSe)$_2$ indicou que esse composto não alterou as funções renal, hepática e cardíaca. Diante disso, há indicativos de que o (*m*-CF_3-PhSe)$_2$ não induz toxicidade sistêmica e, portanto, sugere-se que o uso contínuo deste composto seja relativamente seguro (ROSA et al., 2017). Entretanto, o papel do (*m*-CF_3-PhSe)$_2$ sobre os processos adaptativos relacionados com a síndrome de abstinência induzida pela morfina ainda é desconhecido.

Figura 3 – Efeitos farmacológicos do (*m*-CF_3-PhSe)$_2$ elucidados em diferentes modelos experimentais em roedores.



Representação esquemática dos componentes intracelulares envolvidos no fenótipo do tipo antidepressivo e na prevenção ao recondicionamento à anfetamina em modelos animais.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial efeito do tratamento com o (*m*-CF_3-PhSe)$_2$ nos sinais físicos e no fenótipo do tipo depressivo induzidos pela abstinência espontânea à morfina em camundongos.

2.2 OBJETIVOS ESPECÍFICOS

De acordo com os aspectos mencionados anteriormente, os objetivos específicos dessa dissertação abrangem:

- ✓ Avaliar o efeito do (*m*-CF_3-PhSe)$_2$ sobre os sinais físicos de abstinência induzidos pela retirada da morfina;
- ✓ Avaliar a ação do tipo antidepressiva do (*m*-CF_3-PhSe)$_2$ em camundongos abstinentes à morfina;
- ✓ Investigar o efeito do (*m*-CF_3-PhSe)$_2$ nos níveis de peroxidação lipídica e de tióis não proteicos (NPSH) no hipocampo de camundongos abstinentes à morfina;
- ✓ Investigar o efeito do (*m*-CF_3-PhSe)$_2$ nos níveis das proteínas relacionadas à sinalização das defesas antioxidantes no hipocampo de camundongos abstinentes à morfina;
- ✓ Investigar o papel do (*m*-CF_3-PhSe)$_2$ sobre os níveis das subunidades dos receptores glutamatérgicos do tipo NMDA no hipocampo de camundongos abstinentes à morfina;
- ✓ Investigar o papel do (*m*-CF_3-PhSe)$_2$ sobre os níveis das proteínas relacionadas à via de sinalização neurotrófica do *m*BDNF no hipocampo de camundongos abstinentes à morfina;
- ✓ Investigar o papel do (*m*-CF_3-PhSe)$_2$ sobre os níveis das proteínas relacionadas à via de sinalização apoptótica do proBDNF no hipocampo de camundongos abstinentes à morfina.

3 DESENVOLVIMENTO

O desenvolvimento dessa dissertação está apresentado na forma de um manuscrito. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se descritos no próprio manuscrito, o qual está estruturado de acordo com as normas da revista ao qual foi submetido.

m-Trifluoromethyl-diphenyl diselenide (*m*-CF_3-PhSe)$_2$ modulates the hippocampal neurotoxic adaptations and abolishes a depressive-like phenotype in a short-term morphine withdrawal in mice

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Abstract

The opioid withdrawal syndrome is defined as a complex phenomenon involving multiple cellular adaptations, which leads to the emergence of aversive physical and affective signs. The *m*-trifluoromethyl-diphenyl diselenide (*m*-CF_3-PhSe)$_2$ elicits an antidepressant-like effect by modulating the opioid system in different animal models of mood disorders. Notably, repeated exposure to (*m*-CF_3-PhSe)$_2$ did not develop neither tolerance nor withdrawal signs. The aim of the present study was to investigate whether (*m*-CF_3-PhSe)$_2$ attenuates the physical signs and the depressive-like stereotype during morphine withdrawal through its neuroprotective effects on oxidative stress, the NMDA receptor and the proBDNF/*m*BDNF signaling in the hippocampus of mice. In the first six days, adult Swiss mice received scalating doses (20-100 mg/kg, s.c.) of morphine. For the next three days, the animals were treated with (*m*-CF_3-PhSe)$_2$ (5 and 10 mg/kg, i.g.) whereas the morphine injections were discontinued. On day 9, physical withdrawal signs and depressive-like behavior were assessed thirty min after de last adminitration of (*m*-CF_3-PhSe)$_2$. Although short-term treatment of (*m*-CF_3-PhSe)$_2$ at both doses suppressed the aversive physical and affective signs in morphine withdrawn-mice, the highest dose of (*m*-CF_3-PhSe)$_2$ *per se* induced an increased in teeth chattering manifestation. The intrinsic antioxidant property of (*m*-CF_3-PhSe)$_2$ modulated oxidative stress and restored the NMDA receptor levels during morphine withdrawal in the hippocampus of mice. Besides, (*m*-CF_3-PhSe)$_2$ inhibited the proBDNF/p-75^{NTR}/JNK pro-apoptotic pathway without affecting the mBDNF/TrkB/ERK/CREB pro-survival signaling in hippocampus of morphine withdrawn-mice. In conclusion, (*m*-CF_3-PhSe)$_2$ modulated the hippocampal neurotoxic adaptations and abolished the depressive-like phenotype following morphine withdrawal.

Keywords: opioid withdrawal syndrome, depressive-like behavior, stress oxidative, synaptic plasticity, organoselenium.

1. Introduction

The withdrawal syndrome, a classical hallmark of opioid use disorder, manifests itself through the physical signs and an aversive emotional state when opioid use is discontinued. Indeed, the desire to relieve these negative aspects in chronic opiate users may drive them to compulsive drug seeking behavior, regardless of the adverse consequences [1]. In this context, the abusive use of opioids has been identified as a socio-economic and public health concern in the USA and wordwide because opioid overdose is one of the major cause of accidental deaths in adults [2].

The neurobiological basis for withdrawal and negative affective state is mediated by the interconnections and neurocircuitry within mesocorticolimbic system, including nucleus accumbens, hippocampus, prefrontal cortex and caudate putamen [3]. Notably, the hippocampus plays a key role in the processes associated to relapse and addictive behaviors [4]. Morphine withdrawal, a μ opioid receptor agonist widely prescribed for clinical pain management, alters the consolidation of long-term memory information and disrupts the neurogenesis in the hippocampus of rats [5,6]. Besides, it has been suggested that the adaptative changes in neurotransmitters and signal transduction systems in the hippocampus are related to the physical signs and the depressive-like phenotype during morphine withdrawal in rodents [7,8]. In this way, animal studies have shown that neurotoxic events, such as oxidative stress, alterations in the glutamatergic system and in sinaptic plasticity through the opposit effects related to processing brain-derived neurotrophic factor (BDNF), might contribute to withdrawal syndrome [9,10].

Organoselenium compounds have been effectiveness in many experimental models of psychiatric disorders due to their numerous pharmacological properties [11]. Particularly, *m*-trifluoromethyl-diphenyl diselenide (*m*-CF_3-PhSe)$_2$ is the least toxic among its structural analogues studied in our research group [12]. Moreover, the antidepressant-like effect of (*m*-

CF_3-PhSe)$_2$ has been proven in different animal models of mood disorders [13,14]. Recent neurochemical findings suggest that (*m*-CF_3-PhSe)$_2$ exerted modulatory effects on μ, κ and δ opioid receptors which may contribute to its antidepressant-like action in rodents [13,14]. Curiously, repeated (*m*-CF_3-PhSe)$_2$ administration did not develop tolerance and physical withdrawal sings although the opioidergic system is required to elicit its pharmacological effect [15]. Also, the antioxidant and anti-inflammatory properties of (*m*-CF_3-PhSe)$_2$ are thought to be involved in its protective effects within the neural central system (NCS) [16]. In this context, it is well-known that free radical scavangers attenuated the physical signs developed during morphine withdrawal by modulating oxidative stress in the brain of rodents [17,18].

Considering that opioid agonists, such as methadone and buprenorphine, are the available therapeutic agents for the treatment of opiate withdrawal syndrome and based on the (*m*-CF_3-PhSe)$_2$ properties, the purpose of the present study was to evaluate the effects of this multitarget compound on the development of physical signs and depressive-like phenotype during morphine withdrawal in mice. In an attempt to better understand the role of (*m*-CF_3-PhSe)$_2$ in the behavior adaptations induced by morphine withdrawal, we investigated the NMDA receptor protein level, the signaling pathways of the proBDNF and the *m*BDNF, as well as the oxidative stress in the hippocampus of mice.

2. Materials and Methods

2.1 Animals

Male Swiss mice (two months old, 25-35g) were provided by the local breeding colony. The animals were housed in groups (five per cage) with free access to food and water. They were

kept in a separate animal room, under a 12-hour light/ 12-hour dark cycle (the lights were turned on at 07:00 AM), in an appropriate temperature environment (22 ± 2°C). A commercial diet (Guaíba, Rio Grande do Sul, Brazil) and filtered water were available *ad libitum*. The animal protocol was approved by the Institutional Ethics Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Rio Grande do Sul, Brazil and registered under the number 8756060317/2017. All procedures were performed according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the suffering and the number of animals utilized.

