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Papel do receptor B2 de cininas na terapia da neurodegeneração dopaminérgica em modelo animal

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Orientador:
Prof. Dr. Alexander Henning Ulrich

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Resumo

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A Doença de Parkinson (DP) é um distúrbio neurodegenerativo, caracterizada em parte pela perda de neurônios dopaminérgicos da via nigroestriatal, originada na substância negra com projeções para o estriado, causando vários déficits motores. Atualmente, o tratamento mais utilizado é a administração de L-DOPA, um análogo da dopamina. Porém, essa droga apresenta eficácia limitada e induz diversos efeitos colaterais. A exploração dos efeitos neuroprotetores, proliferativos e neuroregenerativos da bradicinina (BK) em modelo animal de DP pode conduzir à substituição celular do tecido lesionado pela 6-hidroxidopamina (6-OHDA). De fato, a BK e seus receptores possuem um grande espectro de ações fisiológicas, estando classicamente envolvida no controle da homeostase cardiovascular e inflamação, além de exercer efeitos protetores em fisiopatologias do sistema nervoso, como em modelos de acidente vascular cerebral. Vários tipos celulares têm suas vias de sinalização associadas à ativação do receptor B2 de cininas (B2BKR). Trabalhos anteriores de nosso grupo mostraram que a BK está envolvida na diferenciação neural de células progenitoras neurais por um *loop* autócrino que resulta em ativação do B2BKR. Os resultados apresentados neste trabalho mostram a eficácia do tratamento com BK, um agonista de B2BKR, em animais submetidos à lesão da via nigro-estriatal induzida por 6-OHDA. Além disso, há uma recuperação comportamental e histológica desses animais quando tratados com Captopril®, um potencializador dos efeitos farmacológicos da BK, e com [Phe$^{8}\Psi$(CH-NH)Arg^{9}]-Bradicinina, agonista estável do receptor B2BKR. Assim, concluímos que a ativação de B2BKR pela BK desencadeia

um processo de neuroregeneração dopaminérgica de animais submetidos à lesão por 6-OHDA. Trabalhos recentes mostram que o receptor B2BKR desempenha um importante papel neuroprotetor em modelo animal da Doença de Alzheimer, o que corrobora nossos achados. Juntos, esses resultados contribuem para o estabelecimento da ação neuroprotetora e neurorregenerativa da BK no modelo de animal de neurodegeneração dopaminérgica, tornando-a uma excelente candidata para aplicação em terapias de reparo neuronal.

Palavras-chave: Receptor B2BkR de cininas, sistema calicreína-cininas, Doença de Parkinson, neuroregeneração.

Abstract

Souza, H.D.N. de. **Targeting Kinin-B2 receptors for the treatment of dopaminergic neurodegeneration in an animal model.** 2018. 93p. Tese de Doutorado – Programa de Pós-Graduação em Ciências Biológicas (Bioquímica). Instituto de Química, Universidade de São Paulo, São Paulo.

Parkinson's disease (PD) is a neurodegenerative disorder partially characterized by the loss of dopaminergic neurons from the nigrostriatal pathway, originated in the substantia nigra with projections to the striatum, which causes several motor deficits. Currently, the most commonly used drug for PD treatment is levodopa. However, it has limited efficacy and induces several side effects. Elucidation of the neuroprotective, proliferative and neuroregenerative effects of bradykinin (BK) in animal models of PD can culminate in cellular replacement of the tissue damaged by 6-hydroxydopamine (6-OHDA). In fact, BK and its receptor have several physiological effects, being classically involved in the control of cardiovascular homeostasis and inflammation. Besides, BK exerts protective effects on nervous system pathophysiology, as observed in stroke models. Several cell types have their signaling pathways associated with the B2 kinin receptor (B2BKR) activation. Previous work from our group showed that BK is involved in differentiation of neural progenitor cells by an autocrine loop that results in activation of B2BKR. The results presented in this thesis show the efficacy of treatment with BK, through B2BKR activation, in animals submitted to nigrostriatal pathway injury induced by 6-OH dopamine. Furthermore, behavioral and histological recoveries of these animals were observed when treated with Captopril®, a potentiator of BK pharmacological effects, and with [Phe8Ψ (CH-NH) Arg9] -BK, a stable agonist of the B2BKR receptor. Thus, we conclude that BK activation of B2BKR triggers neuroregenerative processes in animals submitted to 6-OHDA injury. Recent studies showed that the B2BKR receptor plays an important neuroprotective role in an animal model of Alzheimer's disease, which corroborates

our findings. Together, these results contribute to the establishment of the neuroprotective and neuroregenerative actions of BK - an excellent candidate for neural repair therapies.

Keywords: Kinin B2 receptors, kallikrein-kinin system, Parkinson's disease, neuroregeneration.

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1. INTRODUÇÃO

1. INTRODUÇÃO

1.1 A doença de Parkinson: etiologia, sintomatologia e tratamento farmacológico corrente

A Doença de Parkinson (DP) é um distúrbio neurodegenerativo associado a uma progressiva perda de neurônios dopaminérgicos na via nigroestriatal, causando vários déficits motores, manifestados em tremores, rigidez muscular, alteração da marcha, bradicinesia e instabilidade postural (Parkinson, 2002; Santangelo et al., 2017). Os distúrbios motores são acompanhados de distúrbios não-motores, como disfunções cognitivas e psiquiátricas, que se manifestam sobretudo em quadros de depressão, alucinações, além de distúrbios afetivos e comportamentais (Jankovic, 2008). Observa-se também manifestações em outros processos fisiológicos, como alterações no fluxo intestinal, no sono e nos sentidos, sendo os danos ao olfato o mais bem caracterizado até o momento (Rao et al., 2003; Moustafa e Poletti, 2013).

O quadro de sintomas comuns associados à DP foi descrito pela primeira vez em 1817 por James Parkinson, manifestado como uma paralisia agitante caracterizada por tremores involuntários, perda de força muscular, hipotensão postural, bradicinesia, mas sem lesão aos sentidos e ao intelecto (Parkinson, 2002). No entanto, o quadro clínico complexo de sintomas motores e não motores não é apenas diferente entre pacientes com DP, mas também se altera ao longo da progressão da doença em cada paciente (de Lau e Breteler, 2006).

Segundo a Organização Mundial da Saúde (OMS), cerca de 1% da população mundial acima de 65 anos é acometida pelos sintomas da DP. A DP é a segunda doença neurodegenerativa mais comum, ficando atrás apenas da

Doença de Alzheimer (Arnold, et al., 2016). Estima-se que o número de pessoas afetadas em 2030 nos EUA e na Europa estará na ordem de 5 milhões (Bach, et al. 2001).

A etiologia da DP ainda não está completamente esclarecida. Em parte, a morte neuronal na DP está associada à inclusão de emaranhados da proteína *Tau* nos neurônios e células gliais e acúmulo de proteínas alfa-sinucleína ubiquitinizadas, originando os chamados corpúsculos de Lewy, que levam a distúrbios metabólicos celulares (Nussbaum e Ellis, 2003), seguido pela perda de neurônios dopaminérgicos da porção compacta da substância negra e suas projeções para o estriado. Além da degeneração dos neurônios dopaminérgicos na substância negra, verificam-se a presença de corpúsculos de Lewis, positivos para alfa-sinucleína, no troco encefálico e no neocórtex de pacientes *post-mortem*.

A perda de neurônios dopaminérgicos na via nigroestriais pode estar relacionada ao mau funcionamento de uma série de processos celulares, tais como a perda do controle de processos redox, alteração da atividade lisossomal, a mecanismos anormais de metabolismo proteico no retículo endoplasmático (Olanow, 2008).

Essas anormalidades celulares são os principais fatores que levam ao acúmulo de agregados proteicos com dobragem incorreta (Soto, 2003). Corpos de Lewy constituem um achado patológico característico resultante da agregação de proteínas, principalmente constituído por alfa-sinucleína (Spillantini, 1997).

A manifestação dos sintomas clínicos ocorre somente após a degeneração de 60 a 80% dos neurônios dopaminérgicos nas regiões citadas,

dificultando o tratamento, uma vez que, quando diagnosticada, a doença já se apresenta em estágios bastante avançados (Rugberg et al, 1995).

Atualmente o tratamento mais utilizado é a administração de L-DOPA (L-3,4-dihidroxifenilalanina, L-DOPA). Porém, essa droga apresenta eficácia limitada e diversos efeitos colaterais, tais como alterações motoras e discinesia (Hassin-Baer, et al., 2011). Foi apenas nos anos 60, após a identificação de alterações patológicas e bioquímicas no cérebro de pacientes com DP, que o tratamento com L-DOPA foi introduzido na clínica, o que representou um grande avanço na terapia sintomática da DP.

A L-DOPA é um aminoácido que ocorre naturalmente, sendo encontrado em vegetais, como vários tipos de feijão. L-DOPA é o precursor das catecolaminas dopamina, norepinefrina e epinefrina. Enquanto a própria dopamina não é capaz de atravessar a barreira hematoencefálica, a L-DOPA o faz. Depois de entrar no sistema nervoso central, a L-DOPA é convertido em dopamina pela descarboxilase de L-aminoácidos aromáticos, também chamada DOPA descarboxilase (DDC). Usando inibidores das enzimas conversoras de dopamina, a L-DOPA é capaz de atingir o cérebro e, subsequentemente, ser convertida em dopamina, que é liberada na fenda sináptica. A L-DOPA tem uma meia-vida de 90 minutos, o que alguns casos representa um desafio para o tratamento de determinados pacientes, pois leva a um pico de liberação de dopamina na fenda sináptica pouco tempo após a sua administração (Okun, et al. 2009).

Os benefícios clínicos trazidos pelo uso da L-DOPA são observados em praticamente todos os pacientes, reduzindo a mortalidade precoce pela DP. No entanto, as complicações causadas pelo tratamento a longo prazo, como efeitos

adversos, incluindo flutuações motoras, discinesia e complicações neuropsiquiátricas, representam ainda um limitante na terapia desse distúrbio (Lang, 2009; Olanow, 2009). Assim, torna-se necessário o estudo de terapias efetivas na reversão da morte dopaminérgica da via nigro-estriatal, além do desenvolvimento de modelos que mimetizem os sintomas e fisiopatologia desse distúrbio em animais.

1.2 Os modelos animais da Doença de Parkinson

Entende-se que um modelo ideal e relevante da DP humana deve ter pelo menos as seguintes características: primeiro, quantidades normais de neurônios dopaminérgicos no nascimento do animal utilizado no modelo, com perda seletiva e gradual desses neurônios no início na idade adulta. As perdas devem exceder 50% e ser detectáveis usando técnicas de bioquímicas e de neuropatologia. O modelo deve produzir déficits motores facilmente mensuráveis. Por último, os sintomas devem manifestar-se, ou serem induzidos, em um período relativamente, permitindo uma triagem rápida e menos onerosa de agentes terapêuticos (Beal, 2001).

O primeiro modelo animal utilizado em estudos da DP foi a administração sistêmica de reserpina em ratos. A administração de reserpina resulta em redução aguda da síntese de dopamina. Essa droga leva a um estado de acinesia e rigidez muscular que pode ser reduzido pela administração de L-DOPA. Porém, esse modelo apresenta uma importante limitação, pois não desencadeia a perda de neurônios dopaminérgicos da via nigroestriatal. Além desse modelo, foram desenvolvidos outros mais específicos a partir do uso de

neurotoxinas de ação dopaminérgicas, como a 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) e o a 6-hidroxidopamina (6-OHDA) (figura 1) (Betarbet et al., 2002; Deumens et al. 2002).

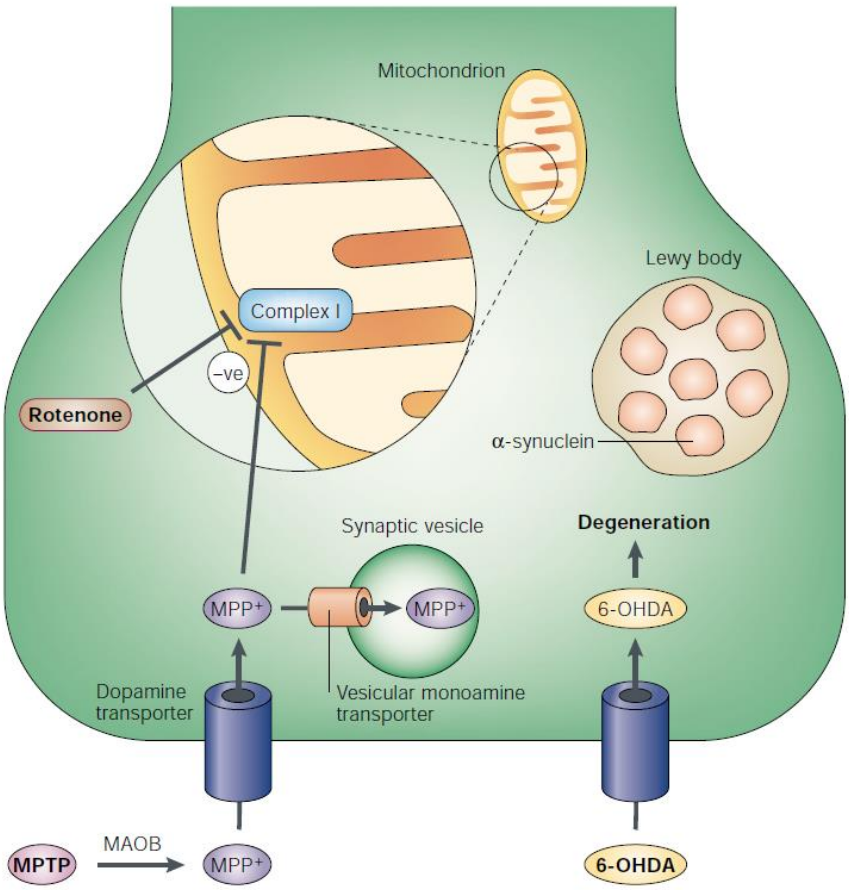

Figura 1: Patogênese da disfunção neuronal produzida por neurotoxinas que afetam neurônios dopaminérgicos. Os mecanismos pelos quais as neurotoxinas matam os neurônios dopaminérgicos envolvem disfunção mitocondrial e dano oxidativo. A 6-OHDA é absorvida pelo transportador de dopamina e então gera radicais livres. Reproduzido de Beal, 2001.

A 6-OHDA é uma toxina análoga à dopamina, o que a torna capaz de alcançar o espaço intracelular pelos transportadores de dopamina, em seguida acumula-se nos neurônios dopaminérgicos e noradrenérgicos, alterando a homeostasia celular, induzindo disfunção mitocondrial e causando a morte

neuronal devido à toxicidade que se acredita ser desencadeada pela geração de radicais livres. Mais detalhadamente, sabe-se que após ser retirada do espaço extracelular pelos transportadores de membrana DAT (*Dopamine membrane transporters*) e NAT (*Noradrenaline membrane transporters*), a 6-OHDA é armazenada em neurônios catecolaminérgicos. Dentro desses neurônios, a 6-OHDA sofre degradação enzimática pela MAO-A (*Monoamine Oxidase*) e por auto oxidação, gerando espécies citotóxicas que produzem danos neuronais. Além disso, a 6-OHDA pode induzir neurotoxicidade ao comprometer a atividade do complexo I da mitocôndria (figura 2). (Beal, 2001; Deumens et al. 2002; Simola et al. 2007).

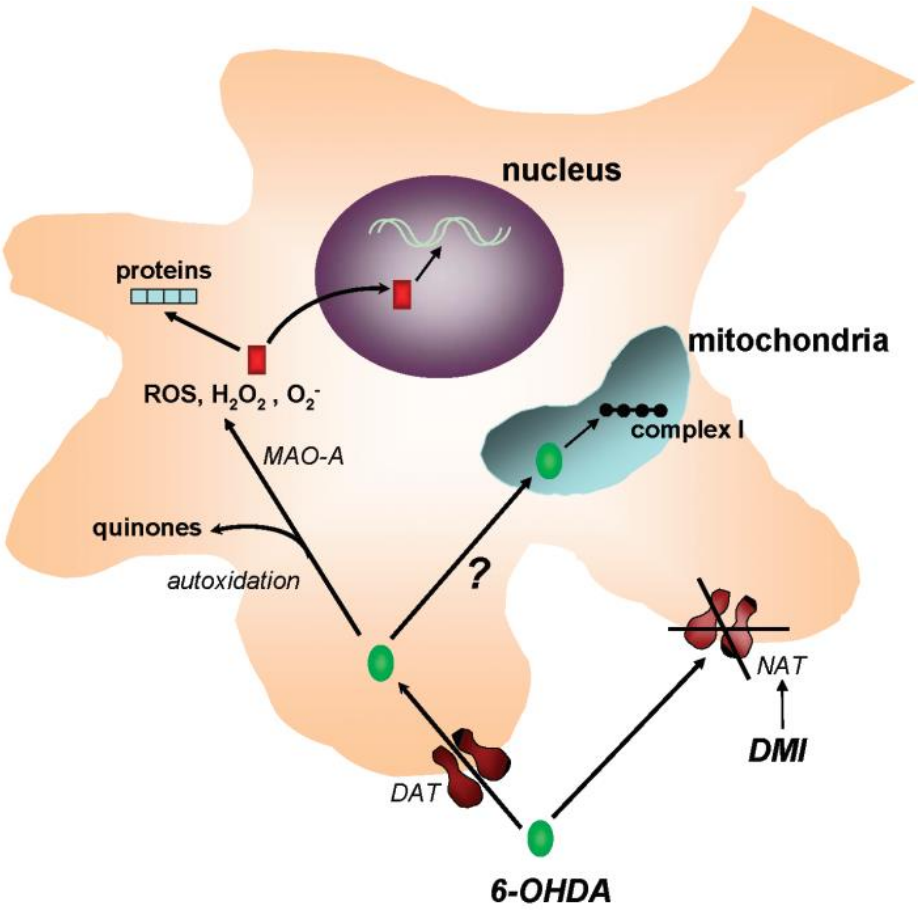

Figura 2: Mecanismos da neurotoxicidade induzida pela 6-OHDA. Reproduzido de Simola et al., 2007.

A 6- OHDA é eficaz em produzir lesões nigroestriatais em ratos, camundongos, gatos e primatas. Em ratos, a extensão da depleção da dopamina pode ser avaliada examinando o comportamento rotatório em resposta a anfetaminas e apomorfina. Portanto, provou ser útil para no desenvolvimento de agentes farmacológico que têm efeitos sobre a ação da dopamina e nos seus receptores (Beal, 2001).

Em contrapartida, as lesões causadas pela 6- OHDA não resultam na formação de Corpos de Lewy na substância negra e podem causar danos não específicos a outros neurônios (Schober, 2004) e a morte neuronal e o aparecimento dos sintomas ocorrem de forma aguda, diferente do processo gradual de neurodegeneração característico da DP. Por outro lado, uma grande vantagem desse modelo, no entanto, é um déficit motor quantificável (rotação) (Beal, 2001).

1.3 A bradicinina e o receptor B2BkR: componentes do Sistema Calicreína-cininas

A bradicinina (BK) é um oligopeptideo composto por nove aminoácidos (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9), que faz parte do sistema calicreina-cininas. Este sistema inclui as enzimas calicreinas (serino-proteases) tecidual e plasmática, cininogênios de alto e baixo peso molecular (precursores das cininas clivadas pelas calicreinas) e as cininas bradicinina (BK), calidina e seus metabolitos, des-Arg^9-BK e des-Arg^9-calidina, todas biologicamente ativas (Bhoola et al., 1992; Moreau, 2005).

As cininas são oligopeptídeos gerados pela clivagem proteolítica dos cininogênios de baixa e alta massa molecular por serino-proteases da família

calicreína. Cininogênios de alta massa molecular são precursores da bradicinina (BK), enquanto cininogênios de baixa massa molecular originam a calidina (figura 3).

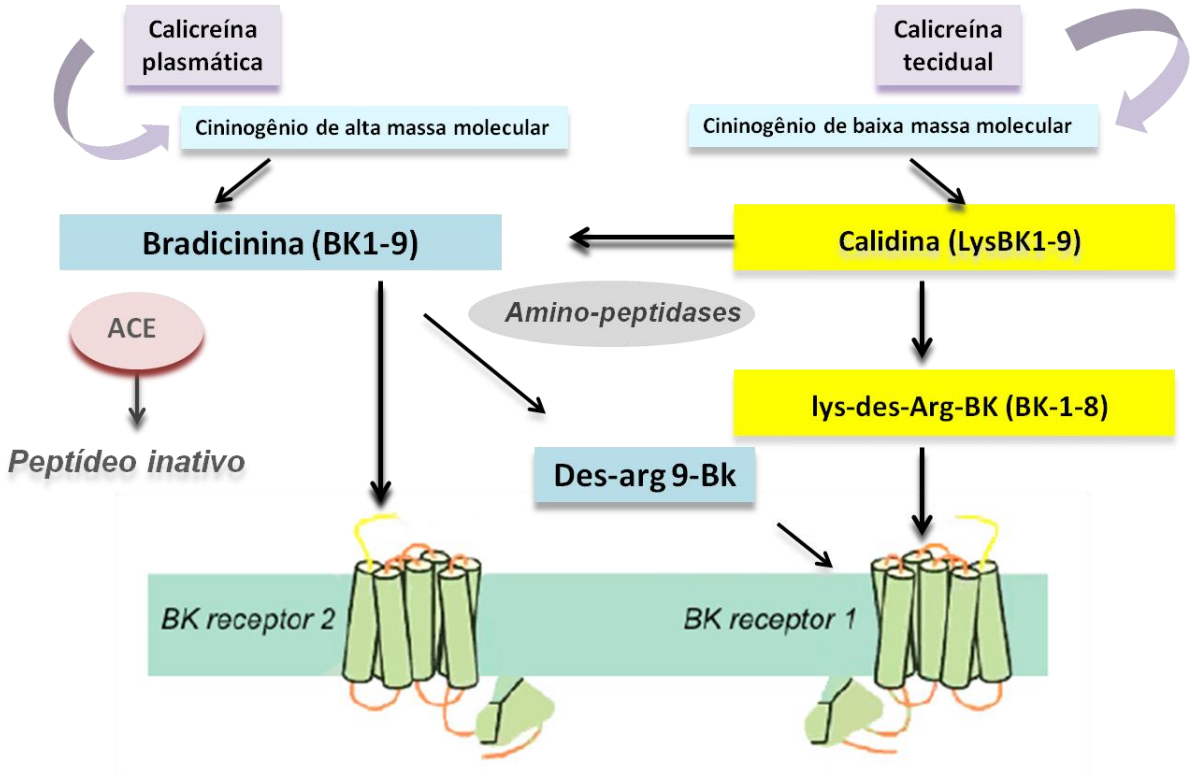

Figura 3: As vias de produção e degradação de cininas e ativação dos seus receptores. Geração das cininas pelas calicreínas teciduais e plasmáticas. Nos tecidos, a calicreína gera calidina; enquanto a calicreína plasmática gera bradicinina (BK) a partir do cininogênio de alta massa molecular. A Bradicinina [BK-(1-9)] e a calidina [Lys-BK-(1-9)] são agonistas mais potentes do receptor B2BKR. Já o BK-1-8, calidina-(Lys-BK1-8) e Des-arg9-Bk são agonistas do B1BKR. Baseada em El Dahr, 1997.

As cininas atuam na regulação de diversos processos fisiológicos como, homeostase cardiovascular, angiogênese, dor, inflamação, coagulação e desenvolvimento (Leeb-Lundberg et al, 2005; Moreau et al, 2005). Além disso, o sistema calicreína-cininas é ativado após injúria tecidual, onde regula a função vascular, crescimento celular, diferenciação e angiogênese (Marceau *et al.*, 1998; Calixto *et al.*, 2000).

Cada vez mais acumulam-se evidências de que o sistema nervoso contém todos os componentes do sistema calicreína-cininas e também de que as cininas podem atuar como neuromediadores em diversos processos (Bhoola *et al*., 1992; Borkowski *et al*. 1995), incluindo diferenciação neuronal e neuroproteção (Martins *et al* 2012; Trujillo et al.; 2012, Pillat et al. 2015).

A expressão de calicreínas no desenvolvimento encefálico de ratos e camundongos (Iwadate *et al*., 2002; Trujillo et al. 2012), e na diferenciação neuronal *in vitro* de células progenitoras neurais de ratos e camundongos (Martins *et al*., 2008; Trujillo et al. 2012, Pillat et al. 2015) dá suporte à ideia de que receptores ativados por cininas podem ser necessários durante a diferenciação neuronal.

Os receptores B1BkR e B2BkR, ambos acoplados a proteína G, apresentam propriedades farmacológicas diferentes. Como já foi dito, há fortes evidências de que o sistema nervoso contenha todos os componentes do sistema calicreína-cininas. A calicreína tecidual foi encontrada em várias regiões do cérebro (córtex, cerebelo, hipotálamo e glândula pineal) (Fujieda et al., 1993), o fato de ter sido encontrada ao longo do desenvolvimento indica que existe uma regulação da expressão dos componentes desse sistema durante a diferenciação neural (Iwadate et al., 2002). O cininogênio de alto peso molecular também foi detectado em regiões específicas do cérebro (Damas et al., 1992).

Resultados anteriores obtidos pelo nosso grupo (Martins et al., 2005 e 2008; Trujillo et al. 2012, Nascimento et al., 2015; Pillat et al., 2015) comprovaram a participação do receptor B2BkR durante a diferenciação neuronal em células de carcinoma embrionário P19 e neuroesferas do telencéfalo embrionário de ratos, respectivamente. Estes trabalhos mostraram

que a expressão gênica do receptor B2BkR aumenta após a indução da diferenciação neuronal desencadeada por ácido retinóico, juntamente com um aumento da secreção do peptídeo bradicinina no meio de cultura. Observou-se também que a inibição do receptor B2BkR por HOE-140, uma antagonista deste receptor, comprometeu a diferenciação terminal das células P19 para neurônios, diminuindo a expressão e funcionalidade dos receptores muscarínicos de acetilcolina, demonstrando uma inter-relação entre esses receptores durante a diferenciação neuronal e indicando que o receptor B2BkR possui uma função essencial durante a diferenciação *in vitro*.

Uma relevante quantidade de trabalhos demonstrou que a expressão do receptor B2BkR é importante para o desenvolvimento do sistema cardiovascular e urinário e que a inibição da atividade da sua atividade em embriões de rato em desenvolvimento resultava em animais com distúrbio no desenvolvimento do fígado (El-Dahr, 1997). Trujillo e colaboradores (2012), demonstrou que o receptor B2BkR tem sua expressão regulada durante o desenvolvimento. Especificamente, observou-se um padrão de expressão desse gene (mRNA) ao longo do desenvolvimento embrionário de camundongos nos estágios E9,5, E11,5 e E12,5 nas regiões do telencéfalo, mesencéfalo, rombencéfalo e medula espinhal. Estes resultados passaram a ser a primeira descrição da presença do receptor B2BkR no sistema nervoso durante do desenvolvimento embrionário do camundongo (Trujillo et al*.* 2012).

