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MODULAÇÃO DO PADRÃO CONVULSIVO E DAS ALTERAÇÕES CEREBRAIS E COMPORTAMENTAIS EM CRISES EPILÉPTICAS ATRAVÉS DO REPOSICIONAMENTO COM MENTANTINA

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Bioquímica.

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"O que sabemos é uma gota; o que ignoramos é um oceano." **Isaac Newton**

" – Você pode me ajudar?

– Sim, pois não.

– Para onde vai essa estrada?

– Para onde você quer ir?

– Eu não sei, estou perdida.

– Para quem não sabe para onde vai, qualquer caminho serve."

Lewis Carroll – Alice no país das maravilhas

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SUMÁRIO

RESUMO

A crise epiléptica prolongada é acompanhada por neurodegeneração, alterações nos biomarcadores relacionados ao dano celular e prejuízos comportamentais, e os antiepilépticos clássicos geralmente não são eficazes em proteger contra estas consequências. O reposicionamento de fármacos, com foco em um medicamento com ação multialvo, é uma solução viável para lidar com a epilepsia refratária e pode fornecer uma alternativa para o gerenciamento das consequências indesejáveis das crises. Antagonistas do receptor de N-metil-D-aspartato exibem ação anticonvulsivante e atuam como neuroprotetores. No entanto, estes compostos demonstraram muitos efeitos adversos inaceitáveis. Apesar disso, a memantina (MN), um antagonista do NMDAR de baixa afinidade, é clinicamente bem tolerado e não produz efeitos colaterais consideráveis. Portanto, o objetivo da presente tese foi investigar o efeito protetor da MN contra a degeneração neuronal, padrão convulsivo, marcadores neuroquímicos e alterações comportamentais a longo prazo, induzidos por modelos químicos de crises prolongadas. Ratos Wistar filhotes receberam MN (20 mg / kg i.p.) em diferentes regimes de tratamento antes e após a indução da crise no modelo de LiCl-pilocarpina. A neurodegeneração foi quantificada por fluoro jade C 24 horas após a crise. Os peixes-zebra foram pré-tratados com veículo ou MN (20 ou 50 mg / kg; i.p.) 1 h ou 2 h antes da imersão em solução de PTZ a 10 mM. As análises comportamentais e neuroquímicas foram realizadas 24 horas após a crise. Em CA1, a neurodegeneração diminuiu significativamente quando os ratos jovens receberam MN nos tempos de 0h e 0,25 h. Além disso, o pós-tratamento foi capaz de proteger contra a neurodegeneração na amigdala. O pré-tratamento com MN mostrou uma redução na morte celular no tálamo nos roedores. Já em peixe-zebra, a administração de MN reduziu a expressão relativa do gene grin2A. As crises induzidas com PTZ aumentam a carbonilação de proteínas e a atividade da SOD, e o tratamento com MN impediu essa alteração no estado redox. O peixe-zebra submetido às crises com PTZ apresentou aumento no tempo gasto na zona branca do aparato claro / escuro e a MN preveniu essa anormalidade comportamental. Nossos resultados demonstraram o efeito neuroprotetor da MN na morte neuronal induzida pelas crises no início da vida em ratos. Este efeito foi dependente do tempo e da região cerebral estudada. De fato, a ação anticonvulsivante da MN previne as alterações comportamentais e neuroquímicas relacionadas ao PTZ em peixe-zebra, reforçando o seu uso *off-label* para tratar as consequências neurocomportamentais da epilepsia.

ABSTRACT

Prolonged epileptic seizure is accompanied by neurodegeneration, damage-related biomarkers alterations and impaired behavior, and the classical antiepileptic drugs are not effective in protect these consequences. Repurposing with focus in a multi-target drug is a viable solution to deal with refractory epilepsy and may provide an alternative for management of undesirable consequences of seizures. Typical N-methyl-D-aspartate receptor antagonists exhibit anticonvulsant action and are currently able to provide neuroprotection against neuronal death. However, these compounds have demonstrated many side effects. Despite this, memantine (MN), a low-affinity NMDA antagonist, is clinically well tolerate and does not induce considerable side effects. Therefore, the aim of the present thesis was to investigate the protectives effect of the MN against neuronal degeneration, seizures pattern, neurochemical seizure markers and long-term behavioral alterations induced by chemical models of prolonged seizures. Wistar pup rats received MN (20 mg/kg i.p.) at different treatment regimens before and after seizure induction in Li-pilocarpine model. Neurodegeneration was quantified by fluoro jade C staining. Zebrafish were pre-treated with vehicle or MN (20 or 50 mg/kg; i.p.) 1h or 2h before immersion into PTZ solution at 10 mM. Behavioral and neurochemical analyses was performed 24 h after seizure. In CA1 subfield neurodegeneration was significantly decreased when animals received MN at 0h and 0.25h post-treatment regimens. Additionally, post-treatment was able to protect against neurodegeneration in amygdala region. MN pre-treatment showed a reduction of cell death in thalamus. MN reduced the relative grin2A gene expression. PTZ-seizure induction increase protein carbonylation and SOD activity, and MN treatment prevented this state redox imbalance. PTZ-challenged zebrafish presented increases in time spent in white zone into light/dark apparatus and MN prevented this behavioural abnormality. Our results demonstrated the neuroprotective effect of MN on neuronal loss induced by seizure early in life. This effect was time- and region-dependent. Indeed, anticonvulsant action of MN ameliorates behavioral and neurochemical PTZ-related changes in zebrafish seizure model, reinforcing the off-label use to treat neurobehavioral consequences of epilepsy.

Lista de abreviaturas

AMPARs – receptores amino-3-hidroxi-S-metil-isoxazol-4-propionato

GABA – ácido γ-aminobutírico

GD – giro denteado

iGluRs – receptores glutamatérgicos ionotrópicos

ILAE – International League Against Epylepsy

KARs – receptores cainato

mGluRs – receptores glutamatérgicos metabotrópicos

MN – Memantina

NMDARs – receptores N-metil-D-aspartato

SE – Status epilepticus

SNC – sistema nervoso central

PARTE I - INTRODUÇÃO

1. Epilepsias

As epilepsias são uma condição neurológica que afeta 2 a 3 % da população mundial, definindo-se como um distúrbio crônico do sistema nervoso central (SNC) e caracterizada por crises epilépticas recorrentes e espontâneas (Abramovici and Bagić, 2016; Engel and International League Against Epilepsy (ILAE), 2001; Fisher et al., 2014). Ela acomete pessoas de todas as faixas etárias, sendo mais prevalente em crianças e idosos (Abramovici and Bagić, 2016). Nos Estados Unidos são gastos anualmente cerca de 15,5 bilhões de dólares para o tratamento de pacientes epilépticos, e estima-se uma prevalência de 50 milhões de pacientes no mundo e 2,4 milhões de novos casos ao ano (Theodore et al., 2006). Os indivíduos com epilepsia apresentam inúmeros prejuízos neurobiológicos, tais como morte neuronal, alterações comportamentais, prejuízos na memória e aprendizado, entre outros. Desta forma, a importância médica das epilepsias se relaciona principalmente às consequências psicopatológicas e sociais (Fisher, 2015a).

O tratamento das epilepsias é fundamentalmente realizado através de terapia medicamentosa. A escolha do medicamento é complexa, e sem dúvida, o tipo de crise ou síndrome epiléptica é crucial nessa decisão. Ciente dessa importância, a *International League Against Epylepsy* (ILAE), órgão internacional que congrega profissionais voltados para o desenvolvimento de conhecimento e tratamento da epilepsia, buscou nos últimos anos aperfeiçoar a sua caracterização clínica (Fisher et al., 2014; Fisher, 2015a). Uma crise epiléptica é o resultado de uma descarga elétrica excessiva, anormal e geralmente hipersincrônica de um grupo de neurônios, que podem estar localizados em qualquer região cerebral. As manifestações clínicas destas crises estão relacionadas com a região onde a descarga se inicia e da sua possível propagação para áreas cerebrais adjacentes. Suas causas podem ser passíveis de identificação, como nos casos de tumores, trauma crânio-encefálicos, disfunção metabólica, infecção e doença vascular. Entretanto, em muitos casos, as causas destas crises são de etiologia desconhecida e com dificuldades para a escolha do melhor regime terapêutico (Engel, 2011; Fisher et al., 2014; Trinka et al., 2015).

Apesar dos avanços dos exames complementares em todas as especialidades médicas, o diagnóstico das epilepsias é primariamente clínico. O histórico, os exames físicos e neurológicos conduzem ao diagnóstico, enquanto os exames complementares confirmam e auxiliam na definição dos diagnósticos etiológicos e sindrômicos. Assim, a

caracterização da crise epiléptica é crucial para definir diagnóstico, tratamento e prognóstico. Em vias gerais, no que se refere à localização das crises epilépticas, elas podem ser classificadas em dois grandes grupos: crises epilépticas focais, que se restringem a uma região específica do SNC a qual origina e mantêm a crise epiléptica; e crises epilépticas generalizadas, nas quais ambos hemisférios cerebrais apresentam hiperatividade, acometendo o cérebro em sua totalidade (Berg and Millichap, 2013). As crises epilépticas do tipo tônico-clônicas são um exemplo do segundo grupo, caso em que o paciente alterna entre episódios de tonia e extensão muscular com contrações musculares involuntárias (Fisher et al., 2014; Panayiotopoulos, 2011). O *Status epilepticus* (*SE*) por sua vez é caracterizado por uma crise epiléptica generalizada de longa duração (Trinka et al., 2015) ou várias crises sucessivas sem que haja recuperação da consciência entre uma crise e outra. Indivíduos acometidos pelo *SE* durante a infância podem apresentar, na idade adulta, alterações no comportamento emocional, esclerose hipocampal, déficit cognitivo e outras complicações tais como comorbidades psiquiátricas, com altos índices de evolução para epilepsia do lobo temporal na vida adulta (Fisher, 2015b; Hingray et al., 2019; Stewart et al., 2019; Tellez-Zenteno et al., 2007).

Outra classificação ocorre mediante a presença (sintomática) ou ausência (criptogênica) de etiologia definida (Berg and Millichap, 2013; Engel, 2011). Os dados epidemiológicos apontam que 60% dos pacientes epilépticos possuem epilepsia criptogênica. Além disso, 30% dos indivíduos epilépticos são refratários aos tratamentos farmacológicos disponíveis (Xiao et al., 2015) e 75% vivem em países em desenvolvimento e não possuem acesso a tratamentos adequados (Nwani et al., 2013). Dessa forma, a busca por novos tratamentos mais acessíveis e capazes de solucionar a refratariedade e a falha terapêutica (Newton and Garcia, 2012) impulsiona estratégias inovadoras de pesquisa, tais como, a busca por novos modelos animais que mimetizem as consequências das crises, bem como a diversificação das abordagens para a escolha de novos potenciais candidatos à terapia.

As epilepsias humanas englobam uma infinidade de manifestações clínicas, comportamentais e elétricas, caracterizando-se como um distúrbio multifatorial. Dessa forma, estudar esta condição neurológica em animais não humanos possibilitou a criação de uma série igualmente ampla de modelos animais; isto é, espécies e protocolos de indução de crises agudas ou crônicas dos mais variados, a fim de sanar as limitações um

do outro (Grone and Baraban, 2015). Neste contexto, a utilização de modelos animais em epilepsia auxilia na compreensão dos mecanismos de epileptogênese e suas implicações em longo prazo, bem como na descoberta de novos medicamentos. Por exemplo, permitem avaliações morfológicas como: verificação da redução do volume hipocampal, amigdalar e do córtex perirrinal de ratos que foram induzidos ao SE aos 12 dias de vida (Nairismagi et al., 2006); presença massiva de morte neuronal no núcleo mediodorsal do tálamo em crises convulsivas prolongadas (Kubová et al., 2001); e a relação de muitas dessas alterações com prejuízos cognitivos na vida adulta (de Oliveira et al., 2008; Loss et al., 2012a; Mussulini et al., 2018). Além disso, as crises epilépticas podem ser estudadas em modelos genéticos e/ou modelos não genéticos, podendo-se fazer uso de mais de um modelo a depender da pergunta do estudo (Grone and Baraban, 2015; Todorova et al., 2000).

Os modelos não genéticos de crises epilépticas se baseiam na administração de compostos pró-convulsivos, os quais alteram o balanço entre os sistemas excitatórios e inibitórios do SNC (Leke and Schousboe, 2016; Zhou and Danbolt, 2014). A pilocarpina, um agonista colinérgico, quando administrada em alta dose, induz uma crise epiléptica causada, inicialmente, pela ação estimulatória da pilocarpina sobre o sistema colinérgico, enquanto a manutenção da crise se deve a ativação do sistema glutamatérgico, caracterizando um modelo de SE (McDonough and Shih, 1997). Este modelo foi aprimorado por Clifford e colaboradores (1987), os quais demonstraram que um pré-tratamento com cloreto de lítio (LiCl) potencializa a ação epileptogênica da pilocarpina, possibilitando uma redução na dose administrada, o que diminui consideravelmente os efeitos colinérgicos periféricos e consequentemente a mortalidade, sem alterar, no entanto, o padrão convulsivo. O composto pentilenotetrazol (PTZ) inibe os receptores ionotrópicos do tipo $GABA_A$, reduzindo a capacidade do sistema inibitório, resultando em hiperexcitabilidade que culmina na geração das crises epilépticas agudas ou prolongadas. O PTZ é o agente pró-convulsivo mais utilizado na indústria farmacêutica para o desenvolvimento de novas terapias (Baraban et al., 2005). O modelo animal ideal deve apresentar os mecanismos causais da patologia, o perfil fenotípico da crise e a resposta anticonvulsiva frente a tratamentos clínicos já validados (Grone and Baraban, 2015). Assim, ambos agentes pró-convulsivantes possuem semelhanças cruciais para o estudo das epilepsias, porém mecanismos distintos para iniciação e propagação das crises,

bem como desfechos neuroquímicos e comportamentais de longo prazo diferentes um do outro.

1.1. Sistema glutamatérgico, neurotoxicidade e crises epilépticas

O glutamato é um aminoácido distribuído amplamente no cérebro de mamíferos e desempenha inúmeras funções essenciais em diversas regiões encefálicas, particularmente como um neurotransmissor excitatório e precursor do neurotransmissor inibitório ácido γ-aminobutírico, o GABA (Sanacora et al., 2008). O sistema glutamatérgico é o principal mecanismo de neurotransmissão excitatório no cérebro de mamíferos e regula inúmeros eventos fisiológicos, tais como: memória e aprendizado (Izquierdo and Medina, 1997), desenvolvimento e envelhecimento (Segovia et al., 2001), proliferação e migração celular (McDonald and Johnston, 1990). Seu papel crucial na fisiologia cerebral faz com que distúrbios na homeostase glutamatérgica estejam envolvidos em diversas patologias do SNC, sendo responsáveis por alterações comportamentais, eventos de morte celular, dentre outros (Matsui et al., 2005; Nedergaard et al., 2002; Pál, 2018). Para que a neurotransmissão glutamatérgica ocorra, as vesículas sinápticas contendo glutamato liberam o seu conteúdo na fenda sináptica e o neurotransmissor liga-se aos receptores expressos na membrana neuronal: receptores do tipo ionotrópicos (iGluRs) e/ou metabotrópicos (mGluRs). Por isso, é a concentração de glutamato no fluido extracelular circundante que determina a extensão da estimulação dos receptores e por conseguinte seu efeito fisiológico ou tóxico (Yin and Niswender, 2014; Zhou and Danbolt, 2014).

Os iGluRs compreendem três tipos de receptores: cainato (KARs), amino-3-hidroxi-S-metil-isoxazol-4-propionato (AMPARs) e N-metil-D-aspartato (NMDARs). Diferentemente dos AMPARs e KARs que são permeáveis majoritariamente a Na^{+2}, os NMDARs são canais dependentes de voltagem altamente permeáveis a Ca^{+2} e possuem como co-agonistas glicina e D-serina (Henneberger et al., 2013; Johnson and Ascher, 1987; Kemp and Leeson, 1993; Mothet et al., 2000). Estes receptores têm um papel central na plasticidade sináptica, com efeitos diretos na potenciação de longa duração e na depressão de longa duração. Em contrapartida, a hiperestimulação de NMDARs está diretamente relacionada a um evento patofisiológico denominado de excitotoxicidade neurodegenerativa (Mehta et al., 2013).

Há cerca de cinco décadas surgiu a primeira evidência de que o glutamato possui efeitos tóxicos para o SNC, podendo ser capaz de causar a morte de células da retina (Lucas and Newhouse, 1957). Posteriormente, John Olney (1969) propôs o termo excitotoxicidade e demonstrou que o excesso de glutamato desencadeia um fenômeno nas células neuronais dependente do influxo de íons Ca^{+2} (Choi, 1987) através de NMDARs (Choi et al., 1988). Atualmente, a modulação da neurotransmissão mediada pelos NMDARs tem importantes implicações na origem da lesão e na morte celular observadas em um amplo espectro de condições neurológicas diferentes, incluindo acidente vascular cerebral, hipóxia, isquemia, doença de Alzheimer, e epilepsias (Flores-Soto et al., 2012; Hardingham, 2009). Estas condições compartilham características patognomônicas, incluindo perda neuronal gradual e seletiva, principalmente devido à hiperatividade de NMDARs, com um aumento na concentração de Ca^{+2} citosólico e a geração de espécies reativas de oxigênio (Betzen et al., 2009; Di Maio et al., 2011; Flores-Soto et al., 2012). Os NMDARs, assim como demais iGluRs, são complexos heteromultiméricos, os quais são constituídos por quatro subunidades formadoras do poro do canal, permitindo assim, a passagem de íons. Foram identificadas 3 famílias de subunidades dos NMDARs: NR1, NR2 e NR3 (recentemente renomeados, GluN1, GluN2 e GluN3, respectivamente) (Paoletti, 2011). Tipicamente os NMDARs são formados por duas subunidades constitutivas GluN1 e duas regulatórias subunidades GluN2 (Dingledine et al., 1999; Watkins and Jane, 2009). Menos comumente, os NMDARs podem conter subunidades GluN3. Desta forma, levando-se em consideração que a subunidade GluN1 é constitutiva e, portanto, presente em todos os NMDARs, existem diferentes combinações de subunidades ao longo das diferentes regiões cerebrais (Henson et al., 2010; Paoletti et al., 2013).

O glutamato desempenha um papel fundamental na iniciação e na propagação da atividade convulsiva. Sabe-se que a administração focal de glutamato em diferentes estruturas cerebrais, bem como a administração focal ou sistêmica de agonistas glutamatérgicos como NMDA, AMPA ou ácido caínico causam convulsões e alterações eletroencefalográficas em animais, e atividade epileptiforme em preparações de células e fatias *in vitro* (Chapman, 1998; Hayashi, 1952). Além disso, os NMDARs demonstraram estar intimamente relacionados ao processo de epileptogênese. Os antagonistas competitivos e não competitivos dos NMDARs inibem crises convulsivas *in vivo* e atividade epileptiforme em modelos *in vitro* (Gilbert, 1988; Manjarrez et al., 2001). Existe

uma hipótese de que os NMDARs estejam envolvidos com o desenvolvimento de da refratariedade em crises convulsivas, e que o tratamento com antagonistas NMDA seria efetivo em pacientes multirresistentes (Fang and Wang, 2015). Ademais, em modelos de *kindling* utilizando eletrochoque, a eficácia da transmissão sináptica excitatória de longa duração está intimamente relacionada com a concentração de glutamato extracelular, com a ativação dos NMDARs e com a inibição de receptores do tipo GABA (Babb et al., 1998; Morimoto, 1989; Ying et al., 1999). Também já foi demonstrado que existem mudanças na expressão das subunidades de NMDARs e AMPARs em roedores e humanos epilépticos (Babb et al., 1998; Doi et al., 2001; Mathern et al., 1998; Mikuni et al., 1999). Postnikova e colaboradores (2017), demonstraram que a alteração na expressão das subunidades do NMDAR depois do SE reduz a magnitude da potenciação de longa duração em sinapses hipocampais, e isto poderia explicar algumas das alterações comportamentais relacionadas ao SE. Todos estes achados corroboram para elucidar os benefícios da modulação do sistema glutamatérgico no tratamento das crises refratárias e para o estudo dos mecanismos de epileptogênese.

1.2. Abordagens farmacológicas para o tratamento das epilepsias

O primeiro fármaco antiepiléptico a ser comercializado, o brometo de potássio, foi introduzido por um obstetra inglês chamado Charles Locock, em 1857, ao observar sua eficiência no controle das crises em 14 mulheres com epilepsia catamenial (Pearce, 2002). A partir de então, medicamentos antiepiléticos são a principal abordagem para o tratamento das epilepsias e apresentam eficácia terapêutica em cerca de dois terços dos pacientes. Assim, surgiram diversos outros medicamentos de primeira, segunda ou terceira geração, sendo classificados de acordo com a época em que os fármacos foram introduzidos no mercado e com as suas características moleculares e mecanismos de ação. Mais de 15 deles (de segunda geração) foram introduzidos durante os anos 90, ampliando as oportunidades de tratamento para cada paciente. Considerando que atualmente estão disponíveis cerca de 27 fármacos antiepilépticos licenciados, os resultados gerais obtidos até então não foram alterados substancialmente, e cerca do um terço dos pacientes continuam não respondendo à terapia medicamentosa. Assim, a cirurgia de epilepsia ainda continua sendo o tratamento mais eficaz para epilepsias refratárias (Perucca et al., 2018).

Neste cenário, os benzodiazepínicos, moduladores alostéricos positivos de receptores GABA$_A$, são a primeira linha de tratamento nos protocolos atuais de

intervenção. Entretanto, são menos efetivos em bloquear as crises do que os tratamentos de segunda linha. A fenitoína é classicamente considerada como tratamento de segunda linha, e atua como um estabilizador dos canais de sódio voltagem-dependentes. O fenobarbital por sua vez aumenta a inibição mediada por GABA e também antagoniza os AMPARs. Embora esses dois medicamentos sejam muito eficazes, eles estão relacionados a inúmeros efeitos adversos periféricos e centrais. Já o ácido valpróico aumenta a síntese de GABA, inibe a GABA transaminase, atua como estabilizador de canais de sódio voltagem-dependentes e como inibidor de canais de cálcio tipo T. Assim como os demais, apresenta numerosos efeitos adversos e elevadas taxas de interação medicamentosa, dificultando o seu uso em casos de polifarmácia, muito comum no tratamento de epilepsias. A lacosamida também atua nos canais de sódio voltagem-dependentes. O levetiracetam inibe a liberação de neurotransmissores atuando na fusão das vesículas e modula os receptores de glutamato, sendo o tratamento que apresenta menor efeitos adversos e menos interações medicamentosas. Ainda, existem alguns casos onde são adotados protocolos de infusão contínua, nos quais são utilizados midazolam, tiopental, pentobarbital ou propofol (Amengual-Gual et al., 2019). Além dessas abordagens clássicas, novas estratégias estão em pauta atualmente, como inibidores da glicoproteína-P, terapia gênica, esquemas de associação medicamentosa, uso de canabinóides e utilização de células tronco. Todos estes esforços se devem ao elevado nível de pacientes refratários a terapia medicamentosa, números estes que permaneceram inalterados ao longo dos anos, apesar das novas opções disponíveis no mercado (Mesraoua et al., 2019).

Todos este medicamento são aplicados na clínica com boas taxas de sucesso para controlar crises epilépticas, embora os mecanismos de ação se sobreponham, o que limita o espectro de eficácia (Glauser et al., 2016). Entretanto, a maior parte deles são ineficazes no controle dos desbalanços neuroquímicos que estão associados ao dano cerebral, sendo incapazes de proteger contra a morte neuronal, além de apresentarem uma infinidade de efeitos adversos sobre a cognição e o comportamento (Abdalla et al., 2014; Ahmed et al., 2011; Kalemenev et al., 2016; Trinka et al., 2015). Ademais, não possuem atividade antiepileptogênica, ou seja, são incapazes de bloquear as alterações cerebrais implicadas na gênese das crises convulsivas (Loscher and Brandt, 2010; Pitkänen et al., 2013). Por conseguinte, não são capazes de prevenir futuras condições psiquiátricas e demais comorbidades associadas às epilepsias (Holmes and Noebels, 2016). Além disso, alguns

dos medicamentos utilizados no tratamento das epilepsias demonstraram induzir uma frequência bastante alta de sintomas depressivos em ensaios clínicos, e também se relacionam com um aumento do risco de autoagressão ou comportamento suicida na prática clínica (Andersohn et al., 2010).

Efeitos anticonvulsivos e efeitos neuroprotetores não são sinônimos, embora algumas vezes, se uma crise convulsiva severa ou do tipo *SE* são prevenidos ou significativamente atenuados através de uma terapia antiepiléptica bem-sucedida, o dano neuronal pode não ocorrer (Velísek and Velísková, 2008). Neste aspecto, Hoffman e colaboradores (2003) demonstraram que a administração de progesterona antes da crise convulsiva foi capaz de proteger contra o dano neuronal, mas somente nos animais onde houve prevenção das crises concomitantemente. Quando o comportamento convulsivo não foi diferente de um animal não tratado, a progesterona não foi efetiva para o bloqueio do dano neuronal. Esses resultados mostraram que o efeito neuroprotetor da progesterona foi resultado das propriedades anticonvulsivantes, muito embora, na maioria das terapias convencionais para o tratamento das crises, os efeitos neuroprotetores não sejam comumente observados (Velísek and Velísková, 2008). Assim, uma terapia anticonvulsivante sintomática que previna a morte neuronal, ou a combinação de terapias neuroprotetoras e anticonvulsivantes constituem a melhor abordagem farmacológica atual para o tratamento das epilepsias.