2.2 Drugs

The compound (*m*-CF_3-PhSe)$_2$ was synthesized in accordance with the method previously described [19]. The ^{1}H NMR and ^{13}C NMR (Nuclear Magnetic Resonance) spectral data were in full agreement with (*m*-CF_3-PhSe)$_2$ assigned structure. The chemical purity of (*m*-CF_3-PhSe)$_2$ (99.9%) was determined by gas chromatography-mass spectrometry (GC-MS; Shimadzu QP2010PLUS GC/MS combination). Morphine sulfate and methadone hydrochloride were purchased from medicine distributor MCW (Santa Cruz, Rio Grande do Sul, Brazil). The opioid drugs were dissolved in saline solution (sodium chloride NaCl 0.9%) whereas the (*m*-CF_3-PhSe)$_2$ was diluted in canola oil. The mice received all drugs at a constant volume of 10 ml/kg body weight.

2.3 Experimental Design

Intermittent escalating-dose morphine administration paradigm and withdrawal

The experimental design of this study is depicted in Fig. 1. Firstly, mice were randomly separate in two groups, saline and morphine. Escalating-dose morphine administration paradigm was performed by using a previously established procedure [20,21]. Briefly, mice received increasing doses of morphine (20, 40, 60, 80, 100 mg/kg) or saline solution twice a day at 09:00 AM and 05:00 PM, by the subcutaneous (sc) route, for five consecutive days. On the sixth day, animals received a single and last dose of morphine (100 mg/kg) or saline solution.

To determine the (*m*-CF_3-PhSe)$_2$ effect on depressive-like behavior induced by morphine withdrawal, mice were subdivided into seven different groups (n = 8/group) as described below. Saline and morphine treated groups were left in their home cages without receiving any injections for three consecutive days to induce spontaneous withdrawal syndrome. Other set of mice underwent morphine or saline administration and received (*m*-CF_3-PhSe)$_2$ at different doses (5 and 10 mg/kg) once a day, by the intragastric (ig) route, for the same time. In order to validate the experimental model, a separate set of mice, previously treated with morphine, received methadone (5 mg/kg, ig), a positive control [22], during morphine withdrawal period.

Thirty minutes after the last administration of (*m*-CF_3-PhSe)$_2$ on day nine, autonomic withdrawal behaviors were observed and recorded. Thereafter, the tail suspension test and the forced swim test were performed to evaluate the depressive-like behaviors. The doses and time intervals of treatment with (*m*-CF_3-PhSe)$_2$ were selected based on previous reports [23,15].

After the behavior tests, mice were killed by cervical dislocation, the brain was collected and hippocampus was dissected. The samples were quickly frozen at -80°C for further neurochemical analyses.

2.4 Behavior Experiments

2.4.1 Locomotor activity

Mice were exposed to the locomotor activity monitor in order to evaluate locomotor coordination and exploratory capacity. This behavior test was performed in a clear acrylic plastic box (50 cm x 48 cm x 50 cm) connected to a monitor with photocell beams and equipped with 16 infrared sensors for the automatic recording of animal position and the general locomotor activity (Insight, Ribeirão Preto, SP, Brazil). Mice were placed in the center of the apparatus and allowed to freely explore the arena. The number of crossings, rearings, total distance and average speed were recorded during 4 min.

2.4.2 Assessment of spontaneous withdrawal signs

Animals underwent morphine withdrawal displayed characteristic autonomic withdrawal signs. Mice were individually placed in a transparent acrylic cylinder (diameter 11 cm and height 30 cm) to monitor physical withdrawal signs. Each behavior observation session lasted 30 min and was recorded by a blind observer. Behaviors including jumping, rearing, paw tremor and teeth chattering were quantified.

2.4.3 Tail suspension test

The tail suspension test was performed in a quiet experimental environment wherein the total immobility duration is considered the major parameter measured to assess the “behavioral despair” rodents [24]. Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. For the next 6 min, the latency for the first

immobility episode and the immobility time were recorded. Mice were only considered immobile when passively hung and completely motionless.

2.4.4 Forced swim test

Originally described by Porsolt et al., 1977, forced swim test is the most sensitive behavior test to evaluate antidepressant properties of new compounds. In this test, mice were individually forced to swim in an open cylindrical container (diameter 10 cm and height 25 cm) containing 19 cm of water at 25 ± 1°C. Latency for the first immobility episode and total immobility duration (escape related mobility behavior) were monitored during 6 min. Each mouse was considered immobile after it ceased struggling and began floating passively on the water.

2.5 *Ex vivo* analyses

2.5.1 Tissue preparation

Samples of hippocampus (n = 6/group) were homogenized (1:5, w/v) in 50 mM of Tris-HCl at pH 7.4. The homogenates were then centrifuged at 2,500 x g for 10 min at 4ºC and the low-speed supernatant (S_1) was used for thiobarbituric acid reactive substances (TBARS) and non-protein thiol (NPSH) assays.

2.5.2 TBARS assay

TBARS assay was performed to indirectly determine the malondialdehyde (MDA) levels, an important lipid peroxidation marker. As previously described by Ohkawa et al. [26], MDA reacts with 2-thiobarbituric acid (TBA) under acidic conditions and high temperatures to yield

the chromogen. The S_1 aliquots were incubated with 0.8% TBA, acetic acid buffer (pH 3.4) and 8.1% sodium dodecyl sulfate (SDS) for 2h at 95ºC. The color reaction was measured at 532 nm and the results were expressed as nmol of MDA/mg protein.

2.5.3 NPSH content

NPSH content, a non-enzymatic antioxidant defense, was determined by Ellman's method [27]. Briefly, S_1 was mixed (1:1) with 10% trichloroacetic acid (TCA). After centrifugation (3000 x g for 10 min), an aliquot of supernatant containing free SH-groups was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid). The color reaction was measured at 412 nm and NPSH levels were expressed as nmol of NPSH/g tissue.

2.5.4 Protein determination

The protein concentration was estimated according to the method described by Bradford [28], using a bovine serum albumin (1 mg/ml) as a standard. The color was measured spectrophotometrically at 595 nm.

2.5.5 Western blot assay

Samples of hippocampus (n = 5/group) were lysed in appropriate Radioimmunoprecipitation assay buffer (RIPA buffer) solution containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), in which it was added commercial phosphatase and protein inhibitor cocktail (Sigma-Aldrich Company, St. Louis, Missouri, United States). The homogenates were diluted and the final protein concentration was adjusted to 2 µg/µl. The samples (30 µg protein/well) and pre stained molecular weight standards (Sigma-Aldrich Company, St. Louis, Missouri, United States) were separated on

SDS-polyacrylamide gel by electrophoresis. Subsequently, proteins were transferred to nitrocellulose membrane (0.45 µm, Bio-Rad) using Transfer-Blot® Turbo™ Transfer System (1.0 mA, 45 min, Bio-Rad) and the protein transfer was confirmed by Ponceau Red. Then blots were blocked with 3% bovine serum albumin (BSA) solution for 1h at room temperature (RT) and incubated overnight at 4ºC inappropriate primary antibodies, as shown in Table 1. β-actin was stained as a constitutive protein. After primary antibody incubation, membranes were washed and incubated in peroxidase-labeled secondary antibodies (Bio-Rad Laboratories, Hercules, CA, USA) for 1h at RT. Bands were detected using a chemiluminescence kit (Amersham, São Paulo/Brazil) and the signals were captured exposing the blots to Amersham Imager 600 (GE healthcare life sciences). The intensity of optical density (OD) was quantified using Image J (NIH, Bethesda, MD, USA) software for Windows. The relative density of all analyzed proteins was normalized to β-actin levels.

2.6 Statistical Analysis

All experimental results are expressed as means ± standard error medium (SEM), as well all statistical analyses were performed using STATISTICA (StatSoft, Oklahoma, USA). A Gaussian distribution was tested using D'Agostino-Pearson omnibus normality test. A two-way analysis of variance (ANOVA) was performed to compare saline x (m-CF_3-PhSe)$_2$ and morphine withdrawal x (m-CF_3-PhSe)$_2$ treatments as independent variables in both behavior and neurochemical analyses. A one-way ANOVA was used to compare different treatment effects (saline, morphine withdrawal and methadone) in behavior experiments. The Newman-Keuls post hoc test was used for individual group comparisons. To autonomic signs of opiate withdrawal, 1 was added to all values and log10 was calculated in order to convert non-parametric data into parametric. Then statistical analyses previously described were applied for each treatment.

3. Results

Behavior studies

(m-CF_3-PhSe)$_2$ treatment attenuates spontaneous withdrawal signs in morphine-dependent mice

The two-way ANOVA analyses revealed a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction on jumping behavior [$F_{(2,42)} = 3.52$, $P < 0.05$], paw tremor [$F_{(2,42)} = 4.97$, $P < 0.05$] and teeth chattering [$F_{(2,42)} = 5.47$, $P < 0.05$]. Morphine withdrawal induced an increase in jumping behavior ($P < 0.05$) as well in teeth chattering and paw tremor manifestations ($P < 0.001$) when compared to the vehicle group. Although the highest dose of (*m*-CF_3-PhSe)$_2$ alone increased teeth chattering ($P < 0.05$) manifestation in mice, (*m*-CF_3-PhSe)$_2$ treatment at both doses attenuated opiates withdrawal syndrome ($P < 0.05$) in mice previously treated with morphine (Fig. 2A-C). In contrast, there was no statistical significance on the number of rearings [$F_{(2,42)} = 1.14$, $P > 0.05$] among the groups (Fig. 2D).

