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A interação de guanosina com os receptores A1 e A2A de adenosina e sua implicação em modelos da doença de Parkinson

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Tese submetida ao Programa de Pós-graduação em Bioquímica da Universidade Federal de Santa Catarina para a obtenção do título de doutor em Bioquímica.

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em bioquimica.

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RESUMO

A doença de Parkinson (PD) é a segunda doença neurodegenerativa de maior prevalência mundial, caracterizada pela perda progressiva dos neurônios dopaminérgicos na parte compacta da substância negra. Os principais sintomas da PD são: tremor, bradicinesia, instabilidade postural e rigidez muscular. Apesar dos tratamentos atuais serem efetivos para esses sintomas motores, os mesmos apresentam efeitos colaterais severos e não impedem a progressão da doença. Diante disso, se faz necessária a busca por novas estratégias terapêuticas. A guanosina, um nucleosídeo endógeno com ação neuromodulatória, já demonstrou eficácia como um agente antiparkinsoniano em modelos animais de indução de sintomas motores da PD, além de apresentar efeitos neuroprotetores em protocolos *in vitro*. No entanto, ainda não existe um receptor clonado e sequenciado específico para esse nucleosídeo e seus mecanismos de ação ainda não estão totalmente compreendidos. Sendo assim, o objetivo desse trabalho foi avaliar o efeito antiparkinsoniano da guanosina e a sua relação com os receptores A_1 e A_{2A} de adenosina (A_1R e $A_{2A}R$). Em um protocolo *in vivo* com a administração de reserpina em camundongos, o aumento de tremores orofaciais foi revertido pela guanosina e dependente da ativação do A_1R e independente da expressão do $A_{2A}R$. O bloqueio farmacológico do A_1R também impediu que a guanosina diminuísse os níveis de espécies reativas de oxigênio (EROs) no estriado desses animais. Em um protocolo *in vitro* que mimetiza eventos celulares iniciais da PD com a toxina 6-hidróxi-dopamina (6-OHDA) em fatias estriatais, observamos que a guanosina preveniu o aumento da geração de EROs, alteração do potencial de membrana mitocondrial, e a depleção dos níveis de ATP. Estes efeitos da guanosina foram abolidos pelo bloqueio farmacológico dos A_1R e a ativação dos $A_{2A}R$. Também *in vitro*, foi investigado a possível interação da guanosina com o heterômero A_1R-$A_{2A}R$ através do uso de transfecção heteróloga desses receptores em células HEK293. Guanosina não alterou a união e sinalização celular em células transfectadas somente com A_1R ou $A_{2A}R$. Porém, em células co-transfectadas com A_1R e $A_{2A}R$ a guanosina não exerceu efeito sobre a funcionalidade do A_1R, mas reduziu a união e a ativação do $A_{2A}R$, e reduziu a impedância celular aumentada por um agonista do $A_{2A}R$. Em síntese, concluímos que: (i) o efeito da guanosina em diminuir tremores orofaciais em um modelo da PD é dependente do A_1R; (ii) a guanosina exerce efeito neuroprotetor frente a danos oxidativos e mitocondriais através da modulação dos A_1R e $A_{2A}R$; (ii) a guanosina interage com os heterômeros A_1R-$A_{2A}R$, potencial alvo molecular responsável pelo seu mecanismo de neuroproteção.

Palavras chave: Doença de Parkinson, guanosina, reserpina, 6-OHDA, receptores de adenosina, oligomerizaçao, heterômeros.

ABSTRACT

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease in the world, characterized by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta*. The main symptoms of PD are tremor, bradykinesia, postural instability and muscle stiffness. Although the current treatments are effective for these motor symptoms, they have severe side effects and do not stop the progression of the disease. Therefore, the search for new therapeutic strategies is necessary. Guanosine, an endogenous nucleoside with neuromodulatory action, has already demonstrated efficacy as an antiparkinsonian agent in animal models of PD, besides presenting neuroprotective effects in *in vitro* protocols. However, there is not yet a specific receptor characterized for this nucleoside and its mechanisms of action are not yet fully understood. Therefore, the objective of this study was to evaluate the antiparkinsonian effect of guanosine and its relationship with adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$). In an *in vivo* protocol with reserpine administration in mice, the increase in orofacial tremors was reversed by guanosine and dependent on activation of A_1R and independent of $A_{2A}R$ expression. The pharmacological blockage of A_1R also prevented guanosine from decreasing the levels of reactive oxygen species (ROS) in the striatum of these animals. In an *in vitro* protocol with 6-hydroxydopamine (6-OHDA) that mimics initial PD cellular events in striated slices, we observed that guanosine prevented the increase in ROS generation, alteration on mitochondrial membrane potential, and depletion of ATP levels. These effects of guanosine were abolished by the pharmacological blockade of A_1R and activation of $A_{2A}R$. Also *in vitro*, the possible interaction of guanosine with the A_1R- $A_{2A}R$ heteromer through the use of heterologous transfection in HEK293 cells was investigated. Guanosine did not alter the binding affinity and cellular signaling in cells transfected only with A_1R or $A_{2A}R$. However, in cells co-transfected with A_1R and $A_{2A}R$, while guanosine had no effect on the functionality of A_1R, it reduced the binding and activation of $A_{2A}R$, and reduced increased cell impedance by an $A_{2A}R$ agonist. In summary, we conclude that: (i) the effect of guanosine on reducing orofacial tremors in a PD model is dependent on A1R; (ii) guanosine exerts a neuroprotective effect against oxidative and mitochondrial damage through modulation of A_1R and $A_{2A}R$; (ii) guanosine interacts with A_1R-$A_{2A}R$ heteromer, the potential molecular target responsible for its neuroprotective mechanism.

Keywords: Parkinson’s disease, guanosine, reserpine, 6-OHDA, adenosine receptors, GPCR oligomerization, heteromers.

LISTA DE ABREVIATURAS

6-OHDA 6-hidróxi-dopamina
A_1R Receptor de adenosina 1
A_{2A}R Receptor de adenosina 2A
ADA Adenosina desaminase
ADP Adenosina-5'-difosfato
Akt Proteína cinase B
AMP Adenosina-5'-monofosfato
AMPc Adenosina-5'-monofosfato cíclico
ATP Adenosina-5'-trifosfato
BK Canal de Potássio de alta condutância dependente de Cálcio
D1R Receptor de dopamina tipo 1
D2R Receptor de dopamina tipo 2
DAT Transportador de dopamina
DBS do inglês, *Deep brain stimulation*
DG Derivados da guanina
DPCPX 8-Ciclopentil-1,3-dipropilxantina
EROs Espécies reativas de oxigênio
GABA Ácido gama-aminobutírico
GDP Guanosina-5'-difosfato
Gi Proteína G do tipo inibitória
GMP Guanosina-5'-monofosfato
GPCR G protein-coupled receptors
GTP Guanosina-5'-trifosfato
iNOS Óxido nítrico sintase induzível
L-DOPA L-diidroxifenilalanina
LID do inglês, *L-DOPA-Induced Dyskinesia*
LPA Ácido lisofosfatídico
LRRK2 do inglês, *Leucine-rich repeat kinase 2*
MAO-B Monoamina oxidase B
MPP+1-metil-4-fenilpiridinio
MPTP 1-metil-4-fenil-1,2,3,6-tetrahidropiridina
NMDA N-metil-D-aspartato
NST Núcleo subtalâmico
PD Doença de Parkinson
PGO Privação de glicose e oxigênio
PINK1 do inglês, *PTEN-induced kinase 1*
PTx Toxina Pertussis
SNC Sistema nervoso central
SNpc Parte compacta da substância negra
TJM Tremoulus jaw movements
TMs Domínios transmembrana
TNF Fator necrose tumoral
VMAT-2 Transportador vesicular de monoaminas 2

SUMÁRIO

1 INTRODUÇÃO

1.1 DOENÇA DE PARKINSON

Histórico e características

A doença de Parkinson (*do inglês* Parkinson's disease, PD) é caracterizada pela perda progressiva dos neurônios dopaminérgicos na parte compacta da substância negra (SNpc), região cerebral mesencefálica envolvida no controle dos movimentos (Hirsch, et al. 1992). Essa perda neuronal na SNpc resulta na diminuição dos níveis de dopamina na região do estriado (via nigroestriatal dopaminérgica). A primeira descrição da PD foi há pouco mais de 200 anos, quando James Parkinson publicou a clássica monografia intitulada "Essay on the Shaking Palsy" em 1817. Posteriormente, Jean-Martin Charcot adicionou importantes detalhes às observações de Parkinson, descrevendo os quatro principais sinais cardinais da PD: tremor de repouso, bradicinesia (lentidão em iniciar o movimento), instabilidade postural e rigidez muscular. Charcot também foi o responsável por nomear a doença em homenagem a James Parkinson (Goetz 2002).

Em 1912, Fritz Henrich Lewy identificou agregados proteicos que até hoje são a principal característica histopatológica da PD (Przedborski 2017). Lewy identificou os agregados no cérebro, e em 1919, Konstantin Nikolaevich Tretiakoff foi o primeiro a identificar essas alterações neuropatológicas na SNpc de pacientes com PD, sendo resposável por nomear esses agregados em homenagem a Lewy. Os corpos de Lewy, então, são inclusões citoplasmáticas eosinofílicas compostos de agregados fibrilares com grande presença da proteína α-sinucleína. A α-sinucleína é altamente expressa em terminais pré-sinápticos e sua função biológica ainda não está totalmente clara, mas acredita-se que sua função esteja associada à sua incorporação nas membranas de vesículas sinápticas (Burré, Sharma, and Südhof 2018). O dobramento incorreto dessa proteína leva a formação oligômeros e agregados que podem ser tóxicos e são

relacionados a doenças neurodegenerativas. Sabe-se também que, à medida que a PD progride e ocorre degeneração neuronal, os corpos de Lewy se aglomeram em grande quantidade nos neurônios e em diversas regiões cerebrais (Markesbery, et al. 2009; Braak, et al. 2004).

Porém, só no final da década de 1950 que foi identificado o papel da dopamina na PD. Carlsson e colaboradores (1957) demonstraram o papel da dopamina utilizando a administração de reserpina, um inibidor do transportador vesicular de monoaminas 2 (VMAT-2). Neste, foi demonstrado que a administração de L-diidroxifenilalanina (L-DOPA), um precursor da dopamina, revertia a redução dos níveis de dopamina e da atividade motora induzida pela reserpina (Carlsson, Lindqvist, and Magnusson 1957). Essas evidências foram cruciais para elucidar o papel da dopamina na sinalização dos gânglios basais e no controle motor.

A PD é normalmente caracterizada como um distúrbio motor, uma vez que a região cerebral afetada está diretamente relacionada a esta função, sendo assim, seu diagnóstico médico é baseado pela presença de dois ou mais sinais motores: rigidez muscular, bradicinesia, tremor de repouso e instabilidade postural (Yamanouchi and Nagura 1997; Cutsuridis and Perantonis 2006; Hirsch, et al. 1992; van der Burg, et al. 2006). Porém, tais sinais motores somente aparecem quando aproximadamente 60–70% dos neurônios da substância negra já se encontram degenerados (Carvey, Punati, and Newman 2006). Atualmente, há consideráveis evidências demonstrando que o processo neurodegenerativo que leva à PD começa anos antes do aparecimento dos sintomas motores, não estando somente restrito aos neurônios dopaminérgicos da via nigroestriatal. Outras áreas cerebrais também estão envolvidas, como as estruturas olfatórias anteriores, núcleo motor dorsal do vago, núcleo caudal da *raphe*, *locus coeruleus*, sistema nervoso autônomo, hipocampo e córtex cerebral (Braak, et al. 2004).

De acordo, neurônios colinérgicos, adrenérgicos e serotoninérgicos também sofrem degeneração, e parecem ser os principais responsáveis pelos sintomas não-motores da PD, incluindo prejuízos olfativo e de memória, distúrbios de sono, ansiedade e depressão (Chaudhuri, et al. 2006). Por essa evolução da PD, Braak e colaboradores desenvolveram um sistema de estagiamento para a PD de causa idiopática (Braak, et al. 2004). Esse estudo também demonstrou que a presença de agregados de α-sinucleína primeiramente acontece no núcleo dorsal motor e então ocorre a progressão para outras regiões como a SNpc.

Etiologia e epidemiologia

A etiologia da PD ainda é tida como idiopática, sendo heterogênea, multifatorial e complexa, mas estudos sugerem que a PD pode ser decorrente de um conjunto de fatores, sejam eles genéticos, toxinas ambientais, estresse oxidativo, anormalidades mitocondriais e/ou alterações do envelhecimento (Pereira and Garrett 2010). Os principais mecanismos bioquímicos evidenciados como possíveis responsáveis pela neurodegeneração na PD são o estresse oxidativo, o dano mitocondrial, uma resposta inflamatória exacerbada e a excitotoxicidade glutamatérgica (Dexter and Jenner 2013). Todos os dias, humanos são expostos a milhares de xenobióticos no ar, água e comida, incluindo agentes químicos de roupas, tintas, plásticos, perfumes, cosméticos, comidas, bebidas, pesticidas, herbicidas e emissões gasosas de veículos e indústrias. Tais químicos podem estar envolvidos na etiologia da PD (Uversky 2004). Em relação aos casos genéticos, algumas mutações são consideradas de grande risco para desenvolvimento da PD, porém tais mutações são responsáveis somente por 5–10% dos casos da doença (Lesage and Brice 2009). Entre as mutações, as mais comuns são as presentes nos genes que codificam para as proteínas LRRK2 (*do inglês*, leucine-rich

repeat kinase 2), Parkina, PINK1 (*do inglês*, PTEN-induced kinase 1), DJ1 (do inglês, protein deglycase DJ-1, ou Parkinson disease protein 7) - sendo que estas mutações estão relacionadas a danos/disfunções mitocondriais - e α-sinucleína (Kim and Alcalay 2017).

A PD é o segundo transtorno neurodegenerativo de maior prevalência (estando atrás da doença de Alzheimer) e seu início raramente se dá antes dos 50 anos de idade, sendo observado um aumento acentuado na sua incidência a partir dos 60 anos, afetando 1 - 2% da população (de Lau and Breteler 2006). Estimou-se que, em 2016, 6.1 milhões de pessoas ao redor do mundo tinham PD, das quais mulheres e homens representavam 2.9 milhões e 3.2 milhões, respectivamente, e mais de 200 mil mortes ocorridas pela doença (Global Burden Disease Collaborators 2018). No mesmo ano, no Brasil, foi estimado 128 mil casos e mais de 4 mil mortes pela PD (Global Burden Disease Collaborators 2018). Como a incidência da doença aumenta com a idade (fator de risco mais importante), e levando em consideração o aumento da expectativa de vida da sociedade atual, é provável que o número de pessoas que sofrem da PD tenda a aumentar constantemente no futuro.

Tratamentos

Embora ainda não exista cura para a PD, existem estratégias para atenuar os sintomas, porém esses tratamentos apresentam limitações. A droga mais utilizada como tratamento na PD é a mesma desde os anos 60, a L-DOPA, um precursor da dopamina, que tem como objetivo restabelecer os níveis de dopamina, porém sua eficácia diminui com os anos de tratamento e altas doses podem levam às discinesias (Hardie, Lees, and Stern 1984).

Uma outra abordagem que trouxe um avanço importante para o tratamento da PD foi a implantação da técnica de DBS, do inglês "deep brain stimulation". Em 1994, Benabid demonstrou os efeitos benéficos da estimulação de alta frequência no núcleo subtalâmico (NST) de pacientes com PD (Benabid, et al. 1994). A cirurgia de DBS já foi realizada em milhares de pessoas ao redor do mundo e os resultados mostraram a eficácia e segurança do método. Entretanto, a cirurgia de DBS é bem invasiva e de alto custo, além de não reverter ou mesmo parar a neurodegeneração, mesmo assim é uma importante estratégia terapêutica no tratamento paliativo de sintoma motores na PD.

Limitações no tratamento farmacológico atual para a PD têm levado a uma crescente investigação sobre drogas que possam promover um tratamento alternativo para sintomas motores e não motores, reduzindo os efeitos colaterais, para assim modificar o curso da doença (Schapira, et al. 2006; Obeso, et al. 2010). Testes clínicos estão avaliando novas drogas com potencial neuroprotetor frente à PD. Estes testes incluem principalmente agonistas de receptores dopaminérgicos, inibidores da monoamina oxidase B (MAO-B), antioxidantes, agentes anti-apoptóticos e antagonistas de receptores A_{2A} de adenosina ($A_{2A}R$) e de receptores N-metil-D-aspartato (NMDA) de glutamato (Schapira, et al. 2006; Obeso, et al. 2010; Dawson and Dawson 2002).

Modelos da PD

O estudo e o desenvolvimento de novas terapias para a PD dependem da existência de modelos animais que apresentem características comportamentais e/ou histopatológicas da enfermidade, bem como modelos celulares, com respectivas alterações bioquímicas similares à doença, permitindo a avaliação de novas drogas e estratégias terapêuticas (Gerlach, Foley, and Riederer 2003). Consideráveis evidências demonstram que diversas drogas podem ser utilizadas para induzir sintomas e as

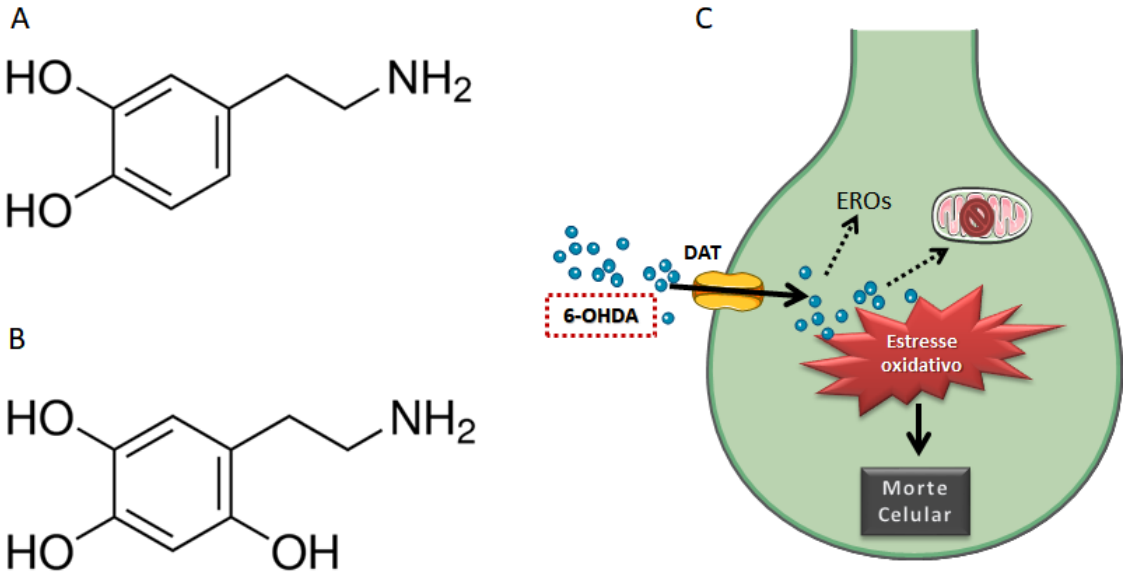
características bioquímicas do parkinsonismo, entre eles o tratamento com as toxinas 6-hidróxi-dopamina (6-OHDA) e com 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) ou seu metabólito ativo 1-metil-4-fenilpiridinio (MPP+), utilização de pesticidas como Paraquat e Rotenona, e depleção ou antagonismo de dopamina com a reserpina e o haloperidol, respectivamente (Duty and Jenner 2011).

A 6-OHDA é a toxina mais utilizada em modelos experimentais da PD *in vivo* (Gomez-Lazaro, et al. 2008; Ikeda, et al. 2008; Mu, et al. 2009). A primeira demonstração dos efeitos biológicos da 6-OHDA foi relatada há mais de 40 anos, demonstrando que esse agente era capaz de induzir depleção de noradrenalina em nervos simpáticos do coração de camundongos (PORTER, TOTARO, and STONE 1963). Anos depois, Ungerstedt demonstrou que uma injeção de 6-OHDA na SNpc era capaz de causar degeneração dopaminérgica do sistema nigroestriatal, gerando assim o primeiro modelo animal da PD (Ungerstedt 1968).

Devido a sua estrutura similar à da dopamina, ela apresenta grande afinidade com o transportador de dopamina e por essa razão seletivamente destrói neurônios dopaminérgicos e monoaminérgicos (Fig 1) (Lehmensiek, et al. 2006). Uma vez dentro do neurônio, a 6-OHDA é acumulada e sofre uma auto-oxidação não enzimática e produz radicais livres (Blandini, Armentero, and Martignoni 2008). Um efeito inibitório sobre a atividade do complexo I da mitocôndria também já foi demonstrado, o que pode ser responsável pela geração de espécies reativas de oxigênio (EROs) ocasionada por essa neurotoxina (Chin, et al. 2008; Inden, et al. 2006; Lehmensiek, et al. 2006). A alteração mitocondrial é apontada como um dos mecanismos responsáveis pela neurodegeneração dopaminérgica na PD. Uma conexão entre a disfunção mitocondrial e o estresse oxidativo em doenças neurodegenerativas já vem sendo postulada há tempos (Przedborski and Ischiropoulos 2005). A excessiva geração de espécies reativas de

oxigênio e nitrogênio ativam sinalizações celulares acarretando em danos celulares e morte (Beal 2005).

Figura 1. Mecanismo de ação da 6-OHDA em neurônios dopaminérgicos



A estrutura da 6-OHDA é muito similar à da dopamina, sendo assim ela tem grande afinidade com o transportador de dopamina. Molécula da dopamina **(A).** Molécula da 6-OHDA (**B**). Sem o armazenamento da dopamina, sua metabolização no citoplasma é aumentada, gerando EROs e quinonas reativas, e assim, resultando em estresse oxidativo **(C).**

Neste estudo foi utilizado um protocolo de toxicidade *in vitro* induzida pela 6-OHDA. Apesar de a 6-OHDA ser classicamente utilizada *in vivo* para estudo dos efeitos motores da PD, nosso grupo em 2016 padronizou um protocolo *in vitro* para avaliar possíveis estratégias de neuroproteção (Massari, et al. 2016). Já foi visto que a incubação com 6-OHDA causa redução na viabilidade celular de fatias estriatais, além induzir o aumento de geração de EROs e diminui o potencial de membrana mitocondrial nessa mesma região (Massari, et al. 2016). Além disso, também foi visto no estriado uma permeabilização de membrana celular e uma diminuição do consumo de oxigênio pela mitocôndria (Marques, Massari, and Tasca 2019; Gonçalves, et al. 2019). Sendo

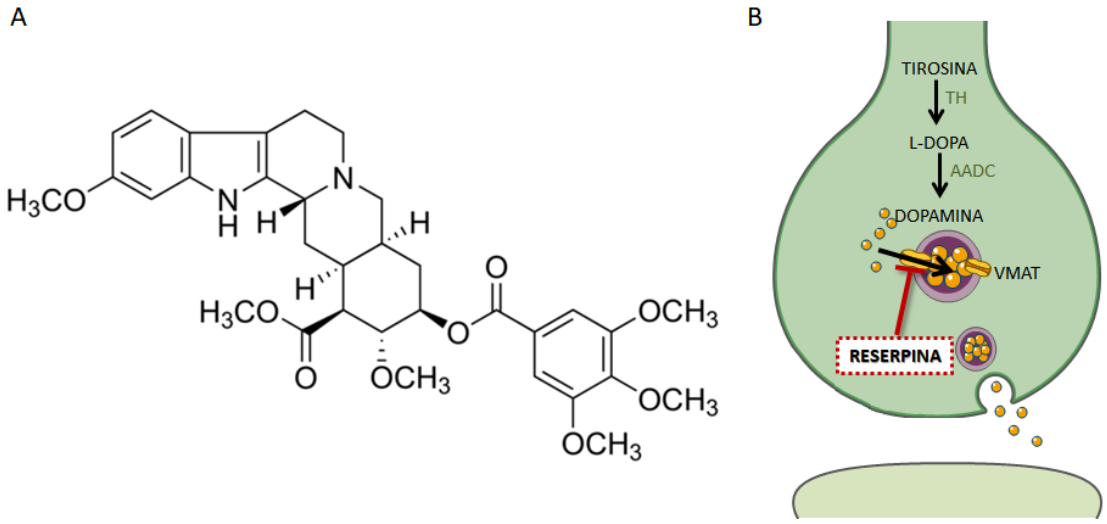
assim, ao estabelecer essa relação da toxicidade da 6-OHDA em fatias estriatais com danos acometidos na PD, esse protocolo tem sido usado para avaliação de moléculas com potencial neuroprotetor e propicia estudo dos mecanismos relacionados à patologia da PD.

