Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-graduação em Biologia Celular e Molecular

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Avaliação do sistema purinérgico em modelos de déficit cognitivo e doenças neurodegenerativas em peixe-zebra (*Danio rerio*)

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Orientadora: Profª Drª Carla Denise Bonan

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Porto Alegre

2015

Dedico esta tese a minha maior incentivadora,

que considerava o estudo uma mola mestra no desenvolvimento de uma pessoa, minha mãe.

AGRADECIMENTOS

Muito especialmente, desejo agradecer a minha orientadora Dra Carla D. Bonan, pela disponibilidade, atenção dispensada, paciência, dedicação, exemplo e profissionalismo, um Muito Obrigado.

Ao meu esposo, Pablo de Pietro, pelo incentivo, compreensão e principalmente por entender minhas ausências frequentes durante todo este período.

À minha família, em particular, ao meu pai e minha mana pelo amor e compreensão durante todo esse percurso

Aos meus amigos, em especial à Stefani Altenhofen, Laura R. Nery, Fernanda F. Zimmermann e Giana P. Cognato pelo seu apoio, café, auxílio e principalmente pelos laços de amizades que compartilhamos.

Aos meus colegas de laboratório, pelos momentos de entusiasmo partilhados em conjunto.

Aos demais professores do programa, em especial a Dra. Mônica R. M. Vianna, ao Dr. Maurício Reis Bogo e a Dra. Rosane Souza da Silva, pelos ensinamentos compartilhados.

A Universidade de Cruz Alta pelo incentivo e por acreditar no meu profissionalismo.

A todos os demais ... .

RESUMO

As doenças neurodegenerativas mais prevalentes são a doença de Alzheimer (DA) e a doença de Parkinson (DP) afetando cerca de 35 e 10 milhões de pessoas no mundo, respectivamente. Essas patologias são caracterizadas pelo depósito de proteínas em células neuronais e pela perda progressiva da função ou estrutura no sistema nervoso central (SNC). Estudos têm mostrado que o sistema purinérgico está envolvido nos mecanismos relacionados a DA e DP. A sinalização purinérgica é mediada pela ação do ATP e da adenosina através da ativação dos receptores P2 e P1, respectivamente. Os níveis de nucleotídeos e de nucleosídeos são modulados pela ação enzimática das ectonucleotidases, especialmente pela ecto-nucleosídeo trifosfato difosfoidrolases (NTPDases) e ecto-5'-nucleotidase (EC-5´-Nt). Estas enzimas hidrolisam ATP em adenosina, que é subsequentemente desaminado pela enzima adenosina deaminase (ADA) a inosina. A adenosina é descrita como importante neuromodulador e neuroprotetor do SNC e sua modulação tem se mostrado uma alternativa promissora para o tratamento de doenças neurodegenerativas. Com isso, este estudo visa avaliar parâmetros comportamentais em modelos de déficit cognitivo induzidos por escopolamina, por 6-hidroxidopamina e por paraquat em peixe-zebra, e estudar o possível efeito desses modelos sobre o sistema purinérgico. Nossos resultados, utilizando o modelo farmacológico de déficit cognitivo induzido por escopolamina em peixe-zebra adulto, mostraram que os inibidores seletivos e não seletivos dos receptores de adenosina (Cafeína, ZM241385 e DPCPX) preveniram o déficit causado pela escopolamina. Os mesmos resultados foram encontrados para o inibidor do transportador de adenosina (dipiridamol) e inibidor da ADA (EHNA). Esses dados suportam a hipótese de que a sinalização adenosinérgica pode modular o processamento da memória. Nosso estudo também caracterizou um novo modelo experimental de neurodegeneração, tratando peixes-zebra adultos cronicamente com paraquat (Pq), herbicida associado aos sintomas da DP. O tratamento consistiu de seis injeções intraperitoniais nas doses de 10 ou 20 mg/kg a cada três dias. O comportamento locomotor foi avaliado 24 horas após cada injeção e mostrou uma diminuição em ambas as doses. O ângulo de giro também foi avaliado 24 horas após cada injeção e demonstrou diferença entre as doses administradas. Comportamentos não motores como ansiedade e interação social não apresentaram diferença significativa após o tratamento crônico com Pq. No entanto, os animais tratados com Pq demonstraram déficit de memória em tarefa de labirinto em Y comparados com o grupo controle. Além dos dados comportamentais, nossos resultados mostraram um aumento nos níveis de dopamina, enquanto os de DOPAC diminuíram nos animais tratados com Pq, mostrando mudança no metabolismo. Já a tirosina hidroxilase não apresentou diferença significativa entre animais do grupo controle e tratados, porém a expressão do transportador de dopamina diminuiu no grupo tratado com 10 mg/Kg de Pq, não havendo alteração na maior dose. Nosso estudo também investigou os efeitos da exposição crônica do Pq e da neurotoxina 6-hidroxidopamina (6-OHDA), comumente usada como modelo animal de DP, sobre o metabolismo extracelular de nucleosídeos e nucleotídeos. Duas doses de 6-OHDA foram testadas, 25 e 50 mg/kg, e os animais foram eutanasiados seis dias após a exposição. Nossos dados mostraram que o Pq não alterou a atividade das NTPDases e EC-5´-Nt ou os níveis extracelulares de ATP, ADP e AMP. No entanto, o tratamento crônico com Pq diminui os níveis extracelulares de adenosina e aumentou os níveis de inosina. Os

peixes tratados com 6-OHDA não apresentaram alterações na atividade das NTPDases e EC-5´-Nt, bem como nos níveis de ATP extracelular nas duas doses testadas. Porém, houve mudanças nos níveis de ADP e uma diminuição, em ambas as doses, nos níveis de AMP. Já os níveis extracelulares de adenosina e inosina apresentaram um aumento. Estes dados sugerem que o sistema purinérgico pode ser modulado de forma diferente em animais experimentais com sintomas de Parkinson. Além disso, este trabalho também apresentou um novo modelo de neurodegeneração utilizando o Pq além de reforçar o envolvimento do sistema purinérgico e o seu potencial farmacológico sobre doenças neurodegenerativas.

Palavras-chaves: adenosina. déficit cognitivo. doenças neurodegenerativas. peixe-zebra. sistema purinérgico.

ABSTRACT

Alzheimer disease (AD) and Parkinson disease (PD) are the two most common neurodegenerative disorders affecting around 35 and 10 million people worldwide, respectively. These disorders are characterized by neuronal protein deposits and progressive loss of function or structure of central nervous system (CNS). Studies have shown that purinergic system is involved in mechanisms associated with neurodegenerative diseases such as AD and PD. The purinergic signaling is meditated by ATP and adenosine through activation of purinoceptors P2 and P1, respectively. The nucleotide and nucleoside levels are modulated by the action of the ectonucleotidase family, especially by ecto-Nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5´-nucleotidase (EC-5´-Nt). These enzymes hydrolyze ATP to adenosine, which is subsequently deaminated by the enzyme Adenosine Deaminase (ADA) to inosine. Adenosine is described as an important neuromodulator and neuroprotective of CNS, and its modulation has proven to be a promising alternative for the treatment of neurodegenerative diseases. Thus, this study aims to evaluate behavioral parameters in models of cognitive impairment induced by scopolamine, 6-hydroxydopamine, and paraquat in zebrafish and study the possible effect of these models on the purinergic system. Our results, using a model of pharmacological cognitive impairment induced by scopolamine in adult zebrafish, showed that selective and non-selective inhibitors of adenosine receptors (Caffeine, ZM241385, and DPCPX) prevented the cognitive deficit induced by scopolamine. The same results were found for the adenosine transporter inhibitor (dipyridamole), and ADA inhibitor (EHNA). These data support the hypothesis of adenosinergic signalling can modulate memory mechanisms. We also developed a new experimental model of neurodegeneration by treating adult zebrafish chronically with paraquat herbicide (Pq) that results in symptoms of PD. Treatment consisted of six ip. injections of Pq in doses of 10 or 20 mg/kg and each injection was administered every three days. Locomotor behavior was assessed 24 hours after each injection and showed a decrease in both doses. The turn angle was also evaluated and showed difference between the doses administered compared to control group. Non-motor behaviors such as anxiety and social interaction were not significantly different after chronic treatment with Pq. However, after Pq exposure, the animals showed a deficit in Y-maze task memory. Apart from the behavioral data, our results presented an increase on dopamine levels, whereas DOPAC decreased in the experimental group showing change in dopamine metabolism. The amount of tyrosine hydroxylase demonstrated no significant difference between control and treated fish; however, dopamine transporter expression decreased in the group treated with 10 mg/kg Pq, and there was no change at the highest dose. Our study also evaluated the effect of chronic exposure of Pq and 6-hydroxydopamine (6-OHDA) neurotoxins, commonly used as an animal model of PD, on extracellular nucleotide and nucleoside metabolism. Two doses of 6-OHDA were tested, 25 and 50 mg/kg, and the animals were sacrificed at six days after exposure. Our data showed no changes in ectonucleotidase activities or ATP, ADP and AMP levels after exposure to Pq. However, a decrease of extracellular adenosine and increase in inosine levels were observed when compared to the control group. The 6-OHDA treatment did not affect the activity of NTPDase, EC-5´-Nt, and ATP levels in both tested doses. In contrast to the previous results, ADP levels were different for each dose used, while the AMP levels

decreased in both doses. The adenosine and inosine showed an increase in both doses of 6-OHDA tested in zebrafish brains. These data suggest that the purinergic system can be modulated differently in experimental animals with symptoms of Parkinson's disease. In addition, this study also presented a new model of neurodegeneration using the Pq and confirms the involvement of purinergic system on neurodegeneration as well as its pharmacological potential in neurodegenerative diseases.

Keywords: adenosine. cognitive impairment. neurodegenerative diseases. zebrafish. purinergic system.

LISTA DE ABREVIATURAS

Ach - acetilcolina

AchE - acetilcolinesterase

AcheI - inibidores da acetilcolinesterase

AD - Alzheimer disease

ADA - adenosina desaminase

ADAl - adenosina desaminase-*like*

ADP - adenosina 5´difosfato

AK - adenosina quinase

AMPc - adenosina 5´ monofosfato cíclico

AMP - adenosina 5´ monofosfato

APOE - apoliproteína E

APP - proteína precursora amiloide

ATP - adenosina 5´ trifosfatado

Aβ - peptídeo β amiloide

BACE1 - Enzima de clivagem de APP com sítio beta

DA - doença de Alzheimer

DP - doença de Parkinson

DPCPX - 8-Ciclopentil-1,3-dipropilxantina

DOPAC - ácido 3,4-dihidroxifenilacético

EHNA - erytro-9-(2-hidroxi-3-nonil)adenina)

E-NPP - ectonucleotídeo pirofosfatase/fosfodiesterase

E-5´-Nt - Ecto-5'-nucleotidase

GABA - ácido gama aminobutírico

GPI - glicosilfosfatidilinositol

MPTP - 1-metil-4-phenil-1,2,3,6-tetrahidropiridina

MPP - 1-metil-4-fenilpiridina

NTPDase - Ecto-nucleosídeo trifosfato difosfoidrolases

PD - Parkinson disease

Pq - paraquat

PSEN 1 - presenilina 1

PSEN 2 - presenilina 2

SNC - sistema nervoso central

SNc - substância negra parte compacta

6-OHDA - 6-hidroxidopamina

SUMÁRIO

1. INTRODUÇÃO

1.1 Doenças neurodegenerativas

O termo doenças neurodegenerativas descreve uma variedade de condições hereditárias e esporádicas caracterizadas por disfunção progressiva do sistema nervoso, resultante da degeneração de neurônios no encéfalo ou na medula espinhal. O início da degeneração neuronal é marcado por sintomas leves, como problemas relacionados à coordenação ou à memória. À medida que a neurodegeneração acelera, os sintomas pioram progressivamente (Migliore e Coppedè, 2009). O envelhecimento é o maior fator de risco para o desenvolvimento de doenças neurodegenerativas, pois não somente torna os pacientes mais propensos a estas desordens, como também prejudica a capacidade de reparo celular neuronal (Hindle et al., 2010). Além do envelhecimento, a agregação anormal de proteínas no citoplasma ou no núcleo das células do encéfalo também representa uma característica comum dessas patologias (Woulf, 2008). As doenças neurodegenerativas mais comuns são a doença de Alzheimer e a doença de Parkinson, descritas a seguir.

1.1.1 Doença de Alzheimer

A doença de Alzheimer (DA), caracterizada pela primeira vez por Alois Alzheimer em 1907, é uma neurodegeneração progressiva associada à deterioração cognitiva e à demência (Goeder e Guetti, 2007; Selkoe, 2001). A prevalência global da demência foi avaliada pelo estudo Delphi, o qual mostrou que em 2001 mais de 24 milhões de pessoas possuíam demência e que este número aumentaria para 42,3 milhões em 2020 (Ferri et al., 2005). Uma revisão recente, realizada com dados da literatura global entre os anos de 1980-2009, estimou que a demência afeta cerca de 35,6 milhões de indivíduos no mundo, e que este número poderá chegar a 115,4 milhões em 2050 (Prince et al., 2013).

A DA é neuropatologicamente caracterizada pela presença de dois tipos de depósitos de proteínas: β amiloide (Aβ) e TAU. Os peptídeos Aβ são produtos naturais do metabolismo, originados através da proteólise da proteína precursora amiloide (APP) pelas enzimas α-secretase, β-secretase e γ-secretase. A α-secretase compõe a via não amiloidogênica, produzindo o peptídeo Aβ solúvel, por clivar o APP no domínio amiloide. As outras duas

enzimas compõem a via amiloidogênica, produzindo o peptídeo Aβ patogênico; a β-secretase com atividade que se origina de uma aspartil protease chamada enzima de clivagem de APP com sítio beta (BACE1); e a γ-secretase, uma proteína com sítio catalítico complexado com presenilina (Hass e Selkoe, 2007; Vassar et al., 1999). Estas enzimas produzem os peptídeos Aβ com diferentes números de aminoácidos, sendo os monômeros com 40 aminoácidos (Aβ40) mais abundantes do que os com 42 aminoácidos (Aβ42) (Blennow et al., 2006; Jarrett et al., 1993). Na DA, há um desequilíbrio no metabolismo dos peptídeos Aβ, ocorrendo acúmulo e depósito desses peptídeos, principalmente o Aβ42, em placas fibrilares, também chamadas de placas amiloide (Hardy e Selkoe, 2002; Hass e Selkoe, 2007).

Outra proteína associada à DA é a TAU. A TAU é uma proteína axonal normal, solúvel, que se liga a microtúbulos através dos seus domínios promovendo, assim, a montagem e a estabilidade dos microtúbulos. A fosforilação da TAU é regulada pelo balanço entre quinases e fosfatases (Iqbal, 2005). Em níveis normais, a TAU contém de 2 a 3 mols de fosfato por mol de proteína para sua atividade ótima (Köpke et al., 1993). Na DA, ocorre a formação de agregados insolúveis da TAU que rompem a estrutura dos neurônios. Os monômeros de TAU primeiramente se ligam para formar oligômeros, que então se agregam em folhas β, formando os emaranhados neurofibrilares. Nesses emaranhados, a TAU se encontra hiperfosforilada chegando a 6-9 mols de fosfato por mol de proteína (Ksiezak-Reading et al., 1992). O mecanismo envolvido nesse aumento da fosforilação ainda não foi elucidado, mas há evidências de que a hiperfosforilação parece ser importante na redução da afinidade da TAU pelos microtúbulos (Meraz-Rios, 2010).

Além da deposição de proteínas, a DA vem sendo caracterizada como de início precoce ou familiar e de início tardio ou esporádico. A DA de início precoce representa de 5 a 10% dos casos de Alzheimer acometendo uma população em idade adulta inferior a 65 anos. Três genes são considerados os principais fatores de risco para o desenvolvimento da DA familiar: proteína precursora amiloide (*APP*), presenilina 1 (*PSEN1*) e presenilina 2 (*PSEN2*). Mutações nesses genes podem resultar em alterações na produção da proteína β amiloide, levando à apoptose de neurônios e à demência (Bertan e Tanzi, 2005; Tanzi et al., 1992). Por outro lado, a DA de início tardio ou esporádico, principal forma da DA, que acomete indivíduos com mais de 65 anos, possui diversos genes já identificados como potencial risco para desenvolvimento da doença (Barral et al., 2012; Bettens et al., 2013; Hollingworth et al., 2011). Dentre eles, o de maior destaque é o gene da apolipoproteína E (*APOE*), maior carreador de colesterol no cérebro, envolvido na manutenção e no reparo neuronal. Pacientes com DA apresentam alta frequência do alelo ε4 da *APOE*. Este alelo está associado a

mudanças na estrutura da *APOE*, resultando em mudanças conformacionais associadas à morte neuronal e à neurodegeneração (Bu, 2009; Mahley e Huang, 2009).

Além dos fatores genéticos, a DA esporádica está associada a outras anormalidades como disfunção mitocondrial, aumento do estresse oxidativo, falha no metabolismo energético, neuroinflamação e distúrbios em vários sistemas de neurotransmissão (Ferrer, 2012). Dentre os sistemas de neurotransmissão, o sistema colinérgico está fortemente relacionado com os sintomas de DA (Giacobini, 2003).

O sistema colinérgico tem um papel importante nas funções de aprendizado e de memória, na manutenção da atividade cortical e no fluxo sanguíneo cerebral (Schlieb e Arendt, 2006). Estudos mostraram que, na DA, ocorre perda precoce e progressiva de neurônios colinérgicos, principalmente no prosencéfalo basal e no hipocampo (Auld et al., 2002; Schliebs, 2005), diminuição da liberação de acetilcolina (ACh), principal neurotransmissor do sistema colinérgico, bem como da expressão dos seus receptores muscarínicos e nicotínicos em pacientes com DA (Court et al., 2001; Mulugeta et al., 2003). Esses estudos estão de acordo com a hipótese colinérgica que associa a deterioração cognitiva com a diminuição dos níveis de acetilcolina, devido à sua rápida hidrólise pela acetilcolinesterase (AChE) (Bartus et al., 1982).

A partir da hipótese colinérgica, estratégias para aumentar a disponibilidade de acetilcolina na fenda sináptica começaram a ser estudadas. Com isso, os inibidores da acetilcolinesterase (AcheI) foram introduzidos na prática clínica para o tratamento sintomático da DA. Esses inibidores aumentam a função colinérgica central pela inibição das enzimas que degradam a acetilcolina, aumentando, assim, a disponibilidade da ACh para estimular os receptores nicotínicos e muscarínicos no cérebro (Casey et al, 2010).

Além dos inibidores da acetilcolinesterase, a memantina, um antagonista não competitivo dos receptores NMDA, também é usada no tratamento da DA (Wilcock, 2003). Apesar dos investimentos em pesquisa para o desenvolvimento de novos fármacos para diminuir os sintomas ou a cura da DA, moléculas e imunoterapias não têm mostrado efetividade nos ensaios clínicos tardios (Cummings et al., 2014), sendo esta uma área de grande interesse para a pesquisa e desenvolvimento de novos medicamentos.

1.1.2 Doença de Parkinson

A doença de Parkinson (DP) é uma desordem neurodegenerativa de alta prevalência. Estima-se que mais de 10 milhões de indivíduos no mundo vivam com essa patologia (Hindle

2010). A DP, segunda desordem neurodegenerativa mais comum após a DA, afeta cerca de 2% da população com 65 anos ou mais, aumentando para 3 a 5% da população em indivíduos acima dos 85 anos (De Lau e Breteler, 2006; Hindle, 2010).

A DP tem como principal característica neuropatológica a lesão dos neurônios dopaminérgicos, localizados na substância negra parte compacta (SNc), os quais enviam projeções para os gânglios da base, ocasionando uma redução nos níveis de dopamina no estriado, uma das estruturas cerebrais essenciais no controle da função motora (Braak et al., 2005; Forno 1996).

A neurodegeneração observada na DP não é restrita aos neurônios dopaminérgicos, uma vez que os neurônios não dopaminérgicos, como colinérgicos, serotoninérgicos e noradrenérgicos também podem ser afetados (Brichta et al., 2013; Jellinger, 1991). Recentemente, o sistema adenosinérgico também foi implicado na patofisiológica da DP, devido à sua interação com o sistema dopaminérgico e a seus efeitos sobre a função motora (Canals et al., 2003; Fenu et al., 1997; Morelli et al., 2010).