As influências da BK no desenvolvimento neural, aumentando a neurogênese e a migração celular durante a diferenciação e suprimindo a gliogênese e a proliferação, foram recentemente descritas pelo nosso grupo de pesquisa (Trujillo et al. 2012, Pillat et al. 2016). Ainda não se tem conhecimento

dos mecanismos envolvidos e das vias de sinalização da BK que resultam na modulação da diferenciação neural. A ativação de receptores B2BkR de cininas garante a viabilidade de neurônios hipocampais devido à ativação da via anti-apoptótica Akt. Por sua vez, a sinalização via MEK/ERK não é relacionada aos efeitos neuroprotetores (Pillat et al. 2016).

Tendo em vista sua atividade neuroprotetora, a bradicinina – ou algum dos seus agonistas mais estáveis – pode ser uma importante ferramenta no tratamento de danos cerebrais decorrentes de isquemia (Martins, et al. 2012) ou da morte celular de tipos neuronais específicos, como na DP, onde a perda de neurônios dopaminérgicos na substância negra resulta na perda de função motora.

Diante destes resultados, cria-se a necessidade um possível potencial na neurorregeneração desencadeado por BK em modelos animais de distúrbio neurodegenerativos. Nesse sentido, o uso de agonistas e antagonistas do receptor B2BkR apresenta-se como uma estratégia para a elucidação dos mecanismos pelos quais a bradicinina exerce seus efeitos. Duas perguntas são fundamentais: 1) A BK é capaz de promover a reversão da lesão por 6-OHDA via receptor de B2BkR? 2) É possível potencializar os efeitos da BK mediante inibição da sua degradação?

1.4 O Captopril: um potencializador farmacológico dos efeitos da BK

A curta meia-vida de BK *in vivo* tem sido atribuída à rápida degradação enzimática por várias peptidases (coletivamente conhecidas como cininases) presentes no plasma e nos tecidos. O papel que uma cininase particular

desempenha no metabolismo da BK depende da sua localização e de outras peptidases presentes no plasma ou nos tecidos. As cininases que degradam predominante a BK são as cininase II, uma metalopeptidase (Enzima Conversora da Angiotensina [ECA]), e a cininase I (Carboxipeptidase M e N, CPM e CPN, respectivamente). A ECA é primariamente uma enzima de membrana, que remove o dipeptídeo C-terminal de BK, o que leva a sua completa inativação. A ECA finalmente cliva BK^{1-7} no fragmento menor, BK^{1-5}. As carboxipeptidases do tipo cininase I clivam o aminoácido básico C-terminal arginina da BK, gerando BK^{1-8}, enquanto a CPN está presente no plasma, a CPM está amplamente distribuída na membrana plasmática. A localização da ECA membrana celular, torna-a ideal como alvo da regulação da atividade da BK (Altura, 1981; Resende et al. 1998).

A potencialização bem conhecida das ações da BK pelos inibidores da ECA tem sido atribuída a uma proteção da BK contra a degradação enzimática da ECA, um efeito que parece contribuir para o amplo espectro terapêutico dos inibidores da ECA (Resende et al. 1998). A abordagem chave para estes estudos controversos foi a utilização de análogos de BK contendo substituições de aminoácidos que conferem um grau variado de resistência à degradação pela ECA e afinidade para o receptor B2BkR. Um exemple de molécula com essa característica é $Phe^{8}\Psi(CH\text{-}NH)Arg^{9}$]-Bradicinina, uma molécula similar à BK, mas com alterações química na ligação peptídica do C-terminal do peptídeo que o torna resistente à clivagem.

No âmbito dos distúrbios neudegenerativos, um dos dados mais consistes utilizando inibidores da ECA na literatura é o apresentado por Munoz e colaboradores em 2006, os quais observaram uma notável redução da

neurodegeneração dopaminérgica e do estresse oxidativo em um modelo animal de neurodegeneração dopaminérgica induzido por MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) pela inibição da ECA em modelo desenvolvido em camundongos. Além disso, os efeitos neuroprotetores do Captopril foram explorados em modelos de 6-OHDA em ratos, onde captopril preveniu a perda de neurônios dopaminérgicos. Porém, nenhum dado descreve os efeitos neuregenerativos dessa droga em modelos animais de neurodegeneração dopaminérgica.

1.5 O problema das estratégias correntes para terapia da Doença de Parkinson e o potencial do sistema calicreínas-cininas na terapêutica das doenças neurodegenerativas

Várias abordagens têm sido feitas para o desenvolvimento de novas terapias para a Doença de Parkinson nas últimas décadas. No entanto, existem diversos problemas que limitam a execução dessas terapias, por questões éticas, técnicas e financeiras. Por exemplo, a utilização de neurônios dopaminérgicos obtidos a partir de tecido fetal para transplantes em pacientes com Parkinson envolve sérios problemas éticos, uma vez que são necessários muitos fetos para obter-se o número de neurônios desejável para um transplante bem-sucedido. Além disso, grande parte dos neurônios transplantados morre após o transplante, diminuindo a eficiência da terapia (Deierborg et al. 2008). Recentemente vários grupos conseguiram diferenciar neurônios dopaminérgicos *in vitro* a partir de células-tronco neurais e embrionárias, mas, a integração desses neurônios e a sua sobrevivência após o transplante, continua sendo um desafio a ser vencido (Lindvall et al., 1990; Brundin et al., 2000).

Alternativamente à substituição celular, desenvolveu-se a abordagem da neuroproteção, ou seja, a prevenção da morte dos neurônios remanescentes poderia deter a progressão da doença. Testes clínicos utilizando o fator trófico e neuroprotetor GDNF (*glial cell line-derived neurotrophic factor*) mostraram-se eficazes não só na prevenção da morte neuronal, como no surgimento de novos neurônios, e causaram melhora sintomática e funcional dos pacientes. A proteína recombinante foi liberada diretamente no cérebro através de uma bomba implantada intraperitonealmente (Gill et al., 2003; Patel et al., 2005). No entanto questões técnicas e financeiras envolvendo o uso de GDNF levaram à interrupção destes testes clínicos (Peck, 2005).

Uma alternativa interessante ao uso dos fatores de crescimento é a terapia celular associada à terapia gênica, isto é, o transplante de células infectadas com vetores virais que expressem proteínas específicas. Esta terapia tem sido amplamente utilizada em estudos pré-clínicos para terapias na Doença de Parkinson. No entanto, as dificuldades de se controlar as quantidades de proteínas expressas, bem como delimitar o local da expressão, são inúmeras. Além disso, o uso de vetores virais envolve riscos de mutações que acarretariam efeitos colaterais, além de desencadearem reações imunológicas indesejadas (Connor et al., 1999). Outro problema é que os sistemas virais promovem a liberação contínua do fator neuroprotetor, fato não desejável, devido aos efeitos colaterais do GDNF quando secretado continuamente (Zhang et al., 1997).

Por tudo isso, o tratamento mais utilizado ainda é a administração de L-DOPA, porém, como já foi dito, essa droga apresenta eficácia limitada e diversos efeitos colaterais. As estratégias de neuroproteção e neuroregeregeneração para doenças neurodegenerativas têm avançado bastante, sendo a bradicinina um

candidato promissor para o tratamento de doenças neurodegenerativas, como a DP.

Em resumo, este capítulo apresentou um novo panorama sobre o papel da bradicinina na neuroproteção e na neuroregeneração *in vivo* em modelos animais de doenças neudegenerativas. As terapias atualmente disponíveis para a DP permanecem puramente sintomáticas, enquanto a procura de drogas neuroprotectoras seguem como um ideal ainda distante. Assim, a bradicinina apresenta-se como um candidato de grande potencial na terapia de doenças neurodegenerativas.

2. OBJETIVOS

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A perda de neurônios dopaminérgicos na PD ocorre por um longo, quase silencioso, período de tempo. Porém, os sintomas só se tornam evidentes após a perda de, pelo menos, 50% dos neurônios na substância negra, resultando em uma drástica redução do conteúdo de dopamina no estriado. Essa lenta e progressiva perda de neurônios dopaminérgicos sugere que uma abordagem visando a estimulação da proliferação neuronal, pode apresentar-se como uma promissora estratégia para o tratamento da neurodegeneração dopaminérgica na DP. Assim, a exploração dos efeitos neurorregenerativos da bradicinina pode teoricamente conduzir à substituição celular do tecido lesionado pela 6-OHDA, essencialmente, neurônios dopaminérgicos. Além disso, existe a possibilidade de proteger os neurônios dopaminérgicos remanescentes. De fato, a bradicinina e seus receptores possuem um grande espectro de ações fisiológicas, estando classicamente envolvida no controle da homeostase cardiovascular e inflamação. Desse modo, o presente trabalho apresenta-se como uma proposta para investigar os mecanismos envolvidos no processo de regeneração neural desencadeado por BK na terapia da neurodegeneração dopaminégica em modelo animal.

2. OBJETIVO GERAL

Investigar os mecanismos envolvidos e o papel do receptor B2 de cininas (B2BkR) no processo de regeneração neuronal desencadeado pela bradicinina na terapia da neurodegeneração dopaminérgica em modelo animal de ratos submetidos à lesão da via nigro-estriatal por 6-OHDA.

2.1 OBJETIVOS ESPECÍFICOS

1. Caracterizar o efeito neuroregenerativo da bradicinina sobre a morte neuronal induzida por 6-OHDA *in vivo*;

2. Avaliar o perfil comportamental dos animais pós-lesão e injeção de BK, [Phe$^{8}\Psi$(CH-NH)-Arg^{9}]-Bradykinin e HOE-140, um antagonista do receptor B2 de cininas;

3. Caracterizar por imuno-histoquímica o efeito neuroregenerativo da BK dos cérebros de animais pós-lesão por 6-OHDA e injeção de BK;

4. Investigar o possível efeito neuroregenerativo do Captopril®, um potencializador dos efeitos endógenos da BK, sobre a morte neuronal induzida por 6-OHDA *in vivo*;

5. Avaliar por ensaios de PCR em tempo real a expressão de marcadores neurais específicos na via nigro-estriatal lesada por 6-OHDA. Os marcadores de padrão utilizados serão: βIII-tubulina (marcador neuronal), e GFAP (marcador de células gliais). Além disso, avaliaremos a expressão do receptor do sistema calicreína-cininas B2BkR;

6. Avaliação *in vitro* do efeito neuroprotetor de BK sobre neurônios com fenótipo dopaminérgico a partir de culturas de células SH-SY5Y.

3. MATERIAIS E MÉTODOS

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3.1 Animais

Ratos Sprague Dawley machos foram utilizados no estabelecimento do modelo de neurodegeneração dopaminérgica no presente trabalho. Os experimentos foram realizados com animais de 60 dias de idade no início dos experimentos, com livre acesso à água e comida. Os animais foram mantidos em ciclo claro e escuro de 12 h:12 h. Os experimentos foram conduzidos em conformidade com as Normas de Bem Estar de Animais de Laboratório, segundo o procedimento submetido e aprovado pela Comissão de Ética do IQ-USP.

3.2 Lesão da via nigro-estriatal com 6-OHDA e procedimento cirúrgico

Para a esterotaxia, os ratos foram anestesiados com 1,4 ml/kg de Ketamina/Xilazina e colocados no estereotáxico. Em seguida, foram injetados com 7 µg/µl de 6-hidroxidopamina (6-OHDA, calculado como base livre) em solução salina 0,9% contendo 0,02% de ácido ascórbico, no estriado direito dos animais (AP -4,4; ML 1,2; DV -8.2, conforme descrito anteriormente por Kirik et al., 1998 (Figura 4) (Paxinos, 2009). Realizamos uma injeção de 7,0 µg de 3,0 µL cada. A agulha foi mantida no local da injeção por 3 min. antes do início da injeção. Após a administração das 6-OHDA, a agulha foi mantida por mais 5 minutos no local da injeção (Ebert et al., 2008).

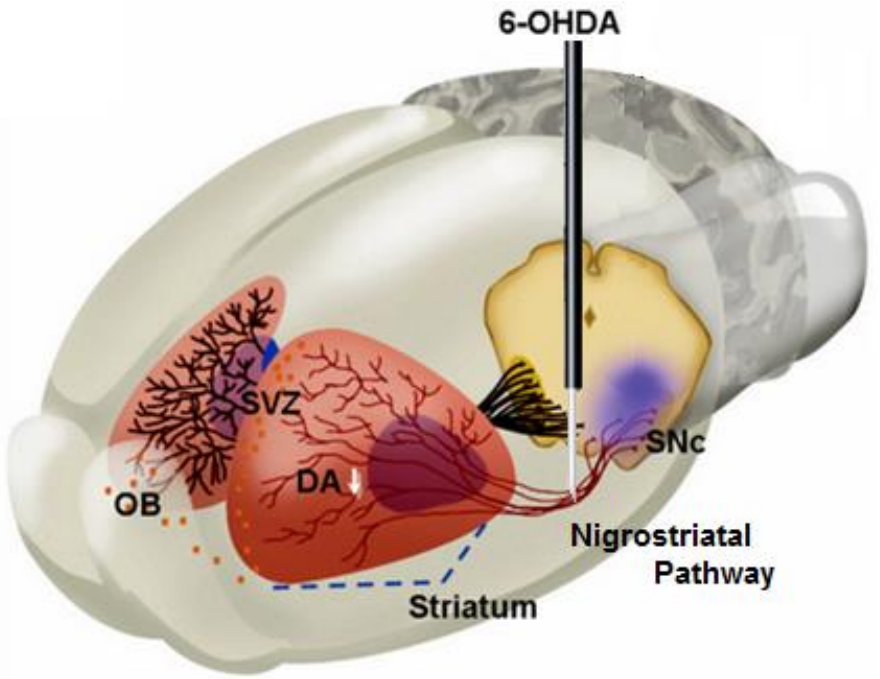

Figura 4: Representação esquemática do protocolo de injeção da 6-OHDA na via nigro-estriatal.

3.3 Injeções de BK, [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina e HOE-140

Uma semana após a lesão, a solução de BK (Tocris, Cat. No. 3004), de [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina (Tocris, Cat. No. 3229) e de HOE-140 (Tocris, Cat. No. 3014) foram injetada no estriado do hemisfério direito dos animais (calculado a partir do bregma), seguindo as coordenadas estereotáxicas: AP-0.4; ML-3.3; DV-5.2 (figura 5) (Paxinos, 2009). Os animais foram anestesiados como descrito no item anterior. Os três compostos foram preparados em solução salina.

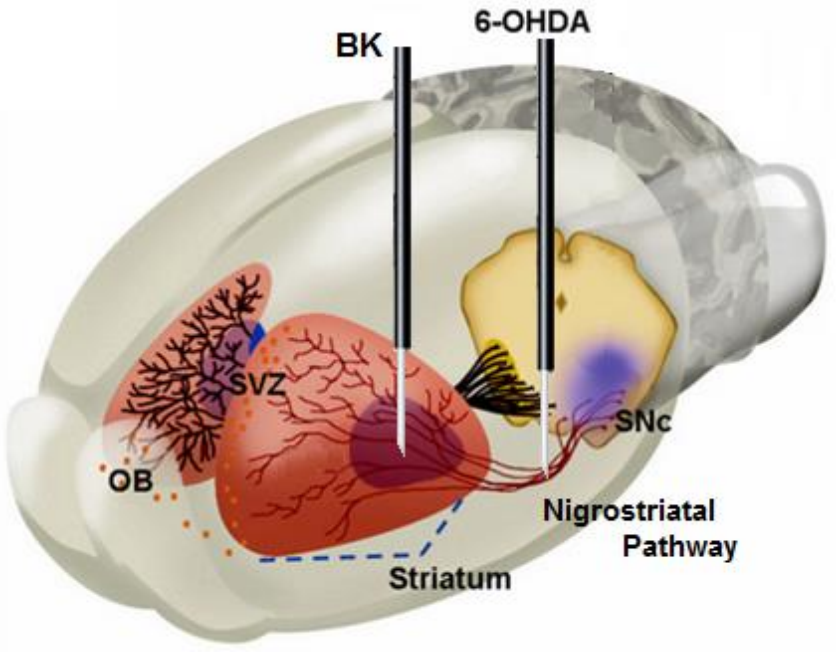

Figura 5: Representação esquemática do protocolo de injeção da BK, da Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina e do HOE-140 no estriado.

3.4 Teste rotacional induzido por anfetamina

Os animais foram testados 7 dias após a lesão da via nigro-estriatal com 6-OHDA e 7 semanas após o tratamento com BK, *[Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina e HOE-140*. Os animais foram pesados e injetados com uma dose de apomorfina recém preparada de 0,5 mg/kg, em solução salina 0,9% estéril contendo ácido ascórbico 0,02%. O número de rotações no sentido anti-horário, isto é, para o lado oposto ao hemisfério do lado onde se produziu injetou a 6-OHDA, foi utilizado para se avaliar o grau da lesão. O critério para a rotação foi um giro de 360 para um dos lados. Esperava-se que o animal lesado rodasse para o lado contralateral ao hemisfério lesado, ou seja, para o lado esquerdo (Henderson et al., 2003; Kirik et al. 1998). Estes testes foram realizados um dia

antes do sacrifício dos animais, de acordo com o protocolo de tratamento com os compostos aqui avaliados.

3.5 Imuno-histoquímica e ensaio de Fluoro Jade B

Foram feitas secções coronais do encéfalo de 30 µm de espessura e armazenadas em placas *multiwell* com solução anticongelante à -20°C. Para visualização de células TH utilizamos anti-TH (Sigma). Os cortes foram selecionados visando as áreas de interesse (estriado e substância negra) e processados *free floating* em placas *multiwell*. Forsam realizados três lavagens de 10 minutos cada em PBS, incubamos em anticorpos primários TH (1:500), onde permaneceram por 12 horas.

Em seguida, os cortes foram lavados em tampão fosfato à temperatura ambiente e incubados por duas horas com anticorpo secundário biotinilado contra as imunoglobulinas do animal no qual foi feito o anticorpo primário (-ms dk b) em uma concentração de 1:200. Após nova série de lavagens à temperatura ambiente, os cortes foram colocados por duas horas numa solução de Triton X-100 0,3% em tampão fosfato 0,1 M com 0,4 M de NaCl, contendo o complexo avidina-biotina-peroxidase (ABC ELITE kit, Vector Labs., Burlingame, CA, EUA). Após nova série de lavagens, os cortes foram imersos num meio contendo 3- a (DAB-Sigma-Aldrich, Saint Louis, MO, EUA) 0,05% em tampão fosfato 0,1 M por 5 minutos. Foram acrescentados a seguir 3 ml de uma solução de H2O2 a 0,1% em água destilada, mantendo-se os cortes neste banho até que a reação fosse revelada. Para interromper essa reação, os cortes foram removidos da solução com DAB e lavados em tampão PB 0,1 M.

As marcações foram analisadas em microscópio óptico EVOS XL Cell Imaging System (Thermo-Fisher, Waltham, MA, USA) e quantificações da marcação foram realizadas com o programa ImageJ (NIH). Obtivemos imagens da substância negra *pars* compacta e do estriado de diferentes alturas anatômicas da estrutura de 5 animais de cada grupo. A quantificação da densidade de fibras TH positivas foi realizada utilizando o software Image J.

As regiões do estriado e da substância negra foram quantificadas separadamente. Para a substância negra, os corpos celulares positivos para TH foram contados usando o próprio Image J. A análise no estriado foi procedida pela quantificação da densidade integrada da marcação obtida em um campo de 2500 μm^2, no Image J. Os valores foram obtidos após realizarmos medidas do corte no estriado, e destas obteve-se uma média. As medidas foram repetidas em 5 cortes para cada animal e destes valores obteve-se uma média para cada animal. Finalmente obtém-se uma razão (média da densidade integrada do hemisfério experimental/média da densidade integrada do hemisfério controle), e, assim, obteve-se um valor final para cada animal, expresso em percentual de marcação, no caso da quantificação no estriado, sendo os hemisférios controle o valor correspondente a marcação máxima (100%).

Para realizar o ensaio do Fluoro Jade B, um modelo de fluorocromo que de detecção de degeneração neuronal, utilizamos cortes da região da substância negra. As lâminas foram lavas na seguinte sequência: etanol absoluto por 3 minutos, etanol a 70% por 1 minuto e água deionizada por 1 minuto. Depois, as lâminas foram imersas em solução de 0,06% permanganato de potássio em água deionizada, por 15 minutos sob agitação. Após nova lavagem das lâminas em água deionizada por 1 minuto, estas foram incubadas na solução de 0,001%

de Fluoro-Jade B (Histochem; Jefferson, AK) em água deionizada com 0,1% de ácido acético por 30 minutos sob agitação, em câmara escura. Por último, as lâminas foram lavadas três vezes em água deionizada e banhadas 2 vezes em xilol, por 1 minuto, sendo então cobertas com DPX (EMS; Fort Washington, PA) e lamínulas. As lâminas foram analisadas em microscópio de fluorescência. As iImagens foram capturadas para ilustração.

3.6 Perfusão dos animais

Os animais foram anestesiados com Ketamina/Xilazina, como descrito no item 3.2, a cavidade torácica será aberta e o coração será exposto de modo a visualizar a aorta ascendente. Inseriremos então uma agulha de 18-gauge no ventrículo esquerdo, até a introdução na aorta ascendente. Efetuamos um corte na aurícula direita e, com o auxílio de uma bomba peristáltica, seguido de lavagem com PBS (200 ml) para retirada de todo a sangue. Logo em seguida iniciou-se a infusão de solução de PBS/PFA 4% a 4°C (250 ml), até total fixação dos tecidos, lentamente, para melhor fixação e preservação dos tecidos. Ao final da perfusão, o cérebro será removido e colocado em solução de PBS/PFA 4% a 4°C por 2 dias. Os cérebros foram transferidos para solução de PBS/sacarose 30% a 4ºC por 48h e seccionados em criostato (Leica).

3.7 Cultura de células da linha SH-SY5Y

As células SH-SY5Y (*LGC Promochem*) foram cultivadas em meio DMEM/F12, na proporção de 1:1 (Meio essencial mínimo modificado por

Dulbeco: mistura de nutrientes de Ham F12, Gibco), suplementado com 10% de soro fetal bovino (FBS) e antibióticos (Invitrogen), em atmosfera a 37ºC com 5% de CO^2. As células foram mantidas em garrafas plásticas (75 cm3, volume de 250 mL) até confluência de 80%, sendo depois tripsinizadas com 2 mL de tripsina.

As células em suspensão foram contadas para determinar a viabilidade pelo azul de Tripan, e posteriormente, repassadas para placas *multi-well* de 24 poços em uma concentração de 1 x 10^4 células/poço. Os ensaios foram condizidos com células em estado de indiferenciação, uma vez que já apresentam o fenótipo dopaminérgico (Lopes et al., 2010). As células foram tratadas com BK, HOE-140 por 30 minutos antes da exposição 6-OHDA 30 µM durante 24 horas. A 6-OHDA foi diluída, minutos antes da incubação, em solução salina com 0,1% de ácido ascórbico para evitar oxidação.

3.8 Avaliação da citotoxicidade

Para avaliar a citotoxicidade dos compostos ensaiados, foram utilizadas duas técnicas. Primeiro a da redução do MTT (brometo de [3-(4-5-dimetiltiazol-2-il]-2-5-difeniltetrazolium), que é um simples método colorimétrico que mede a proliferação ou citotoxicidade *in vitro* (Monsmann, 1983; Talorete, *et al*., 2006).

O ensaio colorimétrico do MTT tem sido amplamente empregado na avaliação da proliferação e toxicidade celular. O MTT é um sal de tetrazólio solúvel em água, possui cor amarela e é convertido em cristais de formazan púrpura, insolúveis em água, após clivagem do anel de tretazólio por desidrogenases mitocondriais e lisossomais. Ao final da exposição a drogas utilizadas neste estudo, as células foram incubadas com 70 µL da solução de

MTT (5 mg/mL em PBS, preparada minutos antes) e acondicionadas estufa por 3 horas para permitir a redução do MTT. Após incubação, os cristais de formazan foram dissolvidos em 700 µL de isopropanol durante 30 minutos sob agitação, sendo a solução púrpura resultante medida em espectrofotômetro a 540 nm.

Os cristais de formazan são dissolvidos com adição de SDS 10% em HCl 0,01N, formando um composto colorido cuja densidade óptica pode ser medida por meio de espectrofotometria. Para tanto, utilizou-se um leitor do tipo ELISA (λ=540 nm). A atividade enzimática de células vivas é diretamente proporcional à capacidade redutora sobre o MTT (Liu et al., 1997).

3.9 PCR em tempo real

A análise dos marcadores presentes ao longo da diferenciação neurais βIII-tubulina e GFAP (*Glia Fibrillary Acidic Protein*), marcador de células gliais, e dos transcritos do receptor B2BkR de cininas, foi realizada através de PCR em tempo real com equipamento ABI-PRISM 7000 utilizando SYBR®-Green Dye como sistema de detecção. Cada reação será feita em duplicata num volume final de 12 µL. Para o PCR em tempo real foram utilizados 1 µl de cDNA , 3,0 µl de *primer Forward e Reverse* e 6,0 µl de SYBR®-Green Dye. As amplificações serão realizadas em termociclador - PRISM 7000 nas seguintes condições: 40 ciclos de 95°C por 2 minutos, 95°C por 30 segundos, 60°C por 30 segundos, 72°C por 30 segundos, seguido de curva de dissociação. Após esse processo, será feita a verificação da especificidade do *amplicon* através de uma curva de dissociação.

Para avaliar a especificidade das reações de amplificação devido a ligação inespecífica do SYBR®-Green as duplas fitas de DNA foram feitas reações de dissociação do produto amplificado de cada reação. Assim, a temperatura das amostras será elevada gradativamente e a fluorescência será medida. Os dadosforam normalizados e, portanto, para cada amostra de cDNA foram feitas duas reações: uma com *primers* para o gene alvo e outra com *primers* para genes com expressão constitutiva como a GAPDH (Gliceraldeído 3-fosfato desidrogenase).

Cada reação foi feita em triplicata num volume final de 25 µL, usando-se o cDNA transcrito reversamente a partir de 3 µg de RNA, 12,5 µL de SYBR-Green (Applied Biosystems) e 200 ηM de cada *primer* (tabela 1). A PCR em tempo real será feita nas condições: 95 °C por 10 min e 40 ciclos de 95 °C por 15 s e 60 °C por 1 min; seguidos pela verificação da especificidade do *amplicon* pela curva de dissociação. Para verificar se a eficiência de todas as reações foi maior que 98%, o parâmetro de expressão 2-ΔCt foi utilizado para avaliar a expressão relativa a GAPDH (*Glyceraldehyde 3-phosphate dehydrogenase*), controle endogeno. ΔCt = variação de ciclos acima do limiar. A análise das expressões relativas de cada transcrito será conduzida conforme Ueno e colaboradores (2002).