Como mencionado acima, um protocolo largamente utilizado *in vivo* é a administração de reserpina, como um modelo para a indução de discinesias orofaciais e outros déficits motores relacionados à PD. Este é um protocolo agudo de alta reprodutibilidade, de baixa toxicidade e que abrange importantes características da PD (Leão, et al. 2015). A reserpina é um alcalóide isolado das raízes da planta *Rauwolfia serpentina* e atua como inibidor do VMAT-2 no sistema nervoso central (Figura 2). Ela foi utilizada inicialmente como uma potente droga anti-hipertensiva, devido a sua capacidade em depletar o conteúdo monoaminérgico (FREIS and ARI 1954; McQUEEN, DOYLE, and SMIRK 1954). Seu uso clínico para esse fim levou a observações de letargia, depressão e discinesias em pacientes que a utilizavam cronicamente, demonstrando, assim, o papel do sistema monoaminérgico em distúrbios afetivos e motores (FREIS 1954; Kane and Smith 1982). Após essa constatação ela foi rapidamente introduzida como um modelo animal para mimetizar os efeitos motores e não-motores da PD. A alta afinidade da reserpina pelo VMAT-2 impede a ligação das monoaminas ao sítio de interação com este transportador, inibindo o seu armazenamento vesicular e impossibilitando a liberação dessas moléculas na fenda sináptica através do processo de exocitose. Desta forma, a reserpina leva à depleção destes neurotransmissores nos terminais nervosos e, como consequência, induz hipolocomoção, rigidez muscular transitória e movimentos involuntários, sendo estas respostas dependentes da dose e tempo de tratamentos utilizados (Gerlach and Riederer 1996; Dawson, et al. 2000). Atualmente, a reserpina não é mais utilizada clinicamente

devido aos seus efeitos colaterais, como cansaço, hipotensão, impotência sexual e depressão.

De acordo com a literatura, o estresse oxidativo está envolvido na patofisiologia das discinesias orofaciais (Abílio, et al. 2003; Burger, et al. 2003; Faria, et al. 2005; Leão, et al. 2015). Na depleção de monoaminas pelo tratamento com reserpina há aumento de EROs e de nitrogênio (Spina and Cohen 1989). Nesse sentido, sabe-se que o metabolismo das catecolaminas resulta na formação de EROs, o que pode ser aumentado pelo tratamento com a reserpina, com a presença de mais catecolaminas livres no citoplasma. Assim, o estresse oxidativo se soma à depleção de monoaminas prejudicando o desempenho motor.

Figura 2. Mecanismo de ação da reserpina em neurônios dopaminérgicos



A depleção de dopamina resulta do efeito inibitório da reserpina no transportador vesicular. Molécula da reserpina **(A).** Sem o armazenamento da dopamina, sua metabolização no citoplasma é aumentada, gerando EROs e quinonas reativas, e assim, resultando em estresse oxidativo **(B).** TH, tirosina hidroxilase; AADC, L-aminoácidos aromáticos descarboxilase; VMAT-2, transportador vesicular de monoaminas 2.

Como já dito, a relação entre a reserpina e a PD foi primeiramente elucidada em 1957 por Carlsson e colaboradores, relatando que o estado acinético (ou seja, ausência de movimento) induzido pela reserpina em roedores, era aliviado pela L-DOPA. A administração aguda da reserpina em roedores, além de mimetizar padrões bioquímicos da PD (depleção de dopamina, estresse oxidativo), induz um comportamento chamado de '*tremoulus jaw movements'* – movimentos de tremores de mandíbula, na tradução literal - (TJM) (Steinpreis and Salamone 1993). Os TJMs são caracterizados como movimentos produzidos por uma deflexão vertical rápida do maxilar inferior que se assemelha à mastigação, porém não é uma resposta a nenhum estímulo específico (Salamone, et al. 1998). Consideráveis evidências apontam que esses movimentos em roedores, induzidos pela reserpina, compartilham diversas características com os tremores observados em pacientes acometidos pela PD. Salamone e Baskin (1996) demonstraram, ao analisar a inter-resposta dos movimentos (ou seja, o tempo entre cada movimento do maxilar), que o tratamento com reserpina gerava tremores com os picos de frequência entre 3-7 Hz, semelhante à frequência dos tremores registrados na PD. Por conta dessa semelhança entre os tremores, este modelo é considerado de extrema importância para estudar esse distúrbio motor relacionado à PD (Salamone, et al. 1998).

Discinesias

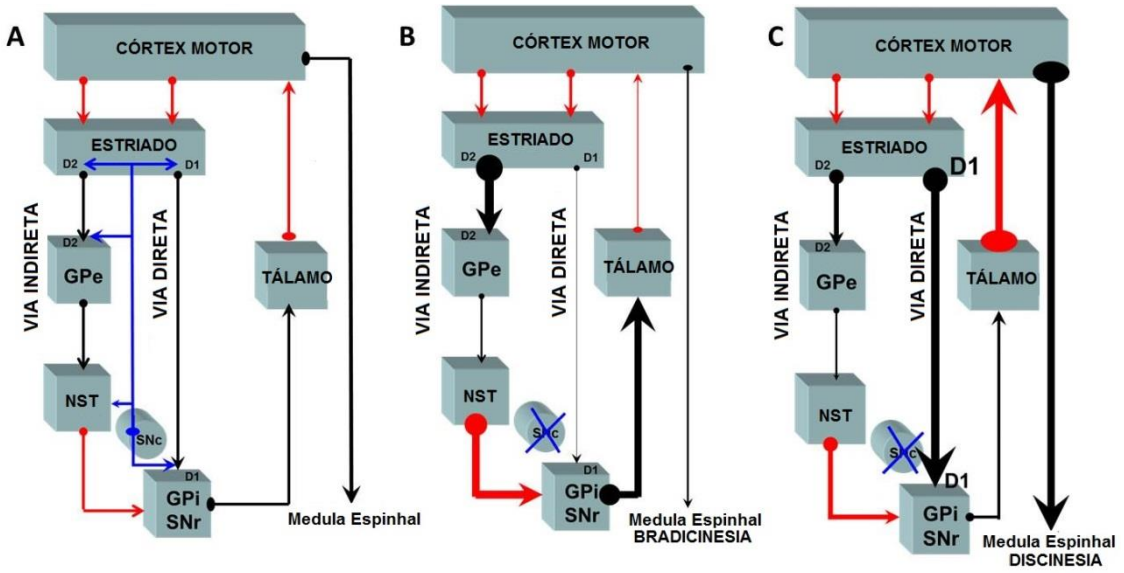
Os mecanismos envolvidos nos sintomas motores são complexos, abrangendo diversos sistemas de neurotransmissão. Normalmente, o controle dos movimentos pela dopamina é realizado através da ativação dos neurônios GABAérgicos da via direta, que expressam receptores dopaminérgicos do tipo D1 (D1R) e pela inibição dos neurônios GABAérgicos da via indireta, que expressam receptores dopaminérgicos do tipo D2 (D2R) (Heiman, et al. 2014) (figura 3). Na PD há alterações na coordenação do

movimento devido à falta de projeções dopaminérgicas da SNpc, aumentando assim a desinibição da via indireta sob o núcleo subtalâmico, inibindo o tálamo e consequentemente o movimento.

Discinesia é um termo amplo para caracterizar diversos movimentos involuntários, tendo como base um desbalanço neuroquímico do circuito motor que envolve os gânglios da base. As discinesias podem ser classificadas em tremores, coreia, distonia, de acordo com sua frequência e abrangência. Diversas doenças neurológicas podem apresentar discinesias, incluindo a discinesia tardia, PD, Huntington (Aquino and Lang 2014; Kobylecki, et al. 2014; Aquino and Fox 2015). Na PD, as discinesias, aparecem como resultado de uma hiperativação da via direta pelos D1R, desinibindo o tálamo e resultando em movimentos involuntários. As discinesias também podem ser induzidas através da administração de várias classes de fármacos, de antagonistas dopaminérgicos, como o haloperidol, de depletores de dopamina, como a reserpina, de colinomiméticos e de agentes anticolinesterásicos, como a pilocarpina e a tacrina, respectivamente (Steinpreis and Salamone 1993; Mayorga, et al. 1997; Collins, et al. 2010).

De grande importância, ainda, o uso crônico da L-DOPA, o principal fármaco usado para aliviar os sintomas motores em pacientes com a PD, pode induzir essas flutuações motoras, mais conhecidas como discinesias induzida por L-DOPA, do inglês *L-DOPA-Induced Dyskinesias* (LID) (Bhide, et al. 2013). Por essa limitação no tratamento com a L-DOPA é interessante investigar, não só opções terapêuticas para a PD, como substâncias que diminuam as discinesias para serem usadas concomitantes à L-DOPA, a fim de prolongar seu uso.

Figura 3. **Os circuitos neurais dos gânglios basais responsáveis pela movimentação.**

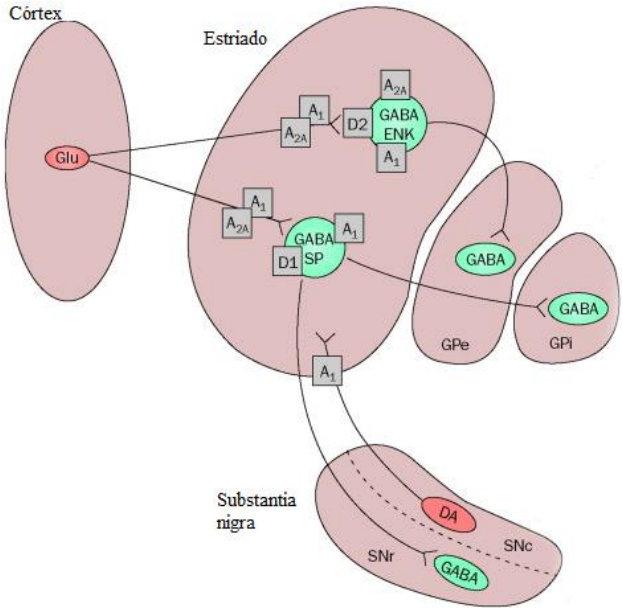


Normal (A), na PD (B) e nas discinesias (C). Gpe, globo pálido externo; NST, núcleo subtlâmico; GPi, globo pálido interno; SNr, substância negra parte reticulada; SNc substância negra parte compacta; D1, receptor de dopamina D1R; D2, receptor de dopamina D2R. Flechas azuis = via Dopaminérgica. Flechas pretas = via Glutamatérgica. Flechas vermelhas = via GABAérgica. Adaptado de (Bravo et al., 2014).

Como comentado anteriormente, a PD não envolve somente a transmissão dopaminérgica, e a modulação do sistema adenosinérgico foi recentemente apontada como uma potencial estratégia de atenuação dos sintomas parkinsonianos. Uma explicação para esse potencial terapêutico baseia-se na distribuição cerebral dos receptores do nucleosídeo adenosina, A_1 e A_{2A}, que são amplamente expressos no estriado (Palmer and Stiles 1995) e na capacidade desses receptores em formar oligômeros entre si e entre receptores de outros neurotransmissores, como os de dopamina e os de glutamato (Fuxe, et al. 2005; Ciruela, et al. 2006; Fernández-Dueñas, et al. 2012). Os receptores A_1R e $A_{2A}R$ modulam antagonisticamente a união dos ligantes e características funcionais dos receptores dopaminérgicos D1R e D2R, respectivamente (Ferré, et al. 1992; Ferre, et al. 1996). Os receptores $A_{2A}R$ estão co-localizados com os receptores D2R nos neurônios GABAérgico da via indireta enquanto

os A_1R e o receptor D1R são co-localizdos nos neurônios GABAérgicos da via direta (figura 4) (Ferre, et al. 1996).

Figura 4. **Co-localização dos receptores de adenosina e dopamina.**



Os receptores de dopamina e adenosina encontram-se co-localizados no estriado onde podem modular respostas motoras. A_1, receptor de adenosina A_1; A_{2A}, receptor de adenosina $A_{2A;}$ D1, receptor de dopamina D1; D2, receptor de dopamina D2; DA, dopamina; ENK, encefalina; GABA, ácido gamma-aminobutírico; Glu, glutamato; GPe, globo pálido externo; GPi, globo pálido interno; SP, substânica P. Adaptado de (Blum et al., 2003).

Sendo assim, crescentes evidências demonstram que o desbalanço entre esses receptores está intimamente ligado ao desenvolvimento de algumas enfermidades, como a doença de Parkinson e a esquizofrenia. Dessa forma, o estudo da interação dos receptores de adenosina constitui uma nova oportunidade no desenvolvimento farmacológico para intervenção terapêutica.

1.2 TRANSMISSÃO PURINÉRGICA

As purinas são uma classe de moléculas orgânicas essenciais para as células e são constituídas pelas bases nitrogenadas adenina e guanina. Compõem a estrutura das purinas, um açúcar de cinco carbonos, a ribose, desta forma constituindo um nucleosídeo (base nitrogenada e ribose) e um, dois ou três fosfatos, constituindo então os nucleotídeos. Portanto, as purinas compreendem os nucleotídeos adenosina-5'-trifosfato (ATP), adenosina-5'-difosfato (ADP), adenosina-5´-monofosfato (AMP), guanosina-5'-trifosfato (GTP), guanosina-5'-difosfato (GDP), guanosina-5'-monofosfato (GMP), os nucleosídeos adenosina, inosina e guanosina, as bases nitrogenadas adenina, guanina, hipoxantina e ainda seus metabólitos xantina e ácido úrico. São moléculas amplamente encontradas dentro das células de animais e plantas. Os nucleotídeos purínicos intracelulares foram identificados primeiramente como componentes estruturais dos ácidos nucléicos, mas também apresentam funções importantes no metabolismo energético, na biossíntese de macromoléculas e na constituição de coenzimas. Além disso, os nucleotídeos cíclicos desempenham papeis como segundos-mensageiros (Lippman 1941).

As purinas também apresentam efeitos extracelulares e no cérebro já foram evidenciadas por ter ação como neurotransmissores e neuromoduladores. Ainda que existam evidências sobre os efeitos extracelulares das purinas derivadas da guanina (DG), mecanismos de captação e liberação, e metabolização extracelular das DG, não há receptores identificados para as DG (detalhado a seguir). Por outro lado, os efeitos extracelulares e os alvos moleculares das purinas derivadas da adenina estão bem estabelecidos e caracterizados. Para as purinas derivadas da adenina já estão descritas duas famílias de receptores, os do tipo P1 e P2. Os receptores pertencentes à família P2 são receptores de ATP e ADP, e são subdivididos em P2X (receptores ionotrópicos) e

P2Y (receptores metabotrópicos acoplados às proteínas G) (Burnstock 2007). Neste estudo, iremos abordar com mais detalhes as funções e modulação dos receptores P1.

Os receptores da família P1 são receptores metabotrópicos para adenosina, e são divididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3 (Fredholm, et al. 2005). Os receptores A_1 são acoplados à proteína Gi levando a uma diminuição da atividade da adenilato ciclase e consequente diminuição nos níveis de AMP cíclico intracelular e são expressos em todo o Sistema Nervoso Central (SNC), com grande densidade no estriado, hipocampo, córtex cerebral e tálamo, enquanto que os receptores A_{2A} são acoplados à proteína Gs, levando ao aumento dos níveis de AMP cíclico. São principalmente expressos no estriado, núcleo accumbens, hipocampo e córtex cerebral. Os receptores A_{2B} também são acoplados à proteína Gs, mas são pouco expressos no cérebro, enquanto os receptores A_3 são moderadamente expressos no cerebelo e hipocampo (Palmer and Stiles 1995; Burnstock 2007).

Dos receptores de adenosina conhecidos, os receptores A_1 e A_{2A} são os principais responsáveis pelos efeitos centrais da adenosina (Dunwiddie and Masino 2001). A estimulação do A_1R pré-sináptico diminui a excitabilidade neuronal e a atividade sináptica, além de diminuir a probabilidade de liberação de neurotransmissores como o glutamato, dopamina, serotonina, noradrenalina e acetilcolina. Por outro lado, o $A_{2A}R$ é um receptor excitatório e está expresso principalmente em regiões dopaminérgicas. Alguns estudos prévios demonstraram evidências de uma interação antagônica entre o A_1R e o $A_{2A}R$ ao modular a liberação de glutamato no estriado e hipocampo (O'Kane and Stone 1998; Lopes, et al. 2002; Quarta, et al. 2004).

Oligomerização dos receptores de adenosina

O entendimento sobre a fisiologia e farmacologia dos receptores acoplados à proteína G (do inglês, *G protein-coupled receptors*, GPCR) tem mudado nas últimas duas décadas. Isso se deve às crescentes evidências de que eles formam homômeros (homo-oligomerização, de mesmo GPCRs) e heterômeros (hetero-oligmerização de GPCRs diferentes). Essa oligomerização induz mudanças nas propriedades bioquímicas dos GPCRs. Já está bem estabelecido que os receptores de adenosina podem formar oligômeros entre si e com receptores para outros neurotransmissores, como receptores de dopamina (Agnati, et al. 2005; Fuxe, et al. 2013; Ciruela, et al. 2006). Sabe-se que os receptores A_1 e A_{2A} formam oligômeros funcionais entre si, e esse heterômero A_1R-$A_{2A}R$ tem um papel importante modulando o controle da função córtico-estriatal (Ciruela, et al. 2006). Esse controle se dá através da ativação dos A_1R ou $A_{2A}R$ pré-sinápticos, que depende da concentração de adenosina, sendo que uma concentração baixa ativaria o A_1R enquanto uma concentração alta ativaria o $A_{2A}R$, resultando em uma menor ou maior liberação de glutamato, respectivamente (Ciruela, et al. 2006).

Entretanto, o heterômero mais estudado é o que envolve os receptores A_{2A} e os receptores D2 de dopamina. Existem evidências que indicam que o oligômero $A_{2A}R$-D2R possui a estrutura de um heterotetrâmero, formado por dois homômeros de $A_{2A}R$ e dois homômeros de D2R. GPCRS contém estruturalmente 7 domínios transmembrana (TMs), e já foi demonstrado que peptídeos que se ligam nos TMs acabam interferindo na formação de oligômeros. Dessa forma, foi demonstrado que homodímeros de $A_{2A}R$ e D2R interagem entre si pelo TM6, enquanto o heteromêro $A_{2A}R$-D2R apresentam uma interação simétrica com os TM4-5/TM5-4 (Navarro, et al. 2018). Essa formação explicaria porque tanto agonistas como antagonistas de $A_{2A}R$ além de afetar alostericamente a união (*binding)*, também modulam a eficácia intrínseca (capacidade

de um agonista de induzir resposta, independente da afinidade do receptor) de um agonista D2R (Bonaventura, et al. 2015).

Por essa interação com os D2R, antagonistas de $A_{2A}R$, principalmente, têm sido sugeridos como um potencial tratamento para os danos motores relacionados à PD. Diversos estudos pré-clínicos demonstraram a eficácia de antagonistas $A_{2A}R$ em variados modelos *in vitro* e *in vivo* da PD (Vallano, et al. 2011; Jenner 2014; Pinna 2014). Além disso, estudos utilizando modelos animais que mimetizam tremores relacionados à PD demonstram que a utilização farmacológica de antagonistas de receptores de adenosina provocam melhoras nesse aspecto motor (Gandía, et al. 2015; Salamone, et al. 2013). Dentre os antagonistas $A_{2A}R$, a Istradefilina (KW6002) já foi aprovada para uso clínico no Japão e, recentemente, nos Estados Unidos da América, após resultados de ensaios clínicos até a fase III (Kondo, Mizuno, and Group 2015; Mizuno, et al. 2010; Hussar 2020; Dungo and Deeks 2013).

Guanosina

As purinas derivadas da guanina também foram inicialmente identificadas pelos seus papeis intracelulares como a constituição de estrutura de ácidos nucleicos e participação no metabolismo energético. No entanto, grande destaque ao papel dos DG foi devido à identificação de sua função como moduladores da atividade das proteínas-G. As proteínas-G desempenham um papel fundamental na transdução de sinal, acoplando receptores transmembrana, ou GPCR aos seus efetores intracelulares. Além das proteínas-G heterotriméricas, os nucleotídeos GTP e GDP modulam também a atividade das proteínas-G monoméricas (*small G-proteins*).

Além desta importante função intracelular, foi identificado principalmente no SNC, que também os nucleosídeos e os nucleotídeos derivados da guanina poderiam

exercer uma função extracelular, atuando como mediadores da sinalização intercelular. Sobre as purinas DG, apesar da guanina também apresentar efeitos como um neuromodulador (Di Liberto, et al. 2016), muita atenção tem sido dada ao efeito biológico da guanosina (Lanznaster, et al. 2016; Tasca, et al. 2018). Ainda que não tenha sido completamente caracterizado um possível receptor específico para a guanosina, são notáveis as evidências que demonstram os efeitos neuroprotetores dessa molécula.

Diversos estudos vêm descrevendo efeito protetor da guanosina em modelos de neurotoxicidade e de doenças neurológicas, tanto *in vitro* quanto *in vivo*. A guanosina é considerada um neuromodulador e tem ação sobre a excitotoxicidade glutamatérgica, já que uma das suas ações bem evidenciadas é promover o aumento da captação de glutamato, desta forma reduzindo os níveis excitotóxicos de glutamato na fenda sináptica (para revisão, (Schmidt, et al. 2010; Lanznaster, et al. 2016; Tasca, et al. 2018). Estudos *in vivo*, demonstram a guanosina exercendo efeitos anti-convulsivante, antinociceptivo, ansiolítico e antidepressivo (Lara, et al. 2001; Bettio, et al. 2012; Bettio, et al. 2014; Almeida, et al. 2016). Os estudos *in vitro* contribuíram para a demonstração dos mecanismos pelos quais a guanosina exerce suas ações, como o aumento da captação de glutamato (Frizzo, et al. 2005), a redução do dano oxidativo causados pelo peptídeo β-amiloide (Tarozzi, et al. 2010) e pelo bloqueio da atividade dos complexos da cadeia respiratória mitocondrial (Dal-Cim, et al. 2012). Além disso, a guanosina apresenta efeitos anti-inflamatórios, exercidos pela inibição da expressão do fator nuclear kappa B (NFk-B) e da óxido nítrico sintase induzível (iNOS) (Dal-Cim, et al. 2013). A guanosina não apresenta toxicidade às células neurais mesmo quando em altas concentrações (Molz, Dal-Cim, and Tasca 2009). Interessantemente, os principais efeitos neuroprotetores e tróficos da guanosina não são afetados por uma prévia

incubação de bloqueadores do transporte de nucleosídeos, mostrando que o efeito não depende do transporte ou do acúmulo intracelular desse nucleosídeo (Giuliani, et al. 2015; Decker, et al. 2019; Oleskovicz, et al. 2008), ou seja, que a guanosina exerce suas ações protetoras e tróficas através da interação com alvos moleculares com um sítio de interação extracelular.

Guanosina e os receptores de adenosina

Mesmo com crescente evidência dos efeitos extracelulares e neuroprotetores da guanosina, ainda não existe um receptor clonado e sequenciado específico para essa purina e seus mecanismos de ação ainda não estão totalmente compreendidos. Estudos de união de guanosina a proteínas em preparações de membrana de cérebro de ratos sugeriram que a guanosina interage com um receptor diferente dos conhecidos receptores de adenosina (Traversa, et al. 2002; Traversa, et al. 2003). No entanto, diversos efeitos biológicos da guanosina são afetados por ligantes seletivos dos receptores de adenosina.

Em cultura de neuroblastoma SH-SY5Y, demonstrou-se que o efeito da guanosina frente ao estresse oxidativo mitocondrial é dependente da ativação dos receptores purinergicos A_1 e A_{2A}, uma vez que os antagonistas destes receptores, DPCPX (8-Ciclopentil-1,3-dipropilxantina) e ZM241385, respectivamente, aboliram o efeito protetor observado (Dal-Cim, et al. 2012). Em estudo avaliando o efeito neuroprotetor da guanosina em fatias hipocampais submetidas à privação de glicose e oxigênio (PGO), foi observado que o bloqueio de A_1R com o antagonista DPCPX reverteu o efeito da guanosina em diminuir a produção de EROs e manter o potencial de membrana mitocondrial, porém não teve efeito sobre a captação de glutamato recuperada pela guanosina (Dal-Cim, et al. 2013). Recentemente, em um modelo de

traumatismo encefálico em ratos, foi demonstrado que a guanosina protege a disfunção mitocondrial, e que esse efeito é abolido com um pré-tratamento com DPCPX (Gerbatin, et al. 2019). Interessantemente, quando o pré-tratamento é feito com SCH58261, um antagonista $A_{2A}R$, o efeito da guanosina não é alterado. Esses resultados sugerem que o A_1R, e não o $A_{2A}R$ estaria relacionado ao efeito da guanosina. Como ainda há controvérsias entre os efeitos da guanosina e os receptores de adenosina, e os mesmos estão implicados em abordagens terapêuticas na PD, é de grande importância investigar essa interação. Além disso, outra forma de encarar esses resultados seria através da possibilidade da guanosina modular os oligomêros dos receptores de adenosina (Tasca, et al. 2018), principalmente no heterômero A_1R-A_2A. De fato, já foi proposto que o heterômero A_1R-A_2A pode ser um alvo terapêutico em potencial para a PD (Fernández-Dueñas, et al. 2017).