Outra importante característica neuropatológica da DP é o aparecimento de inclusões eosinofílicas citoplasmáticas denominadas corpos de *Lewy*, compostas principalmente pelo acúmulo da proteína α-sinucleína na forma agregada, oligomérica ou fibrilar (Dickson, 2001; Miklya et al., 2014; Spillantini et al., 1998). A α-sinucleína é uma proteína pequena, solúvel em seu estado nativo, com aproximadamente 14 KDa e composta de 140 aminoácidos (Beyer, 2006; Pihlstrom e Toft, 2011). Sua função ainda não foi totalmente elucidada, porém evidências sugerem que a α-sinucleína tem um papel no tráfego de vesículas, na liberação de neurotransmissores e no recrutamento de vesículas sinápticas no hipocampo, além de regular diversos fatores-chave envolvidos na homeostase da dopamina (Burré et al., 2010; Cabin et al., 2002; Yavich et al.,2004).

A DP é clinicamente caracterizada pelo início tardio e pela ocorrência progressiva de quatro sintomas principais que incluem rigidez, tremor de descanso, bradicinesia e instabilidade postural (Djaldetti et al., 2006). A bradicinesia é a principal manifestação clínica da DP e indica a existência de distúrbios dos gânglios da base. Caracteriza-se pela lentidão de movimentos e pela dificuldade para planejar, iniciar e executar o movimento (Beradelli et al., 2001). O tremor em repouso, por sua vez, é o sintoma mais característico e mais fácil de ser reconhecido na DP. Em geral, os tremores são unilaterais e proeminentes nas partes distais dos membros. Já a rigidez ocorre pelo aumento do tônus muscular, enquanto a instabilidade postural é desenvolvida nos estágios avançados da doença (Hartelius et. al.,1994; Skodda et. al., 2011).

Além das manifestações clínicas ligadas ao movimento na DP, manifestações clínicas não motoras potencialmente incapacitantes também são relatadas. Entre elas, estão comprometimento cognitivo e demência, distúrbios do sono, disfunções olfatórias, constipação, depressão, entre outros (Chaudhuri e Schapira, 2009; Janvin et. al., 2006). Alguns desses sintomas foram descritos, em 1817, por James Parkinson, primeiro autor a descrever esta patologia, a qual leva seu nome. Além dos sintomas motores, Parkinson relatou a ocorrência de constipação, sialorreia, delírio e insônia em seus pacientes (Alves et al., 2008; Fahn, 2014). Outros pesquisadores da época também relataram a presença de sintomas não motores (Fahn, 2014). Atualmente, sabe-se que os sintomas não motores possuem um impacto negativo na qualidade de vida dos pacientes com DP e estão associados com a progressão da doença. Além disso, um estudo multicêntrico mostrou que 98,6% dos pacientes com DP possuem sintomas não motores, com uma média de 7,8 sintomas por paciente (Barone et al., 2009).

A maioria dos casos de DP é esporádica e está associada ao envelhecimento, sendo que aproximadamente 10% dos casos ocorrem devido a mutações genéticas (Hindle, 2010). Diversos genes têm sido associados à DP, entre eles destacam-se as mutações, duplicações e triplicações do gene da α-sinucleína, proteína com papel central nas inclusões eosinófilas (Appel-Cresswell et al., 2013; Ozansoy e Basak, 2013; Pihlstrom e Toft, 2011; Polymeropoulos et al., 1997). Genes associados a vias que se relacionam com o metabolismo mitocondrial como a *PINK1* e *PARK2* (Ishihara-Paul et al., 2008; Kitada et al., 1998), gene com sensor redox para estressse oxidativo como *DJ-1* (Bonifati et al., 2003) e genes associados à autofagia lisossomal como *ATP13A2*, *GBA*, *LRRK2* (Kachergus et al., 2005; Ramirez et al., 2006) foram identificados na DP. Além disso, recentemente mutações de genes associados à neuroinflamação (*HLA-DR*), à reciclagem de vesículas sinápticas, endocitose mediada por clatrina, processamento da membrana (*DNAJ* e *GAK-DGKQ*) e transporte de endossomos (*VPS35*) também foram identificados na DP (Edvardson et al., 2012; Hamza et al., 2010; Vilariño-Guell et al., 2011).

Além da predisposição genética, estudos epidemiológicos sugerem que a exposição a agentes ambientais, como pesticidas, metais e solventes, pode também aumentar o risco de desenvolvimento de DP (Baltazar et al., 2014; Campdelacreu, 2014; Cannon e Greenamyre, 2011). O interesse pelos pesticidas na DP aumentou a partir da década de 80 após Langston e colaboradores (1983) descreverem os sintomas da DP em jovens usuários de droga injetável. A droga utilizada era uma preparação de um análogo da meperidina contendo o 1-metil-4-phenil-1,2,3,6-tetrahidropiridina (MPTP). O MPTP cruza a barreira hematoencefálica e é

metabolizado pela monoamino oxidase B, produzindo o seu metabólito ativo o 1-metil-4-fenilpiridina (MPP). O MPP, quando carreado por transportadores de dopamina, bloqueia o complexo I mitocondrial, causando dano em neurônios dopaminérgicos (Przedborski et al., 2000). A similaridade estrutural de certos pesticidas, como o paraquat (Pq), ao MPTP e seu metabólito MPP, somados ao dano em cérebros de indivíduos que morreram por intoxicação com Pq (Grant et al., 1980; Hughes, 1988), despertaram o interesse de pesquisadores. Estudos populacionais têm demonstrado que a exposição ao paraquat isolado ou em associações com fungicidas aumenta o risco de desenvolver DP (Moretto e Colosio, 2013; Tanner et al., 2011).

Pesticidas e neurotoxinas são comumente utilizados como modelo animal para mimetizar os sintomas da DP, como bradicinesia, rigidez e tremor de repouso (Bové e Perier, 2012). Compostos reversíveis, como a reserpina, e compostos irreversíveis como o MPTP, 6-hidroxidopamina (6-OHDA), Pq e rotenona vêm sendo utilizados no desenvolvimento de modelos animais para melhor entender a fisiopatologia da doença e buscar novos alvos terapêuticos (Blandine e Armentero, 2012; Bové e Perier, 2012; Santos et al., 2013).

Atualmente, a estratégia farmacoterapêutica para DP visa restabelecer os níveis de dopamina pelo uso de um precursor de dopamina, agonistas dopaminérgicos e inibidores da degradação enzimática de dopamina (Olanow et al., 2004; Stayte e Vissel, 2014). No entanto, esse tratamento não impede a progressão da doença, apenas trata os sintomas motores por um período de tempo. Após esse período, os pacientes desenvolvem sintomas não dopaminérgicos, tais como distúrbio da marcha e demência. Um objetivo crucial é o desenvolvimento de um tratamento neuroprotetor que retarde a progressão da doença e tenha efeitos benéficos sobre as características dopaminérgicas e não dopaminérgicas (Schapira et al., 2014).

1.2 Sistema purinérgico

O sistema purinérgico constitui uma rota comum de comunicação célula-célula, envolvido em muitos mecanismos neuronais e não neuronais, como respostas imunes, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (Abbrachio et al., 2009).

A neurotransmissão purinérgica, proposta pela primeira vez por Geoffrey Burnstock em 1972 (Burnstock, 1972), possui como mensageiros nucleosídeos e nucleotídeos derivados de purina, entre eles o ATP (adenosina 5’-trifosfato) e a adenosina (Burnstock, 2008). O ATP é reconhecido como neurotransmissor, pois é sintetizado e armazenado em terminais

sinápticos e liberado após estímulo desses terminais. Além disso, este neurotransmissor pode ser coliberado juntamente com vários outros neurotransmissores, como acetilcolina, glutamato, dopamina, noradrenalina, serotonina e GABA (Burnstock, 2004; Zimmerman, 2008).

O ATP exerce seus efeitos através da ativação de receptores purinérgicos do tipo P2, divididos em dois grupos de acordo com o mecanismo de ação, a farmacologia e a clonagem molecular: P2X e P2Y (Puchalowicz et al., 2014; Ravelic e Burnstock, 1998; Skaper et al., 2010). A família P2X consiste em receptores ionotrópicos que apresentam permeabilidade rápida e seletiva para cátions (Na^+, K^+ e Ca^{2+}) e está dividida em sete membros (P2X1-7), distribuídos em neurônios, células gliais e no músculo liso (Burnstock, 2004, Burnstock, 2012). A família P2Y consiste em oito membros de receptores metabotrópicos, acoplados à proteína G, funcionalmente descritos (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14), que apresentam uma ampla distribuição nos tecidos e sistemas, tais como: vascular, epitelial, nervoso e cardíaco (Burnstock, 2007; Zimmermann 2011).

O ATP, após a sua liberação, pode ser metabolizado pela ação de ectonucleotidases que fazem a conversão de ATP até adenosina (Robson et al., 2006; Zimmermann et al., 2012). As ectonucleotidases se encontram ancoradas na membrana celular, possuindo seu sítio ativo voltado para o meio extracelular ou presentes na forma solúvel no meio intersticial. As ectonucleotidases são constituídas por quatro famílias de enzimas: Ecto-nucleosídeo trifosfato difosfoidrolases (NTPDases), Ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPPs), fosfatases alcalinas e Ecto-5'-nucleotidase (E-5´NT) (Bonan, 2012; Schetinger et al., 2007; Zimmermann, 2012).

A família das E-NTPDases (EC 3.6.1.5) são responsáveis pela hidrólise de nucleotídeos 5´-trifosfatados em 5´-difosfatados (Zimmermann, 2001). Os membros da família das NTPDases são codificados por oito genes diferentes chamados *entpd*. Quatro dessas enzimas (NTPDases 1, 2, 3 e 8) estão localizadas na membrana celular com o sítio ativo voltado para o meio extracelular; duas (NTPDases 4 e 7) estão localizadas intracelularmente com o sítio ativo voltado para o lúmen de organelas citoplasmáticas e duas (NTPDases 5 e 6) podem ser excretadas de forma heteróloga após expressão intracelular (Robson et al., 2006). Em termos de hidrólise, as NTPDases hidrolisam tanto ATP como ADP (adenosina difosfato), formando AMP (adenosina monofostato) na presença de íons Ca^{+2} e Mg^{+2} (Robson et al., 2006).

A E-5´-NT (EC 3.1.3.5) possui distribuição tecidual ampla, sendo responsável pela desfosforilação de nucleosídeos monofosfatados. Esta enzima é ancorada na membrana

plasmática por um glicosilfosfatidilinositol (GPI) e tem seu sítio catalítico voltado para o meio extracelular. O ancoramento por GPI pode ser clivado dando origem a formas solúveis dessa enzima (Bianchi e Spychala, 2003; Zimmermann, 1992; Zimmermann, 2012). Dentre os nucleotídeos, o AMP (adenosina monofosfatado) tem a hidrólise mais eficiente pela E-5´-NT, formando adenosina. A participação da E-5´-NT na via das ectonucleotidases exerce um papel regulatório sobre a produção de adenosina extracelular, sendo a enzima marca-passo desta cascata enzimática (Zimmermann, 2001; Knapp et al., 2012).

A adenosina é um metabólito constituinte de todas as células, desenvolvendo diversos papéis no organismo, tais como síntese de ácidos nucleicos, metabolismo de aminoácidos e modulação do estado metabólico da célula. No sistema nervoso central (SNC), a adenosina atua como neuromodulador e neuroprotetor, não sendo descrita como neurotransmissor por não ser armazenada em grânulos sinápticos ou liberada de forma quântica (Fredholm et al., 2005, Shen e Chen, 2009). Devido a seu papel de neuromodulação, a adenosina, está envolvida na regulação de importantes mecanismos do SNC, como estados de ansiedade (El Yacoubi et al., 2000; Maximino et al., 2011), sono (Carús-Cadavieco e de Andrés, 2012), cognição e memória (Chen et al., 2014; Shen et al., 2012), entre outros.

A adenosina exerce seus efeitos através da ativação de receptores de membrana específicos do tipo P1. Esses receptores, descritos há mais de 40 anos, são divididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3, e identificados como membros da família de receptores acoplados a proteína G (Fredholm et al., 2001; Fredholm et al., 2011). Os receptores A_1 e A_3 se ligam à família das proteínas $G_{i/o}$, responsáveis pela inibição da produção do segundo mensageiro AMPc. Os receptores A_{2A} e A_{2B} estimulam a produção de AMPc via ativação de proteínas G_s (Fredholm et al., 2001, Fredholm et al., 2011; Ralevic e Burnstock, 1998).

A concentração de adenosina pode ser controlada a partir de sua hidrólise pela Adenosina deaminase (ADA), pela fosforilação a AMP pela adenosina quinase (AK) ou através de sua liberação e recaptação celular através de transportadores de nucleosídeos (Bonan, 2012; King et al., 2006; Latini e Pedata, 2001). A ADA (EC 3.5.4.4) é uma enzima envolvida no metabolismo das purinas por catalisar a desaminação hidrolítica da adenosina e da 2-deoxiadenosina a seus produtos inosina e deoxiinosina, respectivamente (Zavialov e Engström, 2005). A ADA é encontrada tanto no meio citosólico quanto na membrana celular e está presente em diferentes tecidos, entre eles, sistema gastrointestinal, encéfalo e timo. Dois membros dessa família já foram descritos, sendo eles denominados como ADA1 e ADA2 (Hirschhorn e Ratech, 1980; Zavialov e Engström, 2005), além de um grupo similar dessa família de proteínas denominado ADAL (*adenosine deaminase-like*). Todos esses membros

foram classificados como subfamílias pertencentes ao grupo das adenil-desaminases. Por apresentar sítios de aminoácidos importantes relacionados à desaminação de adenosina e motivos conservados entre as subfamílias da ADA, é possível que a ADAL também realize a desaminação hidrolítica de adenosina a inosina (Maier et al., 2005).

O sistema purinérgico, através de seus receptores P2 e P1, está envolvido com os mecanismos relacionados a doenças neurodegenerativas, entre elas a DA (Burnstock 2008; Burnstock et al., 2011; Erb et al., 2014). Dados da literatura demonstram uma mudança na expressão de receptores P2Y em humanos e em modelos animais da DA (Erb et al., 2014; Lai et al., 2008). Os receptores P2Y também parecem participar da regulação e produção da APP, na neuroinflamação e nas funções neurovasculares envolvidas na DA, e os peptídeos Aβ aumentam a liberação de ATP e/ou modulam a expressão de receptores P2Y em modelos *in vitro* e *in vivo* (Erb et al., 2014; Peterson et al., 2010). Interessantemente, a eliminação do receptor P2Y2 em um modelo roedor de DA acelera a mortalidade e modifica o acúmulo de Aβ (Ajit et al., 2014). Do mesmo modo, a expressão reduzida do receptor P2Y2 foi relatada em amostras de cérebro *post-mortem* de pacientes com DA, sugerindo que a diminuição da expressão desse receptor está correlacionada com o fenótipo da DA (Lai et al., 2008). Além dos receptores P2Y, os receptores P2X também estão associados à DA, em especial o receptor P2X7, que tem sua regulação aumentada em torno das placas senis em modelos animais da DA (McLarnan et al., 2006; Parvathenani et al., 2003). A ativação desse receptor em neurônios está associada à ativação da via não amiloidogênica da APP (α-secretase), tendo assim um possível efeito neuroprotetor na DA (Allinson et al., 2003; Delarasse et al., 2011).

Além dos receptores P2, os receptores P1 também estão envolvidos na DA. Análises *post-mortem* do córtex frontal de pacientes com DA mostraram que o número total e os níveis de receptores A_1 e A_{2A} estão aumentados nessa patologia (Albasanz et al., 2008). Ângulo e colaboradores (2003) também mostraram uma redistribuição dos receptores de adenosina no hipocampo e no córtex de pacientes com DA e demonstraram a colocalização do receptor A_1 com placas senis contendo peptídeos Aβ. Além disso, estudos têm mostrado que a modulação desses receptores pode contribuir para a diminuição da neurodegeneração relacionada à DA. Estudos com modelos animais de indução do déficit cognitivo observaram que a administração de antagonistas seletivos e não seletivos dos receptores de adenosina promoveram benefícios na cognição (Arendash et al., 2006; Arendash et al., 2009; Cunha, 2008). Em roedores, o uso de cafeína, antagonista não seletivo dos receptores de adenosina, preveniu o acúmulo de peptídeo Aβ no cérebro e em torno de vasos sanguíneos cerebrais (Cupino e Zabel, 2013; Gahr et al., 2013). Estudos com modelos da DA têm verificado que o

consumo de cafeína está relacionado com a reversão do déficit cognitivo e diminuição dos níveis de peptídeos Aβ em encéfalos (Cao et al., 2009; Chu et al., 2012). Em humanos, um estudo sobre "Fatores de Risco Cardiovasculares, Envelhecimento e Demência" (CAIDE study) observou que o consumo de 3 a 5 xícaras de café por dia foi associado com uma diminuição de 65% no risco de desenvolver demência ou DA (Eskelinen e Kivipelto, 2010). Outro estudo analisou os níveis de cafeína e o estado cognitivo em 124 idosos diagnosticados com demência leve. Os dados mostraram que o aumento da cafeína no plasma foi associado a não conversão para demência (Cao et al., 2012). Além disso, estudos em humanos e modelos animais mostraram que o prejuízo da memória causado pela administração de escopolamina (um antagonista dos receptores muscarínicos) pode ser prevenido pelo tratamento com cafeína (Bortolotto et al., 2015; Botton et al., 2010; Riedel et al., 1995).

O sistema purinérgico também está envolvido em processos relacionados com a DP. Em particular, os receptores de adenosina, principalmente o receptor A_{2A}, vêm chamando atenção como uma promessa para a terapia da DP (Jenner et al., 2009; Morelli et al., 2009; Zhu et al., 2014). A adenosina está associada ao controle da função motora devido à colocalização e à heterodimerização dos receptores de dopamina D_2 e adenosina A_{2A} no estriado, tanto na membrana como intracelularmente (Ferré et al., 2004; Fuxe et. al., 2010; Schiffmann et al., 2007). Esses receptores possuem ação antagônica, ou seja, a ativação do receptor A_{2A} causa mudanças conformacionais no receptor D_2 levando a uma redução do reconhecimento do sinal. O mesmo ocorre durante a ativação do receptor D_2 (Ferré et al., 1997; Fuxe et al., 2007). Assim, o resultado da depleção de dopamina no estriado causa o aumento da sinalização do receptor A_{2A}, resultando nos sintomas hipolocomotores da DP (Gomes et al., 2011).

Estudos com antagonistas não seletivos dos receptores A_{2A} em pacientes com DP mostraram sua habilidade em controlar o comprometimento motor e induzir neuroproteção (Ascherio et al., 2001; Ross et al., 2000). Em modelos animais de DP, os antagonistas de receptores A_{2A}, administrados isoladamente ou coadministrados com fármacos dopaminomiméticos ou agonistas de dopamina, melhoraram a função motora (Cerri et al., 2014; Koga et al., 2000). Porém, estudos clínicos em pacientes com DP e antagonistas de A_{2A} geraram dados inconclusivos devido à manutenção dos efeitos motores desta patologia (Fernandez, 2008; Morelli et al., 2009). Um dos antagonistas de receptores A_{2A} testados, o KW-6002, foi rejeitado pelo FDA (*Food and Drug Administration*) nos Estados Unidos uma vez que os ensaios clínicos demonstraram que os pacientes apresentavam discinesia como efeito adverso. Já, no Japão, esse antagonista foi recentemente aprovado para uso como

auxiliar no tratamento para DP. Atualmente, o preladenante (antagonista de receptor A_{2A}) encontra-se em ensaios clínicos fase II e fase III para o tratamento da DP com modestos, mas promissores resultados positivos para esta patologia (Hauser et al., 2011; Jenner et al., 2009; Stay e Vissel, 2014).

Estudos indicam também que a adenosina antagoniza a transmissão mediada pelo receptor D_1 e estimula os receptores A_1 (Ferré, 1997; Ferré et al., 2001), e estes estão colocalizados no estriado (Ferré et al., 1991). Um estudo com modelos de DP mostrou que o antagonismo do receptor A_1 aumenta o efeito de ativação motora do SK 38393, um agonista de D_1, em ratos (Popoli et al., 1996). Outro estudo utilizando ratos *knockout* para o receptor, A_1, A_{2A}, A_1-A_{2A} e ratos pré-tratados com cafeína (antagonista não seletivo de receptores de adenosina) demonstraram integridade neuronal avaliada pelo teor de dopamina no estriado nos *knockout* e pré-tratados com cafeína após lesão com 6-OHDA (Xiao et al., 2011). Estudos sugerem um efeito sinérgico do antagonismo dos receptores A_1 e A_{2A}, ou seja, o antagonismo do receptor A_1 parece facilitar a liberação de dopamina, enquanto o antagonismo do receptor A_{2A} potencializa a resposta deste neurotransmissor (Moore et al., 2003; Rebola et al., 2003).