Tabela 1 - Sequências de *primers* utilizados nas reações de PCR em tempo real

cDNA	*Primer Forward (5'- 3')*	*Primer Reverse (5'- 3')*	**Amplicon (pb)**
B2BkR	CCCTTCCTCTGGGTCCTCTT	CAGAACACGCTGAGGACAAAGA	105
β3-tubulina	GAGACCTACTGCATCGACAATGAAG	GCTCATGGTAGCAGACACAAGG	111
GFAP	AAGAGTGGTATCGGTCCAAGTTTG	CAGTTGGCGGCGATAGTCAT	107

3.10 Estatística

Nos experimentos com animais, as variáveis foram comparadas entre os diversos grupos com uma análise de One-way ANOVA, seguida de teste *post hoc* de Tukey. Os valores foram expressos com *Standard Error of Means* (SEM), considerando valores com $p<0,05$ como significativos.

Todos os ensaios para a técnica do MTT foram realizados em triplicatas tendo sido realizados três experimentos. Os resultados obtidos foram analisados pelo programa GraphPad Prism versão 6.0, por meio de análise de variância One-way ANOVA utilizando o teste de Bonferroni a partir dos dados normalizados, os resultados foram considerados significativos quando $p < 0,05$.

3.11 Comitê de ética

Este trabalho foi realizado com a aprovação do Comitê de Ética em Pesquisa (CEP) do Instituto de Química da Universidade São Paulo (IQ-USP) sob os protocolos: 15/2013 e 4/2014.

4. RESULTADOS

4. RESULTADOS

4.1 Lesão da via nigro-estriatal com 6-OHDA e tratamento com bradicinina

A primeira etapa consistiu na padronização das lesões com 6-OHDA, gerando lesões extensas. As coordenadas utilizadas para as injeções de 6-OHDA foram: dorso-ventrais [AP -4,4; ML -1,2; DV e -8,2 (Paxinos, 2009)] com lesões superiores 90% das regiões da substância negra e do estriado. A extensão das lesões foi avaliada por testes rotacionais induzidos pela injeção de apomorfina. Animais que apresentaram mais que 4 rotações/minuto foram considerados com lesão >90%. Animais com lesões <90% não apresentaram rotações. A extensão das lesões também foi avaliada por imunohistoquímica para TH e coloração de Fluoro Jade, que marca neurônios em degeneração (Figuras 6 e 7).

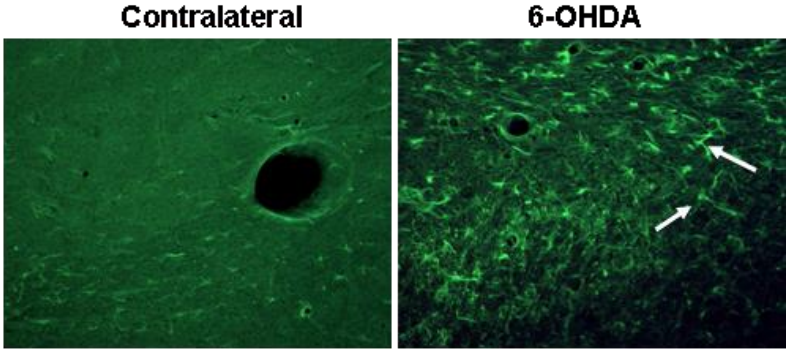

Figura 6: A marcação no lado experimental (6-OHDA) mostrando processos de degeneração 7 dias após a injeção da neurotoxina. Representação de cortes da região da substância negra de animais que sofreram lesão com 6-OHDA. A marcação para Fluoro Jade B, indica degeneração neuronal. A marcação de Fluoro Jade B nos cortes correspondentes à região da lesão foi comparada à marcação da mesma região do hemisfério não lesado (Contralateral).

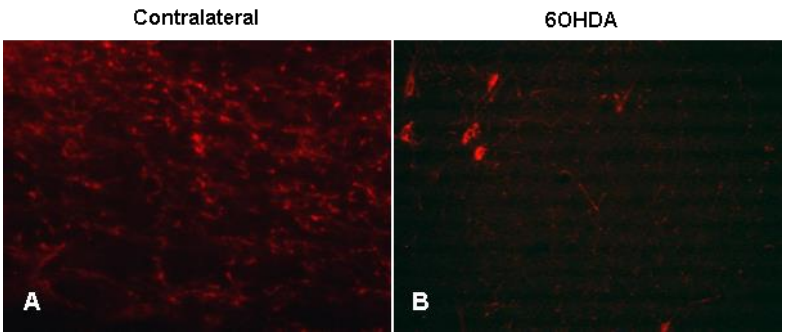

Figura 7: A marcação para neurônios dopaminérgicos (TH$^+$) no hemisfério contralateral, não lesado (A) difere da marcação no hemisfério lesado, onde há pouca marcação para TH (B). Avaliação qualitativa da degeneração de neurônios dopaminérgicos após lesão com 6-OHDA na substância negra por ensaios imuno-histoquímicas.

O primeiro protocolo realizado após o estabelecimento da lesão da via nigro-estriatal pela 6-OHDA, foi o estabelecimento a obtenção de uma dose de BK capaz efetiva para a reversão da lesão da lesão por 6-OHDA em modelo animal de DP. Nesse procedimento, a lesão é estabelecida no hemisfério direito e o hemisfério esquerdo utilizado como controle. Após 7 dias, os animais que apresentaram comportamento rotacional quando estimulados com apomorfina (0,5 mg/kg), foram selecionados para a continuidade do estudo.

Por se tratar de uma metodologia ainda não descrita na literatura, iniciou-se a aplicação de uma dose única de BK (2,0 µg/animal) na região do estriado [AP-0.4; ML-3.3; DV-5.2 (Paxinos, 2009)] e os testes rotacionais foram realizados novamente para avaliar se houve melhora motora (comportamental) entre os animais que receberam BK e aqueles tratados com salina. Nos primeiros testes, observamos que a BK interfere no comportamento rotacional típico do modelo animal de DP, enquanto animais tratados com salina não apresentaram alterações comportamentais (figura 8, tabela 2). Ou seja, nos animais do grupo tratado com BK, observamos uma redução do comportamento rotacional. Observando a tabela 2, nota-se que alguns animais dentro do grupo tratado com BK, não apresentam resposta ao tratamento com BK na dose utilizada, ou seja,

apresentam respostas no teste com apomorfina maiores do que 4 rotações por minuto.

Levando em consideração os promissores resultados obtidos, posteriormente, testamos doses maiores de BK (4 µg e 6 µg/animal). Novamente, podemos afirmar que a BK interfere no comportamento rotacional típico do modelo animal de 6-OHDA, a ponto de, no grupo tratado, os animais cessarem as rotações após a indução por apomorfina. Animais tratados com salina não apresentaram alterações comportamentais 8 semanas após o tratamento (figura 8, tabelas 3 e 4).

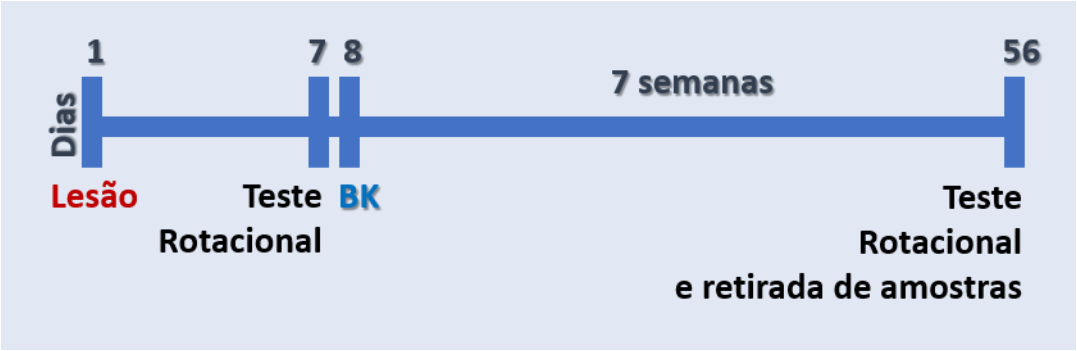

Figura 8a: Resumo esquemático do procedimento de estabelecimento da lesão e tratamento com bradicinina.

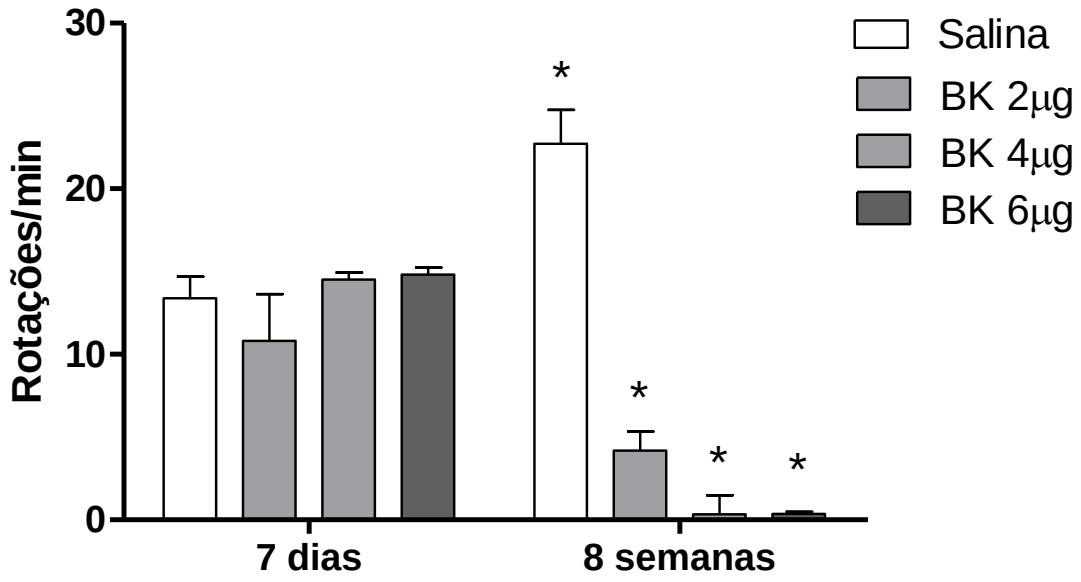

Figura 8b: Comparação entre o número de rotações antes (7 dias pós-lesão) e após o tratamento com Bk ou salina (8 semanas). * p<0,05, indica significância entre o grupo que recebeu BK e o grupo que recebeu apenas salina. (n: para ver conferir o tamanho dos grupos experimentais ver tabelas 2, 3 e 4).

Tabela 2: Número de rotações/minuto induzidas por apomorfina em cada animal antes e após o tratamento com Bk (2µg) ou salina. * tabela indica que alguns animais dentro do grupo tratado com BK, não apresentam resposta ao tratamento com BK na dose utilizada, ou seja, mais de 4 rotações por minuto.

Tratamento	nº de rotações por minuto 7 dias pós-lesão	nº de rotações por minuto 8 semanas pós tratamento com Bk
Bk	09	0
Bk	18	0
Bk	20	0
Bk	5	0
Bk	14	28*
Bk	6	10
Bk	4	8
Bk	8	0
Bk	5	3
Bk	15	0
Bk	14	3
Bk	12	12*
Salina	9	10
Salina	12	10
Salina	8	10
Salina	15	16
Salina	11	11
Salina	17	11
Salina	15	18
Salina	17	17
Salina	09	19
Salina	10	20

Tabela 3: Número de rotações/minuto induzidas por apomorfina em cada animal antes e após o tratamento com Bk (4,0 µg) ou salina.

Tratamento	nº de rotações por minuto 7 dias pós-lesão	nº de rotações por minuto 8 semanas pós tratamento com Bk
Bk	8	0
Bk	12	0
Bk	23	1
Bk	20	0
Bk	17	2
Bk	5	1
Bk	20	3
Bk	20	2
Bk	20	0
Salina	16	26
Salina	13	32
Salina	19	13
Salina	12	20
Salina	14	22
Salina	15	19

Tabela 4: Número de rotações/minuto induzidas por apomorfina em cada animal antes e após o tratamento com Bk (6,0 µg) ou salina.

Tratamento	nº de rotações por minuto 7 dias pós-lesão	nº de rotações por minuto 8 semanas pós tratamento com Bk
Bk	19	0
Bk	17	1
Bk	22	0
Bk	19	1
Bk	23	0
bk	21	2
Salina	23	21
Salina	19	29
Salina	26	15
Salina	22	24
Salina	18	20

4.2 Lesão da via nigro-estriatal com 6-OHDA e tratamento com [Phe^{8}y(CH-NH)Arg^{9}]-Bradicinina

Nesta seção o objetivo era avaliar os efeitos do agonista estável do receptor B2BKR [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina, com o objetivo de esclarecer os mecanismos pelos quais a BK exerce o seu papel. Se de fato a ação é via ativação do receptor B2BkR.

Como no caso da BK, depois do período de 7 semanas após as injeções de 6-OHDA e 6 semanas após a injeção de 4,0 µg de [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina no estriado, ou seja, a mesma dose da bradicinina, um novo teste comportamental rotacional induzido por apomorfina foi realizado. Os dados, de modo geral, revelaram mudanças no comportamento rotacional dos animais que receberam [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina, nos quais não observamos comportamento rotacional assimétrico.

Aqui podemos afirmar que o agonista estável do receptor B2BKR [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina (4,0 µg) interfere no comportamento rotacional típico do modelo animal de 6-OHDA, com resultados similares àqueles observados quando os animais foram tratados com BK. Por outro lado, animais tratados com salina, não apresentaram alterações comportamentais 6 semanas após o tratamento (figura 9, tabela 5).

Figura 9a: Resumo esquemático do procedimento de estabelecimento da lesão e tratamento com (4,0 µg) [Phe^{8}Ψ(CH-NH)Arg^{9}]-bradicinina.

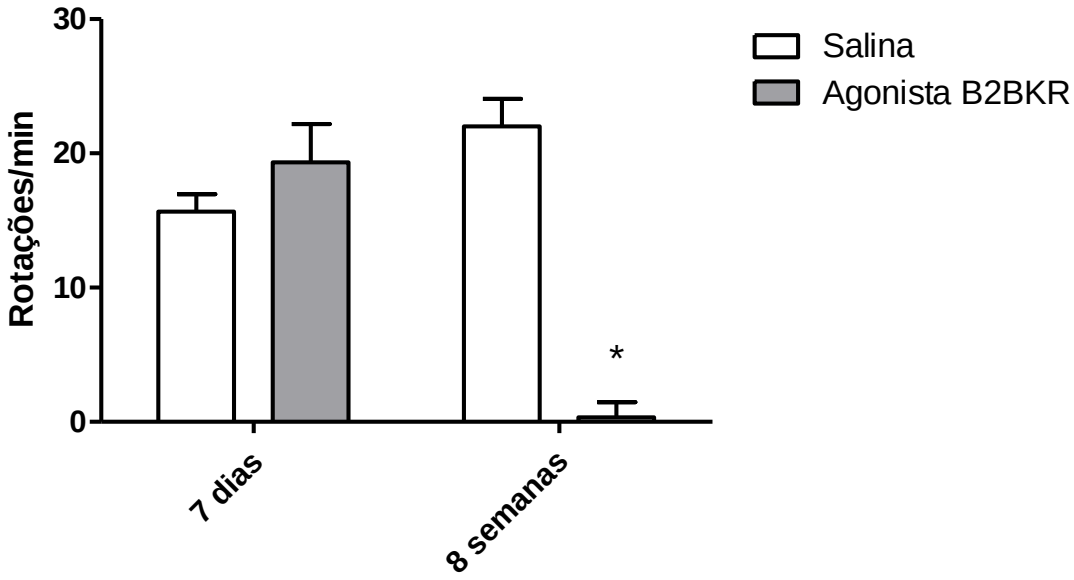

Figura 9b: Comparação entre o número de rotações antes (7 dias pós-lesão) e após o tratamento com 4,0 µg [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina ou salina (7 semanas). * p<0,05 em relação ao grupo [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina 7 dias pós-lesão. (n: 5 animais por grupo).

Tabela 5: Número de rotações/minuto induzidas por apomorfina em cada animal antes e após o tratamento com 4 µg de Bk-Phe^{8}Ψ(CH-NH)Arg^{9} (4,0 µg) ou salina.

Tratamento	nº de rotações por minuto 7 dias pós-lesão	nº de rotações por minuto 8 semanas pós tratamento com Bk Phe^{8}Ψ(CH-NH)Arg^{9}
Bk Phe^{8}Ψ(CH-NH)Arg^{9}	19	0
Bk Phe^{8}Ψ(CH-NH)Arg^{9}	17	1
Bk Phe^{8}Ψ(CH-NH)Arg^{9}	22	0
Bk Phe^{8}Ψ(CH-NH)Arg^{9}	21	2
Bk Phe^{8}Ψ(CH-NH)Arg^{9}	17	1
Salina	19	21
Salina	13	23
Salina	15	22
Salina	23	17
Salina	21	25

4.3 Lesão da via nigro-estriatal com 6-OHDA e tratamento com HOE-140

Nesta seção o objetivo era avaliar os efeitos do antagonista estável do receptor B2BKR HOE-140 (4,0 µg), com o objetivo de esclarecer os mecanismos pelos quais a BK exerce o seu papel. Se de fato a ação é via ativação do receptor B2BkR.

Aqui podemos afirmar que o antagonista estável do receptor B2BKR HOE140 não interfere no comportamento rotacional típico do modelo animal de 6-OHDA, com resultados similares àqueles observados quando os animais foram tratados com salina (figura 10, tabela 6).

Figura 10a: Resumo esquemático do procedimento de estabelecimento da lesão e tratamento com 4 µg de HOE-140. (n: 5 animais por grupo).

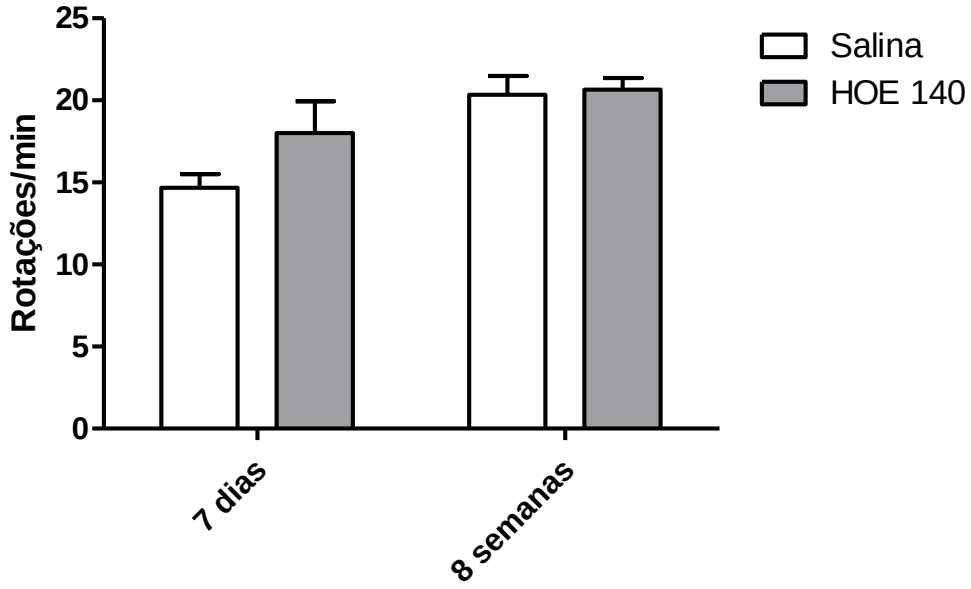

Figura 10b: Comparação entre o número de rotações antes (7 dias pós-lesão) e após o tratamento com 4 µg HOE-140 (ou salina). (n: 5 animais por grupo).

Tabela 6: Número de rotações/minuto induzidas por apomorfina em cada animal antes e após o tratamento com HOE-140 (4,0 µg) ou salina.

Tratamento	nº de rotações por minuto 7 dias pós-lesão	nº de rotações por minuto 8 semanas pós tratamento com HOE-140
HOE-140	14	20
HOE-140	14	22
HOE-140	16	19
HOE-140	15	18
HOE-140	18	19
Salina	13	20
Salina	19	27
Salina	22	15
Salina	18	24
Salina	11	15

4.4 Lesão da via nigro-estriatal com 6-OHDA e tratamento com Captopril®

O Captopril®, inibidor da ACE (*Angiotensin conversion Enzyme*), um potencializador dos efeitos fisiológicos da BK, também foi efetivo no tratamento de animais submetidos à lesão da via nigro-estriatal por 6-OHDA. Os animais receberam salina ou Captopril® por 7 dias consecutivos via oral, numa dose de 3,0 mg/Kg (Munoz, 2006). No grupo tratado, os animais também cessarem as rotações, enquanto que animais tratados com salina não apresentaram alterações comportamentais (Figura 11 e tabela 7). Estes dados são absolutamente inéditos, não havendo qualquer relato na literatura sobre tais efeitos.

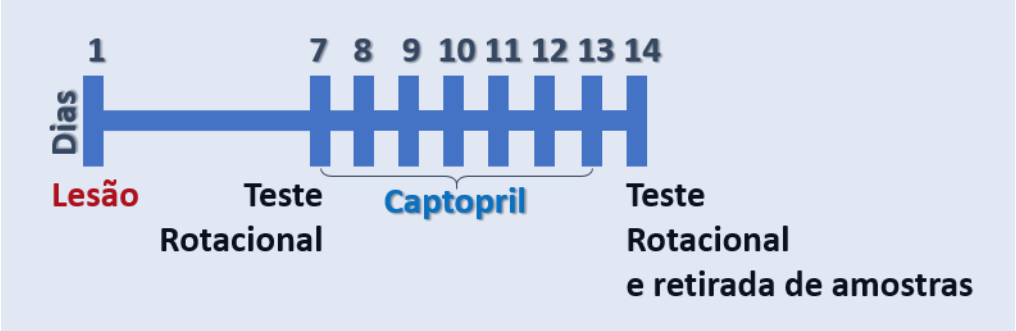

Figura 11a: Resumo esquemático do procedimento de estabelecimento da lesão e tratamento com 3mg/kg de Captopril®.

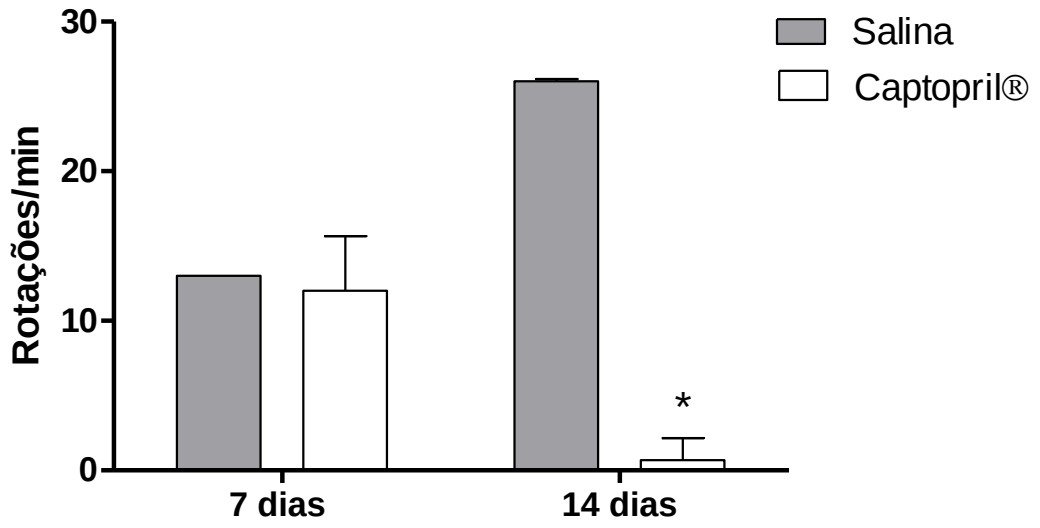

Figura 11b: Comparação entre o número de rotações antes (7 dias pós-lesão) e após o tratamento com 3mg/kg de Captopril® ou salina (14 dias após a lesão). * p<0,05 em relação ao grupo Captopril® 14 dias pós-lesão. (n: 5 animais por grupo).

Tabela 7: Número de rotações/minuto induzidas por apomorfina em cada animal antes e após o tratamento com Captopril (3,0 mg/kg) ou salina.

Tratamento	nº de rotações por minuto 7 dias pós-lesão	nº de rotações 14 dias após tratamento com Captopril®
Captopril	14	2
Captopril	14	0
Captopril	16	0
Captopril	12	1
Captopril	10	4*
Salina	13	20
Salina	19	27
Salina	22	25
Salina	22	26
Salina	24	25

4.5 Análise histológica do marcador de neurônios dopaminérgicos tirosina hidroxilase

Também avaliamos o padrão de fibras positivas para tirosina hidroxilase (TH) (específica para neurônios dopaminérgicos) nas regiões da substância negra e do estriado. Essa quantificação foi realizada por análise de intensidade de marcação com o uso do software Image J para as fibras no estriado e contagem de corpos celulares para as marcações na substância negra.

4.5.1 Análise histológica do marcador de neurônios dopaminérgicos tirosina hidroxilase após lesão da via nigro-estriatal com 6-OHDA e tratamento com bradicinina

A quantificação dos cortes da região do estriado mostrou que o tratamento com BK, 7 dias após a lesão por 6-OHDA, resultou em maior número de fibras TH^+, não diferindo significativamente do hemisfério contra-lateral, correspondente ao controle, considerado como 100% da marcação. Por outro lado, cortes da região da substância negra não apresentaram diferença significativa na porcentagem de fibras TH^+ entre os grupos BK e salina. A porcentagem de fibras TH^+ na região do estriado de animais do grupo BK foi significativamente maior que o grupo salina. Esses resultados demonstram que a BK apresenta efeito neuroregenerativo no estriado de animais que sofreram lesão da via nigro-estriatal por 6-OHDA, com porcentagens de fibras TH^+ semelhantes às regiões contra-laterais, o que em parte também foi observado na região da substância negra (figuras 12, 13, 14 e 15).