Guanosina e modelos da PD

Em relação a estudos abordando os efeitos da guanosina sobre modelos da PD, Giuliani e colaboradores (2012) mostraram um efeito neuroprotetor da guanosina, *in vitro*, frente à toxicidade induzida pela 6-OHDA em células SH-SY5Y, porém sem evidenciar os mecanismos envolvidos. Em outro estudo *in vitro*, utilizando células neuronais PC12, a guanosina reverteu a disfunção mitocondrial causada pelo MPP+, metabólito ativo do MPTP, uma neurotoxina amplamente utilizada em modelos de PD (Li, et al. 2014). Dados do nosso laboratório também mostraram evidências do papel neuroprotetor da guanosina em um modelo *in vitro* da PD. A incubação de fatias de estriado com 6-OHDA induz à redução da viabilidade celular, aumento de EROs, perda do potencial de membrana mitocondrial e diminuição dos níveis de ATP, parâmetros

estes que foram prevenidos com a co-incubação de guanosina (Marques, Massari, and Tasca 2019).

Porém, ainda há poucos estudos mostrando um potencial *in vivo* da guanosina frente a modelos da PD. Su e colaboradores foram os primeiros a demonstrar que a guanosina teria efeito em um modelo de parkinsonismo. Neste estudo, usaram como modelo de PD a administração de um inibidor de proteassoma, e trataram os ratos diariamente com guanosina (8 mg/kg) durante 8 semanas. Após o tratamento a guanosina foi capaz de melhorar a bradiscinesia e aumentar as células positivas para tirosina hidroxilase (marcador de células dopaminérgicas) no estriado (Su, et al. 2009). Em 2017 nosso grupo também evidenciou efeitos da guanosina *in vivo* em distintos modelos de parkinsonismo. No modelo de indução de discinesias orofaciais pela reserpina, a guanosina administrada oral e agudamente (7,5 mg/kg), reverteu os tremores orofaciais e o estado cataléptico induzido pela reserpina (Massari et al., 2017). Com o mesmo protocolo de tratamento, a guanosina também foi capaz de exercer uma significante atenuação das discinesias provocadas pelo tratamento com L-DOPA em animais injetados unilateralmente com 6-OHDA na região estriatal (Massari, et al. 2017). E no protocolo de infusão estriatal unilateral de 6-OHDA, a administração de guanosina aumentou de forma dependente de dose as contrações contralaterais induzidas por doses sub-ativas de L-DOPA, ou seja, guanosina potencializou os efeitos da L-DOPA, indicando uma ação pró-dopaminérgica sem aumentar os efeitos colaterais da L-DOPA (Massari et al., 2017). Além disso, em um protocolo para estudo de sintomas não-motores da PD, nosso grupo demonstrou que o tratamento com guanosina (7,5 mg/kg) previne o comportamento tipo-antidepressivo em ratos infundidos com 6-OHDA no estriado dorsolateral (Marques, et al. 2019).

Desta forma, considerando o potencial da guanosina em atenuar danos que estão bem caracterizados na PD e a possível interação da guanosina com os receptores A_1 e A_{2A}, pretendemos com este estudo contribuir para a compreensão dos mecanismos relacionados a esta purina e seus efeitos neuroprotetores e antiparkinsonianos.

2 JUSTIFICATIVA

Com um grande índice de prevalência da PD em toda população mundial e maior ainda na população acima de 60 anos, tendo em vista que a incidência da doença aumenta com a idade (fator de risco mais importante), a projeção é que o número de pessoas que sofrem dessa doença vá aumentar constantemente no futuro. A terapia dominante para PD se dá através da reposição de dopamina (L-DOPA), utilizada desde os anos 60. Apesar de apresentar alívio dos prejuízos motores, sua eficácia é gradualmente perdida, além de apresentar um grande número de efeitos colaterais como desenvolvimento de movimentos involuntários (discinesias), distúrbios psíquicos e comportamentais, o que justifica a busca por novos alvos e novos fármacos para o tratamento.

Considerando a alta incidência da PD e os debilitantes efeitos colaterais impostos pelo tratamento de escolha, é de grande importância o estudo de uma possível estratégia terapêutica de atenuação dos distúrbios motores e dos processos neurodegenerativos referente aos mecanismos patológicos da PD. Nesse contexto, sabe-se que a guanosina tem demonstrado efeito neuroprotetor em modelos experimentais de diversas doenças neurodegenerativas. Em modelos da PD, a guanosina já demonstrou diminuir sintomas motores induzidos pela reserpina ou contra a discinesia induzida por L-DOPA, além de mostrar proteção contra danos celulares induzidos por toxinas como a 6-OHDA e o MPTP. Apesar desse potencial antiparkinsoniano da guanosina em modelos experimentais da PD, ainda não há um estudo que avalie o(s) alvo(s) molecular(es) dos efeitos da guanosina nessa doença.

Sendo assim, neste tese foi utilizado o modelo de indução de tremores orofaciais para avaliar o papel dos receptores A_1 e A_{2A} de adenosina no efeito motor da guanosina *in vivo* e a incubação com 6-OHDA em fatias de estriado para avaliar, *in vitro*, o

envolvimento desses receptores no efeito neuroprotetor da guanosina. Além disto, esta Tese utilizou um protocolo de transfecção heteróloga dos receptores de interesse para estudar a interação dos oligômeros de receptores de adenosina. A compreensão de como a guanosina interage com o os receptores de adenosina e com o heterômero A_1R-$A_{2A}R$ contribui para o entendimento dos mecanismos de desenvolvimento da PD e a indicação de um possível alvo farmacológico.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Estudar os efeitos da guanosina sobre alterações comportamentais e bioquímicas em modelos experimentais que mimetizam eventos celulares e sintomas motores da doença de Parkinson, avaliando a participação dos receptores de adenosina A_1R e $A_{2A}R$ e a interação da guanosina com o heterômero A_1R-$A_{2A}R$.

3.2 OBJETIVOS ESPECÍFICOS

3.2.1 Investigar o papel dos A_1R e $A_{2A}R$ nos efeitos antiparkinsonianos da guanosina no modelo de tremor orofacial induzida pela reserpina;

3.2.2 Avaliar o papel dos A_1R e $A_{2A}R$ no efeito neuroprotetor da guanosina no protocolo *in vitro* de toxicidade da 6-OHDA em fatias estriatais de rato;

3.2.3 Avaliar a modulação da união e/ou sinalização celular dos A_1R e/ou $A_{2A}R$ e do heterômero A_1R-$A_{2A}R$ como possível sítio de interação da guanosina, em um sistema de transfecção heteróloga de receptores.

4 MATERIAL E MÉTODOS

Os materiais e métodos utilizados para realização dos experimentos contidos nesta tese encontram-se nos respectivos capítulos da mesma, descritos na sessão dos resultados.

5 RESULTADOS

Os resultados desta tese estão divididos na forma de capítulos e serão apresentados na forma de 3 artigos científicos, seguindo a distribuição abaixo:

•Capítulo I: "*O papel dos receptores A_1 e A_{2A} de adenosina na redução de tremores orofacias pela guanosina em animais tratados com reserpina"*;

•Capítulo II: *"Os receptores A_1 e A_{2A} de adenosina estão envolvidos na proteção pela guanosina contra o dano oxidativo e a disfunção mitocondrial induzida pela 6-OHDA em fatias de estriado";*

•Capítulo III: "*Avaliação do heterômero dos receptores A_1 e A_{2A} de adenosina como o alvo molecular para a guanosina".*

CAPÍTULO I

O papel dos receptores de adenosina na redução de tremores orofacias pela guanosina em animais tratados com reserpina

Este capítulo apresenta os resultados relacionados ao objetivo específico I desta tese. Avaliamos o papel dos receptores de adenosina no efeito neuroprotetor e anti-tremor da guanosina em um modelo *in vivo* da PD. Parte deste capítulo (resultados com os animais geneticamente modificados A_{2A}R-KO) foi desenvolvido durante o estágio de doutorado sanduíche (PDSE - CAPES) realizado no *Neuropharmacology and Pain Research Group,* na *Universitat de Barcelona*, em Barcelona – Espanha, sob orientação do Prof. Dr. Francisco Ciruela.

Os resultados estão apresentados em forma de artigo científico intitulado *"Involvement of adenosine A1 and A2A receptors on guanosine-mediated anti-tremor effects in reserpinized mice"* aceito para publicação no periódico *Purinergic Signalling.*

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ORIGINAL ARTICLE

Involvement of adenosine A_1 and A_{2A} receptors on guanosine-mediated anti-tremor effects in reserpinized mice

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Abstract

Parkinson's disease (PD) signs and symptoms regularly include tremor. Interestingly, the nucleoside guanosine (GUO) has already proven to be effective in reducing reserpine-induced tremulous jaw movements (TJMs) in rodent models, thus becoming a promising antiparkinsonian drug. Here, we aimed at revealing the mechanism behind GUO antiparkinsonian efficacy by assessing the role of adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) on GUO-mediated anti-tremor effects in the reserpinized mouse model of PD. Reserpinized mice showed elevated reactive oxygen species (ROS) production and cellular membrane damage in striatal slices assessed ex vivo and GUO treatment reversed ROS production. Interestingly, while the simultaneous administration of sub-effective doses of GUO (5 mg/kg) and SCH58261 (0.01 mg/kg), an $A_{2A}R$ antagonist, precluded reserpine-induced TJMs, these were ineffective on reverting ROS production in ex vivo experiments. Importantly, GUO was able to reduce TJM and ROS production in reserpinized mouse lacking the $A_{2A}R$, thus suggesting an $A_{2A}R$-independent mechanism of GUO-mediated effects. Conversely, the administration of DPCPX (0.75 mg/kg), an A_1R antagonist, completely abolished both GUO-mediated anti-tremor effects and blockade of ROS production. Overall, these results indicated that GUO anti-tremor and antioxidant effects in reserpinized mice were A_1R dependent but $A_{2A}R$ independent, thus suggesting a differential participation of adenosine receptors in GUO-mediated effects.

Keywords Guanosine · Tremor · Reserpine · Adenosine receptors

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. It is mainly characterized by the progressive loss of dopaminergic neurons within the nigrostriatal pathway, which leads to a debilitating motor dysfunction [1]. The cardinal motor symptoms of Parkinsonism include akinesia, bradykinesia, rigidity, and a resting tremor [1]. Tremor can be defined as "a rhythmic and involuntary oscillation of a body part, caused by reciprocal innervations of a muscle, which leads to repetitive, stereotyped contractions with regular frequency and amplitude" [2]. The tremulous jaw movement (TJM) behavior is an extensively validated rodent model of tremor [3]. TJM is characterized by rapid vertical deflections of the lower jaw that resemble chewing but are not directed at any particular stimulus [4]. TJMs are induced by conditions that also lead to parkinsonism in humans (i.e., striatal dopamine depletion, dopamine antagonism, and cholinomimetic activity) [5]. Among them, reserpine, an inhibitor of vesicular monoamine transporter (VMAT-2) that causes monoamine neurotransmitters depletion, induces motor disturbances as hypolocomotion, muscle rigidity, and TJM. Therefore, reserpine administration can be used as a model for screening drugs with potential antiparkinsonian effect [6].

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The purine nucleoside guanosine (GUO), which is able to cross the blood-brain-barrier [7], is an important extracellular signaling molecule at the central nervous system [8]. Accordingly, GUO has been shown to display trophic effects in neural cells and significant neuroprotective effects [9]. Nonetheless, GUO also exerts some behavioral effects in rodents. In line with this, it has been reported that GUO can display anticonvulsive [10], antinociceptive [11], anxiolytic-like [12], and antidepressant-like effects [13]. For these reasons, we have investigated the potential effect of GUO in animal models of parkinsonism. Interestingly, in unilaterally 6-hydroxidopamine-(6-OHDA)-lesioned rats, GUO increased L-DOPA sub-maximal response and decreased L-DOPA-induced dyskinesia (LID). Similarly, GUO also reversed reserpine-induced TJM and catalepsy in mice [14], showing it may be effective for reversing parkinsonian motor impairments. Besides that, GUO also showed protective effects against in vitro cellular models of PD [15–17].

Although the antiparkinsonian-like effects of GUO have been already evaluated, the mechanism of action of this molecule is still unknown. Based on some data reporting anti-ischemic effects of GUO in hippocampal slices and cortical astrocytes, a possible role for adenosine receptors has been suggested [18, 19]. In fact, adenosinergic transmission has been pointed out as a promising therapeutic strategy for motor symptoms of PD [20, 21]. This therapeutic potential is mainly due to the fact that adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) are largely expressed in the striatum and have a key role in modulating dopaminergic neurotransmission [22–28].

Here, we aimed to investigate the potential role of A_1R and $A_{2A}R$ mediating GUO effects in the reserpinized mice by evaluating the behavioral and biochemical effects of GUO in the presence of selective A_1R and $A_{2A}R$ antagonists.

Materials and methods

Animals

Male Swiss mice (central animal facility of Federal University of Santa Catarina) and $A_{2A}R$ knock-out ($A_{2A}R^{-/-}$) mice developed in a CD-1 genetic background (animal facility of University of Barcelona) (30–50 g) were used. Animals were housed and tested in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals [29] and following the European Union directives (2010/63/EU), FELASA and ARRIVE guidelines. The animals were conventionally housed in groups of 4 or 5 in a temperature-controlled (22 °C) and humidity-controlled (66%) environment under a 12-h/12-h light/dark cycle, where food and water intake was ad libitum. The study protocol was approved by the Ethical Committee on Animal Use and Care of the University of Barcelona (CEEA/UB) and Federal University of Santa Catarina (CEUA/UFSC, Protocol PP00955).

Drugs

Reserpine, guanosine (GUO), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) - A_1R antagonist, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH58261) - $A_{2A}R$ antagonist, were from Sigma Chemical, St. Louis, MO.

Reserpine treatment

To induce the TJM behavior, we use a previous standardized protocol [30, 31], where mice were injected twice (every other day) with reserpine (1.0 mg/kg) subcutaneously (s.c.). Reserpine was dissolved in glacial acetic acid and then diluted to a final concentration of 0.1% acetic acid with saline (NaCl 0.9%). Controls were injected with a saline in 0.1% acetic acid solution.

Pharmacological treatment

Guanosine treatment

GUO was dissolved in saline (NaCl 0.9%) and administered in effective or sub-effective doses (7.5 or 5 mg/kg, respectively; [14]) by oral route (p.o.) 20 min prior the behavioral tests and 24 h after the last injection of reserpine. GUO doses were selected from our own group experience [14]. Controls were treated with saline (p.o.).

$A_{2A}R$ experiments

To evaluate the involvement of $A_{2A}R$ on GUO-induced antidyskinetic effect, a dose response of SCH58261 was initially performed. SCH58261 was dissolved in dimethylsulfoxide (DMSO) then in saline to the final desired concentrations, and the behavioral analysis was carried out after 30 min. To analyze a putative potentiation effect with GUO and SCH58261 treatment, they were administered in their sub-effective doses (5 mg/kg p.o. and 0.01 mg/kg i.p., respectively) with 10 min treatment interval. Behavioral tests were conducted 30 min after SCH58261 and 20 min after GUO treatments and 24 h after the last injection of reserpine.

In the $A_{2A}R$-KO mice protocol, mice were treated with GUO (5 or 7.5 mg/kg) 20 min prior the tests and 24 h after the last injection of reserpine.

A_1R experiments

To evaluate the involvement of A_1R on GUO-induced antidyskinetic effect, DPCPX (0.75 mg/kg; dissolved in

DMSO then in saline) was injected via intraperitoneal (i.p.) 30 min prior the GUO active dose administration (7.5 mg/kg, p.o.). The dose of DPCPX was selected on the basis of literature data on oral tremor [32].

Tremulous jaw movements

Tremulous jaw movements (TJMs) were defined as rapid vertical deflections of the lower jaw that resembled chewing but were not directed at any particular stimulus [4]. This protocol was initially standardized to rats [4, 33] and we adapted the protocol to mice based on previous published studies [30, 31]. To quantify the occurrence of this orofacial dyskinesia, mice were placed individually in a glass cylinder (13 cm diameter) and hand-operated counters were employed to score TJM frequencies. Mirrors were placed under the floor and behind the back wall of the cylinder to allow observation when the animal was faced away from the observer. If TJM occurred during a period of grooming, they were not taken into account. The incidence of these oral movements was measured continuously for 10 min.

Brain slices

Animals were euthanized by decapitation and brains were quickly removed and the cerebral cortex, hippocampus, and striatum were rapidly dissected in ice-cold KREBS ringer buffer (KRB) (122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM D-glucose, bubbled with 95% O_2/5% CO_2 up to pH 7.4) [19]. For the biochemical assays, slices (0.4 mm) were prepared using a Mcllwain Tissue Chopper (The Mickle Laboratory Engineering Co. ltd., England) and separated in KRB at 4 °C. After sectioning, slices were incubated in KRB for 30 min, at 37 °C, for recovery.

ROS levels

ROS production was measured by using the molecular probe 2,7-dichlorofluorescein diacetate (H_2DCFDA, Sigma Chemical, St. Louis, MO.). H_2DCFDA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent form 2′,7′-dichlorofluorescein (DCFH). DCFH reacts with intracellular ROS (such as H_2O_2) to form dichlorofluorescein (DCF), a green fluorescent dye. DCF fluorescence intensity is proportional to the amount of ROS. Brain slices were incubated with 80 μM of H_2DCFDA for 30 min at 37 °C and then washed in KRB. Fluorescence was read with the multifunctional microplate reader Infinite M200 (Tecan Group Ltd., Mannedorf, Switzerland), using excitation and emission wavelengths of 480 and 525 nm, respectively [19].

Membrane integrity evaluation

Membrane integrity was assessed by evaluating the uptake of the fluorescent exclusion dye, propidium iodide (PI, Sigma Aldrich, St Louis, MO, USA), which is a polar compound that enters only in cells with damaged membranes. Once inside the cells, PI complexes with DNA and emits an intense red fluorescence (630 nm) when excited by green light (495 nm) [34]. Slices were incubated with PI (7 μg/mL) for 30 min at 37 °C, and then washed with KRB for analysis on fluorescence microplate reader Infinite M200 from Tecan®.

Mitochondrial membrane potential

Mitochondrial membrane potential was measured by using the molecular probe tetramethylrhodamine ethyl ester (TMRE, Sigma Chemical, St. Louis, MO.) 100ηM for 30 min at 37 °C. Fluorescence was measured with the multifunctional microplate reader Infinite M200 from Tecan®, using wavelengths of excitation and emission of 550 and 590 ηm, respectively [35].

MTT reduction assay

Cellular viability in slices was quantified by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical, St. Louis, MO.) to a dark violet formazan product by dehydrogenases. Slices were incubated with MTT (0.5 mg/mL) in KRB buffer for 20 min at 37 °C, the formazan produced was solubilized by replacing the medium with 200 μL of DMSO, resulting in a colored compound which was quantified spectrophotometrically at a wavelength of 550 ηm. Absorbance was measured with the multifunctional microplate reader Infinite M200 from Tecan®. The results are expressed and normalized as percentages relative to the control conditions.

Data analysis

Data are represented as means ± S.E.M. Normalized data from multiple experiments were averaged and statistical analysis was carried out as described in the figure legends. Data with two groups were analyzed by Student's *t* test, and other data used one-way or two-way ANOVA followed by Tukey's post hoc. Statistical difference was accepted when $P < 0.05$.

Results

Guanosine effects on the reserpinized mice

Initially, cortical, hippocampal, and striatal slices were used to biochemical evaluations, as cellular viability of

slices, ROS production, mitochondrial membrane potential, and cell membrane permeabilization. Reserpine administration caused no alteration in cortical or hippocampal slices (Fig. 1a–d). In striatal slices, reserpine administration did not alter cellular viability and mitochondrial membrane potential (Fig. 1a, c). However, striatal slices showed an increase of 50% in ROS production (P = 0.017) and an increase of 58% in PI incorporation (P = 0.046) by reserpine treatment as compared with control (Fig. 1b, d).

Accordingly, we obtained striatal slices and assessed GUO effects reversing reserpine-induced ROS production, and PI incorporation. The dose of 7.5 mg/kg of GUO was used based on a previous study, in which we described that GUO (7.5 mg/kg) decreased TJM frequency in reserpinized mice [14]. Interestingly, we could observe that GUO totally reversed reserpine-induced ROS production in striatal slices (P = 0.031), while it failed to block PI incorporation (P = 0.982) (Fig. 2a, b).

Involvement of adenosine A_{2A}R on guanosine-mediated TJM and ROS decrease

It is known that A_{2A}R antagonism has effects on motor disturbances related to PD [32, 36–38]. Therefore, we aimed to see if GUO effect of reducing TJM and ROS generation in the striatum could be related to antagonism of A_{2A}R. Firstly, a *dose-response curve* was performed with SCH58261 (Fig. 3a). The highest dose of SCH58261 (0.1 mg/kg) fully reversed the TJM by reserpine (P = 0.042) while the lowest dose (0.001 mg/kg) had no effect (P = 0.893). The 0.01 mg/kg dose was sub-effective (i.e., presented statistical difference either from control or to reserpine group). Then, we evaluated the effect of co-treatment of SCH58261 and GUO sub-effective doses. We previously showed that 5 mg/kg GUO presented a sub-effective effect on reserpine-induced TJM [14]. The co-administration of sub-effective doses of SCH58261 (0.01 mg/kg) and GUO

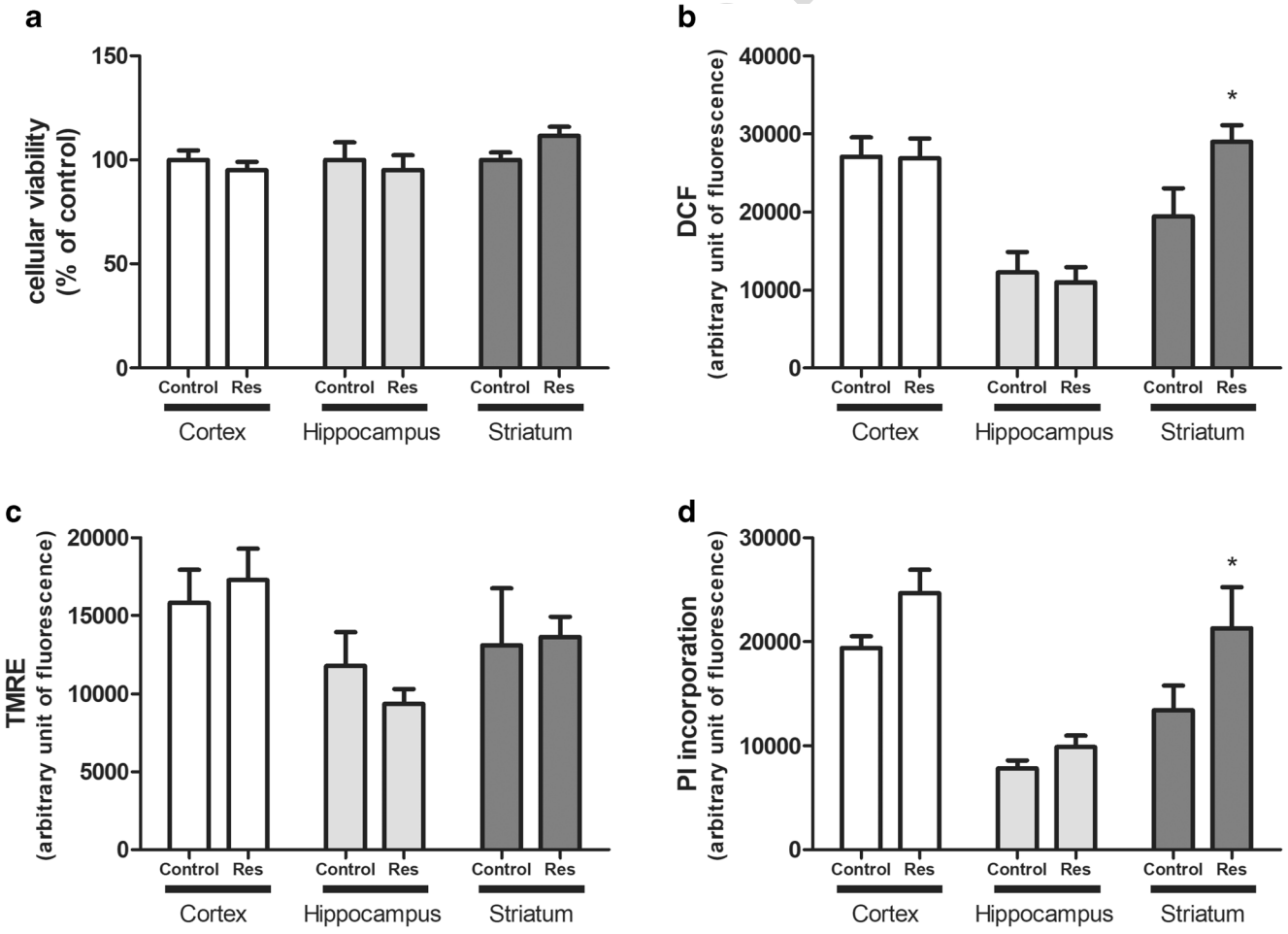

Fig. 1 Evaluation of reserpine (Res, 1 mg/kg) neurotoxicity in cortical, hippocampal, and striatal slices. **a** Cellular viability measured by MTT reduction, expressed as percentage of control. **b** ROS measurement through fluorescence of DCF dye. **c** Evaluation of mitochondrial membrane potential with TMRE fluorescent dye. **d** Membrane integrity evaluation due to PI incorporation. Fluorescence data are shown as arbitrary fluorescent unit. Results are presented as means ± SEM (*P < 0.05 vs control; Student's t test; n = 7)

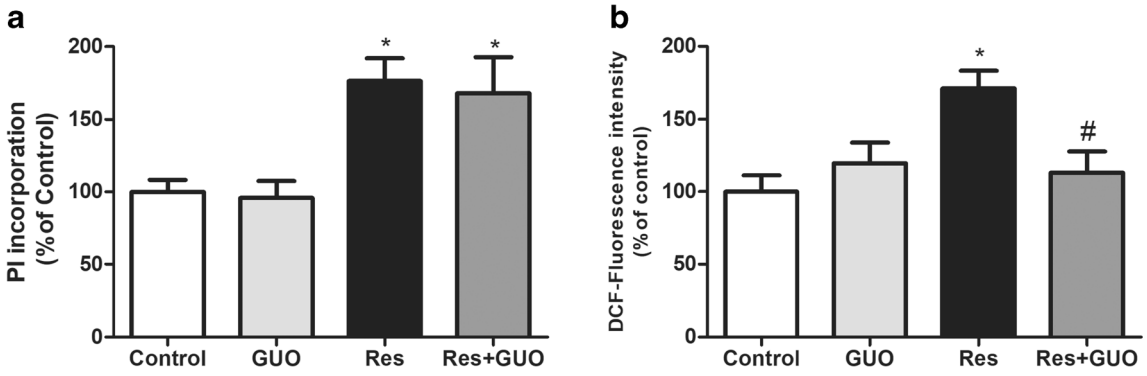

Fig. 2 Guanosine protective ex vivo effect in reserpinized mice. **a** GUO (7.5 mg/kg) effect on ROS production and **b** cellular membrane permeability through PI incorporation in striatal slices of mice treated with reserpine. Results are presented as means ± SEM.; $^{\#}P < 0.05$ vs reserpine (two-way ANOVA with Tukey's post hoc test; $n = 5$)

(5 mg/kg) completely reversed the reserpine-induced orofacial tremor ($P = 0.004$) (Fig. 3b). Regarding ROS generation, treatment with sub-effective dose of SCH58261 (0.01 mg/kg), or with sub-effective GUO dose (5 mg/kg), displayed no statistically significant effect of decreasing ROS production ($P = 0.111$ and 0.553, respectively). Co-administration of SCH58261 and GUO also showed no significant difference from reserpine-treated animals ($P = 0.287$) (Fig. 3c).