The inserts in Fig. 2 represent the effects of methadone, a positive control, on jumping behavior (Fig. 2A), paw tremor (Fig. 2B), teeth chattering (Fig. 2C) and number of rearing (Fig. 2D) in morphine withdrawn-mice. The one-way ANOVA analyses showed a significant difference among the experimental groups on paw tremor manifestations [$F_{(2,21)} = 32.18$, $P <$ 0,001] and teeth chattering [$F_{(2,21)} = 30.51$, $P < 0.001$]. Methadone treatment reduced the appearance of paw tremor and teeth chattering ($P < 0.01$) in morphine withdrawn-mice and thus, alleviated the emergence of physical withdrawal signs.

(m-CF_3-PhSe)$_2$ treatment abolishes depressive-like phenotype induced by morphine withdrawal in mice

The two-way ANOVA analyses of immobility time demonstrated a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction in both TST [$F_{(2,42)} = 9.14$, $P < 0.001$] and FST [$F_{(2,42)} = 4.44$, $P < 0.05$]. Morphine withdrawn-mice remained more immobile in both behavior tests than vehicle-treated mice ($P < 0.01$). In contrast, (*m*-CF_3-PhSe)$_2$ treatment at both doses abolished the increase of immobility time ($P < 0.05$) induced by morphine withdrawal in mice (Fig. 3A-B).

The effect of methadone on this same parameter is shown in the inserts of Fig. 3A-B. The one-way ANOVA analyses of immobility time in TST [$F_{(2,21)} = 16.78$, $P < 0.001$] and FST [$F_{(2,21)} = 4.57$, $P < 0.05$] revealed a significant difference among the experimental groups. However, methadone treatment reversed the increase of immobility time in morphine withdrawn-mice only in TST ($P < 0.001$).

Table 2 shows the effect of (*m*-CF_3-PhSe)$_2$ treatment and morphine withdrawal on latency time for the first episode of immobility in both TST and FST. The two-way ANOVA analyses of latency time [$F_{(2,42)} = 9.87$, $P < 0.001$] indicated a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction only in the TST. Interestingly, latency time was similar for morphine withdrawal and vehicle groups ($P > 0.05$), whereas mice underwent morphine withdrawal and treated with (*m*-CF_3-PhSe)$_2$ at the dose of 5 mg/kg had this parameter increased ($P < 0.05$) when compared to morphine withdrawn-mice.

Similarly, the one-way ANOVA analyses of latency time [$F_{(2,21)} = 4.71$, $P < 0.05$] revealed a significant difference among the experimental groups only in the TST. Mice from the methadone group improved this parameter ($P < 0.05$) when compared to those of morphine withdrawal group.

(m-CF_3-PhSe)$_2$ treatment and morphine withdrawal do not alter locomotor activity in mice

Table 3 shows the effect of (*m*-CF_3-PhSe)$_2$ treatment and morphine withdrawal on parameters of locomotor activity in mice. The two-way ANOVA analyses of number of crossings [$F_{(2,42)} = 0.17$, $P > 0.05$], distance traveled (mm) [$F_{(2,42)} = 0.30$, $P > 0.05$] and speed (mm/s) [$F_{(2,42)} = 0.25$, $P > 0.05$] revealed a non-significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction.

The administration of methadone did not alter locomotor activity in mice. The one-way ANOVA analyses of number of crossings [$F_{(2,21)} = 1.87$, $P > 0.05$], distance traveled (mm) [$F_{(2,21)} = 1.57$, $P > 0.05$] and speed (mm/s) [$F_{(2,21)} = 2.64$, $P > 0.05$] showed no statistical significance among the groups.

Neurochemical studies

The (*m*-CF_3-PhSe)$_2$ dose of 5 mg/kg was chosen for *ex vivo* assays because this was effective in all behavior tests and did not induce any undesirable characteristic effects of opioid drugs.

(m-CF_3-PhSe)$_2$ treatment regulates the hippocampal oxidative stress in morphine withdrawn-mice

The two-way ANOVA analyses of TBARS levels [$F_{(1,20)} = 8.85$, $P < 0.01$] showed a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction. Mice underwent morphine withdrawal increased hippocampal lipid peroxidation levels, indirectly measured by the TBARS assay, when compared to those of vehicle group ($P < 0.001$). (*m*-CF_3-PhSe)$_2$ treatment at the dose of 5 mg/kg reduced TBARS levels ($P < 0.01$) in morphine withdrawn-mice (Fig. 4A).

The two-way ANOVA analyses of NPSH content [$F_{(1,20)} = 0.20$, $P > 0.05$] revealed a non-significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction. The hippocampal non-

enzymatic antioxidant defense remained unchanged ($P > 0.05$) in all experimental groups (Fig. 4B).

The two-way ANOVA analyses of Nrf2 [$F_{(1,16)} = 5.54$, $P < 0.05$] and Keap-1 [$F_{(1,16)} = 7.73$, $P < 0.05$] protein levels demonstrated a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction. Morphine withdrawal induced an increase of hippocampal Nrf2 and Keap-1 levels when compared to those of vehicle group ($P < 0.05$), whereas (*m*-CF_3-PhSe)$_2$ at the dose of 5 mg/kg reduced levels of these proteins ($P < 0.05$) in morphine withdrawn-mice (Fig. 4 C-E).

(m-CF_3-PhSe)$_2$ treatment restores the impaired levels of NMDA receptor subunits in hippocampus of morphine withdrawn-mice

The two-way ANOVA analyses of NR2A [$F_{(1,16)} = 23.56$, $P < 0.001$] and NR2B [$F_{(1,16)} = 19.11$, $P < 0.001$] levels demonstrated a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction. The hippocampal NR2A and NR2B subunits were reduced in morphine withdrawn-mice when compared with the vehicle group ($P < 0.001$). The (*m*-CF_3-PhSe)$_2$ treatment increased the levels of both NMDA receptor subunits ($P < 0.001$) in the hippocampus of mice underwent morphine withdrawal (Fig. 5A-B).

(m-CF_3-PhSe)$_2$ treatment and morphine withdrawal do not affect hippocampal neural plasticity markers

The two-way ANOVA analyses of *m*BDNF [$F_{(1,16)} = 0.78$, $P > 0.05$] and TrkB [$F_{(1,16)} = 2.79$, $P > 0.05$] protein levels as well as *p*-ERK/ERK [$F_{(1,16)} = 0.46$, $P > 0.05$] and *p*-CREB/CREB ratios [$F_{(1,16)} = 0.65$, $P > 0.05$] revealed no statistically significant effects of morphine withdrawal and (*m*-CF_3-PhSe)$_2$ treatment. Neither (*m*-CF_3-PhSe)$_2$ treatment nor morphine withdrawal modulated the *m*BDNF/TrkB/*p*-ERK/*p*-CREB signaling pathway ($P > 0.05$) in hippocampus of mice (Fig. 6A-D).

(m-CF_3-PhSe)$_2$ treatment has neuroprotective effects on hippocampus of morphine withdrawn-mice

The two-way ANOVA analyses for proBDNF [$F_{(1,16)} = 5.69$, $P < 0.05$] and p-75^{NTR} [$F_{(1,16)} = 14.20$, $P < 0.01$] protein levels revealed a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction. Morphine withdrawn-mice increased hippocampal levels of proBDNF and p-75^{NTR} when compared with the vehicle-treated mice ($P < 0.05$). (*m*-CF_3-PhSe)$_2$ treatment in morphine withdrawn-mice reversed this increase to the levels of vehicle group ($P < 0.05$) (Fig. 7A-B).

The two-way ANOVA analysis of *p*-JNK/JNK ratio [$F_{(1,16)} = 8.14$, $P < 0.05$] showed a significant morphine withdrawal and (*m*-CF_3-PhSe)$_2$ interaction. Morphine withdrawal induced an increase in the ratio of *p*-JNK/JNK in hippocampus of mice when compared with the vehicle-treated group ($P < 0.05$). (*m*-CF_3-PhSe)$_2$ treatment was partially effective against this increase ($P > 0.05$) in morphine withdrawn-mice (Fig. 7C).

4. Discussion

The present study demonstrates that (*m*-CF_3-PhSe)$_2$ treatment alleviated the physical withdrawal syndrome and a depressive-like phenotype in a short-term morphine withdrawal in mice. Furthermore, molecular analyses performed in this study provided evidence that (*m*-CF_3-PhSe)$_2$ treatment modulated oxidative stress, the levels of NMDA receptor subunits and the proBDNF/p-75^{NTR} signaling pathway in the hippocampus of mice, suggesting the involvement of these neuroadaptations in morphine withdrawal related to negative affective state.

Manifestations such as paw tremor, teeth chattering and jumps are the most prevalent behavior markers of morphine withdrawal in rodents and were clearly measurable within one week [29]. Besides, previous studies suggested that protracted morphine withdrawal induced a

depressive-like stereotype in mice. Therefore, motivational and physical aspects may be involved in different periods of morphine withdrawal [21,20,7]. Inconsistent with these findings, the current study demonstrates that mice exhibited manifestations of physical withdrawal syndrome and a depressive-like phenotype following three days of morphine withdrawal. Although this mouse model of opioid abstinence mimics the negative emotional state usually displayed by human opioid-withdrawn addicts [30,31], the discrepancies among these studies may be partially explained due to modifications in experimental protocols such as dose, strain and time and route of administration.