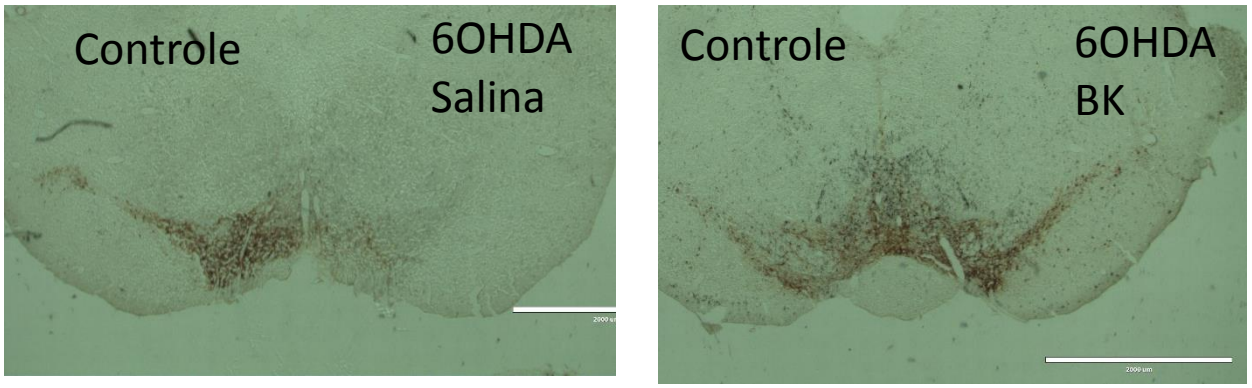

Figura 12: Cortes representativos da marcação para TH das regiões da da substância negra (SN) de animais lesados por 6-OHDA e tratados com injeção de 4µg de BK 8 semanas após a lesão. Aumento de 4x. (Crt: controle; Bk: bradicinina). A barra branca indica em escala uma área de 2000 µm.

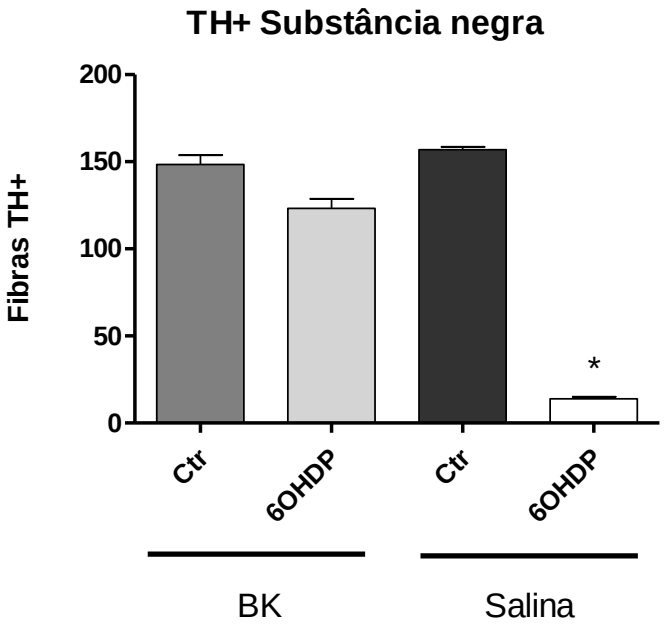

Figura 13: Médias das quantificações de células positivas para TH em cortes das regiões da substância negra (SN) tratados com 4µg de BK e do hemisfério lesado em relação ao hemisfério não lesado contra-lateral (Controle). Resultados expressos como média ± SEM. n=11 cortes por animal, $p<0,05$.

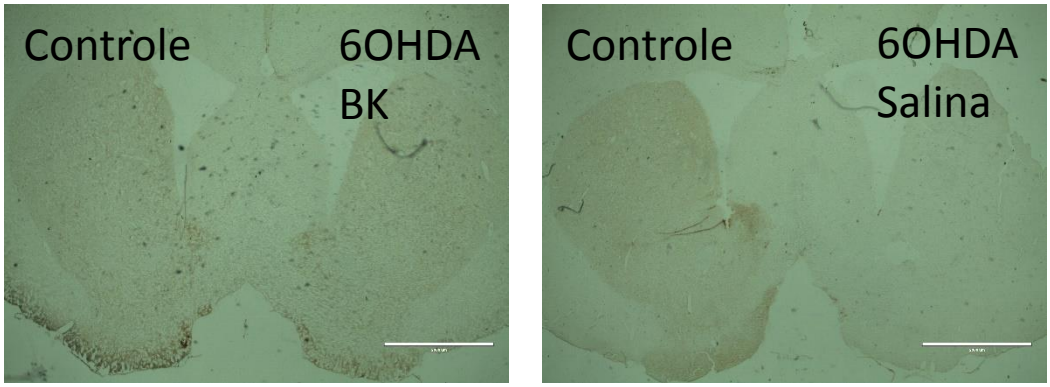

Figura 14: Cortes representativos da marcação para TH em cortes das regiões do estriado e da substância negra (SN) de animais lesados por 6-OHDA e tratados com injeção de 4µg de BK 8 semanas após a lesão. Aumento de 4x. (Bk: bradicinina). A barra branca indica em escala uma área de 2000 µm. (n: 5 animais por grupo)

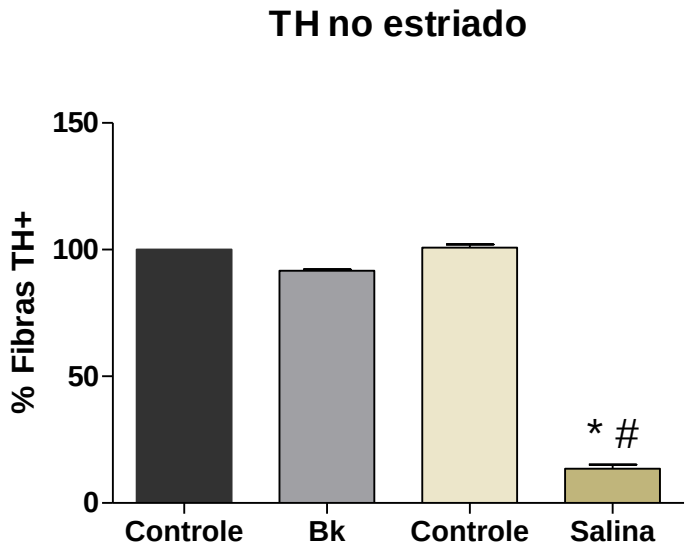

Figura 15: Médias das quantificações da densidade de fibras positivas para TH em cortes das regiões do estriado do hemisfério lesado em relação ao hemisfério não lesado contra-lateral (Controle). Resultados expressos como média ± SEM. n=5 cortes por animal, p<0,05. (n: 5 animais por grupo)

4.5.2 Análise histológica do marcador de neurônios dopaminérgicos tirosina hidroxilase após lesão da via nigro-estriatal com 6-OHDA e tratamento com Captopril®

Também avaliamos o padrão de fibras positivas para TH nas regiões da substância negra e do estriado após o tratamento com Captopril®. A quantificação em cortes da região do estriado mostrou que o tratamento com Captopril®, 7 dias após a lesão por 6-OHDA, resultou em maior número de fibras TH^+, não diferindo significativamente do hemisfério contra-lateral. Novamente, cortes da região da substância negra não apresentaram diferença significativa na porcentagem de fibras TH^+ entre os grupos BK e salina. A porcentagem de fibras TH^+ na região do estriado de animais do grupo Captopril® foi significativamente maior que o grupo salina. Esses resultados demonstram que o Captopril® apresenta efeito neuroregenerativo no estriado de animais que sofreram lesão da via nigro-estriatal por 6-OHDA, com porcentagens de fibras TH^+ semelhantes às regiões contra-laterais, o que em parte também foi observado na região da substância negra (figuras 16, 17, 18 e 19).

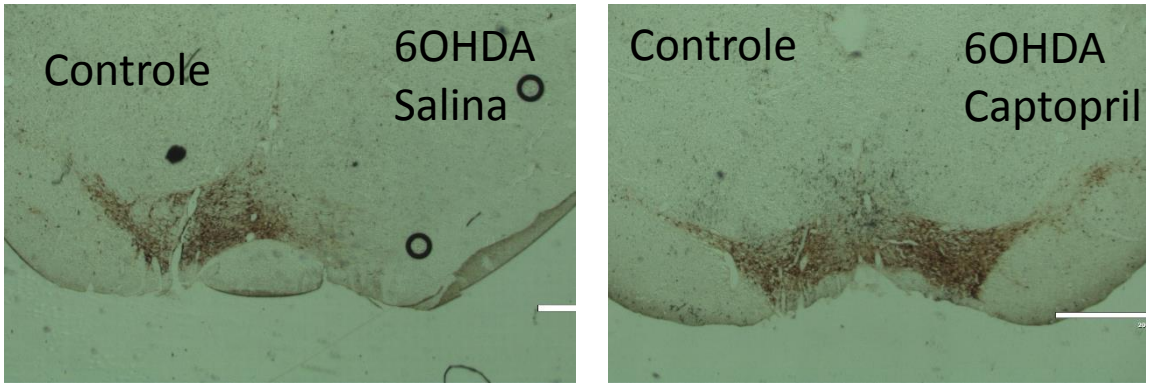

Figura 16: Cortes representativos da marcação para TH em cortes da região da substância negra (SN) de animais lesados por 6-OHDA e tratados com 3,0 mg/kg Captopril. Aumento de 4x. A barra branca indica em escala uma área de 2000 µm.

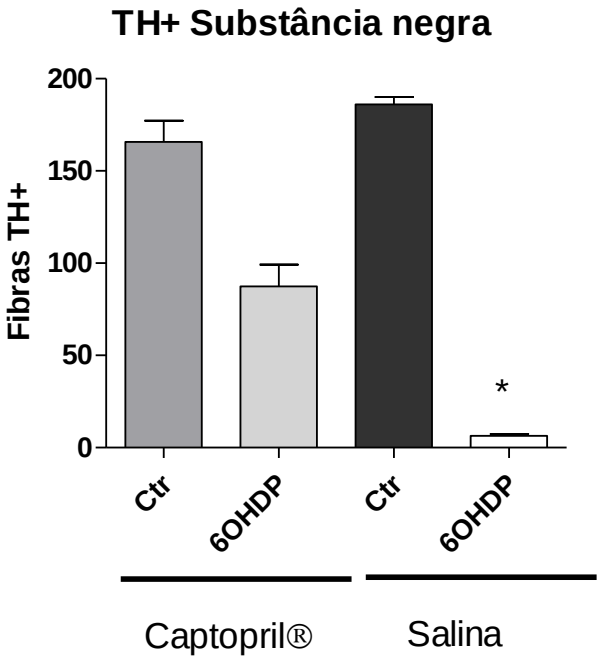

Figura 17: A: Médias das quantificações de células positivas para TH em cortes da região da substância negra (SN) do hemisfério lesado em relação ao hemisfério não lesado contra-lateral (Controle) de animais tratados com Captopril (3 mg/kg) e salina. Resultados expressos como média ± SEM. n=11 cortes por animal, $p<0,05$. (n: 5 animais por grupo)

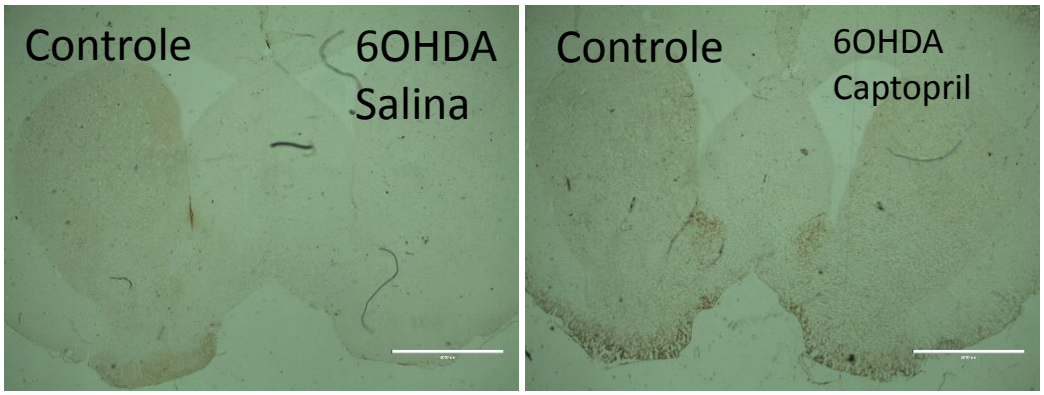

Figura 18: Cortes representativos da marcação para TH em cortes da região do estriado de animais lesados por 6-OHDA e tratados com 3,0 mg/kg Captopril. Aumento de 4x. (Crt: controle; Bk: bradicinina). A barra branca indica em escala uma área de 2000 µm.

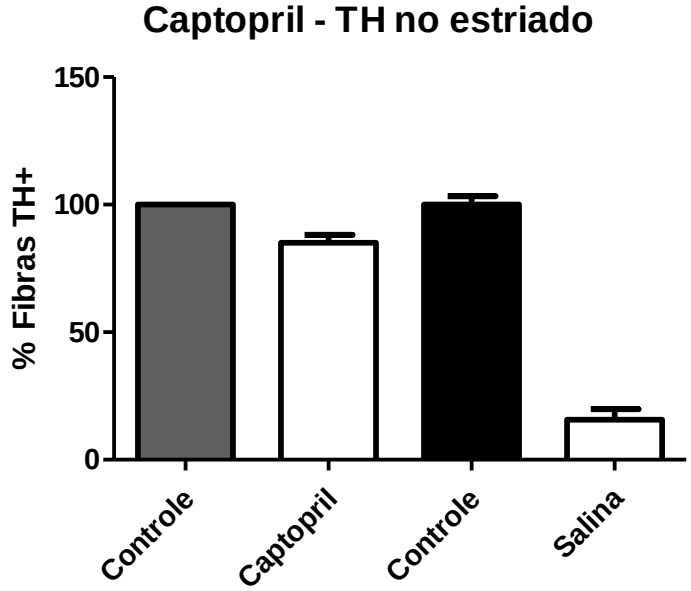

Figura 19: Médias das quantificações da densidade de fibras positivas para TH em cortes da região do estriado do hemisfério lesado em relação ao hemisfério não lesado contra-lateral (Controle) de animais tratados com Captopril (3 mg/kg). Resultados expressos como média ± SEM. n=5 cortes por animal, $p<0,05$.

4.6 Expressão dos genes dos marcadores neurais e do receptor B2BKR

Paralelamente determinamos o perfil de expressão gênica dos genes das proteínas utilizadas como marcadores neuronais (β3-tubulina) e gliais (GFAP) e do receptor B2BKR de cininas no estriado de animais lesionados por 6-OHDA e tratados com BK ou salina 8 semanas após os tratamentos (figuras de 20-22).

A expressão desses genes foi analisada quantitativamente através da técnica de PCR em tempo real. Os níveis de expressão foram normalizados pela expressão de GAPDH, cuja expressão é estável, constitutiva, isenta de alterações nestas condições de análise.

A expressão de β3-tubulina e GFAP foi regulada durante o processo de regeneração ao final das 8 semanas de tratamento, entre os hemisférios controle e tratados com BK ou salina. Verifica-se uma diminuição da expressão dos transcritos do marcador neural β3-tubulina nos animais lesionados que receberam apenas salina. Nos tratados com BK, verifica-se um aumento da expressão dos transcritos de β3-tubulina em relação aos hemisférios controles (figura 20).

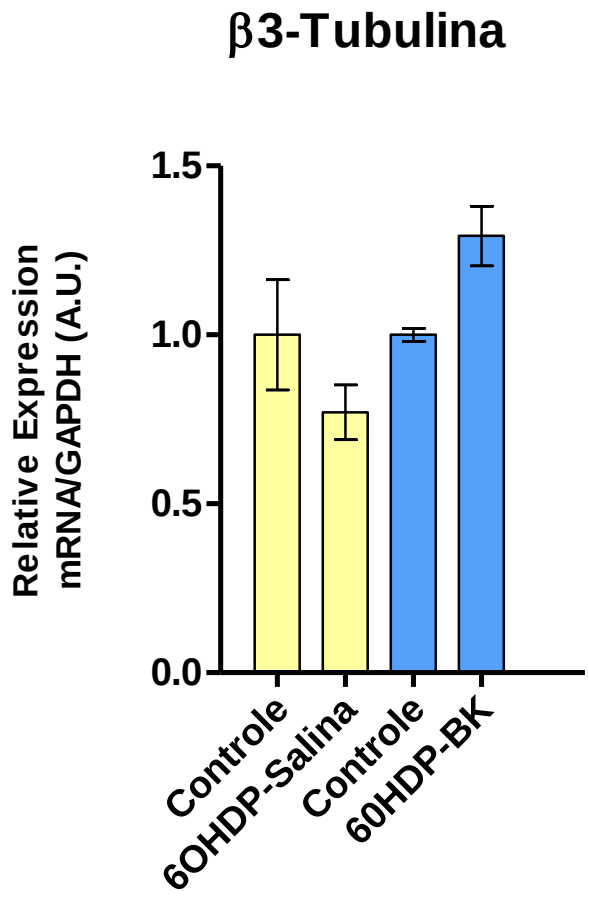

Figura 20: Expressão relativa dos RNAm do marcador neuronal β3-tubulina nos hemisférios controle e tratados com salina e Bk no estriado. As análises quantitativas da expressão do marcador neuronal β3-tubulina do estriado de animais adultos foi feita por PCR em tempo real tendo como referência a expressão do GAPDH para quantificação relativa. Quando comparamos os níveis de expressão do gene nos lados controle e tratados (salina e Bk) verifica-se uma diminuição da expressão deste marcador neural nos animais que receberam apenas salina. Nos animais cujos hemisférios lesados foram tratados com bradicinina, verifica-se um aumento da expressão deste marcador. Onde: Bk: bradicinina 4,0 µg. (n: 3 animais por grupo)

O marcador glial GFAP também teve sua expressão regulada durante o processo de regeneração ao final das 8 semanas pós-tratamento entre os hemisférios controle e tratados com salina ou Bk. O padrão de expressão gênica do marcador glial GFAP é distinto daquele observado para o marcador neuronais β3-tubulina. Quando comparamos os níveis de expressão do gene nos lados controle e tratados (salina e Bk) verifica-se um aumento da expressão deste

marcador neural nos animais que receberam salina e também nos animais tratados com Bk em relação aos hemisférios controles (Figura 21).

No entanto, é maior a expressão dos transcritos do GFAP nos animais tratados com BK em relação aos animais que receberam apenas salina.

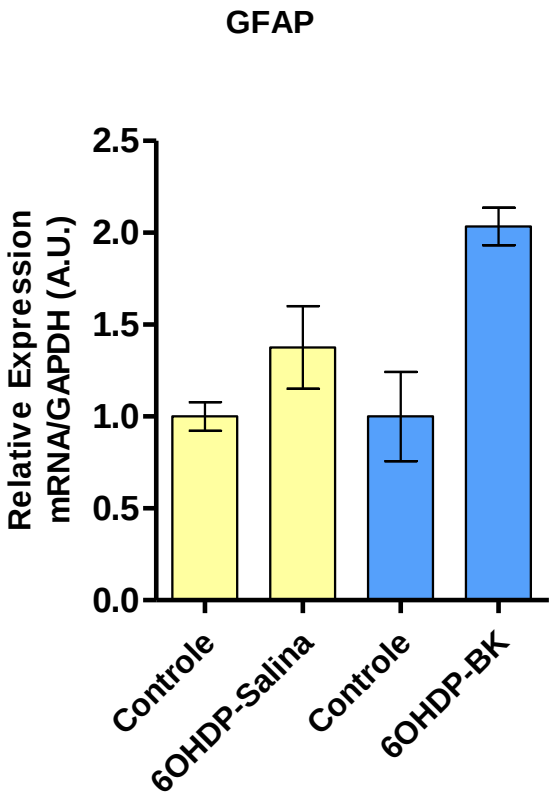

Figura 21: Expressão relativa dos RNAm do marcador neuronal GFAP nos hemisférios controle e tratados com salina e Bk no estriado. As análises quantitativas da expressão do marcador neuronal GFAP do estriado de animais adultos foi feita por PCR em tempo real tendo como referência a expressão do GAPDH (*housekeeping gene*). Quando comparamos os níveis de expressão do gene nos lados controle e tratados (salina e Bk), verifica-se um aumento da expressão deste marcador neural nos animais que receberam salina e Bk em relação ao lado controle. Onde: Bk: bradicinina 4,0µg. (n: 3 animais por grupo)

Finalmente, analisamos a expressão do receptor B2BKR, alvo do nosso estudo. Observa-se que a expressão do receptor está alterada nos hemisférios lesados em relação ao hemisfério controle. A análise quantitativa mostra um

aumento na expressão dos transcritos do receptor B2BkR nos hemisférios lesionados. Além disso, o animal tratado com Bk tem um aumento mais acentuado na expressão dos transcritos deste receptor (figura 22).

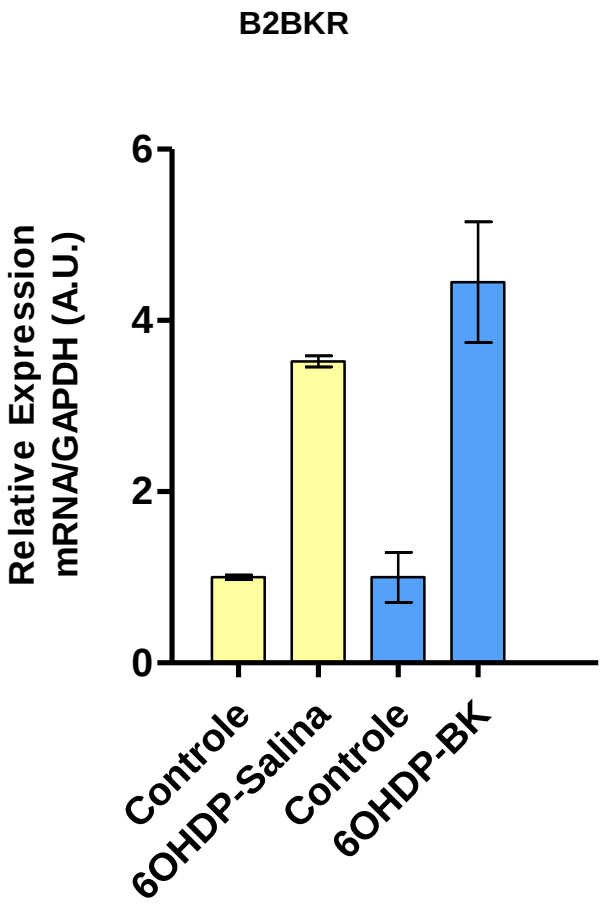

Figura 22: Expressão relativa dos RNAm do marcador do receptor B2BKR de cininas nos hemisférios controle e tratados com salina e Bk no estriado. As análises quantitativas da expressão do receptor B2BKR de cininas do estriado de animais adultos foi feita por PCR em tempo real tendo como referência a expressão do GAPDH (*housekeeping gene*). Quando comparamos os níveis de expressão do gene nos lados controle e tratados (salina e Bk), verifica-se uma diminuição da expressão deste marcador neural nos animais que receberam apenas salina em relação aos animais tratados com BK. Nos animais cujos hemisférios lesados foram tratados com bradicinina, verifica-se um aumento da expressão deste marcador em relação ao lado controle. Onde: Bk: bradicinina 4,0µg. (n: 3 animais por grupo)

4.7 Efeitos citotóxicos da BK e do antagonista do receptor B2BKR HOE-140 e da 6-OHDP sobre a linhagem SHSY5Y

Em outra etapa, fizemos a avaliação *in vitro* dos efeitos da BK sobre neurônios com fenótipo dopaminérgico. Os potenciais citotóxicos da BK e do antagonista do receptor B2BKR (HOE-140) e da 6-OHDA foram avaliados sobre a linhagem SH-SY5Y por meio de ensaios de redução do sal de Tretrazólio (MTT) por períodos de 48 horas de incubação (figura 23).

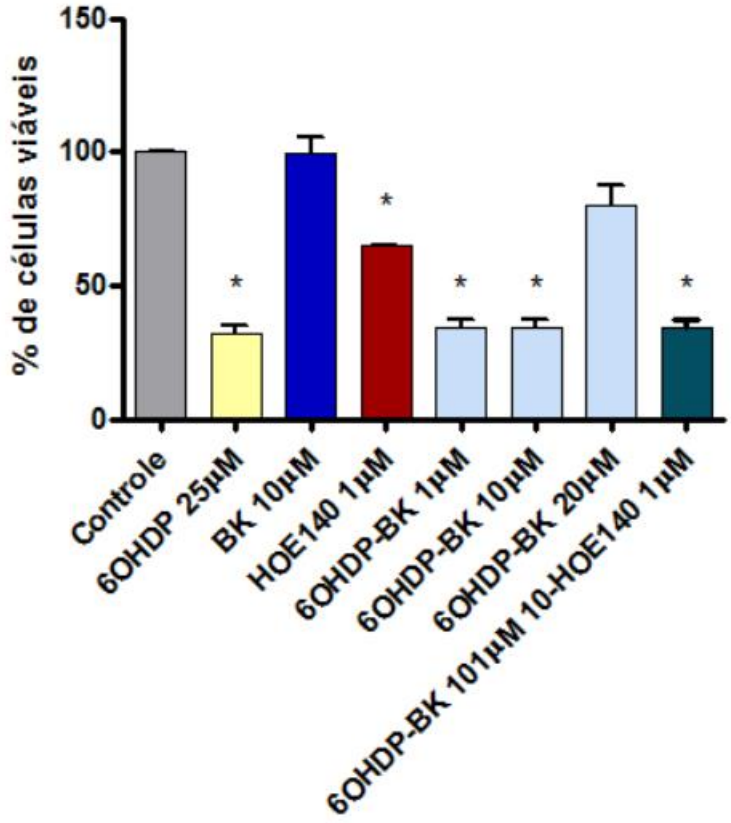

Figura 23: **Efeitos citotóxicos da Bk, do antagonista do receptor B2BKR HOE-140 e da 6-OHDP sobre a linhagem SHSY5Y**. As concentrações estão indicadas na figura. Foram registrados os gráficos de absorbância versus as concentrações dos compostos. (*) P < 0,05. (n: média de três experimentos, cada condição em triplicata).

De fato, observa-se uma redução da viabilidade celular significativa quando as células são expostas à 6-OHDA. A BK, por sua vez, isoladamente não apresenta toxicidade para as células desta linhagem. A BK, quando aplicada 30 minutos antes da aplicação de 6-OHDA, foi capaz de aumentar a viabilidade celular. Porém, quando aplicada após a 6-OHDA, não evitou a diminuição na morte celular.

5. DISCUSSÃO

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Trabalhos anteriores do nosso grupo mostraram que a bradicinina está envolvida na diferenciação neural de células progenitoras neurais por um *loop* autócrino que resulta em ativação do receptor B2BKR de cininas (Martins et al., 2008; Trujillo et al. 2012). Além do envolvimento no processo de diferenciação neural, a BK também atua no processo de proteção microvascular endotelial em modelos de acidente vascular cerebral (Bovenzi et al.; 2010) além de proteção de danos cerebrais decorrentes de isquemia em modelo *in vitro* (Martins, *et al*.; 2012). Assim, decidimos investigar os possíveis efeitos neurorregenerativos da BK na lesão de neurônios dopaminérgicos induzida por 6-OHDA *in vivo* e *in vitro*.