Além dos receptores P1, os receptores P2 também estão envolvidos na DP. Estudos têm mostrado que um subtipo de receptor P2, o receptor P2X7, pode parcialmente prevenir a diminuição de dopamina em modelo de roedores de DP (Carmo et al., 2014; Marcellino et al., 2010). Dados também indicam que o receptor P2X7 é expresso em áreas dopaminérgicas afetadas pela DP (Able et al., 2011; Yu et al., 2008) e que ele pode estar envolvido no controle do dano de células dopaminérgicas (Jun et al., 2007; Orellano et al., 2010). Recentemente, o receptor P2X1 também foi associado à DP, uma vez que Gan e colaboradores (2014) mostraram em modelos de células neuronais que o receptor P2X1 pode estar associado ao mecanismo de agregação protéica na DP. Além disso, os experimentos sugerem que o ATP tem um papel na agregação da α-sinucleína. Esses dados somados aos demais colocam o sistema purinérgico, em especial seus receptores, como alvos potenciais no tratamento terapêutico da DP.

1.3 Sistema colinérgico

A acetilcolina (ACh) foi descoberta em meados de 1920 e representa o primeiro neurotransmissor descoberto na história da neurociência (Brown, 2006). A acetilcolina é sintetizada a partir de acetil-CoA e colina e é transportada para o interior de vesículas sinápticas. Sua liberação depende das variações no potencial elétrico das membranas dos

terminais nervosos, e esse processo é dependente da concentração de cálcio intracelular. Ao ser liberada, a ACh interage com receptores específicos, causando despolarização e propagação do potencial de ação na célula pós-sináptica (Brown, 2006; Oda, 1999).

Na fenda sináptica, a ACh ativa duas classes de receptores: muscarínicos (metabotrópicos) e nicotínicos (ionotrópicos). Os receptores muscarínicos são membros da superfamília de receptores acoplados à proteína G (Wess, 1996). Cinco subtipos desse receptor são descritos na literatura pelas distintas propriedades moleculares e funcionais. Os receptores M1, M3 e M5 são acoplados à proteína G_q e sua estimulação promove a sinalização por meio da hidrólise do fosfoinositídeo e a ativação da fosfolipase C. Por sua vez, os receptores M2 e M4 inibem a atividade da adenilato ciclase via proteína G_i (Bymaster et al., 2003; Wess, 2004).

Os receptores nicotínicos são considerados receptores excitatórios e membros da superfamília de receptores acoplados a canais iônicos. Apresentam-se como proteína transmembrana pentamérica composta de duas subunidades α e uma subunidade β, γ e δ. Uma variedade de estudos mostra a expressão desse receptor em vertebrados e invertebrados e cita ainda mudanças na estrutura das subunidades, gerando um total de 17 subunidades derivadas da α, β, γ e δ (Millar e Harkness, 2008; Millar e Gotti, 2009). Estudos estruturais mostram que as subunidades estão arranjadas ao redor de uma cavidade central, com uma grande porção de proteína voltada para a superfície extracelular. A ACh se liga normalmente à subunidade α, produzindo mudanças conformacionais que permitem a passagem principalmente de cátions (Brejc et al., 2001). No SNC, a ACh desempenha um papel fundamental em diversas funções biológicas relacionadas ao comportamento, ao aprendizado, à memória, à organização cortical do movimento e ao controle do fluxo sanguíneo cerebral (Moretto et al., 2004; Ness, 2014).

A ACh, que permanece na fenda sináptica, é hidrolisada por duas colinesterases diferentes: a acetilcolinesterase (AChE; E.C.3.1.1.7) e a butirilcolinesterase (BuChE; E.C.3.1.1.8), formando colina e acetato. A AChE pode ser usada como um marcador da função colinérgica, uma vez que mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (Soreq e Seidman, 2001). Além disso, é também conhecido o importante papel da acetilcolinesterase em doenças cuja incidência se eleva com o aumento da idade, como a DA (Bartus et al., 1982; Casey et al., 2010; Singh et al., 2013).

1.4 Sistema dopaminérgico

A dopamina é um neurotransmissor do tipo catecolaminérgico. Cerca de 80% do total da dopamina no SNC encontram-se no estriado, enquanto que o restante está distribuído difusamente pelo córtex e outras regiões cerebrais. A dopamina é responsável por regular a locomoção, a cognição, a emoção e o sistema de recompensa (Bjorklund e Dunnett, 2007; Goldman-Rakic, 1998; Schultz, 2002).

A neurotransmissão dopaminérgica ocorre por meio de vários processos relacionados, incluindo síntese, liberação, captação, armazenamento, catabolismo, e ativação do receptor de dopamina (Jones e Miller, 2008). A enzima tirosina hidroxilase (TH), quando ativada, converte o aminoácido L-tirosina em L-DOPA, que é descarboxilado para formar a dopamina (Missale et al, 1998). Uma vez sintetizada, a dopamina é armazenada em vesículas pré-sinapticas. Dois modos distintos de liberação de dopamina são promovidos pelos neurônios dopaminérgicos, um com atividade fásica, representado pelo padrão explosivo de disparos que ocorrem em resposta a estímulos comportamentais e outro com atividade tônica que ocorre através de disparos espontâneos desses neurônios. A liberação fásica de dopamina ativa receptores pós-sinápticos e é rapidamente removida da fenda por mecanismos de recaptação, enquanto a liberação tônica determina os níveis extracelulares desse neurotransmissor em estruturas sub-corticais (Missale et al., 1998).

Existem cinco receptores de dopamina D_1, D_2, D_3, D_4 e D_5 que podem ser divididos em receptores D_1-*like* (D_1,D_5) e D_2-*like* (D_2, D_3, D_4), agrupados por suas propriedades farmacológicas e por similaridade de sequência. Estes receptores são metabotrópicos e acoplados a proteína G. A ativação dos receptores de dopamina D_1-*like* estimula à proteína G_s, que, por sua vez, ativa a adenilato ciclase, aumentando AMPc, enquanto os receptores do D_2-*like* inibem a proteína $G_{i,}$ exercendo influência negativa sobre a atividade da adenilato ciclase, diminuindo o AMPc (Hurley e Jenner, 2006; Missale et al., 1998; Sokoloff e Schwartz, 1995). Os receptores de dopamina são bem distribuídos pelo cérebro, porém cada subtipo possui distribuição única. Os receptores D_1 possuem alta densidade no núcleo caudado, no putamen e no núcleo accumbens em ratos (Gerfen et al., 1995). Além disso, os receptores D_1 também foram encontrados em terminais pré-sinápticos com projeções glutamatérgicas para córtex e tálamo em humanos (Hurley et al., 2003). Os receptores D_5 possuem distribuição restrita e foram localizados no estriado ventral, no glóbulo pálido e no hipocampo em cérebro de ratos e de humanos (Khan et al., 2000).

O receptor D_2 é o mais abundante da família D_2-*like*, sendo encontrado em alta densidade no núcleo caudado, no putâmen e no núcleo accumbens. Ele está localizado nos dendritos estriatopalidais gabaérgicos. Níveis moderados deste receptor foram detectados no tubérculo olfatório e nas ilhas de Calleja, e baixos níveis estão presentes na substância negra compacta reticulada (Gurevich e Joyce, 1999). Os receptores D_3 possuem distribuição mais restrita que o D_2, sendo que altos níveis deste receptor foram encontrados nas ilhas de Calleja e, em níveis menores, em cerebelo, tálamo e hipotálamo (Gurevich e Joyce, 1999; Hurley et al., 1996). Por fim, os receptores D_4 são encontrados no córtex e regiões não estriadas, com baixos níveis de expressão em regiões estriadas (Primus et al., 1997).

1.5 Peixe-zebra

O *Danio rerio*, popularmente conhecido como *zebrafish* ou peixe-zebra, é um pequeno teleósteo de água doce proveniente do sudeste da Ásia, Índia e Nepal. Seu habitat natural é a água lentamente fluída, como pequenos riachos, lagoas, córregos e campos de arroz (Engeszer et al., 2007). Seu pequeno tamanho, a capacidade de viver em confinamento e a alta produção de ovos transparentes e grandes, variando de 2 a 300 ovos por fêmea, chamaram a atenção dos cientistas para o emprego deste peixe na investigação da biologia do desenvolvimento (Lieschke e Currie, 2007; Shin e Fishman, 2002). Concomitante aos estudos da biologia do desenvolvimento, diversas ferramentas genéticas foram desenvolvidas para o peixe-zebra (Davis et al., 2014; Patton e Zon, 2001); e, recentemente, seu genoma foi completamente sequenciado, demonstrando que 70% dos genes deste teleósteo têm um ortólogo humano identificável (Howe et al., 2013).

O acúmulo de conhecimento genético aliado à descrição da organização geral e de circuitos neuronais semelhantes aos observados em mamíferos, presença dos principais neurotransmissores, hormônios e receptores (Arenzana et al., 2005; Boehmler et al., 2004; Boehmler et al., 2007; Panula et al., 2006; Rink e Wullimann, 2001; Zirger et al., 2003) destacaram o peixe-zebra como um importante modelo para estudos comportamentais (Sager et al., 2010; Stewart et al., 2014). O repertório comportamental do peixe-zebra é complexo. Essa espécie vive em cardumes tanto no seu habitat natural quanto em laboratório. Indivíduos exibem preferências sociais pelos seus semelhantes formando grupos, sendo este comportamento efetivo contra predadores em diversas espécies de peixes (Gerlai et al., 2000; Miller e Gerlai., 2007). O comportamento locomotor também desempenha um importante papel na atividade social do peixe-zebra, na alimentação e na defesa contra predadores

(Gerlai, 2000; Granado et al., 1996). Além disso, nos últimos anos, outras características comportamentais desse teleósteo como agressividade (Jones et al., 2015), ansiedade (Maximino et al., 2014; Puty et al., 2014), memória e aprendizado vêm sendo estudadas (Blank et al., 2009; Gerlai, 2011).

O peixe-zebra tem sido cada vez mais utilizado para a análise de aprendizado e de memória (Colwill et al., 2005; Cognato et al., 2012; Gerlai et al., 2011). No entanto, na última década, houve um aumento do desenvolvimento de tarefas de memória específicas para o peixe-zebra. Essas tarefas demonstraram que esse pequeno vertebrado tem a capacidade de associar uma variedade de estímulos condicionados e não-condicionados (Fernades et al., 2014; Sison e Gerlai, 2011; Zala e Määttänen, 2013), possui memória espacial (Cognato et al., 2012; Spence et al., 2012), memória aversiva (Blank et al., 2009) e visual (Avdesh et al., 2012). Nesse contexto, o peixe-zebra tem se tornado uma excelente ferramenta de pesquisa da memória e da cognição, com possibilidade de realizar triagens genéticas e farmacológicas (Gerlai, 2010; Hicks et al., 2006).

Além de estudos comportamentais, o peixe-zebra vem se destacando como um bom modelo para estudo de doenças do SNC, entre elas as doenças neurodegenerativas como DA e DP (Newman et al., 2014; Xi et al., 2011). Na DA, modelos transgênicos e *knockout* do peixe-zebra vêm sendo utilizados na literatura (Xi et al., 2011). O gene da APP em peixe-zebra possui dois genes associados à APP humana, *APPa* and *APPb* (Musa et al., 2001). O bloqueio da *APPa* por morfolino no peixe-zebra tem poucos efeitos em lavas, porém o bloqueio de *APPb* causa defeito no desenvolvimento neuronal do peixe (Song e Pimplikar, 2012), incluindo defeito no desenvolvimento axonal e na formação de sinapses (Abramsson et al., 2013). Outro estudo realizado com este teleósteo expressando um gene humano mutado da TAU associado a promotor fluorescente mostrou um crescimento axonal defeituoso, hiperfosforilação da TAU, permitindo a triagem de novas moléculas terapêuticas (Paquet et al., 2009). Além dos modelos genéticos, o modelo de déficit cognitivo farmacológico de DA, utilizando a escopolamina, um antagonista dos receptores muscarínicos, apresentou prejuízo cognitivo e no processamento da memória aversiva e de reconhecimento no peixe-zebra (Cognato et al., 2012; Kim et al., 2010; Richetti et al., 2011).

Na DP, genes ortólogos associados a esta patologia como *pink 1*, *dj-1*, *parkin* e *lrrk2* foram encontrados em peixe-zebra. Da mesma forma que na DA, modelos transgênicos e *knockout* do peixe-zebra para DP vêm sendo utilizados na literatura (Anichtchik et al., 2008; Flinn et al., 2009; Xi et al., 2011). Recentemente, um estudo utilizando peixe-zebra mutante para *pink 1* apresentou perda de neurônios dopaminérgicos, além do comprometimento

precoce da função mitocondrial e morfologia (Flinn et al., 2013). Já o *knockout* de *dj-1* em peixe-zebra não afetou o número de neurônios dopaminérgicos, mas os embriões ficaram mais susceptíveis ao estresse oxidativo (Bretaud et al., 2007). Além disso, a anulação da atividade de *parkin* em peixe-zebra leva a uma diminuição significativa no número de neurônios dopaminérgicos ascendente no tubérculo posterior (homólogo à substantia nigra em seres humanos), um efeito aumentado pela exposição a MPP (Flinn et al., 2009). Exposição a neurotoxinas como o MPTP, seu metabólito MPP e a 6-OHDA mostrou um efeito hipolocomotor e uma diminuição dos níveis de dopamina no cérebro de peixe-zebra (Anitchtchik et al., 2004; Bretaud et al., 2004; Feng et al., 2014; Panula et al., 2010). Exposição a pesticidas, como paraquat e rotenona, também tem demonstrado diminuição na locomoção (Bretaud et al., 2004), na modulação dos níveis de dopamina, e comprometimento na aquisição e consolidação da memória espacial em peixe-zebra (Bortolotto et al., 2014).

A sinalização purinérgica, um possível alvo terapêutico para DA e DP (Burnstock, 2011; Zhu et al., 2014), já foi caracterizada em peixe-zebra. Estudos realizados pelo nosso laboratório identificaram a presença de NTPDases e EC-5´NT em membranas cerebrais, as quais são dependentes de cátions, apresentando atividade ótima à temperatura de 37 ºC, pH ótimo entre 7,2 e 8,0, K_M na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (Rico et al., 2003; Senger et al., 2004). Rico e colaboradores (2006) identificaram três isoformas da NTPDase 2, que foram nomeadas como NTPDase2mv, NTPDase2mq e NTPDase2mg em peixe-zebra. Recentemente, um estudo caracterizou o padrão de expressão da NTPDase 3 em peixe-zebra (Appelbaum et al., 2007), assim como Rosemberg e colaboradores (2010) demonstraram a expressão de NTPDases 1,2,3,4,5,6 e 8 em cérebro, coração e fígado de peixe-zebra. O mapeamento do padrão de expressão de genes relacionados à adenosina deaminase (ADA1, ADAL e dois ortólogos da ADA2) foi realizado por nosso laboratório em vários tecidos de peixe-zebra (Rosemberg et al., 2007), bem como a caracterização cinética da atividade da ADA em encéfalo de peixe-zebra (Rosemberg et al., 2008).

Receptores do sistema purinérgico, P2 e P1, também foram clonados e identificados no peixe-zebra (Appelbaum et al., 2007; Diaz-Hernandes et al., 2002; Kucenas et al., 2003; Young, 2010). A família P2X possui nove membros. Desses seis ortólogos a genes dos receptores P2X de mamíferos, dois parálogos e um gene ainda precisam ser devidamente classificado (Kucenas et al., 2003; Kucenas et al., 2009). Os subtipos dos receptores P2X do peixe-zebra contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos (Ralevic e Bursntock, 1998). Dos receptores P2Y, somente os

receptores P2Y1 foram identificados em trombócitos de peixe-zebra (Gregory e Jagadeeswaran, 2002). Dos receptores P1, foi demonstrada a presença de duas formas de receptores A_{2A} em cérebros de embriões de peixe-zebra e uma forma de A_{2B} (Boehmler et al. 2009). Um estudo realizado pelo nosso grupo também mostrou que as isoformas A_1, $A_{2A.1}$, $A_{2A.2}$ e A_{2B} são expressas desde 24 horas após a fertilização em peixe-zebra (Capiotti et al., 2011).

2 OBJETIVOS

2.1 Objetivo geral

Considerando que a adenosina possui um importante papel neuromodulador e que sua modulação tem se mostrado uma alternativa promissora para o tratamento de doenças neurodegenerativas, este estudo visa avaliar parâmetros comportamentais em modelos de déficit cognitivo induzidos por escopolamina, e neurodegeneração induzidos por 6-hidroxidopamina ou por paraquat em peixe-zebra, bem como, estudar o possível efeito desses modelos sobre o sistema purinérgico e dopaminérgico.

2.2 Objetivos específicos

- Avaliar os efeitos de antagonistas dos receptores de adenosina A_1 e A_{2A} no modelo de déficit cognitivo induzido por escopolamina, utilizando parâmetros comportamentais;
- Avaliar os efeitos de inibidores de transportadores de nucleosídeos e da adenosina deaminase no modelo de déficit cognitivo induzido por escopolamina, utilizando parâmetros comportamentais;
- Avaliar parâmetros comportamentais, tais como atividade locomotora, ansiedade, interação social e memória após indução do modelo de déficit cognitivo induzido por paraquat;
- Caracterizar o modelo de neurodegeneração induzido por paraquat utilizando medidas de dopamina e seu metabólito DOPAC, tirosina hidroxilase e níveis de mRNA do transportador de dopamina em encéfalos de peixe-zebra adulto.
- Analisar a atividade enzimática e a expressão gênica das enzimas ecto-5'-nucleotidase, NTPDases e adenosina deaminase nos modelos de neurodegeneração induzidos por 6-hidroxidopamina e paraquat em peixe-zebra.

3. RESULTADOS

CAPÍTULO I

Artigo científico

Modulation of adenosine signaling prevents scopolamine-induced cognitive impairment in zebrafish

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Artigo publicado no periódico
Neurobiology of Learning and Memory 2015; 118: 113–119
(doi: 10.1016/j.nlm.2014.11.016)

Neurobiology of Learning and Memory 118 (2015) 113–119

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Modulation of adenosine signaling prevents scopolamine-induced cognitive impairment in zebrafish

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ARTICLE INFO

Article history:
Received 19 September 2014
Revised 21 November 2014
Accepted 27 November 2014
Available online 6 December 2014

Keywords:
Adenosine
Inhibitory avoidance
Memory
Scopolamine
Zebrafish

ABSTRACT

Adenosine, a purine ribonucleoside, exhibits neuromodulatory and neuroprotective effects in the brain and is involved in memory formation and cognitive function. Adenosine signaling is mediated by adenosine receptors (A_1, A_{2A}, A_{2B} and A_3); in turn, nucleotide and nucleoside-metabolizing enzymes and adenosine transporters regulate its levels. Scopolamine, a muscarinic cholinergic receptor antagonist, has profound amnesic effects in a variety of learning paradigms and has been used to induce cognitive deficits in animal models. This study investigated the effects of acute exposure to caffeine (a non-selective antagonist of adenosine receptors A_1 and A_{2A}), ZM 241385 (adenosine receptor A_{2A} antagonist), DPCPX (adenosine receptor A_1 antagonist), dipyridamole (inhibitor of nucleoside transporters) and EHNA (inhibitor of adenosine deaminase) in a model of pharmacological cognitive impairment induced by scopolamine in adult zebrafish. Caffeine, ZM 241385, DPCPX, dipyridamole, and EHNA were acutely administered independently via i.p. in zebrafish, followed by exposure to scopolamine dissolved in tank water (200 μM). These compounds prevented the scopolamine-induced amnesia without impacting locomotor activity or social interaction. Together, these data support the hypothesis that adenosine signaling may modulate memory processing, suggesting that these compounds present a potential preventive strategy against cognitive impairment.

1. Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder characterized by memory loss and cognitive deficits (Selkoe, 2001). AD etiology is not yet fully understood, though hallmark neuropathological features have been identified and include the deposition of senile plaques, neurofibrillary tangles, and progressive synaptic and neuronal loss (Haass & Selkoe, 2007). Additionally, cholinergic transmission deficiencies have been implicated in the cognitive decline observed in AD patients. According to the cholinergic hypothesis, cognitive deterioration is associated with a decrease in acetylcholine (ACh) levels due to its rapid hydrolysis by acetylcholinesterase (AChE) (Bartus, Dean, Beer, & Lippa, 1982). This hypothesis was the foundation for the use of acetylcholinesterase inhibitors (AChEi) for the treatment of cognitive deficits in AD patients (Casey, Antimisiaris, & ÓBrien, 2010; Small, 2005).