During the past years, previous studies carried out by our research group investigated the role of (*m*-CF_3-PhSe)$_2$, an organoselenium compound, in mouse models of psychiatric disorders [23,32,14]. Recent findings reported the involvement of the opioid system in the antidepressant-like effects elicited by (*m*-CF_3-PhSe)$_2$ in mice and interestingly, repeated administration of this compound did not induce neither physical withdrawal signs nor tolerance, the most prevalent undesirable effects related to long-term use of opioid drugs [15]. However, whether (*m*-CF_3-PhSe)$_2$ affects physical withdrawal syndrome and depressive-like behaviors in mice following morphine withdrawal remains unknown. Thus, we used the TST and FST because they are the most used tools for screening antidepressant drugs in rodents [25,24]. Our results showed that the administration of (*m*-CF_3-PhSe)$_2$ during morphine withdrawal decreased the immobility time in both behavior tests without changing the locomotor activity of mice.

Therefore, the present study is the first, to our knowledge, to provide compelling evidence that (*m*-CF_3-PhSe)$_2$ treatment reduced the appearance of withdrawal signs and blocked the expression of depressive-like phenotype in morphine withdrawn-mice. As expected, most of (*m*-CF_3-PhSe)$_2$ effects were similar to those exerted by methadone, a μ opioid receptor agonist widely prescribed for opioid dependence treatment. However, methadone treatment was

ineffectiveness to abolish the depressive-like stereotype in the FST, following morphine withdrawal in mice. This result is consistent with the findings of previous studies about aversive motivational signs, including depressive and anxiety-like behaviors, and methadone treatment [33]. Taken together, our findings suggest that (*m*-CF_3-PhSe)$_2$ suppressed physical and negative affective sings induced by morphine withdrawal and might avoid the aversively motivated drug seeking.

Several studies reported the central role of oxidative stress in pathophysiological conditions, including opiate withdrawal state and depressive-like behavior [34,35]. An imbalance on redox signaling may trigger structural and functional damages of macromolecules, resulting in a dysregulation of cell proliferation/differentiation and neurotransmission [36,37]. On the other hand, the Nrf2/Keap-1 system, widely expressed in the CNS, represents one of the most important endogenous defenses in response to oxidative and electrophilic environments. Nrf2 is sequestered and form a dimer with its repressor Keap-1 in the cytoplasm, which mediates the rapid ubiquination and proteasomal degradation of Nrf2, leading to suppresses the expression of antioxidant and detoxification enzyme genes in the nucleus under unstressed conditions [38].

Our results indicate that morphine withdrawal induced oxidative stress, characterized by incresing levels of TBARS, Nrf2 and Keap-1, whereas the non-enzymatic antioxidant defenses were not altered in the hippocampus of mice. Thus, we hypothesize that morphine withdrawal promotes an oxidative condition within the cell through increased lipid peroxidation. This environmental modifies the cysteine residues of Keap-1, allowing Nrf2 dissociates from Keap-1 and translocates to the nucleus where upregulates the expression of cytoprotective genes, such as thioredoxins (*Trxs*), heme oxygenase-1 (*HO-1*), superoxide dismutase-1 (*SOD-1*) and glutathione peroxidase (*GPx*) [39]. Furthermore, these findings of the current study reinforce the antioxidant property of (*m*-CF_3-PhSe)$_2$ because it might

modulate the redox homeostasis and, as a consequence, reverses the neurobehavioral adaptations in morphine withdrawn-mice. However, it remains unclear the precise effect of (*m*-CF_3-PhSe)$_2$ treatment on oxidative stress induced by morphine withdrawal, thus we acknowledge the lack of antioxidant enzyme activities (catalase and SOD) determination and another markers of oxidative damage (reactive species) as limitations of this study.

Some authors also suggest a relationship among oxidative stress, glutamatergic neurotransmission dysfunctions and opioid addiction processes [40,41]. Several proteins involved in glutamatergic neurotransmission contain modulatory redox sites, including NMDA receptors. Therefore, a redox dysregulation may impair the functionality and structure of NMDA receptors [42]. Moreover, it is well stablished that repeated exposure to morphine increases the extracellular glutamate levels and alters the levels of NMDA receptors in hippocampus of rodents [43]. Our present findings show that the hippocampal levels of NR2A and NR2B were downregulated following morphine withdrawal. According to our previous study, repeated (*m*-CF_3-PhSe)$_2$ treatment alone had no effect on extracellular glutamate levels in the hippocampus of mice and this could be partially related to the absence of behavior adaptations characteristic of opioids drugs [15]. However, our results demonstrate that (*m*-CF_3-PhSe)$_2$ promoted an increase in both subunits of NMDA receptor in morphine withdrawn-mice. Together, these data suggest that the modulatory effects on oxidative stress induced by (*m*-CF_3-PhSe)$_2$, restored the subunits of NMDA receptor levels in the hippocampus and then this may be a possible neurochemical mechanism underlying this organoselenium compound suppressed the autonomic and affective signs in morphine withdrawn-mice.

Moreover, the NMDA receptor signaling also plays a fundamental role on synaptic transmission and plasticity, which controls physiological process that include mood, cognition, learning and reward [44,45]. Previous studies support that BDNF signaling induces

persistent alterations in synaptic plasticity that may contribute to behavior and biochemical manifestations in response to repeated morphine exposure [46-48]. In this way, it has been reported that drugs of abuse, such as morphine, increase *m*BDNF levels and require the activation of ERK/CREB signaling pathway to mediate the process related to drug addiction [49]. The findings of our study indicate that *m*BDNF pathway was not affected during morphine withdrawal in hippocampus of mice, because the protein levels of *m*BDNF and its receptor TrkB as well as their downstream proteins, represented by *p*-ERK/ERK and *p*-CREB/CREB ratios, were unchanged.

In an attempt to explain these divergent results, the present study also investigated the role of proBDNF on behavior adaptations during morphine withdrawal. Our data showed that morphine withdrawal increased proBDNF levels in the hippocampus of mice, consistent with a recent study reporting that proBDNF levels are increased in cerebral cortex of rats in reponse to morphine treatment or withdrawal [10]. Taken together, these findings suggest that morphine withdrawal might deregulate the proteolytic processing of proBDNF in *m*BDNF. In this context, similar studies indicated that proBDNF and its signaling via p-75^{NTR} lead to activation of JNK, which is involved in neuronal apoptosis [50,51]. In agreement, our results suggest that morphine withdrawal may upregulate the proBDNF/p-75^{NTR}/JNK neurodegenerative signaling pathway in hippocampus of mice. In fact, it is well stablished that proBDNF pathway decreases spine density and induces long-term depression in hippocampal neurons [52,53]. Therefore, our study provide evidence that the activation of proBDNF signaling pathway induced neurotoxic events, which might be associated to physical withdrawal signs and depressive-like phenotype in morphine withdrawn-mice.

Regarding the organoselenium compound, the lipophilicity of (*m*-CF_3-PhSe)$_2$ allows it to permeate the blood-brain barrier and interacts with multiple biological targets in the central nervous system [16,54]. It is well-known that *m*BDNF promotes neuronal survival via its

receptor TrkB, whereas proBDNF regulates neuronal death via its receptor p-75^{NTR} [55,56]. In this way, our data demonstrate that although the (*m*-CF_3-PhSe)$_2$ treatment was effectiviness against the increase in the levels of proBDNF, p-75^{NTR} and in the ratio *p*-JNK/JNK, this compound did not affect the *m*BDNF signaling in hippocampus of mice following morphine withdrawal. These results suggest that (*m*-CF_3-PhSe)$_2$ restored the balance between the proBDNF/*m*BDNF signaling and inhibited the proBDNF/p-75^{NTR}/JNK pro-apoptotic pathway in hippocampus of morphine withdrawn-mice. The findings of the current study reinforce the neuroprotective effects of (*m*-CF_3-PhSe)$_2$ by modulating oxidative stress, the subunits of NMDA receptor and synaptic plasticity, which leads to suppress both autonomic and affective signs during morphine withdrawal, according to Fig. 8.

In conclusion, (*m*-CF_3-PhSe)$_2$ treatment attenuated physical withdrawal signs and the depressive-like phenotype by modulating oxidative stress, NMDA receptor, the proBDNF signaling in hippocampus of mice and thus, promoting neuroprotective effects during morphine withdrawal.

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Conflict of interest

The authors declare that there is no conflict of interest in the present study.

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Figure captions

Fig. 1. Schematic representation of the experimental design of this study.

Fig. 2. (*m*-CF_3-PhSe)$_2$ attenuated the opiate withdrawal syndrome in mice. The autonomic signs, jumping behavior (A), paw tremor (B), teeth chattering (C) and number of rearings (D), were recorded thirty min after last (*m*-CF_3-PhSe)$_2$ administration at doses of 5 and 10 mg/kg. Inserts indicate the effect of methadone treatment (5 mg/kg) on the jumping behavior (A), paw tremor (B), teeth chattering (C) and number of rearings (D). Data are expressed as mean ± S.E.M of 8 animals/group. Asterisk denotes significant levels when compared to the vehicle group: (***) $P < 0.001$ and (*) $P < 0.05$. Hashtag denotes significant levels when compared with the morphine withdrawal group: ($^{\#\#\#}$) $P < 0.001$, ($^{\#\#}$) $P < 0.01$ and ($^{\#}$) $P < 0.05$ (In order to convert non-parametric data into parametric, it was added 1 to all values and log 10 was calculated. Then two-way or one-way ANOVA followed by the Newman-Keul's test were applied for each treatment).