2. Reposicionamento de fármacos

Disponibilizar um novo medicamento no mercado é uma tarefa muito cara e demorada. As estimativas gerais mostram que os custos capitalizados dessa tarefa variam de aproximadamente 160 milhões a 1,8 bilhão de dólares. Dessas estimativas, os estágios iniciais de desenvolvimento representam cerca de 51% dos gastos. Além do elevado custo, o cronograma desse processo é de cerca de 15 anos, com uma taxa de sucesso máximo de apenas 11,5% (DiMasi, 2001; DiMasi et al., 2003; Morgan et al., 2011). Estes são alguns dos dados que justificam os declínios na produtividade da indústria farmacêutica nas áreas de pesquisa e desenvolvimento de fármacos ao longo dos anos (DiMasi, 2001). Assim, nos últimos anos a pesquisa farmacêutica para desenvolvimento de novos fármacos expandiu suas fronteiras na descoberta e nos estudos fisiológicos do alvo de interesse, aliando técnicas computacionais ao conhecimento molecular dos processos patológicos, o que trouxe inúmeras vantagens para novos candidatos a

fármacos. Entretanto, a descoberta e desenvolvimento de fármacos ainda é um processo complexo, demorado e caracterizado como um empreendimento de alto custo (Barnieh et al., 2014; DiMasi et al., 2003).

Neste contexto, as abordagens comumente utilizadas para o desenvolvimento de medicamentos focam na identificação de um novo alvo de tratamento ('de novo'), seguido da busca por um composto capaz de modular esse alvo e, por último, um estudo de validação. Entretanto, alvos adicionais para esses compostos geralmente não são investigados, e outras aplicações clínicas geralmente não são exploradas. No entanto, esses elementos extras representam uma oportunidade para a identificação sistemática de novas indicações para a terapêutica existente. De fato, alguns dos medicamentos com mais sucesso comercial são administrados para diferentes indicações do que aquela inicialmente prevista em seu desenvolvimento (uso *off-label*) (Dudley et al., 2011; Shameer et al., 2015). Assim, a prática de identificar indicações terapêuticas adicionais para compostos existentes, referida como 'reposicionamento', possui alguns benefícios importantes sobre os métodos tradicionais de desenvolvimento de medicamentos (Ashburn and Thor, 2004; Barratt and Frail, 2012). O reposicionamento de fármacos é uma estratégia que envolve encontrar novas aplicações terapêuticas para fármacos já disponíveis no mercado. Quando comparada à busca por novos medicamentos (utilizando a estratégia 'de novo'), é uma abordagem vantajosa por apresentar maior probabilidade de sucesso e representar uma redução expressiva nos custos e na linha de tempo do desenvolvimento (Ashburn and Thor, 2004; Barratt and Frail, 2012). Esta estratégia de redescobrir o valor de 'moléculas velhas' cresceu nos últimos anos, possibilitando o uso *off-label* de centenas de compostos com eficácia e segurança, o que estimulou a criação de bancos de dados com os potenciais alvos e permitindo uma maior interação entre indústria e academia (Caban et al., 2017; Murteira et al., 2014).

De fato, as receitas obtidas por medicamentos reposicionados podem ser extremamente lucrativas. Por exemplo, as vendas de talidomida, reposicionada para o tratamento de mieloma múltiplo, alcançaram milhões de dólares e o sildenafil, reposicionado para disfunção erétil, obteve vendas anuais de 1,88 bilhão de dólares em 2003 (Ashburn and Thor, 2004; Barratt and Frail, 2012). Além disso, o processo de desenvolvimento de medicamentos para um medicamento reposicionado pode ser encurtado para cerca de 3-10 anos, principalmente porque várias etapas do *pipeline* de desenvolvimento podem ser eliminadas (Dudley et al., 2011; Shameer et al., 2015). Estas

incluem: seletividade para o alvo; estudos de farmacocinética; toxicologia; e efeitos indesejáveis. Estes são obstáculos potenciais que acarretam em divergências nos testes em espécies animais e humanos, os quais são facilmente superados para fármacos reposicionados (Parsons, 2019). Adicionalmente, o reposicionamento de fármacos nos permite racionalizar a pesquisa para uma terapia multialvo, porque possibilita a identificação de compostos que produzem um efeito terapêutico esperado e reconhecido através da observação fenotípica (Kodama et al., 1997). Uma droga multialvo apresenta diferentes farmacóforos em uma única molécula ou um farmacóforo comum para diferentes alvos, ampliando o espectro de aplicabilidade clínica e contornando algumas limitações da farmacoterapia clássica (Morphy et al., 2004).

O reposicionamento é particularmente relevante em áreas terapêuticas menos exploradas, como no tratamento de doenças do SNC, doenças pediátricas ou órfãs, dentre outras (Caban et al., 2017). Sabidamente, no arsenal terapêutico para o tratamento da Doença de Parkinson, existe um medicamento reposicionado denominado amantadina. A amantadina foi inicialmente desenvolvida para o tratamento da gripe e, somente mais tarde, direcionada ao tratamento da Doença de Parkinson por atuar como um antagonista dos receptores de glutamato e por aumentar os níveis de dopamina. No caso da Doença de Alzheimer por exemplo, verificou-se que os medicamentos contra o câncer podem ser reaproveitados em seu tratamento. A lógica por trás dessa ideia vem do fato de que o câncer e a neurodegeneração compartilham vias de sinalização, como disfunção mitocondrial, estresse oxidativo, comprometimento do metabolismo celular e síntese de proteínas malformadas, além de evidências clínicas nas quais pacientes tratados com quimioterapia também apresentam um risco menor de desenvolver Alzheimer em sua velhice (Durães et al., 2018). Assim, a semelhança nos mecanismos patognomônicos é crucial para o desenvolvimento de um reposicionamento de sucesso. Neste aspecto, o ácido valpróico é um exemplo de medicamento antiepiléptico sugerido como um agente neuroprotetor para a doença de Alzheimer, apontando para similaridades entre o Alzheimer e epilepsia (Mark et al., 1995; Tariot et al., 2011). Este candidato ao reposicionamento mostrou redução na formação de placas amiloides, melhoria nos déficits de memória em camundongos transgênicos, inibição da produção de citocinas pró-inflamatórias e aumento da fagocitose microglial (Qing et al., 2008; Smith et al., 2010; Tariot et al., 2011).

Especialmente no caso de fármacos antiepilépticos, a indústria farmacêutica vem diminuindo progressivamente seu interesse no desenvolvimento de novos compostos. Isto se deve ao fato de que os custos são infinitamente maiores quando analisados em separado (cerca de 335 milhões de dólares) e com uma razão de sucesso em torno de 5% (sem mencionar a parcela de pacientes refratários). Estes dados desencorajam e diminuem os investimentos em pesquisa e desenvolvimento de fármacos antiepilépticos. Assim, das condições que afetam o SNC, o reposicionamento de fármacos para o tratamento das epilepsias é uma oportunidade comercial imediata. Selecionar medicamentos de outras áreas terapêuticas que possuam propriedades modificadoras de doença relevantes para as epilepsias, ou um novo mecanismo de ação que forneça substancial eficácia sinérgica contra epilepsia resistente a medicamentos (a fim de encontrar um fármaco que possa ser utilizado combinado com uma terapia existente), pode reduzir drasticamente o nível de investimento necessário para a descoberta e desenvolvimento (Löscher et al., 2013). Especificamente no caso do *SE*, no qual os medicamentos disponíveis têm uma taxa relativamente alta de falha, ampliaram-se as discussões sobre reposicionamento de fármacos, particularmente aplicando-os em polifarmacoterapia. As novas estratégias de tratamento visam direcionar a fisiopatologia do *SE*, a fim de reduzir a recorrência de crises epilépticas e seus impactos/consequências à longo prazo. Alguns dos exemplos de fármacos explorados são os moduladores dos receptores GABA$_A$ sinápticos e extrasinápticos (como a alopregnanolona) e antagonistas de NMDARs (como a cetamina) (Amengual-Gual et al., 2019). Além disso, realizar o reposicionamento da molécula desejada em diferentes modelos de indução e em organismos de diferentes espécies pode providenciar maior chance de sucesso. Assim, selecionamos uma molécula com maior probabilidade de ser efetiva clinicamente.

2.1. Antagonismo de receptores NMDA como estratégia terapêutica para tratamento de crises epilépticas e reposicionamento de fármacos

Os antagonistas competitivos de NMDARs como AP-7, CGP 40116, APV, CPP, dentre outros, demonstraram possuir um amplo espectro de atividade anticonvulsivante em diversos modelos animais de crises generalizadas (PTZ, 4-AP, NMDA, eletrochoque, bicuculina e cocaína), e em modelos de *SE* (pilocarpina e estimulação elétrica hipocampal contínua) (Aldinio et al., 1983; Chapman et al., 1990; Croucher et al., 1982; Czuczwar et al., 1985b, 1985a; Czuczwar and Meldrum, 1982; Eblen et al., 1996; Fujikawa et al., 1994; Ghasemi and Schachter, 2011; Millan et al., 1986; Morales-Villagrán et al., 1996;

Turski et al., 1990; Yen et al., 2004). Além disso, estudos *in vitro* demonstraram que AP-7 e APV reduzem a atividade epileptiforme induzida por baixa concentração de Mg^{+2}, penicilina e picrotoxina em neurônios hipocampais (Ashton et al., 1988; Brady and Swann, 1986). Por outro lado, os antagonistas não competitivos de NMDARs como cetamina, MK-801, ifenprodil, entre outros, demonstraram efeitos anticonvulsivantes em modelos de crises tônico-clônicas generalizadas (induzidas por NMDA, cocaína, bicuculina, PTZ, 4-AP) (Geter-Douglass and Witkin, 1999; Ghasemi and Schachter, 2011; Parsons et al., 1995; Swinyard et al., 1986; Turski et al., 1990; Zaitsev et al., 2015). Em modelos químicos de *SE* (ácido caínico e pilocarpina), cetamina, MK-801 e ifenprodil possuem atividade anticonvulsivante e diminuem os danos cerebrais relacionados às crises (Clifford et al., 1990; Ding et al., 2007; Freitas et al., 2006; Fujikawa, 1995; Halonen et al., 1999; Hughes et al., 1993; Yen et al., 2004). Estudos em modelos animais demonstraram que o *SE* no cérebro em desenvolvimento causa uma morte neuronal expressiva em diversas regiões como hipocampo, córtex, amígdala e tálamo (de Oliveira et al., 2011; Kubová et al., 2004; Sankar et al., 1998a), e que esta neurodegeneração está associada com a hiperestimulação de NMDARs (Holopainen, 2008), o que justifica o efeito neuroprotetor dos antagonistas de NMDAR.

Devido aos resultados promissores dessa classe de compostos, alguns ensaios clínicos foram realizados. O felbamato por exemplo, um bloqueador dos NMDARs altamente seletivo para as subunidades NR1A/NR2B é classificado com um antiepiléptico de segunda geração. Os estudos demonstraram sua eficácia tanto como monoterapia quanto em associações para o tratamento de epilepsias refratárias e em crises de ausência, em doses que variam de 45 a 300 mg/kg (Canger et al., 1999; Devinsky et al., 1994; Theodore et al., 1991). Entretanto sua utilização é limitada e vinculada a acompanhamento por exames laboratoriais devido ao elevado número de efeitos adversos (ataxia, sedação, insônia, anorexia, vômitos, perda de peso, irritabilidade, etc) e pelos riscos de complicações mais graves como anemia aplástica e falência hepática (Pellock, 1999; Pellock et al., 2006). A cetamina é outro antagonista do NMDAR que possui estudos clínicos, e tem sido reportada como eficaz no controle do *SE* refratário (convulsivo e não convulsivo) por via intravenosa e oral. Além disso, seus efeitos adversos aparentam ser raros, embora exista uma inúmera lista de restrições de uso (Fang and Wang, 2015). As restrições incluem um grupo grande de pessoas tendo em vista as sérias complicações que podem ocorrer como sepse, choque, falência de órgãos,

taquicardia, sintomas psiquiátricos, neurotoxicidade, pneumonia, dentre outros (Fang and Wang, 2015; Gaspard et al., 2013). Dessa forma, o tratamento de desordens do SNC com antagonistas do NMDAR foi associado com efeitos colaterais elevados, o que pode se relacionar com o envolvimento paradoxal deste receptor tanto na morte celular quanto na sobrevivência e plasticidade neuronal (Hardingham, 2009; Huang et al., 2012). Estes agentes neuroprotetores bloqueiam completamente o NMDAR, o que garante a prevenção contra a excitotoxicidade, porém causam também prejuízos na transmissão sináptica fisiológica, o que explica os numerosos efeitos indesejados (Danysz and Parsons, 2002; Huang et al., 2012; Loss et al., 2012a; Muir and Lees, 1995).

A memantina (MN) é atualmente empregada na clínica para o tratamento da Doença de Alzheimer, por produzir uma melhora global nos aspectos comportamentais e cognitivos dos pacientes, demonstrando-se eficaz e segura (Huang and Mucke, 2012; Peskind et al., 2006). Neste contexto, a MN representa um exemplo dos potenciais benefícios da utilização clínica de antagonistas NMDAR em desordens do SNC (Lipton, 2006; Zheng et al., 2014). O mecanismo de ação da MN envolve um antagonismo não competitivo de baixa afinidade aos NMDAR. Isso leva a um bloqueio parcial do influxo excessivo de Ca^{+2} decorrente do aumento da atividade do receptor na fenda sináptica, e devido a sua rápida cinética de *turn on/turn off*, a MN não compromete a atividade fisiológica da transmissão glutamatérgica (Johnson and Kotermanski, 2006; Parsons et al., 2008, 2007). Assim, a MN não produz efeitos colaterais tão intensos como aqueles observados no uso de antagonistas seletivos de alta afinidade, como cetamina e outros bloqueadores do NMDAR, sendo melhor tolerada pelos pacientes (Parsons et al., 2008; Rogawski, 1993).

Considerando a eficácia da MN no tratamento da Doença de Alzheimer, e que existem mecanismos na patogênese dessa doença que se assemelham aos encontrados na epilepsia, existe uma possibilidade da aplicação terapêutica da MN reposicionada para pacientes epilépticos (Chin and Scharfman, 2013). Evidências em modelo animal demonstraram que a MN foi capaz de prevenir crises convulsivas na dose de 10 mg/kg/dia durante dois dias, antes da indução da crises epilépticas com NMDA (Chang-Mu et al., 2010). Adicionalmente, em um modelo de crise convulsiva induzida por PTZ em ratos jovens, a administração de MN na dose de 20 mg/kg foi capaz de suprimir as manifestações tônico-clônicas (Mareš and Mikulecká, 2009). Embora os efeitos neuroprotetores da MN não tenham sido investigados em modelos específicos de crises

convulsivas, como o *SE*, pode-se sugeri-la como uma alternativa em potencial para a prevenção de crises e da morte neuronal decorrentes de eventos excitotóxicos. Além disso, os efeitos da MN no controle de crises epilépticas permanece como um tópico controverso na literatura (Creeley et al., 2006; Löscher and Hönack, 1990).

Além da ação sobre os NMDARs, evidências recentes sugerem que a MN atua também no controle do estado redox em diferentes condições patológicas, aumentando seu espectro de ação (efeito multialvo), e em um dos alvos sabidamente envolvidos na patogênese das epilepsias (Bardak et al., 2018; Khalili-Fomeshi et al., 2018; Tanaka et al., 2018).

3. OBJETIVOS

Considerando:

(i) A importância clínica das epilepsias no que se refere às consequências psicopatológicas e sociais, as altas taxas de refratariedade e a necessidade de novas abordagens terapêuticas para contornar esses problemas;

(ii) O envolvimento do sistema glutamatérgico na epileptogênese, particularmente relativo aos NMDARs, e os benefícios da modulação do sistema glutamatérgico no tratamento das crises refratárias;

(iii) A segurança e boa tolerabilidade da MN na prática clínica;

(iiii) As similaridades patognomônicas entre Alzheimer e epilepsia e os potenciais benefícios da utilização da MN em desordens do SNC;

Nós hipotetizamos que a MN poderia ser uma alternativa eficaz no manejo de crises epilépticas, podendo ser reposicionada com sucesso mediante a comprovação de seus efeitos em modelos animais à curto e longo prazos.

3.1. Objetivo geral

Investigar se a MN é capaz de atenuar o fenótipo epiléptico e as alterações cerebrais e comportamentais relacionadas a crises epilépticas.

3.1.1. Objetivos específicos do capítulo I

- Avaliar o efeito do pré-tratamento e do pós-tratamento com MN na morte neuronal induzida pelo *Status epilepticu*s induzido por LiCl-pilocarpina em diferentes regiões cerebrais de ratos jovens.

- Avaliar as diferenças no perfil de neuroproteção de uma única dose e de múltiplas doses de MN nas diferentes regiões cerebrais de ratos jovens submetidos ao *Status epilepticu*s induzido por LiCl-pilocarpina.

3.1.2. Objetivos específicos do capítulo II

- Avaliar o efeito do pré-tratamento com MN sobre o fenótipo de crises epilépticas induzidas com PTZ em peixe-zebra.

- Avaliar o efeito do pré-tratamento com MN sobre a expressão de receptores NMDA e estresse oxidativo em animais submetidos a crises epilépticas induzidas por PTZ.

- Avaliar o efeito do pré-tratamento com MN sobre as alterações comportamentais induzidas por crises epilépticas induzidas por PTZ em peixe-zebra.

PARTE II – ARTIGOS CIENTÍFICOS

4. CAPÍTULO I

Artigo publicado

Título: Memantine decreases neuronal degeneration in young rats submitted to LiCl-pilocarpine-induced *status epilepticus*

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Justificativa: O estudo do papel dos antagonistas do receptor N-metil-D-aspartato demonstra sua ação anticonvulsivante, porém seu papel como neuroprotetores na morte neuronal induzida pelo SE permanece pouco explorado. Além disso, a memantina é clinicamente bem tolerada e não induz tantos efeitos indesejados e a investigação de seus efeitos neuroprotetores é fundamental para a possibilidade de reposicionar este medicamento para o tratamento das epilepsias.

Objetivo geral: O objetivo deste trabalho foi investigar o efeito neuroprotetor da memantina contra a degeneração neuronal induzida pelo SE induzido por LiCl-pilocarpina nos períodos iniciais da vida.

Resultados: Observamos que a neurodegeneração diminuiu significativamente no hipocampo e no tálamo; o pós-tratamento foi capaz de proteger contra a neurodegeneração em uma região da amígdala. Assim, nossos resultados demonstram o efeito neuroprotetor da memantina na morte neuronal induzida pelo SE no início da vida. Este efeito é dependente do tempo de tratamento e da região cerebral.
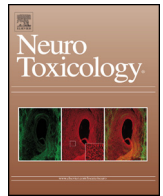
Full Length Article

Memantine decreases neuronal degeneration in young rats submitted to LiCl-pilocarpine-induced *status epilepticus*

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ABSTRACT

Several works have demonstrated that *status epilepticus* (SE) induced-neurodegeneration appears to involve an overactivation of *N*-methyl-D-aspartate receptors and treatment with high-affinity NMDAR antagonists is neuroprotective against this brain damage. However, these compounds display undesirable side effects for patients since they block physiological NMDA receptor dependent-activity. In this context, memantine (MN), a well tolerable low-affinity NMDAR channel blocker, will be a promising alternative, since it does not compromise the physiological role of NMDA receptors on synaptic transmission. The aim of the present study was to investigate if MN could attenuate seizure severity and neuronal cell death caused by SE induced early in life. Wistar rats (15 days old; n = 6–8 per group) received memantine (20 mg/kg i.p.) in six different treatments: 6 and 3 h before SE onset; concomitant with pilocarpine; 15 min and 1 h after SE onset; and four consecutive administrations (15 min, 6 h, 12 h, and 18 h) after pilocarpine injection. Neurodegeneration was quantified by fluoro-jade C staining. Treatment with memantine increase latency to SE onset only in groups treated 3 h before or concomitant with pilocarpine. In CA1 hippocampal subfield, memantine significantly reduced neurodegeneration at the following times: 3 h prior SE-onset, concomitant with pilocarpine, and 15 min after pilocarpine injection. For amygdala and thalamus, all post-SE onset treatments were able to decrease neurodegeneration. In conclusion, the present study showed that MN was neuroprotective against SE-induced neuronal death and this neuroprotection appears to be time- and region-dependent.

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1. Introduction

Status epilepticus (SE) is "a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms, which lead to abnormally, prolonged seizures" (Trinka et al., 2015). It is most prevalent in children than adults and its annual incidence is 10 to 73 episodes/ 100,000 children, being more incident in children younger than two years of age (135/100,000 to 156/100,000 children) (Fencl et al., 2000; Majores et al., 2007; Raspall-Chaure et al., 2006).

One of most remarkable SE-induced consequence early in life is a massive neuronal cell death observed in specific brain areas, such as temporal cortex, hippocampus, amygdala and thalamus, which may lead to a long-term alterations in neural network development and thus functional and cognitive deficits at adulthood (de Oliveira et al., 2008; Kubova et al., 2002; Sankar et al., 1998). Although clinically used antiepileptic drugs, such as midazolam, diazepam, fosphenytoin, valproic acid, levetiracetam, and phenobarbital, have been recommended to stop convulsive SE (Glauser et al., 2016), they have mostly failed to halt SE-induced neuronal

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degeneration (Abdalla et al., 2014; Ahmed et al., 2011; Kalemenev et al., 2016; Trinka et al., 2015).

SE-induced neuronal degeneration appears to involve an overactivation of N-methyl-D-aspartate receptors (NMDARs) (Holopainen, 2008) and treatment with high-affinity NMDAR antagonists, such as TCP, ketamine and MK-801, has been showed to be effective for preventing SE-induced neuronal death (Brandt et al., 2003; Lerner-Natoli et al., 1991; Stafstrom et al., 1997). However, preventive protection of NMDAR antagonists seems to have a short "therapeutic time window", since this drugs were efficacious only when they were administered prior or up to 15 min after SE onset (Fujikawa, 1995; Fujikawa et al., 1994; Loss et al., 2012). Furthermore, clinical use of high-affinity NMDAR antagonists is not recommended. High-affinity NMDAR antagonists cut off normal glutamatergic neurotransmission (Lipton, 2004), causing thus severe and undesirable side effects for patients (Danysz and Parsons, 2002; Muir and Lees, 1995).

In this context, memantine (MN) has been proposed as an effective NMDAR channel blocker (Chen and Lipton, 2006). The pharmacodynamics of MN involves NMDAR fast blocking/unblocking kinetics and strong voltage-dependency. These characteristics culminate in a partial blockage of NMDAR, which does not compromise the physiological and neurochemical role of these receptors on synaptic neurotransmission (Johnson and Kotermanski, 2006; Parsons et al., 2007; Rammes et al., 2008). Moreover, MN appears to be more tolerable for patients than classical high-affinity NMDAR antagonists (Chen and Lipton, 2006; Parsons et al., 1999; Parsons et al., 2007). Recently, memantine was approved by the Food and Drug Administration to treat moderate-to-severe Alzheimer's disease (Parsons et al., 2007).

Taking into account that NMDAR overactivation may be implicated in SE-induced neurodegeneration and memantine is a well tolerable partial NMDAR channel blocker, the aim of the present study was to investigate if MN could attenuate seizure severity and neuronal cell death induced by SE early in life.

2. Material and methods

2.1. Drugs

Pilocarpine hydrochloride and memantine were purchased from Sigma–Aldrich (USA). Fluoro-Jade C was purchased from Chemicon, Inc. (USA). Other chemicals were purchased from Nuclear (Brazil).

2.2. Animals

One hundred and thirtythree young Wistar rats were obtained from local breeding. The litters were culled to 7 pups. Each litter contained animals from all experimental groups and was composed of 1:1 male/female ratio approximately. Animals were housed under controlled environment: light/dark photoperiod cycle of 12/12 h (lights on at 7:00 a.m.) and room temperature of $21 \pm 2\,°C$. N was 6–8 animals/group. All procedures were approved by the Research Ethics Committee from Universidade Federal do Rio Grande do Sul (protocol number #25772).