Scopolamine, a muscarinic cholinergic receptor antagonist, is known to impair learning and memory formation and used to model cognitive deficits in animal models (Klinkenberg & Blokland, 2010). The zebrafish, a small tropical freshwater teleost, has emerged as a prominent animal model for studying complex behaviors including learning and memory. Several memory tasks

Abbreviations: AD, Alzheimer disease; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DMSO, dimethylsulfoxide; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; ZM 241385, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl) phenol.

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http://dx.doi.org/10.1016/j.nlm.2014.11.016
1074-7427/

were established for this model organism in recent years, including the plus maze, Y-maze, and inhibitory avoidance task, which have proven to be useful tools for evaluating several behavioral and pharmacological conditions in this species (Blank, Guerim, Cordeiro, & Vianna, 2009; Cognato et al., 2012; Sison & Gerlai, 2010). The high genomic similarity to humans (Howe et al., 2013) and the identification of classical neurotransmitter systems involved in learning and memory processing, such as glutamatergic (Todd, Slatter, & Ali, 2004) and cholinergic (Clemente et al., 2004) systems, are important aspects for modeling neurological disorders in zebrafish (Best & Alderton, 2008; Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). Previous studies have demonstrated that scopolamine induces cognitive impairment and disrupts aversive and recognition memory processing in zebrafish (Cognato et al., 2012; Kim, Lee, Kim, Jung, & Lee, 2010; Richetti et al., 2011).

Adenosine, a purine ribonucleoside, plays important roles as a homeostatic regulator and neuromodulator, controlling neuronal excitability and, consequently, modulating physiological and pathological conditions of the central nervous system (CNS) (Boison, 2012; Chen, Eltzschig, & Fredholm, 2013; Dias, Rombo, Ribeiro, Henley, & Sebastião, 2013). Adenosine signaling is mediated via four G-protein coupled receptors: A_1, A_{2A}, A_{2B}, and A_3. These receptors were identified in several species including zebrafish (Boehmler et al., 2009; Capiotti et al., 2011) and are linked to a variety of transduction mechanisms (Fredholm, IJzerman, Jacobson, Linden, & Müller, 2011). Extracellular adenosine levels are dynamically controlled by the action of ectonucleotidases, involved in adenosine production by ATP degradation and equilibrative and concentrative nucleoside transporters (Bonan, 2012). In addition, adenosine may be deaminated by adenosine deaminase (ADA), a mainly cytosolic enzyme that is also found in the external cell surface already described in zebrafish (Rosemberg et al., 2007).

The involvement of adenosine receptors in cognitive processes in humans and rodents has been demonstrated (Gomes, Kaster, Tomé, Agostinho, & Cunha, 2011; Sebastiao, Ribeiro, & Ribeiro, 2012). *Post-mortem* analysis of frontal cortex from AD patients revealed a significant increase in A_1 and A_{2A} receptor expression in early and advanced stages of disease (Albasanz, Perez, Barrachina, Ferrer, & Martín, 2008). Epidemiological studies demonstrated that the incidence of AD was inversely associated with consumption of caffeine, which acts as a non-selective adenosine receptor antagonist (Maia & De Mendonça, 2002; Ritchie et al., 2007). Caffeine also prevented the scopolamine-induced disruption of short- and long-term memory in rodents and humans (Botton et al., 2010; Riedel et al., 1995). Studies using the A_1 and A_{2A} dual antagonist ASP5854 exhibited reversion of scopolamine-induced memory deficits in rats, whereas the specific A_{2A} antagonist KW-6002 had no such effect. Similarly, selective A_{2A} antagonist SCH58261 failed to reverse either MK-801- or scopolamine-induced amnesia in rats (Cunha et al., 2008). This evidence supports the specific involvement of A_1 receptor antagonism in cognition (Mihara et al., 2007).

Therefore, considering: (i) the role of adenosine receptors in prevention or reversion of scopolamine-induced memory deficits, (ii) the relevance of zebrafish as a powerful model for modeling neurological disorders and pharmacological studies and, (iii) the modulation of adenosine signaling exerted by nucleotide and nucleoside-metabolizing enzymes and adenosine transporters, this study aimed to investigate the effects of acute administration of caffeine, ZM 241385 (adenosine A_{2A} receptor antagonist), DPCPX (adenosine A_1 receptor antagonist), dipyridamole (inhibitor of nucleoside transporters) and EHNA (inhibitor of adenosine deaminase) in a model of pharmacological cognitive impairment induced by scopolamine in adult zebrafish.

2. Materials and methods

2.1. Animals

Adult wild type zebrafish (0.3–0.5 g) were obtained from a specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept in 30-L housing tanks with unchlorinated water at a targeted temperature of 26 ± 2 °C and continuously aerated under 14:10 h light:dark photoperiod. The fish were acclimated to the laboratory environment for at least 14 days and were fed three times a day with commercial flake food supplemented with brine shrimp. All procedures were approved by the institutional Animal Care Committee (11/00257-CEUA-PUCRS).

2.2. Pharmacological treatments

Caffeine (1,3,7-trimethylxanthine; Sigma Chemical Co., USA), ZM 241385 (4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo-{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl) phenol; Tocris Cookson, USA), DPCPX (8-cyclopentyl-1,3-dipropylxanthine; Tocris Cookson, USA), EHNA (erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; Sigma–Aldrich, St Louis, MO) and dipyridamole (Sigma–Aldrich, St Louis, MO) were used in the study. Caffeine and EHNA were dissolved in saline (0.9% NaCl); ZM 241385, DPCPX and dipyridamole were dissolved in 1% DMSO (dimethylsulfoxide). Caffeine (10 mg/kg), ZM 241385 (10 μg/kg), DPCPX (0.5 mg/kg), dipyridamole (5 mg/kg), and EHNA (100 μg/kg) were administered via intraperitoneal (i.p.) injection in a volume of 10 μL using a 3/10-mL U100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16") × 31G Short Needle (Becton Dickinson and Company, New Jersey, USA) (Kinkel, Eames, Philipson, & Prince, 2010). Drug doses and administration routes were chosen and adjusted based on previous studies demonstrating effects on memory in rodents (Melani, Cipriani, Corti, & Pedata, 2010; Prediger & Takahashi, 2005). Only doses that were unable to alter locomotor activity were used to ensure the effects observed were related to memory processing. Before drug or vehicle administration, fish were anesthetized by immersion in 0.1 g/L tricaine solution (ethyl 3-aminobenzoate methanesulfonate salt; Fluka, Buchs, Switzerland).

After treatment, animals were placed in a separate tank with aerated, unchlorinated tap water to recover from the anesthesia. Caffeine, ZM 241385, DPCPX, dipyridamole, EHNA, and the appropriate vehicle (used as control group) were injected 2 h before the beginning of each experiment. One hour prior to the start of the behavioral assay, animals were transferred to a tank containing 200 μM scopolamine solution (dissolved in aerated, unchlorinated water). Animals that did not receive scopolamine were also transferred to another tank with water to control for handling effects (Kim et al., 2010).

2.3. Behavioral analysis

2.3.1. Inhibitory avoidance task

Long-term memory was evaluated using the inhibitory avoidance (IA) protocol previously described in detail (Blank et al., 2009). After treatment, zebrafish were individually trained and tested in a glass tank (18 cm L × 9 cm H × 7 cm W) divided by a sliding guillotine-type partition (9 cm × 7 cm) in two equally sized compartments, white and dark. During a training session, animals were individually placed in the white side of the tank with the partition closed. After 1 min of habituation, the partition was raised 1 cm, allowing fish to cross to the dark side of the tank. Immediately after crossing and entering the dark side, the slide partition was closed and a pulsed electric shock of 3 ± 0.2 V was administered for 5 s. Fish were then removed from the apparatus and

placed in a temporary tank; they were later returned to their housing tank. Twenty-four hours after a training session, animals were submitted to a test session. The test session repeated the training protocol except that no shock was administered. The latency to completely enter the dark compartment was measured during both sessions.

2.3.2. Exploratory assessment

After animals received the second treatment, they were placed individually in the experimental tanks (30 cm L × 15 cm H × 10 cm W) and habituated for 30 s, as previously described (Gerlai, Lahav, Guo, & Rosenthal, 2000). After the habituation period, locomotor activity was video recorded for 5 min using Logitech Quickcam PRO 9000 and quantitatively analyzed using ANY-Maze recording software (Stoelting Co. Wood Dale, IL, USA). The tank was virtually divided into equal sections with three vertical lines and one horizontal line. The behavioral patterns quantified were the number of sectional line crossings, distance traveled, mean speed, and time spent in each tank section (i.e., the bottom vs. upper levels). Because zebrafish have a natural tendency to spend more time at the bottom of a novel tank before gradually exploring higher portions of the tank over a period of minutes, time spent in each tank section can be used as a measure of anxiety (Levin, Bencan, & Cerutti, 2007). All exploratory assessment was performed between 9:00 a.m. and 1:00 p.m.

2.3.3. Social interaction

The zebrafish is a social animal. To test social interaction, animals from the same shoal were used in each experiment. Five experimental fish were placed in a small experimental aquarium (30 cm L × 15 cm H × 10 cm W). On one side of the experimental aquarium, an empty tank was placed; on the opposing side, a "stimulus tank" of identical size held 15 zebrafish. The behavior of experimental fish was recorded after 30 s of habituation time. In order to quantify any inherent preference for the "stimulus" side, the central tank was in two equal parts; the time that experimental fish spent in the virtual half adjacent to the conspecific school was measured (Gerlai, 2003).

2.4. Statistical analysis

Inhibitory avoidance memory data are presented as mean ± S.E.M. Training and test latencies for each group were compared by Wilcoxon matched pairs test. Latencies of multiple groups were compared using Kruskal–Wallis and Mann–Whitney U tests. Exploratory assessment and social interaction data were analyzed via one-way analysis of variance (ANOVA) followed by post hoc comparisons using Tukey's HSD test and were expressed as the mean ± S.E.M. For all comparisons, $p < 0.05$ was considered significant.

3. Results

3.1. Caffeine, ZM 241385, DPCPX, dipyridamole, and EHNA prevent scopolamine-induced memory deficits

We first analyzed the influence of pretreatment with adenosine receptor antagonists on scopolamine-induced memory impairment using an inhibitory avoidance task. We independently evaluated the effects of caffeine (10 mg/kg), ZM 241385 (10 μg/kg), and DPCPX (0.5 mg/kg) i.p treatment, followed by pre-training scopolamine (200 μM) exposure (Fig. 1). Saline was used as vehicle for caffeine and 1% DMSO was used as vehicle for both ZM 241385 and DPCPX. Vehicle-exposed animals followed by water treatment demonstrated robust retention of memory during the test session

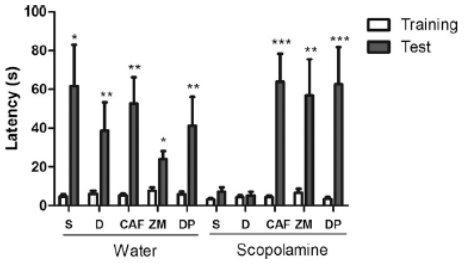

Fig. 1. Pretreatment with adenosine receptor antagonists caffeine (CAF), ZM 241385 (ZM), and DPCPX (DP) prevented scopolamine-induced memory impairment in the inhibitory avoidance task. Animals received a single i.p. injection of saline (S), DMSO (D), caffeine (10 mg/kg), ZM 241385 (10 μg/kg) or DPCPX (0.5 mg/kg) 2 h before the training session. The i.p. treatment was followed by a 1 h exposure to water or scopolamine prior to testing. Effects of scopolamine or water on the latency to enter the dark compartment during training and test sessions in the inhibitory avoidance task were evaluated. Data are presented as mean ± SEM ($n = 12$ per group) $^{*}p < 0.05$ $^{**}p < 0.001$ $^{***}p < 0.0001$ indicate differences between training and test session for each group compared by Wilcoxon matched pair test. No differences were found between training performances among all groups as evaluated by Kruskal–Wallis test.

performed 24 h after training ($p < 0.05$). Pretreatment of caffeine ($p < 0.001$), ZM 241385 ($p < 0.05$) or DPCPX ($p < 0.001$), followed by water treatment, resulted in significant differences between zebrafish training and test sessions, thus suggesting effective learning of the task. However, vehicle-exposed animals subsequently treated with scopolamine did not exhibit memory retention during the test session performed 24 h after training. Interestingly, treatment with either caffeine, ZM 241385 or DPCPX prevented the memory impairment induced by pre-training scopolamine exposure, as observed by the difference in latencies between training and test sessions for each treatment ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$, respectively) (Fig. 1). In addition, there were no significant differences in the latencies for test sessions between the groups pretreated with adenosine receptor antagonists (Caffeine, ZM241385, or DPCPX) followed by water or scopolamine treatment. These results suggest that adenosine A_1 and A_{2A} receptor antagonists ameliorated scopolamine-induced memory impairment.

To elucidate the roles of nucleoside transporters and nucleoside-metabolizing enzymes in scopolamine-induced memory deficit, respectively, we tested an inhibitor of nucleoside transporters, dipyridamole, and an inhibitor of adenosine deaminase, EHNA (Fig. 2). Animals treated with 1% DMSO or dipyridamole (5 mg/kg) followed by water exposure presented robust memory retention, exhibiting significant differences between their training and test sessions ($p < 0.05$ for each group analyzed separately) (Fig. 2A). However, pre-training scopolamine exposure disrupted memory formation in 1% DMSO-treated animals. In contrast, dipyridamole prevented scopolamine-induced impairment, as evident in the significant difference between training and test session latencies ($p < 0.05$) (Fig. 2A). Similarly, pretreatment with EHNA prevented scopolamine-induced memory deficits ($p < 0.001$; Fig. 2B). Taken together, these results suggest that acute treatment of either dipyridamole or EHNA before scopolamine treatment prevents the reliably induced memory impairment that would follow.

3.2. Effects of scopolamine and purinergic modulation on exploratory assessment

Zebrafish exploratory activity was evaluated after i.p. injections of caffeine, ZM 241385, DPCPX, dipyridamole, and EHNA, combined with subsequent water or scopolamine treatment. No significant difference in either distance travelled or mean speed were found in animals that received any of the treatments when

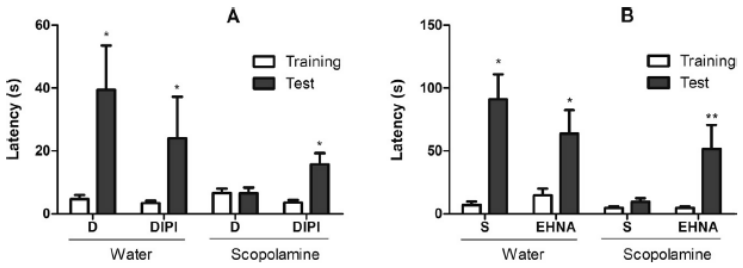

Fig. 2. Pretreatment with dipyridamole (DIPI) or EHNA prevented scopolamine-induced memory impairment during the inhibitory avoidance task. (A) Animals received a single i.p. injection of DMSO (D) or DIPI (5 mg/kg). (B) Animals received a single i.p. injection of saline (S) or EHNA (100 μg/kg). The i.p. treatment, administered 2 h before the training sessions, was followed by a 1 h exposure of either water or scopolamine prior to testing. The effects of scopolamine and water on the latency to enter the dark compartment in training and test sessions in the inhibitory avoidance task were evaluated. Data are presented as mean ± S.E.M ($n = 12$ per group); $^{*}p < 0.05$ $^{**}p < 0.001$ indicate the differences between training and test sessions for each group compared via Wilcoxon matched pair test. No differences were found between training performance among all groups as evaluated by Kruskal–Wallis test.

compared to the control group (saline or 1% DMSO; Fig. 3 and 4). Swimming coordination was also evaluated; none of the treatments altered the absolute body turn angle during swimming (Figs. 3C and 4C). Time spent in the upper and lower portions of the tank was measured to evaluate anxiety; no significant differences were found in treated animals when compared to their respective control group (saline or 1% DMSO; Figs. 3D and E and 4D and E).

3.3. Effects of scopolamine, caffeine, ZM 241385, DPCPX, dipyridamole, and EHNA on social interaction

Social interaction was evaluated after i.p. injections of caffeine, ZM 241385, DPCPX, dipyridamole, and EHNA, followed by water or scopolamine (Fig. 5A and B). There were no significant changes between experimental groups in the time spent near the stimulus tank, indicating that social interaction was not altered by either scopolamine or modulators of adenosine signaling.

4. Discussion

In this study, we evaluated the preventive role of adenosine signaling modulation on scopolamine-induced memory deficits in zebrafish. Previous studies using adult zebrafish showed that scopolamine triggered robust impairment of memory during an inhibitory avoidance task (Kim et al., 2010; Richetti et al., 2011). Our data demonstrated that acute i.p pretreatments with non-selective adenosine receptor antagonist caffeine, and the selective antagonists of A_{2A} (ZM 241385) and A_1 (DPCPX) receptors all prevented the scopolamine-induced memory deficits observed in the inhibitory avoidance task. This study also demonstrates that the inhibition of either nucleoside transporters or nucleoside-metabolizing enzyme adenosine deaminase using dipyridamole and EHNA, respectively, prevents scopolamine-induced memory impairment.

Studies have also demonstrated the prevention of scopolamine-induced cognitive impairment in mice using acute pretreatment with caffeine (10 mg/kg) (Botton et al., 2010). Other studies

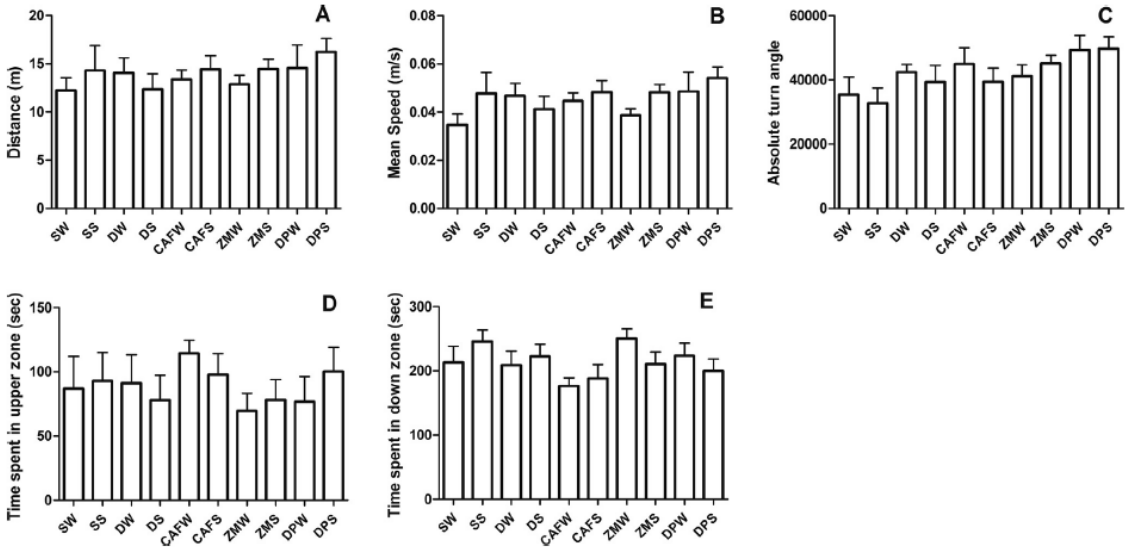

Fig. 3. Locomotor activity was evaluated after an i.p. injection of adenosine receptor antagonist caffeine (CAF), ZM 241385 (ZM) or DPCPX (DP) followed by scopolamine (S) or water (W) exposure. Metrics measured include distance traveled (A), mean speed (B), absolute turn angle (C), time spent in upper zone (D), and time spent in lower zone in (E). No differences were found between groups. Data are expressed as mean ± S.E.M. of 12 different animals for each group and were analyzed by one-way ANOVA test. SW, saline + water; SS, saline + scopolamine; DW, DMSO + water; DS, DMSO + scopolamine; CAFW, caffeine + water; CAFS, caffeine + scopolamine; ZMW, ZM 241385 + water; ZMS, ZM 241385 + scopolamine; DPW, DPCPX + water; DPS, DPCPX + scopolamine.