Fig. 3. (*m*-CF_3-PhSe)$_2$ treatment reversed the depressive-like behavior induced by morphine withdrawal in mice. Effects of (*m*-CF_3-PhSe)$_2$ at doses of 5 and 10 mg/kg on immobility time of mice in both TST (A) and FST (B) respectively. Inserts show the effect of methadone treatment (5 mg/kg) in both behavioral tests related to its antidepressive-like potential in mice. Values are reported as mean ± S.E.M of 8 animals/group. Asterisk denotes significance levels when compared with the vehicle group: (***) $P < 0.001$, (**) $P < 0.01$ and (*) $P < 0.05$. Hashtag denotes significance levels in comparison with the morphine withdrawal group: ($^{\#\#\#}$) $P < 0.001$, ($^{\#\#}$) $P < 0.01$) and ($^{\#}$) $P < 0.05$ (Two-way or one-way ANOVA followed by the Newman-Keul's test).

Fig. 4. (*m*-CF_3-PhSe)$_2$ treatment regulated the hippocampal oxidative stress in morphine withdrawn-mice. Effects of (*m*-CF_3-PhSe)$_2$ treatment on lipid peroxidation (A) and NPSH (B) levels in hippocampus of morphine withdrawn-mice. Qualitative images of western blotting

analyses (C) represent the effect of (*m*-CF_3-PhSe)$_2$ on hippocampal Nrf2 (D) and Keap-1 (E) protein contents in morphine withdrawn-mice. VHC, CF_3, WDW and WDW-CF_3 mean vehicle, (*m*-CF_3-PhSe)$_2$, withdrawal and withdrawal-(*m*-CF_3-PhSe)$_2$, respectively. Values are expressed as mean ± S.E.M of 5 - 6 animals per group. Asterisk denotes significance levels when compared with the vehicle group: (***) $P < 0.001$ and (*) $P < 0.05$. Hashtag denotes significance levels when compared with the morphine withdrawal group: ($^{\#\#}$) $P < 0.01$ and ($^{\#}$) $P < 0.05$ (Two-way ANOVA followed by the Newman-Keul's test).

Fig. 5. (*m*-CF_3-PhSe)$_2$ treatment restored the impaired levels of NMDA receptor subunits in hippocampus of morphine withdrawn-mice. Effects of (*m*-CF_3-PhSe)$_2$ treatment on NMDA receptor subunits NR2A (A) and NR2B (B) in hippocampus of morphine withdrawn-mice. Data are expressed as mean ± S.E.M of 5 animals per group. Asterisk denotes significance levels when compared with the vehicle group: (***) $P < 0.001$. Hashtag denotes significance levels when compared with the morphine withdrawal group: ($^{\#\#\#}$) $P < 0.001$ (Two-way ANOVA followed by the Newman-Keul's test). Qualitative images of western blotting analyses represent one mouse of each group.

Fig. 6. (*m*-CF_3-PhSe)$_2$ treatment and morphine withdrawal did not affect hippocampal neural plasticity markers. Effect of (*m*-CF_3-PhSe)$_2$ treatment on protein contents of *m*BDNF (A), TrkB (B), *p*-ERK/ERK (C) and *p*-CREB/CREB (D) in the hippocampus of morphine withdrawn-mice. Values are expressed as mean ± S.E.M of 5 animals per group. The results were analyzed by two-way ANOVA. The images represent qualitative Western blotting analyses of one mouse of each group.

Fig. 7. (*m*-CF_3-PhSe)$_2$ treatment had neuroprotective effects on hippocampus of morphine withdrawn-mice. Effect of (*m*-CF_3-PhSe)$_2$ treatment on protein levels of pro-BDNF (A), p-75^{NTR}(B) and *p*-JNK/JNK ratio (C) in the hippocampus of morphine withdrawn-mice. Data are expressed as mean ± S.E.M of 5 animals per group. Asterisk denotes significance levels

when compared with the vehicle group: (**) $P < 0.01$ and (*) $P < 0.05$. Hashtag denotes significance levels when compared with the morphine withdrawal mice: ($^{\#\#}$) $P < 0.01$ and ($^{\#}$) $P < 0.05$ (Two-way ANOVA followed by the Newman-Keul's test). The images represent qualitative Western blotting analyses of one mouse of each group.

Fig. 8. Summary of (*m*-CF_3-PhSe)$_2$ effects on neurochemical and behaviors adaptations in morphine withdrawn-mice.

Figures

Fig. 1

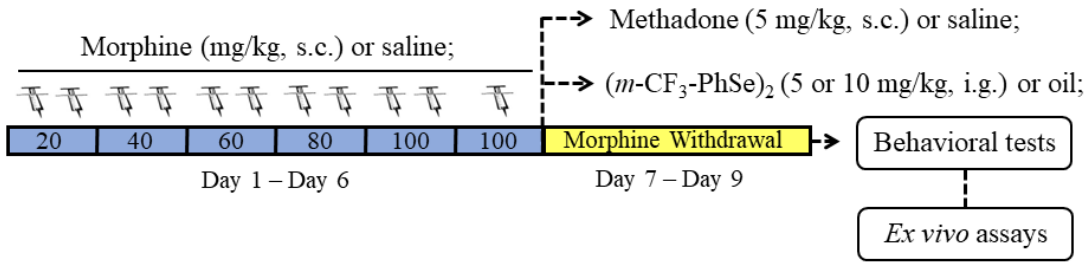

Fig. 2

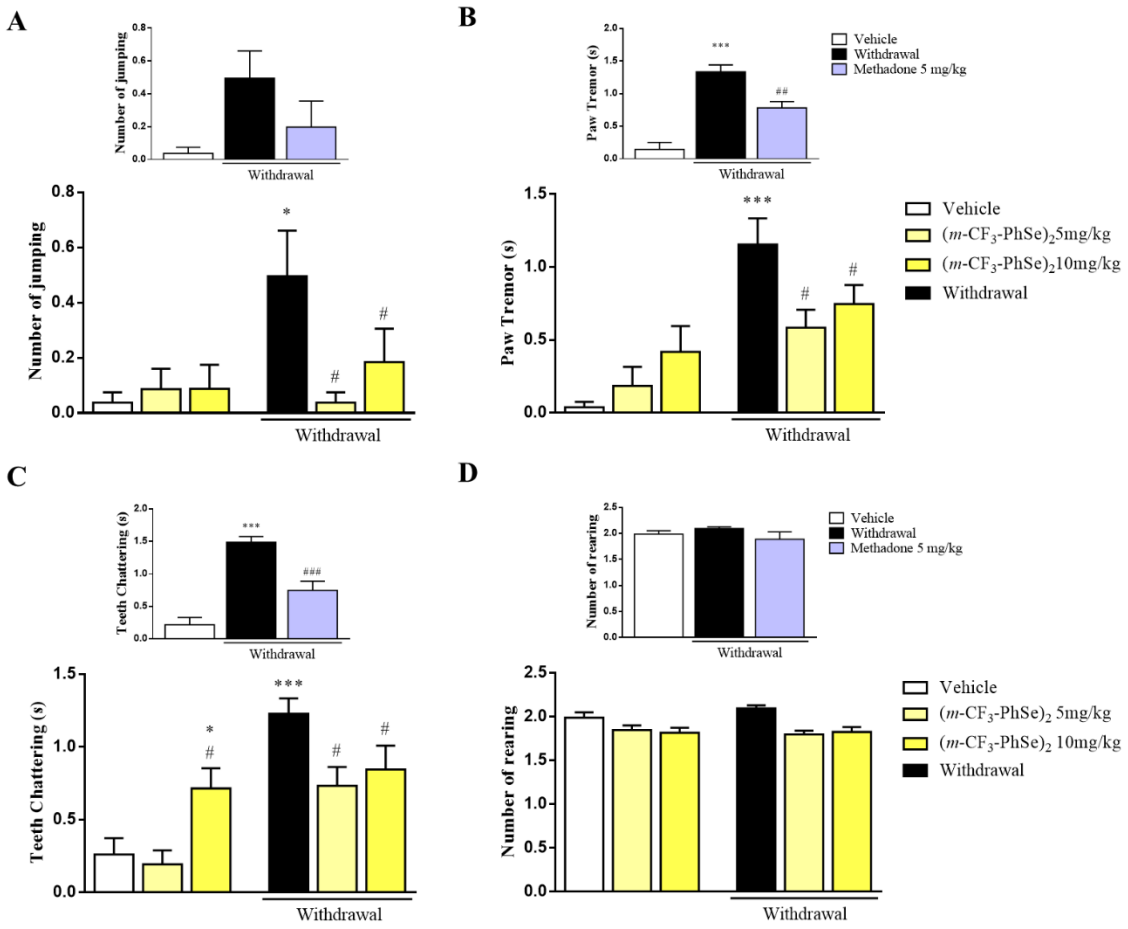
A
Number of jumping
Withdrawal
Vehicle
Withdrawal
Methadone 5 mg/kg
B
Paw Tremor (s)
Withdrawal
Vehicle
(m-CF3-PhSe)2 5mg/kg
(m-CF3-PhSe)2 10mg/kg
Withdrawal
C
Teeth Chattering (s)
Withdrawal
D
Number of rearing
Withdrawal