2.3. Induction of status epilepticus and treatment interventions

Rat pups (15 days old) were injected (10 ml/kg) with LiCl (3 mEq/kg; i.p.) 15 h prior to pilocarpine (60 mg/kg; i.p.; SE group) or saline (0.9% NaCl; i.p.; non-SE group) administrations. According to our previous works (de Oliveira et al., 2011; Loss et al., 2012), this pilocarpine dose causes SE in all animals without induces high mortality rate. Memantine (MN) (20 mg/kg; i.p.) (Chen et al., 1998; Stieg et al., 1999) or saline (0,9%) were administrated in both SE-

and non-SE groups in six different therapeutic regimens: (I) 6 and (II) 3h prior to pilocarpine injection; (III) concomitant with pilocarpine injection; (IV) 15 min and (V) 1h after pilocarpine injection; and (VI) four consecutive administrations of MN (15 min, 6 h, 12 h, and 18 h) after pilocarpine injection (Supplementary Fig. S1). Treated rats were kept in individual plastic cages during 3 h for seizure observation. Behavioral manifestations of seizure were recorded and scored accordingly to Racine scale (Racine, 1972). After this period, animals returned to home cages and were observed for more 1 h for seizure recovery. Animals spontaneously recovered from SE without anticonvulsant administration. Twenty-four hours after SE induction, animals were used to determine neuronal degeneration.

2.4. Fluoro-jade C staining

For evaluating neuronal degeneration, Fluoro-jade C staining (FJC) was conducted as described by Schmued et al. (2005). Briefly, 24 h after SE induction rats were deeply anesthetized i.p. with ketamine (90 mg/kg) and xylazine (12 mg/kg) and sequentially perfused through the heart with 200 mL of ice-cold 0.1 M sodium phosphate buffer, pH 7.4, followed by 100 mL of ice-cold fixative solution (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4). Brains were removed and immersed overnight in fixative solution followed by 30% sucrose until brains sank to the bottom of the chamber. Coronal slices (30 μm) were obtained using a Leica VT 1000S vibroslicer, mounted on to gelatin-coated slides and dried at room temperature overnight. Slides were immersed in alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min. Then, slides were rinsed for 2 min in 70% ethanol, 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution during 10 min. After rinsing with distilled water for 2 min, slides were transferred for 10 min to a 0.0001% FJC solution dissolved in 0.1% acetic acid, washed three times for 1 min with distilled water, dried at room temperature overnight, dehydrated in xylene, and cover slipped. Sections were analysed using a Nikon Eclipse E600 epifluorescence microscope.

Total number of FJC-positive cells per animal corresponds to an average of 3 consecutive coronal brain sections from plates 31–33 of Paxinos and Watson's Atlas (1998). Cells were counted at 200x magnification by using the software NIS-Elements Version 3.10 (Nikon Instruments Inc., USA). Only cells exhibiting bright green fluorescence and neuronal profile were counted (Wang et al., 2008). Brain regions analysed were thalamus, amygdala and hippocampus, which were chosen accordingly to previous studies (de Oliveira et al., 2008; Kubova et al., 2002).

2.5. Statistical analysis

All data were first tested for normality by D'Agostino-Pearson omnibus normality test. Two-way ANOVA followed by Bonferroni post hoc test was used to analyse latency to SE onset and results were expressed as mean ± S.E.M. Mann–Whitney U-test was used to analyse FJC data and results were expressed as median (interquartile range). $P < 0.05$ was considered significant.

3. Results

Systemic administration of LiCl-pilocarpine produced defecation, salivation, body tremor, and scratching within 5 to 10 min. This behavioral pattern progressed within 20 to 45 min to increased levels of motor activity and culminated in SE in all animals. SE was characterized by sustained orofacial automatisms, salivation, chewing, forelimb clonus, loss of the righting reflex and falling, and lasting up to 3-4 h. Treatment with MN increased the latency to generalized motor convulsions (stage 5 of Racine scale)
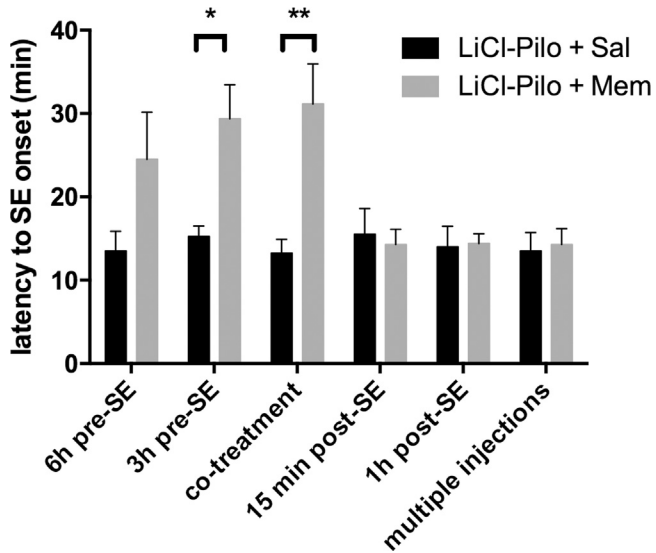
Fig. 1. Latency to SE (*status epilepticus*) in minutes characterizing seizure behavioral pattern. Black bars represented animals that received vehicle and pilocarpine and grey bars animals that received memantine and pilocarpine. Data were expressed with mean + S.E.M. $^* = p < 0.05$; $^{**} = p < 0.01$.

only when it was administered 3h before or concomitant with pilocarpine ($p = 0.0385$ and $p = 0.0055$, respectively; Fig. 1). For the other Racine stages, MN had no effect. Additionally, MN treatment was not able to decrease mortality rates.

FJC-positive cells exhibited a bright green color in the soma and in the fine neuronal processes (Figs. 2 and 3; insets showing higher magnification view). Twenty-four hours after SE onset, animals treated with LiCl-pilocarpine + saline presented an expressive neuronal degeneration in CA1 and DG hippocampal subfields, as well as in posteroventral medial nucleus (MePV) of amygdala and ventral posteromedial nucleus (VPM) of thalamus (Figs. 2 and 3). Pre- and post- therapeutic regimens with MN did not reduce the number of FJC-positive neurons in DG (Fig. 2; Table 1). In contrast, the number of FJC-positive cells in CA1 subfield was significantly reduced when animals were treated with MN 3h before, concomitant or 15 min after pilocarpine administration ($p = 0.008$, $p = 0.024$ and $p = 0.002$, respectively) (Fig. 2; Table 1). At 1h after pilocarpine or multiple injections regimen, MN did not reduce the number of FJC-positive cells in CA1 subfield.

In MePV nucleus of amygdala, MN administered 15 min or 1 h after pilocarpine reduced significantly the number of FJC-positive neurons ($p = 0.001$ and $p = 0.004$, respectively). Moreover, in this brain region a protective effect was also observed when MN was administered in a multiple injections regimen ($p = 0.017$) (Fig. 3; Table 1).

For VPM nucleus of thalamus, MN was protective in all treatment regimens (Fig. 3; Table 1).

None FJC-positive neurons were observed in non-SE animals treated with saline or MN (data not shown).

4. Discussion

Massive neuronal cell death is one of the most remarkable consequences of SE for the developing brain. It has been considered a critical signal to induce abnormal axonal sprouting and synaptic-circuit reorganization, which have been pointed as a structural and functional basis for behavioral and cognitive dysfunctions observed in adult epileptic patients. Neuronal cell death induced by SE appears to involve an overactivation of NMDAR (Holopainen,

2008) and treatment with NMDAR antagonists, such as MK-801 and ketamine, was shown to prevent SE-induced neurodegeneration in several experimental models (Brandt et al., 2003; Lerner-Natoli et al., 1991; Stafstrom et al., 1997). However, clinical use of high-affinity NMDAR antagonists is not recommended, since they can block physiological glutamatergic neurotransmission causing severe and undesirable side effects for patients (Danysz and Parsons, 2002; Lipton, 2004; Muir and Lees, 1995).

In this context, development and testing drugs that potentially prevent SE-induced neurodegeneration without, however, affecting normal neurotransmission can be crucial for clinical management of pediatric SE. In present work, we showed that MN, an effective NMDAR channel blocker with fast blocking/unblocking kinetics, was able to reduce SE-induced neuronal degeneration in the developing brain. MN neuroprotection was time- and region-dependent and may be attributed to its capacity of blocking GluN2A- and GluN2B-containing NMDAR, which are highly expressed in immature brain (Monyer et al., 1994; Paoletti et al., 2013).

MN neuroprotection observed in CA1 subfield may represent the ability of MN bind to channels formed by these both NMDAR subunits, since Bresink et al. (1996) and Parsons et al. (1999) have demonstrated that MN was equipotent for blocking glutamate-induced currents mediated by GluN2A- and GluN2B-containing NMDAR in hippocampal slices. However, we observed a complete lack of neuroprotection in DG. This can be attributed to a later NMDA receptor maturation in this region. According to Bekenstein and Lothman (1991), hippocampal NMDA receptor dependent-LTP reaches the adult values about two weeks after birth in CA1, while for DG this value is reached only about 3 weeks after birth. Since our animals are two-weeks old, NMDAR functional immaturity can confer a reduced sensibility of DG to MN, which would preclude its neuroprotective action (McDonald et al., 1990).

Post-SE onset treatments in amygdala and thalamus showed a highest neuroprotective effect of MN. Comparatively with the hippocampus, there is a massive expression of GluN2B subunits in amygdala and thalamus during the first and second weeks of life in rats (Lopez de Armentia and Sah, 2003). Since MN present high affinity for GluN2B subunits, neuroprotective effects observed in these regions probably can be attributed to this increased expression of GluN2B subunits (Grimwood et al., 1996).

Surprisingly, MN also showed a prominent neuroprotection in VPM. This neuroprotection can be attributed to distinct compositions of NMDAR subunits in thalamus. For instance, Paoletti et al. (2013) have shown that the expression of NMDAR subunits in thalamus during brain development comprises GluN1/GluN2A, GluN1/GluN2B and GluN1/GluN2D and literature have reported that MN is particularly able to interact with all of these functional isoforms (Parsons et al., 2007).

Excitotoxicity caused by excessive neuronal activity has been proposed as molecular mechanism implicated in SE-induced neuronal cell death (Choi, 1992). This pathological process is mediated mainly by an overactivation of NMDA receptors, which can lead to an excessive calcium entry into neuronal cytosol and a subsequent activation of proteases, phospholipases and endonucleases (Szado et al., 2008; Wang et al., 1999). Neuronal cell death can also be induced by an elevation of $[Ca^{+2}]i$, which may be mediated by Ca^{+2} release from IP3- and ryanodine-sensitive calcium stores (Deshpande et al., 2016; Deshpande et al., 2014). Moreover, SE also can disrupt glial glutamate uptake and reduce Na^+/K^+ ATPase activity (de Oliveira et al., 2011), which may lead to an elevation of glutamate levels on synaptic cleft and, subsequently, an exacerbation of excitotoxic neuronal injury (Rothman and Olney, 1995; Yan et al., 2013). Since excitotoxicity has been proposed to be one of the molecular mechanisms involved in SE-induced neuronal cell death, NMDAR channel-blockers that do not
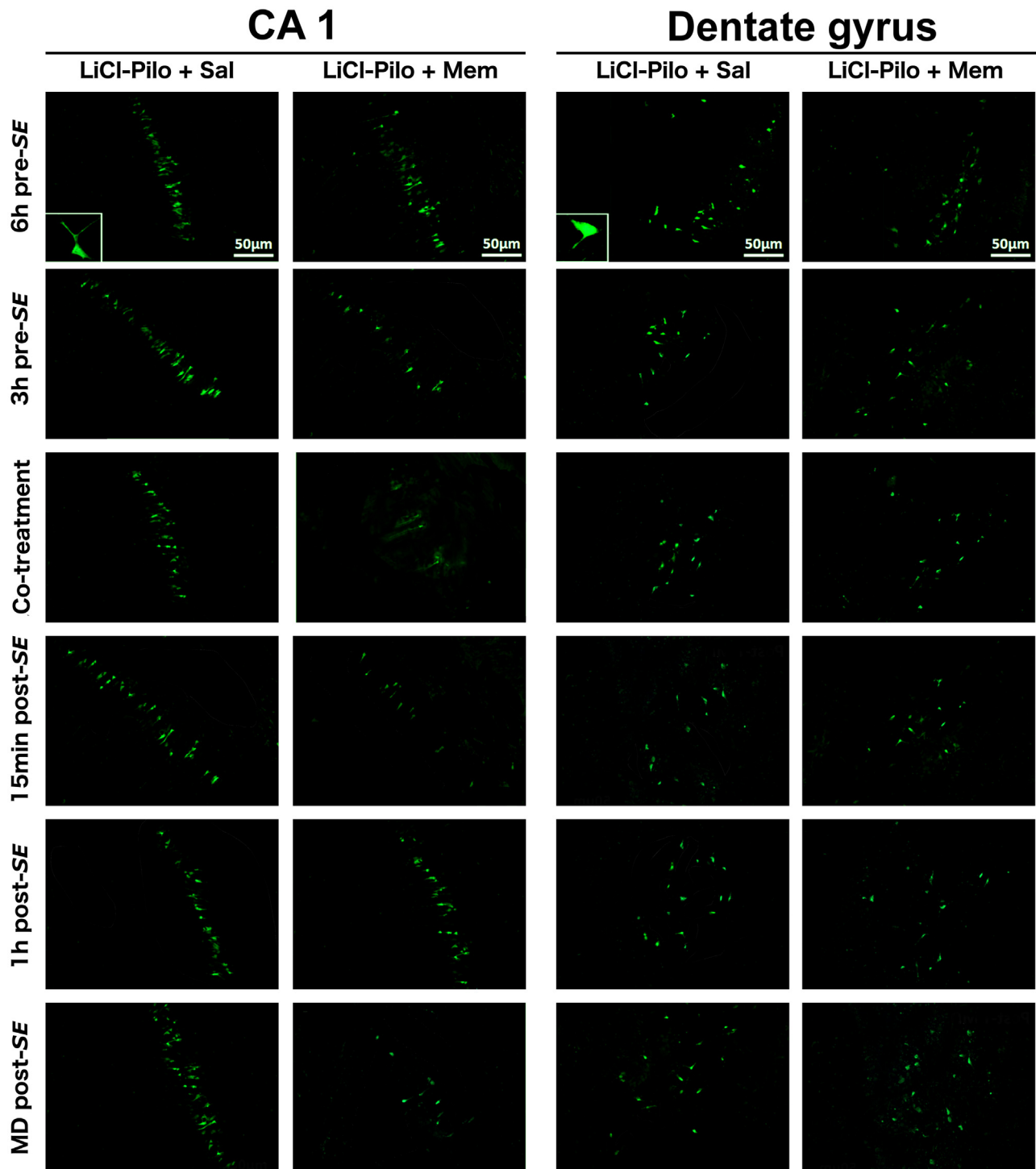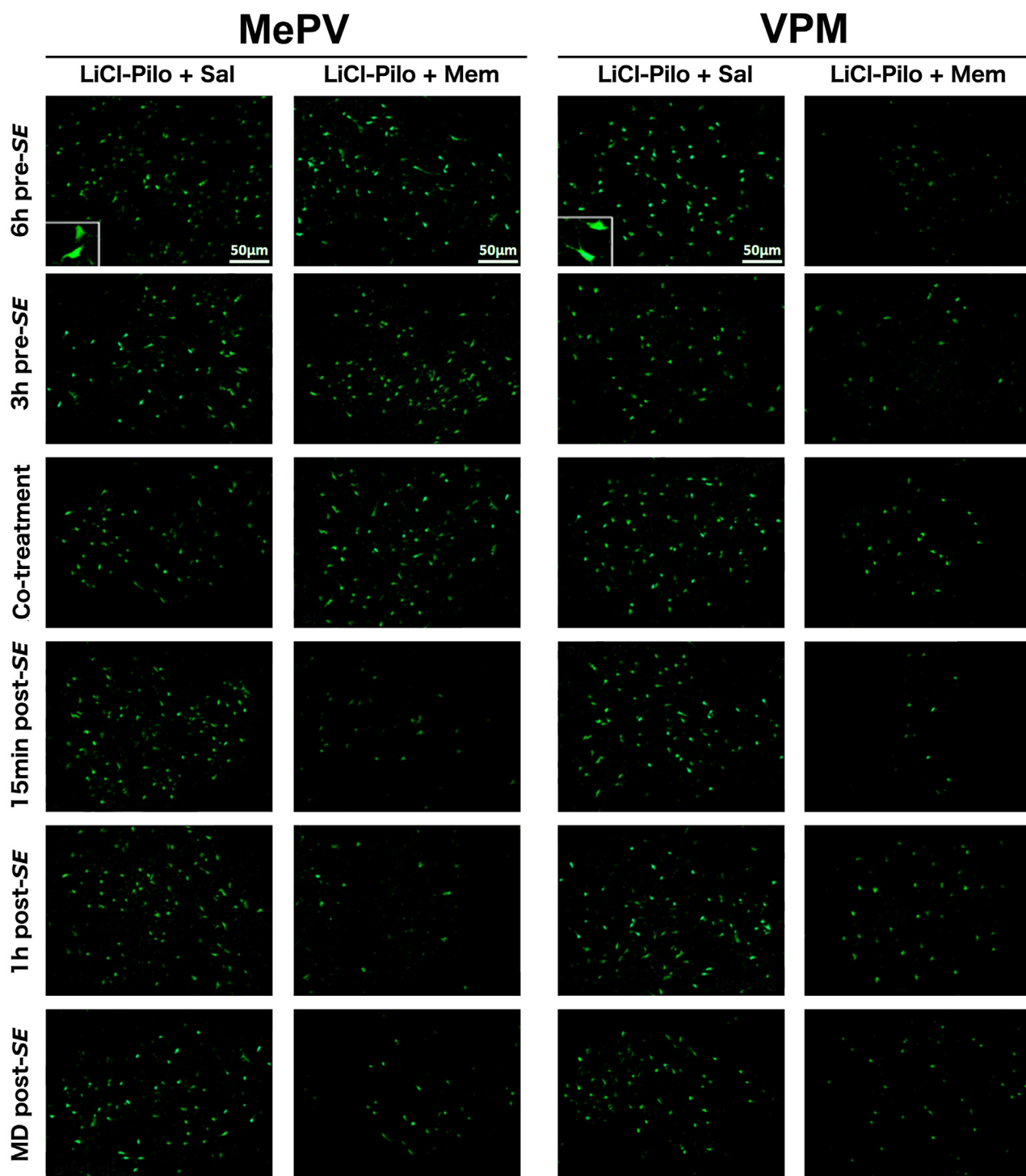
Fig. 2. Representative images of the MN effect on SE-induced neuronal degeneration in hippocampal CA1 and dentate gyrus (DG). FJC-positive cells were quantified 24 h after administration of LiCl-pilocarpine. Images are at 200× magnification.

impair physiological neurotransmission could be considered as an adjuvant therapy for SE-induced neurodegeneration. This notion can be reinforced by the fact that clinically used antiepileptic drugs have a time-dependent loss of effectiveness against SE-induced brain injury, i.e. they are protective only when they are administered early or immediately after SE onset (George and Kulkarni, 1996; Kim et al., 2007; Mazarati et al., 1998; Morrisett et al., 1987; Sofia et al., 1993). Few AEDs exhibit significant neuroprotection against SE-induced neuronal damage when they are administered after SE onset (Abdalla et al., 2014; Kalemenev et al., 2016).

In our study, we showed that MN was able to reduce neuronal damage in thalamus and amygdala even when it was administered at a single injection after SE onset. Neuroprotection was also observed when it was administered at a post-SE multiple injection therapeutic regimen. These findings have a potential clinical value, since classical AEDs failed into protect the brain from SE-induced neuronal damage (Temkin, 2009). Neuroprotection following brain

Fig. 3. Representative images of the MN effects on SE-induced neuronal degeneration. MePV: posteroventral medial amygdaloid nucleus; VPM: ventral posteromedial thalamic nucleus. FJC-positive cells were quantified 24 h after administration of LiCl-pilocarpine. Images are at 200x magnification.

insults does not seem to provide a promising strategy to prevent epilepsy, but may reduce or prevent other consequences of brain insults, such as cognitive deficits or psychopathology in adulthood (Loscher and Brandt, 2010).

Several NMDAR antagonists, including MN, exert anticonvulsant activity in some animal models. Dhir and Chopra (2015) demonstrated that MN pre-treatment delayed the onset of different phases of NMDA-induced convulsions in neonatal rats at dose-dependent manner. Moreover, MN also suppressed

generalized tonic-clonic PTZ-induced seizures in immature rats (Mares and Mikulecka, 2009). However, in our study MN does not seem to be a promising strategy to stop LiCl-pilocarpine-induced seizures, although it increased latency to generalized motor convulsions. This distinct anticonvulsant profile of MN can be associated with peculiarities of each seizure-induced animal model, such as type of induced-seizure and chemoconvulsant target site (Kupferberg, 2001). Despite this controversial anticonvulsant activity, as well as some intolerable side effects (Creeley

Table 1
Number of Fluoro Jade-C positive cells in different brain regions of SE-induced animals treated with saline or memantine.

Brain region	Treatment regimen	SAL + PILO	MN + PILO	Statistical significance
CA1	Pre-T 6 h	16 (14:20)	17 (4:24)	n.s.
	Pre-T 3 h	14 (10:19)	9 (6:12)	P = 0.008
	Co-T 0 h	12 (8:14)	6 (3:8)	p = 0.024
	Post-T 15 min	13 (11:16)	6 (2:9)	p = 0.002
	Post-T 1 h	16 (15:21)	18 (14:27)	n.s.
	Post-T MD	15 (9:24)	9 (7:11)	n.s.
DG	Pre-T 6 h	12 (8:20)	11 (9:18)	n.s.
	Pre-T 3 h	15 (10:21)	14 (9:22)	n.s.
	Co-T 0 h	14 (10:19)	16 (11:18)	n.s.
	Post-T 15 min	12 (7:18)	14 (9:17)	n.s.
	Post-T 1 h	15 (7:22)	11 (6:22)	n.s.
	Post-T MD	13 (6:18)	14 (9:20)	n.s.
MePV	Pre-T 6 h	266 (217:284)	281 (201:361)	n.s.
	Pre-T 3 h	269 (149:400)	201 (154:298)	n.s.
	Co-T 0 h	272 (193:304)	247 (169:410)	n.s.
	Post-T 15 min	325 (161: 425)	43 (0:107)	p = 0.001
	Post-T 1 h	274 (194:415)	49 (18:203)	p = 0.004
	Post-T MD	267 (169:389)	32 (8:196)	p = 0.017
VPM	Pre-T 6 h	114 (92:120)	72 (53:79)	p = 0.001
	Pre-T 3 h	125 (104:132)	46 (40:54)	p = 0.001
	Co-T 0 h	113 (91:166)	41 (25:55)	p = 0.001
	Post-T 15 min	102 (81:137)	19 (0:63)	p = 0.001
	Post-T 1 h	113 (78:159)	49 (18:138)	p = 0.031
	Post-T MD	157 (91:195)	41 (27:130)	p = 0.026

Data were expressed as median (interquartile range).

et al., 2006; Loscher and Honack, 1990), MN may be a very promising therapeutic strategy for ameliorates cognitive impairments associated with early-life SE. Post-treatment with MN prevented impaired exploratory behavior and spatial memory deficits, and a decline of spatial memory-extinction in pilocarpine-induced SE (Kalemenev et al., 2016).

Our data suggest that cellular mechanisms underlying seizure maintenance and neurodegeneration at LiCl-pilocarpine-induced SE involve distinct signaling pathways. To analyse the mechanisms responsible for sustained SE, it is necessary to know exactly the brain regions involved and mechanisms of seizure propagation in pilocarpine model, especially because we demonstrated that MN exerts region-dependent effects. For example, *substantia nigra* is a crucial site of anticonvulsant activity, while forebrain and hindbrain areas had no significant contribution (Iadarola and Gale, 1982; Lado and Moshe, 2008) and this could limit the full effect of MN. Differences between pharmacodynamics of MN and others NMDAR antagonists have been proposed to more potent inhibition of extrasynaptic than synaptic NMDAR by MN (Parsons et al., 2007; Xia et al., 2010; Zhou et al., 2013). MN binding depends upon intracellular calcium concentration and thus on the intensity of NMDAR activation, as well as on duration of glutamate exposure in an NMDAR subtype-dependent manner, and compared to others NMDAR antagonists, differentially alter NMDAR desensitization (Glasgow et al., 2017). Thus, kinetic proprieties also could contribute to less efficiency to MN for stop seizure. Furthermore, MN (6 – 10 μM) did not attenuate LTP in hippocampal slices and substantially spared the NMDA component of excitatory postsynaptic currents, while MK-801 at same concentrations or APV (50 μM) completely blocked these events (Chen et al., 1998). Thus, we hypothesized that MN promotes fewer anticonvulsive effects through NMDAR-mediated overactivity, displaying a less pronounced effect than other NMDAR antagonists, due to the more rapid unblocking rate of MN.

In summary, our results showed that treatment with MN was able to protect the brain against SE-induced neuronal damage in a time- and spatial-dependent manner. Since MN is an FDA-approved drug, our experimental data may point to the putative use of this drug in futures prospective clinical trials involving neuroprotection against SE-induced brain damage.

Conflict of interest statement

There is no conflict of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2018.03.005.

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5. CAPÍTULO II

Artigo submetido

Título: Memantine attenuates seizure severity, reduces oxidative brain damage and prevents anxiety-like behaviors in PTZ-treated adult zebrafish.