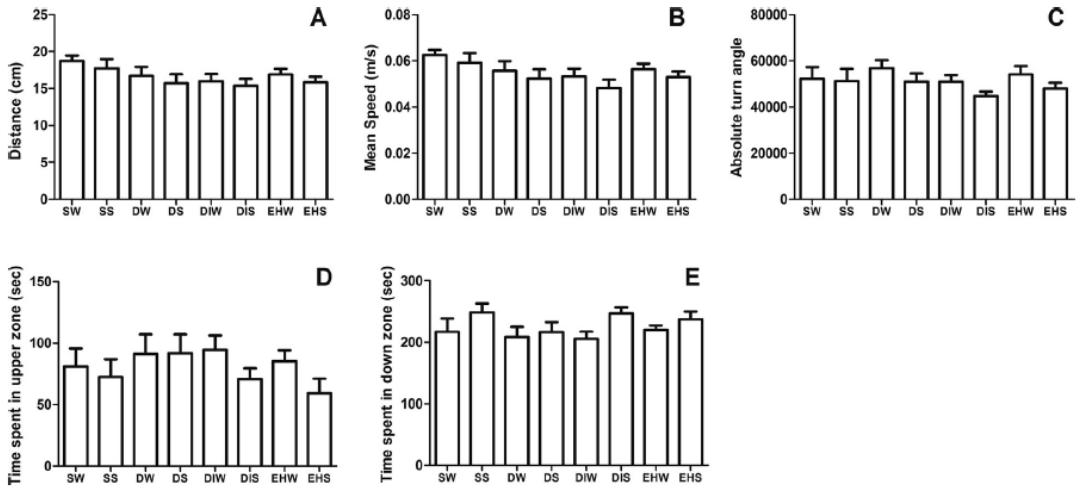

Fig. 4. Locomotor activity was evaluated after an i.p. injection of dipyridamole (DI) or EHNA (EH), followed by scopolamine (S) or water (W) exposure. Analyzed metrics include distance traveled (A), mean speed (B), absolute turn angle (C), time spent in upper zone (D), and time spent in lower zone in (E). No differences were found between groups. Data are expressed as mean ± S.E.M. of 12 different animals for each group and were analyzed by one-way ANOVA test. SW, saline + water; SS, saline + scopolamine; DW, DMSO + water; DS, DMSO + scopolamine; DIW, dipyridamole + water; DIS, dipyridamole + scopolamine; EHW, EHNA + water; EHS, EHNA + scopolamine.

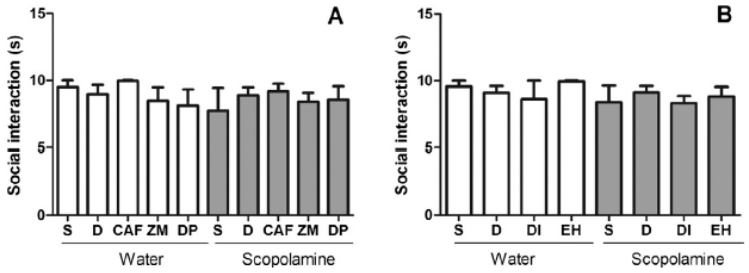

Fig. 5. Social interaction was evaluated after the i.p. injection of adenosine receptor antagonist caffeine (CAF), ZM 241385 (ZM), DPCPX (DP; A) or dipyridamole (DI), EHNA (EH; B), followed by scopolamine or water treatment. Saline (S) or saline plus DMSO (D) was used as a control. No differences were found between groups. Data are expressed as mean ± S.E.M. of 6 different groups and were analyzed via one-way ANOVA.

revealed similar beneficial effects of caffeine in mice with respect to memory deficits induced by a variety of factors such as alcohol (Spinetta et al., 2008) and beta-amyloid (Dall'Igna et al., 2007). Furthermore, chronic intake of caffeine in a transgenic mouse model of AD improved cognition in mice (Arendash et al., 2006) and reduced the deposition of senile plaques (Arendash et al., 2009; Cao et al., 2009). Short and long-term memories formed during an inhibitory avoidance task were improved in middle-aged mice chronically treated with caffeine (Sallaberry et al., 2013). In humans, a placebo-controlled blind crossover study showed that three cups of coffee per day attenuated the scopolamine-induced impairment of several parameters in a word learning task, including free recall from short- and long-term memory, retrieval quality and speed (Riedel et al., 1995). Other studies performed in humans corroborate the potential of caffeine with respect to reducing cognitive decline (Ritchie et al., 2007; van Gelder et al., 2007). Our results are in agreement with previous studies, demonstrating that acute caffeine treatment prevents the memory disruption caused by scopolamine in zebrafish.

The psychostimulant effect of caffeine on the central nervous system (CNS) is primarily due to the antagonism of adenosine receptors A_1 and A_{2A} (Ferré, 2008; Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). We have shown that selective antagonism of adenosine A_{2A} or A_1 receptors by ZM 241385 and DPCPX, respectively, prevented scopolamine-induced memory deficits during an inhibitory avoidance task in zebrafish. Other studies have also described the beneficial effects of A_{2A} antagonism on the synaptic mechanisms of learning and memory in AD animal models and patients (Cunha & Agostinho, 2010; Takahashi, Pamplona, & Prediger, 2008). Rats overexpressing A_{2A} receptors presented memory deficits (Giménez-Llort et al., 2007), while mice lacking adenosine A_{2A} receptors have improved spatial recognition memory (Wang, Ma, & van den Buuse, 2006). Interestingly, the A_{2A} receptor antagonist SCH5861 was unable to prevent the memory deficits induced by scopolamine treatment in rats during a Y-maze task (Cunha et al., 2008). The discrepancies between our work and this result can be attributed to the different memory tasks employed and/or the different methods of scopolamine administration. In addition, we did not observe alterations in locomotion after scopolamine treatment, which differs from the results observed by Cunha et al. (2008). Our results suggest that A_{2A} antagonism can improve memory performance without changes in locomotor activity.

Our results demonstrated that blockade of adenosine A_1 receptor by DPCPX prevents scopolamine-induced memory impairment in zebrafish. Accordingly, previous work demonstrated significant improvement of scopolamine-induced memory impairment using DPCPX in mice (Zhang & Ren, 2003). Another study characterized

the effects of a novel adenosine A_1 antagonist, FR94921, on passive avoidance memory. FR194921 ameliorated scopolamine-induced memory deficits in rats, suggesting this compound harbors a potential for cognitive enhancement (Maemoto et al., 2004). Similar results were observed by Pitsikas and Borsini (1997) using an antagonist of A_1 receptors, BIIP 20, which restored the memory deficits caused by scopolamine in rats (Pitsikas & Borsini, 1997).

To better understand the role of adenosine signaling in memory impairment, we tested the effects of inhibitors of nucleoside transport (dipyridamole) and adenosine deamination (EHNA) on scopolamine-induced memory deficits in zebrafish. Nucleoside transporters were acutely blocked with dipyridamole, resulting in a significant improvement in scopolamine-induced memory impairment. Dipyridamole was previously used in a model of vascular cognitive impairment in rats caused by bilateral carotid artery occlusion (Melani et al., 2010). After one week of intravenous perfusion, it significantly restored spatial memory in the Y-maze test. Dipyridamole is commonly used to inhibit platelet aggregation and reduce thrombi formation *in vivo* (Heptinstall, Fox, Crawford, & Hawkins, 1986). However, few investigations have demonstrated any neuroprotective properties of this compound *in vivo*. *In vitro* studies, however, have revealed a neuroprotective effect of dipyridamole in neuronal cultures, most likely due to its role as an antioxidant (Blake, 2004; Farinelli, Greene, & Friedman, 1998). More recently, studies have suggested that dipyridamole can augment vessel function, restoring blood flow in cerebrovascular disease (Chakrabarti & Freedman, 2008). In blood cells, the antiplatelet mechanism of dipyridamole is due in part to the inhibition of adenosine uptake. The increased extracellular concentration of adenosine results in vasodilation (Geiger, 2001). As adenosine is known for its neuroprotective role in the CNS, it is possible that dipyridamole acts by controlling nucleoside levels and, consequently, inducing an improvement in scopolamine-induced memory deficits in zebrafish.

Adenosine deaminase catalyzes the irreversible deamination of adenosine to inosine (Franco et al., 1997). A previous study demonstrated that scopolamine increased adenosine deaminase activity in rat cerebral cortex and hippocampus synaptosomes. However, adenosine levels measured by HPLC did not change upon scopolamine treatment (Gutierres et al., 2012). Our results demonstrate that acute pretreatment with EHNA, an ADA inhibitor, protected zebrafish from the memory impairment caused by scopolamine. Adenosine has been reported as a neuromodulator, with an important role in synaptic plasticity and memory processing, and its depletion can disrupt memory formation (de Mendonça, Costenla, & Ribeiro, 2002; de Mendonça & Ribeiro, 1997). The data presented here suggests that modulation of adenosine levels via the inhibition of nucleoside transporters or adenosine metabolism can prevent scopolamine-induced effects, reinforcing the proposed involvement of adenosine signaling in cognitive function.

Scopolamine is a classical model of induced amnesia. However, some studies have been questioned the use of scopolamine for inducing cognitive impairment due to differences in data related to locomotor analyses (Klinkenberg & Blokland, 2010). As shown in Figs. 3 and 4, we did not observe changes in locomotor parameters between control and treated fish. In addition, there were no changes in the time spent in the upper or lower portions of the tank, suggesting that scopolamine did not alter anxiety parameters in zebrafish. These data are in agreement with previous studies in both zebrafish and rodents (Botton et al., 2010; Gutierres et al., 2012; Kim et al., 2010; Richetti et al., 2011). However, Cunha and coworkers (2008) observed scopolamine-induced hyperlocomotion that was not prevented by SCH58261 (Cunha et al., 2008).

Zebrafish are a social species and they prefer to swim in groups (Engeszer, Patterson, Rao, & Parichy, 2007; Miller & Gerlai, 2007). Evidence suggests that the cholinergic system participates in social recognition (Wang, Karp, Winblad, & Fratiglioni, 2002; Winslow & Camacho, 1995). Our data revealed no changes in social interaction between the control and treated groups (Fig. 5). In mice, social interaction was not altered by different doses of scopolamine; however, social memory was impaired by scopolamine in a dose-dependent manner (Riedel, Kang, Choi, & Platt, 2009). These results suggest that scopolamine promotes memory disruption without changes in social interaction.

5. Conclusion

In conclusion, our results support the idea that the antagonism of adenosine receptors or a possible increase of adenosine levels induced by inhibition of nucleoside transport or adenosine catabolism prevented scopolamine-induced memory deficits. This data corroborates the hypothesis that adenosine signaling is involved in memory processing in zebrafish and may be a target for the development of preventive strategies against cognitive impairment.

Acknowledgments

This work was supported by DECIT/SCTIE-MS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. 10/0036-5 — PRONEX) and CNPq (Proc. 473275/2011-2). G.P.C, C.D.B and M.R.V. were recipients of fellowships from CNPq. J.W.B was the recipient of a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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CAPÍTULO II

Artigo científico

Long-Term Exposure to Paraquat Alters Behavioral Parameters and Dopamine Levels in Adult Zebrafish (Danio Rerio)

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Artigo publicado no periódico
Zebrafish 2014; 11(2): 142-153
(doi: 10.1089/zeb.2013.0923)

ZEBRAFISH
Volume 11, Number 2, 2014

DOI: 10.1089/zeb.2013.0923

Long-Term Exposure to Paraquat Alters Behavioral Parameters and Dopamine Levels in Adult Zebrafish (*Danio Rerio*)

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Abstract

Chronic exposure to paraquat (Pq), a toxic herbicide, can result in Parkinsonian symptoms. This study evaluated the effect of the systemic administration of Pq on locomotion, learning and memory, social interaction, tyrosine hydroxylase (TH) expression, dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) levels, and dopamine transporter (DAT) gene expression in zebrafish. Adult zebrafish received an i.p. injection of either 10 mg/kg (Pq10) or 20 mg/kg (Pq20) of Pq every 3 days for a total of six injections. Locomotion and distance traveled decreased at 24 h after each injection in both treatment doses. In addition, both Pq10- and Pq20-treated animals exhibited differential effects on the absolute turn angle. Nonmotor behaviors were also evaluated, and no changes were observed in anxiety-related behaviors or social interactions in Pq-treated zebrafish. However, Pq-treated animals demonstrated impaired acquisition and consolidation of spatial memory in the Y-maze task. Interestingly, dopamine levels increased while DOPAC levels decreased in the zebrafish brain after both treatments. However, DAT expression decreased in the Pq10-treated group, and there was no change in the Pq20-treated group. The amount of TH protein showed no significant difference in the treated group. Our study establishes a new model to study Parkinson-associated symptoms in zebrafish that have been chronically treated with Pq.

Introduction

The dopaminergic system mediates several important brain functions, such as motor activity, cognition, emotion, motivation, and reward.[1,2] The degeneration of dopaminergic neurons in the substantia nigra results in the depletion of dopamine (DA) in the striatum, which is associated with a commonly occurring movement disorder called Parkinson disease (PD).[3,4] PD is characterized by motor abnormalities such as resting tremor, rigidity, bradykinesia, and postural instability.[5,6] Nonmotor symptoms, including olfactory deficits, sleep impairment, anxiety, and depression, and at later stages, impaired cognition and dementia are also observed.[7,8]

The majority of PD cases are sporadic, which suggests a role for environmental factors or more complex gene-environmental interactions.[9] Neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), have been widely used in animal models to mimic specific pathogenic events and behavioral features that are observed in PD.[10,11] In addition, pesticides such as paraquat (Pq) or rotenone have been described as potential environmental Parkinsonian toxicants both in humans and in experimental animals.[12,13]

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Some of these neurotoxins have also been used in adult zebrafish. The teleost zebrafish (*Danio rerio)* has become popular for behavioral studies[14–16] and appears to be a good animal model to study movement disorders such as PD,[17] because the basic brain anatomy and neurotransmitter systems in these fish are similar to those found in mammalian brains.[18–20] DA neurons are detected early in zebrafish development in the posterior tuberculum of the ventral diencephalon[21] and project to the prosencephalon, which is anatomically similar to the mammalian striatum. This pathway is comparable to the nigrostriatal network in humans.[19,20] Tyrosine hydroxylase (TH), an important enzyme involved in DA synthesis, also exists in zebrafish, which have two nonallelic TH genes (*th1* and *th2*) due to gene duplication in teleost fish.[22] This enzyme catalyzes the conversion of tyrosine to L-DOPA, which is a precursor for the catecholamines DA, epinephrine, and norepinephrine.[23] Therefore, TH is commonly used as a marker for catecholinergic neurons.[24,25] Zebrafish express the DA receptor subtypes D1-like, D2-like, D3-like, and D4-like. This expression pattern resembles that of mammals, with the exception of the D5-like receptor, which is absent in zebrafish.[26–28] Furthermore, PD-related genes such as *J-1*, *UCH-L1*, *SNCA*, *Pink1, PARK2*, and *LRR2* are evolutionarily conserved in both zebrafish and humans.[17,29,30]

In zebrafish, acute exposure (i.e., intramuscular injection) to either MPTP or 6-OHDA has been shown to diminish DA and noradrenaline levels in the brain without changing TH levels. One day after being exposed to these drugs, the fish exhibit a decrease in both swimming velocity and total distance traveled, and these behaviors remain impaired for 9 days.[31] A previous study has demonstrated that adult zebrafish given a single intraperitoneal injection of MPTP exhibit reduced locomotion but experience no change in the number of TH-positive neurons. In contrast, exposure to rotenone or Pq via immersion (i.e., pesticides diluted in tank water) has not been shown to alter either locomotion or the number of DA neurons in zebrafish.[32] In rodents, the systemic administration of Pq has been shown to affect dopamine transporter (DAT) and DA metabolism in nigrostriatal neurons.[33] The subchronic administration of Pq has also been shown to influence DA metabolism and cause oxidative stress and cell death in rats.[34] Previous studies have also demonstrated that the continuous administration of Pq can result in behavioral changes and modification of DA levels. Pq-treated rats display anxiety-like phenotypes,[35,36] the progressive loss of olfactory discrimination,[36] diminished locomotion,[35,37] and motor deficits in the pole test,[35] all of which mimic PD symptoms. However, little is known about the effects of systemically administering Pq in zebrafish.

The idea that Pq contributes to dopaminergic neurodegeneration is based on its structural similarity to 1-methyl-4 phenylpyridinium (MPP^+), which is the active metabolite of MPTP. Previous studies have demonstrated that Pq in its monovalent state can be absorbed by neurons through the DAT and can induce oxidative stress and cytotoxicity.[38] The role of Pq as a environmental neurotoxin that is associated with an increased risk for neurodegenerative diseases after chronic exposure has been investigated[39] by Costello *et al.*, who used geographic information from the Central Valley of California to demonstrate that exposure to Pq alone or in combination with other pesticides increases the risk for PD.[40] In addition, cumulative exposure to Pq has been shown to significantly augment the risk of developing PD.[41–43] Therefore, this study aimed at evaluating a possible neurotoxic effect of systemically administering Pq on the locomotion profile, learning and memory, social interaction, TH expression, DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels, and DAT gene expression in zebrafish.

Materials and Methods

Animals

Adult male wild-type zebrafish (0.4–0.6 g) were obtained from a commercial supplier (Redfish) and were housed at a controlled temperature (26°C ± 2°C) in a 30 L aquarium at a density of three animals per liter. The fish were allowed to acclimate to the laboratory environment for at least 14 days. They were subjected to a day:night cycle of 14:10 h and were fed thrice per day with commercial flake food that was supplemented with live brine shrimp. All protocols were approved by the Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (11/00257-CEUA- PUCRS) and adhered to all relevant guidelines on the care and use of fish in research, teaching, and testing as specified in Brazilian legislation, the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC).

Treatment

After the acclimation period, the fish were divided into three experimental groups (Pq10, Pq20 and control) and received a total of six i.p. injections (one injection every 3 days; Fig. 1). Before each injection, the fish were anesthetized with 0.1 g/L tricaine solution (ethyl 3-aminobenzoate methanesulfonate salt; Fluka). Two different doses of Pq (10 and 20 mg/kg; Pq dichloride x-hydrate, PESTANAL®) were used, while the control group instead received the same volume of saline.[44] Each injection consisted of a total volume of 10 μL.

Behavioral analyses

Locomotion. Twenty-four hours after each injection (days 1, 4, 7, 10, 13, and 16), the locomotion of each animal was measured between 9:00 and 13:00. Animals were placed individually in the experimental tanks (30 cm long × 15 cm high × 10 cm wide) and habituated for 30 s, after which their behavior was recorded on video for 10 min.[45] The videos were analyzed using ANY-maze software (Stoelting Co.), which virtually divided the tanks into equal sections with three vertical lines and one horizontal line. The behavioral patterns measured in these videos were the number of sectional line crossings, distance traveled, mean speed, and time spent in each tank section (i.e., the bottom vs. upper levels). Since zebrafish have a natural tendency to spend more time at the bottom of a novel tank before gradually exploring higher portions of the tank over a period of minutes,[46] the time spent in each tank section could be used as a measure of anxiety. In addition, we measured the absolute turn angle, which represents a sum of the movement vectors from one position relative to the animal's center point and the next both clockwise and anti-clockwise. The absolute value of this angle is summed for all the positions of the animal throughout the test. To

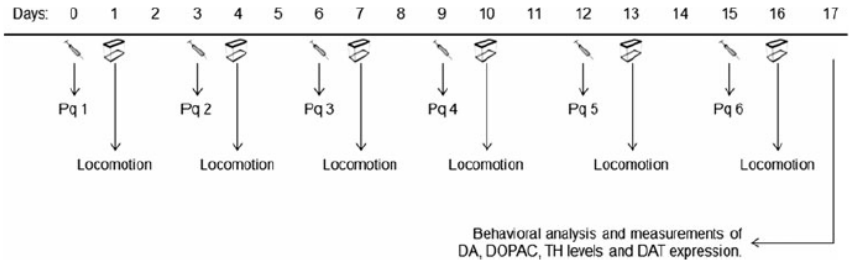

FIG. 1. Timeline of the experimental procedure. Zebrafish received i.p. injections of either saline (control group; Ctrl) or Pq at a dose of 10 mg/kg (Pq10) or 20 mg/kg (Pq20). The injections were administered during every 3 days for a total of six injections over the course of 16 days. On day 17, DA, DOPAC, and TH levels and DAT gene expression were determined. DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; Pq, paraquat; TH, tyrosine hydroxylase.

measure this parameter, each fish was placed in a 20-cm diameter round tank for 10 min, and the data were collected as described earlier.[31]

Social interaction. Zebrafish are a social species, and they prefer to swim in groups.[47,48] To measure their social interaction, we used a protocol previously described by Saverino and Gerlai[49] with some modifications. The apparatus consisted of three tanks (designated as left, central, and right), each with the same dimensions (30 cm long × 15 cm high × 10 cm wide). First, one Pq-treated fish was individually placed into the central tank, and its actions were recorded on video over the course of 8 min (''habituation period''). After the habituation period, 15 female zebrafish of identical size were positioned in the left tank, while the right tank remained empty. Then, a video was recorded for another 8 min (''stimulus period''). The central tank was divided in two equal parts, and the amount of time that the experimental fish spent near the conspecific school was measured during the stimulus period to quantify their preference for the stimulus tank versus the empty tank.