Fig. 3

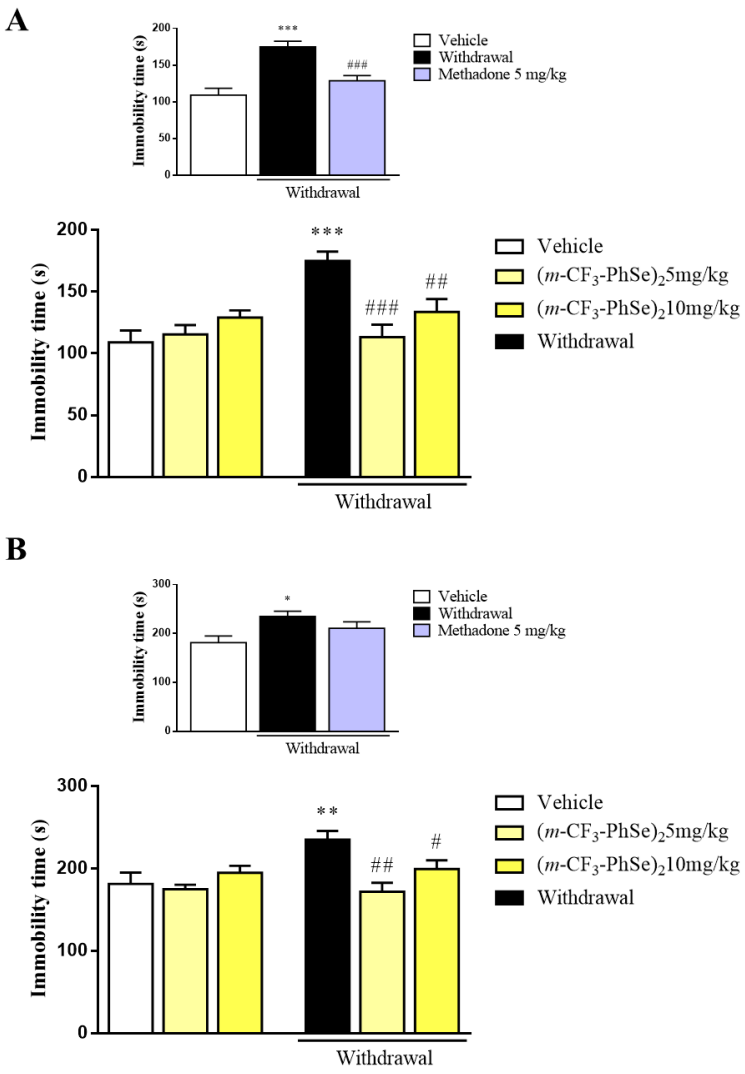
A
Immobility time (s)
200
150
100
50
0
***
###
Vehicle
Withdrawal
Methadone 5 mg/kg
Withdrawal
Immobility time (s)
200
150
100
50
0
***
###
##
Vehicle
(m-CF3-PhSe)2 5mg/kg
(m-CF3-PhSe)2 10mg/kg
Withdrawal
Withdrawal
B
Immobility time (s)
300
200
100
0
*
Vehicle
Withdrawal
Methadone 5 mg/kg
Withdrawal
Immobility time (s)
300
200
100
0
**
##
#
Vehicle
(m-CF3-PhSe)2 5mg/kg
(m-CF3-PhSe)2 10mg/kg
Withdrawal
Withdrawal

Fig. 4

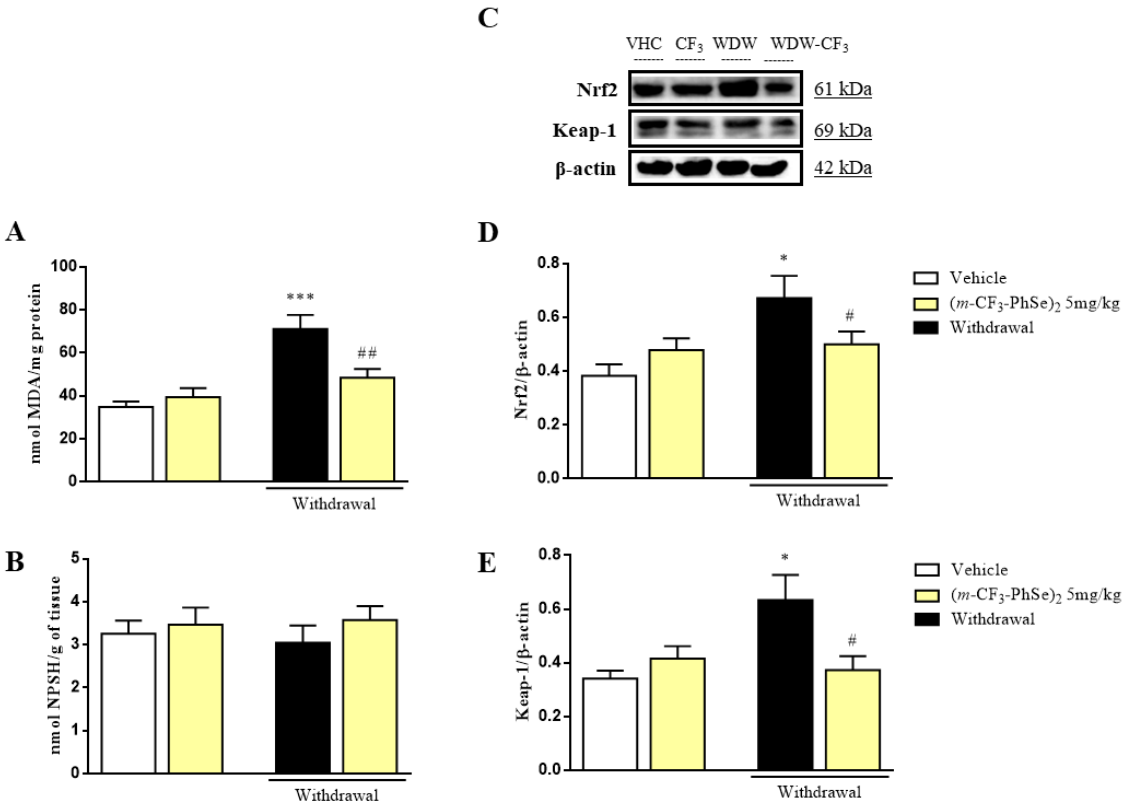
C
VHC
CF3
WDW
WDW-CF3
Nrf2
61 kDa
Keap-1
69 kDa
β-actin
42 kDa
A
nmol MDA/mg protein
***
##
Withdrawal
D
Nrf2/β-actin
*
#
Withdrawal
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal
B
nmol NPSH/g of tissue
Withdrawal
E
Keap-1/β-actin
*
#
Withdrawal
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal

Fig. 5

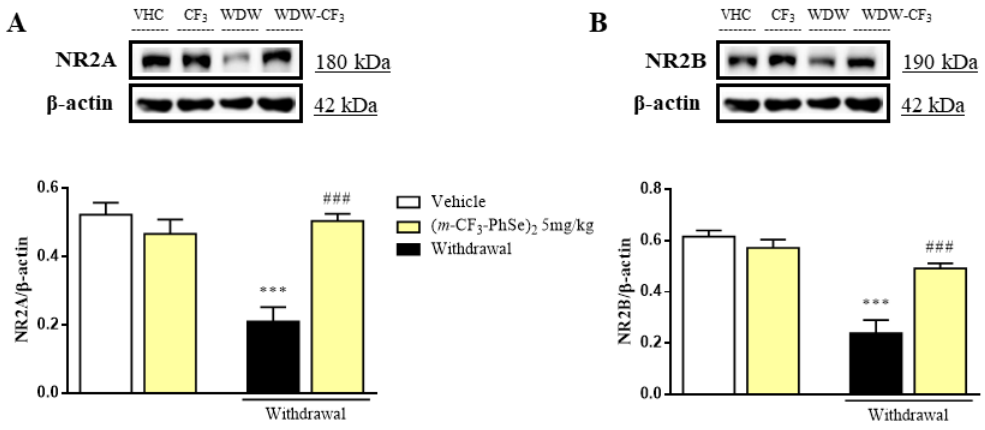
A
VHC
CF3
WDW
WDW-CF3
NR2A
180 kDa
β-actin
42 kDa
NR2A/β-actin
0.6
0.4
0.2
0.0
###
***
Withdrawal
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal
B
VHC
CF3
WDW
WDW-CF3
NR2B
190 kDa
β-actin
42 kDa
NR2B/β-actin
0.8
0.6
0.4
0.2
0.0
###
***
Withdrawal