Revista: Neuropharmacology

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Justificativa: Apesar de algumas evidências apontarem para a eficácia da MN como anticonvulsivante, a investigação de seus efeitos neuroquímicos e comportamentais de longo prazo perante as crises é fundamental para que sua utilização como fármaco reposicionado para o tratamento das epilepsias seja vantajosa. Assim, a comprovação de seus efeitos multialvo e uma melhor compreensão dos mecanismos relacionados a sua eficácia podem reforçar os benefícios

Objetivo geral: O objetivo deste trabalho foi investigar se a MN é capaz de prevenir as crises convulsivas induzidas por PTZ em peixe-zebra adulto, bem como se esse tratamento pode modular as alterações neuroquímicas e comportamentais neste modelo.

Resultados: Observamos que o tratamento com MN reduziu a expressão relativa da subunidade GluN2A do NMDAR. Demonstramos que a convulsão induzida por PTZ aumenta a carbonilação e a atividade da SOD, e o tratamento com MN foi capaz de prevenir esse dano oxidativo. Os animais submetidos às crises com PTZ apresentaram maior tempo gasto na zona branca em no aparato claro/escuros e a MN impediu essa anormalidade comportamental.

1 Memantine attenuates seizure severity, reduces oxidative brain damage and prevents
2 anxiety-like behaviors in PTZ-treated adult zebrafish.
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33 **ABSTRACT**

34 Repurposed drugs allow us to rationalize the research for a multi-target therapy because

35 can identify compounds that produce a therapeutic effect through simultaneous activity

36 at multiple targets using phenotypic screens. Repurposing with focus in a multi-target

37 drug is a viable solution to deal with refractory epilepsy. MN is a multi-target agent, well

38 tolerable partial NMDAR channel blocker, and is imperative to evaluate different

39 preclinical approaches for its repurposing in epilepsy. The aim of the present study was

40 to investigate if MN prevent or worse seizures pattern, neurochemical seizure markers

41 and long-term behavioral alterations in adult zebrafish PTZ-challenged. Animals were

42 pre-treated with vehicle or MN (20 or 50 mg/kg; i.p.) 1h or 2h before immersion into PTZ

43 solution at 10 mM (20 fish/group). Behavioral and neurochemical analyses was

44 performed 24 h after seizure. MN reduced relative expression of GluN2A NMDAR

45 subunit. PTZ-induced seizure increases carbonylation and SOD activity, and MN

46 treatment prevented this oxidative damage. PTZ-challenged zebrafish presented increases

47 in time spent in white zone into light/dark apparatus and MN prevented this behavioural

48 abnormality. Indeed, anticonvulsant action of MN ameliorates behavioral and

49 neurochemical PTZ-related changes in zebrafish seizure model, reinforcing the off-label

50 use to treat neurobehavioral consequences of epilepsy.

51

52 **Keyword:** memantine, repurposing, seizure, anxiety, zebrafish.

53 **1. Introduction**

54   Generalized seizures can lead to long-term and irreversible consequences for the

55 brain (Trinka et al., 2015). One of most remarkable consequence is alteration in neural

56 networks and neuronal death which can lead to epileptogenesis and behavioral deficits

57 (de Oliveira et al., 2008; Kubová et al., 2002; Minjarez et al., 2017; Sankar et al., 1998).

58 Although clinically used antiepileptic drugs have been recommended to stop convulsive

59 seizure (Glauser et al., 2016), they have failed to halt seizure-induced neurochemical

60 alterations associated with brain damage and epileptogenesis and presented undesirable

61 side effects for patients (Abdalla et al., 2014; Ahmed et al., 2011; Kalemenev et al., 2016;

62 Trinka et al., 2015). Moreover, 30% of the patients suffer from drug resistant, refractory

63 or intractable epilepsy and it remains a significant medical and economic problem (Brodie

64 et al., 2012). In addition, there was a decrease in the industrial interest for development

65 of new compounds for epilepsy and one strategy is to investigate the possible application

66 of one already approved drug to treat a different disease as therapy in epilepsy with focus

67 in multi-target agents discovery (Löscher et al., 2013; Morphy et al., 2004).

68   Drug repurposing or drug repositioning involves finding new therapeutic uses for

69 existing drugs. This strategy presents higher probability of success (at preclinical and

70 clinical level) than *de novo* drugs and represents a reduction in costs and development

71 timeline (Ashburn and Thor, 2004; Barratt and Frail, 2012). This strategy has allowed the

72 creation of information-sharing platforms for potential target molecules and repurposing

73 partnerships between academia and private companies (Caban et al., 2017; Murteira et

74 al., 2014). Repositioning is particularly relevant in poorly addressed therapeutic areas,

75 such as central nervous system, pediatric or orphan diseases (Caban et al., 2017).

76 Moreover, repurposed drugs allow us to rationalize the research for a multi-target therapy

77 because it can identify compounds that produce a therapeutic effect through simultaneous

78 activity at multiple targets (Kodama et al., 1997). A multi-target drug presents different

79 pharmacophores in a single molecule or a common pharmacophore for different targets

80 (Morphy et al., 2004), and it is a viable solution to deal with refractory epilepsy (Rocha

81 L, 2012). In this context, Memantine (MN) represents an outstanding example

82 repositioning drug of a multi-target ligand to treat CNS disorders (Lipton, 2006; Zheng

83 et al., 2014). It is an effective N-methyl-D-aspartate receptor (NMDAR) blocker (Chen

84 and Lipton, 2006) that possesses a fast blocking/unblocking kinetic and a partial blockage

85 of NMDAR, which does not compromise the physiological and neurochemical role of

86 these receptors on synaptic transmission (Johnson and Kotermanski, 2006; Parsons et al.,

87 2008, 2007). MN has been approved by the Food and Drug Administration to treat
88 moderate-to-severe Alzheimer's disease (Parsons et al., 2007). MN also shows
89 uncompetitive antagonism on serotonin 5-HT3 receptors (Rammes et al., 2001) and acts
90 as agonist on dopamine D2 receptors (Seeman et al., 2008), with similar affinity for the
91 NMDAR. In addition, recent evidences suggest that MN acts in redox imbalance in
92 different pathological conditions, increasing their potential as multi-target drug (Bardak
93 et al., 2018; Khalili-Fomeshi et al., 2018; Tanaka et al., 2018).

94   More recently, MN has also been used as an anticonvulsant agent in seizure-
95 induced animal models. MN at 20 mg/kg shown to be neuroprotective and attenuated
96 seizure severity in immature rats submitted to LiCl-pilocapine-induced *status epilepticus*
97 (Zenki et al., 2018). This study showed that MN presented a singular profile of
98 neuroprotection across immature brain, even when it was administered after seizure onset
99 (Zenki et al., 2018).

100   Considering that seizures alters several biomarkers associated to NMDAR
101 overactivation and MN is a multi-target agent, well tolerable partial NMDAR channel
102 blocker, the aim of the present study was to investigate if MN prevents PTZ-induced
103 seizures in adult zebrafish, as well as reduces the neurochemical and behavioral
104 alterations caused by PTZ-induced seizures.

105   **2. Material and methods**

106   **2.1. Drugs**

107   Pentylenetetrazole (#P6500), tricaine (#A5040) and memantine hydrochloride

108   (#M9292) were purchased from Sigma-Aldrich (USA).

109   **2.2. Animals**

110   Male adult zebrafish (*Danio rerio*) (short fin wild-type strain; 4-6 months old)

111   were obtained from a local commercial supplier (Aquazoo, SE, Brazil). Fish were

112   acclimated for at least 2 weeks prior the experiments. Animals were kept in 20 L aerated

113   tanks (3 animals/L) filled with distilled water under mechanical and chemical filtration.

114   Water temperature, pH and conductivity were adjusted to 26±2° C, 7.0-8.0, and 500

115   µS/cm, respectively. Ceiling-mounted fluorescent white lamps provided room

116   illumination (600 lux) and light/dark cycle was 14/10 h (lights on at 7:00 am). Only

117   animals with 0.25-0.35 g of body weight were used. All animals were fed four times a

118   day with ALCON BASIC™ flake food (ALCON, Brazil; twice a day) and *Artemia sp.*

119   nauplii (twice a day). All procedures were performed accordingly Brazilian's Law for

120   Care and Use of Laboratory Animals (Law 11794/2008) and were previously approved

121   by the Committee for Animal Care and Use from Universidade Federal de Sergipe

122   (number 57/2015).

123   **2.3. Memantine treatment and PTZ-induced seizure model**

124   Animals were anesthetized by immersion in tricaine solution (160 µg/mL) (Alfaro

125   et al., 2011) and further injected i.p. with memantine (MN) at 20 and 50 mg/kg or vehicle

126   (saline solution 0.9%). Maximal volume of injection was 10 µL/g of body mass (Kinkel

127   et al., 2010). Tricaine immersion lasted for 1 to 2 min, until fish were completely

128   immobile and decreased operculum opening. Initially, a set of animals was divided in 4

129   subsets which were treated with MN 20 mg/kg, MN 50 mg/kg or vehicle (N= 10

130   fish/group; supplementary Figure 1A). This evaluation was performed in order to access

131   the sedative or hypolocomotory effects of MN. Since memantine reduced the fish distance

132   traveled immediately or 30 min after its administration (Supplementary Fig. 1B), we

133   chose 1 and 2 h after MN injection as a time-points to perform the further analyses.

134   To investigate the putative anticonvulsant action of memantine, another set of

135   animals were pre-treated with vehicle or MN (20 or 50 mg/kg; i.p.) 1h or 2h before

136   immersion in 10 mM of pentylenetetrazole (PTZ) solution (20 fish/group). Fish were

137   observed during 20 min for seizure evaluation (Mussulini et al., 2013). After, animals

138   returned to their home-tanks and twenty-four hours after, animals were divided in two

139   subsets for behavioral and neurochemical analyses (Supplementary Fig. 2). For all

140   neurochemical analyses fish brain were removed and stores in liquid nitrogen. All

141   analyses were performed by 3 independent experiments.

2.4. Quantitative real time RT-PCR

143   Total RNA was extracted from frozen brains using Trizol™ (Invitrogen Life

144   Technologies, USA) (N= 6 samples/group; 2 fish's brain/sample). Samples were

145   homogenized with Trizol, chloroform was added (1:5, v/v) and then they were centrifuged

146   at 12000*g* (15 min, 4°C). Aqueous phase was collected and total RNA was precipitated

147   with isopropanol for 10 min at room temperature followed by centrifugation (12000*g*, 10

148   min, 4°C). Supernatant was discarded, pellet was washed two times with 75% ethanol

149   and subsequently centrifuged at 12000*g* (5 min, 4° C). Pellet was dissolved in DEPC

150   water, and the total RNA was quantified for purity at 230, 260 and 280 nm (NanoDrop

151   ND-1000). Total RNA was stored at -80° C.

152   Total RNA (1 μg) was used as template to synthesize cDNA. It was incubated for

153   5 min at 65° C with 1 μL of oligo (dT) (0.5mg/mL, Invitrogen), 1 μL of 10 mM dNTPs

154   and DEPC water for a final volume of 13 μL. The following reagents were subsequently

155   added to a final volume of 19 μl: 4 μl of RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM

156   KCl, 3 mM MgCl2), and 2 μl of 0.1 M DTT. After 2 min of incubation at 37°C, 1 μL of

157   M-MLV RT (200 U/μL) was added. cDNA synthesis was performed at 37° C for 50 min

158   and reaction was stopped by incubation at 70° C for 15 min.

159   The qPCR was performed for the relative quantification of gene expression using

160   SYBR-Green™ (Applied Biosystems), and specific primers (Invitrogen) were used to

161   evaluate the expression of NMDA receptor subunits: *grin1a* (GluN1), *grin2a* (GluN2a),

162   and *grin2b* (GluN2b). β-actin was used as the housekeeping gene since it shows highest

163   expression stability in zebrafish brain (Casadei et al., 2011).

164   Table 1 showed the sequences of each primer used. Primers were designed using

165   Primer3Plus software (*www.bioinformatics.nl/primer3plus*) based on zebrafish

166   sequences on GenBank database (*www.ncbi.nlm.nih.gov*) and on previous work by Hunt

167   et al. (2012). Specificity of each primer was verified by BLAST on the NCBI database.

168   For each set of primers, the standard curve was prepared using serial dilutions (1:1, 1:5,

169   1:25, 1:125, 1:625, 1:3125). The efficiency was calculated according to the formula

170   E=10(-1 slope)-1. Primers with slope standard curve values ranging from 90 to 110%

171   were accepted (Svec et al., 2015). Amplification was performed using 7.5 μL of SYBR

172   Green PCR Master Mix™ (Applied Biosystems, São Paulo, Brazil), 0.5 μL of forward

173   and reverse primers (10 μM each), 1 μL of cDNA, and nuclease-free water for a total

174   volume of 15 μL. Samples without RNA were included as a negative control. Reactions

175   were performed on a 96-well optical plate using a StepOnePlus™ Thermocycler (Applied

176   Biosystems, USA). After an initial denaturation step at 95°C for 10 min, the amplification

177   was carried out for 40 cycles of denaturation (95°C for 30 s), hybridization (60°C for 40

178   s), and extension (72°C for 40s).

179   The analysis of the melting curves was used to confirm the specificity of the PCR

180   product (Wilhelm and Pingoud, 2003). No signals were detected in the negative controls.

181   The experimental CT (Cycle Threshold) was calculated using algorithms provided by the

182   equipment (Schmittgen and Livak, 2008). All samples were tested in duplicate and the

183   mean value of each duplicate was used for calculations. The method of evaluating the

184   quantification of gene expression was $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

185   **2.5. Oxidative stress assessment**

186   Frozen brains (N= 4 samples/group; 6 fish's brain/sample) were homogenized into

187   1000 µL of lysis buffer (Igepal 0.5%, Tris HCl 50 mM) and centrifuged at 600g during 2

188   min. Supernatant was collected and protein content was measured. Total protein was

189   normalized to 1 µg/µL.

190   **2.5.1. Total Reduced Thiol (-SH) assay**

191   Total reduced thiol levels (-SH) were determined at 50 µL (duplicate samples) by

192   measuring absorbance of DTNB at 412 nm (Ellman, 1959) and expressed as µmol of

193   −SH/mg of protein.

194   **2.5.2. Protein Carbonyl content assay**

195   Protein carbonylation was estimated by protein precipitation in the presence of

196   trichloroacetic acid and dinitrophenylhydrazine (DNPH) with modifications (Yan et al.,

197   1995). Brain samples (200 μL) were mixed with 0.15 mL of 10 mM DNPH and incubated

198   for 1 h. After, 0.125 mL of SDS (3.0%), 0.5 mL of heptane (99.5%), and 0.5 mL of ethanol

199   (99.8%) were added and mixed for 30 s. Samples were centrifuged at 1000g for 15 min

200   and supernatant was discarded. Pellet was homogenized in 0.25 mL of 3% SDS and the

201   amount of carbonylated proteins was determined at 370 nm. Results were expressed as

202   nmol carbonyl/mg protein and calculated using a molar extinction coefficient of 22.000

203   M/cm.

204   **2.5.3. Superoxide Dismutase (SOD) activity**

205   Superoxide dismutase (SOD) activity was measured in a spectrophotometric assay

206   at 480 nm by inhibition of epinephrine oxidation by superoxide radical. Brain samples

207 (10, 20 and 40 µL for activity curve) were homogenized with 50 mM glycine-NaOH

208 buffer pH 10. Epinephrine was added to start the reaction. The kinetic curve was read

209 immediately at 480 nm during 20 minutes at 32° C and the results were expressed as SOD

210 units/mg of protein. One unit was defined as the enzyme amount that inhibits the rate of

211 reaction by 50% (Misra and Fridovich, 1972).

212   **2.5.4. Catalase activity**

213   Catalase (CAT) activity was determined in a spectrophotometer at 240 nm by

214 measuring the decomposition rate of hydrogen peroxide (Aebi, 1984). Brain samples

215 (duplicates of 10 µL) were mixed with 50 mM phosphate buffer pH 7.0 and read

216 immediately at 25ºC. Hydrogen peroxide (10-50 mM) was added to start the kinetic

217 reaction, which was read at 240 nm. To perform calculation, we assume that one unit of

218 catalase decomposes 1 mol of peroxide per minute at 25°C at pH 7

219   **2.5.5. Glutathione Peroxidase activity**

220   Glutathione Peroxidase activity was measured spectrophotometrically at 340 nm

221 by measuring the oxidation of reduced glutathione (GSH) by tert-butil hydroperoxide.

222 Substrate is maintained at a constant concentration by the addition of reduced

223 nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction mixture containing

224 1 mM sodium azide, 0.5 mM tert-butylhydroperoxide, 50 mM phosphate buffer pH 7.0

225 at 30ºC in the presence of 1mM reduced glutathione and 0.25 U/ml glutathione reductase.

226 Brain samples (duplicates of 30 µL) were added into the mix reaction medium. Reduction

227 in absorbance of reaction mixture at 340 nm is a measure of the NADPH oxidation

228 according to the method of Lawrence and Burk (1976). One enzymatic unit was defined

229 as 1 µmol of NADPH oxidized/min, which correspond to 1 µmol of glutathione

230 oxidized/min (Günzler et al., 1974).

231   **2.5.6. GSH concentration assay**

232   GSH concentrations were measured according to Browne and Armstrong (1998)

233 with minor modifications. Brain samples (1µg protein/µL; duplicates of 30 µL) were first

234 deproteinized with metaphosphoric acid, centrifuged at 7000g for 10 min and

235 immediately used for GSH quantification. One hundred and eighty-five microliters of 100

236 mM sodium phosphate buffer, pH 8.0, containing 5 mM ethylenediaminetetraacetic acid,

237 and 15 µl of o-phthaldialdehyde (1 mg/mL) were added to 30 µL of deproteinized brain

238 samples. This mixture was incubated at RT in a dark room for 15 min. Fluorescence was

239 measured using excitation and emission wavelengths of 350 and 420 nm, respectively.

240 Calibration curve was prepared with standard GSH (0.001–1 mM).

2.6. Protein quantification

Protein content was measured according to the protein assay method of Lowry with modifications (Peterson, 1977). Aliquots of 5 μL protein were used to perform the assay (Zenki et al., 2014).

2.7. Behavioral tests

All behavioral tests performed after PTZ-induced seizures were conducted between 9:00 a.m. and 4:00 p.m. All tests were conducted under white illumination with light intensity of 600 lux. All putative environmental interferents (such as noise, vibration, movement in front of the tank) were avoided in order to reduced potential experimental bias. Fish were transferred to experimental room 30 min before the beginning of the behavioral tests. After, animals were gently netted and transferred to the behavioral apparatuses. Fish behavior was analyzed by ANY-maze® video-tracking system (Stoelting CO, EUA). Each animal was tested only once.

2.7.1. Open tank test

Animals (10 fish/group) were placed individually in a 1.5 L trapezoidal tank (23.9 cm along the bottom X 28.9 cm at the top X 15.1 cm high and 15.9 cm along the diagonal side) similar to those previously described by Rosemberg et al. (2011). Tank was filled with 1.5 L home-tank water. It was divided into three equally virtual horizontal areas (named bottom, middle and top). The locomotor and exploratory activities were recorded for 6 min. The following parameters was evaluated: total distance traveled (m), mean speed (m/s), total mobile and immobile time (s), time spent (s) in bottom, middle and top areas. A video camera located approximately 40 cm above the tank was used to record the fish activity.

2.7.2. Social preference test

Social preference was conducted in a glass tank divided into three zones: center zone (which consisted in testing area), opposite zone (which consisted in an empty area), and the conspecific zone (which contained three stimulus zebrafish) (Saverino and Gerlai, 2008). Animals (n=10 animals/group) were introduced in the center zone and were allowed to explore it for 6 min (Baggio et al., 2018). Stimulus fish were moved to the experimental room 24 h before the beginning of experiments for acclimation.

2.7.3. Light/dark test

Animals (n=10 animals/group) were introduced into apparatus to light/dark test accordingly to Maximino et al. (2010) with modifications. Apparatus consisted in a tank (15 × 10 × 45 cm, height × depth × length) equally divided in two distinct compartments

275 (one covered black and other covered white). Opaque plastic self-adhesive films were
276 used for covering walls and floor of both compartments. Apparatus was filled with 1.5 L
277 home-tank water. Animals were individually placed in the lit area of the tank and were
278 allowed to explore it for 15 min. The following behavioral parameters were recorded: the
279 time spent in each compartment and the number of crossings.

280 **2.8. Statistical analysis**

281 Data were expressed as mean ± standard error of the mean (S.E.M.), except for
282 seizure score which was expressed as median with interquartile range. All data were
283 tested for normality by D'Agostino-Pearson omnibus normality test. Normal data were
284 analyzed by two-way ANOVA followed by Tukey's *post hoc* test. $P \leq 0.05$ was
285 considered significantly.

286

3. Results

Latency to seizure onset (score 4) in control animals was similar to the original work described by Mussulini et al. (2013) (Figure 1A). MN 50 mg/kg increased the latency to score 4 in both pre-treated groups (P = 0.0328 and P = 0.0007 for 1 and 2h, respectively) (Figure 1A). In addition, animals treated with MN 50 mg/kg 2h before PTZ showed a decreased time to return to score 0 (Figure 1B; P = 0.0007). There was no difference between MN 20 mg/kg and PTZ-treated groups.

Vehicle-treated animals exposed to PTZ showed a rapid seizure progression in the first 300s of test. In contrast, pre-treatment with MN attenuated this progression by reducing seizure scores (Figure 2A and B). In comparison with vehicle-PTZ group, quantitative analysis of these data showed that all pre-treatments reduced the seizure severity in all time intervals tested (Figure 2C-E). Animals treated with vehicle only did not show any seizure-like behavior (data not shown).

All animals from control group underwent tonic-clonic seizures (score 4) during the first 300s of observation (Figure 3A and B). However, in MN treated animals this time was increased up to 600s (Figure 3C-F).

There was no difference between MN and control groups regarding the expression of NMDA receptor subunits NR1 and NR2B (*grin1 and grin2b* genes, respectively) (Figures 4A C, respectively). However, vehicle animals submitted to PTZ-induced seizure showed an increase in relative expression of NR2A NMDA receptor subunit (*grin2a* gene) in both pre-treatment times when compared to vehicle group (P = 0.0057 for 1h and P = 0.0436 for 2h of pre-treatment) (Figure 3B). Pre-treatment with MN 20 mg/Kg 1h or 2h before seizures induced by PTZ prevented the PTZ-induced augmentation of NR2A expression (P = 0.0004 and P = 0.0028, respectively). MN 50 mg/Kg partially reduced the PTZ-induced NR2B increased expression.

There were no differences among groups regarding reduced thiol levels (Figure 5A), catalase activity (Figure 5D), GPx activity and GSH content (Figures 5E and 5F, respectively). However, PTZ-induced seizure increased protein carbonylation and reduced SOD activity when compared to vehicle group in both pre-treatment times (protein carbonylation: P = 0.0067 for 1h and P = 0.0021 for 2h, Figure 5B; SOD activity: P = 0.0273 for 1h and P = 0.0152 for 2h, Figure 5C). MN pre-treatments only prevented partially these alterations, however without statistical significance.

There were no differences among groups regarding the exploratory and locomotory profile in the open tank test (Figure 6). In addition, MN and PTZ treatments

321   did not alter the sociability of zebrafish in the social preference test (Figure 7). In contrast,

322   PTZ exposure increased the time spent in white compartment in vehicle animals pre-

323   treated 1 and 2h before PTZ (P < 0.0001 and P = 0.003 Figure 8A). Treatment with MN

324   1h before PTZ in both doses prevented this alteration (p < 0.0001 for 20 mg/Kg and P =

325   0.0161 for 50 mg/Kg when compared to PTZ group). MN administered 2h before PTZ

326   did not prevent PTZ-induced increase in the time spent in white compartment. Treatments

327   did not alter the number of crossings of animals in the light/dark test (Figure 8B).

4. Discussion

In the present work we report a preventive effect of MN on neurobehavioral parameters in PTZ-induced seizures in adult zebrafish. MN attenuated seizure-like behaviors and decreased seizure intensity. Particularly, MN 50 mg/Kg abolished seizure score 5 in 20% of animals when administered 1 and 2 h before PTZ. Moreover, MN prevented the increase in the expression of NMDA-NR2A subunit as well as protein carbonylation. These effects were followed by an increase in SOD activity and a reduction of PTZ-induced anxiety like-behaviors in animals. Overall, we suggest a protective role of MN against PTZ-induced behavioral and neurochemical changes in adult zebrafish.

MN effects on seizure control or psychoemotional state of animals remains a controversial topic (Creeley et al., 2006; Löscher and Hönack, 1990), although it has been demonstrated a preventive effect against NMDA-induced seizures in neonatal rats (Dhir and Chopra, 2015) and a suppression of generalized tonic-clonic PTZ-induced seizures (Mareš and Mikulecká, 2009). Additionally, MN administration reduced seizure intensity from Stage 5 to Stage 0 in seizures produced by a single sublethal injection of soman in rats (McLean et al., 1992). In fact, NMDAR antagonists are powerful anticonvulsants and possess neuroprotective effects in several animal models (Fujikawa, 1995; Loss et al., 2012; Stafstrom et al., 1997). MN is thought to play a pivotal role in controlling the inhibitory/excitatory balance since it may act as a NMDAR blocker, attenuating seizure-like behaviors. Although in our work only MN 50 mg/Kg increased latency to reach score 4 and decreased the time to return to normal swim behavior (Figures 1A B), all MN treatments decreased seizure intensity (Figure 2), indicating that MN could be considered for seizure/epilepsy treatment.