O modelo de degeneração dopaminérgica na via nigroestriatal foi desenvolvido em ratos por injeção de 6-OHDA, após 7 dias, foram feitos os testes rotacionais induzidos por apomorfina, para avaliação da extensão da lesão. Observamos que no grupo que recebeu BK (2,0 µg, 4,0 µg e 6,0 µg por animal), apenas um animal apresentou rotações 8 semanas após a injeção, os demais não apresentaram rotações (figuras 8 e 9, tabela 2). A avaliação do perfil médio de comportamento rotacional de cada grupo (BK e salina), mostrou que apenas o grupo que recebeu injeção de BK apresentou diminuição significativa do número de rotações (figura 8 e 9).

Podemos afirmar também que o agonista estável do receptor B2BKR [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina (4,0 µg por animal) interfere no comportamento rotacional típico do modelo animal de 6-OHDA, com resultados similares àqueles observados quando os animais foram tratados com BK (figura 10). Por outro lado, animais tratados com HOE-140, antagonista do receptor B2BKR, não

apresentaram alterações comportamentais 8 semanas após o tratamento (figura 11). Esses achados confirmam a hipótese inicial de trabalho, na qual sugeríamos que os efeitos neurorregenerativos resultantes do tratamento dos animais com tratados com BK seriam via ativação do receptor B2BKR.

Além disso, o Captopril®, inibidor da ACE (*Angiotensin Converting Enzyme*), um potencializador dos efeitos fisiológicos da BK, também foi efetivo no tratamento de animais submetidos à lesão da via nigro-estriatal por 6-OHDA. No grupo tratado com este fármaco, os animais também cessarem as rotações, enquanto que animais tratados com salina não apresentaram alterações comportamentais (figura 12). Os dados aqui apresentados representam a primeira descrição dos efeitos do Captopril® sobre fenômenos neuroregenerativos em modelo de neurdegeneração dopaminérgica induzida por 6-OHDA após estabelecimento da lesão.

Atribuímos essa melhora comportamental, detectada pelo teste rotacional, e a melhora histológica (aumento da porcentagem de fibras TH+) à neurorregeneração induzida por BK e não à neuroproteção. Uma vez que a BK foi adicionada após o estabelecimento completo da lesão, não podemos discutir a hipótese de neuroproteção. No entanto, nos experimentos *in vitro* realizados com a linhagem SH-SY5Y (figura 23), um neuroblastoma de fenótipo dopaminérgico, a BK não foi efetiva na neuroproteção destes neurônios frente a indução da morte celular induzida por 6-OHDA. Assim, acreditamos na hipótese da neurorregeneração devida à melhora do microambiente e, possivelmente, recrutamento e ativação de células-tronco endógenas.

Nesse sentido, os dados de PCR quantitativos indicam que há um aumento na expressão do marcador neural B3-tubulina no estriado dos

hemisférios lesionados dos animais tratados com Bk (4,0 µg por animal) e que apresentaram melhora comportamental, mas não em animais que receberam apenas salina. Recentemente, Luzatti e colaboradores (2014), demonstraram que células progenitoras neurais, residentes na zona subventricular (SVZ) e no giro-dentado do hipocampo (DG), migram para estriado, formando áreas de neurogênese e maturação neuronal ativas (Luzatti et al., 2014).

Vários tipos celulares têm suas vias de sinalização associadas a ativação do receptor B2BKR de cininas, além de seu envolvimento no processo de diferenciação neural. A BK também atua no processo de proteção microvascular endotelial em modelos de acidente vascular cerebral (AVC) (Bovenzi e cols., 2010). Todos os componentes do sistema calicreina-cinina estão presentes no SNC de adultos (Damas et al, 1992; Fujieda et al, 1993) e muitos deles também foram encontrados no cérebro em desenvolvimento de camundongos e ratos (Iwadate et al, 2002); (dados do nosso laboratório ainda não publicados).

A BK conhecidamente atua estimulando a liberação de neurotransmissores, como a noradrenalina e neuropeptideo Y em neurônios simpáticos, células PC12 e cromafins (Appell & Barefoot, 1989; Kansui et al, 2002; Kurz et al, 1997). Níveis extremamente elevados dessa cinina são observados no sistema nervoso central em condições de dano, como isquemias, acidentes vasculares encefálicos (AVE), traumas e na doença de Alzheimer (Groger et al, 2005; Myagkova et al, 2003). Nesse sentido, Xia e colaboradores (2004; 2006) observaram a influência da BK em processos pós-isquêmicos na região danificada do cérebro de ratos adultos.

De fato, estudos recentes demonstraram o papel da bradicinina/B2BKR, favorecendo a neurogênese durante a diferenciação de células-tronco e

progenitoras neurais. Estes resultados foram obtidos em diversas modelos de diferenciação neural: com células embrionário pluripotentes E14Tg2a (Nascimento et al., 2015), células de carcinoma embrionários de rato P19 (Trujillo et ai., 2012), células progenitoras neurais de camundongo (NPCs) (Pillat et al., 2015; Trujillo et al., 2012), células humanas pluripotentes induzidas (iPS) (Trujillo et al., 2012).

Os insultos do microambiente ativam funções fagocitárias que por sua vez liberam citocinas e interleucinas ativando astrócitos e micróglias, e estes regulam respostas imunológicas do SNC o que modula a sobrevivência dos neurônios, bem como altera a sua migração e plasticidade neuronal (Hanisch 2002).

Nossos resultados demostraram a eficácia do tratamento com bradicinina, um agonista de B2BKR, nos animais submetidos à lesão da via nigro-estriatal induzidos por 6-OHDA. Recentemente, Caetano e colaboradores mostraram que o receptor B2BKR desempenha um importante papel neuroprotetor em modelo animal da Doença de Alzheimer (Caetano et al*.,* 2015) o que corrobora nossos achados, possibilitando investigações adicionais sobre o efeito do receptor B2BKR e da bradicinina no modelo animal de Doença de Parkinson.

Embora não tenhamos observado o efeito neuroprotetor da BK *in vitro* quando esta foi adicionada após a adição de 6-OHDA na cultura de do neuroblastoma SH-SY5Y, quando já ocorreu a morte neuronal, resolvemos investigar se em animais com neurodegeneração dopaminérgica induzida por 6-OHDA a BK exerceria efeito neurorregenerativo quando injetada após estabelecimento da lesão (7 dias pós-lesão). A justificativa para esta abordagem é que quando os pacientes com Parkinson são diagnosticados pela sintomatologia, já ocorreu >50% de morte neuronal. Por essa razão optamos por

injetar a BK somente após o estabelecimento da neurodegeneração dopaminérgica induzida por 6-OHDA.

A morte de populações neuronais específicas no sistema nervoso central e na medula espinal pode ser responsável por doenças neurológicas como: Alzheimer, acidente vascular encefálico, epilepsia e doença de Parkinson (Rao e Mattson 2001). Já é conhecida a existência de populações de células progenitoras neuronais em áreas específicas do encéfalo como no hipocampo e na zona subventricular (Gage 2000). Poderíamos diferenciar essas novas células incorporando Bromodeoxyuridina (BrdU) em seu DNA, e posteriormente marcando-as por ensaios de imunofluorescência. Uma dupla marcação para BrdU e TH deve nos responder a origem de uma possível neurogênese promovida pela BK. Aqui deixamos em aberto um caminho para futuro esclarecimento do mecanismo pelos quais a BK exerce o seu efeito neuregenerativo no modelo de neurodegeneração dopaminérgica desencadeado pela 6-OHDA.

Hoje sabe-se que na região do estriado há a geração novos neurônios durante a vida adulta, o que pode servir como uma base para a recuperação após acidente vascular cerebral e mesmo para o desenvolvimento de novos tratamentos para doenças neurodegenerativas. Kempermann e coleboradores (2018), propõem que esses novos neurônios alcançam o estriado, região envolvida no controle motor e nas funções cognitivas a partir de células progenitoras neurais, residentes na zona subventricular (SVZ) e no giro-dentado do hipocampo (DG), como já citado.

Nossa hipótese está apoiada em dados que sugerem neurogênese na substância negra e nas suas projeções pelo estriado desencadeadas pelo

tratamento com BK e pelo Captopril, que potencializa os efeitos fisiológicos da BK por preservá-la da degradação. Embora, este campo esteja ainda sendo desvendado, há uma série de evidências que corroboram a hipótese de neurogênese no cérebro adulto.

Boldrini e colaboradores (2018), avaliaram por autópsia os hipocampos de indivíduos humanos saudáveis variando de 14 a 79 anos de idade. Eles encontraram um semelhante de progenitores neurais intermediários e de neurônios imaturos no giro-dentado do hipocampo (região neurogênica), e comparáveis números de glia e neurônios maduros, e volume equivalente de hipocampo entre indivíduos nas diversas faixas etárias. No entanto, indivíduos mais velhos têm menos angiogênese e neuroplasticidade e uma menor região progenitora na porção anterior-média do hipocampo, sem alterações na região posterior da mesma estrutura. Assim, eles concluem que indivíduos idosos saudáveis sem doenças neuropsiquiátricas exibem o potencial de neurogênese preservado. Eles teorizam assim que seja possível que a neurogênese no hipocampo sustente a função cognitiva específica ao longo da vida e que a perda desse potencial neurogênico pode estar associada ao comprometimento cognitivo (Boldrini et al. 2018).

Porém, aplicar a BK ou qualquer outro composto em um eventual protocolo de tratamento clínico parece pouco viável, uma vez que seria um método muito invasivo. Como alternativa, demonstramos que o Captopril administrado sistemicamente exerce efeitos equivalente aos da BK no tratamento da neurodegeneração dopaminérgica desencadeado pela 6-OHDA.

Interessantemente, Borlongan e colaboradores (2002), demonstraram que a administração sistêmica de BK resultou ineficiente na reversão da

neurodegeneração dopaminérgica induzida por 6-OHDA em ratos. A BK facilitou a atividade neurogênica da Ciclosporina A, sabidamente um agente neurogênico, porém incapaz de atravessar a barreira hematoencefálica. Quando administrado junto á BK sistemicamente, a Ciclosporina A reverteu a neurodegeneração dopaminérgica induzida por 6-OHDA, isso por que é conhecida a capacidade da BK de abrir a barreira hematoencefálica (Borlongan et al. 2002).

Nesse sentido, várias abordagens têm sido feitas para o desenvolvimento de novas terapias para neurodegeneração dopaminérgica nas últimas décadas. Por exemplo, a utilização de neurônios dopaminérgicos obtidos a partir de tecido fetal para transplantes em pacientes com Parkinson envolve sérias questões éticas, uma vez que são necessários muitos fetos para se obter o número de neurônios desejável para se obter sucesso no transplante. Além disso, grande parte dos neurônios transplantados morre após o transplante, diminuindo a eficiência da terapia. Recentemente vários grupos conseguiram diferenciar neurônios dopaminérgicos *in vitro* a partir de células-tronco neurais e embrionárias, mas, a integração desses neurônios e a sua sobrevivência após o transplante, continua sendo um desafio a ser vencido (Lindvall et al., 1990; Brundin et al., 2000).

Alternativamente à substituição celular, desenvolveu-se a abordagem da neuroproteção, ou seja, a prevenção da morte dos neurônios remanescentes poderia deter a progressão da doença. Testes clínicos utilizando o fator trófico e neuroprotetor GDNF mostraram-se eficazes não só na prevenção da morte neuronal, como no surgimento de novos neurônios, e causaram melhora sintomática e funcional dos pacientes. A proteína recombinante foi liberada

diretamente no cérebro através de uma bomba implantada intraperitonealmente (Gill et al., 2003; Patel et al., 2005). No entanto questões técnicas e financeiras envolvendo o uso de GDNF levaram à interrupção destes testes clínicos (Peck, 2005).

Uma alternativa interessante é a terapia celular associada à terapia gênica, isto é, o transplante de células infectadas com vetores virais que expressem proteínas específicas. Este tipo de terapia tem sido amplamente utilizada em estudos pré-clínicos para terapias na Doença de Parkinson. No entanto as dificuldades de se controlar as quantidades de proteínas expressas, bem como delimitar o local da expressão, são inúmeras. Além disso, o uso de vetores virais envolve riscos de mutações (Donsante et al., 2007), que acarretariam efeitos colaterais, além de desencadearem reações imunológicas indesejadas (Connor et al, 1999). Outro problema é que os sistemas virais promovem a liberação contínua do fator neuroprotetor, fato não desejável, devido aos efeitos colaterais do GDNF quando secretado continuamente (Nutt et al., 2003; Zhang et al., 1997).

Vetores virais recombinantes têm sido utilizados para produzir fatores tróficos *in situ*. Faz-se a infecção de células com os vetores virais (adenovírus e lentivírus) induzíveis e, após o transplante dessas células, o fator trófico de interesse é produzido. A terapia gênica é bastante eficaz na doença de Parkinson, uma vez que a liberação contínua de fatores neuroprotetores, como o GDNF é necessária para que se interrompa a perda neuronal. No entanto, sistemas virais têm limitações, como a possibilidade de mutações que podem alterar a expressão das proteínas e até mesmo tornar os vírus tumorigênicos. A

injeção de células também pode causar uma resposta imune que leva à rejeição e, por essa razão, é necessário fazer uso de imunossupressores.

Alternativamente, desenvolveu-se a tecnologia de encapsulamento das células para serem transplantadas. Dessa forma, as microcápsulas atuam como barreiras para as reações do sistema imunológico. Porém, verificou-se também que a liberação continua de GDNF está associada a efeitos colaterais (Nutt et al., 2003; Zhang et al., 1997). A remoção de microcápsulas que liberavam GDNF nos cérebros de roedores 7 semanas após o transplante resultou na manutenção dos efeitos benéficos deste fator neuroprotetor até o momento do sacrifício dos animais, mostrando assim que a administração de GDNF, mesmo transiente, tem efeitos prolongados (Sajadi et al., 2006). Dessa forma ainda não se sabe ao certo se a liberação contínua de GDNF no cérebro de ratos modelo de Parkinson é necessária e/ou desejada. As microesferas cumpririam o papel de liberarem transitoriamente os fatores tróficos, apenas pelo período necessário à integração e diferenciação das células transplantadas, auxiliando mais como favorecedor do microambiente. A partir deste ponto as células recém integradas poderiam restaurar a função e os fatores tróficos não seriam mais necessários.

Dessa forma, diante dos resultados apresentados, a exploração dos efeitos terapêuticos da BK e do Captopril® apresentam potencial para serem utilizados em futuros estudos clínicos. O Captopril® apresenta a vantagem de já ser uma droga de uso clínico. Para tanto, seria necessário seguir todo o protocolo estabelecido na pré-clínica, prévia a testes em grupos humanos. Isso significa, em resumo, ampliar os estudos para outras espécies animais.

No caso do Captopril, teoricamente para esta aplicação, os estudos de toxicidade de doses repetidas seriam a etapa posterior, consistindo em

caracterizar o perfil toxicológico da substância pela administração repetida. A partir deles é possível a obtenção de informações sobre os efeitos tóxicos, identificação de órgãos alvos, efeitos na fisiologia do animal, hematológicas, bioquímicas, anátomo e histopatológicas, além de informações sobre a indicação do NOEL e NOAEL (*Nível de dose sem observação de efeito* e *Nível de dose sem observação de efeito adverso*, respectivamente).

Como já dissemos, os estudos deveriam ser conduzidos com no mínimo duas espécies de mamíferos, incluindo uma espécie não roedora. As espécies devem ser selecionadas com base em sua relevância para a extrapolação de dados para seres humanos, considerando a farmacocinética, farmacodinâmica e biodisponibilidade da substância teste, incluindo sua biotransformação. (ANVISA, 2013).

Assim, os resultados obtidos no presente estudo demonstram que o Captopril® é uma droga promissora para a pesquisa translacional na neurodegeneração dopaminérgica, embora os mecanismos celulares pelos quais a BK exerça os seus efeitos não esteja ainda esclarecido.

6. CONCLUSÕES

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O conjunto de resultados permite-nos propor que:

- ✓ A injeção de BK e Captopril® levou à neuroregeneração pela indução da neurogênese na via nigro-estriatal em animais submetidos à lesão desta via por 6-OHDA.
- ✓ A injeção de BK, de Captopril® e [Phe^{8}Ψ(CH-NH)Arg^{9}]-Bradicinina levou à melhora comportamental de animais submetidos à lesão desta via por 6-OHDA;
- ✓ A injeção de HOE-140 não levou à melhora comportamental de animais submetidos à lesão desta via por 6-OHDA e não induziu neurogênese no modelo de lesão da via nigroestriatal;
- ✓ A BK não foi capaz de aumentar a viabilidade celular em ensaios de redução do sal de MTT em células com fenótipo dopaminérgico da linhagem SH-SY5Y quando expostas juntamente à 6-OHDA;
- ✓ A hipótese da neuroregeneração é corroborada adicionalmente pelo fato de que há aumento da expressão dos transcritos dos genes do marcador neural β3-tubulina.
- ✓ O receptor do sistema calicreína-cininas B2BkR tem sua expressão relativa aumentada quando comparamos os hemisférios controle dos animais tratados com bradicinina.

7. REFERÊNCIAS

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Altura BM, Chand N. Bradykinin-induced relaxation of renal and pulmonar arteries is dependent upon intact endothelial cell. *Br J Pharmacol*. 1981;74:10 – 11.

Appell KC, Barefoot DS (1989) Neurotransmitter release from bradykinin-stimulated PC12 cells. Stimulation of cytosolic calcium and neurotransmitter release. *The Biochemical journal* 263: 11-18

Arnold AC, Okamoto LE, Gamboa A, et al. Mineralocorticoid Receptor Activation Contributes to the Supine Hypertension of Autonomic Failure. Hypertension 2016;67:424-9

Bach JP, Ziegler U, Deuschl G, et al. Projected numbers of people with movement disorders in the years 2030 and 2050. Mov Disord 2011;26:2286-90

Beal MF[1.] Experimental models of Parkinson's disease. Nat Rev Neurosci. 2001 May;2(5):325-34.

Betarbet R[1], Sherer TB, Greenamyre JT. Animal models of Parkinson's disease. Bioessays. 2002 Apr;24(4):308-18.

Bhoola KD, Elson CJ, Dieppe PA. (1992). Kinins-key mediators in inflammatory arthritis? *Br J Rheumatol.* 31, 509-18.

Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, Rosoklija GB, Stankov A, Arango V, Dwork AJ, Hen R, Mann JJ. Human Hippocampal Neurogenesis Persists throughout Aging.
Cell Stem Cell. 2018 Apr 5;22(4):589-599.e5. doi: 10.1016/j.stem.2018.03.015.

Borkowski, J.A., Ranson, R.W., Seabrook, G.R., Trumbauer, M., Chen, H., Hill, R.G., Strader, C.D. AND Hess, J.F. (1995). Targeted disruption of a B_2 bradykinin receptor in mice eliminates bradykinin action in smooth muscle and neurons. *J. Biol. Chemistry* 270, 13706–13710

Borlongan CV[1], Emerich DF, Hoffer BJ, Bartus RT. Bradykinin receptor agonist facilitates low-dose cyclosporine-A protection against 6-hydroxydopamine neurotoxicity. Brain Res. 2002 Nov 29;956(2):211-20.

Bovenzi V, Savard M, Morin J, Cuerrier CM, Grandbois M, Gobeil F Jr (2010) Bradykinin protects against brain microvascular endothelial cell death induced by pathophysiological stimuli. J Cell Physiol 222:168-176.

Brundin P, Karlsson J, Emgard M, Schierle GS, Hansson O, Petersen A, Castilho RF (2000) Improving the survival of grafted dopaminergic neurons: a review over current approaches. Cell Transplant 9:179-195.

Caetano AL[1], Dong-Creste KE[1], Amaral FA[2], Monteiro-Silva KC[2], Pesquero JB[3], Araujo MS[4], Montor WR[2], Viel TA[5], Buck HS[1]. Kinin B2 receptor can play a neuroprotective role in Alzheimer's disease. Neuropeptides. 2015 Oct;53:51-62. doi: 10.1016/j.npep.2015.09.001.

Calixto, J.B., Cabrini, D.A., Ferreira, J., Campos, M.M. (2000). Kinins in pain and inflammation. *Pain* 87, 1-5.

Connor B, Kozlowski DA, Schallert T, Tillerson JL, Davidson BL, Bohn MC (1999) Differential effects of glial cell line-derived neurotrophic factor (GDNF) in the striatum and substantia nigra of the aged Parkinsonian rat. Gene Ther 6:1936-1951.

de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. Lancet Neurol. 2006 Jun;5(6):525-35.

Deumens R[1], Blokland A, Prickaerts J. Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. Exp Neurol. 2002 Jun;175(2):303-17.

Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, Sands MS (2007) AAV vector integration sites in mouse hepatocellular carcinoma. Science 317(5837):477

Damas J, Delrée P, Bourdon V (1992) Distribution of immunoreactive T-kininogen in rat nervous tissues. Brain Res 569: 63-70.

El-dahr, S.S.(1997). Ontogeny of the intrarenal kallikrein-kinin system: proposed role in renal development. *Microsc. Res. Tec*.39,222-232.

Fortunati, E.; Bianchi, V. Spectrophotometric determination of total macromolecules for the evaluation of cellular density in cytotoxicity tests. Alternatives to Laboratory Animals, n. 19, 1991

Fujieda H, Sato T, Wake K, Takaoka M, Morimoto S (1993) Tissue kallikrein in the rat pineal gland: an immunocytochemical study. J Pineal Res 14: 84-88.

Gage FH (2000) Mammalian neural stem cells. *Science* 287: 1433-1438

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med 9:589-595.

Gould E, Tanapat P, Hastings NB, Shors TJ (1999) Neurogenesis in adulthood: a possible role in learning. Trends Cogn Sci 3:186-192.

Gröger M, Lebesgue D, Pruneau D, Relton J, Kim SW, Nussberger J, Plesnila N (2005) Release of bradykinin and expression of kinin B2 receptors in the brain: role for cell death and brain edema formation after focal cerebral ischemia in mice. J Cereb Blood Flow Metab 25: 978-989.

Hanisch UK. Microglia as a source and target of cytokines. Glia. 2002 Nov;40(2):140-55.

Hassin-Baer, S., I. Molchadski*, et al.* Gender effect on time to levodopa-induced dyskinesias. J Neurol, May 1. 2011

Henderson JM, Watson S, Halliday GM, Heinemann T, Gerlach M. Relationships between various behavioural abnormalities and nigrostriatal dopamine depletion in the unilateral 6- OHDA-lesioned rat. Behav Brain Res. 2003;139(1-2):105-13.

Iwadate H, Kudo M, Kizuki K (2002) Expression of tissue kallikrein mRNA in developing rat brains. Can J Physiol Pharmacol 80: 245-248.

Kansui Y, Fujii K, Goto K, Abe I (2002) Bradykinin enhances sympathetic neurotransmission in rat blood vessels. Hypertension 39: 29-34.

Kempermann G, Gage FH, Aigner L, Song H, Curtis MA, Thuret S, Kuhn HG, Jessberger S, Frankland PW, Cameron HA, Gould E, Hen R, Abrous DN, Toni N, Schinder AF, Zhao X, Lucassen PJ, Frisén J. Human Adult Neurogenesis: Evidence and Remaining Questions. Cell Stem Cell. 2018 Apr 18. pii: S1934-5909(18)30166-8.

Kirik, D, et al. Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. Exp Neurol, 152(2):259-77, 1998.

Kurz T, Tölg R, Richardt G (1997) Bradykinin B2-receptor-mediated stimulation of exocytotic noradrenaline release from cardiac sympathetic neurons. J Mol Cell Cardiol 29: 2561-2569.

Lang, A.E., *When and how should treatment be started in Parkinson disease?* Neurology, 2009. 72(7 Suppl): p. S39-43.

Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacological reviews* 57: 27-77

Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD, et al. (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science 247:574-577.

Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. Mechanism of cellular 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Journal of Neurochemistry, v. 69, n. 2, p. 581 - 593, 1997.

Lopes, F.M. *et al.* Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res. v. 1337, p. 85-94, 2010.

Luzzati F. et. (2014) Quiescent neuronal progenitors are activated in the juvenile guinea pig lateral striatum and give rise to transient neurons al.Development 2014 141: 4065-4075.

Marceau, F., Hess, J.F., Bachvarov, D.R. (1998). The B1 receptors for kinins. *Pharmacol. Ver.* 50; 357-86.

Martins AH, Resende RR, Majumder P, et al. (2005) Neuronal differentiation of P19 embryonal carcinoma cells modulates kinin B2 receptor gene expression and function. J Biol Chem. 280, 19576-86.

Martins AH, Alves JM, Trujillo CA, Schwindt TT, Barnabé GF, Motta FL, Guimaraes AO, Casarini DE, Mello LE, Pesquero JB, Ulrich H (2008) Kinin-B2 receptor expression and activity during differentiation of embryonic rat neurospheres. Cytometry A 73: 361-368.

Martins AH, Alves JM, Perez D, Carrasco M, Torres-Rivera W, Eterović VA, Ferchmin PA, Ulrich H. (2012) Kinin-B2 receptor mediated neuroprotection after NMDA excitotoxicity is reversed in the presence of kinin-B1 receptor agonists. PLoS One. 7:e30755

Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunology Methods, v. 65 p. 55 - 63, 1983

Moreau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A (2005) The kallikrein-kinin system: current and future pharmacological targets. *Journal of pharmacological sciences* 99: 6-38

Moustafa, A. A., Poletti, M. Neural and behavioral substrates of subtypes of Parkinson's disease. Frontiers in Systems Neuroscience, 7:117, 2013.

Muñoz A, Rey P, Guerra MJ, Mendez-Alvarez E, Soto-Otero R, Labandeira-Garcia JL.
Reduction of dopaminergic degeneration and oxidative stress by inhibition of angiotensin converting enzyme in a MPTP model of parkinsonism. Neuropharmacology. 2006 Jul;51(1):112-20. Epub 2006 May 5.

Myagkova MA, Gavrilova SI, Lermontova NN, Kalyn YB, Selezneva ND, Zharikov GA, Kolykhalov IV, Abramenko TV, Serkova TP, Bachurin SO (2003) Content of autoantibodies to bradykinin and beta-amyloid(1-42) as a criterion for

biochemical differences between Alzheimer's dementias. *Bulletin of experimental biology and medicine* 136: 49-52

Nascimento IC ; Glaser, t. ; Nery, a. a. ; Pillat, m. m. ; Pesquero, j. b. ; Ulrich, H. . Kinin-B1 and B2 receptor activity in proliferation and neural phenotype determination of mouse embryonic stem cells. Cytometry. Part A, v. 87, p. 989-1000, 2015

Nussbaum, R. L., Ellis, C. E. Alzheimer's disease and Parkinson's disease. N Engl J Med, 348(14):1356-64, 2003.

Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, Laws ER, Jr., Lozano AM, Penn RD, Simpson RK, Jr., Stacy M, Wooten GF (2003) Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60:69-73.

Okun, H.H.F., Donald G Grosset, Katherine A Grosset, *Parkinson's Disease*2009, London.