Y-maze task. The spatial memory of both Pq-treated fish and controls was evaluated using the Y-maze task.[50] For this task, the control, Pq10, and Pq20 animals ($n = 15$) were individually trained and tested in a Y-Maze glass aquarium with three arms (25 cm long, 8 cm wide, and 15 cm high), which were designated as the ''start'' arm, the ''other'' arm (always open), and the ''novel'' arm (open only during the test trial). During the training session (5 min), the fish were individually placed in the start arm, and the novel arm was closed. After 1 h, the test session (5 min) began, and the fish was again placed in the start arm, but this time, the novel arm was open. The time spent in each arm was determined, and locomotion parameters were measured (distance traveled and mean speed). Both the training and test sessions were recorded and analyzed further using the ANY-Maze software (Stoelting Co.).

Western blot analysis of TH levels

Dissected brain tissue from euthanized fish ($n = 5$ per group in sixplicate) was placed in a cooled protease inhibitor solution (Complete Mini; Roche Applied Science) and stored at −80°C for subsequent analysis as previously described[31] with a few modifications. The protein extract was prepared in RIPA buffer (Sigma-Aldrich). Total protein (25 μg) was separated on a 12% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Next, the membrane was blocked with 5% albumin (Sigma-Aldrich) in TBS containing 0.05% Tween-20 and incubated overnight with rabbit polyclonal antibody against ß-actin (ab34731, 1:2000; Abcam) or mouse monoclonal anti-TH antibody (MAB318, 1:1000; Millipore) that served as primary antibodies. Goat anti-mouse (G-21040, 1:2000; Molecular Probes) and goat anti-rabbit (ab97069, 1:2000; Abcam) horseradish peroxidase-conjugated secondary antibodies were used to detect the primary antibodies, and the resulting signal was measured with the Western Lighting-Enhanced Chemiluminescence detection kit (NEL 104001EA; Perkin Elmer). Prestained molecular-weight protein markers (Benchmark marker; Invitrogen) were used to determine the molecular weight of each detected band and to confirm antibody target specificity. Densitometry quantification of each replicated gel ($n = 6$) was performed using Carestream software (Carestream Health). Total protein levls were normalized for each sample relative to ß-actin protein levels.

Determination of DA and DOPAC levels by liquid chromatography-tandem mass spectrometry

The presence of DA and its metabolite DOPAC was analyzed according to a previously described method[51] with some modifications. Five brains were pooled for each of the four replicates. The samples were homogenized in 500 μL of formic acid (0.1 M) and centrifuged at 20,000 *g* for 20 min at 4°C. Next, the supernatant was filtered (0.22 μm filter) and injected into the UHPLC 1290/MS 6460 TQQQ—Agilent (all HPLC components and the MassHunter software were obtained from Agilent Technologies®). Chromatographic separations were performed using a Zorbax Eclipse Plus C18 2.1 × 50 mm 1.8 μm column. The flow rate of methanol (eluent A): 0.05% formic acid with 1 mM of heptafluorobutyric acid (HFBA) (eluent B) in the mobile phase was 0.2 mL/min with a column temperature of 30°C. A gradient of flow was used, which started at 95% of eluent B for 0.5 min and subsequently decreased to 0% over 3.5 min. A total volume of 5 μL was injected into the UHPLC system for each sample. The monitored transitions were as follows: DA (154 > 137

and 154 > 91) and DOPAC (169 > 123 and 169 > 77). The results were expressed as ng of analyte per mg of total protein from brain homogenates.

Gene expression analysis by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Gene expression analysis was carried out only when kinetic alteration occurred. Total RNA was isolated with Trizol® reagent (Invitrogen) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry (A260/A280), and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA, containing a final concentration of 0.2 × SYBR Green I (Invitrogen), 100 μM dNTP, 1 × PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen), and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were as follows: an initial polymerase activation step for 5 min at 95°C, 40 cycles of 15 s at 95°C for denaturation, 35 s at 60°C for annealing, and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included; fluorescence was measured from 60°C to 99°C and showed in all cases one single peak. *Rpl13α* and *EF1α* were used as an endogenous control. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 2012.3 Software (http://LinRegPCR.nl), and the stability of the references genes, *EF1α* and *Rlp13α* (*M-value*) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Differences in locomotor parameters, for a comparison between doses (Pq10 and Pq20) and day after treatment (days 1, 4, 7, 10, 13, and 16), were evaluated by two-way analysis of variance (ANOVA) followed by posthoc comparisons using Bonferroni correction and were expressed as the mean ± S.E.M. Social interaction, spatial memory and DAT gene expression were analyzed by one-way ANOVA followed by *post-hoc* comparisons using Tukey's HSD test and were expressed as the mean ± S.E.M. Western blot results and DA and DOPAC levels were evaluated by one-way ANOVA followed by a *post-hoc* Tukey's HSD test and were expressed as the mean ± S.D. For all comparisons, the significance level was set at $p < 0.05$.

Results

Locomotor activity was measured at 24 h after each i.p. injection of Pq10 or Pq20. Both dosages resulted in marked alterations in zebrafish swimming behavior relative to controls (Fig. 2). Specific parameters of fish swimming activity were measured in the tank test. The total distance traveled was measured 24 h after each Pq20 injection and decreased at all timepoints when compared with the control group ($F_{(2,23)} = 1.621$; $p < 0.05$). In contrast, Pq10-treated fish displayed a difference in the distance traveled only at day 10, 13, and 16 ($F_{(2,23)} = 1.621$; $p < 0.05$; Fig. 2A). Another locomotor parameter analyzed was the absolute turn angle, which calculates the overall movement relative to a center point and also takes into account the direction of the movement. According to Blazina *et al.*,[54] the absolute turn angle is a sensitive measure of motor coordination, as it is affected by drug treatments at doses that failed to alter locomotion. Zebrafish exposed to Pq showed significant changes in the absolute turn angle. Pq10-treated fish increased the turn angle only at day 10 when compared with the control group ($F_{(2,23)} = 7.273$; $p < 0.001$), while Pq20-treated fish demonstrated a decrease in turn angle after all treatments ($F_{(2,23)} = 7.273$; $p < 0.001$) with the exception of day 10 and 16, where no significant differences were observed in comparison to the control group (Fig. 2B). The number of line crossings of Pq10-treated fish decreased at day 10, 13, and 16 ($F_{(2,23)} = 1.974$; $p < 0.05$); while no changes were found at day 1, 4, or 7. The Pq20-treated fish demonstrated a 26% decrease in the number of line crossings at 24 h after the first Pq20 injection. This pattern remained altered at each subsequent timepoint ($F_{(2,23)} = 1.974$; $p < 0.05$) (data not shown). The mean velocity of Pq10-treated fish decreased only at day 10, 13, and 16 ($F_{(2,23)} = 1.372$; $p < 0.05$); this parameter was not significantly different from that of the control group at day 1, 4, and 7. In contrast, Pq20-treated fish demonstrated a decrease in mean velocity at all time points when compared with the control group ($F_{(2,23)} = 1.372$; $p < 0.05$) (data not shown). Taken together, these results suggest a dose-dependent effect on the initiation and maintenance of motor symptoms in zebrafish chronically treated with Pq.

Nonmotor behaviors that are commonly present in PD were also analyzed, such as anxiety, cognition, and social interaction. The same tank used to measure swimming activity was also used to observe the index of anxiety, which was determined by the time spent in the upper portion of the tank at 24 h after each injection.[46] As illustrated in Figure 3,

TABLE 1. PRIMER SEQUENCES FOR REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION EXPERIMENTS

Gene	*Forward primer*	*Reverse primer*
EF1α[a]	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3′
Rpl13α[a]	5′-TCTGGAGGACTGTAAGAGGTATGC-3′	5′-AGACGCACAATCTTGAGAGCAG-3′
dat[b]	5′-AGACATCTGGGAAGGTGGTG-3′	5′-ACCTGAGCATCATACAGGCG-3′

[a]According to Tang *et al.*[52]
[b]According to Barreto-Valer *et al.*[53]

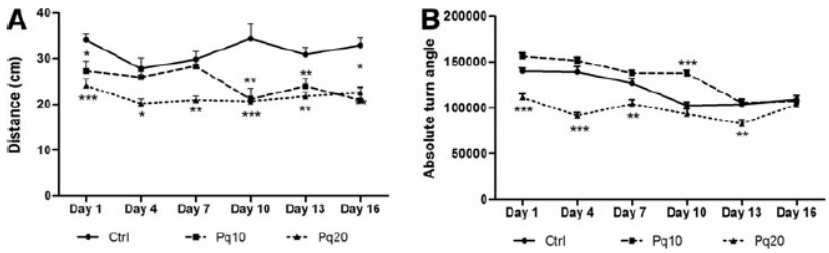

FIG. 2. Locomotion profile of control and Pq-treated zebrafish. Twenty four hours after each injection, total distance traveled **(A)** and absolute turn angle **(B)** were evaluated. The data are expressed as the mean ± S.E.M. from 24 animals for each group and were analyzed by two-way ANOVA followed by Bonferroni *post-hoc* test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ relative to the control group. Ctrl, saline injection; Pq10, 10 mg/kg Pq; Pq20, 20 mg/kg Pq; ANOVA, analysis of variance.

there were no significant differences in the time spent in the upper portion of the test tank among the Pq10- or Pq 20-treated groups at any timepoint when compared with the control group. These results indicate that the repeated i.p. injection of Pq did not induce anxiety in the fish. Spatial memory was also evaluated in Pq-treated fish by subjecting them to the Y-Maze task after all six injections had been given (day 17). In this test, the time spent in each arm (novel, start, and other) was measured 1 h after the training session, and the response to novelty (time spent in novel arm) was determined. Pq10 (32.6% ± 4.2%) and Pq20-treated (26.8% ± 4.3%) fish spent less time in the novel arm compared with the control group (52% ± 5%; $F_{(2,14)}=4.63$; $p<0.01$). There were no significant differences in the time spent in each arm for the treated groups (Fig. 4). These data demonstrate that Pq treatment results in impaired acquisition and consolidation of spatial memory. However, the Pq-treated fish did not demonstrate any locomotion deficits in this task, such as a change in distance traveled and mean velocity, when compared with the control group (data not shown). Finally, there were no significant differences in social interaction between zebrafish treated with Pq 10 mg/kg (331.5 ± 26.7 s) or Pq 20 mg/kg (389.9 ± 17.3 s) and the control group (335.5 ± 21.3 s; $p=0.357$; $F_{(2,19)}=2,38$; Fig. 5). These data suggest that repeated Pq treatment does not disrupt the preference of zebrafish to live in groups.

We also analyzed the levels of DA and DOPAC in zebrafish brains after the sixth i.p. injection of 10 or 20 mg/kg Pq. Changes in both DA and DOPAC levels were observed in the treated groups. Compared with controls, DA levels increased 35.5% ± 5.9% in Pq10-treated fish ($p<0.001$; $F_{(2,3)}=34.09$) and 45.5% ± 5.5% in Pq20-treated fish ($p<0.001$; $F_{(2,3)}=34.09$). In contrast, DOPAC levels decreased in the brains of both Pq10-treated fish (25.3% ± 3.5%; $p<0.004$; $F_{(2,3)}=11.53$) and Pq20-treated fish (17% ± 4.6% $F_{(2,3)}=11.53$; $p<0.004$) (Fig. 6). TH protein levels in Pq-treated zebrafish brain were also measured by western blotting. No changes in the level of total TH protein were observed in Pq10 (1.03 ± 0.24) or Pq 20-treated fish (0.87 ± 0.20) when compared with the control group (0.85 ± 0.24; $p=0.83$; $F_{(2,5)}=0.1781$) on day 17 (Fig. 7). We performed a qRT-PCR analysis in order to evaluate the influence of DAT on DA metabolism after Pq repetitive exposure in zebrafish brain. The Pq10-treated group showed a decrease (1.18 ± 0.1; $F_{(2,6)}=4.79$ $p<0,03$) in DAT gene expression compared with the control group (1.6 ± 0.1), while the Pq20-treated group (1.3 ± 0.1) did not present significant changes in gene expression compared with the control group (Fig. 8).

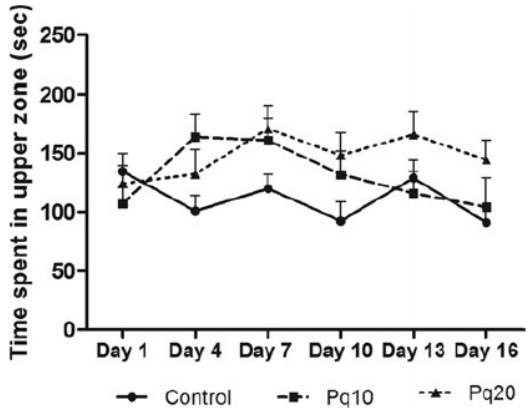

FIG. 3. Time spent in the upper portion of the tank after Pq treatment or control injection ($n=24$ for each group). The data are expressed as the mean ± S.E.M. and were analyzed by a two-way ANOVA followed by a Bonferroni *post-hoc* test.

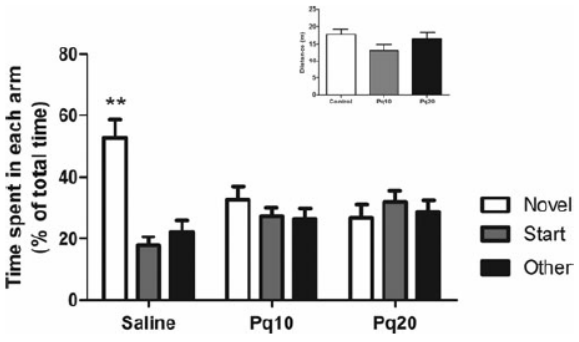

FIG. 4. The Y-maze response to novelty in treated and control zebrafish. The *inset* shows the distance traveled during the test session. The data are expressed as the mean ± S.E.M. ($n=14$) and were analyzed by a one-way ANOVA followed by Tukey's *post-hoc* test. **$p<0.01$.

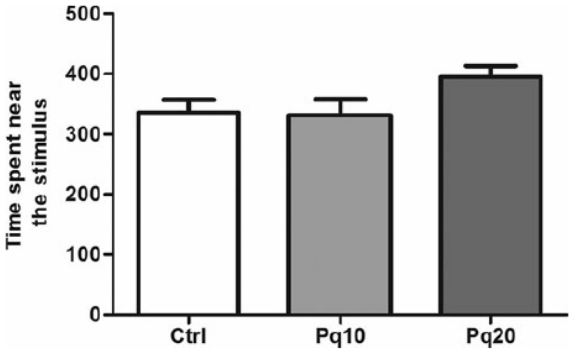

FIG. 5. Time spent near the stimulus screen as a measurement of social interaction. No changes in the time spent near the stimulus were observed in either the control or treated groups ($n=20$ for each group). The data are expressed as the mean ± S.E.M. and were analyzed by a one-way ANOVA followed by Tukey's *post-hoc* test.

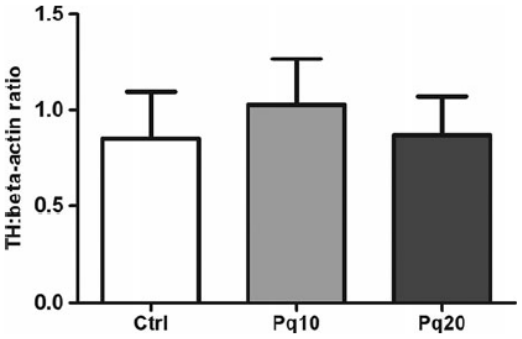

FIG. 7. Western blotting of zebrafish brain homogenates probed with anti-TH antibodies. The results are expressed as the mean ± S.D. ($n=6$) of TH levels relative to beta actin levels.

Discussion

Several studies have demonstrated that prolonged exposure to pesticides is an environmental risk factor for the development of PD. One such pesticide, Pq, has been associated with an increased risk of PD in multiple studies involving humans.[55–57] Indeed, previous studies have shown that dopaminergic neurons are susceptible to Pq[58–60] and that reduced motor activity can result from exposure to this chemical.[35,37,58] Costello *et al.*[40] demonstrated that the exposure to multiple chemicals may potentiate the effect of each chemical as well as the time of exposure is important. In this study, we utilized zebrafish, a vertebrate organism that is commonly used to study both behavioral and neurodegenerative disorders.[61–63] Several studies have already developed different experimental animal models of parkinsonism, with distinct neurochemical and behavioral alterations (for a review, see Table 2) This study is the first which demonstrates that zebrafish exposed to repeated i.p. injections of the pesticide Pq exhibit a decrease in general locomotor activity. One interesting observation is the dose dependence of this phenomenon. While treatment with 10 mg/kg Pq caused a reduction in locomotion starting from the fourth injection (day 10), treatment with 20 mg/kg Pq diminished locomotion from the first injection (day 1) and continued to act until the end of the treatment period (i.e., the sixth i.p. injection on day 16). These results conflict with those of Bretaud *et al.*,[32]

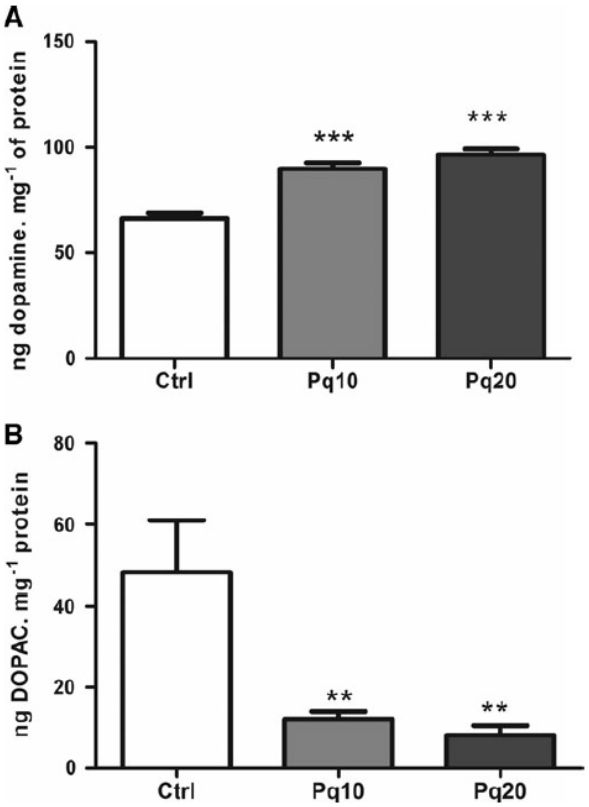

FIG. 6. The influence of Pq on DA levels (**A;** $n=4$) and its metabolite DOPAC (**B;** $n=4$). The results (mean ± S.D.) are shown in ng/mg of protein and were analyzed by a one-way ANOVA followed by Tukey's *post-hoc* test. $^{**}p<0.01$; $^{***}p<0.001$.

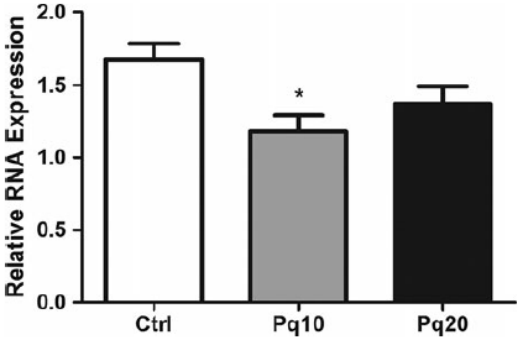

FIG. 8. Effects of Pq repeated treatment in DAT gene expression. Data are expressed as mean ± S.E.M of six independent experiments performed in triplicate. Results were analyzed by a one-way ANOVA followed by Tukey's *post-hoc* test. $^{*}p<0.05$.

where no alteration in the locomotor activity of adult zebrafish was observed after exposure to Pq (5 mg/L) in the water tank over the course of 4 weeks. Similar results were observed in 7 days post-fertilization (dpf) zebrafish larvae, which were exposed to Pq (5 and 10 mg/L) that was also administered directly in their water tank.[32] Therefore, the distinct routes of Pq exposure can explain the differences observed in our results when compared with the literature, which make the Pq-repeated exposure via i.p. more suitable for inducing parkinsonism in this species.