MN is particularly effective to block the effects of glutamate concentration elevation or calcium influx increase, because it antagonizes the NMDAR. Beyond direct antagonism, MN could indirectly modulate others glutamatergic hyperstimulation-related biomarkers (Parsons et al., 2008; Zaitsev et al., 2015). Persistent presence of agonist glutamate can lead for a negative feedback in NMDAR subunit expression may be a defensive mechanism to avoid receptor overactivation and MN could be prevent this event. The treatment of cultured cortical neurons with glutamate or NMDA resulted in a reduction of the GluN1 protein and mRNA (Gascón et al., 2005; Mao et al., 2002), and the treatment with quinolic acid produced the same effect (Kumar, 2004). There have been reports of alterations in NMDAR subunits expression in acute or chronic seizures, using rats or zebrafish models (Hunt et al., 2012; Jensen et al., 1997). In addition,

362 Postnikova et al., (2017) demonstrated significant increases in the expression of GluN1

363 and GluN2A subunits 3 h after PTZ-induced status epilepticus and a 3-fold increase in

364 the expression of the GluN2B subunit 24 h after PTZ-induced status epilepticus. Indeed,

365 these evidences corroborates for multi-target action of MN.

366 We did not observe alterations in GluN1 and GluN2B NMDAR subunits

367 expression when vehicle animals were treated with PTZ (Figure 4). However, PTZ-

368 induced seizure increased the relative expression for GluN2A subunit, which corroborates

369 with previous work of Zhu et al., (2004). Authors demonstrated that in the early phase of

370 epileptogenesis, GluN2A subunit gene was markedly expressed in cortex of rats. Authors

371 also suggested that this increase of GluN2A expression may contribute to elevate the

372 central nervous system excitability, contributing to the seizure generation and

373 epileptogenesis. Our data provided further evidences to support that GluN2A subunit may

374 be involved in regulating epileptic activity during PTZ-induced seizure and that MN may

375 be considered to counterbalance this alteration.

376 In the present work, we showed that PTZ-induced seizure increased protein

377 carbonylation, which suggests an oxidative damage of zebrafish CNS. Moreover, PTZ-

378 treated animals showed increased level of SOD activity, which can indicate an elevated

379 levels of anion superoxide and a putative mechanism to remove it. We also showed that

380 MN was able to prevent this PTZ-induced oxidative damage, suggesting a putative

381 antioxidant effect of MN through a modulatory action on enzymatic antioxidant defenses.

382 In fact, Reactive oxygen species play a pivotal role in seizure-induced neurodegeneration

383 (Patel, 2016; Rao et al., 2006). NMDAR activation induces superoxide-mediated

384 oxidative stress, which lead to an excessive calcium influx and subsequent neuronal

385 degeneration (Gao et al., 2007; Reyes et al., 2012; Singh et al., 2003). Since MN blocka

386 NMDAR, its antioxidant action may be through the inhibition of extracellular calcium

387 influx and subsequent reactive oxygen species production (Glasgow et al., 2017; Jiang et

388 al., 2010; Prentice et al., 2015).

389 Epilepsy is associated with the manifestations of abnormal stereotyped behaviors

390 and cognitive impairments (Choi-Kwon et al., 2003; de Oliveira et al., 2008;

391 Krishnamoorthy et al., 2007; Minjarez et al., 2017). Psychiatric comorbidities are 3-fold

392 more frequent in patients with epilepsy than in normal population (Tellez-Zenteno et al.,

393 2007). Therefore, in the present study were analyzed locomotory, exploratory, social and

394 anxiety-like behaviors after PTZ-induced seizures. We found that PTZ no alters zebrafish

395   locomotion, exploratory profile or social interaction parameters, when endpoint was 24

396   hours.

397   Remarkably, exist also a powerful relationship between epilepsy and anxiety

398   disorders (Choi-Kwon et al., 2003; Hingray et al., 2019), and NMDA antagonists appear

399   to modulate these conditions (Loss et al., 2012). We not observed any indicative of classic

400   anxiety-like behavior in open tank or social tests. Thus, we evaluated this, using the

401   light/dark test (Figure 8), it has been used as an anxiety test in zebrafish and anxiolytics

402   drugs have been found increased time spent in the light zone whereas anxiogenic drugs

403   decreased it (Gebauer et al., 2011; Maximino et al., 2010; Stewart et al., 2012).

404   Surprisingly, we demonstrated that PTZ-treated animals increased the time spent in white

405   compartment. Treatment with MN prevented this behavioral abnormality. This result

406   suggests a reduction of defensive behavior, disrupting innate light avoidance of zebrafish.

407   Detour et al. (2005) suggested that the disruption of neural networks involved in fear

408   expression can causes a misevaluation of threatening stimulus, reducing anxiety and/or

409   enhancing impulsive behaviors. In the pilocarpine-induced seizure model, epileptic rats

410   stayed longer periods in the open arms of elevated-plus maze when compared to control

411   ones (Detour et al., 2005; dos Santos et al., 2005). MN has demonstrated beneficial effects

412   in some neuropsychiatric disorders (Hosenbocus and Chahal, 2013; Koukopoulos et al.,

413   2012). Furthermore, MN prevented impaired exploratory behavior and spatial memory,

414   and a decline of extinction of oriented behavior in Li–pilocarpine model of epilepsy

415   (Kalemenev et al., 2016). The behavioral benefits are extend to humans, which observed

416   beneficial effects such as improvements in awareness, behavior and sleep using MN to

417   treat epileptic encephalopathy (Platzer et al., 2017). Taking together with your results,

418   these emerging the putative off-label use of MN as a co-adjuvant therapy to treat

419   neurobehavioral consequences of seizures.

420 **5. Conclusion**

421   In the present work we showed that MN reduced the behavioral and
422 neurochemical alterations caused by PTZ-induced seizures adult in zebrafish, which
423 indicates that it may be considered as a potential multi-target drug for treatment of
424 neurobehavioral alterations associated to epilepsy.

430

431 **Conflict of Interest Statement**

432 There is no conflict of interest.

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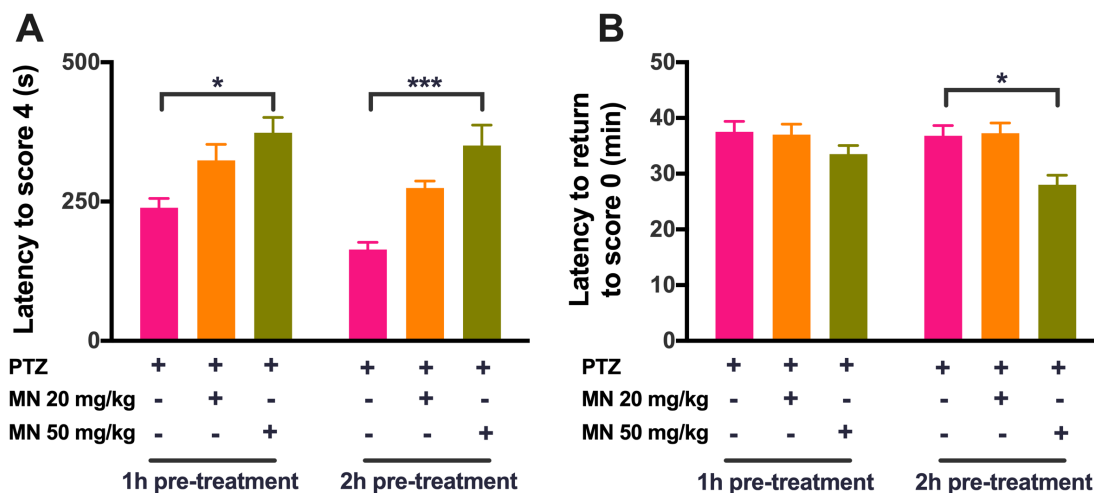
Figure 1. Effect of memantine pre-treatment on PTZ-induced seizure. **A)** Latency to reach score 4 and **B)** Latency to return to score 0. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's *post hoc* test. * = P <0.05 when compared to PTZ-treated group. ** = P <0.01 when compared to PTZ-treated group. *** = P <0.001 when compared to PTZ-treated group. **** = P <0.0001 when compared to PTZ-treated group. N = 24 animals per group.
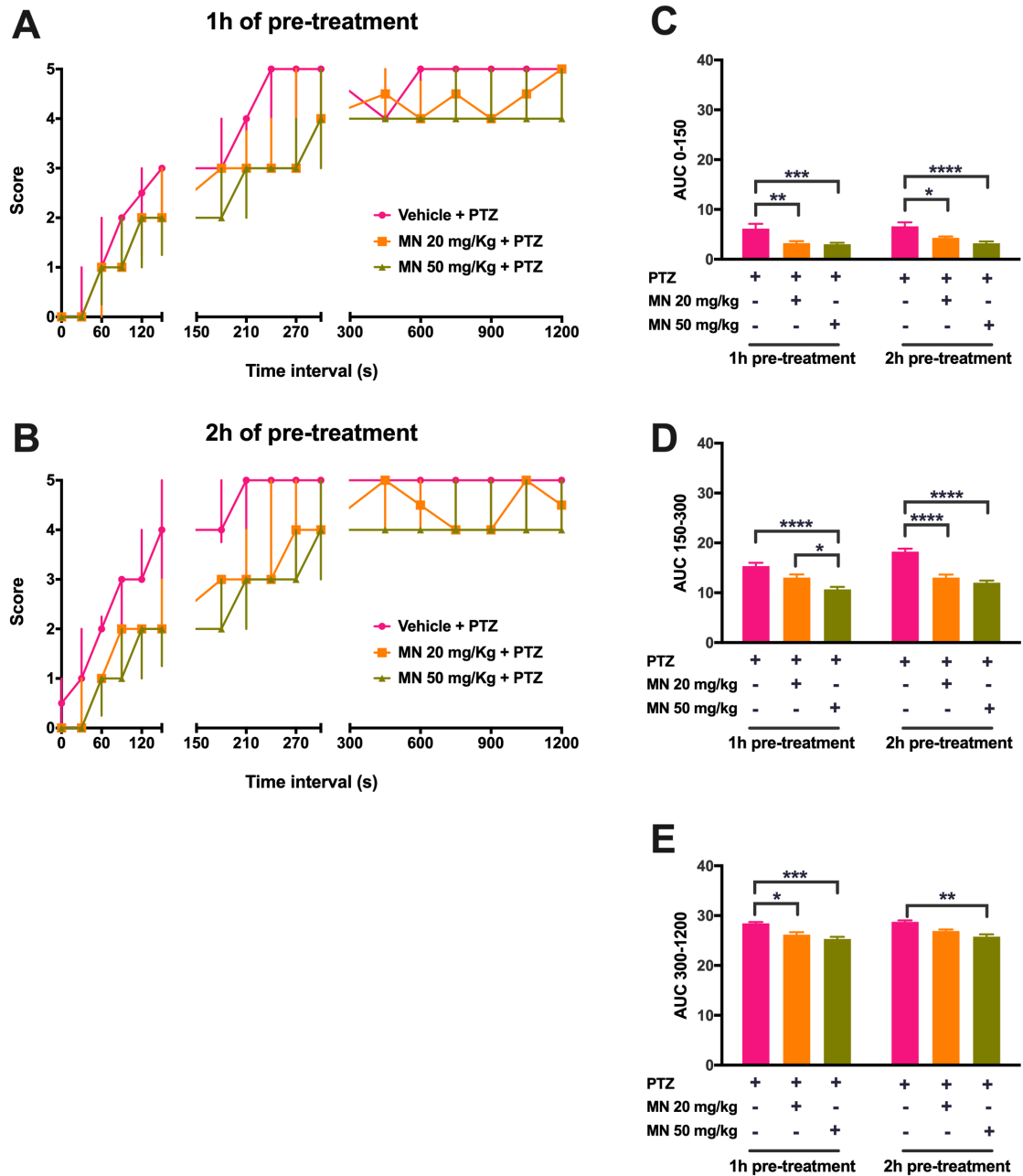
Figure 2. Temporal behavioral seizure profile of adult zebrafish pre-treated with memantine and submitted to PTZ-induced seizure. **A and B)** Seizure scores across time. Data were expressed as median with interquartile range. **C-E)** Seizure severity in specific time intervals (0-150, 150-300, and 300-1200 s). Seizure severity was evaluated as the total area under score curve (AUC) and was expressed as mean ± S.E.M. It was analyzed by two-way ANOVA followed by Tukey's *post hoc* test. * = $P <0.05$ when compared to PTZ-treated group. ** = $P <0.01$ when compared to PTZ-treated group. *** = $P <0.001$ when compared to PTZ-treated group. **** = $P <0.0001$ when compared to PTZ-treated group. N = 24 animals/group.
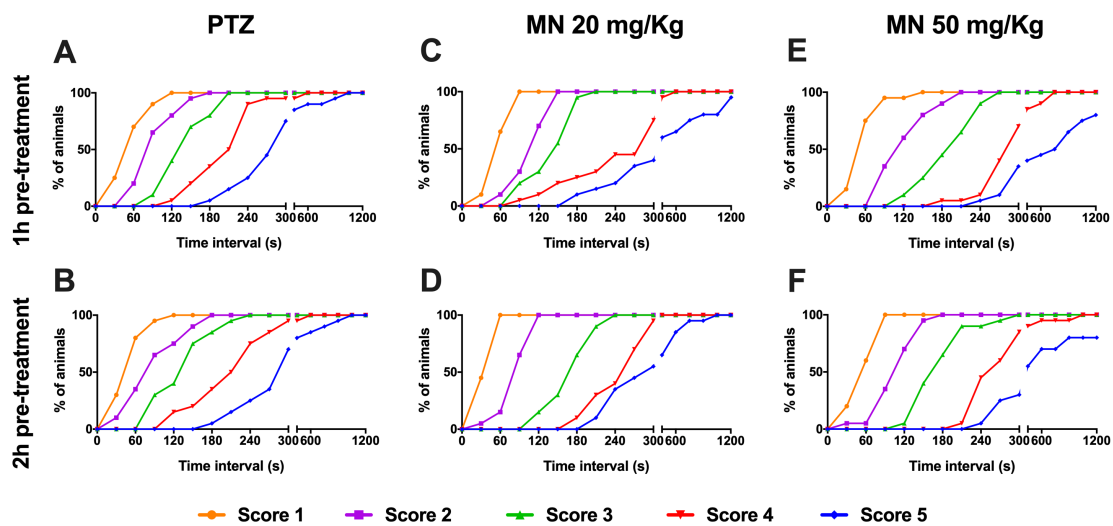
Figure 3. Cumulative frequency of animals that reached each seizure score across time. N = 24 animals/group.
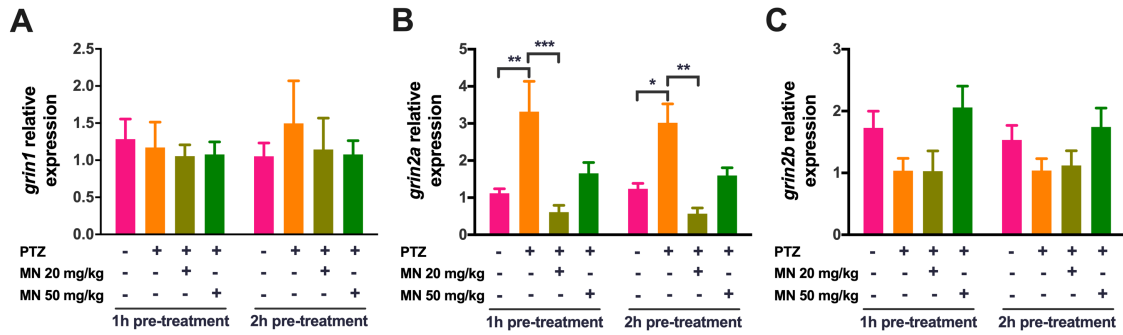
Figure 4. Expression of NMDA receptors in zebrafish brain pre-treated with memantine and submitted to PTZ-induced seizure **A)** Relative expression of GluN1 obligatory subunit; **B)** Relative expression of GluN2A regulatory subunit. **C)** Relative expression of GluN2B regulatory subunit. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's *post hoc* test. * = P<0.05, ** = P<0.01, and *** = P<0.001. N= 6 samples/group, 2 fish's brain/sample.
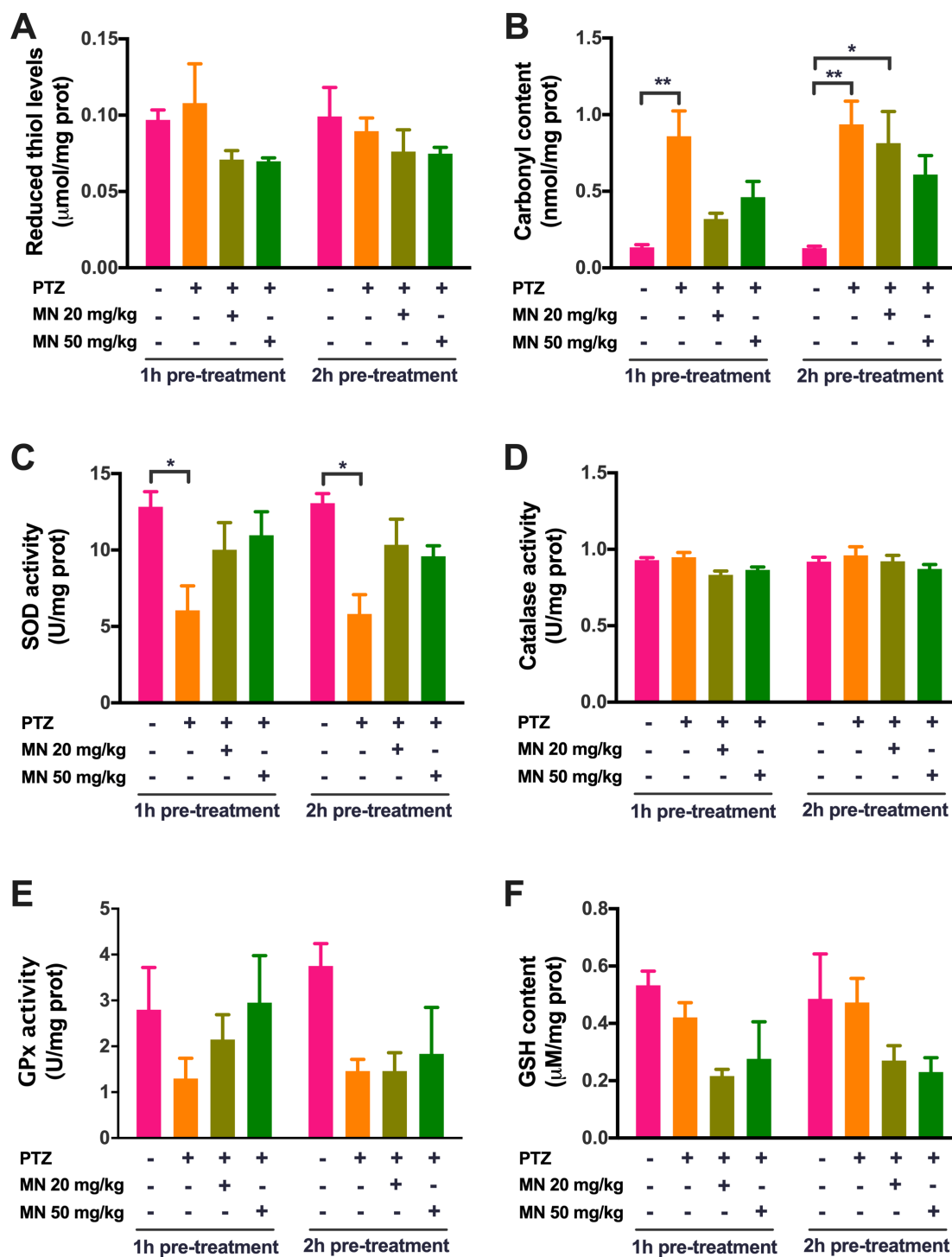
Figure 5. Oxidative stress assessment in adult zebrafish pre-treated with memantine and submitted to PTZ-induced seizure. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's *post hoc* test. *= $P<0.05$, **= $P<0.01$. N= 4 samples/group, 6 fish's brain/sample.