Olanow, C.W., M.B. Stern, and K. Sethi, *The scientific and clinical basis for the treatment of Parkinson disease (2009).* Neurology, 2009. 72(21 Suppl 4): p. S1-136.

Parkinson, J. An essay on the shaking palsy. J Neuropsychiatry Clin Neurosci, 14(2):223-36, 2002.

Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005) Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. Ann Neurol 57:298-302.

Paxinos G, Watson C (2009) The rat brain in stereotaxic coordinates. Compact sixth edition, San Diego: Academic Press

Peck P (2005) AMGEN decision to halt GDNF clinical trials and withdraw the drug triggers protest from researchers and patients. Neurology Today 5(4):4,7,24

Pillat, M. M. ; Lameu C ; Trujillo, C. A. ; Glaser, T. ; Cappellari, A.R. ; Negraes, P.D. ; Battastini, A.M. ; Schwindt Tt ; Muotri, A. R. ; Ulrich, H. . Bradykinin promotes neuron-generating division of neural progenitor cells via ERK activation. Journal of Cell Science, v. 129, p. 3437-3448, 2015.

Pillat, M. M. ; Oliveira, M. ; Motaln, H. ; Breznik, B. ; Glaser, T. ; Lah Tt ; Ulrich, H. . Glioblastoma-mesenchymal stem cell communication modulates expression patterns of kinin receptors: Possible involvement of bradykinin in information flow. Cytometry. Part A, v. 89, p. 365-375, 2016.

Rao MS[1], Mattson MP. Stem cells and aging: expanding the possibilities. Mech Ageing Dev. 2001 May 31;122(7):713-34.

Rao G, Fisch L, Srinivasan S, D'amico F, Okada T, Eaton C, Robbins C. (2003). Does this patient have Parkinson disease? Jama 289: 347-353.

Resende AC, Ballejo G, Salgado MC. Role of non-nitric oxide non-prostaglandin endothelium-derived relaxing factor(s) in bradykinin vasodilation. *Braz J Med Biol Res*. 1998;31:1229 –1235.

Ruberg M, SCHERMAN D, JAVOY-AGID F. (1995). Agid, Dopamine denervation, age of onset, and Parkinson's disease. Neurology 45: 392.

Sajadi A, Bensadoun JC, Schneider BL, Lo Bianco C, Aebischer P (2006) Transient striatal delivery of GDNF via encapsulated cells leads to sustained behavioral improvement in a bilateral model of Parkinson disease. Neurobiol Dis 22:119-129.

Santangelo g, piscopo f, barone p, vitale c. (2017). Personality in Parkinson's disease: Clinical, behavioural and cognitive correlates. J Neurological Sciences. 374: 17-25.

Simola N[1], Morelli M, Carta AR. The 6-hydroxydopamine model of Parkinson's disease. Neurotox Res. 2007 Apr;11(3-4):151-67.

Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci 2003; 4:49-60

Spillantini MG[1], Goedert M Neurodegeneration and the ordered assembly of α-synuclein. Cell Tissue Res. 2017 Nov 8. doi: 10.1007/s00441-017-2706-9. [Epub ahead of print]

Talorete T. P. N..; Bouaziz, M.; Sayadi, S.; Isoda, H. Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells. Cytotechnology. v. 52, p. 189–198, 2006.

Trujillo, C. A. ; Negraes, P.D. ; Schwindt TT ; Lameu C ; Carromeu, C. ; Muotri, A. R. Pesquero, J. B. ; Cerqueira, D. M. ; Pillat, M. M. ; Souza, H. D. N. ; Turaca, L. T. ; Abreu, J. G. ; Ulrich, H. (2012) . Kinin-B2 Receptor Activity Determines the Differentiation Fate of Neural Stem Cells. The Journal of Biological Chemistry (Print), v. 287, p. 44046-44061.

Ueno S, Yamada H, Moriyama T, Honda K, Takano Y, Kamiya HO, Katsuragi T. (2002). Measurement of dorsal root ganglion P2X mRNA by SYBR Green fluorescence. *Brain Res Brain Res Protoc*. 10, 95-101.

Xia CF, Yin H, Borlongan CV, Chao L, Chao J. (2004). Kallikrein gene transfer protects against ischemic stroke by promoting glial cell migration and inhibiting apoptosis. *Hypertension*. 43, 452-9.

Xia CF, Yin H, Yao YY, Borlongan CV, Chao L, Chao J. (2006). Kallikrein protects stroke by inhibiting apoptosis and inflammation and promoting angiogenesis and neurogenesis. *Hum Gene Ther*. 17, 206-19.

Van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034.

Zhang Z, Miyoshi Y, Lapchak PA, Collins F, Hilt D, Lebel C, Kryscio R, Gash DM (1997) Dose response to intraventricular glial cell line-derived neurotrophic factor administration in parkinsonian monkeys. J Pharmacol Exp Ther 282:1396-1401.

8. ANEXOS

SÚMULA CURRICULAR

DADOS PESSOAIS

Nome: Héllio Danny Nóbrega de Souza
Local e data de nascimento: Patos-PB, 13/10/1984

EDUCAÇÃO

- Escola Estadual Monsenhor Manuel Vieira – 2003: Ensino Médio.
- Universidade Federal da Paraíba – João Pessoa – 2009: Graduação em Ciências Biológicas (licenciatura e bacharelado)
- Universidade de São Paulo: mestrado em Ciências Biológicas (Bioquímica)

CURRÍCULO

- Conferir currículo Lattes na continuação dos anexos.

Héllio Danny Nóbrega de Souza

Endereço para acessar este CV: http://lattes.cnpq.br/4719550313759434
Última atualização do currículo em 19/04/2018

Possui graduação em Ciências Biológicas pela Universidade Federal da Paraíba (2009) e mestrado em Bioquímica pela Universidade de São Paulo (2013). Atualmente é aluno de doutorado do Departamento de Bioquímica do Instituto de Química da Universidade de São Paulo. Tem experiência na área de Bioquímica e Biologia Molecular do Desenvolvimento, atuando principalmente nos temas: receptores de neurotransmissores, desenvolvimento do sistema nervoso e participação de receptores de neurotransmissores neste processo. **(Texto informado pelo autor)**

Identificação

Nome Héllio Danny Nóbrega de Souza
Nome em citações bibliográficas DE SOUZA, H. D. N.;H. D. N. SOUZA;HÉLLIO D. N. DE SOUZA;DE SOUZA, HÉLLIO D. N.;Héllio D. N. de Souza;Souza, Héllio Danny Nóbrega de;SOUZA, HELLIO D. N.;DE SOUZA, HÉLLIO D.N.

Endereço

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Formação acadêmica/titulação

2013
Doutorado em andamento em Ciências Biológicas (Bioquímica) (Conceito CAPES 7).
Universidade de São Paulo, USP, Brasil.
Título: Papel do receptor B2 de cininas na terapia da Doença de Parkinson em modelo animal.,
Orientador: Dr. Alexander Henning Ulrich.
Bolsista do(a): Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, Brasil.
Palavras-chave: Bioquímica e Bologia Molecular.
Grande área: Ciências Biológicas

2009 - 2013
Mestrado em Ciências Biológicas (Bioquímica) (Conceito CAPES 7).
Universidade de São Paulo, USP, Brasil.
Título: Padrões de expressão gênica de proteínas marcadoras neurais e dos sistemas purinérgico e cininérgico durante o desenvolvimento encefálico de camundongos knockout para o receptor B2 de cininas.,Ano de Obtenção: 2013.
Orientador: Dr. Alexander Henning Ulrich.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.
Palavras-chave: Bioquímica e Biologia Molecular.
Grande área: Ciências Biológicas
Grande Área: Ciências Biológicas / Área: Morfologia / Subárea: Embriologia.

2005 - 2009
Graduação em Ciências Biológicas.
Universidade Federal da Paraíba, UFPB, Brasil.
Título: Efeito Citotóxico dos Compostos Naturais Isolados: Quercetina, Reserpina, Cumarina e Ácido úsnico sobre Macrófagos Elicitados.
Orientador: Dr. Demetrius Antônio Machado de Araújo.

Formação Complementar

2008 - 2008 Extensão universitária em Bioinformática: Aplicações e Desenvolvimento. (Carga horária: 384h).
Universidade Federal da Paraíba, UFPB, Brasil.

2007 - 2007 Extensão universitária em Bioinformática: Aplicações e Desenvolvimento. (Carga horária: 288h).
Universidade Federal da Paraíba, UFPB, Brasil.

2007 - 2007 II Curso de Verão Bioquímica e Biologia Molecular. (Carga horária: 80h).
Universidade Cidade de São Paulo, UNICID, Brasil.

Atuação Profissional

Instituto de Química - Universidade de São Paulo, IQUSP, Brasil., IQUSP, Brasil.

Vínculo institucional
2013 - Atual Vínculo: Bolsista, Enquadramento Funcional: Doutorando, Regime: Dedicação exclusiva.
Vínculo institucional
2014 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Monitor, Carga horária: 6
Outras informações Monitoria da disciplina Biologia Molecular do Departamento de Bioquímica (IQ-USP)
Vínculo institucional
2014 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Monitor, Carga horária: 6
Outras informações Monitoria da disciplina Bioquímica do Departamento de Bioquímica (IQ-USP).
Vínculo institucional
2009 - 2013 Vínculo: Bolsista, Enquadramento Funcional: Mestrando, Regime: Dedicação exclusiva.

Universidade Federal da Paraíba, UFPB, Brasil.

Vínculo institucional
2008 - 2009 Vínculo: Bolsista, Enquadramento Funcional: Monitor, Carga horária: 12
Outras informações Monitoria da disciplina Fisiologia Animal Comparada do Departamento de Sistemática e Ecologia (DSE-UFPB).

Vínculo institucional
2006 - 2009 Vínculo: Bolsista, Enquadramento Funcional: Iniciação científica, Carga horária: 12
Vínculo institucional
2008 - 2008 Vínculo: Bolsista, Enquadramento Funcional: Monitor, Carga horária: 12
Outras informações Monitoria da disciplina Fisiologia Animal Comparada do Departamento de Sistemática e Ecologia (DSE-UFPB).

Atividades
04/2007 - 12/2008 Extensão universitária , Centro de Ciências Exatas e da Natureza - Campus I, .
Atividade de extensão realizada
Extensionista bolsista do Projeto Bioinformática: Área de Potencial Para Formação de Pessoal, Serviços, Aplicações e Desenvolvimento (Carga Horária - 288 horas). Projeto com o objetivo de capacitação de pessoal através de cursos de Extensão..

01/2006 - 12/2006 Extensão universitária , Centro de Ciências Exatas e da Natureza - Campus I, .
Atividade de extensão realizada
Extensionista Colaboradora do Projeto Bioinformática: Área de Potencial Para Formação de Pessoal, Serviços, Aplicações e Desenvolvimento (Carga Horária - 288 horas). Projeto com o objetivo de capacitação de pessoal através de cursos de Extensão.

Projetos de extensão

2006 - 2009 Desenvolvimento e Aplicação de Recursos Educativos para Difusão da Biotecnologia em Escolas Públicas
Descrição: Nesse projeto, aprovado na chamada CNPq 12/2006, iremos divulgar a biotecnologia em algumas escolas públicas do ensino médio da grande João Pessoa, utilizando material didático que será produzido pela equipe.
Situação: Concluído; Natureza: Extensão.
Alunos envolvidos: Graduação: (9) / Mestrado acadêmico: (1) / Doutorado: (3) .

Integrantes: Héllio Danny Nóbrega de Souza - Integrante / Vinícius Ramos Henriques Maracajá Coutinho - Integrante / Demétrius Antônio Machado de Araújo - Coordenador / João Paulo Di Monaco Durbano - Integrante / Amely Branquinho Martins - Integrante / Luís Henrique M. Melo - Integrante / Luiz Thibério Lira Diniz Rangel - Integrante / Flávio Ribeiro Nóbrega - Integrante / Luís Fernando Marques dos Santos - Integrante / Cosme Rafael Martínez Salinas - Integrante / Aretha Vieira Bezerra - Integrante / Patrícia Keytth Lins Rocha

- Integrante / Renata dos Santos Almeida - Integrante / Itácio Queiroz de Melo Padilha - Integrante.
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico - Auxílio financeiro.

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Bioquímica / Subárea: Biologia Molecular.
2. Grande área: Ciências Biológicas / Área: Bioquímica.
3. Grande área: Ciências Biológicas / Área: Morfologia / Subárea: Embriologia.

Idiomas

Inglês Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.
Espanhol Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.

Prêmios e títulos

2017 Poster Awards in 11th world Congress on Neurology and Therapeutics, 2017, Madrid., OMICS International.
2007 Prêmio Elo Cidadão, Pró-Reitoria de Extensão e Assuntos Comunitários/UFPB., Pró-reitoria de Assuntos Comunitários/ UFPB.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica ▼

1. OLIVEIRA-GIACOMELLI, ÁGATHA ; NAALDIJK, YAHAIRA ; SARDÁ-ARROYO, LAURA ; GONÇALVES, MARIA C. B. ; CORRÊA-VELLOSO, JULIANA ; PILLAT, MICHELI M. ; **DE SOUZA, HÉLLIO D. N.** ; ULRICH, HENNING . Purinergic Receptors in Neurological Diseases With Motor Symptoms: Targets for Therapy. Frontiers in Pharmacology JCR, v. 9, p. 1, 2018.

2. ★ FERRAZOLI, ENÉAS G. ; **DE SOUZA, HÉLLIO D.N.** ; NASCIMENTO, ISIS C. ; OLIVEIRA-GIACOMELLI, ÁGATHA ; SCHWINDT, TELMA T. ; BRITTO, LUIZ R. ; ULRICH, HENNING . Brilliant Blue G, But Not Fenofibrate, Treatment Reverts Hemiparkinsonian Behavior and Restores Dopamine Levels in an Animal Model of Parkinson?s Disease. CELL TRANSPLANTATION JCR, v. 26, p. 669-677, 2017.

3. CAPPELLARI, ANGÉLICA R. ; PILLAT, MICHELI M. ; **SOUZA, HELLIO D. N.** ; DIETRICH, FABRÍCIA ; OLIVEIRA, FRANCINE H. ; FIGUEIRÓ, FABRÍCIO ; ABUJAMRA, ANA L. ; ROESLER, RAFAEL ; LECKA, JOANNA ; SÉVIGNY, JEAN ; BATTASTINI, ANA MARIA O. ; ULRICH, HENNING . Ecto-5?-Nucleotidase Overexpression Reduces Tumor Growth in a Xenograph Medulloblastoma Model. Plos One JCR, v. 10, p. e0140996, 2015.

Citações: WEB OF SCIENCE 1 | SCOPUS 6

4. ★ GONÇALVES, JUAN CARLOS R. ; SILVEIRA, ALETHÉIA L. ; **DE SOUZA, HÉLLIO D. N.** ; NERY, ARTHUR A. ; PRADO, VANIA F. ; PRADO, MARCO A. M. ; ULRICH, HENNING ; ARAÚJO, DEMETRIUS A. M. . The monoterpene (-)-carvone: A novel agonist of TRPV1 channels. Cytometry. Part A JCR, v. 00A, p. n/a-n/a, 2013.

Citações: WEB OF SCIENCE 8 | SCOPUS 8

5. TRUJILLO, CLEBER A. ; NEGRAES, PRISCILLA D. ; SCHWINDT, TELMA T. ; LAMEU, CLAUDIANA ; CARROMEU, CASSIANO ; MUOTRI, ALYSSON R. ; PESQUERO, JOÃO B. ; CERQUEIRA, DÉBORA M. ; PILLAT, MICHELI M. ; **DE SOUZA, HÉLLIO D. N.** ; TURAÇA, LAURO T. ; ABREU, JOSÉ G. ; ULRICH, HENNING . Kinin-B2 Receptor Activity Determines the Differentiation Fate of Neural Stem Cells. JOURNAL OF BIOLOGICAL CHEMISTRY JCR, v. 287, p. 44046-44061, 2012.

Citações: WEB OF SCIENCE 16 | SCOPUS 19

Resumos publicados em anais de congressos

1. ULRICH, H. ; **SOUZA, HELLIO D. N.** ; PILLAT, M. M. . Cytometry of Neural Differentiation in vitro and in vivo: Stimulation of Neurogenesis and Neuroregeneration by Bradykinin. In: 33rd Congress of the International Society for Advancement of

Cytometry - CYTO, 2018, Praga. 33rd Congress of the International Society for Advancement of Cytometry - CYTO, 2018.

2. **Souza, Héllio Danny Nóbrega de**; OLIVEIRA, A. ; ULRICH, H. . BRADYKININ REVERTS CONTRALATERAL ROTATION AND RESTORES DOPAMINE LEVELS IN AN ANIMAL MODEL OF PARKINSON DISEASE.. In: 11th world Congress on Neurology and Therapeutics, 2017, Madrid. Journal of Neurology & Neurophysiology. Westlake, Los Angeles: OMICS International Journals, 2017. v. 8. p. 84-84.
3. **H. D. N. SOUZA**; OLIVEIRA, A. ; ULRICH, H. . ROLE OF KININS RECEPTOR B2 IN THERAPY OF PARKINSON'S DISEASE IN ANIMAL MODEL. In: 11th World Congress on Controversies in Neurology (CONy), 2017, Atenas, Grécia. Neurologia Special Issue. Atenas: Lychnia S.A., 2017. v. 26. p. 95-95.
4. OLIVEIRA, A. ; **DE SOUZA, HÉLLIO D. N.** ; CORREA, J. ; ULRICH, H. ; PEREIRA, R. L. . P2X7 RECEPTOR EXPRESSION PATTERNS IN AN ANIMAL MODEL OF PARKINSON'S DISEASE. In: VI Brazilian Purine Club Meeting, 2016, João Pessoa-PB, Brazil. VI Brazilian Purine Club Meeting, 2016.
5. **Héllio D. N. de Souza**; FERRAZOLI, E. G. ; OLIVEIRA, A. ; ULRICH, H. . Role of brydikinin in therapy of Parkinson's Disease in animal model. In: IBRO - 9th World Congress International Brain Research Organization, 2015, Rio de Janeiro. Abstract book of IBRO - 9th World Congress International Brain Research Organization, 2015.
6. CAPPELLARI, ANGÉLICA R. ; PILLAT, M. M. ; **DE SOUZA, HÉLLIO D. N.** ; DIETRICH, FABRÍCIA ; OLIVEIRA, FRANCINE H. ; ABUJAMRA, ANA L. ; ROESLER, RAFAEL ; ULRICH, H. ; BATTASTINI, ANA MARIA O. . OVEREXPRESSION OF CD73 REDUCES THE MEDULLOBLASTOMA TUMOR GROWTH. In: II International Congress of Purinergic Signalling in South America and V Brazilian Purine Club Meeting, 2015, Maresias, São Paulo-Brazil. II International Congress of Purinergic Signalling in South America, 2015.
7. NAALDIJK, Y. ; CORREA, J. ; FERRAZOLI, E. G. ; GONCALVES, M. C. ; FABIAN, C. ; GLASER, T. ; HEINE, C. ; HOLLAND, H. ; LAMEU, C. ; MELZER, S. ; OLIVEIRA, A. ; PILLAT, M. M. ; NEGRAES, P. ; SARDA, L. ; SAVKOVIC, V. ; STACHNIK, J. ; **DE SOUZA, H. D. N.** ; TARNOK, A. ; SACK, U. ; ULRICH, H. . Bradykinin and purinergic signaling in brain diseases. In: II International Congress of Purinergic Signalling in South America and V Congress of the Brazilian Purine Club, 2015, São Sebastião-SP. Abstract book of II International Congress of Purinergic Signalling in South America, 2015.
8. **DE SOUZA, H. D. N.**; CAMPOS, M. ; Demasi M.A. ; NERY, A.A. ; Negraes, P.D. ; Sogayar. M.C. ; ULRICH, H. . Characterization of Allosteric Effects in Murine Heteromeric P2X4/6 Receptors and its Splicing Variants. In: XL Annual Meeting of The Brazilian of Biochemistry and Molecular Biology Society (SBBq), 2011, Foz do Iguaçi. XL Annual Meeting of The Brazilian of Biochemistry and Molecular Biology Society (SBBq), 2011.
9. GONCALVES, J.C.R. ; **DE SOUZA, H. D. N.** ; NERY, A.A. ; ULRICH, A.H. ; PRADO, V. F. ; PRADO, M. A. M. ; ARAUJO, D. A. M. . (-)-CARVONE ACTIVATES TRPV1 CHANNELS IN DRG NEURONS AND TRPV1-EXPRESSING HEK CELLS. In: XXVI Reunião Anual da FeSBE, 2011, Rio de Janeiro. XXVI Reunião Anual da FeSBE, 2011.
10. GONCALVES, J.C.R. ; SILVEIRA, A.L. ; **DE SOUZA, H. D. N.** ; NERY, A.A. ; ULRICH, A.H. ; ARAUJO, D. A. M. . (-)-Carvone increase intracellular calcium levels in drg neurons. In: XV Meeting of the Brazilian Society for Cell Biology, 2010, São Paulo-SP. XV Meeting of the Brazilian Society for Cell Biology, 2010.
11. **DE SOUZA, H. D. N.**; DURBANO, O. P. M. ; ROCHA, P. K. L. ; PADILHA, I. Q. M. ; ALMEIDA, R. S. ; BEZERRA, A. V. ; RANGEL, L. T. L. D. ; ARAUJO, D. A. M. . CURSO DE CURTA DURAÇÃO EM BIOINFORMÁTICA COMO INSTRUMENTO DE INSERÇÃO DIGITAL E DE DIFUSÃO BIOTECNOLÓGICA. In: X Encontro de Extensão da Universidade Federal da Paraíba, 2008, João Pessoa. Caderno de Resumos do X Encontro de Extensão da UFPB, 2008.
12. BEZERRA, A. V. ; MARTINS, A. B. ; **DE SOUZA, H. D. N.** ; RANGEL, L. T. L. D. ; ROCHA, P. K. L. ; ARAUJO, D. A. M. . AVALIAÇÃO DO POTENCIAL LEISHMANIOSTÁTICO DO COMPOSTO NATURAL ISOLADO QUERCETINA SOBRE LEISHMANIA CHAGASI. In: XX Congresso Brasileiro de Parasitologia, 2007, Recife - PE. Revista de Patologia Tropical. Goiânia - GO: Editora da Universidade Federal de Goiás, 2007. v. 36. p. 286-287.
13. Araujo J.P.C. ; MARTINS, A. B. ; ALMEIDA, R. S. ; **DE SOUZA, H. D. N.** ; COUTINHO, V. R. H. M. ; PADILHA, I. Q. M. ; BEZERRA, A. V. ; ARAUJO, D. A. M. . A BIOINFORMÁTICA COMO INSTRUMENTO DE INSERÇÃO DIGITAL E DE DIFUSÃO DA BIOTECNOLOGIA. In: IX Encontro de Extensão da Universidade Federal da Paraíba, 2007, João Pessoa. Livro de Resumos do IX Encontro de Extesão da UFPB, 2007.

Demais tipos de produção técnica

1. **Souza, Héllio Danny Nóbrega de**. IX Curso de Verão em Bioquímica e Biologia Molecular. 2014. (Curso de curta duração ministrado/Extensão).

Eventos

Participação em eventos, congressos, exposições e feiras

1. 11th World Congress on Controversies in Neurology (CONy). Role of kinins receptor b2 in therapy of Parkinson?s disease in animal model. 2017. (Congresso).
2. 11th World Congress on Neurology and Therapeutic. BRADYKININ REVERTS CONTRALATERAL ROTATION AND RESTORES DOPAMINE LEVELS IN AN ANIMAL MODEL OF PARKINSON DISEASE.. 2017. (Congresso).
3. IBRO - 9th World Congress International Brain Research Organization. Role of brydikinin in therapy of Parkinson's Disease in animal model. 2015. (Congresso).
4. 1º encontro de pesquisadores do NAPNA ? Núcleo de Apoio à Pesquisa em Neurociência Aplicada. 2011. (Encontro).
5.

XL Annual Meeting of The Brazilian of Biochemistry and Molecular Biology Society (SBBq). Characterization of Allosteric Effects in Murine Heteromeric P2X4/6 Receptors and its Splicing Variants. 2011. (Congresso).

6. Simpósio Agenda do Futuro nas Ciências Biomédicas. 2009. (Simpósio).
7. X Encontro de Extensão da Universidade Federal da Paraíba.Curso de Curta Duração em Bioinformática como Instrumento de Inserção e de Difusão Biotecnológica. 2008. (Encontro).
8. IX Encontro de Extensão da Universidade Federal da Paraíba.A Bioinformática como Instrumento de Inserção Digital e de Difusão da Biotecnologia. 2007. (Encontro).
9. XX Congresso Brasileiro de Parasitologia. AVALIAÇÃO DO POTENCIAL LEISHMANIOSTÁTICO DO COMPOSTO NATURAL ISOLADO QUERCETINA SOBRE LEISHMANIA CHAGASI. 2007. (Congresso).

Educação e Popularização de C & T

Cursos de curta duração ministrados

1. **Souza, Héllio Danny Nóbrega de**. IX Curso de Verão em Bioquímica e Biologia Molecular. 2014. (Curso de curta duração ministrado/Extensão).

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RESEARCH ARTICLE

Ecto-5'-Nucleotidase Overexpression Reduces Tumor Growth in a Xenograph Medulloblastoma Model

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Abstract

Background

Ecto-5'-nucleotidase/CD73 (ecto-5'-NT) participates in extracellular ATP catabolism by converting adenosine monophosphate (AMP) into adenosine. This enzyme affects the progression and invasiveness of different tumors. Furthermore, the expression of ecto-5'-NT has also been suggested as a favorable prognostic marker, attributing to this enzyme contradictory functions in cancer. Medulloblastoma (MB) is the most common brain tumor of the cerebellum and affects mainly children.

Materials and Methods

The effects of ecto-5'-NT overexpression on human MB tumor growth were studied in an *in vivo* model. Balb/c immunodeficient (nude) 6 to 14-week-old mice were used for dorsal subcutaneous xenograph tumor implant. Tumor development was evaluated by pathophysiological analysis. In addition, the expression patterns of adenosine receptors were verified.

Results

The human MB cell line D283, transfected with ecto-5'-NT (D283hCD73), revealed reduced tumor growth compared to the original cell line transfected with an empty vector. D283hCD73 generated tumors with a reduced proliferative index, lower vascularization, the presence of differentiated cells and increased active caspase-3 expression. Prominent A_1 adenosine receptor expression rates were detected in MB cells overexpressing ecto-5'-NT.

Competing Interests: The authors have declared that no competing interests exist.

Conclusion

This work suggests that ecto-5'-NT promotes reduced tumor growth to reduce cell proliferation and vascularization, promote higher differentiation rates and initiate apoptosis, supposedly by accumulating adenosine, which then acts through A_1 adenosine receptors. Therefore, ecto-5'-NT might be considered an important prognostic marker, being associated with good prognosis and used as a potential target for therapy.