After administering only one injection of the two neurotoxins known to induce DA deficiency, MPTP, or 6-OHDA, Anichtchik *et al.* observed a similar pattern of diminished locomotion in adult zebrafish, which suggests that the route of exposure can be crucial in causing behavioral effects.[31] Intraperitoneal injections of 75 mg/kg MPP^+, a metabolite of MPTP, did not induce deficits in locomotion in adult zebrafish, whereas MPTP decreased locomotion.[32] However, larval zebrafish exposure to MPP^+ from days 2 to 6 dpf was associated with reduced motor activity and DA neurons[63] and diminished spontaneous propulsive movements.[79] In addition, both systemic and chronic administration of Pq (i.p.) in rodents have been shown to cause a neurobehavioral syndrome that resembles the effects observed in our study. One previous study indicated that 10 doses of Pq (7 mg/kg) can impair swimming behavior when compared with the controls.[80] Another study has demonstrated that using Pq in two different doses (5 or 10 mg/kg) administered in three i.p. injections per week for a total of 22 days results in a pronounced decrease in motor activity compared with the controls. The same decrease in locomotion has also been induced by two injections of MPTP at a dose of either 10 or 30 mg/kg.[58] In addition, mice that received 24 i.p. injections of 10 mg/kg Pq twice a week have been shown to exhibit lower levels of horizontal activity after all doses except for the first one.[44]

We also measured the absolute turn angle, which reveals changes in zebrafish swimming direction. The Pq10 group demonstrated an increased turn angle only at day 10, while the Pq20 group displayed a decreased turn angle at all time points except for days 10 and 16. After administering one injection of either MPTP or 6-OHDA, Anichtchik *et al.* observed an increase in the turn angle 9 days later.[31] Our data have shown a dose dependency for this parameter, because the Pq10 group exhibited increased turns only at one timepoint, whereas the Pq20 group showed a more significant decrease in the turn angles at multiple timepoints. A decrease in turn angle has also been observed in zebrafish that were exposed to endosulfan, an organochlorine pesticide used as insecticide and an acaricide.[81] This evidence reinforces the idea that the swimming activity is a sensitive parameter which is used to evaluate the action of toxic agents that are able to alter the neurotransmitter system, such as dopaminergic and serotonergic signaling[67,82,83] Taken together, our findings demonstrate that Pq exposure can disrupt locomotor activity and motor posture as verified by lower mean speed, distance traveled, line crossings, and change in turn angle of Pq-treated fish when compared with the control group.

In addition to motor alterations resulting from the degeneration of dopaminergic neurons, nonmotor symptoms have also been described in PD. Multiple neuronal systems seem to be involved in PD pathophysiology, which leads to a spectrum of symptoms, including olfactory deficits, affective disorders, memory impairment, and digestive dysfunction.[84] Therefore, we also evaluated nonmotor behaviors in zebrafish exposed to Pq, such as anxiety, cognition, and social interaction. In this study, we evaluated spatial memory using the Y-maze task as previously described by Cognato *et al.*[50] Our results demonstrate for the first time that repeated injections of Pq may impair the acquisition and consolidation of spatial memory in zebrafish. Previous studies have demonstrated that the oral administration of Pq in mice results in an increase in the time required for treated animals to reach the platform in a water maze when compared with the controls. In a probe test, Pq treatment has been shown to significantly decrease the time required to pass the site where the platform had been originally located. Taken together, these data suggest that Pq may influence learning and memory in mice.[85] Impaired learning and memory were also observed in rats after dopaminergic neurons had been destroyed by MPTP treatment.[86] Therefore, our results are in agreement with the literature, which suggests that neurotoxins and pesticides might induce general memory impairment.

In addition, our study found no effect of Pq treatment on the social preference of zebrafish. It has been well established that zebrafish prefer to swim in groups. This behavioral strategy is considered as guarding against predators in several fish species.[45,47] The possible involvement of the dopaminergic system in social preference has previously been analyzed by Scerbina *et al.*[87] Using a D1 receptor antagonist, the authors observed a reduction in the social preference in the AB strain of zebrafish but saw no change in the SF strain. It is interesting to note that these two strains exhibit a decrease in locomotion resulting from the D1 antagonist.[87] Recently, it has been shown that acute social interactions elicit rapid and differential changes in serotonergic and dopaminergic activity across different brain regions in zebrafish.[88] In addition, studies have demonstrated that the maturation of shoaling behavior is followed by changes in serotonergic and dopaminergic systems.[89,90] In our study, wild-type zebrafish treated continuously with Pq showed no alteration in social preference despite a change in DA and DOPAC levels.

When introduced to a novel environment, zebrafish naturally remain at the bottom of the tank and then gradually explore the upper portions.[46] In our study, zebrafish treated with Pq10 and Pq20 showed no changes in the amount of time spent in the upper zone of the test tank, which suggests a nonanxiolytic effect of Pq treatment. This result conflicts with previous studies conducted in rats where four injections of Pq (10 mg/kg) resulted in anxiety-like behavior in the elevated-plus maze test from the first injection onward.[91] In addition, rats treated for 3 weeks with Pq (10 mg/kg) spent less time in the center of an open field, which indicates anxiogenic behavior.[35] Previous studies addressing anxiety-like behavior after MPTP or 6-OHDA treatment in rats have produced conflicting results.[92] Vuckovic *et al.* found that four i.p. injections of MPTP did not result in anxious behavior in the light-dark preference test at 7 or 30 days post-treatment.[93] Gorton *et al.*, using the same regimen of MPTP treatment, reported an anxiety-like response in the marble-burring test but no anxiety-like response in the elevated plus maze test.[94] Branchi *et al.*, using bilateral injections of 6-OHDA, found a significant reduction in anxiety-like behavior in the elevated plus maze test but no difference in the time spent next to the walls of an open field.[95]

TABLE 2. ANIMAL MODELS OF EXPERIMENTAL PARKINSONISM

Agent	*Model*	*Behavioral alterations*	*Neurochemical alterations*	*Ref.*
MPTP	Acute mouse model		Loss of DA neurons and decreased DA content	64
	Subacute mouse model	Decrease in locomotor activity and exhibited akinesia Motor impairment	Decreased DA and DOPAC levels Neuronal degeneration Loss of dopaminergic neurons, induction of apoptosis, and enhanced oxidative stress	65,66
	Acute adult zebrafish model	Motor impairment Rotational behavior	Decreased DA content and no alterations in DA neurons and TH levels	31
	Zebrafish larvae	Motility disorder	Decline of TH 5-HT neurons affected.	67
6-OHDA	Unilateral injection into MFB (medial foreinbrain bundle) model—rodents		Degeneration of neurons in the nigrostriatal system. Loss of DA content, Decreased TH levels	68,69
	Unilateral injection into striatal model—rodents	Rotational behavior	Loss of DA neurons and decreased DA content.	70
	Acute zebrafish Model	Motor impairment Rotational behavior	Decreased DA content and no alterations in DA neurons and TH levels	31
	Zebrafish larvae		Decreased TH levels	71
Rotenone	Systemic administration—rodents	Catalepsy and reduced motor activity Hypokinesia and rigidity	Decreased TH levels and DA content Cytoplasmic inclusions reminiscent of Lewy bodies	72,73
	Chronic zebrafish model-tank water	No motor effects in larval of adult zebrafish	No changes in TH levels	32
	Chronic Drosophila model	Locomotor deficits	DA neuron degeneration	74
Paraquat	Systemic administration—mice model	Divergent effects on horizontal locomotor behavior	Decreased DA and TH levels Loss of DA neurons Increased α-synuclein immunoreactivity	59,60,75
	Acute expose—rat model	Locomotor deficits	Decreased DA in striatal content	76
	Long exposure—rat model	Locomotor deficits	Decreased TH Changes in DA levels.	77
	Chronic zebrafish model-tank water	No motor effects in larval or adult zebrafish	No changes in TH levels	32
	Acute Drosophila model	Parkinsonian syndrome: resting tremor, bradykinesis, rotational behavior	Reduced number of DA neurons Changes in neuronal morphology	78

5-HT, serotonin; 6-OHDA, 6-hydroxydopamine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH, tyrosine hydroxylase.

Our results also demonstrate that repeated i.p. injections of Pq affect the levels of both DA and its metabolite DOPAC in the zebrafish brain. DA levels increased after six injections of Pq at both doses tested, while DOPAC levels in the zebrafish brain decreased after Pq treatment. This result suggests that Pq treatment reduces DA metabolism in the zebrafish brain. These findings support those of Kuter *et al.*, where a similar profile of DA metabolism was observed in rats treated with 10 mg/kg Pq. In that study, a decrease in the DOPAC/DA ratio was detected at 3 days after the treatment ended.[96] Another study in which Pq was administered by microdialysis also suggested that extracellular levels of DA in the striatum increase in a dose-dependent manner.[34] Songin *et al.* administered 10 mg/kg of Pq to rats by an i.p. injection once a week for 37 weeks and found a decrease (13%) in the DOPAC/DA ratio in the striatum.[97] Shepherd *et al.* administered six Pq injections and observed no differences in DA levels when compared with the controls; however, DOPAC levels decreased when compared with the controls.[37] Taken together, these studies suggest that Pq might alter DA release and/or inhibit DA reuptake and that a potential compensatory mechanism may occur after Pq administration to restore impaired dopaminergic transmission. Moreover, a recent study found no difference in DA or DOPAC levels after one, two, or three i.p. administrations of Pq (10 or 15 mg/kg) given 1 week apart.[98] The same study used [C^{14}]-labeled Pq to

demonstrate that this pesticide remained in the rat brain at 7 days after the last dose was received; striatal levels of DA and DOPAC were also calculated at this timepoint using HPLC. The exposure to Pq either twice or thrice per week did not significantly change these levels. The results revealed only a modest increase in DA levels at 7 days after the 12th and 18th doses of Pq compared with controls and a significant decrease in DA levels after the 24th dose compared with all other groups.[98]

Using western blot analysis, we also measured TH levels in the brains of Pq-treated zebrafish. Interestingly, no significant changes in TH expression were observed in zebrafish treated with Pq in comparison to the control group. These results are consistent with those of Anichtchik *et al.*, where zebrafish were given one injection of MPTP or 6-OHDA.[31] In addition, a recent study in rats did not find any consistent stereological evidence of DAB-labeled TH neuronal loss after i.p. treatment with 10 and 15 mg/kg Pq.[98] However, Brooks *et al.* have shown that three doses of Pq can diminish TH levels in the striatum in mice.[58] Reeves *et al.* measured TH levels in striatal sections of rat brains treated chronically with Pq by both HPLC and immunohistochemistry and observed a decrease in TH in both tests.[44] Another study has also demonstrated a decrease in TH immunoreactivity in the midbrain of rats treated with Pq.[99] A similar reduction in TH levels was observed in TH-positive neurons in the substantia nigra in rats that were treated with 10 mg/kg Pq.[80] The conflicting results regarding TH levels after Pq treatment could result from either the different animal models used in these studies or the methodology used to measure TH protein levels.

In this study, we also investigated whether repeated Pq exposure alters DAT gene expression by performing quantitative RT-PCR assays. Our results presented a significant decrease in DAT mRNA levels of Pq10-treated fish, when compared with the control. However, Pq20-treated fish did not show significant changes in mRNA DAT transcript levels. Previous studies have demonstrated that mice treated orally with Pq (10 mg/kg) during 4 months diminished mRNA and protein expression of DAT in substantia nigra.[100] Another study demonstrated that rats exposed to Pq (10 mg/Kg) via i.p. during 2 and 24 h or 7 days after the injection showed a decrease in DAT content, suggesting that even an acute systemic Pq administration affects striatal DAT and DA metabolism.[33] Furthermore, a decrease in DAT content was also seen in the MPTP mouse model of PD.[101,102] Since DAT is responsible for the reuptake of DA into presynaptic neurons and plays a central role in the spatiotemporal buffering of released DA and its recycling,[103] the decreased DAT expression observed in our study might be involved with the increase of DA levels found in zebrafish that are repeatedly exposed to Pq.

In conclusion, our findings indicate that repeated i.p. injections of Pq in zebrafish produce a significantly decreased locomotion profile, dose-dependent alterations in the turn angle, and an impairment of spatial memory with no anxiety-like behavior or alterations in social interaction. Therefore, we have identified a novel animal model to study PD that involves exposing zebrafish to chronic treatment with the Parkinsonian agent Pq.

Acknowledgments

This work was supported by DECIT/SCTIE-MS through the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. 10/0036-5, conv. n. 700545/2008–PRONEX). J.W.B., L.N.R., and L.W.K. received fellowships from CAPES. G.P.C., R.R.C., M.R.B., M.R.V., and C.D.B. received fellowships from CNPq.

Disclosure Statement

No competing financial interests exist.

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4. DISCUSSÃO

As doenças neurodegenerativas são definidas como desordens resultantes da perda progressiva de função ou de estrutura neuronal no sistema nervoso central (Serrano-Pozo et al., 2011). Dentre estas patologias, a DA se caracteriza pelo depósito de agregados proteicos e da perda progressiva de neurônios colinérgicos, levando ao aparecimento de problemas comportamentais como dano cognitivo e demência (Selkoe et al., 2012). Para o tratamento da DA, inibidores da acetilcolinesterase são utilizados até os dias atuais na prática clínica. Esses inibidores aumentam a função colinérgica, tratando apenas os sintomas da DA (Casey, 2010).

A adenosina desempenha um papel importante como neuromodulador, controlando a excitabilidade neuronal e, consequentemente, modulando condições fisiológicas e patológicas no SNC (Boison, 2012; Chen et al., 2013a; Dias et al., 2013). Em ratos e humanos, foi demonstrado o envolvimento dos receptores de adenosina no processo cognitivo (Gomes et al., 2011; Sebastiao et al., 2012). Além disso, estudos mostraram um aumento da expressão dos receptores de adenosina A_1 e A_{2A} em pacientes com DA (Albasanz et al., 2008), e estudos epidemiológicos demonstraram que a incidência de DA é associada inversamente com o consumo de cafeína, antagonista não seletivo de receptores de adenosina (Maia e De Mendonça, 2002; Ritchie et al., 2007).

A escopolamina, um antagonista colinérgico muscarínico, conhecido por causar prejuízo de aprendizado e de memória em animais experimentais (KlinKenberg e Blokland, 2010), vem sendo utilizada no peixe-zebra para comprometer a cognição e perturbar a formação de memória (Cognato et al., 2012; Kim et al., 2010; Richetti et al., 2011). Nosso estudo, apresentado no Capítulo 1 desta tese, utilizou o modelo farmacológico de déficit cognitivo causado por escopolamina em peixe-zebra adulto para avaliar os efeitos neuroprotetores da administração aguda de antagonistas dos receptores de adenosina, como cafeína, ZM 241385 (antagonista seletivo do receptor de adenosina A_{2A}) e DPCPX (antagonista seletivo do receptor de adenosina A_1) na tarefa de esquiva inibitória. Nossos resultados mostraram que o pré-tratamento, pela via i.p., com os antagonistas cafeína, ZM 241385 e DPCPX preveniram o déficit cognitivo causado pela escopolamina, enquanto os animais controles (receberam salina ou DMSO no pré-tratamento) apresentaram déficit cognitivo.

Um estudo, utilizando o modelo de escopolamina em camundongos, mostrou que o pré-tratamento com cafeína na dose de 10 mg/Kg, também preveniu o déficit cognitivo

causado por este fármaco (Botton et al., 2010). Em humanos, o consumo de três xícaras de café por dia atenuou o déficit causado pela escopolamina em vários parâmetros, incluindo evocação livre de memória de curto e longo prazo e a sua qualidade e velocidade de recuperação em tarefa de aprendizado de palavras (Riedel et al., 1995). Outros estudos realizados com humanos e modelos animais de déficit de memória têm mostrado o potencial da cafeína para reduzir o declínio cognitivo (Dall´Igna et al., 2007; Ritchie et al., 2007; Spinetta et al., 2008; van Gelder et al., 2007).

Além da cafeína, antagonistas seletivos dos receptores de adenosina também têm apresentado um papel neuroprotetor. Estudos têm descrito efeitos benéficos dos antagonistas de receptores A_{2A} sobre os mecanismos de aprendizado e de memória em modelos animais (Cunha e Agostinho, 2010; Takahashi et al., 2008). Interessantemente, o antagonismo do receptor A_{2A} pelo SCH5861 não preveniu o déficit de memória induzido por escopolamina em ratos, usando-se o aparato de labirinto em Y (Cunha et al., 2008b), o que se revelou diferente dos nossos resultados após o pré-tratamento com ZM 241385 no aparato de esquiva inibitória. Diferenças entre nosso estudo e o estudo realizado por Cunha e colaboradores (2008) relacionadas com o método de administração da escopolamina, tipo de memória avaliada e, principalmente, diferenças no padrão de locomoção (discutido a seguir) podem justificar os distintos efeitos observados em cada estudo.

Além do receptor A_{2A}, o receptor A_1 também está associado à melhora na cognição e na memória. Estudos com roedores mostraram que os antagonistas do receptor A1 DPCPX, o BIP20 e o FR194921 melhoraram o déficit cognitivo causado pela escopolamina (Pitsikas e Borsini, 1997; Zhang e Ren, 2003). Portanto, nossos resultados estão de acordo com esses achados prévios, demonstrando que o antagonismo seletivo e o não seletivo dos receptores de adenosina A_1 ou A_2A podem prevenir o déficit cognitivo causado pela escopolamina.

O inibidor do transportador de adenosina (dipiridamol) e o inibidor da enzima ADA (EHNA), responsável por degradar adenosina em inosina (Zavialov e Engström, 2005), também foram testados neste estudo. Tanto o transportador de adenosina quanto a ADA estão envolvidos no controle dos níveis de adenosina no encéfalo (Bonan, 2012; Parkinson et al., 2011). Nossos dados revelaram que o pré-tratamento com dipiridamol ou com EHNA preveniu o déficit cognitivo causado pela escopolamina em peixe-zebra. O dipiridamol já foi utilizado anteriormente em um modelo de disfunção cognitiva vascular em ratos. Após uma semana de tratamento intravenoso, houve significativa melhora da memória espacial no aparato de labirinto em Y (Melani et al., 2010). Estudos *in vitro* revelaram o efeito neuroprotetor do dipiridamol em culturas de neurônios, principalmente devido ao seu efeito

antioxidante (Blake, 2004; Farinelli et al., 1998). Além dos efeitos antioxidantes, Chakrabarti e Freedman (2008) sugeriram que o dipiridamol pode aumentar a função dos vasos e reparar o fluxo sanguíneo em doenças cerebrovasculares (Chakrabarti e Freedman, 2008). Em relação à ADA, um estudo demonstrou que a escopolamina aumenta a atividade dessa enzima em sinaptossomas de córtex cerebral e no hipocampo de ratos. Porém, os níveis de adenosina medidos por HPLC não apresentaram diferença com o tratamento (Gutierres et al., 2012). Analisando esses resultados em conjunto, associados à função de cada inibidor supracitado, pode-se sugerir que o dipiridamol ou o EHNA preveniram o déficit cognitivo causado pela escopolamina devido a modular positivamente os níveis de adenosina, reforçando o papel neuroprotetor deste nucleosídeo, aliado ao seu envolvimento na plasticidade sináptica e no processamento da memória (de Mendonça e Ribeiro, 1997; de Mendonça et al., 2002).

Nossos resultados mostraram também que a escopolamina e os inibidores dos receptores de adenosina, do transportador de adenosina ou de ADA não influenciam o comportamento locomotor, a ansiedade ou o comportamento social do peixe-zebra. É importante salientar que a literatura questiona o modelo da escopolamina em função de alguns dados que mostram mudanças em padrões de locomoção em animais experimentais (Klinkenberg e Blokland, 2010). Entretanto, estudos realizados em roedores e no peixe-zebra corroboram nossos achados mostrando que a escopolamina não altera o comportamento locomotor e a ansiedade (Botton et al., 2010; Gutierres et al., 2012; Kim et al., 2010; Richetti et al., 2011). Além disso, um estudo realizado com camundongos expostos a diferentes doses de escopolamina não observou mudanças no comportamento social desses animais (Riedel et al., 2009), o que está de acordo com nossos achados.