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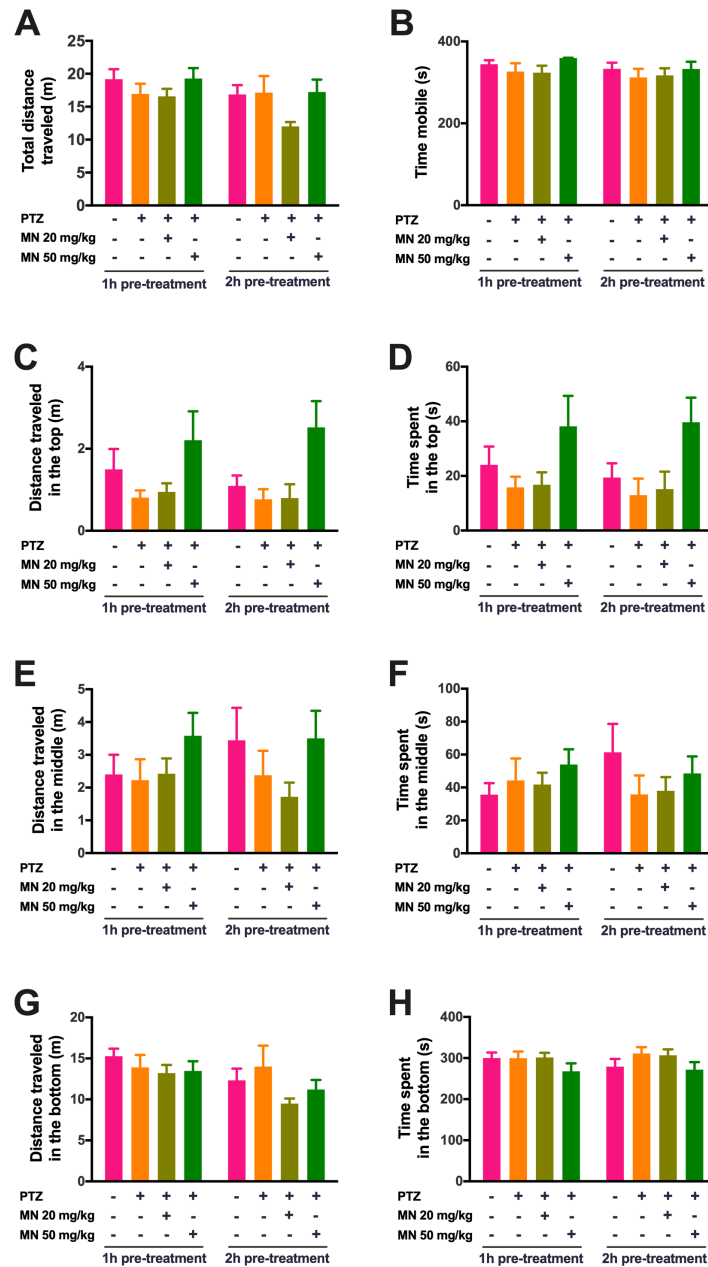
Figure 6. Exploratory and locomotory behavioral profile of adult zebrafish pre-treated
with memantine and submitted to PTZ-induced seizure. Data were expressed as mean ±
SEM and analyzed by two-way ANOVA followed by Tukey's *post hoc* test. N= 10
animals/group.
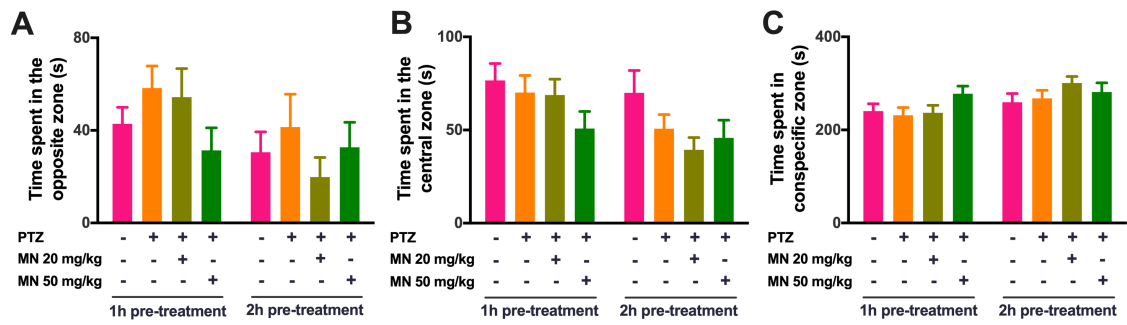
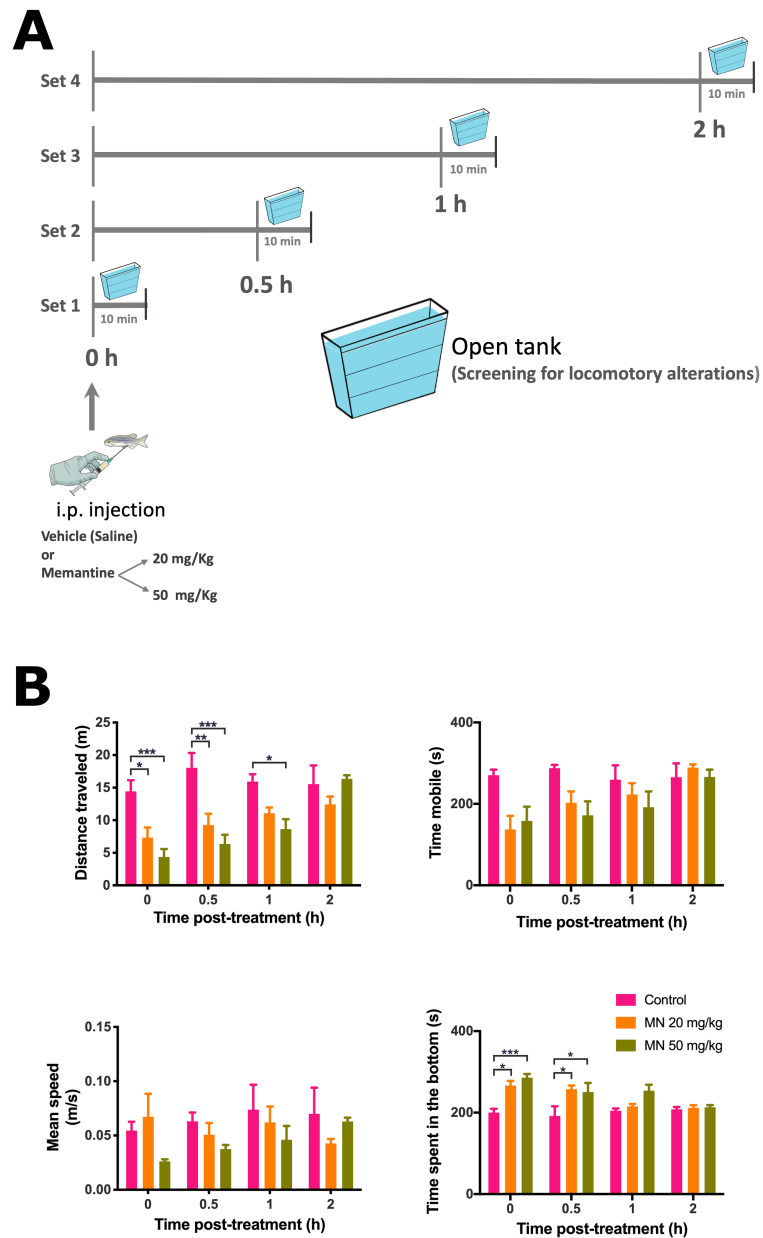**Figure 7.** Behavioral profile of adult zebrafish pre-treated with memantine and submitted to PTZ-induced seizure in the social preference test. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's *post hoc* test. N= 10 animals/group.
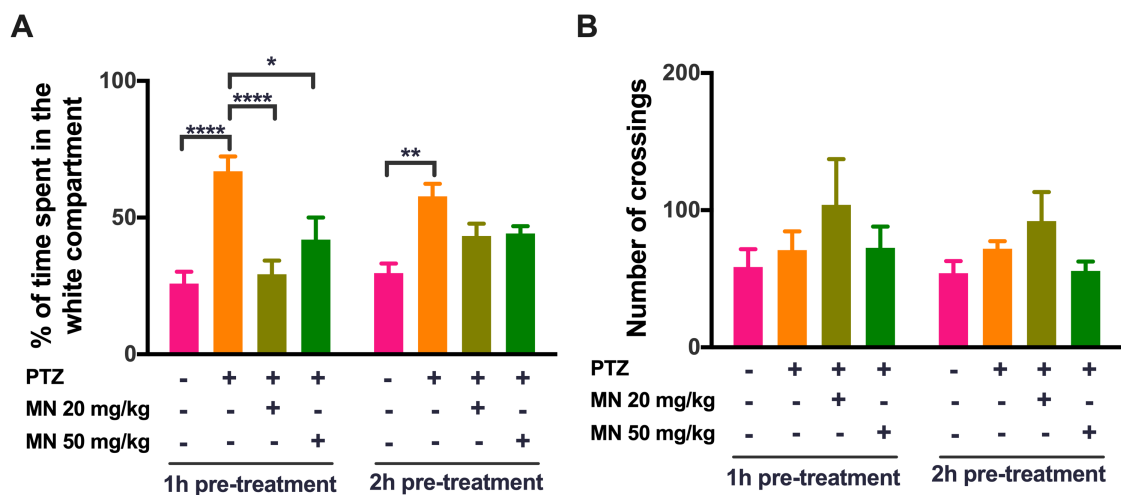
Figure 8. Behavioral profile of adult zebrafish pre-treated with memantine and submitted to PTZ-induced seizure in the light/dark test. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's *post hoc* test. * = P <0.05 when compared to PTZ-treated group. ** = P <0.01 when compared to PTZ-treated group. *** = P <0.001 when compared to PTZ-treated group. **** = P <0.0001 when compared to PTZ-treated group. N= 10 animals per group.

782

783 **Supplementary Figure 1.** Effect of memantine on locomotor and exploratory behavioral
784 profile of adult naïve zebrafish. **A)** Representative illustration of the experimental design.
785 Animals were treated with memantine (20 or 50 mg/kg) and after 0, 0.5, 1 or 2 hours their
786 locomotor and exploratory behavior was analyzed in the open tank test. Locomotor and
787 exploratory profiles were evaluated during 10 min. **B)** Locomotor and exploratory
788 variables evaluated in the open tank test.
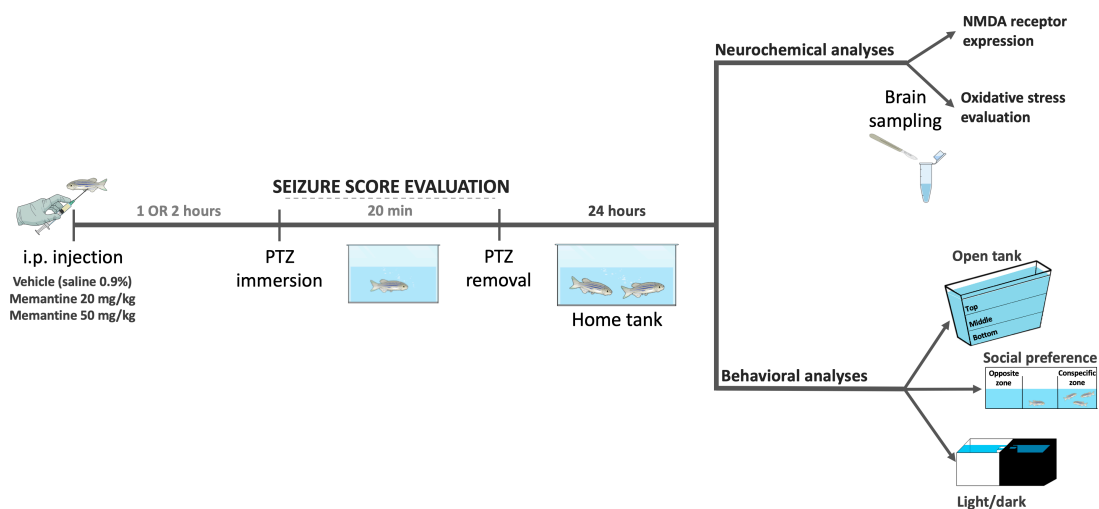
Supplementary Figure 2. Representative illustration of the experimental design for evaluating the anticonvulsant and neuroprotective effect of memantine. Animals were pre-treated with memantine 1 or 2 hours before submitting to PTZ-induced seizures. Seizures were evaluating during 20 min and scored according to Mussulini et al. (2013). After seizures, animals were divided in two independent sets, one for neurochemical analyses and other for behavioral evaluation.

PARTE III – DISCUSSÃO GERAL

6. DISCUSSÃO

No presente trabalho investigamos se a MN foi capaz de atenuar o fenótipo de crises epilépticas bem como reduzir as alterações cerebrais e comportamentais relacionadas a elas. Esta abordagem foi escolhida a fim de proporcionar embasamento que justifique o uso *off-label* reposicionado deste fármaco multialvo no manejo das crises epilépticas. A morte neuronal induzida pelo SE parece envolver uma superativação do NMDAR (Holopainen, 2008) e o tratamento com antagonistas, como MK-801 e cetamina, mostrou prevenir a neurodegeneração induzida por SE em diversos modelos experimentais (Brandt et al., 2003; Lerner-Natoli et al., 1991; Loss et al., 2012b; Stafstrom et al., 1997). Tendo em vista que o uso clínico de antagonistas NMDAR de alta afinidade não é recomendado, uma vez que podem bloquear a neurotransmissão glutamatérgica fisiológica, causando efeitos colaterais graves e indesejáveis para os pacientes (Danysz and Parsons, 2002; Lipton, 2006; Muir and Lees, 1995), nós optamos por utilizar o antagonista MN, de baixa afinidade e com rápida cinética de bloqueio/desbloqueio do NMDAR, providenciando uma alternativa interessante para o manejo clínico do SE.

Apesar das controvérsias acerca dos efeitos benéficos da MN (Creeley et al., 2006; Löscher and Hönack, 1990), já foi demonstrado o seu efeito preventivo contra crises convulsivas induzidas pela administração de NMDA em ratos jovens (Dhir and Chopra, 2015) e a supressão de crises generalizadas induzidas por PTZ (Mareš and Mikulecká, 2009) e por organofosforados (McLean et al., 1992). Nossos dados corroboram com estes achados e suportam a hipótese de que a MN é capaz de atenuar as crises epilépticas por ser efetiva no controle do balanço excitatório/inibitório, atuando como um bloqueador de NMDAR. Nós demonstramos isto no primeiro capítulo, no qual o tratamento com MN foi capaz de aumentar a latência para as convulsões motoras no modelo de pilocarpina em ratos (estágio 5 da escala de Racine). E confirmamos esta propriedade no segundo capítulo, no qual todos os regimes terapêuticos testados diminuíram a intensidade das crises epilépticas induzidas por PTZ em peixe-zebra. Particularmente, MN 50 mg/Kg aumentou a latência para atingir o estágio 4 (fenótipo semelhante à crise clônica), além de diminuir o tempo necessário para o retorno ao nado normal. Além disso, nós reportamos que a MN foi capaz de abolir o estágio 5 em cerca de 20% dos animais.

Como demonstrado pelos nossos dados, o tratamento com MN não parece ser uma estratégia promissora para interromper o *SE*, apesar de atenuar o fenótipo das crises. Assim, nós confirmamos a hipótese de que o efeito anticonvulsivo e a neuroproteção não são eventos interdependentes e interrelacionados. Os efeitos anticonvulsivos e neuroprotetores são comumente dissociados porque, embora convulsões ou SE sejam prevenidos ou significativamente atenuados por uma terapia antiepiléptica bem-sucedida, os efeitos neuroprotetores raramente são satisfatórios (Hoffman et al., 2003; Velísek and Velísková, 2008). Além disso, o uso de MN pode ser muito promissor para correção de prejuízos cognitivos associados ao SE (Kalemenev et al., 2016). No entanto, os efeitos da MN no estado psicoemocional dos animais permanece como um tópico controverso, a qual se atribui efeitos indesejáveis nas doses neuroprotetoras (Creeley et al., 2006).

No primeiro capítulo desta tese, mostramos que a MN foi capaz de reduzir a degeneração neuronal induzida pelo SE no cérebro em desenvolvimento e que este perfil de neuroproteção foi dependente do tempo e da região cerebral. A neuroproteção da MN observada na região CA1 pode estar associado à sua capacidade de ligar-se a NMDARs formados pelas subunidades GluN2A e GluN2B, as quais estão relacionados a eventos excitotóxicos. Bresink e colaboradores (1996) e Parsons e colaboradores (1999) demonstraram que a MN é equipotente para bloquear as correntes induzidas por glutamato via NMDARs contendo GluN2A e GluN2B em fatias do hipocampo. Entretanto, observamos uma completa falta de neuroproteção no DG. Isso pode estar relacionado à imaturidade dos NMDARs nos animais de 15 dias de vida nesta região. Diferentemente do CA1, no qual a completa a maturação funcional ocorre 2 semanas após o nascimento, no DG ocorre cerca de 3 semanas após o nascimento (Bekenstein and Lothman, 1991). Assim, a imaturidade funcional dos NMDARs no DG seria consequência do desenvolvimento ontogênico do NMDAR, o que modula diferencialmente a sensibilidade do NMDAR ao glutamato, e explica as diferenças no perfil de excitotoxicidade e neuroproteção em CA1 e DG (McDonald and Johnston, 1990). A excitotoxicidade mediada por glutamato pode ter relação com o aumento da produção de espécies reativas de oxigênio ou redução das defesas antioxidantes que levam ao estresse oxidativo, e por conseguinte culminam na morte neuronal (Ambrogini et al., 2019; Puttachary et al., 2015). Além disso, já foi demonstrado que a ativação do NMDAR induz o estresse oxidativo mediado pelo ânion superóxido gerado pelo excessivo influxo de íons cálcio através deste receptor, levando a morte neuronal (Gao et

al., 2007; Reyes et al., 2012; Singh et al., 2003). O papel crucial das espécies reativas de oxigênio nos desfechos de morte neuronal associados à crises epilépticas fazem pensar que uma terapia multialvo interessante e promissora para epilepsia poderia contribuir para a redução da produção destas espécies reativas (Patel, 2016; Rao et al., 2006). Assim, no segundo capítulo desta tese nós demonstramos que as crises epilépticas induzidas por PTZ aumentaram a carbonilação de proteínas e a atividade da enzima SOD, um indicativo de dano oxidativo. A MN apresentou um efeito antioxidante, evitando essas alterações.

Os resultados dessa tese demonstram que a MN é um fármaco multialvo capaz de providenciar neuroproteção contra o evento excitotóxico e de proteger contra o estresse oxidativo relacionado às crises. Outros trabalhos já demonstraram que a MN é capaz de proteger contra a excitotoxicidade glutamatérgica inibindo o excesso de cálcio intracelular, pois diminui o influxo através do NMDAR e modula a liberação de 'pools' internos (Glasgow et al., 2017; Jiang et al., 2010; Prentice et al., 2015), e/ou modulando parâmetros relacionados ao estresse oxidativo *in vivo* (Bardak et al., 2018; Khalili-Fomeshi et al., 2018; Tanaka et al., 2018). Surpreendentemente, a MN apresentou efeito neuroprotetor no tálamo e na amígdala nos regimes de pós tratamento. Nessa perspectiva, a utilização de antagonistas do NMDAR que não prejudicam a neurotransmissão fisiológica, como a MN, pode ser considerada promissora como uma terapia adjuvante para a morte neuronal induzida pela SE. Isto é particularmente importante pois as drogas antiepilépticas usadas clinicamente têm uma perda de eficácia dependente do tempo contra lesão cerebral induzida pelo SE, ou seja, são protetoras apenas quando administradas precocemente ou imediatamente após o início da crise (George and Kulkarni, 1996; Kim et al., 2007; Mazarati et al., 1998; Sankar et al., 1998b; Sofia et al., 1993). Poucos antiepilépticos exibem neuroproteção significativa contra danos neuronais, especialmente quando administrados após o início da SE (Abdalla et al., 2014; Kalemenev et al., 2016). Dessa forma, nossos achados têm um impacto clínico potencial, uma vez que os antiepilépticos clássicos falharam em proteger o cérebro dos danos neuronais induzidos pelo SE (Temkin, 2009). A neuroproteção após insultos cerebrais não parece fornecer uma estratégia promissora para prevenir a epilepsia, mas pode reduzir ou prevenir outras consequências dos insultos cerebrais, como déficits cognitivos ou psicopatologia na idade adulta (Loscher and Brandt, 2010).

Comparativamente com o hipocampo (onde só houve proteção no pré-tratamento), há uma expressão massiva de subunidades GluN2B nessas regiões durante as primeiras

semanas de vida (Lopez de Armentia and Sah, 2003). Este perfil de neuroproteção particularmente importante da MN pode ser explicado pela sua alta afinidade pelas subunidades GluN2B. Assim, os efeitos neuroprotetores observados em tálamo e amigdala provavelmente podem ser atribuídos a essa expressão aumentada das subunidades GluN2B (Grimwood et al., 1996). Dessa forma, estes resultados apontam para uma possível capacidade da MN de modular eventos tóxicos a partir da atividade diferencial dos NMDARs de acordo com as subunidades expressas. Além disso, a ação multialvo da MN já é um indicativo de que ela pode ser capaz de atuar indiretamente sobre biomarcadores relacionados à hiperestimulação glutamatérgica e que seu efeitos vão além do simples antagonismo que bloqueia o excesso de cálcio intracelular (Parsons et al., 2008; Zaitsev et al., 2015). Nesse contexto, a presença de agonistas de NMDARs leva a um feedback negative na expressão das subunidades e a MN pode atenuar esse evento (Gascón et al., 2005; Mao et al., 2002). Ademais, já foi reportado que crises convulsivas agudas ou crônicas modulam a expressão das subunidades do NMDAR (Hunt et al., 2012; Jensen et al., 1997). Assim, nós avaliamos o efeito das crises induzidas por PTZ e do tratamento com MN sobre as subunidades do NMDAR.

Nós não observamos alterações na expressão dos genes que codificam as subunidades GluN1 e GluN2B em peixe-zebra submetido às crises com PTZ. Entretanto, esses animais apresentaram um aumento na expressão relativa para o gene *grin2a*, corroborando com Zhu e colaboradores (2004). Eles demonstraram que nos primeiros eventos que ocorrem para o desencadeamento das crises, o gene que codifica GluN2A aumenta expressivamente no córtex. Assim, sugerem que o aumento na expressão de GluN2A contribui para alterações na excitabilidade do SNC, tendo relações com o aumento do tônus excitatório e com o mecanismo de propagação da crise (Mathern et al., 1997; Postnikova et al., 2017). Postnikova e colaboradores (2017) demonstraram que o aumento na expressão de GluN2A é precoce, sendo verificado 3 h após o SE induzido com PTZ, e que mais tardiamente (24h após o SE) a expressão de GluN2B também aumenta. Nós demonstramos que a administração de MN 20 mg/Kg reduziu a expressão da subunidade GluN2A para os níveis do controle. Isto pode ter relação com o potencial efeito da MN em modular a função aberrante do NMDAR demonstrada por Platzer e colaboradores (2017), que investigaram as respostas *in vitro* da MN para controlar o ganho de função tóxica de NMDARs com variantes em GluN2A. Com base nesses achados, a MN foi utilizada *off-label* para tratar um paciente com encefalopatia epiléptica

causada pela função aberrante da subunidade GluN2A (Pierson et al., 2014), sugerindo que a MN é capaz de bloquear a hiperfunção de NMDAR em condições específicas na epilepsia. Assim, nossos dados providenciam evidências para o envolvimento da subunidade GluN2A na regulação da atividade epiléptica das crises induzidas por PTZ e para a capacidade da MN de controlar as alterações do NMDAR durante os processos relacionados à epileptogênese.

Tanto nos modelos animais de epilepsia como nos pacientes, anormalidades comportamentais e prejuízos cognitivos são comumente observados (Choi-Kwon et al., 2003; de Oliveira et al., 2008; Krishnamoorthy et al., 2007; Minjarez et al., 2017). As comorbidades psiquiátricas são mais frequentemente observadas em pacientes com epilepsia do que na população em geral (Tellez-Zenteno et al., 2007). Dessa forma, nós avaliamos o perfil locomotor, exploratório, social e ansioso dos animais submetidos às crises com PTZ. Quando colocado no aparato open tank, o peixe-zebra inicialmente permanece o fundo do tanque, e gradualmente se habitua ao aparato, explorando as porções superiores do tanque com o decorrer do tempo (Maximino et al., 2012; Rosemberg et al., 2011). O teste de preferência social permite acessar parâmetros relacionados à necessidade de interação e também relacionados à ansiedade ou medo (Barba-Escobedo and Gould, 2012). No claro/escuro, o lado branco exerce um estimulo aversivo, e é um aparato classicamente utilizado para acessar parâmetros relacionados ao fenótipo semelhante à ansiedade (Blaser and Gerlai, 2006; Blaser and Peñalosa, 2011; Maximino et al., 2010a; Stephenson et al., 2011). Classicamente, o peixe-zebra aumenta sua permanência na região clara do aparato quando lhe são administrados compostos ansiolíticos, enquanto compostos ansiogênicos diminuem o tempo nessa região (Gebauer et al., 2011; Maximino et al., 2010b; Stewart et al., 2012). Nós não observamos nenhuma alteração no perfil locomotor/exploratório ou no perfil de interação social dos animais expostos ao PTZ. Apesar de existir uma forte relação entre epilepsia e ansiedade (Choi-Kwon et al., 2003; Hingray et al., 2019), nós não observamos nenhum indicativo clássico desse fenótipo nos testes de open tank e sociabilidade. Todavia, nós demonstramos que os animais expostos ao PTZ permanecem mais tempo na região clara do aparato. Nós não acreditamos que isso seja decorrente de um efeito ansiolítico do PTZ. Na verdade, este resultado pode sugerir uma diminuição do comportamento defensivo inato dos animais, fazendo com que eles percam a aversão natural aos ambientes claros. Detour e colaboradores (2005) sugerem que o mau funcionamento das vias encefálicas

relacionadas à expressão do medo causam um prejuízo na avaliação de situações de risco, o que poderia ser confundido com uma diminuição da ansiedade e/ou aumento da impulsividade. Esse fenótipo de "redução da ansiedade" já foi observado no modelo de pilocarpina, nos quais os ratos epilépticos permaneciam mais tempo nos braços abertos do aparato *plus maze* (Detour et al., 2005; dos Santos et al., 2005).

Dessa forma, a ansiedade desempenha um papel crucial na avaliação de exposição ao risco e na defesa inata dos animais, a fim de avaliar a iminência de um perigo (Fanselow, 2018), e prejuízos nesse comportamento defensivo inato podem ser um consequência negativa das crises. Além disso, o comportamento suicida é frequentemente relatado em pacientes que fazem uso de fármacos antiepilépticos (cerca de 15 medicamentos apresentaram aumentos nestes índices em ensaios clínicos). Diversas publicações sugerem que ambos, depressão e suicídio, apresentam taxas expressivas em pacientes com epilepsia. Por esta razão, o FDA recomenda que os fabricantes dessa classe de medicamentos incluam informações nos rótulos sobre o aumento nos riscos de pensamentos suicidas, a fim de alertar médicos e pacientes para um acompanhamento psiquiátrico adequado (e se necessário suspender a medicação) (Andersohn et al., 2010; Mula and Hesdorffer, 2011). O comportamento suicida pode ter relação com a diminuição do comportamento defensivo inato observado em nossos animais, tendo em vista que ambos envolvem a diminuição do senso de autopreservação. Além disso, nós demonstramos que o tratamento com MN foi capaz de prevenir esta anormalidade comportamental. Assim, nossos dados sugerem que a associação da MN com os antiepilépticos clássicos poderia fornecer subsídios para diminuir este tipo de efeito adverso. A literatura reporta inúmeros efeitos benéficos da MN em desordens neuropsiquiátricas (Hosenbocus and Chahal, 2013; Koukopoulos et al., 2012). Além disso, a administração de MN mostrou prevenir alterações no perfil exploratório e na cognição no modelo de Li–pilocarpina (Kalemenev et al., 2016). Esses benefícios também são observados em humanos com epilepsia encefalopática, nos quais o tratamento com MN melhora a qualidade do sono, o humor e outros aspectos gerais da cognição (Platzer et al., 2017). Dessa forma, a MN parece providenciar um bom manejo das consequências comportamentais negativa relacionadas às crises e sua associação com os tratamentos clássicos poderia melhorar o prognóstico dos pacientes epilépticos.

7. CONCLUSÕES

Nossos resultados mostram que o tratamento com MN foi capaz de proteger o cérebro contra a morte neuronal induzida por crises epilépticas prolongadas do tipo *Status epilepticus* bem como prevenir as alterações neuroquímicas relacionadas ao dano celular associados às crises epilépticas, demonstrando sua eficácia como uma terapia multialvo. Além disso, a MN parece ser uma estratégia eficaz para a prevenção das alterações comportamentais decorrentes de crises epilépticas. Dessa forma, estudos clínicos futuros que visem a neuroproteção e a redução das comorbidades associadas às epilepsias podem indicar o uso *off-label* da MN como terapia adjuvante (associada aos fármacos clássicos) para o tratamento das consequências neurocomportamentais de crises epilépticas.

8.   ANEXO I

Artigo Científico produzido durante o período em que atuei com docente substituta na Universidade Federal de Sergipe (UFS) durante os dois primeiros anos do doutorado.