Introduction

Ecto-5'-nucleotidase/CD73 (ecto-5'-NT) is expressed by various human tissues and considered the main producer of extracellular adenosine [1]. Adenosine activates P1 metabotropic receptors, subdivided into A_1, A_{2A}, A_{2B} and A_3 receptors, which participate in the control of intracellular cAMP levels [2]. Ecto-5'-NT influences cancer progression in different types of tumors, including bladder and breast cancer, melanomas and gliomas [1]. Sadej and co-workers (2006) [3] demonstrated that ecto-5'-NT expression increased with the degree of malignancy of human melanoma cell lines, where higher expression levels were measured in a metastatic melanoma cell line. In breast cancer, the involvement of ecto-5'-NT in invasiveness and its interaction with extracellular matrix proteins were demonstrated [4]. Previous studies from our laboratory have shown a role of ecto-5'-NT in glioma progression and tissue invasiveness events. First, different glioma cell lines expressed prominent levels of ecto-5'-NT compared to normal astrocytes [5]. Second, increased cellular confluence was accompanied by enhanced ecto-5'-NT expression and activity [6]. Third, diminished ecto-5'-NT activity affected glioma cell adhesion and reduced cell proliferation [7, 6], suggesting the importance of ecto-5'-NT enzymatic activity for glioma cell survival.

However, immunohistochemistry and microarray analysis of human breast cancer samples revealed that ecto-5'-NT overexpression, which was observed in 74% of analyzed tissues, was correlated with the disease-free state and overall survival, suggesting that the expression of this enzyme is associated with good prognosis [8]. Ecto-5'-NT expression levels in medulloblastoma (MB) cell lines were reported in our previous paper. While the primary MB cell lines (Daoy and ONS76) expressed this enzyme, the metastatic MB cell line (D283) did not [9]. This difference was attributed to the regulation of ecto-5'-NT expression by β-catenin nuclear immunoreactivity [10], which has been suggested to predict a favorable prognostic for MB [11]. Unlike gliomas, MB mainly affects children with a median age of 9 years, and the median survival of the patient is approximately 5 years [12]. These tumors occur preferentially in the cerebellum and are considered the most common brain tumors in children, classified by the World Health Organization (WHO) as fourth degree tumor the highest malignant grade [13]. Considering the supposed crucial and contradictory functions of ecto-5'-NT in tumor growth, we investigated the role of ecto-5'-NT in MB progression. The enzyme was overexpressed in the D283 human MB cell line in order to evaluate its participation in tumor growth in an *in vivo* nude mice model. Here, we demonstrated that the overexpression of ecto-5'-NT promotes a reduction of tumor growth; interferes with Ki67, CD31 and caspase-3 immunolabeling; and promotes an increase in differentiated tumor cells. In addition, we showed that the expression of A1 adenosine receptor was enhanced, suggesting the participation of adenosine signaling in MB tumor progression.

Materials and Methods

Cell culture

Daoy (representative of a human primary MB) and D283 (representative of a secondary or metastatic human MB) cell lines (generated by American Type Culture Collection, ATCC) were kindly donated by the Laboratório de Pesquisa em Câncer Infantil of Hospital de Clínicas de Porto Alegre in Rio Grande do Sul, Brazil. The cells were cultured in low glucose Dulbecco ´s Modified Eagle´s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 0.5 U/mL penicillin/streptomycin antibiotics. The cells were kept at 37°C in an incubator with a minimum relative humidity of 95% and 5% of CO_2.

Transient D283 cell line transfection

The D283 MB cell line was seeded in culture flasks, and after reaching 80% confluence, the cells were transfected with Lipofectamine® 2000 Transfection Reagent (Invitrogen *by* Life Technologies—Carlsbad, CA, United States). We used 1 μg of pcDNA3.1/V5-His plasmid as a transfection control (D283 empty vector—D283ev) or pcDNA3.1/V5-His containing the human ecto-5'-NT gene sequence (D283hCD73) and incubated for 4 h [14]. Then, the cells were kept in DMEM with 10% FBS for 24 h. The transfected cells were selected based on their resistance to the antibiotic G418 (1.0 mg/mL). The functionality of the pcDNA3.1/V5-His plasmid (D283ev) or pcDNA3.1/V5-His encoding ecto-5'-NT (D283hCD73) sequences was confirmed by evaluating the ecto-5'-NT mRNA and protein expression and ecto-5'-NT activity.

RT-PCR and Real-Time PCR

Total RNA from MB cell lines was isolated with Trizol® Reagent (Invitrogen *by* Life Technologies—Carlsbad, CA, United States). The cDNA was synthesized with RevertAid reverse transcriptase (Fermentas *by* Life Technologies—Carlsbad, CA, United States) from 3 μg of total RNA in a final volume of 20 μL in the presence of oligo dT primer. The following PCR reaction was performed in a total volume of 25 μL, which included 1 μL of each forward and reverse primers to ecto-5'-NT coding sequences (S1 Table) and 1 μL of Taq DNA polymerase enzyme (Fermentas *by* Life Technologies—Carlsbad, CA, United States). The PCR products were analyzed on a 2.0% agarose gel containing ethidium bromide and visualized under ultraviolet light. The plasmid containing the sequences for ecto-5'-NT was used as a positive expression control for this enzyme. A negative control reaction was performed by substituting the templates for DNase/RNase-free distilled water.

Real-time PCR analysis was performed in the ABI Step One Plus Instrument using the SYBR Green amplification System (Applied Biosystems, Foster City, CA). Each reaction was performed with 0.25 μL of each forward and reverse primer (10 μM) (S1 Table). Because the efficiency of all of the reactions was >95%, the ΔΔCt parameter was used to determine the relative expression levels, using GAPDH gene expression as an endogenous control for normalization.

Flow Cytometry

Ecto-5'-NT. For flow cytometry analysis, one million cells were washed twice with phosphate buffered saline (PBS) plus 1% fetal calf serum (FCS) and centrifuged. The pellets were resuspended and incubated for 1 h with purified mouse anti-human CD73 antibody (1:10, BD Pharmingen TM) for 1 h at 4°C. Next, all of the samples were washed and incubated for 1 h with Alexa Fluor 555 rabbit anti-mouse (1:100) at room temperature. Then, the labeled cells were washed with PBS and immediately analyzed by flow cytometry (Beckman Coulter Fc500).

Fifty thousand events in the cell gate were collected and further analyzed using the FlowJo® 7.6.3 software.

Active caspase-3 measurement. To evaluate caspase 3 immunolabeling, MB cell lines were seeded and cultivated until 70% confluence. For sequencing, the cells were washed twice and then resuspended in BD Cytofix/Cytoperm™ solution at a concentration of 3×10^5 cells per 150 μL and incubated for 20 min at 4°C. Afterward, the cells were washed twice with BD Perm/Wash™ buffer (1×) at room temperature. Finally, the cells were incubated in BD Perm/Wash™ buffer (1×) containing an antibody against active caspase-3 for 30 min at room temperature in the dark and then analyzed by flow cytometry (FACS Caliber, BD Biosciences, San Jose, CA, USA). The data were analyzed using FlowJo® software (USA).

Ecto-5'-NT enzymatic assay and protein determination

For the determination of AMP hydrolysis, MB tumor cells were incubated for 10 min with 2 mM AMP diluted in incubation buffer, followed by the determination of the released inorganic phosphate (Pi) [9]. The specific activity was expressed as nmol Pi released/min/mg of protein (nmol Pi/min/mg). The protein concentration was determined by the Coomassie blue method using bovine serum albumin (BSA) as a standard. To determine the effect of APCP on AMPase activity, the cell lines were pre-incubated for 10 min with different APCP concentrations (1, 5, 10, 20 and 50 μM) diluted in incubation buffer. At sequencing, MB tumor cells were incubated with 2 mM AMP diluted in incubation buffer plus the respective APCP concentrations, and the protocols were performed as described above.

Cell counting

Cells were washed twice with PBS, and 100 μL of 0.25% trypsin/EDTA solution was added to detach the cells prior to counting them with a hemocytometer. According to specific protocols, the cells were prepared as follows: to evaluate the transfection effects on cell proliferation, 10^4 cells per well were seeded in 24-well plates and allowed to grow for 5 days. A cell count was performed daily. To determine the APCP effect of cell line proliferation, the cells were seeded, and when they reached 60% confluence, the cells were treated. After 24 and 48 h of treatment, cell counting was performed.

Animal care and tumor implant

All of the experiments were performed in accordance with Ethical Principles in Animal Research as adopted by the Brazilian College Laboratory of Animal Experimentation (COBEA) with prior approval by the Internal Animal Care and Use the Ethical Committee of the Instituto de Química at Universidade de São Paulo (Protocol number 15/2013, 09/20/2010). The animals were maintained in the Animal Facility of the Instituto de Química at the Universidade de São Paulo in Brazil. Balb/c immunodeficient (nude) mice that were 6 to 14 weeks old were used to perform the dorsal subcutaneous xenograph tumor implant model. One million cells were diluted in 200 μL of DMEM and supplemented with 10% FBS and 200 μL of BD Matrigel™ Basement Membrane Matrix (BD PharmingenTM—San Jose, CA) and then injected into the dorsal flank of nude mice. The animal groups were set up as follows: control (injected with 200 μL of DMEM with 10% FBS plus 200 μL of Matrigel without cells), Daoy (injected with wild type cells expressing ecto-5'-NT), D283ev (injected with cells with transfection control—empty vector) and D283hCD73 (injected with cells overexpressing ecto-5'-NT). Animals were monitored weekly regarding their weight and tumor growth. Tumor growth measurements performed with a pachymeter twice a week were based on the biggest and smallest diameter of the tumor mass. The tumor size was determined with the equation $TS = (\pi/6) \times A \times B^2$ (*TS*—

tumor size; *A*—greater tumor mass diameter; *B*—smaller tumor mass diameter). After the first tumor of each group reached approximately 18 mm at the largest diameter of the tumor mass, all the animals in the group were euthanized by cervical dislocation. The tumors were excised, and their weights and sizes were determined. Tumor masses were fixed with formalin for posterior analysis. To maintain the physical integrity of animals after tumor development, pain signals were monitored, as well as weight loss and apathy. In the case of any of these symptoms, the animals were euthanized. The observation of the animals did not showed that any of them became severely ill or injured during the course of the study. These parameters were established in accordance with the Tumor Policy for Mice and Rats, as described by Boston University Research Compliance—Research Committees, which is accepted by IACUC (Institutional Animal Care and Use Committee), and analyzed and approved by the local Committee as described above.

In vivo nude mice imaging

Nude mice received an injection containing IRDYE® 800 CW PEG Contrast Agent (1.5 nmol) into the tail artery. After 24 h, the animals were anesthetized with flank injections of ketamine and xylazine (6.67 μL/g) and imaged using the Odyssey®CLx Infrared Imaging System plus MousePOD *in vivo* Imaging Accessory. The images were analyzed by the Odyssey®CLx software.

Histopathology and immunohistochemical staining

For histopathological analysis, samples of MB tumors were formalin-fixed, paraffin-embedded and sectioned at a 5-μm thickness. For each sample, a section was stained using standard hematoxylin and eosin (H&E) protocols. Other sections of these samples were used for immunohistochemistry protocols. For this, sections of 5 μm thickness were processed using antigenic Tris/EDTA recuperation at pH 9.0 and high temperature and blocked with methanol (3%) and FBS (5%), followed by overnight incubation at 4°C with the following specific antibodies: anti-human CD31, anti-synaptophysin, anti-enolase, anti-Ki67 (Cell Marque, Rocklin, CA), anti-human CD73 (Santa Cruz Biotechnology, INC.—Dallas, Texas) and polyclonal rabbit antibody against active Caspase 3 (Abcam, Cambridge, MA). Next, tissue sections were incubated with the Reveal Complement secondary antibody (Spring Bioscience). The H&E and Immunoblotting slides were analyzed by a pathologist in a blind manner. Each positive endothelial cell cluster of immunoreactivity in contact with the selected field was considered an individual vessel. For all of the antigens analyzed here, the expression and localization were considered positive only when the cells were clearly immunoreactive. Pathological evaluations were performed for ten randomly chosen fields (× 200) per tumor, using an Olympus BH-2 microscope. Blind quantitative analyses of five randomly chosen images of each tumor sample were performed for Ki67 and CD31 immunolabeling, using ImageJ as the image analysis program.

Statistical Analysis

Data were expressed as the mean value ± S.D. of at least three independent experiments and were subjected to a One-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. The differences between the mean values were considered significant when $p<0.05$.

Results

Analysis of ecto-5'-NT expression and activity in MB transfected cells

The expression of ecto-5'-NT in transfected cells (D283hCD73), as well as in Daoy, D283 and D283ev (empty vector control), was detected on the gene and protein expression levels (Fig 1).

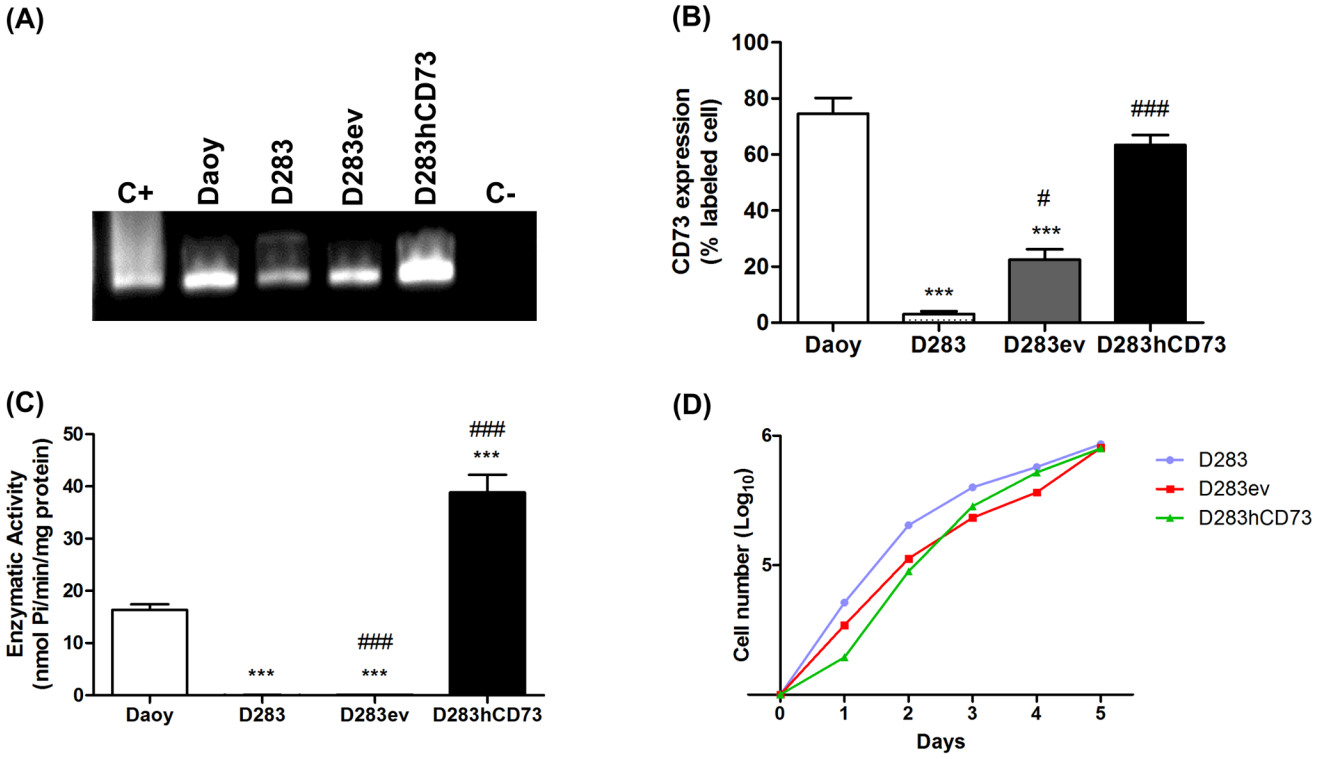

Fig 1. Ecto-5'-NT expression and activity following transfection of the D283 MB cell line. Ecto-5'-NT expression was determined by **(A)** RT-PCR analysis, **(B)** flow cytometry and **(C)** enzymatic activity as described in the Materials and Methods. **(D)** Cell proliferation indices for each cell line were obtained during five days of culture. (*) $p < 0.05$; (**) $p < 0.01$; and (***) $p < 0.001$ indicate significant differences compared to the Daoy cell line, and (#) $p < 0.05$; (##) $p < 0.01$; and (###) $p < 0.001$ indicate significant differences compared to the D283 cell line.

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The Daoy MB cell line was used as a positive control for ecto-5'-NT expression and D283 as a cell line expressing low levels of this enzyme [9]. Gene expression analysis revealed that the D283hCD73 MB cell ecto-5'-NT expression levels are low in D283ev cells (Fig 1A), in agreement with the flow cytometry results (Fig 1B). Daoy and D283hCD73 cells were positive for anti-ecto-5'-NT immunofluorescence staining, with no significant differences (Fig 1B). The obtained expression profiles were in agreement with the enzymatic activity levels, revealing the enhanced functionality of ecto-5'-NT in the D283hCD73 cell line in converting AMP into adenosine (Fig 1C). Ecto-5'-NT overexpression and the transfection process *per se* did not affect the *in vitro* proliferation of MB cells (Fig 1D).

Evaluation of AMPase activity and cell proliferation after ecto-5'-NT inhibition

First, we determined the optimal concentration of APCP, a specific ecto-5'-NT inhibitor that inhibits the enzymatic activity in the cell lines Daoy, D283, D283ev and D283hCD73 (Fig 2). We could observe that D283 and D283ev demonstrated a very low enzymatic activity, corresponding to the low ecto-5'-NT expression that is presented by these cell lines. Thus, it is possible to infer that APCP did not alter this behavior. Daoy and D283hCD73 presented a prominent AMP hydrolysis, and APCP efficiently inhibited ecto-5'-NT activity at all of the tested concentrations (Fig 2). During sequencing, to determine the influence of ecto-5'-NT on MB cell proliferation, we used 5 μM APCP for 24 and 48 h. After 48 h, APCP stimulated the

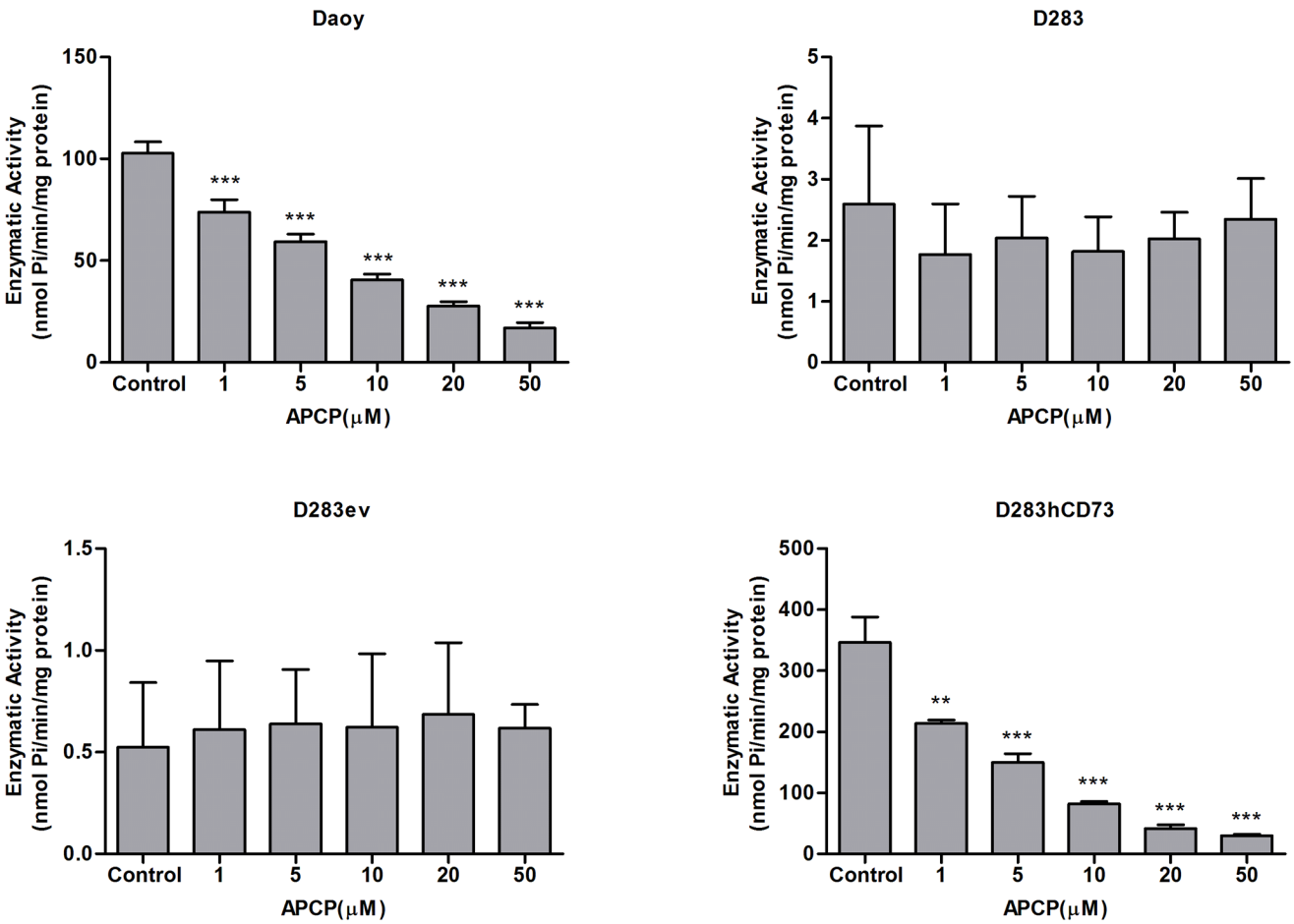

Fig 2. Effect of APCP on AMPase activity in human MB cell lines. After reaching confluence, MB cell lines were pre-incubated for 10 min with APCP at the following concentrations: 1, 5, 10, 20 and 50 μM. At sequencing, AMP was added as a substrate at 2 mM for all cell lines. For Daoy and D283hCD73, the cells were incubated for 10 min and for D283 and D283ev, 30 min. The control did not receive APCP at any time. Specific activities were expressed as nmol/Pi/mg of protein. (*) $p < 0.05$; (**) $p < 0.01$; and (***) $p < 0.001$ indicate significant differences compared to the control of each respective cell line.

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cell proliferation in the Daoy (54%) and D283hCD73 (41%) cell lines (Fig 3). These data suggest that the inhibition of ecto-5'-NT promotes cell proliferation in MB cell lines that express this enzyme. No difference was observed in D283ev cell proliferation at any time evaluated. D283 was not tested because it does did show a significant difference compared to D283ev, the transfection control cell line.

The effect of ecto-5'-NT overexpression on the MB xenograph in vivo model

The effects of ecto-5'-NT overexpression on *in vivo* MB growth were assessed after the subcutaneous injection of Daoy, D283ev or D283hCD73 cells in a nude mouse xenograph model. No changes in the body weight of the animals were noted during tumor growth (S1 Fig). Measurements of the maximal and minimal diameters showed that the D283ev cell line produced larger tumors compared to the other transplanted tumor type (Fig 4A and 4B and S2 Fig). The Daoy cell line generated the lowest mass within the studied tumors (S2 Fig). An important result that

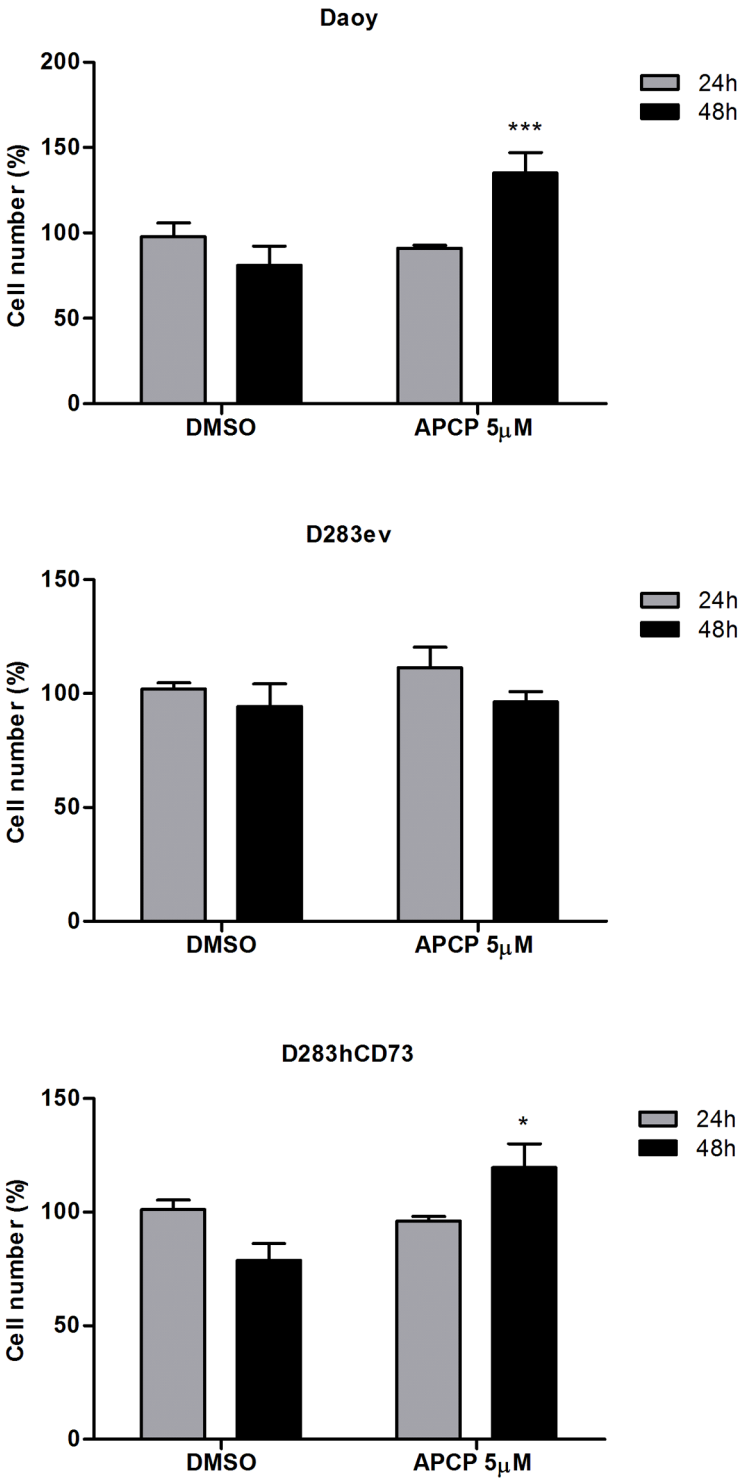

Fig 3. Effect of APCP on MB cell proliferation. At 60% confluence, the cells were treated with 5 μM APCP for 24 and 48 h, and cell counting was performed as described in the Materials and Methods. Controls were considered 100%. The data were analyzed by a Student t-test, and (*) $p < 0.05$ and (***) $p < 0.001$ indicate significant differences compared to the control of each respective cell line.