To clarify the role of $A_{2A}R$ on GUO effect in the reserpine-induced TJM and striatal ROS production, genetic-modified mice $A_{2A}R$ deficient ($A_{2A}R$-KO) were used. Animals were subjected to the same protocol of reserpine and GUO treatment. The sub-effective GUO dose (5 mg/kg) had no effect against reserpine in these animals, both in the TJM quantification ($P = 0.362$) and ROS measurement ($P = 0.807$) (Fig. 4a). On the other hand, GUO at 7.5 mg/kg dose presented an effect of reversing the reserpine induction of TJM ($P = 0.017$) and ROS increase ($P = 0.040$) (Fig. 4b), indicating that presence of $A_{2A}R$ is not necessary to GUO effect in reserpinized mice.

Involvement of adenosine A_1R on guanosine-mediated TJM and ROS decrease

To test the involvement of A_1R, 24 h after the last reserpine administration, mice were treated with the A_1R antagonist DPCPX (0.75 mg/kg i.p. [32], 30 min prior the GUO administration. DPCPX treatment did not alter reserpine-induced TJM ($P = 0.999$), but it completely blocked the effect of GUO on TJM frequency ($P = 0.0003$) (Fig. 5a).

As GUO (7.5 mg/kg) showed effect through reverting ROS increase by reserpine, we aimed to see if this effect is related to A_1R. Prior treatment with the A_1R antagonist DPCPX (0.75 mg/kg) did not significantly alter ROS increase induced by reserpine ($P = 0.383$) but it prevented the reversion of GUO ($P = 0.912$) (Fig. 5b). These data suggest a strong dependence of A_1R for GUO behavioral and biochemical effects.

Discussion

GUO treatment shows a promising effect on animal models of motor disorders. We already shown that in unilaterally 6-

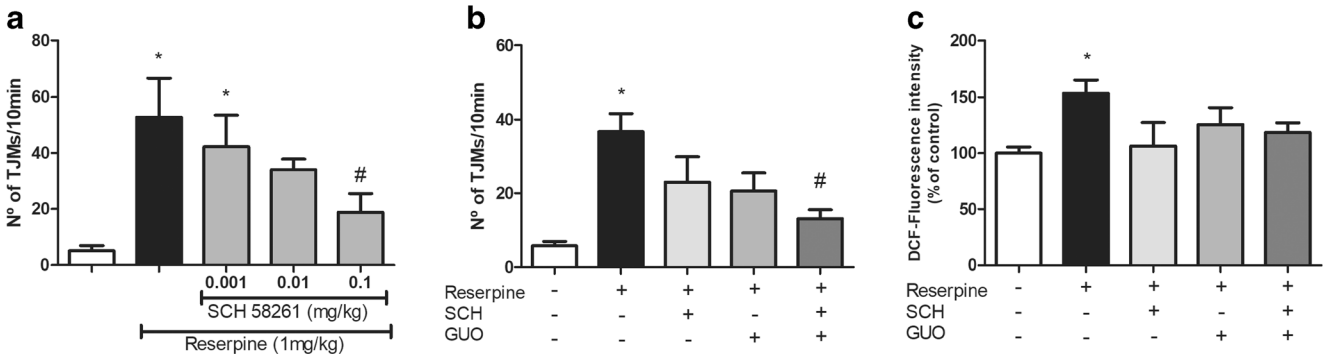

Fig. 3 Involvement of $A_{2A}R$ on reserpine-induced TJM and ROS production. **a** Dose-response curve of $A_{2A}R$ antagonist SCH58261 (0.001; 0.01 and 0.1 mg/kg) in reserpine-induced oral tremor (TJM). **b** SCH58261 (0.01 mg/kg) plus GUO (5 mg/kg) effect on reserpine-induced TJM. **c** SCH58261 (0.01 mg/kg) and GUO (5 mg/kg) effect on ROS increase in striatal slices of reserpinized mice. Results are presented as means ± SEM ($^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs reserpine; one-way ANOVA with Tukey's post hoc test; $n = 8$–10)

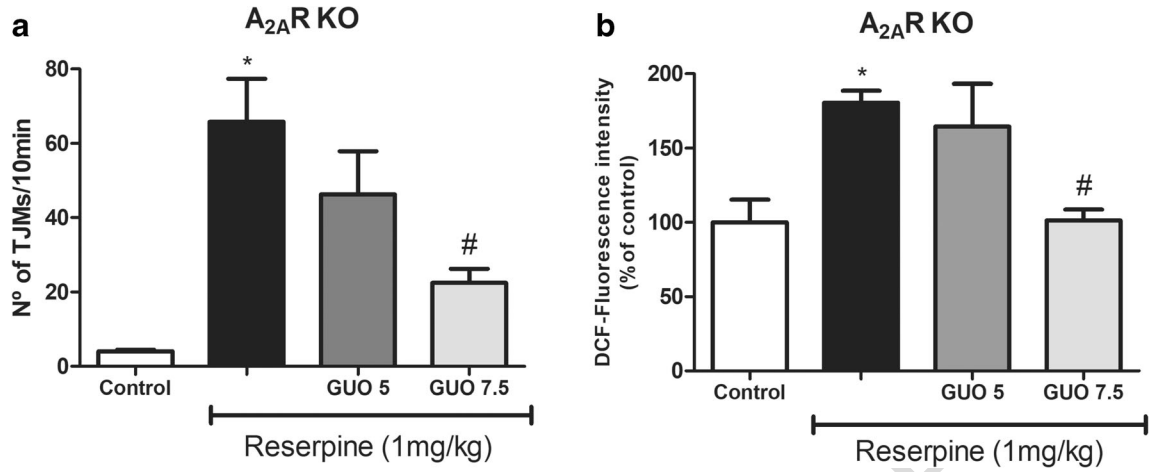

Fig. 4 Effect of guanosine on reserpine-induced TJM and ROS production in A_{2A}R-deficient (A_{2A}R-KO) mice. **a** TJM in mice treated with GUO sub-effective (5 mg/kg) or effective (7.5 mg/kg) doses ($n = 6$). **b** ROS production in striatal slices of mice treated GUO (5 mg/kg or 7.5 mg/kg, $n = 3$). Results are presented as means ±SEM (*$P < 0.05$ vs control; #$P < 0.05$ vs reserpine; one-way ANOVA with Tukey's post hoc test)

hydroxidopamine-(6-OHDA)-lesioned rats, GUO increased L-DOPA sub-maximal response and decreased LID. Also, GUO reversed reserpine-induced TJM and catalepsy in mice [14]. In this study, we investigated the mechanisms behind this GUO effect of reducing the orofacial tremor and the striatal oxidative damage evoked by reserpine, by assessing the possible involvement of adenosine receptors in the GUO effects.

The mechanism related to the induction of oral tremor is multifaceted, with multiple neurotransmitters, including GABA, serotonin, adenosine, and acetylcholine, interacting with dopamine in the regulation of basal ganglia motor functions [4, 39–43]. In this study, we focused in a possible therapeutic approach towards adenosinergic transmission.

A_{2A}R antagonists have emerged as a potential treatment of parkinsonian motor impairments as they can exert allosteric modulations upon D2R ligands [44]. Also, A_{2A}R is highly expressed in the striatum and it was shown that its antagonism reduces oral tremor in different rodent models [32, 36, 45–48]. Accordingly, SCH58261, the A_{2A}R antagonist tested in this study, exhibited effect in the reserpine-induced TJM in mice. Moreover, SCH58261 and GUO sub-effective doses potentiated each other's effect on TJM behavior. Despite the potentiated effect observed with SCH58261 and GUO co-treatment on reserpine-induced TJM, when GUO was tested in genetic-modified animals lacking adenosine A_{2A} receptors (A_{2A}R-KO), it was observed that this receptor is not essentially involved in the GUO antidyskinetic effect. Therefore, we decided to test the participation of A_1R on behavioral and biochemical GUO effects.

It is known that adenosine A_1R can antagonistically modulate D1R responses and that the stimulation of A_1R could inhibit the D1R stimulation [25, 49–51]. Interestingly, rats treated with reserpine (1 mg/kg; s.c.) for 5 days showed an

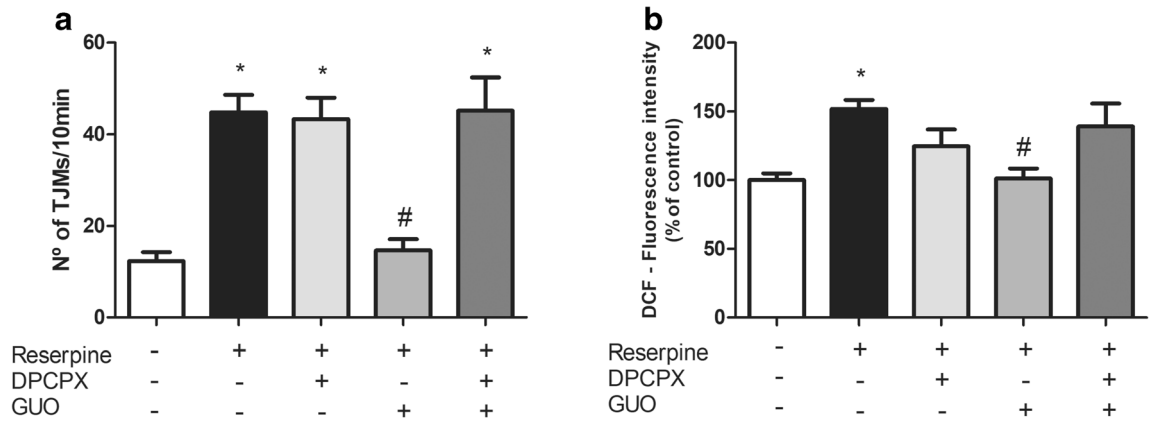

Fig. 5 Effect of A_1R blockade on reserpine-induced TJM and ROS production in mouse striatal slices. **a** Effect of A1R antagonist DPCPX (0.75 mg/kg i.p.) plus GUO (7.5 mg/kg) on TJM of mice. **b** Evaluation of GUO (7.5 mg/kg) plus DPCPX (0.75 mg/kg) effect on ROS increase in striatal slices of reserpinized mice. Results are presented as means ± SEM (*$P < 0.05$ vs control; #$P < 0.05$ vs reserpine; one-way ANOVA with Tukey's post hoc test; $n = 5$–6)

increase in the responsiveness of adenylate cyclase after D1R stimulation [52]. Also, rats treated with one single dose of reserpine (1 mg/kg; i.p.) showed upregulation of the transduction mechanism associated with D1R, without changing the activity of D2R [53]. Evidence from our results suggests that GUO motor effect could be through A_1R stimulation. In fact, as results with A_1R stimulation for oral tremor are lacking in the literature, we tested reserpinized mice with 2-chloro-N-6-cyclopentyladenosine (CCPA), an A_1R agonist, and a potent antidyskinetic effect was observed (in the doses 0.0125, 0.025, and 0.05 mg/kg). However, we also observed that CCPA promotes an important sedative response in these animals (data not shown) that precludes its use as a treatment against motor impairments. In this sense, A_1R stimulation promoted by GUO could be inhibiting the overstimulation of D1R in reserpinized mice, and then decreasing the oral tremor.

Besides the motor disturbance, we also investigated biochemical changes in reserpinized mice in different cerebral structures (i.e., cerebrocortex, hippocampus, and striatum). It is well known that the inhibition of dopamine vesicular storage leads to an increase in ROS; this occurs because dopamine metabolism intrinsically results in ROS formation [54]. The major area affected was the striatum, where it was seen an increase in ROS production and permeabilization of the cellular membrane. Thus, oxidative and cell membrane damage in the striatum might sum up to the monoamine depletion to impair motor performance. This increase in ROS nearby the cell membrane could cause its oxidation and lead to an injury in the membrane lipids as it was seen on incorporation of PI. In fact, some studies with the same reserpine protocol have already shown an increase on lipid peroxidation in striatum [30, 55]. This increase in ROS production and cell membrane permeabilization may reflect early events of toxicity by reserpine but surprisingly, we did not see alteration in the cell reductive capacity, assessed by MTT reduction method. Accordingly, the reserpine toxicity in this protocol does not affect the mitochondrial membrane potential. More important, GUO acutely administrated was able to reverse ROS increase induced by reserpine, and this effect was dependent on A_1R and not $A_{2A}R$.

To our knowledge, our previous study was the first to identify GUO treatment as an antiparkinsonian agent in a rodent model of orofacial tremor [14]. Although other studies have shown the protective effect of GUO in cellular models of PD [15, 17] or in vivo rodent models of PD [56], none of them has assessed the molecular targets related to GUO effects. Despite this, evidence from other brain disease models has pointed to GUO effect via adenosine receptor modulation. In an in vitro ischemia model, hippocampal slices subjected to oxygen/glucose deprivation presented increased ROS production prevented by GUO, but this effect is abolished by preincubation with DPCPX [19]. These data corroborate with the idea that GUO effect of preventing an oxidative damage is A_1R dependent. Notwithstanding, in the same ischemia protocol, not only DPCPX but also an $A_{2A}R$ agonist (CGS21680) blunted the protective effect of GUO in hippocampal slices [19] and in cortical astrocytes [18]. Likewise, the ischemia model in $A_{2A}R$-KO animals implies GUO-protective effects upon $A_{2A}R$ in the hippocampus [57]. As different results obtained with $A_{2A}R$-KO mice may be dependent on the cerebral area analyzed, there is still controversy regarding GUO effects via adenosine A_1R or $A_{2A}R$ interaction, and additionally, the possibility of GUO interaction with adenosine receptor heteromers. More important, a recent study from our group shed some light on this issue of GUO interaction on A_1R and/or $A_{2A}R$. We showed that GUO-induced effects may require both A_1R and $A_{2A}R$ co-expression in transfected HEK293 cells, indicating that GUO acts on adenosine receptors in an oligomeric conformation, i.e., the A_1R-$A_{2A}R$ heteromer [57]. Once GUO acts upon A_1R-$A_{2A}R$ heteromer formation, its effects on other receptor oligomeric organization of A_1R and/or $A_{2A}R$ are possible and were still not evaluated. Thus, it is feasible to speculate that in the striatum, GUO can interact with A_1R and then modulated D1R or A_1R-D1R heteromer interaction, and further investigations are necessary to clarify GUO mechanism on motor control.

In conclusion, our results strengthen the demonstration of extracellular actions of GUO and the dependence of adenosine A_1R activation to the motor-related effect of GUO. Considering the GUO-mediated motor improvement differs mechanistically from classic adenosine receptor modulators, it is important to understand the mechanisms behind GUO effects.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Ethical approval The study protocol was approved by the Ethical Committee on Animal Use and Care of the University of Barcelona (CEEA/UB) and Federal University of Santa Catarina (CEUA/UFSC, Protocol PP00955).

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CAPÍTULO II

Os receptores A_1 e A_{2A} de adenosina estão envolvidos na proteção pela guanosina contra o dano oxidativo e a disfunção mitocondrial induzida pela 6-OHDA em fatias de estriado

Este capítulo apresenta os resultados relacionados ao objetivo específicos II desta tese. Avaliamos o efeito neuroprotetor da guanosina em um modelo *in vitro* que foi padronizado em nosso laboratório como objetivo de identificar possíveis agentes neuroprotetores para os eventos celulares relacionados a PD. Os resultados referente ao efeito da guanosina em fatias de estriado de ratos expostos à 6-OHDA *in vitro* e estão apresentados como o artigo científico intitulado *"Adenosine A_1 and A_{2A} receptors are involved on guanosine protective effects against oxidative burst and mitochondrial dysfunction induced by 6-OHDA in striatal slices"*.

Adenosine A_1 and A_{2A} receptors are involved on guanosine protective effects against oxidative burst and mitochondrial dysfunction induced by 6-OHDA in striatal slices

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Running title: Guanosine prevents striatal damage via A_1R and $A_{2A}R$

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Abstract

6-Hydroxydopamine (6-OHDA) is the most used toxin in experimental Parkinson's disease (PD) models. 6-OHDA shows high affinity for the dopamine transporter and once inside the neuron, it accumulates and undergoes non-enzymatic auto-oxidation, promoting reactive oxygen species (ROS) formation and selective damage of catecholaminergic neurons. In this way, our group has established a 6-OHDA *in vitro* protocol with rat striatal slices as a rapid and effective model for screening of new drugs with protective effects against PD. We have shown that co-incubation with guanosine (GUO, 100 μM) prevented the 6-OHDA-induced damage in striatal slices. The exactly GUO mechanism of action remains unknown. The aim of this study was to investigate if adenosine A_1 (A_1R) and/or A_{2A} receptors ($A_{2A}R$) are involved on GUO protective effects on striatal slices. Pre-incubation with DPCPX, an A_1R antagonist, prevented guanosine effects on 6-OHDA-induced ROS formation and mitochondrial membrane potential depolarization, while CCPA, an A_1R agonist, did not alter GUO effects. Regarding $A_{2A}R$, the antagonist SCH58261 had similar protective effect as GUO in ROS formation and mitochondrial membrane potential. Additionally, SCH58261 did not affect GUO protective effects. The $A_{2A}R$ agonist CGS21680, although, completely blocked GUO effects. Finally, the A_1R antagonist DPCPX, and the $A_{2A}R$ agonist CGS21680 also abolished the preventive guanosine effect on 6-OHDA-induced ATP levels decrease. These results clearly indicate a dependence on adenosine receptors modulation to GUO protective effects and also point to a putative interaction with A_1R-$A_{2A}R$ heteromer as its molecular target.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by bradykinesia, tremor and rigidity, caused by the progressive loss of the dopaminergic neurons in the nigrostriatal pathway (Hirsch et al. 1992). Although the etiology of PD is considered idiopathic, it can be related to other factors as genetics, environmental toxins, oxidative stress or mitochondrial abnormalities (Pereira and Garrett 2010). The main therapeutic approach is based on dopamine replacement to promote motor symptoms relief, however it do not stop the progression of the disease (Poewe 2009). The molecular trigger to neuronal degeneration may involve the oxidative burst, mitochondrial dysfunction and bioenergetics impairment (Beal 2005, Lin and Beal 2006, Johri and Beal 2012), thus new strategies of treatment aiming protection of dopaminergic neurons are necessary.

The experimental study of PD relies, mainly, in the use of animal models administered with toxins to mimic the neurodegeneration in the nigrostriatal pathway. 6-hydroxydopamine (6-OHDA) was the first discovered drug that has specific toxicity for dopaminergic neurons (Ungerstedt 1968) and, it is the most used toxin in experimental PD models (Blandini, Armentero, and Martignoni 2008, Gomez-Lazaro et al. 2008, Mu et al. 2009). Because of the similar structure, 6-OHDA also shows affinity for the dopamine transporters (Lehmensiek et al. 2006) and it accumulates inside the neurons, where undergoes an auto-oxidation, promoting selective damage of catecholaminergic neurons (Lehmensiek et al. 2006, Blandini, Armentero, and Martignoni 2008).

In this way, our group has established a 6-OHDA *in vitro* model with rat brain slices, showing that *in vitro* incubation with 6-OHDA induced a decrease in cellular viability, increase in ROS production and a disruption in mitochondrial membrane

potential in striatal slices (Massari et al. 2016). Moreover, we have shown that co-incubation with guanosine (GUO) prevented the 6-OHDA-induced damage in striatal slices (Marques, Massari, and Tasca 2019). GUO is a purine nucleoside, which has demonstrated neuroprotective effects in several animal and cellular models of neurotoxic conditions and neurodegenerative diseases (Lanznaster et al. 2016).

Regarding PD, it is already known that GUO exerts protective effects against *in vitro* 6-OHDA toxicity in two cell lines (C6 glioma and dopaminergic human SH-SY5Y neuroblastoma cells) (Giuliani et al. 2015, Giuliani et al. 2012). Besides that, GUO also have effects on *in vivo* PD models. In unilaterally 6-OHDA-lesioned rats, GUO acutely administered increased L-DOPA sub-maximal response and decreased L-DOPA-induced dyskinesia, i.e. GUO potentialized the L-DOPA effects diminishing its side effects. In the same way, GUO also reversed reserpine-induced motor disturbance in mice (Massari et al. 2017). In search for the molecular target of GUO, our group has already implied adenosine receptors modulation with GUO effects in an ischemic-like damage, in hippocampal slices and cortical astrocytes (Dal-Cim et al. 2019, Dal-Cim et al. 2013). In fact, adenosinergic transmission has been pointed out as a promising therapeutic strategy for motor symptoms of PD (Yabe et al. 2017, Suzuki et al. 2018). This therapeutic potential is mainly due the fact that adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) are largely expressed in the striatum and have a key role in modulation of dopaminergic transmission (Palmer and Stiles 1995, Krügel et al. 2003).

Since the GUO mechanism of action it is still not clearly identified, it is of great interest to understand the signaling behind its effects acting as neuroprotective agent. Therefore, the aim of this study was to investigate if adenosine A_1R and/or $A_{2A}R$ are involved on GUO protective effects on striatal slices against oxidative damage, mitochondrial dysfunction and ATP depletion due to 6-OHDA-induced toxicity *in vitro*.

Materials and methods

Animals

Male Wistar rats (3 months old, 350–400 g) were obtained from our local colony, maintained in a 12-h dark/light cycle, at constant room temperature at 23 ± 1 °C and with food and water *ad libitum*. Experiments followed the ARRIVE Guidelines published in 2010 and were approved by the local Ethical Committee for Animal Research (CEUA/UFSC PP00955).

Brain slices

Animals were euthanized by decapitation and the whole brain were quickly removed and the striatum was rapidly dissected in ice-cold Krebs Ringer buffer (KRB) (122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM D-glucose, bubbled with 95% O_2/ 5% CO_2 up to pH 7.4). Slices (0.4 mm) were prepared using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. ltd., England) and separated in KRB at 4°C. After sectioning, slices were randomly selected (3 slices per group) and incubated in 24-well cell culture plate with KRB (1 mL) for 30 minutes, at 37ºC, for metabolic recovery from slicing procedure.