Fig. 6

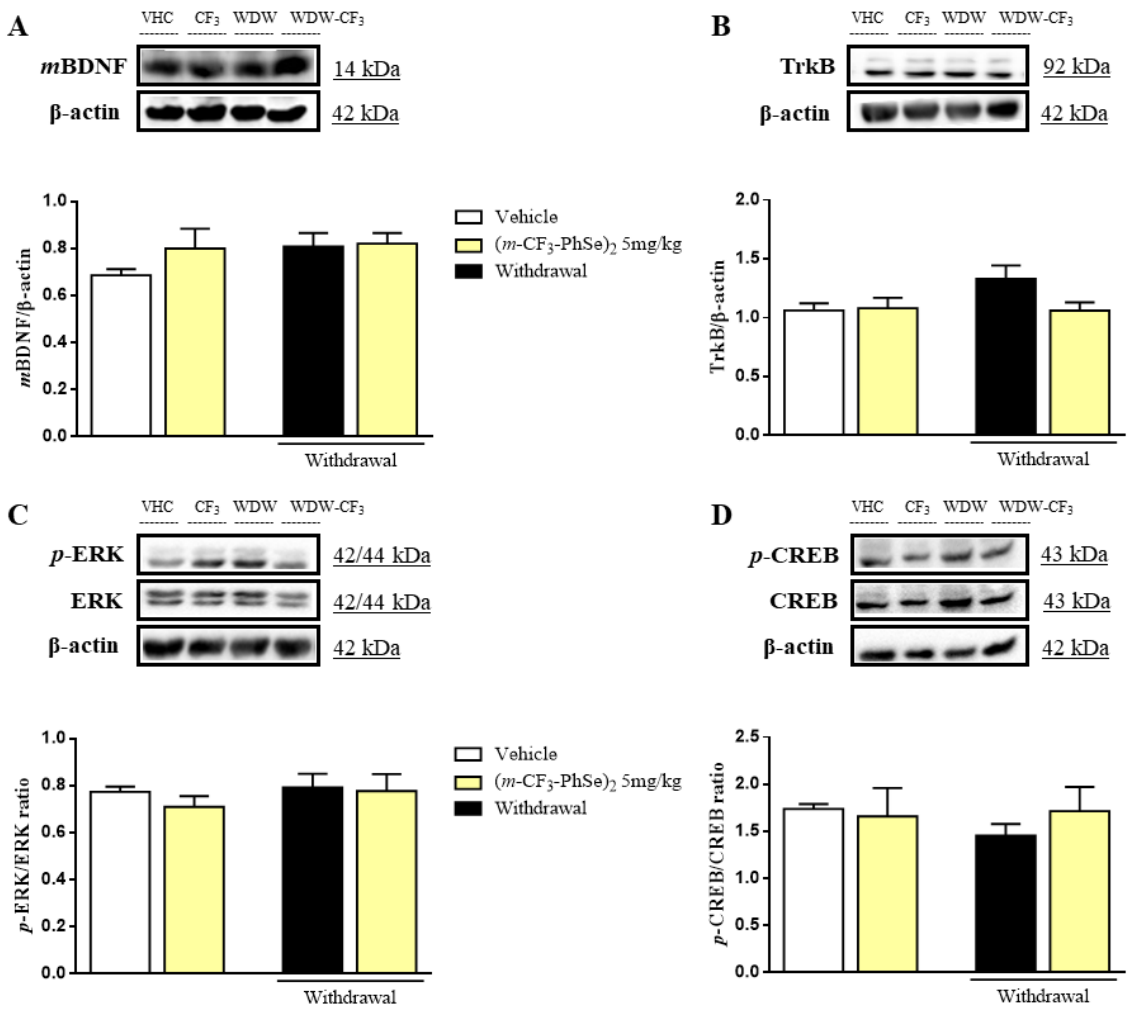
A
VHC CF3 WDW WDW-CF3
mBDNF
14 kDa
β-actin
42 kDa
mBDNF/β-actin
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal
B
VHC CF3 WDW WDW-CF3
TrkB
92 kDa
β-actin
42 kDa
TrkB/β-actin
Withdrawal
C
VHC CF3 WDW WDW-CF3
p-ERK
42/44 kDa
ERK
42/44 kDa
β-actin
42 kDa
p-ERK/ERK ratio
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal
D
VHC CF3 WDW WDW-CF3
p-CREB
43 kDa
CREB
43 kDa
β-actin
42 kDa
p-CREB/CREB ratio
Withdrawal

Fig. 7

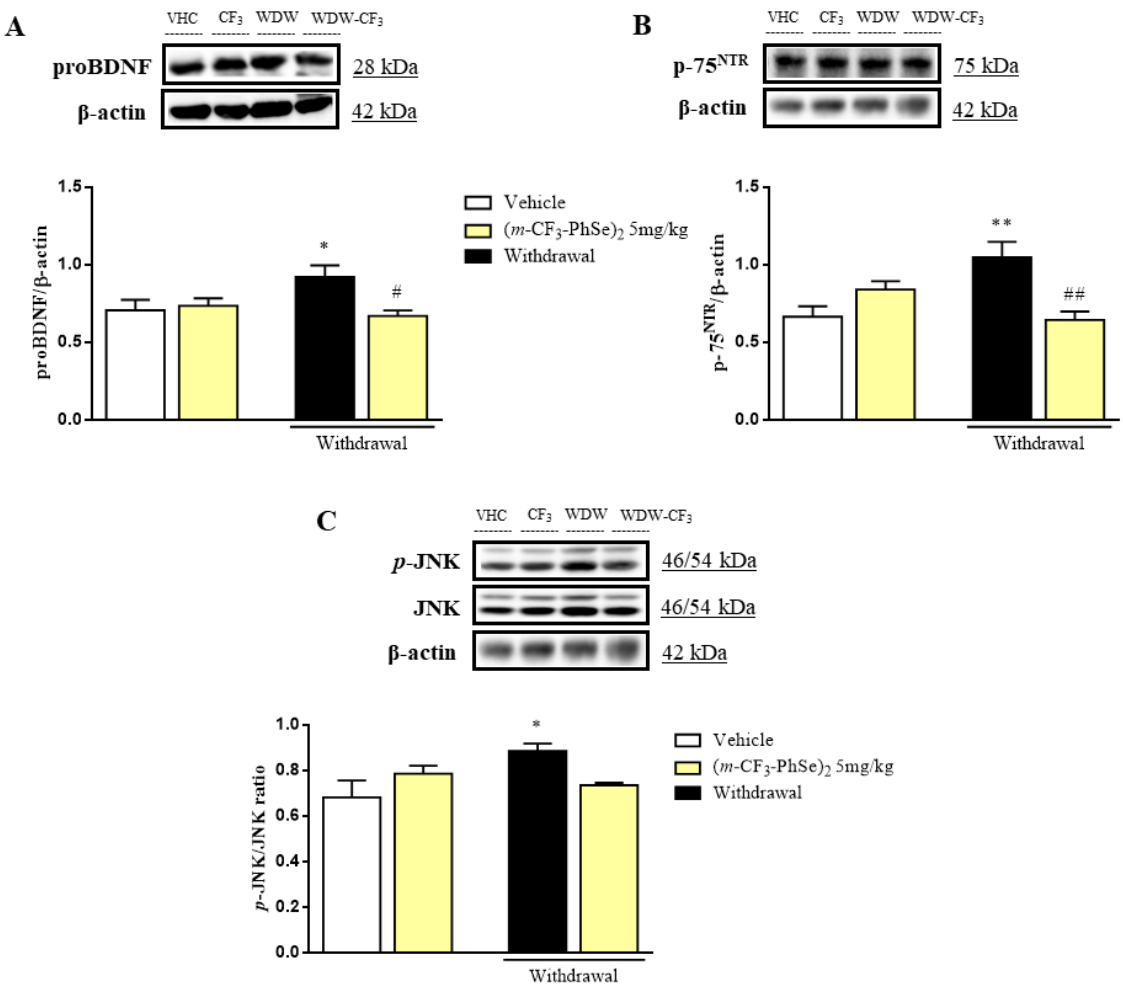
A
VHC
CF3
WDW
WDW-CF3
proBDNF
28 kDa
β-actin
42 kDa
proBDNF/β-actin
1.5
1.0
0.5
0.0
*
#
Withdrawal
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal
B
VHC
CF3
WDW
WDW-CF3
p-75NTR
75 kDa
β-actin
42 kDa
p-75NTR/β-actin
1.5
1.0
0.5
0.0
**
##
Withdrawal
C
VHC
CF3
WDW
WDW-CF3
p-JNK
46/54 kDa
JNK
46/54 kDa
β-actin
42 kDa
p-JNK/JNK ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
Withdrawal
Vehicle
(m-CF3-PhSe)2 5mg/kg
Withdrawal