Título: Coriandrum sativum Extract Prevents Alarm Substance-Induced Fear- and Anxiety-Like Responses in Adult Zebrafish

Revista: Zebrafish

Qualis-CAPES-CBII: B2

Fator de Impacto: 1.75

Atividades exercidas na UFS:

- Professora substituta 40 horas;

- Orientação de TCC do aluno Lucas Santos Souza;

- Implementação de um laboratório para estudos com peixe-zebra e neurociência que culminou neste artigo anexado.

Coriandrum sativum Extract Prevents Alarm Substance-Induced Fear- and Anxiety-Like Responses in Adult Zebrafish

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Abstract

Anxiety disorders appear to involve distinct neurobiological mechanisms and several medications are available against this mental health problem. However, pharmacological therapeutic approaches display undesirable side effects for patients, particularly when long-term therapy is required. Some evidences have suggested that *Coriandrum sativum* extract (CSE) provide sedative and anxiolytic effects. We investigate if CSE could attenuate anxiety-like behaviors induced by novelty and alarm substance exposures in zebrafish. Adult zebrafish were injected with vehicle, clonazepam, or CSE (25, 50 or 100 mg/kg) and submitted to novel tank test. At the end, saline or alarm substance was added and anxiety-like responses were recorded. Twenty-four hours after, fish were submitted to the light/dark test. Novelty associated with alarm substance exposure decreased distance traveled and total time mobile in novel tank, and CSE (at 50 and 100 mg/kg) prevented these alterations similarly to clonazepam. Alarm substance reduced the time spent in white compartment ($p = 0.0193$ as compared with vehicle group). Clonazepam and CSE prevented this anxiogenic effect of alarm substance. CSE presents anxiolytic effects against alarm substance-induced locomotor and anxiogenic responses similarly to clonazepam. These data corroborate with the use of this plant in traditional medicine and provides a putative new pharmacological intervention for anxiety disorders.

Keywords: anxiety, zebrafish, alarm substance, *Coriandrum sativum*

Introduction

Anxiety disorders are among the most prevalent and disabling mental health conditions worldwide.[1] They are considered risk factors for the development of additional mental health disorders and they are associated with an increased risk of suicide in adulthood.[2] Current pharmacological anxiety treatments include the use of selective serotonin reuptake inhibitors, benzodiazepines, alpha/delta calcium channel blockers, azapirones, and beta blockers.[3] Although clinical trials have showed the efficacy of the mentioned drugs, they have displayed a long list of undesirable side

effects for patients, including sedation, muscle relaxation, amnesia, tolerance, and dependence, particularly when long-term therapy was required.[4] These unwanted adverse effects have propelled scientists to search new chemical entities, mainly those from traditional medicine.[5]

In this context, several plants from different parts of the world, such as *Passiflora coerulea*, *Matricaria recutita*, *Valeriana officinalis, Salvia guaranitica*, *Tilia europeae*, and *Tilia tormentosa*, have been employed to discover new chemicals with putative sedative or anxiolytic effects.[6,7]

Another plant with reported anxiolytic and sedative effects in folk medicine is *Coriandrum sativum* (Apiaceae).

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Popularly known as ''coentro'' in Brazil and coriander in other places, *C. sativum* has been used as a culinary and medicinal plant over the years.[8,9] Their leaves and seeds are widely used by folk medicine for treatment of digestive disorders, appetite loss, diabetes, and other diseases.[8,10] Regarding its biological activity, this plant has hypoglycemic, anti-inflammatory, antioxidant, and antimicrobial effects.[8,11,12]

Moreover, other additional effects, such as hypolipidemic, antimutagenic, antihypertensive, diuretic, carminative, antispasmodic, and relaxant, were observed.[13–20] In Iranian traditional medicine, the coriander aerial parts have been used for relief of anxiety and insomnia because of its sedative and hypnotic effects.[16,21,22] The traditional dose of seed powder is from 1 to 5 g, three times per day. This translates to a 14–71 mg/kg dose, three times per day.[21,23] Importantly, coriander decoction to compose polyherbal traditional remedies from Ksar Lakbir district (NW Morocco) are used as neurosedative and to calm.[24]

On the brain, *C. sativum* extracts (CSEs) have been reported to exhibit sedative and anxiolytic effects. Mahendra and Bisht[9] showed that hydroalcoholic extract of coriander (100 and 200 mg/kg) produced anxiolytic effects similar to diazepam in different animal models of anxiety in mice. In addition, Emamghoreishi *et al.*[16] and Latha *et al.*[25] demonstrated that aqueous extract of coriander (100 and 200 mg/kg, respectively) induced an anxiolytic activity in mice by increasing both the time spent and the percentage of entries in the open arms of elevated plus maze.

Considering the traditional use of *C. sativum* and its sedative effects reported in rodents, as also the possibility of having high amount of quercetin in coriander extracts, it has been hypothesized that CSE might also possess anxiolytic properties. Thus, the present study aimed to investigate the putative antianxiety and antistress actions of *C. sativum* hydroalcoholic leaf extract in zebrafish. For inducing anxiety and stress responses we exposed zebrafish to two distinct paradigms: novel tank test and alarm substance exposure. Novel tank test is widely used as a tool to induce anxiety responses associated to a novel environment exposure.[26,27]

Materials and Methods

Plant collection

C. sativum L. was collected in Aquidabã (10° 16' 53'' S 37° 01' 07'' W), Sergipe, Brazil, between June and August. A voucher specimen number ASE 35.092 was deposited at Herbarium of the Department of Botany, Federal University of Sergipe (UFS), Brazil.

Extract preparation

C. sativum fresh leaves (282 g) were macerated with 2 L of ethanol (70%) at room temperature. Crude hydroalcoholic extract was concentrated up to dryness in a rotary evaporator (Fisatom®, model 802, Brazil) at 40°C. The product formed was frozen at −20°C for 24 h and lyophilized (Labconco®, FreeZone 4.5 model). Total yield was 3.898%.

High-performance liquid chromatography analysis

Chromatographic analysis was performed using a Shimadzu HPLC system (Shimadzu, Columbia, MD) composed of two pumps with an online degasser, an autoinjector, and a C18 column (4.6 × 250 mm, 5 µm) coupled to a DAD-UV/Vis detector. Sample volume of 20 µL (1 mg/mL) was dissolved in HPLC grade methanol and subjected to gradient elution with mobile phases consisting of water and methanol from 5% to 100% during 60 min in a wavelength range from 220 to 400 nm. Shimadzu LC Solution software (Kyoto, Japan) was used for data acquisition. Identification of the compounds was performed with the coinjection of standard secondary metabolites, according with their retention time (RT) and UV spectra. Gallic acid ($C_7H_6O_5$), caffeic acid ($C_9H_8O_4$), luteolin ($C_{15}H_{10}O_6$), quercetin 3-β-D-glucoside ($C_{21}H_{20}O_{12}$), and rutin ($C_{27}H_{30}O_{16}$), were obtained from Sigma-Aldrich® and diluted with methanol (see chromatogram in Fig. 1). To calculate the concentration of each secondary metabolite, calibration curves for each compound were performed with five different concentrations ranging from 1 to 50 µg/mL. Analyzes were performed in triplicate ($n = 3$).

Animals and treatments

A total of 105 male adult zebrafish (*Danio rerio*) (short fin wild-type strain; 4–6 months old) were used in the present work. They were obtained from a local commercial supplier (Aquazoo, SE, Brazil) and were acclimated for at least 2 weeks prior the experiments. Animals were maintained in ''home tanks,'' which consisted in 20 L aerated tanks (three animals/L) filled with distilled water under mechanical and chemical filtration. Water temperature, pH, and conductivity were adjusted to 26°C ± 2°C, 7.0–8.0, and 500 µS, respectively.

Ceiling-mounted fluorescent white lamps provided room illumination (600 lux). Light/dark cycle was 12/12 h (lights on at 7:00 am). Only animals weighing between 0.25 and 0.35 g were used. All animals were fed four times a day with ALCON BASIC™ flake food (ALCON, Brazil; twice a day) and *Artemia sp.* nauplii (twice a day). All procedures were performed according to the Brazilian's Law for Care and Use of Laboratory Animals (Law 11794/2008) and previously approved by the Animal Research Ethics Committee from UFS/Brazil (protocol number 37/2015).

Alarm substance extraction. Alarm substance was extracted as described previously.[28–30] One set of zebrafish (five animals that were not used for behavioral analysis) were cold anesthetized and quickly euthanized by decapitation. Alarm substance was extracted performing fifteen superficial cuts in the skin of animals and then washed with saline solution (10 mL/animal). During the extraction and further use, alarm substance solution was kept on ice, at 4°C.

Drug administration. Another set of animals ($n = 100$ animals) were anesthetized by tricaine immersion (160 µg/mL)[31] and further injected i.p. with CSE (25, 50 or 100 mg/kg), or clonazepam (0.05 mg/kg) or vehicle (20 animals/group). The maximal volume of injection was 10 µL/g of body mass.[32] Immersion in tricaine lasted 1–2 min, until fish were completely immobile and decreased opercular opening. Vehicle was composed of 40% propylene glycol, 10% ethyl alcohol, 5% sodium benzoate, and 1.5% benzyl alcohol.[33,34] Drugs were administered 30 min before the behavioral evaluations.[9]

FIG. 1. Chromatogram of a fresh leaf aqueous alcoholic extract (70% ethanol) from *Coriandrum sativum* after lyophilization.
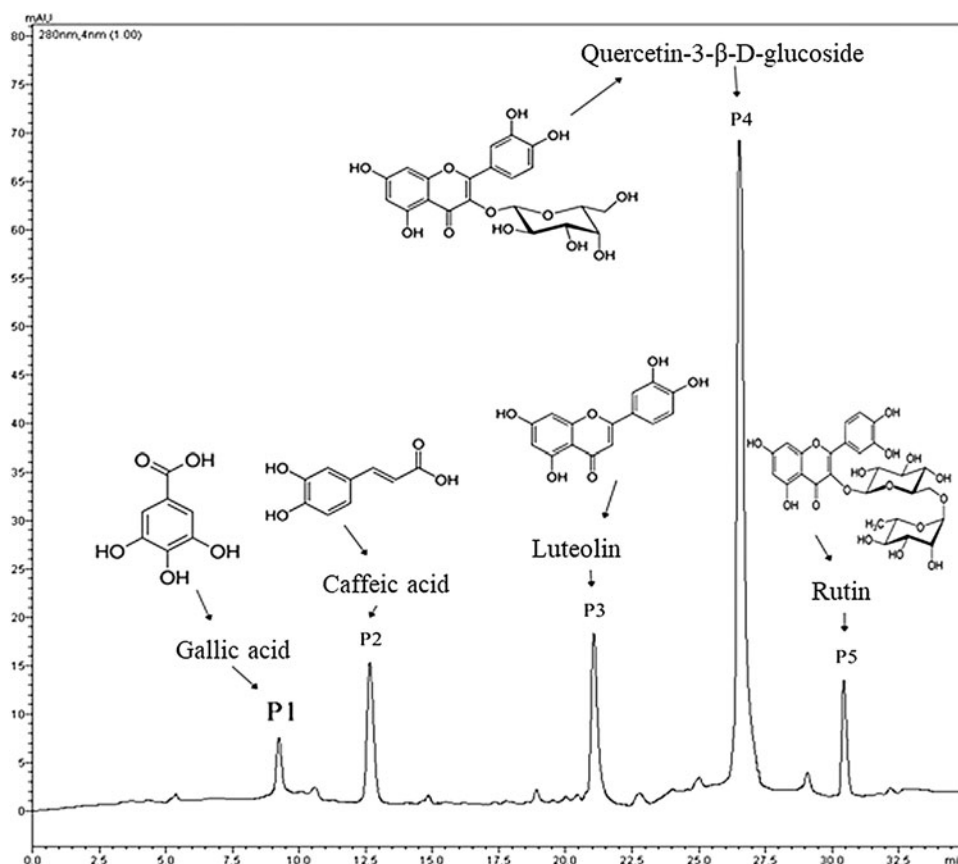
Behavioral procedures and alarm substance exposure

All behavioral tests were performed between 9:00 a.m. and 4:00 p.m. Behavioral analysis was conducted as depicted in Figure 2. Animals treated with vehicle, clonazepam, or CSE were submitted to novel tank test for 6 min. At the end of the novel tank test, animals were exposed to alarm substance. Without removing animals from the novel tank apparatus, alarm substance (3.5 mL of skin washout/L) or saline solution were carefully delivered in the tank through a syringe connected to a polypropylene tube ($n = 10$ animals for each group).

Swimming behavior was recorded for additional 6 min. After this period, fish were removed from the novel tank apparatus and placed, in pairs, in 1.5-L tanks until the beginning of the light/dark test. This strategy was used for keeping all experimental groups for the same time period of 24 h until the beginning of light/dark test. All behavioral tests were conducted under white illumination (800 lux of intensity). Behavioral analysis was performed in four independent experiments (25 animals/experiment). Animals were randomly distributed in each experiment as well as in experimental group by using an online tool for randomization (www.randomizer.org).

Novel tank test. Novel tank consisted of a 1.5-L trapezoidal tank (23.9 cm along the bottom × 28.9 cm at the top × 15.1 cm high × 15.9 cm along the diagonal side) similar to those previously described by Rosemberg *et al.*[35] Tank was filled with 1.5 L home-tank water and was divided in three equally virtual horizontal areas (named bottom, middle, and top). The following behavioral variables were evaluated:

total time mobile (s), distance travelled (m), total time spent (s) in bottom, middle, and top areas. A video camera located ~40 cm ahead the tank was used to monitor the activity of fish. ANY-Maze® software was used to track the swimming pattern of fish. Immobility was previously defined as the absence of movement or fish moving slower than 1 cm/s.

Light/dark test. The light/dark test was performed accordingly to Maximino *et al.*[33] with modifications. Apparatus consisted of a tank (15 × 10 × 45 cm, height × depth × length) equally divided into two distinct compartments (one black and other white covered). Opaque plastic self-adhesive films were used for covering walls and floor of both compartments. Apparatus was filled with 1.5 L home-tank water. Animals were individually placed in the lit area of the tank and were allowed to explore the apparatus for 15 min. The following behavioral parameters were recorded: number of entries and time spent in each compartment, and number of risk assessment behaviors. Risk assessment behaviors were defined as a partial entry in the white compartment followed by a fast return to the dark one.[36]

Statistical analysis

Data were expressed as mean ± standard deviation. All data were first tested for normality by D'Agostino–Pearson omnibus normality test. Basal novel tank data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Behavioral data from light/dark test and alarm substance exposure were analyzed by two-way ANOVA followed by Sidak's *post hoc* test. For each data, Cohen's *d* was shown as
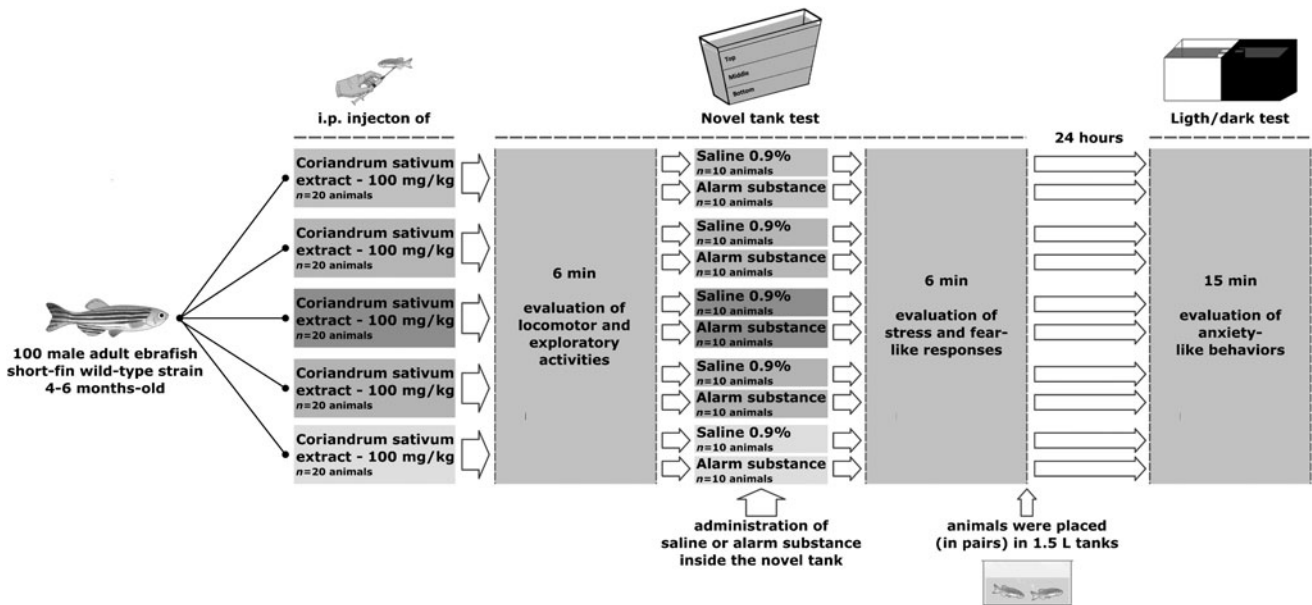
FIG. 2. Diagram illustrating the experimental design. After acquisition, animals were acclimated for 2 weeks before the beginning of the experiments. Animals were injected with vehicle, clonazepam, or CSE and were submitted to novel tank test for evaluating the basal locomotor and exploratory activities. At the end of novel tank test, saline or alarm substance were added to the tank and the locomotor and anxiety-like responses were recorded during 6 min. After this period, animals returned in pairs to the home-tank. Twenty-four hours after, fish were submitted to the light/dark test. CSE, *Coriandrum sativum* extract.

Cumming estimation plot. The raw data were plotted on the upper axes of figures; each mean difference was plotted on the lower axes of figures as a bootstrap sampling distribution. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical error bars. $p \leq 0.05$ was considered significant.

Results

HPLC characterization of CSE

Chromatographic profile of the CSE was shown in Figure 1. Fractions P1 (RT: 9.16 min), P2 (RT: 12.58 min), P3 (RT: 21.09 min), P4 (RT: 26.49 min), and P5 (RT: 30.37 min), were
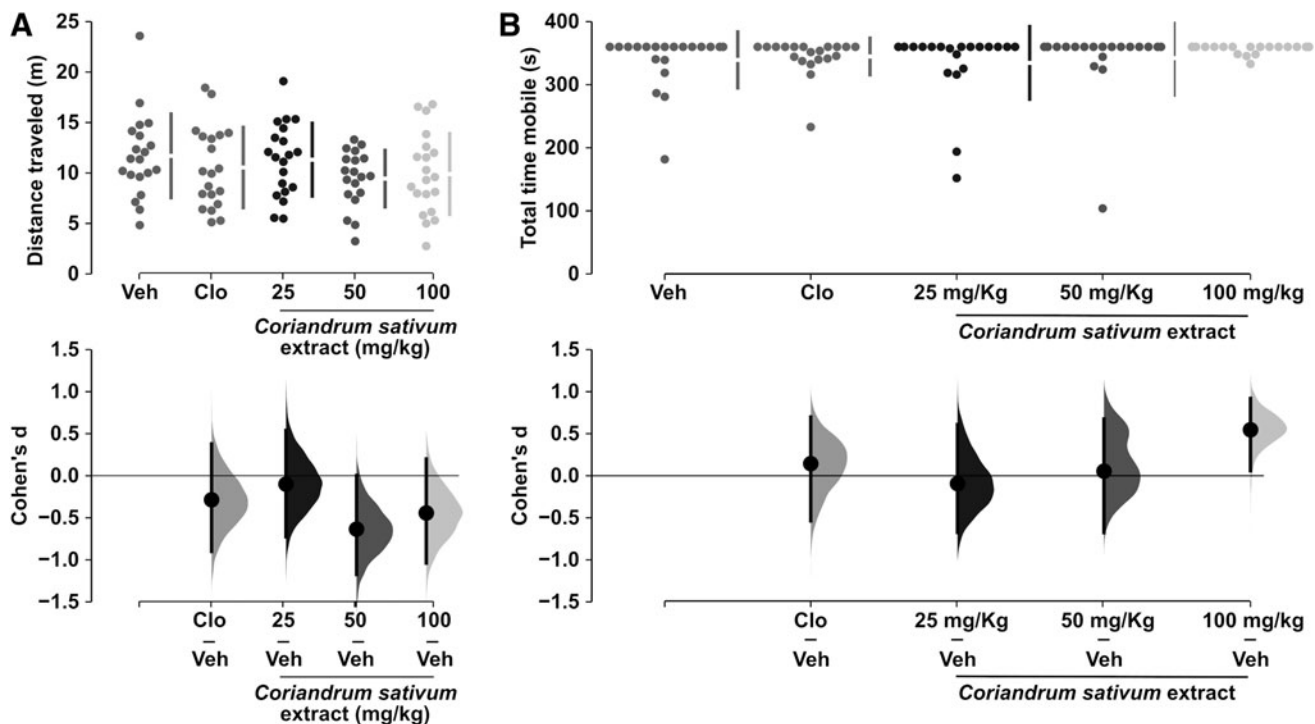
FIG. 3. Baseline locomotor profile of fish treated with vehicle, clonazepam or CSE; $n = 20$ animals/group. **(A)** Total distance travelled (m) in apparatus during 6 min of trial; **(B)** Total mobile time (s) during 6 min. Data expressed as mean ± standard deviation (one-way ANOVA followed by Tukey's *post hoc* test).
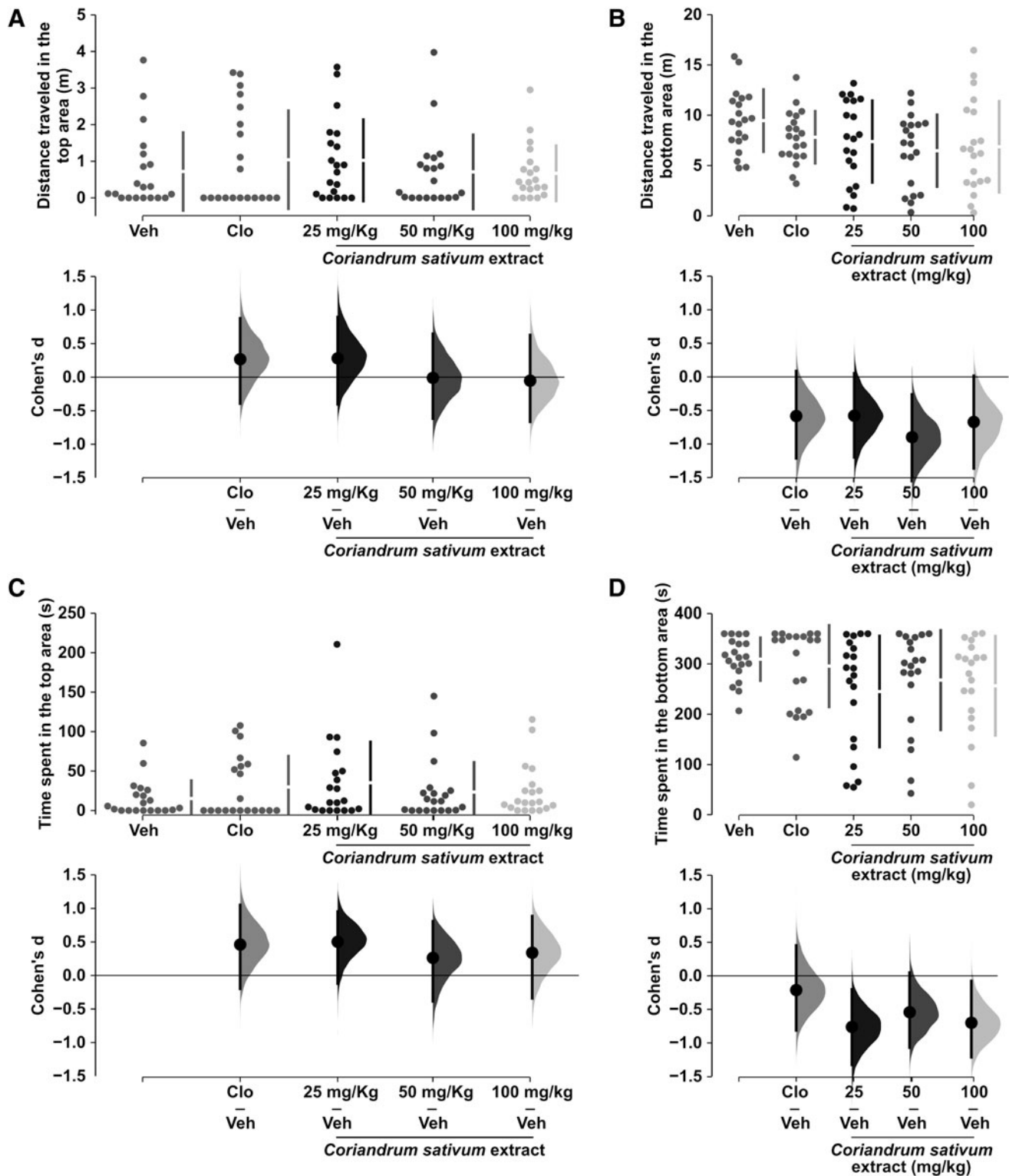
FIG. 4. Baseline exploratory profiles into two horizontal portions of the apparatus: **(A)** Total distance travelled in top area (m); **(B)** Total distance travelled in bottom area (m); **(C)** Total time spent in top area (s); **(D)** Total time spent in bottom area (s) of fish treated with vehicle, clonazepam, or CSE. $n = 20$ animals/group. Data expressed as mean ± standard deviation (one-way ANOVA followed by Tukey's *post hoc* test).

identified as gallic acid, caffeic acid, luteolin, quercetin 3-β-D-glucoside, and rutin, respectively. Quantitative analysis was obtained by using calibration curves of each secondary metabolite. All curves showed a correlation coefficient of $r \geq 0.999$. Concentrations of P1, P2, P3, P4, and P5 were 1.88, 2.32, 5.61, 43.56, and 3.21 $\mu g/mL$, respectively.