Slices treatment

6-OHDA (Sigma, St. Louis, MO, USA) was solubilized in water at 0.1% sodium metabisulfite and stored at –20 °C. For the experiment, 6-OHDA was diluted to 100 μM in KRB. To investigate 6-OHDA-induced damage, slices from the striatum were selected and exposed to 6-OHDA (100 μM) during 1 h (Massari et al. 2016). GUO (Sigma, St. Louis,MO, USA) was freshly prepared and diluted in KRB (100 μM) and co-incubated with 6-OHDA for 1 h to determine its neuroprotective effect. To

investigate the role of A_1R or $A_{2A}R$ in the GUO neuroprotective effect, slices were pre-incubated with agonists or antagonists of A_1R (CCPA and DPCPX, respectively) and $A_{2A}R$ (CGS21680 and SCH58261) 15 min prior to the incubation with 6-OHDA and/or GUO (Fig. 1A). Concentrations of adenosine receptors ligands were selected based on previous studies (Dal-Cim et al. 2013, Almeida et al. 2016). Slices of control group were incubated in a physiological KRB. All experimental groups were assayed in triplicates. After the 1 h of co-incubation with 6-OHDA and GUO, ROS production, mitochondrial membrane potential, or intracellular ATP levels were evaluated.

ROS levels

ROS production was measured by using the molecular probe 2,7-dichlorofluorescein diacetate (H_2DCFDA, Sigma Aldrich, St Louis, MO, USA). H_2DCFDA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent form 2′,7′-dichlorofluorescein (DCFH). DCFH reacts with intracellular ROS (such as H_2O_2) to form dichlorofluorescein (DCF), a green fluorescent dye. DCF fluorescence intensity is proportional to the amount of ROS. Striatal slices were incubated with 80 μM of H_2DCFDA diluted in KRB (1 mL) for 30 min at 37°C. Slices were then washed with and maintained in KRB (1 mL) for fluorescence measurement. Fluorescence was read using excitation and emission wavelengths of 480 and 525 nm, respectively in a fluorescence microplate reader (TECAN®). Results were obtained as arbitrary unit of fluorescence and were expressed in percentage related to control levels.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was measured by using the molecular probe tetramethylrhodamine ethyl ester (TMRE, Sigma Aldrich, St Louis, MO, USA). TMRE is a cell-permeant, cationic, red-orange fluorescent dye that is readily

sequestered by active mitochondria. Slices were incubated with 10 nM TMRE diluted in KRB (1 mL) for 30 min at 37°C. Slices were then washed with and maintained in KRB (1 mL) for fluorescence measurement. Fluorescence was measured using wavelengths of excitation and emission of 550 and 590 nm, respectively. The results are expressed and normalized as percentages relative to the control conditions. Results were obtained as arbitrary unit of fluorescence and were expressed in percentage related to control levels.

ATP levels

After GUO and 6-OHDA treatment brains slices from striatum were homogenized in trichloroacetic acid (TCA) 2% aqueous solution (350 µL). The homogenates were centrifuged at 14000 rpm at 4°C for 3 minutes. The supernatants (100 µL) were used for determination of ATP levels, using bioluminescent assay kit according to the manufacturer's recommendations (#FLAA, Sigma Aldrich, St Louis, MO, USA). The amount of protein in each sample was measured using the method of (LOWRY et al. 1951) and the results are expressed in µmol ATP/µg of protein in each sample (3 slices for group).

Statistical Analysis

Results are expressed as means ± standard error (SEM). Comparisons among experimental and control groups were performed by one-way ANOVA followed by the Tukey post hoc test. Statistical difference was accepted when $p < 0.05$.

Results

A_1R modulation

As we previously shown, GUO (100 μM) protects striatal slices against *in vitro* 6-OHDA-induced mitochondrial membrane depolarization and increased ROS generation (Marques, Massari, and Tasca 2019). So, we aimed to investigate whether these effects were related to A_1R modulation. Slices incubated with 6-OHDA (100 μM) showed a decrease in the florescence of the TMRE dye, that is related to a mitochondrial membrane depolarization, as the same effect was observed when slices were incubated with carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 10 μM), a mitochondrial oxidative phosphorylation uncoupler (Massari et al. 2016). Interestingly, when slices were pre-incubated with the A_1R agonist, CCPA (100 nM), it prevented the 6-OHDA-induced mitochondrial membrane depolarization (Fig. 1A). However, the pre-incubation with CCPA did not affect the GUO protective effect on mitochondrial membrane potential (ΔΨ). Regarding ROS levels, CCPA pre-incubation had no effect on 6-OHDA-induced ROS increase and did not alter the protective effect of GUO. These results suggest that activation of A_1R does not counteract the GUO effects.

On the other hand, pre-incubation with DPCPX (250 nM), an A_1R antagonist, showed to be ineffective in preventing the mitochondrial membrane depolarization and ROS increase caused by 6-OHDA (Fig. 2C, D). Besides that, DPCPX pre-incubation totally abolished GUO effects on 6-OHDA-induced mitochondrial membrane depolarization and ROS increase. These experiments indicate that blocking A_1R also blocks the GUO effects, showing an A_1R dependence on GUO protective effects.

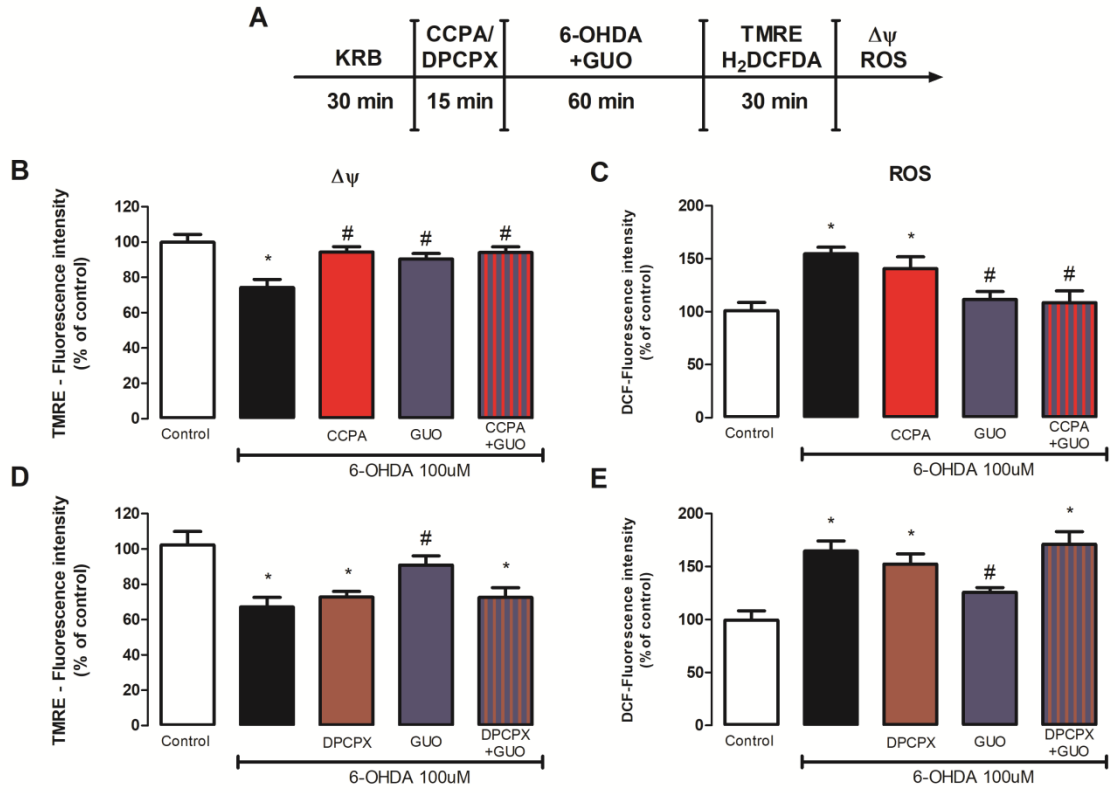

Figure 1. Effects of A_1R modulation on 6-OHDA-induced toxicity in striatal slices. Experimental design is describe in **(A)**. Striatal slices were pre-incubated with A_1R agonist CCPA (100 nM; **B, C**) and A_1R antagonist DPCPX (250 nM; **D, E**) . Evaluation of 6-OHDA (100 μM) incubation and co-incubation with GUO (100 μM) on mitochondrial membrane potential (ΔΨ) **(B, D)** and ROS levels **(C, E)**. Data are expressed as percentage of controls normalized among individual experiments and represent means with SEM (n = 6). (*) when $p < 0.05$ compared with control or (#) compared to 6-OHDA group (one-way ANOVA followed by Tukey's test).

$A_{2A}R$ modulation

A putative $A_{2A}R$ dependence on the GUO protective effects in the 6-OHDA *in vitro* protocol was also assessed in striatal slices (Fig. 2A). The pre-incubation with the $A_{2A}R$ agonist CGS 21680 (30 nM) exerted no effect on 6-OHDA-induced mitochondrial membrane potential (ΔΨ) depolarization and ROS levels increase, however it completely blocked the GUO protective effect in both ΔΨ and ROS levels (Fig. 2 B, C). These results indicate that $A_{2A}R$ activation can affect GUO effects. The blockade of $A_{2A}R$ is already seen as an anti-parkinsonian strategy, as it has protective effects on

many PD models. In this way, pre-incubation of SCH58261 (50 nM), an $A_{2A}R$ antagonist, presented a protective effect upon OHDA-induced ΔΨ depolarization and ROS levels increase (Fig 2 C, D). Moreover, this protective effect of SCH58261 does not affect GUO effects.

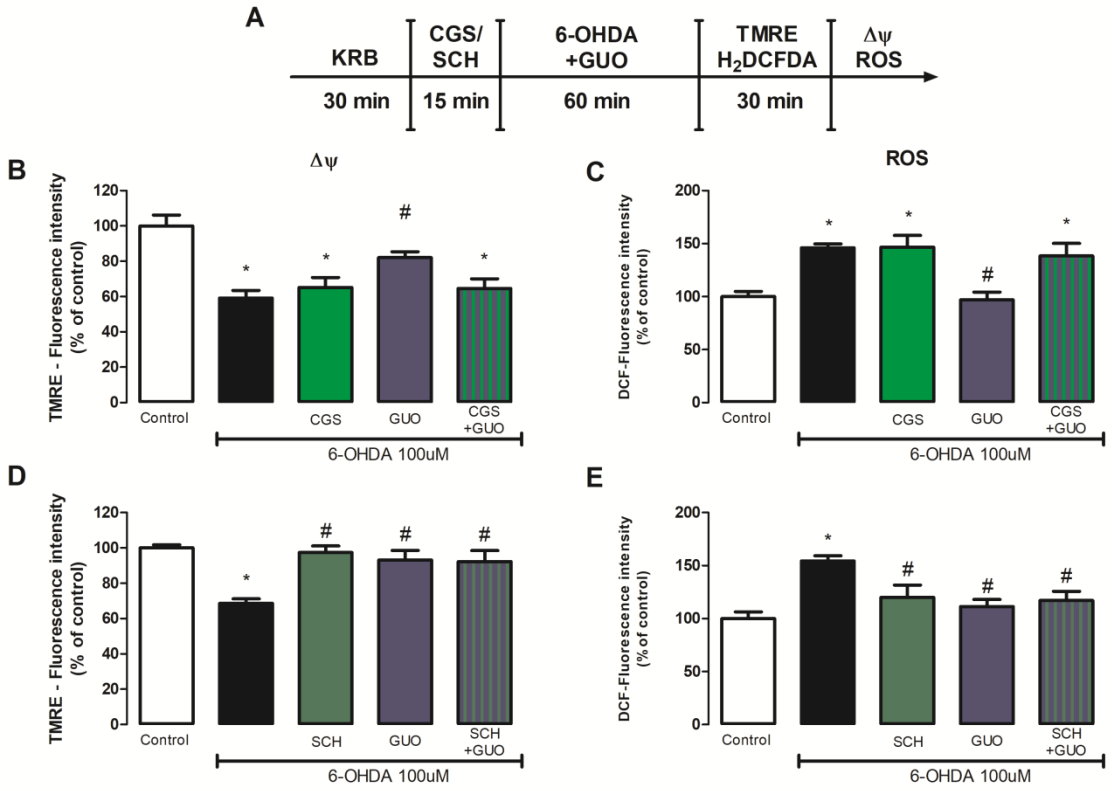

Figure 2. Effects of $A_{2A}R$ modulation on 6-OHDA-induced toxicity in striatal slices. Experimental design is describe in **(A)**. Striatal slices were pre-incubated with of $A_{2A}R$ agonist CGS 21680 (CGS, 30 nM; **B, C**) and $A_{2A}R$ antagonist SCH 58261 (SCH, 50 nM; **D, E**). Slices were incubated with 6-OHDA (100 µM) and/or co-incubated with GUO (100 µM). 6-OHDA-induced mitochondrial membrane depolarization **(B, D)** and ROS levels increase **(C, E)**. Data are expressed as percentage of controls normalized among individual experiments and represent means with SEM (n = 6). (*) when $p < 0.05$ compared with control or (#) compared to 6-OHDA group (one-way ANOVA followed by Tukey's test).

ATP levels

As mitochondrial depolarization might cause ATP depletion and 6-OHDA also causes changes in the cellular energetic balance (Marques, Massari, and Tasca 2019) we

evaluate the effects of adenosine receptors ligands on 6-OHDA and/or GUO incubation, by measuring ATP levels in striatal slices. Since the blockade of A_1R or activation of $A_{2A}R$ interfered on the GUO neuroprotective effect, we performed ATP measurements with DPCPX and CGS21680. As expected, 6-OHDA incubation decreased the ATP levels and GUO co-incubation prevented this decrease (Fig. 3). Although neither DPCPX nor CGS21680 presented effect on ATP levels decrease by 6-OHDA, both ligands impaired the capability of GUO to prevent it. These results are in line with the observed for $\Delta\Psi$ and ROS levels, indicating that GUO effects are related to both A_1R and $A_{2A}R$.

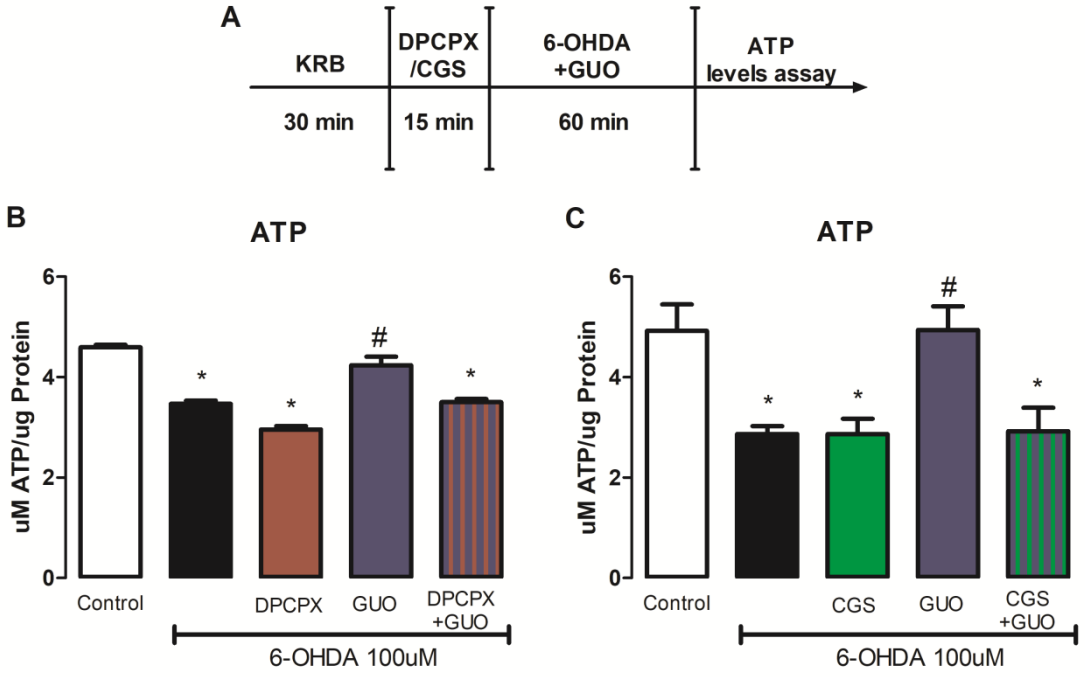

Figure 3. A_1R and $A_{2A}R$ modulation on ATP levels in striatal slices. Experimental design is describe in **(A).** Effect of pre-incubation of A_1R antagonist, DPCPX (250 nM) **(B)** and $A_{2A}R$ agonist, CGS21680 (CGS, 30 nM) **(C)** on 6-OHDA-induced ATP depletion. Data are expressed as µmol ATP/µg of protein of each sample and represent means with SEM (n = 3). (*) when $p < 0.05$ compared with control or (#) compared to 6-OHDA group (one-way ANOVA followed by Tukey's test)

Discussion

In this study, we investigated the modulation of A_1R and $A_{2A}R$ upon GUO effects against the cellular damage caused by *in vitro* incubation with 6-OHDA in *ex vivo* slices obtained from rat striatum. This *in vitro* protocol of 6-OHDA-induced toxicity is a simple and sensitive protocol that completely suits our goal of evaluating the mechanism of neuroprotection afforded by GUO. We have already shown that co-incubation with GUO (100 μM) prevented the striatal slices against oxidative damage, mitochondrial dysfunction and ATP depletion caused by 6-OHDA (Marques, Massari, and Tasca 2019). GUO is a naturally occurring guanine-based purine that has been pointed out to act as a neuromodulator and a neuroprotective agent (Tasca et al. 2018, Lanznaster et al. 2016). Other studies have already shown the protective effect of GUO in models of PD, both *in vitro* (Giuliani et al. 2015, Giuliani et al. 2012, Li et al. 2014) and *in vivo* rodent models of PD (Su et al. 2009, Massari et al. 2017), but none of them have assessed the molecular targets related to GUO effect.

Among the new classes of drugs developed to improve the clinical features of PD, $A_{2A}R$ antagonists appear to be the most promising. $A_{2A}R$ blockade has been demonstrated to be effective in both preclinical and clinical PD studies (Vallano et al. 2011, Pinna 2014, Jenner 2014). Indeed, istradefylline (KW6002), an $A_{2A}R$ antagonist, was already approved for clinical use in Japan, and recently, in USA (Kondo, Mizuno, and Group 2015, Hussar 2020, Dungo and Deeks 2013). Interestingly, the mechanism behind $A_{2A}R$ antagonists in PD may rely in part to the existing functional and molecular interaction (i.e., heteromerization) of $A_{2A}R$ and D_2R within postsynaptic striatal neurons (Fuxe et al. 2005, Fernández-Dueñas et al. 2015). Moreover, a mutual trans-inhibition between these two receptors has been described (Ferré et al. 2016). Corroborating with this, in our results the $A_{2A}R$ antagonist SCH58261 was effective in both parameters

analyzed. More important, the protective effect of SCH58261 does not affect GUO effects, suggesting that they do not interfere in each other mechanism.

In addition to this postsynaptic site of action, $A_{2A}R$ can also form heteromeric complexes with A_1R in presynaptic neurons of the basal ganglia, where they can control glutamate release (Ciruela et al. 2006) and striatal circuits independently of dopaminergic signaling (Schiffmann et al. 2007). Regarding PD, rare mutations on A_1R gene could lead to PD (Blauwendraat et al. 2017). Also, some studies show that A_1R modulation could control and improve motor function associated with PD (Mango et al. 2014, Rivera-Oliver et al. 2019, Cortés et al. 2019). In fact, a lot of data show that A_1R stimulation is neuroprotective (Mitchell et al. 1995, Kawamura, Ruskin, and Masino 2019, Cunha 2016, Duarte, Cunha, and Carvalho 2016). Surprisingly, CCPA was unable to prevent the ROS increase induced by 6-OHDA, but it did protected mitochondrial function. Nevertheless, the potential use of A_1R-based therapies, by using A_1R agonists, could lead to deleterious peripheral side-effects, once A_1R is also expressed in the vascular system.

In relation to GUO, evidences from other disease models have pointed to its effect via adenosine receptors modulation. In an *in vitro* brain ischemia model, hippocampal slices subjected to oxygen/glucose deprivation presented increased ROS production prevented by GUO, but this effect is abolished by pre-incubation with DPCPX (Dal-Cim et al. 2013). Notwithstanding, in the same protocol, not only DPCPX but also CGS21680 blunted the protective effect of GUO in hippocampal slices (Dal-Cim et al. 2013) and in cortical astrocytes (Dal-Cim et al. 2019). The same pattern of results was seen in our study that used an *in vitro* PD model and evaluated other brain area. Moreover, in the ischemia model, hippocampal slices of $A_{2A}R$-knockout animals subjected to oxygen/glucose privation, the GUO-protective effects is abolished,

evidencing the importance of this receptor for GUO effects. Taken together, these observations strengths the possible mechanism of GUO-effects through the dependence of adenosine A_1 and A_{2A} receptors modulation.

Unfortunately, there is still controversy data regarding GUO effect via A_1R or $A_{2A}R$ interaction, and the possibility of GUO interaction with adenosine receptor heteromers appears to be the most likely scenario to explain this debate (Ciruela 2013). In fact, we recently showed that GUO-induced effects require both A_1R and $A_{2A}R$ co-expression in transfected HEK293 cells (Lanznaster et al. 2019). Only in cells expressing both A_1R and $A_{2A}R$, GUO was able to decrease $A_{2A}R$ binding affinity and cAMP response evoked by a selective $A_{2A}R$ ligand. Also, GUO had no effect on A_1R signaling through intracellular calcium increase, even in the presence or absence of $A_{2A}R$ co-expression (Lanznaster et al. 2019). Considering all these evidences, our working hypothesis is that we need to interpret GUO interaction with adenosine receptors results not as separated receptors but as a heteromeric entity. GUO could be acting as a negative modulator of $A_{2A}R$, but only in the presence of A_1R. It is feasible that the physical interaction between A_1R and $A_{2A}R$ could lead to an increase in affinity for GUO. This could explain why GUO effects are blocked by CGS21680 and DPCPX (by causing allosteric modulation of GUO $A_{2A}R$ affinity) and not by SCH58261 and CCPA. Indeed, GUO modulation over A_1R-$A_{2A}R$ heteromer or A_1R or $A_{2A}R$ individual entities could vary among brain structures, once that it may depend on receptors expression. Therefore, we also cannot exclude the possibility of GUO acting through other heteromer, that could modulate or be associated with A_1R or $A_{2A}R$, and further investigations are necessary to detail GUO mechanism of action.

In conclusion, we demonstrated that GUO protective effects on oxidative damage, mitochondrial dysfunction and ATP depletion caused by 6-OHDA in rat

striatal slices are sensitive of both A_1R and $A_{2A}R$ modulation. These results indicated another GUO action through the A_1R-$A_{2A}R$ heteromer and highlight its importance as a neuroprotective agent in PD.

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CAPÍTULO III

Avaliação do heterômero dos receptores A_1 e A_{2A} de adenosina como o alvo molecular para a guanosina

Este capítulo apresenta os resultados relacionados ao objetivo específicos III desta tese. Em um modelo *in vitro,* com expressão heteróloga do A_1R e/ou $A_{2A}R$ e do heterômero A_1R-$A_{2A}R$, avaliamos o efeito de união e sinalização da guanosina em relação a esses receptores. Este capítulo foi desenvolvido durante o estágio de doutorado sanduíche (PDSE - CAPES) realizado no *Neuropharmacology and Pain Research Group,* na *Universitat de Barcelona*, em Barcelona – Espanha, sob orientação do Prof. Dr. Francisco Ciruela. Os resultados estão apresentados como o artigo científico intitulado *"Adenosine A_1-A_{2A} receptor heteromer: contribution to guanosine-mediated effects"* publicado no periódico *Cells.*

Cabe salientar que os resultados da Figura 1 deste artigo são relativos à tese de Doutorado da Drª. Débora Lanznaster pelo programa de Pós-Graduação em Neurociências em 2016, também em colaboração com o Prof. Dr. Francisco Ciruela.

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Adenosine A_1-A_{2A} Receptor-Receptor Interaction: Contribution to Guanosine-Mediated Effects

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Abstract: Guanosine, a guanine-based purine nucleoside, has been described as a neuromodulator that exerts neuroprotective effects in animal and cellular ischemia models. However, guanosine's exact mechanism of action and molecular targets have not yet been identified. Here, we aimed to elucidate a role of adenosine receptors (ARs) in mediating guanosine effects. We investigated the neuroprotective effects of guanosine in hippocampal slices from $A_{2A}R$-deficient mice ($A_{2A}R^{-/-}$) subjected to oxygen/glucose deprivation (OGD). Next, we assessed guanosine binding at ARs taking advantage of a fluorescent-selective $A_{2A}R$ antagonist (MRS7396) which could engage in a bioluminescence resonance energy transfer (BRET) process with NanoLuc-tagged $A_{2A}R$. Next, we evaluated functional AR activation by determining cAMP and calcium accumulation. Finally, we assessed the impact of A_1R and $A_{2A}R$ co-expression in guanosine-mediated impedance responses in living cells. Guanosine prevented the reduction of cellular viability and increased reactive oxygen species generation induced by OGD in hippocampal slices from wild-type, but not from $A_{2A}R^{-/-}$ mice. Notably, while guanosine was not able to modify MRS7396 binding to $A_{2A}R$-expressing cells, a partial blockade was observed in cells co-expressing A_1R and $A_{2A}R$. The relevance of the A_1R and $A_{2A}R$ interaction in guanosine effects was further substantiated by means of functional assays (i.e., cAMP and calcium determinations), since guanosine only blocked $A_{2A}R$ agonist-mediated effects in doubly expressing A_1R and $A_{2A}R$ cells. Interestingly, while guanosine did not affect A_1R/$A_{2A}R$ heteromer formation, it reduced $A_{2A}R$ agonist-mediated cell impedance responses. Our results indicate that guanosine-induced effects may require both A_1R and $A_{2A}R$ co-expression, thus identifying a molecular substrate that may allow fine tuning of guanosine-mediated responses.