Portanto, nossos resultados sugerem que a modulação da adenosina, seja pelo antagonismo de seus receptores ou seja pela inibição do seu metabolismo, pode melhorar a função cognitiva e a memória. Além disso, propõem que a modulação do sistema adenosinérgico pode ser um alvo para o desenvolvimento de estratégias preventivas contra doenças que causam perda de memória como a DA.

A sinalização adenosinérgica também exerce um efeito modulatório sobre a DP (Jenner et al., 2009; Morelli et al., 2009; Zhu et al., 2014). A DP é caracterizada pela perda de neurônios dopaminérgicos associada a disfunções progressivas do sistema nervoso (Morelli et al., 2012; Peterson et al., 2012; Stone et al., 2009). Clinicamente, a DP é caracterizada pela presença de mudanças motoras como rigidez, tremor de descanso, bradicinesia e instabilidade postural (Djaldetti et al., 2006), além de manifestações não motoras como comprometimento

cognitivo e demência, distúrbios do sono, disfunções olfatórias, constipação, depressão, entre outros (Chaudhuri e Schapira, 2009).

Na DP, a adenosina, por seu papel neuromodulador, regula respostas da dopamina e outros neurotransmissores em áreas do cérebro que são responsáveis pela função motora, pelo aprendizado e pela memória (Latini e Pedata, 2001). Além disso, os receptores de adenosina (A_1 e A_{2A}) se colocalizam com receptores de dopamina no encéfalo (Ferré, 1997; Ferré et al., 2001). Estudos têm mostrado também que a modulação dos receptores A_1 e A_{2A} pode contribuir para a diminuição da neurodegeneração relacionada à DA (Moore et al., 2003; Rebola et al., 2003; Xiao et al., 2011).

Fatores ambientais podem provocar uma cascata de degeneração de neurônios, principalmente os neurônios dopaminérgicos (Bové e Perier, 2012; Freire e Koifman, 2012). Compostos químicos, especialmente, pesticidas têm sido propostos como fatores de risco para o desenvolvimento da DP (McCormack et al., 2002; Freire e Koifman, 2012). O paraquat (Pq), um pesticida classificado como herbicida, apresenta efeitos tóxicos em vários órgãos como pulmão, fígado e rins após exposição acidental (Dinis-Oliveira et al., 2008; Yen et al., 2010). Estudos mostraram que o Pq atravessa a barreira hematoencefálica e é transportado pelos transportadores de dopamina, após conversão para sua forma monovalente Pq^{+1} (Rappold et al., 2011). O tratamento repetido com o Pq tem sido utilizado como modelo de DP (Chen et al, 2010; Reeves et al, 2003).

No Capítulo 2 desta tese, apresentamos a caracterização do modelo de neurotoxicidade induzido por Pq em peixe-zebra. Este estudo teve como objetivo avaliar os efeitos da administração repetida de Pq sobre os seguintes parâmetros comportamentais: locomoção, ansiedade, memória e interação social. Além de avaliar os níveis de dopamina e seu metabólito Ácido 3,4-diidroxifenilacético (DOPAC), os níveis de Tirosina Hidroxilase (TH), enzima envolvida na síntese de dopamina (Molinoff, 1971) e a expressão do transportador de dopamina em encéfalo de peixe-zebra também foram anlisados.

Duas doses de Pq foram avaliadas, a dose de 10 mg/kg (Pq10) e a dose de 20 mg/Kg (Pq20), e o grupo controle recebeu salina pela via i.p. O tratamento consistiu em seis injeções, realizadas em um intervalo de dois dias, com experimentos de locomoção avaliados 24 horas após cada injeção (ver Fig 1 do Capítulo 2). Os parâmetros locomotores avaliados foram distância percorrida, número de linhas cruzadas, velocidade média e ângulo de giro. Os resultados mostraram uma diferença no comportamento locomotor entre as doses de Pq testadas e o grupo controle. Todos os parâmetros locomotores diminuíram após 24 horas de

cada injeção na dose de Pq20. Já a dose de Pq10 apresentou diminuição do comportamento locomotor após 24 horas da administração da quarta injeção.

Estudos com roedores expostos ao Pq de forma aguda ou repetida também observaram diminuição na atividade locomotora (Brooks et al., 1999; Chen et al., 2008; Litteljohn et al., 2009; Reeves et al., 2003; Shepherd et al., 2006), corroborando nossos resultados. No entanto, Bretaud e colaboradores (2004) não encontraram diferenças na locomoção em peixes-zebra adultos e larvas expostos ao Pq diluído na água do tanque. As diferentes vias de exposição ao Pq podem explicar as diferenças observadas em nossos resultados, mostrando que a exposição repetida ao Pq pode induzir aos efeitos hipolocomotores encontrados.

No peixe-zebra, a medida do ângulo de giro está relacionada à coordenação motora (Blazina et al., 2013). Nossos resultados apresentaram comportamentos distintos no ângulo de giro nas duas doses de Pq testadas. Na dose Pq10, houve um aumento de giro apenas após a quarta injeção, comparado ao grupo controle, enquanto que os peixes tratados com Pq20 demonstraram um decréscimo no ângulo de giro após as injeções i.p, com exceção da quarta e da sexta administração de Pq em que não foram observadas diferenças significativas em comparação ao grupo controle. Um estudo realizado em peixe-zebra adulto após administração i.p de MPTP ou 6-OHDA, neurotoxinas utilizadas em modelos animais experimentais de DP, observou um aumento neste parâmetro locomotor após nove dias da administração (Anichtchik et al., 2004). Todavia, outro estudo expondo o peixe-zebra ao endossulfan, um herbicida organoclorado, diminuiu o ângulo de giro nos peixes (Pereira et al., 2012). Nossos dados apresentaram resultados distintos por dose, porém a maior dose, Pq20, parece influenciar negativamente o ângulo de giro nos animais corroborando os dados de Pereira e colaboradores (2012).

Além das alterações motoras, nosso estudo também avaliou alterações não motoras como memória espacial, ansiedade e interação social. Nossos resultados apresentaram que o Pq, nas duas doses, pode prejudicar a memória espacial em peixe-zebra. Em concordância com nossos achados, foi observado que camundongos que receberam Pq pela via oral apresentaram mudança no tempo de chegada e de troca de local no labirinto de Morris (*Water maze*), indicando que o Pq influencia na memória e no aprendizado (Chen et al., 2010). O comprometimento da memória e de aprendizado também foi observado em ratos tratados com a neurotoxina MPTP (Moriguchi et al., 2012).

Apesar do envolvimento do sistema dopaminérgico na preferência social do peixe-zebra (Mahabir et al., 2013; Teles et al., 2013), nossos resultados não apresentaram diferença estatística significativa na interação social nos peixes tratados com Pq em relação aos

controles. Também não encontramos diferença no tempo decorrido nas zonas inferior ou superior do aquário nos peixes tratados com Pq, indicando que o Pq não influenciou na ansiedade do peixe-zebra. Interessantemente, dados da literatura com ratos tratados com Pq mostraram um aumento no comportamento de ansiedade (Litteljohn et al., 2009; Prediger et al., 2012), sendo que efeitos similares também foram observados em ratos tratados com MPTP ou 6-OHDA (Branchi et al., 2008; Gorton et al., 2010; Kuter et al., 2007; Prediger et al., 2012).

Para uma melhor caracterização do modelo descrito no capítulo 2, os níveis de dopamina e DOPAC foram avaliados após as seis injeções de Pq. Interessantemente, nossos resultados apresentaram um aumento nos níveis de dopamina e uma diminuição nos níveis de DOPAC após o tratamento com Pq nas duas doses. Esse resultado caracteriza uma possível redução no metabolismo da dopamina no encéfalo de peixe-zebra. Kuter e colaboradores (2007) e Songin e colegas (2011) obtiveram o mesmo perfil na concentração de dopamina e DOPAC em ratos tratados com Pq. Além disso, outro estudo, administrando Pq por microdiálise também observou um aumento nos níveis extracelulares de dopamina no estriado (Breckenridgea et al., 2013). Esse aumento nos níveis de dopamina e a diminuição de DOPAC podem sugerir que o Pq influencia a liberação e/ou recaptação de dopamina, ativando um mecanismo compensatório visando restaurar a transmissão sináptica após o tratamento com Pq.

Os níveis de TH e do transportador de dopamina também foram avaliados neste estudo a fim de melhor entender o metabolismo da dopamina após o tratamento com Pq,. O tratamento com Pq não alterou os níveis de TH no encéfalo de peixe-zebra comparado ao grupo controle. Dados da literatura com diferentes tratamentos utilizando o Pq em roedores mostraram uma diminuição nos níveis de TH (Brooks et al., 1999; Mori et al., 2005; Reeves et al., 2003; Zhang et al., 2013), contrariando o resultado encontrado neste estudo. Uma pesquisa realizada com peixe-zebra utilizando as neurotoxinas MPTP e 6-OHDA também não observou mudanças na expressão de TH (Anichtchik et al., 2004), mostrando que há diferenças na expressão de TH em diferentes modelos animais experimentais. Por outro lado, nossos resultados mostraram uma diminuição na expressão do transportador de dopamina na dose de Pq10 comparado com o controle, o que não foi observado na dose de Pq20. Estudos prévios em roedores mostraram uma diminuição nos níveis do transportador de dopamina (Ossowka et al., 2005; Uhl, 2003). Possivelmente, a diminuição na expressão do transportador de dopamina pode aumentar os níveis de dopamina, uma vez que o transportador é responsável por regular a liberação e a reciclagem de dopamina em neurônios (Uhl, 2003).

Em conclusão, no Capítulo 2, nossos achados indicam que injeções consecutivas de Pq em peixe-zebra produzem uma redução no perfil locomotor e na memória espacial, além de alterar o metabolismo da dopamina e a expressão do transportador de dopamina em encéfalos de peixe-zebra; caracterizando-se como um novo modelo para estudo da DP.

Modelos animais gerados a partir de toxinas, como o descrito no capítulo 2, são bastante utilizados para estudar a fisiopatologia da DP e na triagem de compostos com potencial para neuroproteção ou tratamento dos sintomas da DP (Blandini e Armentero, 2012; Bové e Perier, 2012). Atualmente, a estratégia farmacoterapêutica para DP visa restabelecer os níveis de dopamina com a L-Dopa (Olanow et al., 2004; Stayte & Vissel, 2014), porém diversos estudos têm mostrado que o uso prolongado desta estratégia farmacoterápica causa complicações, incluindo discinesias e flutuações motoras (Hametner et al., 2010; Stayte e Vissel, 2014). Com isso, terapias não dopaminérgicas, como o antagonismo dos receptores adenosinérgicos, em especial o A_{2A}, resultaram em possíveis alvos terapêuticos para DP (Jenner et al, 2009; Hauser et al, 2011; Stay e Vissel, 2014).

Os receptores da adenosina são classificados como receptores purinérgicos do tipo P1. Essa família de receptores também conta com os do tipo P2, divididos em P2Y e P2X. Esses receptores, P2, estão envolvidos no controle da libertação de dopamina e dano a neurônios do estriado (Franke e Illes, 2006). Estudos têm indicado que a inibição de um subtipo de receptor de P2, do receptor P2X7, pode prevenir em parte a depleção de adenosina em um modelo de rato de DP (Carmo et al., 2014; Marcellino et al., 2010). Em cultura de neurônios, outro subtipo do receptor P2, o P2X1 foi associado ao mecanismo de agregação proteíca da DP, e os experimentos também sugerem que o ATP tem um papel na agregação da sinucleína (Gan et al., 2014). Além disso, um estudo, realizado em roedores expostos a 6-OHDA, mostrou um aumento na hidrólise de ADP e AMP em fatias de estriado (Oses et al., 2011).

No decorrer desta tese também foi avaliada a influência da exposição repetida do Pq e da exposição aguda a 6-OHDA sobre a atividade das ectonucleotidases, sobre o metabolismo extracelular do ATP, bem como os níveis de mRNA destas enzimas em encéfalos de peixe-zebra adulto. Estes resultados adicionais foram compilados em um manuscrito que será submetido para publicação.

Nossos resultados após o tratamento com Pq, nas doses de 10 mg/kg (Pq10) e 20 mg/kg (Pq20), não apresentaram diferença significativa na hidrólise das ectonucleotidases e no metabolismo extracelular do ATP, ADP e AMP. O tratamento com 6-OHDA, nas doses de 25 mg/kg e 50 mg/kg, também não apresentou diferença significativa na hidrólise das ectonucleotidases e no metabolismo extracelular do ATP. Porém, os níveis de ADP

extracelular apresentaram diferença nas duas doses, com diminuição dos níveis na menor dose e um aumento dos níveis na dose de 50 mg/kg. O AMP extracelular apresentou uma diminuição nas duas doses de 6-OHDA utilizadas.

Os resultados gerados pelo tratamento com a 6-OHDA corroboram parcialmente os encontrados por Oses e colaboradores (2011), quando foi verificado que roedores expostos a 6-OHDA apresentaram um aumento na hidrólise de ADP e AMP, com consequente diminuição nos níveis destes nucleotídeos no estriado. Em peixe-zebra, não houve mudanças nas atividades das ectonucleotidases. Porém, houve uma diminuição nos níveis de AMP, enquanto os níveis de ADP apresentaram um aumento na dose de 50 mg/kg após 180 minutos de análise.

Alterações nos níveis de ADP e AMP poderiam ocorrer devido a mudanças transcricionais na expressão das enzimas NTPDAse e ecto-5´-nucleotidase Nossos dados de PCR quantitativo mostraram que não houve diferença na expressão das ectonucleotidades em encéfalos de peixe-zebra após o tratamento com a 6-OHDA. O mesmo resultado foi descrito em ratos por Oses e colegas (2011). Estes dados em conjunto sugerem que o modelo da 6-OHDA não induz uma modulação transcricional das ectonucleotidases.

Neste estudo, os níveis de adenosina extracelular também foram avaliados em encéfalos de peixe-zebra após tratamento com Pq ou 6-OHDA. Nossos dados demonstram que há uma diminuição dos níveis de adenosina extracelular após o tratamento com as duas doses de Pq, enquanto o tratamento com 6-OHDA, nas duas doses, apresentou um aumento de adenosina extracelular. Estudos prévios utilizando o modelo da 6-OHDA apresentaram tanto uma redução (Pinna et al., 2002) quanto um aumento (Wojcik e Neff, 1983) dos níveis de adenosina no estriado. Além disso, estudos realizados com outra neurotoxina, o MPTP, demonstraram um aumento dos níveis de adenosina (Ballarin et al.,1987; Nomoto et al., 2000). Apesar destes resultados controversos, é importante salientar que a adenosina atua no encéfalo em receptores distintos, A_1 e A_{2A}, e estes receptores interagem de forma antagônica com receptores do sistema dopaminérgico, D_1 e D_2, respectivamente (Ferré et al., 1991; Ferré et al., 1997; Ferré et al., 2001). Estudos mostraram que a interação A_1-D_1 participa da ativação motora enquanto a interação A_{2A}-D_2 participa da inibição motora (Agnati et al., 2003; Ferré et al., 1997; Fuxe et al., 1998;). Então, considerando que, em baixos níveis, a adenosina ativa receptores A_1 e quantidades altas de adenosina ativam os receptores A_{2A} (Cunha et al, 1996; Pinna et al. 2002), nós sugerimos que os baixos níveis de adenosina encontrados após o tratamento com o Pq podem estar ativando o receptor A_1, e com isso inibindo o receptor D_1, enquanto o contrário ocorre com os animais que receberam 6-OHDA,

ou seja, o aumento de adenosina está associado à ativação do receptor A_{2A} e à consequente inibição do receptor D_2. Esse cenário pode estar contribuindo para os efeitos hipolocomotores destas neurotoxinas no peixe-zebra. Esta sugestão também corrobora estudos que mostram um papel neuroprotetor da cafeína em modelos animais tratados com Pq ou 6-OHDA (Acuña-Lizama et al., 2013; Kachroo et al., 2010; Machado-Filho et al., 2014; Yadav et al., 2012) e ratifica as pesquisas com inibidores duplos do receptor A_1 e A_{2A} para o tratamento do Parkinson (Shook et al., 2010; Shook et al., 2012).

Nosso estudo também avaliou a expressão das isoformas de ADA: ADA1, ADA 2.1, ADA 2.2, ADAasi e ADAL no encéfalo de peixe-zebra tratados com Pq ou 6-OHDA, mostrando uma diminuição nos níveis de RNAm na isoforma ADA 2.1 nos peixes tratados com Pq comparados ao controle. Já os animais tratados com 6-OHDA apresentaram um aumento na expressão da isoforma ADAasi. Esses dados são contrários à observação de que houve um aumento nos níveis de adenosina, conforme descrito acima. Um mecanismo que poderia explicar as diferenças encontradas na regulação enzimática e de expressão transcripcional é conhecido como feedback negativo autoregulatório, que permite que genes que não são fatores de transcrição possam regular a sua própria síntese (Krishna et al., 2006).

Por fim, nosso estudo analisou o produto da degradação da adenosina, a inosina, considerada hoje um potencial alvo farmacológico para a DP. Estudos demonstraram que a inosina ministrada pela via oral aumenta os níveis de urato na circulação (Spitsin et al., 2001; Yamamoto et al., 2002). O urato, um produto final do metabolismo das purinas, confere neuroproteção contra o estresse oxidativo em modelos de DP (Chen et al., 2013b; Cipriani et al., 2012; Gong et al., 2012) e previne a perda de neurônios dopaminérgicos em ratos tratados com 6-OHDA (Wang et al., 2010). Em humanos, níveis elevados de urato no soro estão associados a uma diminuição da progressão da DP (Ascherio et al., 2009; Schwarzschild et al., 2008). Nosso estudo encontrou um aumento dos níveis de inosina extracelular nos dois tratamentos, Pq e 6-OHDA, em ambas as doses. Estes dados confirmam os achados de Nomoto e colaboradores (2000) que mostraram um aumento de inosina no estriado de animais tratados com MPTP. É importante salientar que o tratamento com 6-OHDA em peixe-zebra apresentou um aumento de adenosina e do seu produto inosina, em menor escala. Esse resultado discrepante pode ser explicado pelo aumento da expressão da ADAasi, isoforma de ADA, ou ainda pela da hiperatividade da E-5´-NT apresentada, resultando no aumento da produção de adenosina, culminando com o aumento dos níveis de inosina em menor escala.

Estes dados sugerem que o Pq e a 6-OHDA modulam diferentemente o sistema purinérgico. Contudo, os dois tratamentos mostraram um aumento nos níveis de inosina, um

candidato para o tratamento da DP (Parkinson Study Group SURE-PD Investigation, 2014), reforçando o potencial deste sistema como um alvo farmacológico para a DP.

De forma conjunta, os resultados apresentados nos dois capítulos desta tese, somados aos resultados adicionais ainda não publicados, podem contribuir para um melhor entendimento da sinalização purinérgica sobre as duas patologias neurodegenerativas mais prevalentes, DA e DP, além de apresentar um modelo de neurodegeneração induzido por exposição consecutiva de Pq em peixe-zebra adulto. Nossos dados também mostraram uma modulação distinta do sistema purinérgico após tratamento com 6-OHDA ou paraquat, reforçando o potencial farmacológico deste sistema sobre as neurodegenerações estudas.

6. PERSPECTIVAS

Os resultados gerados nesta tese apresentaram o envolvimento do sistema purinérgico no modelo de déficit cognitivo induzido por escopolamina e degeneração neuronal induzida por Pq e 6-OHDA. Estes resultados apontam o sistema purinérgico como um potencial alvo farmacológico para o tratamento de doenças neurodegenerativas. Tendo em vista as recentes publicações e nossos achados propomos as seguintes perspectivas nos modelos apresentados nesta tese:

- Avaliar o potencial neuroprotetor da inosina nos modelos de déficit cognitivo induzido por escopolamina e degeneração neuronal induzida por Pq e 6-OHDA;

- Avaliar os efeitos de antagonistas dos receptores de adenosina nos modelos de degeneração neuronal induzida por Pq e 6-OHDA, utilizando parâmetros comportamentais;

- Avaliar o possível efeito neuroprotetor da inibição do transportador de adenosina e da ADA nos modelos de neurodegeneração neuronal induzida por Pq e 6-OHDA, utilizando parâmetros comportamentais;

- Avaliar o efeito da exposição do Pq em larvas e sua influência em parametos comportamentais na vida adulta do peixe-zebra

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