### Effect of CSE on alarm substance-induced alterations in locomotor and exploratory activities

Baseline locomotor and exploratory activities did not differ among experimental groups (Figs. 3–5). Animals spent more time in the bottom area. Clonazepam or CSE administration did not alter distance traveled or time spent in homebase area when compared with control group, suggesting a classical homebase formation (Fig. 5). In control groups, alarm substance exposure reduced distance traveled and total time mobile (Fig. 6A $F[1,90] = 7.638$ and B $F[1,90] = 3.997$; $p = 0.0257$ and 0.0343, respectively). To distance parameter, the unpaired Cohen's d between vehicle–saline and vehicle–alarm is -1.27 [95.0% CI -2.55, -0.242]. The two-sided p value of the Mann–Whitney test is 0.0257; the unpaired Cohen's d between clonazepam-saline and clonazepam-alarm is -0.512 [95.0% CI -1.45, 0.454]. The two-sided p value of the Mann–Whitney test is 0.273; the unpaired Cohen's d between 25 mg/kg-saline and 25 mg/kg-alarm is -1.5 [95.0% CI -2.6, -0.368]. The two-sided p value of the Mann–Whitney test is 0.00911; the unpaired Cohen's d between 50 mg/kg-saline and 50 mg/kg-alarm is 0.0705 [95.0% CI -0.864, 1.0]. The two-sided p value of the Mann–Whitney test is

0.734; the unpaired Cohen's d between 100 mg/kg-saline and 100 mg/kg-alarm is 0.0891 [95.0% CI -0.862, 1.0]. The two-sided p value of the Mann–Whitney test is 0.734.

Only treatment with 25 mg/kg of CSE ($p = 0.0322$) did not prevent the alarm substance-induced behavioral changes. In contrast, 50 and 100 mg/kg of CSE prevented the reduction in distance travelled and total time mobile induced by alarm substance (Fig. 6A, B, respectively). There was no difference between groups regarding distance traveled and time spent in top and bottom areas (Fig. 7). Although distance traveled in top area was not different among groups, alarm substance exposure appears to reduce suddenly the distance traveled in vehicle group ($p = 0.2621$) (Fig. 7A).

Effect of CSE on anxiety-like behaviors

Alarm substance exposure reduced the time spent in white compartment when compared with fish from saline group ($p = 0.0193$). Treatment with clonazepam and CSE in all tested concentrations prevented this reduction (Fig. 8A; $F[1,90] = 5.514$). The unpaired Cohen's d between vehicle-saline and vehicle-alarm is -1.35 [95.0% CI -2.53, -0.2]. The two-sided p value of the Mann–Whitney test is 0.0173; the unpaired Cohen's d between clonazepam–saline and clonazepam–alarm is 0.704 [95.0% CI -0.236, 1.78]. The two-sided p value of the Mann–Whitney test is 0.186; the unpaired Cohen's d between 25 mg/kg-saline and 25 mg/kg-alarm is -2.06 [95.0% CI -2.98, -1.03]. The two-sided p value of the Mann–Whitney test is 0.00131; the unpaired
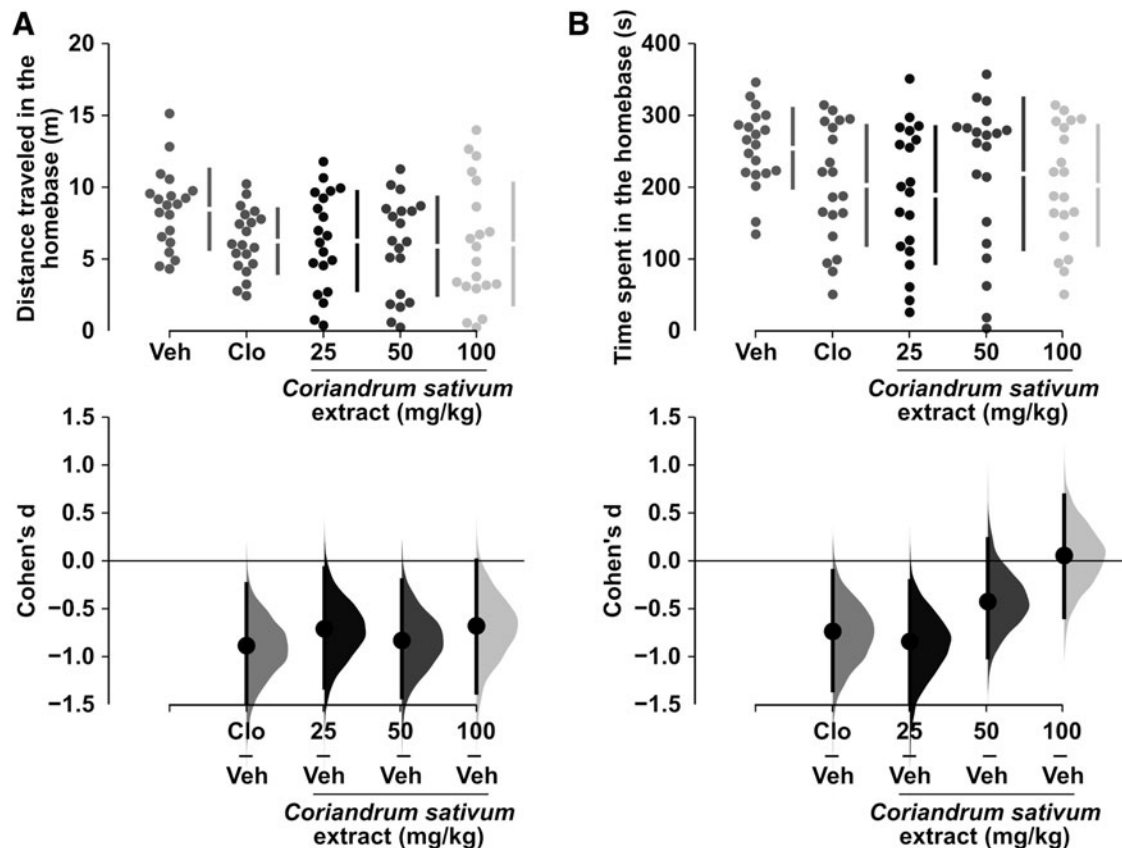

FIG. 5. Locomotor profiles in homebase area of fish treated with vehicle, clonazepam or CSE. **(A)** Total distance traveled and **(B)** total time spent in the home base area. $n = 20$ animals/group. Data expressed as mean ± standard deviation (one-way ANOVA followed by Tukey's *post hoc* test). Data expressed as mean ± standard deviation.
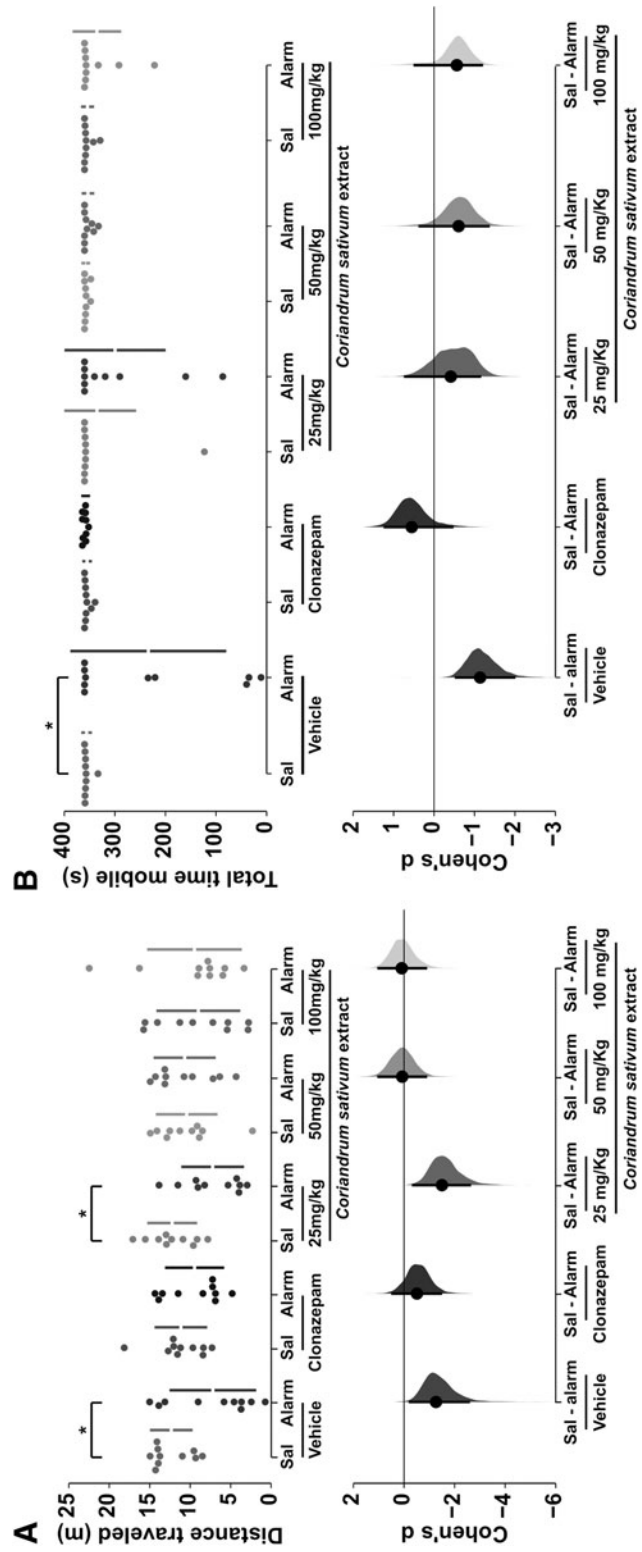
FIG. 6. Locomotor and exploratory profiles of vehicle, clonazepam-, or CSE-treated groups after alarm substance exposure. $n = 10$ animals/group. (**A**) Total distance traveled and (**B**) total time mobile in the apparatus. $*p \leq 0.05$ differs significantly when compared with the respective control group (two-way ANOVA followed by Sídak's *post hoc* test). Data were expressed as mean ± standard deviation.

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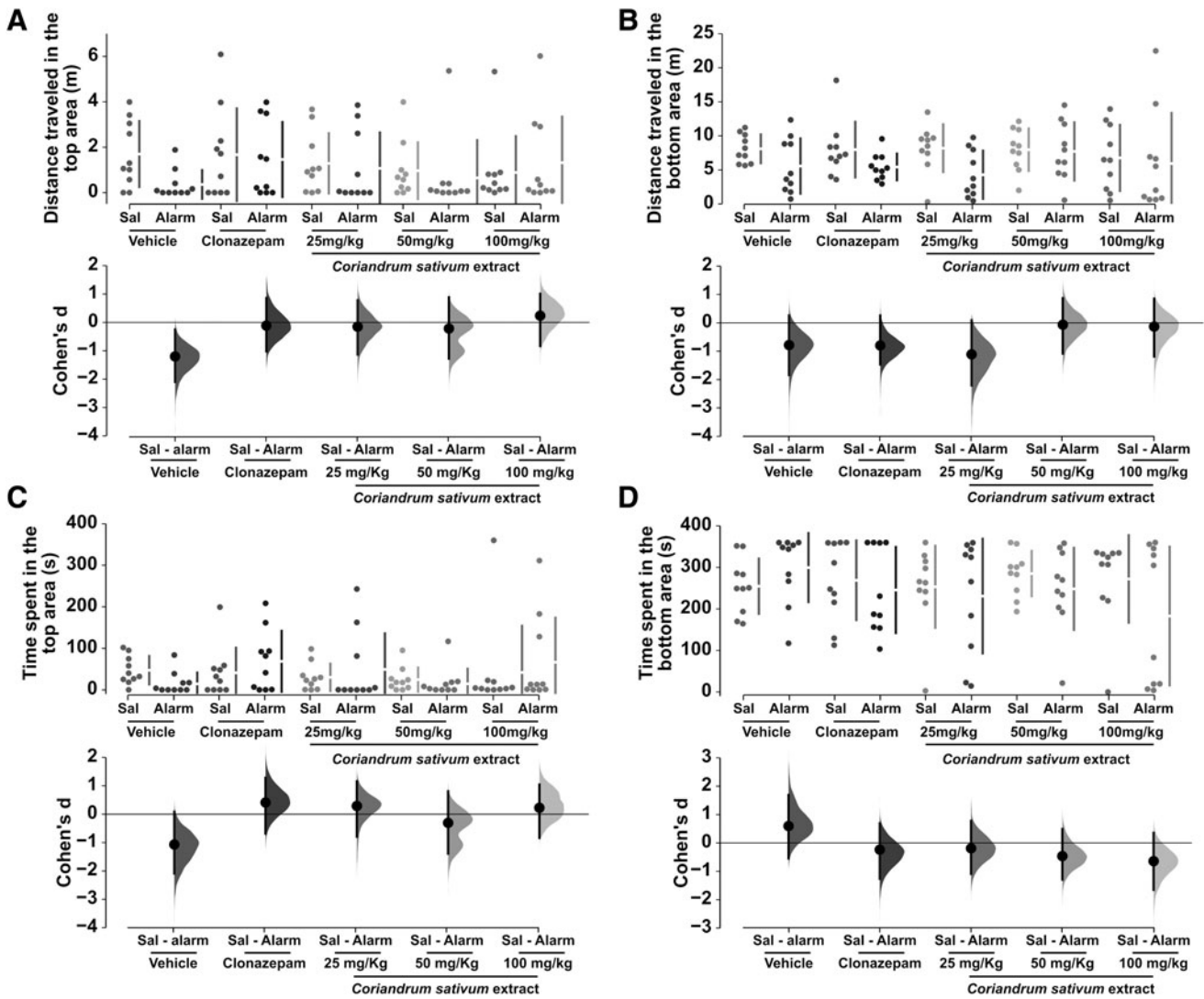
FIG. 7. Locomotor and exploratory profiles of vehicle, clonazepam-, or CSE-treated groups after alarm substance exposure. $n = 10$ animals per group. **(A)** Total distance traveled in the top area; **(B)** total distance traveled in the top bottom; **(C)** total time spent in the top area; and **(D)** total time spent in the top area of the apparatus. Data are expressed as mean ± standard deviation and were analyzed by two-way ANOVA followed by Sidak's *post hoc* test.

Cohen's *d* between 50 mg/kg-saline and 50 mg/kg-alarm is −0.253 [95.0% CI −1.22, 0.736]. The two-sided *p* value of the Mann–Whitney test is 0.623; the unpaired Cohen's *d* between 100 mg/kg-saline and 100 mg/kg-alarm is −0.498 [95.0% CI −1.39, 0.489]. The two-sided *p* value of the Mann–Whitney test is 0.345.

Moreover, alarm substance induced a significant increase in the risk assessment behaviors in animals treated with vehicle ($p < 0.0001$) and clonazepam ($p = 0.0173$). There was no difference in risk assessment behaviors in CSE-treated animals (Fig. 8C; $F[1,90] = 5.514$). The unpaired Cohen's *d* between vehicle-saline and vehicle-alarm is 6.6 [95.0% CI 4.81, 8.81]. The two-sided *p* value of the Mann–Whitney test is 0.000176; the unpaired Cohen's *d* between clonazepam–saline and clonazepam–alarm is 1.43 [95.0% CI 0.667, 2.31]. The two-sided *p* value of the Mann–Whitney test is 0.009; the unpaired Cohen's *d* between 25 mg/kg-saline and 25 mg/kg-alarm is 0.688 [95.0% CI −0.293, 1.72]. The two-sided *p* value of the Mann–Whitney test is 0.0818; the unpaired Cohen's *d* between 50 mg/kg-saline and 50 mg/kg-alarm is 0.751 [95.0% CI

−0.211, 1.46]. The two-sided *p* value of the Mann–Whitney test is 0.172; The unpaired Cohen's *d* between 100 mg/kg-saline and 100 mg/kg-alarm is 0.944 [95.0% CI −0.158, 1.85]. The two-sided *p* value of the Mann–Whitney test is 0.0885. The number of crossing was not altered in any groups (Fig. 8B).

Discussion

To evaluate the locomotor and exploratory activities in fish treated with CSE, we tested it at concentrations ranging from 25 to 100 mg/kg. After treatments, animals performed novel tank test before (baseline) and after (post stress) saline or alarm substance exposure. We observed that CSE-treated animals did not present alterations in baseline behavior. In the present study motor and nonmotor patterns associated with anxiety-like behaviors after stressful paradigm were analyzed, to investigate the putative CSE anxiolytic effect. In poststress session, total distance traveled and total time mobile decreases after alarm substance administration when compared with saline administration in vehicle-treated animals,

whereas treatment with clonazepam and CSE (50 and 100 mg/kg) did not alter this parameter. We also found that acute exposure to alarm substance inhibited transitions to the top of the tank. These data corroborate with previous findings in which alarm substance exposure disrupts rapid habituation response of zebrafish in the novel tank test[37] and suggest that alarm substance is effective in inducing fear responses in zebrafish.[28] Thus, CSE-treated animals presented an anxiolytic response similar to clonazepam-treated animals into stressful paradigm used in the present study.

Situations like threat or danger cause fear and/or anxiety that may elicit innate defensive behaviors. Pharmacological studies show that alarm substance released from injured skin cells may elicit defensive behaviors characterized by increased speed of movement, erratic movements, freezing bouts, and agglomeration in the bottom zone of the tank.[27,28,30,38,39] Therefore, decrease in movement observed in the poststress session in novel tank may be related to alarm substance exposure added to new environment interaction, considering that first novel tank session provides a stressor stimulus.

In fact, besides alarm substance exposure, we explore the novel tank test to propitiate stressful condition, because it is known as a novel tank that is being utilized as a tool to reveal the animal's interaction with a novel environment.[26,27] Several studies justified this test as a model of anxiety based on the animal's natural instinct to seek protection in an unfamiliar environment presented exploration, freezing, or immobility and top entries,[26,29,40] and it is a behavioral tool that is used to measure novelty-associated behavioral stress responses.[41]

To confirm the anxiolytic action of CSE demonstrated in novel tank test, we evaluated the light/dark test. It has been used as an anxiety test and anxiolytic drugs have been found to increase the time spent in the light zone, whereas anxiogenic drugs decreased it.[33,34,42] We demonstrated for the first time that novel tank associated with alarm substance administration produces an anxiogenic effect that persists during 24 h. Importantly, CSE and clonazepam administration provide a control of this long-term behavior alteration. Our results demonstrated that CSE prevents anxiety-like phenotype produced by novelty response associated to alarm substance exposure, as well as exploratory and locomotor influences produced by acute alarm substance administration.

C. sativum, popularly known as "*coentro*" or coriander, is traditionally used for gastrointestinal and cardiovascular disorders.[43] Additionally, some evidence supports this plant use in convulsions, anxiety, insomnia, because of its sedative property.[16,17,44] Also, in the Indian traditional medicine, it is used in the disorders of digestive, respiratory, and urinary systems, as it has diaphoretic and diuretic effects.[9]

Several compounds have been isolated from coriander oil, such linalool, α-pirene, γ-terpinene, geranyl acetate, camphor,

and geraniol,[14,15] although many biologically active compounds are also soluble in water.[45] CSE was characterized for LC-MS screening, which revealed the presence of a high amount of quercetin. There are several studies, which have shown that quercetin produces a variety of anxiolytic-like behavioral effects.[46–48] Nevertheless, it has been reported that
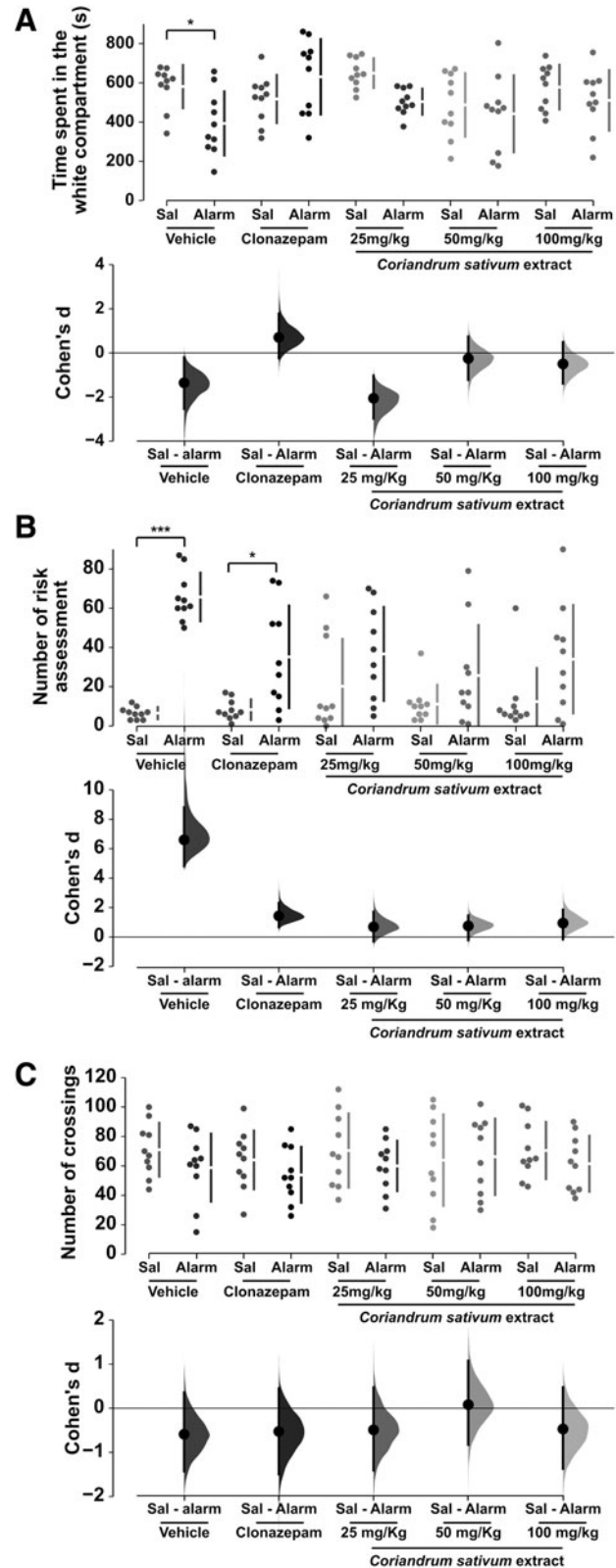
FIG. 8. Anxiety-like behavioral profile of vehicle, clonazepam, or CSE-treated groups after alarm substance exposure. **(A)** Total time spent in the white compartment of black/white apparatus; **(B)** number of risk assessment behaviors; and **(C)** number of crossings between white and black compartment. $n = 10$ animals per group. $*p \le 0.05$ and $***p \le 0.0001$ differs significantly when compared with respective to control group (two-way ANOVA followed by Sidak's *post hoc* test).

quercetin present good potential to reduce stress, decreased plasma cortisol levels, and improve memory performance.[49,50] Moreover, quercetin could prevent the impairment of the enzymes that regulate the purinergic and cholinergic extra-cellular signaling as adenosine deaminase and acetylcholinesterase improving the memory and anxiety-like behavior induced by streptozotocin-induced diabetic rats.[51] Taken together, these results may suggest that quercetin might be responsible for improving behavioral dysfunction during anxiety episodes. Although quercetin is the major compound in CSE, its anxiolytic effect is probably related to polyphenol mixture, considering the potential of these compounds for modulation of the anxiety-like phenotype.[52,53]

In the present work, treatment with clonazepam prevented the alarm substance-induced decrease in time spent in white compartment. Another study demonstrated a similar result for clonazepam (0.3 mg/L) in light/dark test.[42] Studies verified that treatment with anxiolytic drugs as fluoxetine, clonazepam, buspirone, and ethanol reduced fear- and anxiety-like behavior.[34,40,42] Doses of CSE administrated in this work did not alter the number of crossing responses in the light/dark test, suggesting a potential anxiolytic effect without producing effects on locomotion. Indeed, vehicle- and clonazepam-treated animals increased the number of risk assessment episodes, whereas CSE prevent this effect in the light/dark test. This parameter indicates a potential anxiolytic activity to CSE. Due to this, CSE represents a new potential candidate for treatment of anxiety disorders without producing effects on locomotion.

Conclusion

Our findings suggest that long-term anxiety behavior may be achieved using a combination of novelty response in novel tank and alarm substance exposure in zebrafish. In this approach, CSE promoted anxiolytic effect. Thus, this work assists in the efficacy confirmation of this plant used in traditional medicine and can provide a new pharmacological intervention in anxiety disorders.

Disclosure Statement

No competing financial interests exist.

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