Keywords: guanosine; neuroprotection; oxygen/glucose deprivation; NanoBRET; A_1R/$A_{2A}R$ heteromer

1. Introduction

Guanosine is a guanine-based purine nucleoside that has been shown to exert neuroprotective and neurotrophic effects in both in vitro and in vivo studies (for review, see [1]). Thus, it has been postulated as a good candidate for the management of several central nervous system (CNS) disorders, including neurodegenerative diseases (i.e., Parkinson's, Alzheimer's) or ischemia [1,2]. Brain ischemia is one of the major health disability conditions worldwide [3]. It occurs after a blood supply collapse that leads to a reduced level of oxygen and glucose within the affected brain area. Similarly, upon excitotoxicity and oxidative stress a failure of cellular bioenergetics occurs [4]. Importantly, a neuroprotective role of guanosine has been extensively investigated in animal and cellular models of ischemia, excitotoxicity and oxidative stress [5–10]. Indeed, we have demonstrated that guanosine prevents reactive oxygen species (ROS) generation and cell death in hippocampal slices subjected to the oxygen/glucose deprivation (OGD) [11].

The mechanism by which guanosine exerts its neuroprotective effects is still intriguing. Despite the identification of a putative guanosine binding site in rat brain membranes [12], a specific guanosine receptor has not yet been discovered. Importantly, it has been hypothesized that adenosine receptors (ARs) may play a role in mediating guanosine effects, although with some controversy. For instance, it has been reported that AR selective ligands do not compete for guanosine binding to rat brain membranes [13,14], whereas AR ligands were able to block some of the guanosine-dependent neuroprotective effects [15]. In line with this, a selective adenosine A_1 receptor (A_1R) antagonist (DPCPX, 8-cyclopentyl-1,3-dipropylxanthine) and a selective A_{2A} receptor ($A_{2A}R$) agonist (CGS21680, 2-(4-(2-carboxyethyl)phenethylamino)-5'-*N*-ethylcarboxamidoadenosine) inhibited guanosine-mediated neuroprotection in hippocampal slices subjected to OGD [11]. Overall, these findings, including those using multimodal A_1R and $A_{2A}R$ ligand treatments, supported the notion that both A_1R and $A_{2A}R$ would participate in guanosine-mediated effects.

Interestingly, it has been hypothesized that adenosine A_1 and A_{2A}receptor-receptor interactions (i.e., heteromerization) might be behind some of the guanosine-mediated effects, thus pointing to the $A_1R/A_{2A}R$ heteromer as a putative molecular target for guanosine [16]. Indeed, the existence of $A_1R/A_{2A}R$ heteromers has been demonstrated in presynaptic terminals of striatal neurons controlling glutamate release [17], thus acting as an adenosine concentration-dependent switch [18]. Consequently, low to moderate concentrations of adenosine predominantly activate A_1R within the $A_1R/A_{2A}R$ heteromer (i.e., inhibiting glutamate release), whereas moderate to high concentrations of adenosine also activate $A_{2A}R$, which, by means of the A_1R-$A_{2A}R$ intramembrane negative allosteric interaction, antagonizes A_1R function, therefore facilitating glutamate release. Altogether, in view of the already known experimental indications, the $A_1R/A_{2A}R$ heteromer might be viewed as a potential target for guanosine, thus deserving further attention. Here, we aimed to assess the role of A_1R and $A_{2A}R$ interaction in guanosine-mediated effects. First, we studied the neuroprotective effects of guanosine in an ex vivo model of brain ischemia, both in wild-type and $A_{2A}R$ deficient ($A_{2A}R^{-/-}$) mice; subsequently, we aimed to elucidate, in vitro, both the putative guanosine binding and activation of the $A_1R/A_{2A}R$ heteromer.

2. Materials and Methods

2.1. Chemicals

The ligands used were: adenosine and guanosine from Sigma-Aldrich (St. Louis, MO, USA); CGS21680 and SCH442416 (2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo [4,3-*e*]-[1,2,4]triazolo [1,5-*c*]pyrimidin-5-amine) from Tocris Bioscience (Ellisville, MI, USA). Adenosine deaminase (ADA) was purchased from Roche Diagnostics (GmbH, Mannheim, Germany) and zardaverine from Calbiochem (San Diego, CA, USA). MRS7396, which is a selective fluorescent antagonist at the $A_{2A}R$ derived from SCH442416, was previously described [19].

2.2. Animals

Wild-type and $A_{2A}R^{-/-}$ CD-1 male and female mice [20] weighing 25–50 g were used at 2–3 months of age. The University of Barcelona Committee on Animal Use and Care (CEEA-UB) approved the protocol (Code 10033, 04/02/2018). Animals were housed and tested in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals [21] and following the European Union directives (2010/63/EU), FELASA and ARRIVE guidelines. Mice were housed in groups of five in standard cages with ad libitum access to food and water and maintained under a 12-h dark/light cycle (starting at 7:30 AM), 22 °C temperature, and 66% humidity (standard conditions).

2.3. OGD Protocol

Mice were euthanized by cervical dislocation and hippocampi rapidly removed and placed in an ice-cold Krebs-Ringer bicarbonate buffer (KRB) (composition in mM: 122 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 0.4 KH_2PO_4, 25 $NaHCO_3$ and 10 D-glucose). The buffer was bubbled with 95% O_2/5% CO_2 up to pH 7.4. Slices (0.3 mm) were prepared using a Leica VT1200 vibrating blade microtome (Leica, Wetzlar, Germany) in KRB at 4 °C, and one slice per tube was allowed to recover for 30 min in KRB at 37 °C. Control hippocampal slices were incubated until the end of the experiment (15 min plus 2 h) in oxygenated KRB. OGD was induced by incubating the slices for a 15 min period in an OGD buffer in Hank's balanced salt solution (HBSS; composition in mM: 1.3 $CaCl_2$, 137 NaCl, 5 KCl, 0.65 $MgSO_4$, 0.3 Na_2HPO_4, 1.1 KH_2PO_4, and 5 HEPES), where 10 mM D-glucose was replaced by 10 mM 2-deoxy-glucose and equilibrated with a 95% N_2/5% CO_2 gas mixture, as described previously [5] After 15 min of OGD the media of the slices was replaced by oxygenated KRB and maintained for 2 h for evaluation of cellular viability and ROS generation. Guanosine (100 μM), when present, was added 15 min before (in KRB) and during OGD (in OGD buffer), and maintained in the re-oxygenation period (2 h), when the OGD buffer was replaced by physiological KRB.

2.4. Cellular Viability Evaluation

For cellular viability assessment, slices were incubated in 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) for 20 min at 37 °C, as previously described [22]. In brief, the tetrazolium ring of MTT is first cleaved by active dehydrogenases to produce a precipitated formazan. Then, precipitated formazan can be solubilized with 200 μL of dimethyl sulfoxide (DMSO) and cellular viability quantified spectrophotometrically at a wavelength of 550 nm by means of a POLARstarplate-reader (BMG Labtech, Durham, NC, USA).

2.5. Measurement of ROS Production

For evaluating ROS generation, slices were incubated with 80 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) for 30 min [23]. Then, subsequent to the OGD/reoxygenation protocol, slices were washed twice with KRB and maintained for 15 min before adding DCFH-DA. H_2DCFDA diffuses through the cell membrane, and it is hydrolyzed by intracellular esterases to the non-fluorescent form dichlorofluorescin (DCFH). Afterwards, DCFH can react with intracellular H_2O_2 to form dichlorofluorescein (DCF), a green fluorescent dye. Slices were then transferred to a 96-well black plate containing 200 μL of KRB, and fluorescence was read (excitation 480 nm, emission 525 nm) using a POLARStar plate reader (BMG Labtech).

2.6. Plasmid Constructs

The cDNA encoding the human A_1R tagged at its N-terminal tail with the O6-alkylguanine-DNA alkyltransferase (i.e., A_1R^{SNAP}) cloned in pRK5 vector (BD PharMingen, San Jose, CA, USA) was a gift from Prof. Jean-Philippe Pin (CNRS, Montpellier, France). Thus, to perform functional assays $A_{2A}R^{SNAP}$ [24] and A_1R^{SNAP} were used. Also, $A_{2A}R^{RLuc}$ and A_1R^{YFP} constructs [17] were used to perform classical BRET (Bioluminescence Resonance Energy Transfer) assays. Finally, to perform

NanoBRET experiments with the MRS7396 fluorescent antagonist, we created an $A_{2A}R$ NanoLuc sensor ($A_{2A}R^{NL}$). To this end, the cDNA encoding the human $A_{2A}R$ was amplified by polymerase chain reaction from the pECFP-$A_{2A}R$ vector using the primers: FA2AEco (5′-GCCG**GAATTC**CCCATCATGGGCTCC TCGGTGTAC-3′) and RA2ANot (5′-CGCG**GCGGCCGC**tcaggacactcctgctccatcctggg-3′). The amplified $A_{2A}R$ insert was then cloned into the *Eco*RI/*Not*I sites of pNLF1-secN vector (Promega, Stockholm, Sweden) containing a hemagglutinin (HA) epitope tag. All the constructs were verified by DNA sequencing.

2.7. Cell Culture and Transfection

Human embryonic kidney (HEK)-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL streptomycin, 100 mg/mL penicillin and 5% (*v*/*v*) fetal bovine serum at 37 °C and in an atmosphere of 5% CO_2. HEK-293T cells growing in 60 cm^2 plates were transfected with the cDNA encoding the different plasmids using linear PolyEthylenImine reagent (PEI) (Polysciences Inc., USA).

2.8. NanoBRET Experiments

The NanoBRET assay was performed on stably expressing ($A_{2A}R^{NL}$) HEK-293T cells, transiently transfected (or not) with A_1R^{SNAP}, according to [25]. In brief, cells were re-suspended in HBSS, and seeded into poly ornithine coated white 96-well plates. After 24 h, cells were challenged with/without the non-labelled $A_{2A}R$ antagonist (SCH442416) or guanosine and incubated for 1 h at 37 °C. Subsequently, the fluorescent ligand (MRS7396) was added and the plate and returned to 37 °C for 1 h. Finally, coelenterazine-h (Life Technologies Corp.) was added at a final concentration of 5 μM, and readings were performed after 5 min using a CLARIOStar plate reader (BMG Labtech). The donor and acceptor emissions were measured at 490–510 nm and 650–680 nm, respectively. The raw NanoBRET ratio was calculated by dividing the 650 nm emission by the 490 nm emission. In competition studies, results were expressed as a percentage of the maximum signal obtained (mBU; milliBRET Units).

2.9. cAMP Assay

cAMP accumulation was measured using the LANCE® Ultra cAMP Kit (PerkinElmer, Waltham, MA, USA) as previously described [26]. In brief, transfected ($A_{2A}R^{SNAP}$ or $A_{2A}R^{SNAP}$ + A_1R^{SNAP}) HEK-293T cells were firstly incubated for 1 h at 37 °C with stimulation buffer (BSA 0.1%, ADA 0.5 units/mL, zardaverine 2 μM; in serum-free DMEM) and later on with CGS21680 for 30 min at 37 °C. Thereafter, cells were transferred to a 384-well plate in which reagents were added following manufacturer's instructions. After 1 h at room temperature, Time-Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) was determined by measuring light emission at 620 nm and 665 nm by means of a CLARIOstar plate reader (BMG Labtech).

2.10. Intracellular Calcium Determinations

The A_1R-mediated intracellular Ca^{2+} accumulation was assessed by means of a luciferase reporter assay based on the expression of the nuclear factor of activated T-cells (NFAT), as previously described [27]. In brief, cells were transfected with the cDNA encoding the A_1R, the NFAT-luciferase reporter (pGL4-NFAT-RE/luc2p; Promega) and the yellow fluorescent protein (pEYFP-N1; Promega). After 36 h post-transfection, cells were incubated with the indicated drugs for 6 h. Subsequently, cells were harvested with passive lysis buffer (Promega), and the luciferase activity of cell extracts was determined using a luciferase Bright-Glo™assay (Promega) in a POLARStar plate-reader (BMG Labtech) using a 30-nm bandwidth excitation filter at 535 nm.

2.11. Label-Free Cellular Impedance Assay

The xCELLigence Real-Time Cell Analyzer (RTCA) system (ACEA Biosciences, San Diego, CA, USA) was employed to measure changes in cellular impedance correlating with cell spreading and tightness, thus being widely accepted as a morphological and functional biosensor of cell status [28–30]. Thus, 16-well E-plates (ACEA Biosciences) were coated with 50 µL fibronectin (10 µg/mL) at 37 °C for 1 h before being washed three times with 100 µL MilliQ-water before use. The background index for each well was determined with 90 µL of stimulation buffer (supplemented DMEM with ADA 0.5 U/mL and zardaverine 10 µM) in the absence of cells. Data from each well were normalized to the time point just before compound addition using the RTCA software providing the normalized cell index (NCI). Subsequently, HEK-293T cells permanently expressing the $A_{2A}R^{SNAP}$ construct [31] in the absence or presence of A_1R^{SNAP} (90 µL resuspended in stimulation buffer) were then plated at a cell density of 40,000 cells/well and grown for 18 h in the RTCA SP device station (ACEA Biosciences) at 37 °C and in an atmosphere of 5% CO_2 before ligand (i.e., CGS21680 and/or guanosine) addition. Cell index values were obtained immediately following ligand stimulation every 15 s for a total time of at least 50 min. For data analysis, the area under the curve (AUC) for each NCI trace response was quantified and normalized to the basal.

2.12. Statistics

Data are represented as mean ± standard error of mean (SEM). The number of samples/animals (n) in each experimental condition is indicated in the corresponding figure legend. Comparisons among experimental groups were performed by Student's t-test and ANOVA, using GraphPad Prism 6.01 (San Diego, CA, USA), as indicated. Statistical difference was accepted when $p < 0.05$.

3. Results

3.1. Guanosine-Mediated Neuroprotection in Hippocampal Slices Depends on $A_{2A}R$ Expression

It has been postulated that ARs might be involved in guanosine-mediated responses in vivo [16]. Within this line of inquiry, we first interrogated whether $A_{2A}R$ expression is necessary for guanosine-mediated neuroprotection, a well-known guanosine effect in vivo [1]. To this end, we subjected hippocampal slices from wild-type (i.e., $A_{2A}R^{+/+}$) and $A_{2A}R^{-/-}$ mice to an OGD protocol in the presence or absence of guanosine. Indeed, significant cell death ($p < 0.001$) and ROS production ($p = 0.0359$) were observed in $A_{2A}R^{+/+}$ hippocampal slices subjected to the OGD protocol (Figure 1A,B). Interestingly, guanosine (100 µM) was able to prevent these effects, thus cellular viability significantly increased ($p = 0.0012$) and ROS production decreased ($p = 0.0389$) (Figure 1A,B), as previously reported [5,11]. Importantly, under the same experimental conditions, in hippocampal slices obtained from $A_{2A}R^{-/-}$ mice, guanosine failed to prevent OGD-mediated cell death ($p = 0.005$) and ROS production ($p = 0.0279$) (Figure 1A,B), thus losing its neuroprotective effect. Overall, these results suggested that $A_{2A}R$ expression was necessary for guanosine-mediated neuroprotection.

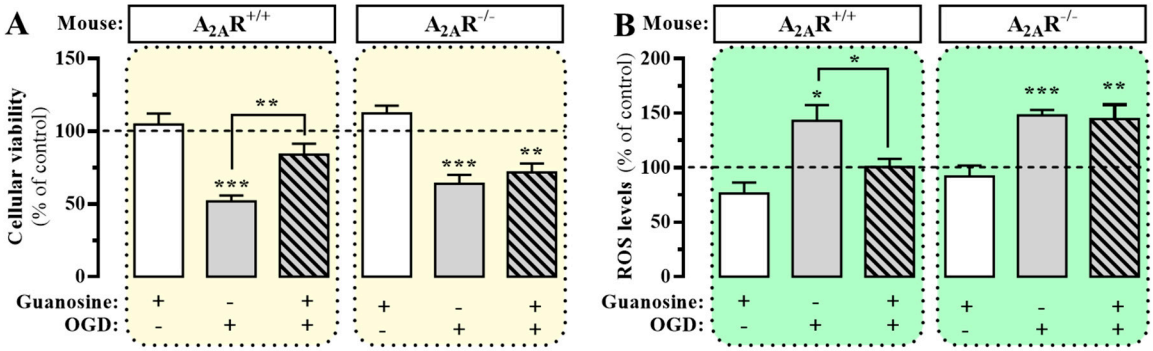

Figure 1. Guanosine-mediated neuroprotection in mouse hippocampal slices. Hippocampal slices from $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice were subjected to oxygen/glucose deprivation (OGD) in the absence or presence of guanosine (100 μM) for 15 min before, and during OGD and re-oxygenation. The cellular viability (**A**) was assessed by MTT reduction whereas ROS levels (**B**) were measured after incorporation of the DCFDA fluorescent probe. Results were normalized to the control slices (vehicle-treated slices, dashed line) and expressed as mean ± SEM of three independent experiments performed in triplicate. The asterisks indicate statistically significant differences (* p <0.05, ** p < 0.01 and *** p < 0.001; one-way ANOVA with Tukey's post-hoc test).

3.2. *$A_{2A}R$ Ligand Binding is Affected by Guanosine upon A_1R Coexpression*

Once we demonstrated that the neuroprotective effect of guanosine was $A_{2A}R$-dependent, we aimed to assess the putative direct interaction of guanosine with $A_{2A}R$ through ligand binding studies. To this end, we engineered a fluorescent ligand BRET-based assay to assess $A_{2A}R$ ligand binding in living cells (Figure 2A). We used a fluorescent $A_{2A}R$ antagonist (MRS7396) that is able to engage in a BRET process upon interacting with a cell surface $A_{2A}R$ tagged with the NanoLuciferase (NL) at its N-terminus (i.e., $A_{2A}R^{NL}$) (Figure 2A). MRS7396 is a BODIPY630/650 derivative of SCH442416 [19], which upon $A_{2A}R$ binding can act as an acceptor chromophore for NanoLuciferase emission (490 nm) in a BRET process. Thus, we challenged stable $A_{2A}R^{NL}$-expressing cells with increasing concentrations of MRS7396, in the presence/absence of non-labelled SCH442416. Interestingly, a bell-shaped binding saturation hyperbola, with a K_D = 4.8 ± 2.7 nM, was obtained for MRS7396, while in the presence of a saturating concentration of SCH442416 (1 μM) the binding was displaced (Figure 2B). Our results showed that the NanoBRET binding assay was a robust and reliable way to assess $A_{2A}R$ ligand binding. Accordingly, we next assessed possible guanosine effects on $A_{2A}R$ orthosteric binding by performing a competition assay with a fixed concentration of MRS7396 (10 nM) (occupying ~80% of receptors at equilibrium) and increasing concentrations of guanosine. Interestingly, under these experimental conditions, guanosine was unable to alter MRS7396 binding to $A_{2A}R^{NL}$ (Figure 2C), thus indicating that guanosine does not orthosterically bind to $A_{2A}R$, as previously reported [12,13].

Since $A_{2A}R$ heteromerizes with A_1R [17], and some of the physiological effects of guanosine were modulated by A_1R ligands [32,33], we investigated whether $A_1R/A_{2A}R$ heteromer formation affected AR-related guanosine-dependent effects. To this end, we first recreated the formation of $A_1R/A_{2A}R$ heteromers in HEK-293T cells by transfecting $A_{2A}R^{RLuc}$ and A_1R^{YFP} constructs and monitoring $A_{2A}R/A_1R$ heteromerization by a classical BRET approach (Figure A1). Interestingly, neither adenosine nor guanosine incubation altered $A_1R/A_{2A}R$ heteromer formation (Figure A1). Subsequently, we assessed the impact of A_1R co-expression in $A_{2A}R$ binding of MRS7396 using our NanoBRET binding assay. Notably, in A_1R-$A_{2A}R$ doubly expressing cells, guanosine (100 μM) was able to significantly reduce by 19 ± 4% (p = 0.0138) the binding of MRS7396 to the $A_{2A}R^{NL}$, thus indicating that the $A_1R/A_{2A}R$ heteromer might play a potential role in AR-related guanosine-dependent effects (Figure 2C).

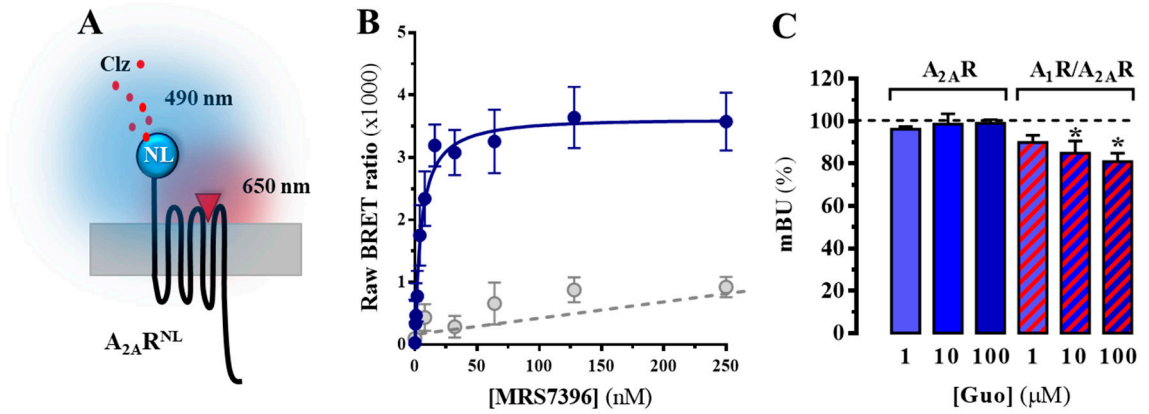

Figure 2. NanoBRET-based $A_{2A}R$ binding determinations. (**A**) Schematic representation of the NanoBRET-based assay using $A_{2A}R^{NL}$ stably expressing cells and the fluorescent MRS7396 ligand (red triangle). When the coelenterazine (Clz) substrate is metabolized by NanoLuciferase (NL), its 475 nm light emission may engage in a BRET process with MRS7396 given the close proximity (i.e., bound to $A_{2A}R^{NL}$). (**B**) NanoBRET signal for $A_{2A}R^{NL}$ with increasing MRS7396 concentrations in the absence (solid line) and presence (dotted line) of 1 μM SCH442416. (**C**) Guanosine (Guo) effects on MRS7396 binding to cells expressing $A_{2A}R^{NL}$ (blue bars) or $A_{2A}R^{NL}$ plus A_1R^{SNAP} (red dashed bars). Cells were incubated with MRS7396 (10 nM) and increasing guanosine concentrations (1–100 μM) in the presence or absence of 1 μMSCH442416 to allow specific binding calculations. Results were normalized to the MRS7396 specific binding in the absence of guanosine for each transfection set and expressed as mean ± SEM of four independent experiments performed in triplicate. The asterisks indicate statistically significant differences * $p < 0.05$, one-way ANOVA followed by Dunnett's post-hoc testwhile compared to control (dashed line).

3.3. $A_{2A}R$ Signalling, but Not A_1R, is Modulated by Guanosine in an A_1R Coexpression-Dependent Manner

Given that guanosine reduced $A_{2A}R$ binding in an A_1R-expression-dependent manner, we next aimed to determine whether guanosine also impinged into $A_{2A}R$ signaling. Accordingly, we determined the effects of guanosine in $A_{2A}R$-mediated cAMP accumulation upon agonist incubation. In $A_{2A}R$-expressing cells, the selective $A_{2A}R$ full agonist CGS21680 induced a concentration-dependent cAMP accumulation (pEC_{50} = 7.98 ± 0.08), indicating that the receptor was expressed and functional at the plasma membrane (Figure 3A). Subsequently, we challenged cells with a fixed concentration of CGS21680 (200 nM) and evaluated the effects of increasing concentrations of guanosine in $A_{2A}R$-dependent cAMP accumulation. As shown in Figure 3B, guanosine did not preclude $A_{2A}R$-mediated cAMP accumulation. Conversely, in cells doubly expressing A_1R and $A_{2A}R$, guanosine (100 μM) was able to significantly reduce, by 19 ± 3% (p = 0.0460), the $A_{2A}R$-mediated cAMP accumulation (Figure 3B). These results supported the hypothesis that the effects of guanosine might be dependent on an A_1R-$A_{2A}R$ interaction.