Fig. 8

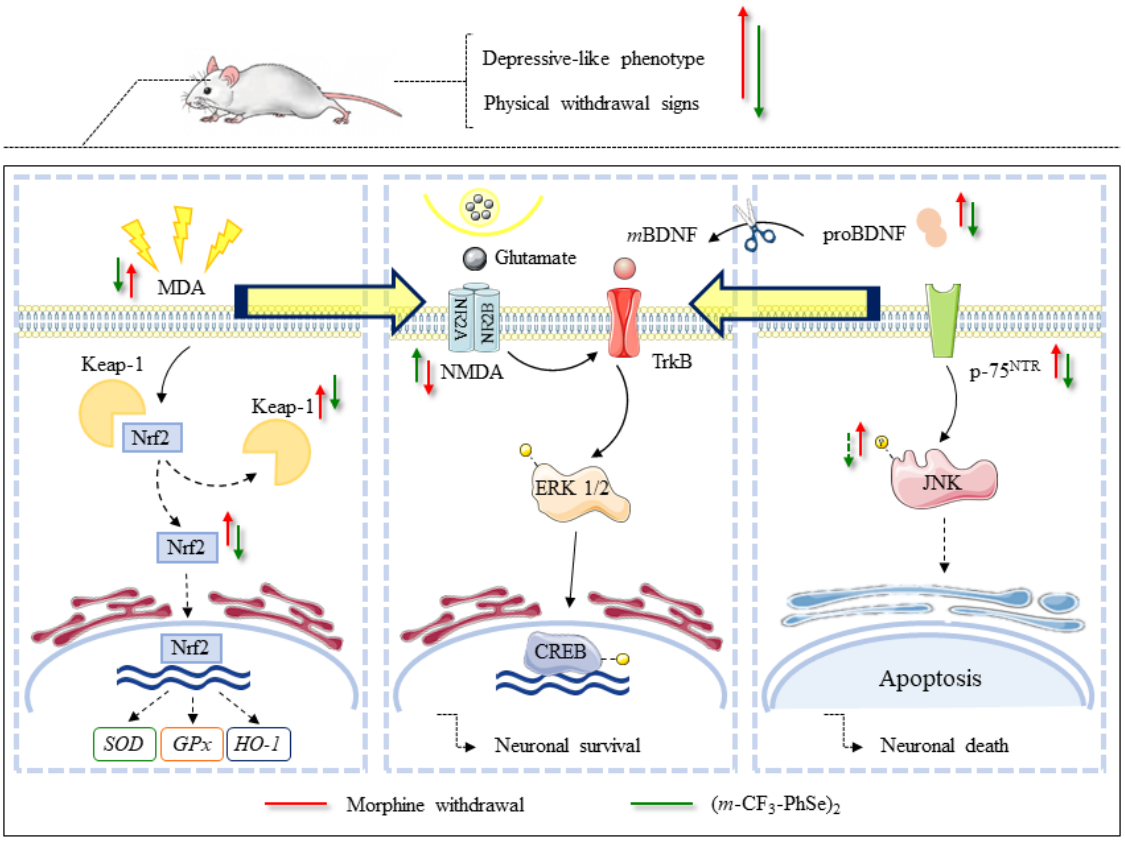
Depressive-like phenotype
Physical withdrawal signs
MDA
Keap-1
Nrf2
Keap-1
Nrf2
Nrf2
SOD
GPx
HO-1
Glutamate
NR2A
NR2B
NMDA
mBDNF
proBDNF
TrkB
p-75NTR
ERK 1/2
JNK
CREB
Apoptosis
Neuronal survival
Neuronal death
Morphine withdrawal
(m-CF3-PhSe)2

Table 1: List of primary antibodies and their properties.

Antibody	Molecular weight	Type	Company	Dilution
Nrf2	61 kDa	Rabbit	Santa Cruz Biotechnology	1:1000
Keap-1	69kDa	Goat	Santa Cruz Biotechnology	1:1000
NMDAR2A	180kDa	Rabbit	CellSignaling Technology	1:1000
NMDA2RB	190kDa	Rabbit	CellSignaling Technology	1:1000
*m*BDNF	28kDa	Rabbit	Abcam	1:1000
TrkB	92kDa	Rabbit	Abcam	1:1000
p-ERK	42/44kDa	Rabbit	CellSignaling Technology	1:1000
ERK	42/44kDa	Rabbit	CellSignaling Technology	1:1000
p-CREB	43kDa	Rabbit	CellSignaling Technology	1:1000
CREB	43kDa	Rabbit	CellSignaling Technology	1:1000
proBDNF	14kDa	Rabbit	Abcam	1:1000
p-75^{NTR}	75kDa	Rabbit	CellSignaling Technology	1:1000
p-JNK	46/54kDa	Mouse	CellSignaling Technology	1:1000
JNK	46/54kDa	Mouse	CellSignaling Technology	1:1000
β-actina	42kDa	Mouse	CellSignaling Technology	1:5000

Nrf2 (nucleus factor erythroid 2-related factor2); Keap-1 (kelch-like ECH associated protein 1); NMDAR 2A (N-methyl-D-aspartate receptor subunit 2A); NMDAR 2B (N-methyl-D-aspartate receptor subunit 2B); BDNF (brain-derived neurotrophic factor); TrkB (tropomyosin receptor kinase B); ERK (extracelular signal-regulated kinase); CREB (cAMP response elemento-binding protein); p-75^{NTR} (p75 neurotrophin receptor); JNK (c-Jun amino terminal kinase).

Table 2: Effect of (*m*-CF_3-PhSe)$_2$ or methadone treatment on latency for the first episode of immobility in mice.

Groups	**Latency to immobility (s)**	
	TST	FST
Vehicle	79.9 ± 10.8	69.6 ± 2.9
(*m*-CF_3-PhSe)$_2$ 5 mg/kg	47.4 ± 8.9	73.1 ± 6.1
(*m*-CF_3-PhSe)$_2$ 10 mg/kg	98.4 ± 13.0	68.5 ± 3.5
Withdrawal	49.0 ± 10.5	71.3 ± 5.8
Withdrawal - (*m*-CF_3-PhSe)$_2$ 5 mg/kg	94.1 ± 14.0$^{\#}$	65.1 ± 3.7
Withdrawal - (*m*-CF_3-PhSe)$_2$ 10 mg/kg	55.1 ± 7.2	69.4 ± 4.3
Withdrawal - Methadone 5 mg/kg	95.8 ± 11.5$^{\#}$	62.8 ± 3.0

Data are expressed as means ± S.E.M for 8 animals per group. In order to determine the effects of (*m*-CF_3-PhSe)$_2$ or methadone treatment on morphine-withdrawn-mice, data were analyzed by two-way and one-way ANOVA respectively, followed Newman-Keul's multiple comparison test when appropriate. (#) denotes $P < 0.05$ when compared to morphine withdrawal mice.

Table 3: Spontaneous locomotor activity of mice underwent morphine withdrawal and (*m*-CF_3-PhSe)$_2$ or methadone treatments.

Groups	**Spontaneous Locomotor Activity**		
	Crossing[a]	*Distance*[b]	*Speed*[c]
Vehicle	619.4 ± 37.8	11033.0 ± 487.0	46.6 ± 2.1
(*m*-CF_3-PhSe)$_2$ 5 mg/kg	619.4 ± 35.6	11077.0 ± 726.4	47.7 ± 2.9
(*m*-CF_3-PhSe)$_2$ 10 mg/kg	691.5 ± 33.3	10344.0 ± 576.0	44.0 ± 2.6
Withdrawal	550.6 ± 33.7	9101.1 ± 537.5	38.7 ± 2.3
Withdrawal - (*m*-CF_3-PhSe)$_2$ 5 mg/kg	530.4 ± 49.9	8753.0 ± 984.0	39.6 ± 3.8
Withdrawal - (*m*-CF_3-PhSe)$_2$ 10 mg/kg	580.3 ± 19.8	9066.4 ± 690.9	39.3 ± 2.2
Withdrawal - Methadone 5 mg/kg	627.3 ± 116.3	13648.2 ± 3065.0	57.6 ± 9.7

Data are reported as means ± S.E.M. for 8 animals per group. In order to determine the effects of (*m*-CF_3-PhSe)$_2$ or methadone treatment on morphine withdrawn-mice, statistical analyses were performed by two-way and one-way ANOVA respectively, followed Newman-Keul's multiple comparison test when appropriate.

[a] Data are expressed as number of crossings;

[b] Data are expressed as mm;

[c] Data are expressed as mm/s.

4 CONCLUSÃO

Os dados deste estudo demonstraram que a retirada da morfina à curto prazo induziu a síndrome de abstinência, caracterizada pela manifestação dos sinais físicos aversivos e pelo fenótipo do tipo depressivo em camundongos. Tais adaptações comportamentais podem estar relacionadas aos eventos neurotóxicos desencadeados no hipocampo durante o estágio de abstinência, tais como o desequilíbrio redox, a redução nos níveis das subunidades de receptores NMDA e as alterações na plasticidade sináptica através da estimulação da via pró-apoptótica do proBDNF em relação a via neurotrófica do *m*BDNF. O tratamento com (*m*-CF_3-PhSe)$_2$ reverteu as adaptações moleculares e comportamentais induzidas durante a abstinência à morfina em camundongos. Os efeitos neuroprotetores do (*m*-CF_3-PhSe)$_2$, mediados especialmente pela propriedade antioxidante intrínseca deste composto, podem modular os processos adaptativos relacionados ao reforço negativo induzidos durante o estágio da abstinência e assim, atenuar os sinais físicos aversivos e o fenótipo do tipo depressivo durante a retirada da morfina em camundongos.

5 REFERÊNCIAS BIBLIOGRÁFICAS

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ANEXO A - CARTA DE APROVAÇÃO DO PROJETO DE PESQUISA PELA COMISSÃO DE ÉTICA NO 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 "Estudo da ação do disseleneto de m-trifluormetil difenila (m-CF3-PhSe)2 em modelos experimentais de dependência, tolerância e depressão induzidas por morfina em camundongos.", protocolada sob o CEUA nº 8756060317, sob a responsabilidade de **Gilson Rogério Zeni** - 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 05/04/2017.

We certify that the proposal "Study of the m-trifluoromethyl diphenyl diselenete (m-CF3-PhSe)2 action on experimental models of morphine-induced dependence, tolerance and depression in mice.", utilizing 240 Heterogenics mice (240 males), protocol number CEUA 8756060317, under the responsibility of **Gilson Rogério Zeni** - 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 04/05/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2017 a 04/2018 Área: Bioquímica E Biologia Molecular

Origem:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	8 a 10 semanas	N:	240
Linhagem:	Swiss			Peso:	25 a 35 g		

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

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
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