Interestingly, our NanoBRET-based binding results and cAMP determinations in the absence and presence of A_1R suggested a direct involvement of this receptor in guanosine-mediated blockade of $A_{2A}R$ ligand binding and signaling. Thus, to ascertain whether guanosine would directly interact with A_1R we assessed its impact on A_1R-dependent signaling. To this end, A_1R-mediated calcium responses in HEK-293T cells were determined through a homogenous bioluminescence reporter assay system using a NFAT response element controlling luciferase gene expression. While the activation of A_1R, via application of the agonist N^6-*R*-phenylisopropyladenosine (*R*-PIA, 50 nM), increased intracellular Ca^{2+}, the incubation with guanosine (100 μM) did not promote intracellular Ca^{2+} mobilization (Figure 4A). Similarly, when A_1R-expressing cells were treated with R-PIA in the presence of increasing concentrations of guanosine, A_1R-dependent intracellular Ca^{2+} mobilization was not affected, as observed in doubly A_1R and $A_{2A}R$ transfected cells (Figure 4B). Overall, these results

indicated that guanosine did not interact with A_1R, thus ruling out any orthosteric A_1R-dependent trans-inhibition of $A_{2A}R$ function in A_1R-$A_{2A}R$ expressing cells.

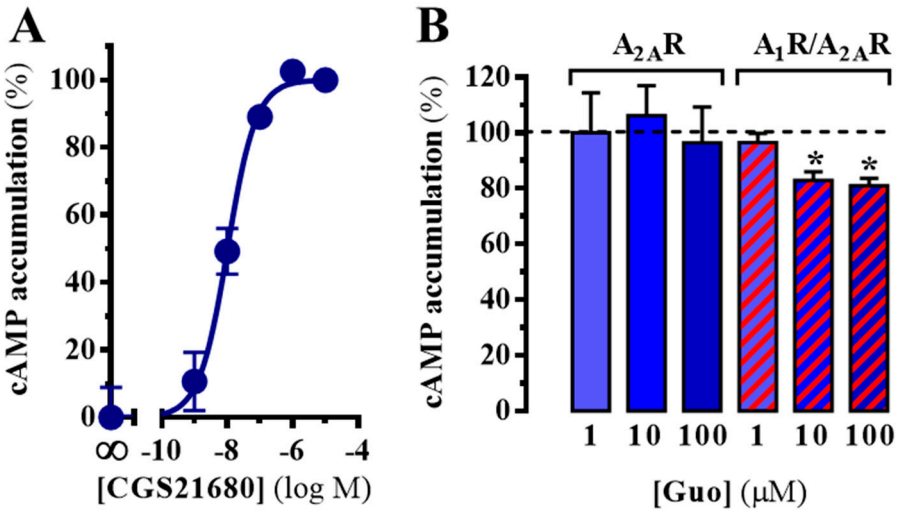

Figure 3. $A_{2A}R$-dependent cAMP accumulation. (**A**) Concentration-dependent effects of CGS21680 in cAMP accumulation in singly $A_{2A}R$ expressing cells. The signal was normalized by assigning the 100% to the maximum signal obtained and 0% to cells without ligand. The data are expressed as the mean ± SD of a representative experiment performed in triplicate. (**B**) Guanosine effects on CGS21680-mediated cAMP accumulation in cells expressing $A_{2A}R^{SNAP}$ (blue bars) or $A_{2A}R^{SNAP}$ plus A_1R^{SNAP} (red dashed bars). Results were normalized to the specific cAMP accumulation in the absence of guanosine for each transfection set and are expressed as mean ± SEM of four independent experiments performed in triplicate. The asterisks indicate statistically significant differences * $p < 0.05$, one-way ANOVA followed by Dunnett's post-hoc testwhile compared to control (dashed line).

Finally, we assessed the functional activity of guanosine using the label-free technology. To this end, the whole-cell guanosine-mediated impedance responses were monitored in living cells expressing $A_{2A}R$ in the absence or presence of A_1R using a biosensor method, as previously reported [34]. First, we tested CGS21680-mediated changes in morphology (i.e., impedance) of $A_{2A}R^{SNAP}$ expressing HEK-293T cells, which were recorded in real-time. Interestingly, addition of CGS21680 resulted in a significant ($p = 0.015$) increase of impedance, which was blocked by incubation with the selective $A_{2A}R$ antagonist ZM241385 (Figure 5A,B). In addition, guanosine did not affect the cell basal morphology ($p = 0.6105$) nor its CGS218680-mediated changes ($p = 0.1217$) (Figure 5B). However, in doubly expressing $A_1R/A_{2A}R$ cells guanosine significantly reduced ($p < 0.0106$) cell basal morphology and precluded ($p < 0.0001$) the CGS218680-induced increase in cellular impedance (Figure 5B). Again, these results indicated that the A_1R-$A_{2A}R$ co-expression may play a potential role in AR-related guanosine-dependent cellular effects.

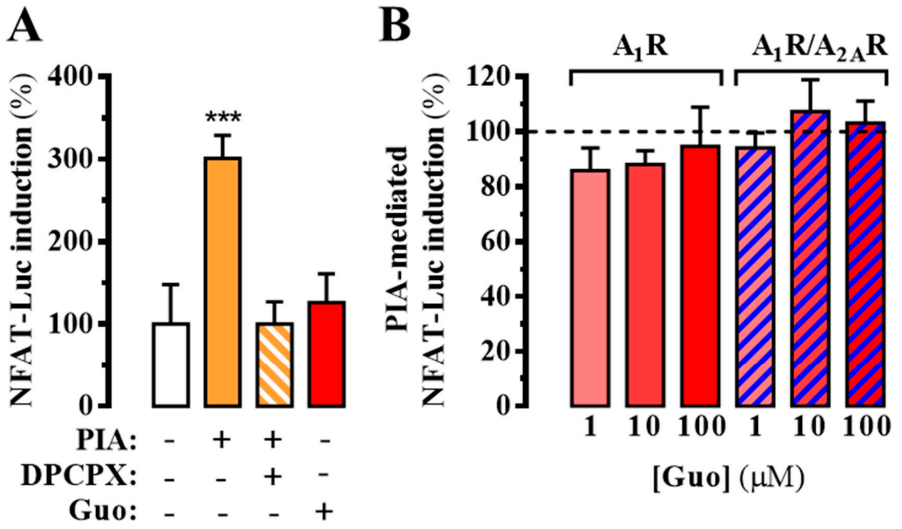

Figure 4. A_1R-dependent intracellular Ca^{2+}mobilization. (**A**) Determination of A_1R-mediated intracellular calcium accumulation by means of a luciferase reporter assay system. HEK-293T cells were transiently transfected with the firefly luciferase-encoding plasmid (pGL4-NFAT-luc2p) and the cDNAs encoding the A_1R^{SNAP} and the YFP. Thirty-six hours after transfection, cells were treated 6 h with the A_1R agonist R-PIA (PIA, 50 nM) in the absence or presence of DPCPX (500 nM) or guanosine (Guo, 100 μM). Light emission is presented as the percentage increase over basal levels. The data are expressed as the mean ± SEM of three independent experiments performed in triplicate. The asterisks indicate statistically significant differences *** $p < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test when compared to control. (**B**) Guanosine modulation of R-PIA-mediated intracellular Ca^{2+}mobilization (PIA-mediated NFAT-Luc induction) in cells expressing A_1R^{SNAP} (red bars) or A_1R^{SNAP} plus $A_{2A}R^{SNAP}$ (blue dashed bars). The dotted line represents the Ca^{2+} mobilization induced by R-PIA in the absence of guanosine within each cell transfection group. The data are expressed as the mean ± SEM of three independent experiments performed in triplicate.

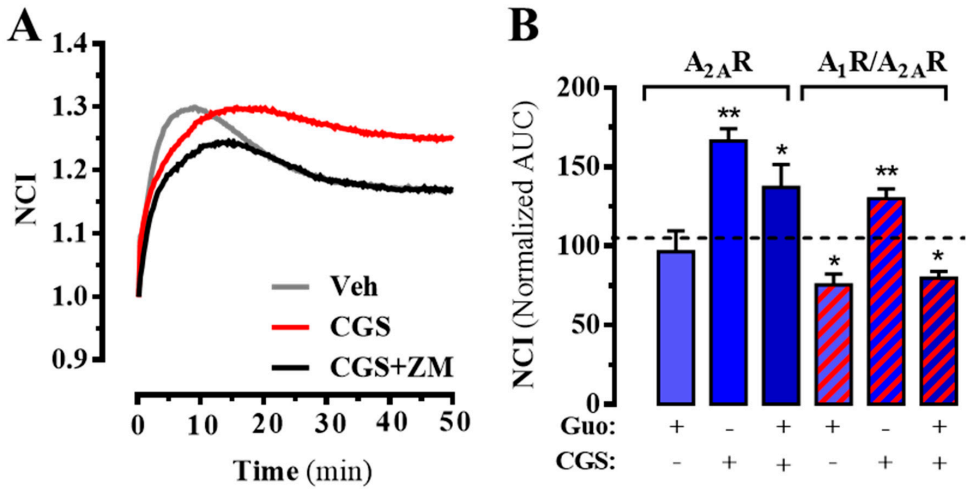

Figure 5. $A_{2A}R$-mediated whole-cell label-free responses. (**A**) Real-time cellular impedance changes upon CGS21680 (200 nM) incubation in the absence or presence of ZM241385 (1 μM). The signal was normalized when the ligand was added. (**B**) Guanosine (100 μM) effects on CGS21680-mediated cellular impedance changes in cells expressing $A_{2A}R^{SNAP}$ (blue bars) or $A_{2A}R^{SNAP}$ plus A_1R^{SNAP} (dashed red bars). Results are presented as area under the cure (AUC) and normalized to the AUC in the basal condition (i.e., absence of any drug) for each transfection set and expressed as mean ± SEM of three independent experiments performed in duplicate. * $p < 0.05$ and ** $p < 0.01$, one-way ANOVA followed by Dunnett's post-hoc test while compared to control (dashed line).

4. Discussion

Guanosine is a purine nucleoside with widely demonstrated extracellular neuromodulatory effects in the CNS, but so far without an identified receptor. Based on the use of selective ligands, ARs have been proposed as possible targets to explain guanosine-mediated effects in animal and cellular models of ischemia. However, at present, the mechanism of action of guanosine is not clear. Here, we show that $A_{2A}R$ expression was crucial for guanosine-mediated protective effects in an ex vivo model of brain ischemia. In addition, when examining guanosine effects in a controlled heterologous system, we were able to reveal the importance of a proposed A_1R-$A_{2A}R$ interaction mediating guanosine effects, both in $A_{2A}R$-ligand binding and in receptor function.

In the OGD ischemia model in hippocampal slices, we previously showed that guanosine induced a neuroprotective effect (increase of glutamate uptake) that was inhibited by activation of $A_{2A}R$ by CGS2180 [11]. This effect of CGS21680 in abolishing a guanosine-evoked increase in glutamate uptake in an OGD protocol was also observed in cultured astrocytes expressing the astrocytic glutamate transporter Glt-1 [15]. Therefore, here we evaluated guanosine's neuroprotective effects in $A_{2A}R^{-/-}$ mice and revealed an important role for this receptor. Thus, in $A_{2A}R^{-/-}$ hippocampal slices, we observed a loss of the neuroprotective effects of guanosine (increasing viability and controlling ROS production in OGD conditions) that were observed in slices from wild-type mice (Figure 6A). This result, consistent with previous data, pointed to ARs as possible targets for guanosine [35,36], prompting us to further explore the mechanism by which guanosine might act.

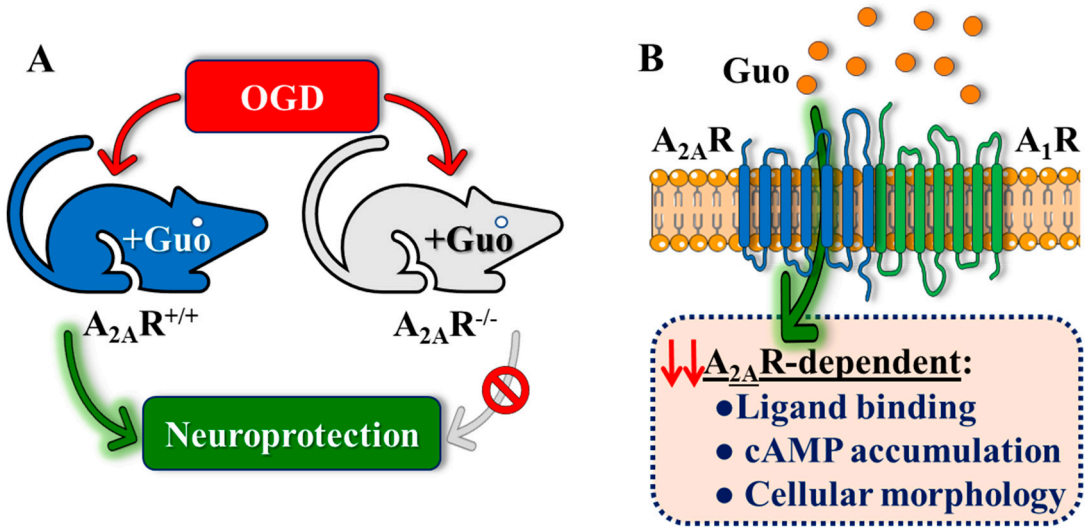

Figure 6. Schematic summary of the overall findings. (**A**) Guanosine-mediated neuroprotection in mouse is dependent on $A_{2A}R$ expression. Thus, guanosine fails to neuroprotect from OGD damage in $A_{2A}R^{-/-}$ mouse hippocampal slices. (**B**) Guanosine modulates $A_{2A}R$ functionality in living cells in an A_1R-dependent manner. While guanosine does not interfere with A_1R-dependent signaling, it modulates $A_{2A}R$ binding and intracellular signaling (i.e., cAMP accumulation and cellular morphology) only in A_1R-$A_{2A}R$ co-expressing cells. Therefore, A_1R and $A_{2A}R$ may constitute a molecular substrate involved in guanosine-mediated effects, but the precise mechanism of action of guanosine involving ARs is still lacking.

Our NanoBRET-based sensor data suggested that, as previously reported [13], guanosine apparently does not bind directly to the $A_{2A}R$. However, in $A_1R/A_{2A}R$ cells, it was possible to observe a guanosine-mediated partial displacement of $A_{2A}R$-ligand binding (Figure 6B). Together with the ex vivo data, this result would indicate that the mechanism of action of guanosine would be mediated by this receptor–receptor entity. Indeed, previous data showing both DPCPX- and pertussis toxin-dependent blockade of protective effects of guanosine in hippocampal slices subjected

to OGD [11], supported the dependence on functional A_1Rs coupled to a G-protein to mediate guanosine effects.

We found that guanosine reduced A_{2A}R orthosteric binding only in A_1R-A_{2A}R expressing cells. Thus, we evaluated whether guanosine could modulate A_{2A}R-dependent signaling under the same experimental conditions. Interestingly, while guanosine did not preclude CGS21680-induced cAMP accumulation in A_{2A}R-expressing cells, it reduced A_{2A}R-mediated cAMP accumulation in doubly A_1R-A_{2A}R transfected cells, as observed in the ligand-binding assay (Figure 6B). Additionally, the evaluation of guanosine effects on the functional activity of ARs using the label-free technology confirmed that guanosine-mediated cell impedance responses were dependent on A_1R-A_{2A}R co-expression. Hence, our results indicate that guanosine could attenuate A_{2A}R signaling (i.e., agonist-mediated cAMP accumulation and cell impedance responses) in an A_1R-dependent manner (Figure 6B). On the other hand, when the A_1R-dependent signaling (i.e., intracellular Ca^{2+} mobilization) was assessed, guanosine was unable to modulate receptor's function both in singly and doubly A_1R-A_{2A}R transfected cells. Taken together, our results suggest that while guanosine did not signal through A_1R, it requires this receptor to exert its A_{2A}R modulatory effect, which could indicate that the A_1R/A_{2A}R heteromer might be a molecular substrate for guanosine.

The A_1R/A_{2A}R heteromer displays some functional characteristics similar to that reported for other AR-containing oligomers, for instance A_{2A}R combined with the dopamine D_2 receptor (D_2R) or the cannabinoid CB_1 receptor (CB_1R) [37]. Interestingly, these receptor heteromers have been shown to exert reciprocal receptor-receptor allosteric antagonistic interactions [38]. Precisely, an A_1R/A_{2A}R heteromer-mediated transmembrane-dependent negative allosteric interaction at the ligand-receptor binding level has been described [39]. In addition, co-activation of both receptors led to a canonical protein Gs-Gi antagonistic interaction at the level of the adenylyl cyclase [40]. This situation makes it difficult to conclude whether an effect in a given signaling pathway is caused by either the allosteric or the canonical interaction. Thus, our data showing that guanosine was able to modulate AR functioning (i.e., cAMP assay) only in cells expressing A_1R and A_{2A}R do not permit a clear determination of the interaction at the intracellular level (i.e., canonical protein Gs-Gi antagonistic interaction). However, considering the whole picture, it seems likely that guanosine effects in the physiological context may depend on the co-expression of both receptors and their and interaction. Indeed, guanosine did not disrupt the A_1R/A_{2A}R heteromer, as observed by a saturable BRET signal, similar to that obtained following adenosine treatment, and by membrane co-localization of A_1R and A_{2A}R in guanosine-treated cells (Figure A1).

Overall, our data suggest an important role for the A_1-A_{2A} receptor–receptor interaction in guanosine-mediated effects. Thus, while our results seem to rule out an eventual guanosine-mediated A_1R-A_{2A}R canonical antagonistic interaction, further investigation is needed to ascertain whether guanosine may either modulate the well-known A_1R-A_{2A}R allosteric interaction or an indirect mechanism of action yet to be discovered.

5. Conclusions

In summary, our results revealed that certain AR-related guanosine-mediated effects rely on A_1R and A_{2A}R co-expression. Indeed, in ex vivo experiments, the well-known guanosine-mediated neuroprotective effect depends on A_{2A}R expression. Thus, guanosine failed to protect $A_{2A}R^{-/-}$ mouse hippocampal slices from ischemia-induced damage. In addition, while guanosine did not interfere with A_1R-mediated signaling, it modulated A_{2A}R binding and intracellular signaling only in A_1R-A_{2A}R co-expressing cells. Overall, our results suggest that A_1R and A_{2A}R may constitute a molecular substrate involved in guanosine effects, but the precise mechanism of action of guanosine involving ARs still is intriguing.

Author Contributions: D.L., C.M.M., V.M. and T.Š. performed experiments and analyzed results. R.D. and K.A.J. synthesized the fluorescent ligand and analyzed results. V.F.-D. performed experiments, analyzed results and

wrote the paper. C.I.T. and F.C. conceived the project, analyzed results and wrote the paper. All authors read and approved the final manuscript.

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Appendix A

Appendix A.1 Materials and Methods

Appendix A.1.1 Immunocytochemistry

Transfected HEK-293T cells growing on coverslips were fixed in 4% paraformaldehyde for 15 min and exposed to goat anti-$A_{2A}R$ antibody (1 µg/mL; Santa Cruz Biotechnology Inc., Dallas, TX, USA) plus a rabbit anti-A_1R antibody (1 µg/mL; Millipore, Billerica, MA, USA). Primary antibodies were detected using a Cy3-conjugated donkey anti-goat antibody (1/200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and Cy2-conjugated donkey anti-rabbit antibody (1/200; Jackson ImmunoResearch Laboratories Inc.). Coverslips were rinsed for 30 min, mounted with Vectashield immunofluorescence medium (Vector Laboratories, Peterborough, UK) and examined using a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik GmbH, Heidelberg, Germany).

Appendix A.1.2 BRET

BRET saturation experiments were performed as previously described [41]. In brief, HEK-293T cells were transiently transfected with a constant amount of the $A_{2A}R^{Rluc}$ and increasing amounts of A_1R^{YFP}. After 48 h, cells were rapidly washed twice in PBS, detached and resuspended in Hank's balanced salt solution buffer (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, pH 7.4), containing 10 mM glucose. Cell suspensions were distributed in triplicate into 96-well microplate black plates (Corning, Stockholm, Sweden) for fluorescence measurement or white plates (Corning 3600) for BRET determination. For BRET measurement, 5 µM benzyl-coelenterazine (NanoLight Technology, Prolume Ltd., Pinetop, AZ, USA) was added, and readings were performed 1 min after substrate addition using the POLARstar Omega plate-reader (BMG Labtech, Durham,NC, USA), which allows the simultaneous integration of the signals detected with two filter settings [485 nm (440–500 nm) and 530 nm (510–560 nm)]. The BRET ratio was defined and represented as previously described [41].

Appendix A.2 Results

We aimed to assess whether guanosine treatment modulated the $A_1R/A_{2A}R$ heteromerization status. To this end, we performed immunocytochemistry analyses and constructed classical A_1R-$A_{2A}R$ heteromer-based BRET saturation curves (Figure A1). Our immunocytochemistry experiments revealed that $A_{2A}R$ and A_1R co-distributed in transiently transfected HEK-293T cell, as previously reported [17], and that 2 h incubation with guanosine did not alter their apparent co-distribution (Figure A1A). Subsequently, the close proximity of the two receptors was monitored through BRET saturation analysis in cells transiently expressing $A_{2A}R^{Rluc}$ and increasing concentrations of A_1R^{YFP}showing a

bell-shaped BRET saturation curve ($BRET_{50}$ = 0.38 ± 0.07 and $BRET_{max}$ = 90 ± 6), thus indicating the formation of constitutive A_1R-$A_{2A}R$ complexes in living cells (Figure A1B). Importantly, under the same experimental conditions, the treatment with either adenosine (100 μM) or guanosine (100 μM) for 2 h did not alter the physical proximity of A_1R and $A_{2A}R$. Thus, neither the $BRET_{50}$ [$F_{(2,30)}$ = 1.524, *p*-value = 0.2343] nor the $BRET_{max}$ [$F_{(2,30)}$ = 0.3135, *p*-value = 0.7333] was significantly affected by adenosine or guanosine incubation (Figure A1B). Overall, these results corroborated the formation of $A_1R/A_{2A}R$ heterocomplexes in living cells, as previously described [17], and that these complexes were not affected by adenosine or guanosine, consistent with the general notion that GPCR homo- and heteromerization is often constitutive.

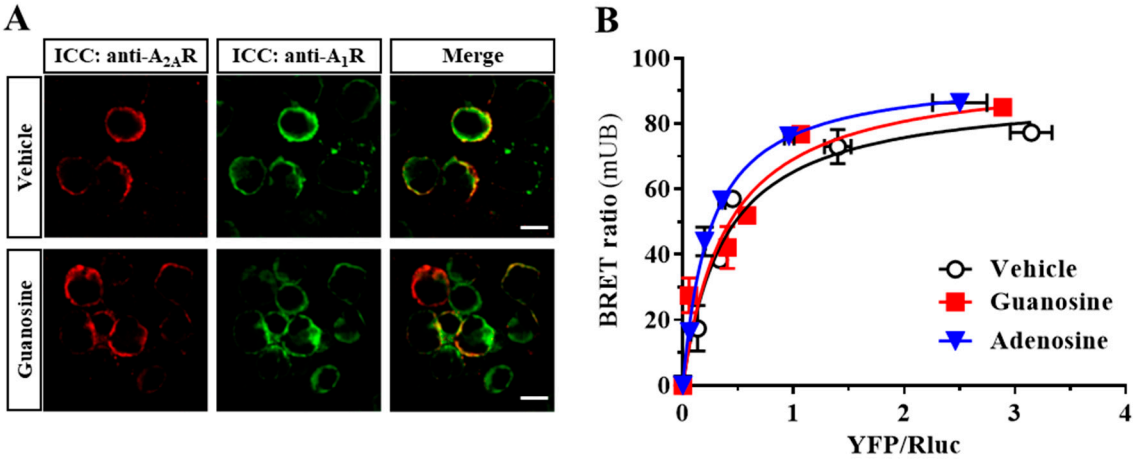

Figure A1. A_1R and $A_{2A}R$ interaction in HEK-293T cells. (**A**) Co-distribution of $A_{2A}R$ and A_1R in HEK-293T. Cells transiently transfected with $A_{2A}R^{SNAP}$ and A_1R^{SNAP} and incubated with vehicle or guanosine (100 μM) for 2 h. Cells were processed for immunocytochemical (ICC) detection of $A_{2A}R$ (red) and A_1R (green) using specific antibodies (see Appendix A.1). Merged images reveal co-distribution of $A_{2A}R^{SNAP}$ and A_1R^{SNAP} (yellow). Scale bar: 100 μm. (**B**) BRET saturation curve between $A_{2A}R$ and A_1R. BRET was measured in HEK-293T cells co-expressing $A_{2A}R^{Rluc}$ and A_1R^{YFP} constructs and incubated with vehicle, adenosine (100 μM) or guanosine (100 μM) for 2 h. Cells were co-transfected with a fixed amount of $A_{2A}R^{Rluc}$ and increasing amounts A_1R^{YFP}. Plotted on the *X*-axis is the fluorescence value obtained from the YFP, normalized with the luminescence value of the *R*luc constructs 10 min after coelenterazine h incubation and in the *Y*-axis the corresponding BRET ratio (×1000). mBU: mBRET units. Results are expressed as mean ± SEM of four independent experiments grouped as a function of the amount of acceptor fluorescence.

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6 DISCUSSÃO

Os resultados obtidos no presente estudo demonstram que os efeitos da guanosina avaliados, *in vitro* e *in vivo*, estão relacionados, direta ou indiretamente, com os receptores de adenosina. Foram utilizadas três abordagens distintas para avaliar os efeitos da guanosina, sendo duas delas relativas à PD e uma específica para estudar a interação da guanosina com o heterômero A_1R-$A_{2A}R$. O protocolo *in vivo* com a administração de reserpina evidenciou que o efeito da guanosina em diminuir o aumento de EROs no estriado e os tremores orofaciais em camundongos são dependentes do A_1R. Ao avaliar o efeito da toxicidade da 6-OHDA *in vitro* em fatias estriatias, foi constatado que a modulação tanto do A_1R quanto do $A_{2A}R$, interferem no efeito da guanosina em prevenir o aumento de EROs, a perda do potencial de membrana mitocondrial e o decréscimo nos níveis de ATP, induzidos pela 6-OHDA. Por fim, em um estudo com uma abordagem farmaco-genômica, com a transfecção heteróloga dos A_1R e $A_{2A}R$, ficou evidente que a guanosina atua sobre o heterômero A_1R-$A_{2A}R$, promovendo uma alteração na afinidade e funcionalidade do $A_{2A}R$, somente na presença concomitante do A_1R.

Como já descrito, a guanosina é uma molécula endógena com alto potencial terapêutico e, ainda, sem alvo molecular (receptor) e sinalização celular totalmente elucidados. Mesmo sem essa caracterização de receptores de membrana, tanto para guanosina quanto para os outros DG, existe a proposta de um sistema purinérgico dos DG, uma vez que os mecanismos de captação e liberação dos DG e sua metabolização extracelular já foram descritos (Schmidt, Lara, and Souza 2007). Já foi evidenciado que o GTP pode ser captado e armazenado em vesículas sinápticas, por um sistema de transporte dependente de gradiente eletroquímico como observado para outros neurotransmissores (Santos, Souza, and Tasca 2006), e com uma cinética de captação

similar ao do neurotransmissor ATP (Gualix, Pintor, and Miras-Portugal 1999). A presença de GTP co-acumulado com neurotransmissores em vesículas sinápticas também já havia sido previamente demonstrada (Wagner, Carlson, and Kelly 1978). Portanto, sugere-se que o GTP desempenhe um papel como co-transmissor e a sua liberação vesicular e posterior hidrólise pela atividade de ecto-nucleotidases, levará à formação do nucleosídeo guanosina no espaço sináptico, onde poderá desempenhar seu papel neuromodulador e neuroprotetor (Lanznaster, et al. 2016).

Em relação à metabolização dos DG, já foi descrita que ela pode ser tanto intracelular, quanto extracelular. As enzimas envolvidas no metabolismo extracelular dos nucleotídeos são chamadas de ectonucleotidases, que hidrolisam ATP e GTP a ADP e GDP, respectivamente, ou que hidrolisam tanto ATP/GTP ou ADP/GDP a AMP/GMP; e, por fim, hidrolisam AMP/GMP aos nucleosídeos adenosina/guanosina (Zimmermann 1996; Schadeck, et al. 1989). Em cultura de astrócitos, a inibição da atividade da ecto-5'-nucleotidase reduz significativamente o acúmulo extracelular de guanosina indicando que, assim como a adenosina, a guanosina extracelular também é derivada principalmente da hidrólise extracelular dos nucleotídeos (Ciccarelli, et al. 2001). Após a ocorrência de uma lesão cerebral, os nucleotídeos liberados podem ser hidrolisados aos seus respectivos nucleosídeos que por sua vez desempenham papéis protetores ou mesmo restaurativos. Por isso há a necessidade de estudos para identificar os alvos moleculares dos DG, e principalmente, da guanosina.

Estudos prévios indicam alguns prováveis alvos moleculares para a guanosina. Os primeiros estudos avaliando os efeitos dos DG como mediadores de sinalização intercelular, demonstraram que os DG são moduladores da transmissão glutamatérgica (Sharif and Roberts 1981). Foi observado que os nucleotídeos GTP, GDP e GMP podem se ligar na região N-terminal extracelular dos receptores de glutamato,

deslocando a interação do glutamato com seus receptores e inibindo as respostas intracelulares induzidas pelo glutamato (Paz, et al. 1994; Tasca, et al. 1995). No entanto, a guanosina não desloca a união do glutamato e seus análogos aos receptores glutamatérgicos (Souza and Ramirez 1991). Diversos estudos já demonstraram o efeito da guanosina sobre o transporte de glutamato (Dal-Cim et al., 2013; 2016), porém ainda não há evidência de interação direta da guanosina com os transportadores de glutamato. Outro possível candidato ao alvo de membrana para a guanosina foi identificado como sendo um receptor acoplado à proteína G (GPCR) de 23 kDa, o receptor GPR23. O GPR23 é um dos receptores para o ácido lisofosfatídico (LPA), identificado como LPA4. Uma comunicação em evento científico apresentou resultados em que o silenciamento gênico do GPR23 em culturas de glioma U87 diminuiu o efeito da guanosina em inibir a proliferação celular, enquanto que sua superexpressão aumentou o efeito anti-proliferativo da guanosina (Di Liberto, et al. 2012). Porém, a interação direta da guanosina com este receptor ainda não foi demonstrada, não descartando a possibilidade de que a guanosina interaja com outras proteínas de membrana (R. Volpini, et al. 2011). Além da possível interação com GPCRs, nosso grupo de pesquisa evidenciou que o efeito protetor da guanosina envolve a ativação de canais de potássio (K^+) (Oleskovicz, et al. 2008). O uso de inibidores farmacológicos seletivos para diferentes canais de K^+ sugeriu uma interação da guanosina com o canal de K^+-dependente de Ca^{+2} de alta condutância (BK), pois o bloqueio deste canal abole os efeitos benéficos da guanosina sobre a redução da viabilidade celular e da captação de glutamato em um modelo de isquemia cerebral *in vitro* (Dal-Cim, et al. 2011; Oleskovicz, et al. 2008). Portanto, é possível que a guanosina interaja com o canal BK, ou promova a ativação do canal através da interação com algum GPCR. Outro estudo sugere a interação da guanosina com o receptor CD40, um membro da superfamília dos

receptores de fator necrose tumoral (TNF), em cultura primária de microglia, que participaria no processo inflamação em doenças neurodegenerativas (D'Alimonte, et al. 2007).

Apesar dos dados da interação com a guanosina com receptores já descritos, não se pode ignorar que existem resultados sugerindo sítios seletivos para a ligação da guanosina. Em membranas isoladas obtidas de cérebro de ratos foi demonstrado um suposto sítio de união seletiva (afinidade na ordem de nM) para guanosina (Traversa, et al. 2002; Traversa, et al. 2003). Essa seletividade também se baseia no resultado de que outras purinas, como adenosina, hipoxantina, xantina, GDP, GMP e ATP não deslocaram a união da guanosina, assim como os antagonistas não-seletivos dos receptores de adenosina, cafeína e teofilina (Traversa, et al. 2002). Em outro estudo, utilizando um ensaio de ligação de GTP à proteína-G, foi observada que a interação da guanosina seria um receptor da grande família dos GPCRs, e que seria diferente dos conhecidos receptores de adenosina, já que os agonistas dos receptores de adenosina não tiveram nenhum efeito sobre a ligação da guanosina (Rosaria Volpini, et al. 2011). De acordo, a incubação das membranas de cérebro de ratos com a toxina Pertussis (PTx), um inibidor da família das proteínas Gi, foi capaz de reduzir a capacidade de união da guanosina (Traversa, et al. 2003). Também nesse estudo, a guanosina induziu, de maneira dose-dependente, um aumento de AMPc intracelular em fatias corticais de ratos. Além disso, o pré-tratamento com a ADA (adenosina desaminase, enzima que converte adenosina em inosina), ou com antagonistas A_1R e $A_{2A}R$ não modificaram o acúmulo de AMPc induzido pela guanosina (Traversa, et al. 2003).

Apesar das evidências supracitadas e os diversos possíveis alvos da guanosina, este estudo investigou o possível papel dos receptores de adenosina nos efeitos da guanosina. A identidade e estrutura dos receptores de adenosina estão bem estabelecidas

e caracterizadas e crescentes evidências implicam uma possível interação da guanosina com estes receptores. A modulação dos A_1R e dos A_2R está relacionada a diversos efeitos demonstrados para a guanosina, como: anti-apoptótico (Di Iorio, et al. 2004); proliferativo (Jackson and Gillespie 2013; Decker, et al. 2019); tipo-ansiolítico (Almeida, et al. 2016; Almeida, et al. 2017); anticonvulsivante (Kovács, et al. 2015; Lakatos, et al. 2016); antinociceptivo (Schmidt, et al. 2010); neuroprotetor nos modelos de traumatismo crânio-encefálico (Gerbatin, et al. 2019) e de isquemia (Dal-Cim, et al. 2013; Dal-Cim, et al. 2019b); e até citotóxico em células tumorais em cultura (Oliveira et al., 2017). Além disso, há crescentes evidências da guanosina como potencial agente terapêutico na PD, demonstradas em melhoras motoras (Massari, et al. 2017; Su, et al. 2009) e em parâmetros bioquímicos relacionados a essa enfermidade (Giuliani, et al. 2012; Giuliani, et al. 2015; Marques, Massari, and Tasca 2019). Sendo assim, foi de interesse deste estudo entender se e como a modulação do A_1R e do A_2R poderia afetar os efeitos da guanosina em modelos da PD. Além disto, avaliamos os efeitos de união e sinalização da guanosina sobre os A_1R e A_2R em um modelo de expressão heteróloga desses receptores.

Como resultados de neuroproteção obtidos, tanto no protocolo *in vitro* de toxicidade estriatal da 6-OHDA, quanto no protocolo *in vivo* de indução de tremor associado à PD com a reserpina, foi demonstrado que o antagonismo do A_1R com DPCPX bloqueia o efeito protetor da guanosina frente à geração de EROs e perda do potencial de membrana mitocondrial. A capacidade da guanosina de atenuar os níves de EROs e manter o potencial de membrana mitocondrial já foi previamente demonstrada em outros estudos (Dal-Cim, et al. 2019a; Marques, Massari, and Tasca 2019; Dal-Cim, et al. 2012; Dal-Cim, et al. 2013), e já se excluiu a possibilidade desse efeito estar relacionado a um efeito de *scavenger* de espécies reativas pela guanosina (Thomaz, et

al. 2016). Corroborando com os resultados desta tese, também já se evidenciou que a diminuição de EROs pela guanosina foi abolida pelo DPCPX (Dal-Cim, et al. 2013). Sendo assim, a guanosina poderia atuar ativando o A_1R. De fato, a estimulação do A_1R tem efeito neuroprotetor em diversos protocolos de neurotoxoicidade (Mitchell, et al. 1995; Kawamura, Ruskin, and Masino 2019; Cunha 2016; Duarte, Cunha, and Carvalho 2016). De forma similar à guanosina, observamos que a incubação com CCPA, um agonista A_1R, também teve efeito em manter o potencial de membrana mitocondrial diminuído pela incubação com 6-OHDA, além de não afetar os efeitos da guanosina. Também já foi demonstrado que o uso PTx pode abolir os efeitos da guanosina (Traversa, et al. 2003; D'Alimonte, et al. 2007; Dal-Cim, et al. 2013; Di Iorio, et al. 2004). Uma vez que os A_1R são acoplados à proteína Gi isso contribuiria para fortalecer essa proposta. Porém, nesse estudo, o CCPA não teve um efeito semelhante ao da guanosina em relação aos níveis de EROs. No protocolo da 6-OHDA *in vitro*, o aumento dos níveis de EROs foi prevenido pela guanosina e não pelo CCPA, o que colocaria em dúvida se a guanosina atuaria por esse receptor, uma vez que a estimulação do A_1R não é capaz de produzir efeito similar ao da guanosina. Mais importante, nos resultados obtidos com células HEK293 transfectadas, somente com A_1R não foi visto uma mobilização de cálcio relativa à estimulação desse receptor, seja pela guanosina sozinha ou pela guanosina junto à estimulação com o CCPA.

Por outro lado, o papel do $A_{2A}R$ nos efeitos protetores da guanosina também é um pouco conflitante. Ficou evidenciado que a ativação do $A_{2A}R$, com o uso do CGS21680 - um agonista $A_{2A}R$, efetivamente bloqueia os efeitos da guanosina. No protocolo *in vitro* com 6-OHDA, a pré-incubação com CGS21680 foi capaz de impedir a prevenção dos níveis de EROs, do potencial de membrana mitocondrial e dos níveis intracelulares de ATP pela guanosina. Esse mesmo efeito do CGS21680 sobre efeitos da

guanosina já foi demonstrado em um modelo de isquemia, tanto em fatias de hipocampo, como em cultura cortical de astrócitos de ratos (Dal-Cim, et al. 2013; Dal-Cim, et al. 2019b). Enquanto isso, a pré-incubação com SCH58261, um antagonista do $A_{2A}R$, não causou interferência nos efeitos da guanosina sobre a toxicidade da 6-OHDA *in vitro*. Já se tem bem estabelecido na literatura que o antagonismo do $A_{2A}R$ está ligado a efeitos benéficos em diversos modelos de PD (Vallano, et al. 2011; Jenner 2014; Preti, et al. 2015). De acordo, no protocolo *in vitro,* o SCH58261 também se mostrou efetivo em prevenir o dano causado pela 6-OHDA no estriado de ratos. Essa semelhança nos efeitos poderia demonstrar que a guanosina teria um papel antagonístico sobre o $A_{2A}R$. No entanto, no protocolo *in vivo* de administração da reserpina, o tratamento com doses sub-efetivas de SCH58261 e guanosina não apresentou um efeito somatório em reverter o aumento de EROs no estriado. Ademais, o efeito da guanosina em diminuir os níveis de EROs no estriado foi visto em camundongos que não expressam o $A_{2A}R$ ($A_{2A}R$-KO). Contrastando diretamente com esse resultado, em fatias hipocampais de camundongos $A_{2A}R$-KO submetidos ao protocolo de PGO, a guanosina perdeu seus efeitos, tanto de diminuição de EROs como de atenuação da viabilidade celular. Demonstrando assim que alguns efeitos da guanosina são dependentes da expressão do $A_{2A}R$. Apesar dos resultados em animais $A_{2A}R$-KO citados acima, é importante ressaltar que os experimentos refletem diferentes estruturas cerebrais, que apresentam diferentes níveis de expressões do receptor, bem como podem apresentar padrões de localizações neuronais distintas (isto é, pré- e pós-sináptico).

Além dos efeitos neuroprotetores, também foi feito um estudo da guanosina em relação a um dos principais sintomas motores da PD. Nosso grupo foi o primeiro a demonstrar um efeito anti-discinético da guanosina em modelos da PD (Massari, et al. 2017). Nesse estudo, seguimos com o protocolo da reserpina para tentar evidenciar

como a modulação dos receptores de adenosina afeta os efeitos da guanosina. Salamone e colaboradores (1998) defendem que as discinesias induzidas pela reserpina compartilham características com os tremores acometidos em pacientes com PD. Assim como nos resultados anteriores, vimos o mesmo padrão nos sintomas motores, em que o DPCPX também bloqueia o efeito da guanosina em diminuir os TJM induzidos pela reserpina. Sabe-se que o A_1R pode modular antagonisticamente as respostas do D1R (Ferré, et al. 1994; Ferre, et al. 1996; Popoli, et al. 1996; Ismayilova, et al. 2004). Além disso, ratos tratados com reserpina apresentaram um aumento da expressão e da resposta associada ao D1R (Missale, et al. 1989; Liberini, et al. 1989). Não há, até o presente momento, na literatura o uso de agonistas de A_1R para o tratamento de tremores relacionados à PD. Existem dois recentes estudos que mostram um efeito benéfico do agonista do A_1R, 5'-Cloro-5'-Deoxi-(±)-ENBA, em modelos de tremor essencial em ratos (Kosmowska, et al. 2017; Kosmowska, et al. 2020), o que evidencia que esse receptor pode estar associado a este controle motor. Os poucos estudos relacionados ao estímulo do A_1R se devem, em parte, pela ampla expressão desse receptor, tanto no SNC como perifericamente, e aos possíveis efeitos colaterais (como sedação, bradicardia), o que inviabilizaria o uso de agonistas A_1R. Neste estudo testamos o CCPA, e em doses muito baixas (0,0125 mg/kg), ele demonstrou efeito em diminuir os TJM induzidos pela reserpina (dados não mostrados). No entanto, os animais apresentavam uma visível sedação, o que claramente limitaria esta estratégia como uma boa alternativa terapêutica.

Nas últimas décadas, antagonistas do $A_{2A}R$ surgiram como um possível tratamento para os sintomas motores da PD, e parte desse potencial terapêutico se deve ao fato desses receptores poderem modular alostericamente os D2R (Ferré, et al. 2016). Os $A_{2A}R$ são altamente expressos no estriado e já foi demonstrado que seu antagonismo

é benéfico em modelos de tremor relacionado à PD (Svenningsson, et al. 1997; Dixon, et al. 1996; Pinna, et al. 2016; Salamone, et al. 2013; Collins, et al. 2010; Collins-Praino, et al. 2013). De acordo, neste estudo o antagonismo do $A_{2A}R$ com o SCH58261 teve efeito em diminuir os TJM induzidos pela reserpina. Foi feita uma curva de doses para estabelecer doses ativas e sub-ativas do SCH58261 nesse protocolo. Em uma associação farmacológica, foram testadas doses sub-ativas de guanosina e de SCH58261, e quando administradas juntas tiveram efetivo efeito em diminuir os TJM. Porém, utilizando o mesmo protocolo da reserpina em camundongos $A_{2A}R$-KO foi demonstrado que esse efeito da guanosina é mantido mesmo sem a expressão desse receptor. Evidenciando assim, que o efeito motor da guanosina independe do receptor $A_{2A}R$.

Uma possível explicação para os dados conflitantes com os camundongos $A_{2A}R$-KO pode estar relacionada a diferentes perfis de afinidade descritos para este receptor. Em 1996, (Cunha, et al.) demonstraram a existência de pelo menos dois sítios de interação de alta afinidade para o [^{3}H]CGS21680 no SNC de ratos. Alguns anos depois, foi discutida a existência de dois $A_{2A}R$, que seriam farmacologicamente diferentes entre si (Cunha, Constantino, and Ribeiro 1999). Essa diferença também se encontraria em nível regional no cérebro, sendo que as propriedades de união do $A_{2A}R$ no hipocampo e córtex eram diferentes das propriedades do $A_{2A}R$ no estriado. Apesar de não existir muitos dados na literatura sobre essas diferentes propriedades do receptor A_{2A}, em 2010, um estudo confirmou esses perfis do receptor A_{2A} na região do hipocampo e na região do estriado, propondo que são resultados das interações heteroméricas entre os receptores A_{2A}, A_{2B} e A_1 nas diferentes regiões (Riccioni, Leonardi, and Borsini 2010). Nos camundongos $A_{2A}R$-KO utilizados não sabemos precisar exatamente se existe uma deleção de ambos receptores ou sua prevalência, mas poderia explicar a diferença de

resultados, e a dependência do $A_{2A}R$ no efeito da guanosina no hipocampo e não no estriado.

Mesmo com essas sugestões, nos experimentos pontuais que se analisou o papel de cada receptor de forma individual, os resultados não apontam uma conclusão nítida. Uma outra forma de olhar os resultados poderia ajudar a compreendê-los. Essa nova abordagem pode se basear no fato que os receptores A_1R e $A_{2A}R$ formam heterômeros funcionais, e essa interação pode alterar a farmacologia dos receptores individuais. A investigação da possível modulação do heterômero A_1R-$A_{2A}R$ pela guanosina representa uma abordagem interessante na identificação do possível sítio de interação da guanosina na membrana celular. Desta forma, experimentos foram planejados a fim de investigar a possível interação da guanosina com o heterômero A_1R-$A_{2A}R$ através do uso de transfecção heteróloga em células HEK293. Utilizando a técnica de transferência de energia ressonante vimos que a guanosina reduz a união do MRS7396 (uma molécula fluorescente com ação antagonista sobre o $A_{2A}R$) ao $A_{2A}R$, porém essa diminuição só existe quando também há a presença do A_1R, indicando que a guanosina pode ter efeito sobre o heterômero A_1R-$A_{2A}R$. Também foi demonstrado, através da quantificação de AMPc intracelular que a guanosina só diminui os níveis de AMPc induzidos por CGS21680, em células co-transfectadas com A_1R e $A_{2A}R$. Esses resultados implicam em um papel antagônico da guanosina no $A_{2A}R$, tanto de união quanto de funcionalidade, porém, somente em presença do A_1R. Além disso, ao avaliar as mudanças na impedância elétrica das células induzidas pelo CGS21680, a guanosina foi capaz de bloquear essas respostas somente em células co-transfectadas com A_1R e $A_{2A}R$. Para avaliar um possível efeito da guanosina sobre o A_1R, foi avaliado o aumento de cálcio intracelular nas células HEK. Foi visto que a guanosina não teve efeito em células transfectadas com A_1R ou co-transfectadas com A_1R e $A_{2A}R$. Esse conjunto de

dados demonstra que a guanosina tem efeito sobre os receptores de adenosina, porém somente quando há a presença dos dois receptores (A_1R e $A_{2A}R$) e provavelmente formando o heterômero. Além disto, a ação da guanosina sobre a interação A_1R-$A_{2A}R$ é evidenciada como a de antagonizar os efeitos do $A_{2A}R$.

Importante ressaltar as diferentes metodologias utilizadas no emprego deste estudo. Foi abordado um protocolo *in vivo* com o uso da reserpina, que age em neurônios monoaminérgicos, e vimos danos referentes à região do estriado, porém não podemos excluir totalmente efeitos de outras regiões, tanto pelo dano quanto pelos tratamentos, uma vez que eram administradas sistemicamente. Já no protocolo *in vitro*, tanto o dano com 6-OHDA quanto os tratamentos foram direcionados unicamente para a região do estriado, e obtivemos um resultado similar entre os protocolos. O uso de células HEK transfectadas com os receptores de interesse permite uma avaliação mais direta da sua interação com os ligantes, excluindo as diferenças estruturais, níveis de expressão e localização neuronal. Além disso, a técnica de *label free* permite o estudo de modulação de GPCRs através da análise de mudanças na impedância elétrica das células e sem o uso de nenhuma sonda ou artifício externo que possa interferir no resultado. Essa junção de métodos e complementação de possíveis vieses metodológicos nos permite uma maior credibilidade aos resultados obtidos neste estudo.

Considerando todas as informações descritas acima e os resultados obtidos neste estudo, não podemos precisar exatamente qual receptor é o responsável diretamente pelos efeitos da guanosina. Ainda, não podemos excluir a possibilidade de existir um receptor seletivo para guanosina ainda não conhecido e caracterizado e que poderia estar associado ou modular os A_1R ou $A_{2A}R$. Porém, neste estudo fica claro o envolvimento da modulação dos receptores de adenosina A_1 e/ou A_{2A} nos efeitos da guanosina. Mais importante, esse estudo traz à luz evidências de uma sinalização através da formação de

heterômeros desses receptores. A sinalização celular partindo do princípio de hetêromeros como uma entidade funcional ainda é um tema recente, porém a guanosina emerge como uma molécula de interesse nesse campo e, sendo assim, mais estudos são necessários para elucidar seus mecanismos e transpô-los em medidas terapêuticas.

7 CONLCUSÕES

- O efeito da guanosina em reduzir o tremor orofacial induzida pela reserpina, assim como o aumento da produção de EROs no estriado, dependem da estimulação do A_1R;
- Guanosina previne o aumento de EROs, a alteração do potencial de membrana mitocondrial e a redução dos níveis de ATP induzidos por 6-OHDA em fatias de estriado e este efeito é abolido pelo bloqueio dos A_1R e pela ativação dos $A_{2A}R$;
- A guanosina não interfere com a sinalização celular (isto é, a ativação dos receptores) em células HEK239 que expressam somente A_1R ou $A_{2A}R$;
- Em células HEK239 co-transfectadas com A_1R e $A_{2A}R$ a guanosina interfere na união e na ativação do $A_{2A}R$, bem como em mudanças na morfologia dessas células pela ativação farmacológica do $A_{2A}R$, demonstrando uma interação da guanosina com o heterômero A_1R-$A_{2A}R$.

8 PERSPECTIVAS

- Avaliar alterações na função mitocondrial causadas pela 6-OHDA, e a possível proteção da guanosina, através da técnica de respirometria de alta resolução, bem como o envolvimento dos A_1R e $A_{2A}R$;
- Avaliar a regulação da enzima tirosina hidroxilase no estriado após o tratamento com reserpina *in vivo* e a incubação com 6-OHDA *in vitro*;
- Estudar, *in silico*, a interação físico/química da guanosina com os A_1R e $A_{2A}R$;
- Estudar o possível efeito da guanosina sobre a formação e a sinalização do heterômero $A_{2A}R$- D_2R;

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