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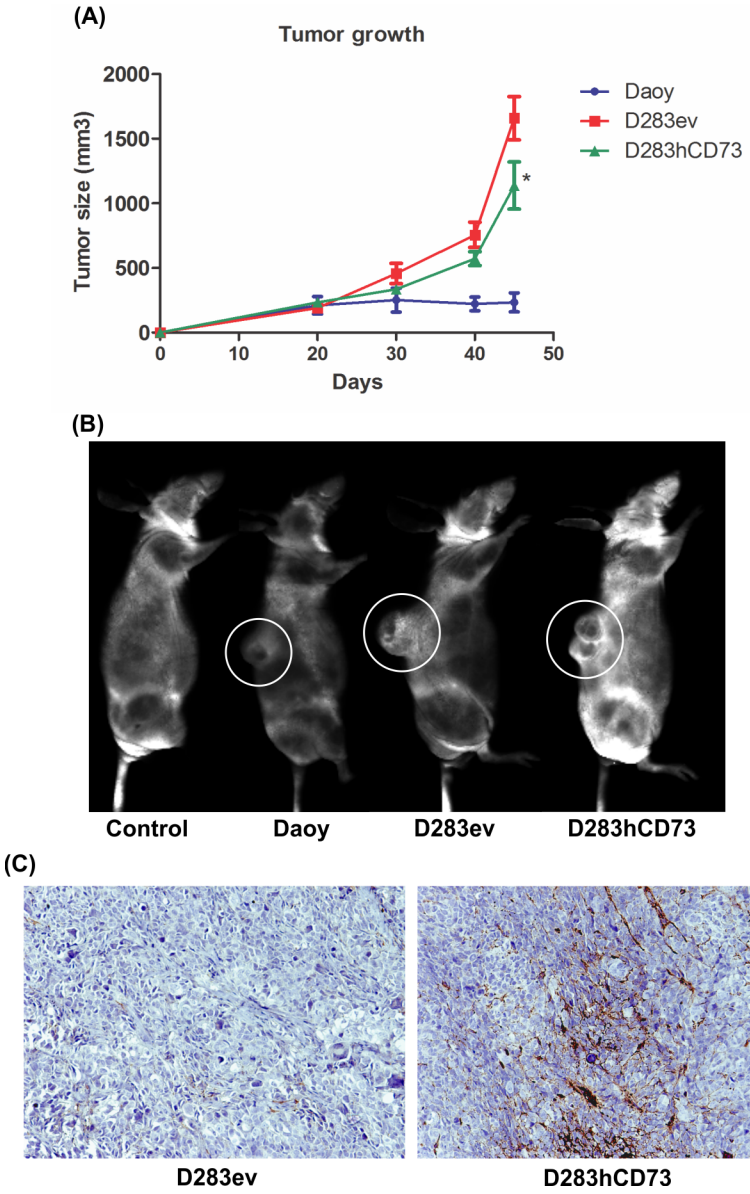

Fig 4. Ecto-5'-NT expression in D283 decreases the growth of tumor cells. To determine human MB tumor growth in nude mice, equal amounts of Daoy, D283ev and D283hCD73 cells (1×10^6 cells) were implanted by subcutaneous injection into the dorsal region of nude mice. During tumor growth, the following data were obtained: **(A)** Measurements of tumor mass, which determine tumor growth (mm^3). **(B)** Prior to euthanasia, nude mice were injected with the IRDYE® 800 CW PEG Contrast Agent, and images were captured in the Odyssey®CLx Infrared Imaging System plus MousePOD *in vivo* Imaging Accessory. Thus, the location and size of the tumor could be qualitatively measured *in vivo*. The white circle highlights the tumor mass in each animal that was examined. **(C)** Detection of ecto-5'-NT immunoreactivity in D283ev and D283hCD73 tumors. The values represent the mean values ± SD (n = 10) for each analyzed group, where (*) $p < 0.05$.

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was obtained in this work was the reduction of the tumor growth presented by the D283hCD73 cell line (Fig 4A and 4B and S2 Fig), suggesting that the overexpression and increased activity of ecto-5'-NT favor the reduction of MB tumor growth in this *in vivo* model. This observation is supported by the final tumor size and weight (S1 Fig). Both results

demonstrate the evident effect of ecto-5'-NT overexpression on promoting tumor reduction compared to the D283ev control. At the same time of tumor growth, Daoy cells with elevated endogenous ecto-5'-NT expression generated a smaller tumor mass. In addition, the immunoreactivity levels for ecto-5'-NT confirmed the differences between ecto-5'-NT expression rates, in accordance with the produced tumor mass, where the tumors that originated by D283hCD73 showed the most prominent reactivity for ecto-5'-NT expression, while the D283ev tumors were negative (Fig 4C).

The characterization of xenograph human MB tumors and histopathological analysis

The pathological analysis of the implanted tumors demonstrated that all of the samples presented characteristics of MB (Fig 5; Table 1). The histopathological analysis showed that Daoy-implanted tumors presented areas of nodularity, accompanied by accentuated cellular atypia and moderate cellularity. Additionally, these tumors presented a low mitotic index and poor vascularization of the tumor tissue. On the other hand, D283ev and D283hCD73-transplanted animals showed augmented cellular atypia and cellularity with extensive necrosis areas. The

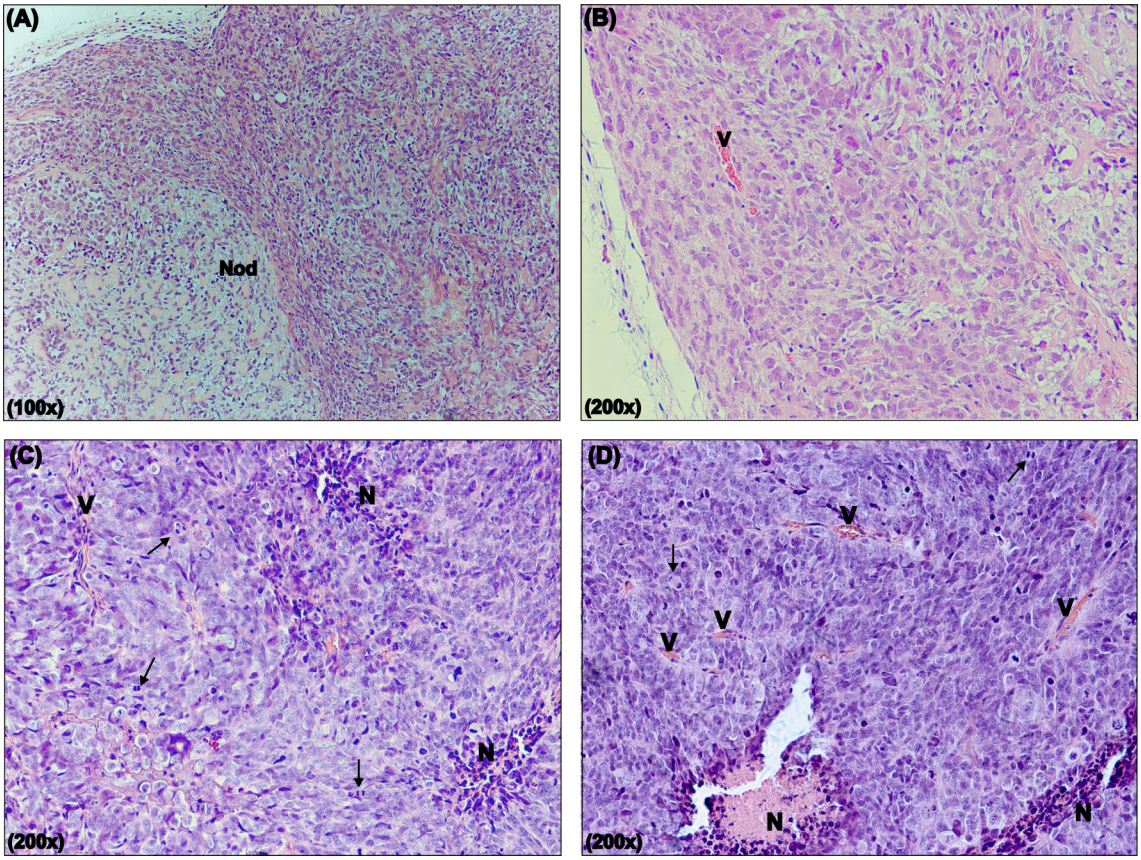

Fig 5. Histopathological analysis of human MB implanted tumors. The implanted tumors were excised, fixed and destined to posterior analysis as described in the Materials and Methods. Representative H&E sections of MB tumors **(A, B)** Daoy, **(C)** D283ev and **(D)** D283hCD73 demonstrate the histopathological characteristics of human MB: the presence of nodularity (Nod), intratumoral vascularization (V), necrotic areas (N) and the presence of mitosis are indicated by arrows. The results of additional analyses are detailed in Table 1. The images were obtained using an inverted fluorescence microscope (Nikon Eclipse TE 300).

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Table 1. Histopathological characteristics of implanted MB.

	Cellularity	Atypia	Necrosis	Mitotic index
Daoy (n = 6)	Moderate (4/6)	Accentuated (3/6)	Absent (6/6)	14.5
D283ev (n = 10)	Accentuated (10/10)	Accentuated (10/10)	Present (10/10)	79.8
D283hCD3 (n = 9)	Accentuated (8/9)	Accentuated (8/9)	Present (8/9)	64.7

The slides of H&E were analyzed by a pathologist in a blinded manner. Analysis was performed using ten randomly chosen fields (x 200) per tumor (Olympus BH-2 microscope).

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mitotic index that was presented by D283hCD73 MB tumor samples was lower than that of D283ev (Table 1). The reduced proliferation and size of the tumors generated by this cell line correlate with the enhanced expression of ecto-5'-NT.

Implanted tumors represented human MB, as revealed by immunohistochemical staining for the specific markers enolase and synaptophysin. In addition, Ki67 and CD31 characterize this tumor type (Fig 6 and Table 2). Intensive and diffuse reactivity to enolase was present in D283ev and D283hCD73 tumor samples. Furthermore, D283ev tumor samples did not present immunoreactivity for synaptophysin, a marker of differentiated cells. The absence of this marker in D283ev demonstrates that this tumor was highly undifferentiated. On the other hand, synaptophysin expression, as observed in D283hCD73 samples, revealed an expressive population of differentiated cells, characterizing it as less aggressive. In agreement, D283hCD73-originated tumors showed less staining for the proliferation antigen Ki67 than did D283ev tumors (Fig 6, Table 2 and S3 Fig). The immunoreactivity for CD31, a vessel marker, was less evident in D283hCD73 tumor samples, indicating that this tumor is also less vascularized than the tumor originated by D283ev (Fig 6, Table 2 and S3 Fig).

In addition, we evaluated the immunolabeling to active caspase-3 in MB cell lines by flow cytometry and in MB tumor samples by immunohistochemistry. We observed that Daoy and D283hCD73 cell lines presented the highest staining for active caspase-3, where D283hCD73 showed a significant difference (Fig 7A). Although the values represented the basal levels of caspase-3 expression, the significant difference presented by D283hCD73 in relation to D283ev reflects elevated apoptosis levels and might be related to the reduced tumor growth. In agreement, with immunohistochemistry labeling for active caspase-3, D283hCD73 and Daoy tumor samples presented the prominent expression of this protein in relation to D283ev (Fig 7B). Given these data, we suggest that the expression of ecto-5'-NT by MB tumor cells can promote alterations in basal apoptosis levels, favoring the reduction of the tumor throughout its growth.

P1 receptor gene expression in human MB cell lines

The expression levels of the A_1 adenosine receptor were higher in Daoy cells than in the D283 cell line (Fig 8). D283ev and D283hCD73 cells also revealed diminished A_1 receptor expression levels compared to Daoy cells. On the other hand, D283 cells showed the most prominent expression of the A_{2A} adenosine receptor. All of the other cell lines presented low expression rates of A_{2B} adenosine receptors. A_3 adenosine receptor expression could not be detected in these cell lines. The altered expression of P1 receptors as observed in transfected cells might be related to the transfection process because A_1 and A_{2A} expression were not different in D283ev and D283hCD73 cells.

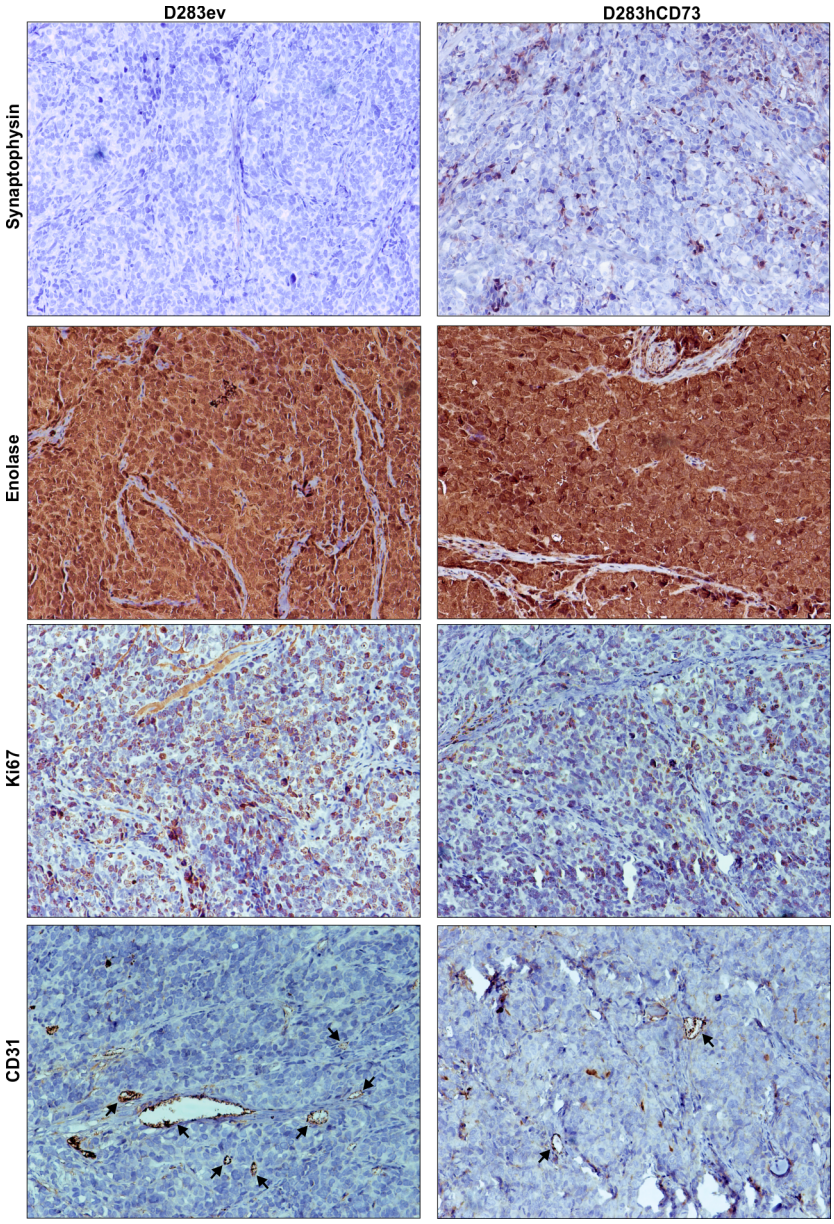

Fig 6. Immunohistochemical characterization of engrafted D283ev and D283hCD73 human MB tumors. Immunohistochemical labeling was performed as described in the Materials and Methods. Anti-synaptophysin and enolase immunostaining were used to evaluate whether the tumor samples were representative of human MB. The proliferation profile was determined using the anti-human Ki67 antibody. Tissue vascularization was visualized with an anti-human CD31 antibody. Labeling is indicated by arrows. Additional analyses are described in Table 2 and S3 Fig.

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Discussion

To investigate the role of ecto-5'-NT in MB tumor progression, we overexpressed this enzyme in D283 MB cells, generating novel tools for investigating the participation of this enzyme in the process: D283ev was used as a transfection control, and D283hCD73 were the cells that received the ecto-5'-NT human sequence. First, we performed experiments to evaluate the

Table 2. Immunohistochemical analysis of implanted MB.

	Enolase	Synaptophysin	Ki67	CD31	Ecto-5'-NT
D283ev (n = 3)	Positive diffuse (3/3)	Absent (3/3)	90% (3/3)	Strong positivity (3/3)	Absent (3/3)
D283hCD7 (n = 3)	Positive diffuse (3/3)	Positive focal (3/3)	75% (3/3)	Weak positivity (3/3)	Positive focal (3/3)

Immunohistochemistry slides were analyzed by a pathologist in a blinded manner. Analysis were performed using ten randomly chosen fields (x200) per tumor (Olympus BH-2 microscope). Ki67 positive cells were quantified by counting labeled cells in each field.

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efficiency of transfection. The obtained results showed that D283hCD73 expressed higher levels of ecto-5'-NT mRNA and translated protein (Fig 1A and 1B). Moreover, this transfected cell line showed higher AMPase activity than did D283 wild type and D283ev cells (Fig 1C), confirming that the ecto-5'-NT transfection was successful. At sequencing, we could see that APCP *in vitro* could efficiently inhibit ecto-5'-NT activity (Fig 2A and 2D) and promote cell proliferation in Daoy and D283hCD73 (Fig 3A and 3C).

The most important result was that the inhibition of ecto-5'-NT *in vitro* promotes cell proliferation, while *in vivo*, its overexpression promotes the reduction of tumor growth, demonstrating that the modulation of this enzyme can promote alterations in the MB cell proliferation levels, where its expression and activity can reduce this cell event. Immunoreactivity for ecto-5'-NT presented by D283hCD73 tumor slices confirms its overexpression in the tumor mass that presented reduced tumor growth (Fig 4). The pharmacological modulation of ecto-5'-NT promotes increased activity and a reduction of glioma cell proliferation *in vitro* [15,

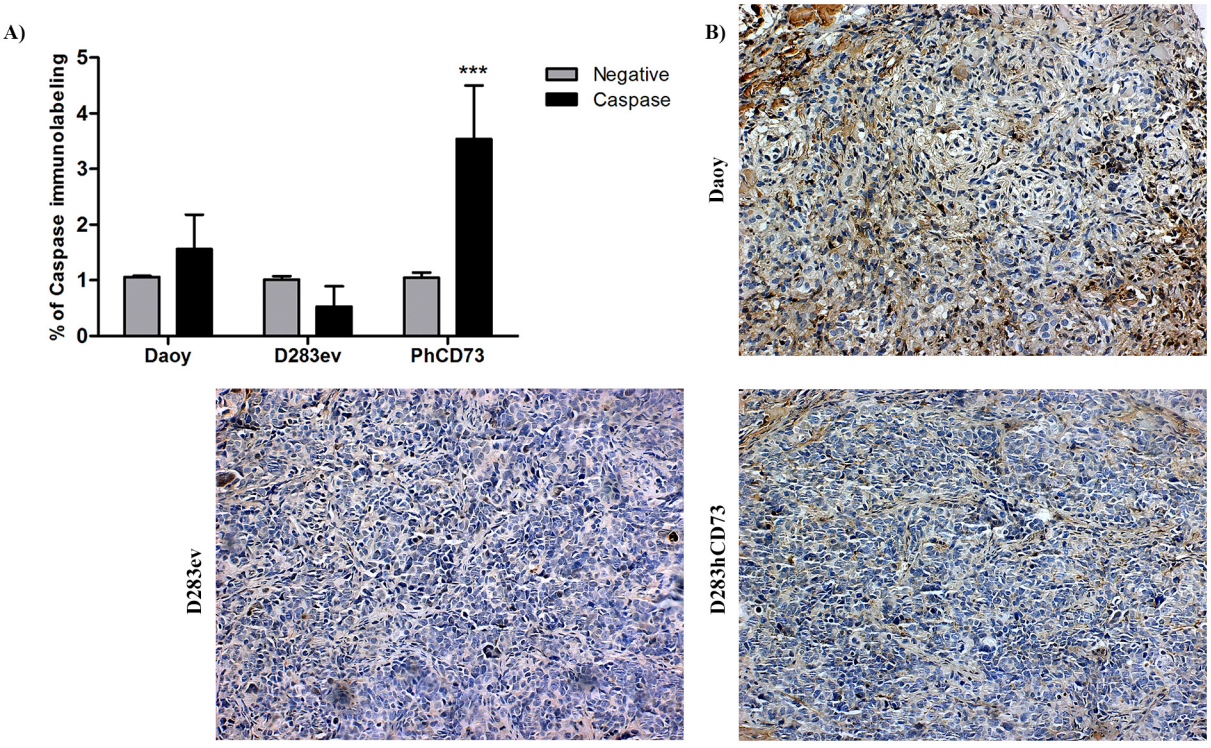

Fig 7. Evaluation of immunolabeling of active Caspase-3. Caspase-3 immunolabeling was evaluated in MB tumor cells lines by flow cytometry (A) and tumor samples by immunohistochemical staining (B) as described in the Materials and Methods. (***) $p < 0.001$ indicates a statistically relevant difference in relation to negative cells. The images were obtained using an inverted fluorescence microscope (Carl Zeiss-Imager M2 microscope) at 20× magnification.

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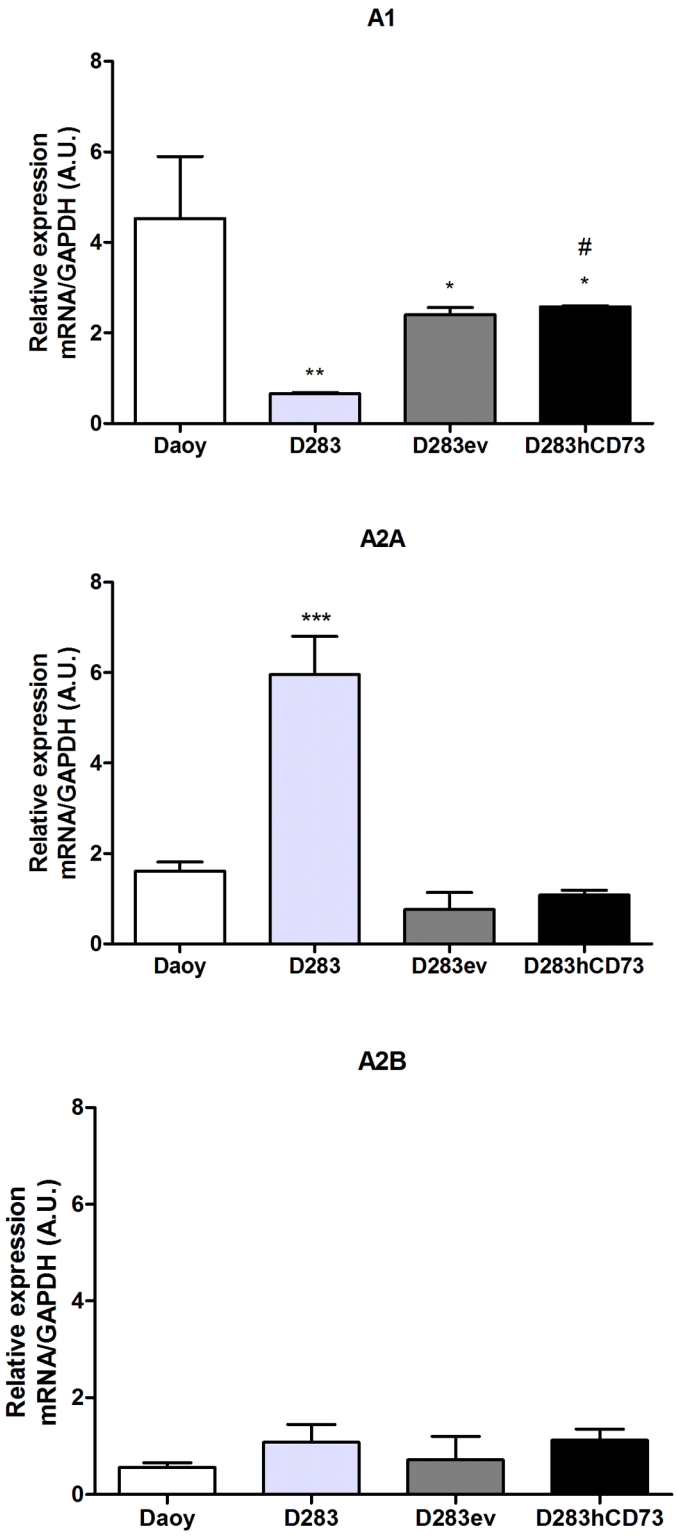

Fig 8. P1 adenosine receptor expression in human MB lines. The relative expression levels of A_1, A_{2A}, A_{2B} and A_3 in Daoy, D283, D283ev and D283hCD73 MB cell lines were assessed by real-time PCR. Endogenous GAPDH expression was used to normalize the adenosine expression levels. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate a significant difference between the analyzed samples and the Daoy cell line; # $p < 0.05$ corresponds to differences between the analyzed samples and the D283 cell line.

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16]. Indomethacin, in addition to activating ecto-5'-NT, also promotes the expression of A_3 adenosine receptor, which induces cell death [16]. In this way, knowing that the main function attributed to ecto-5'-NT is the extracellular production of adenosine [1], which make its function by activation of P1 adenosine receptors, we also determined the expression profile of P1 receptors in human MB cell lines. Daoy, D283ev and D283hCD73 preferentially expressed A_1, and D283 expressed prominent levels of the A_{2A} adenosine receptor (Fig 8). Of note, the cell transfection process appeared to increase the expression of all of the adenosine receptors in both the D283hCD73 and D283ev cell lines; nevertheless, in our tumor model, only Daoy and D283hCD73 were exposed to high adenosine levels, due to ecto-5'-NT expression. In pathological environments, ecto-5'-NT presents an important function to modulate the action of adenosine in tumor progression [1]. This nucleoside, by sensitizing P1 receptors, may favor tumor growth by stimulating angiogenesis, cell proliferation and immune response suppression [2]. However, adenosine is capable of generating different cellular behaviors depending on the expression profile of P1 receptors and the cell type expressing these receptors. It has been suggested that adenosine promotes tumor cell apoptosis by intrinsic [17] and extrinsic pathways [18]. Adenosine is taken up by cells into the intracellular medium, where it activates AMP-activated protein kinase (AMPK) and thus promotes apoptosis by caspase-3/-8, as shown for human hepatoma cells [17]. Saito and co-workers (2010) have shown that adenosine suppresses the growth of CW2 human colonic tumor cells by inducing apoptosis, which is mediated by A_1 receptors [19]. The RCR-1 astrocytoma cell line contains high levels of extracellular adenosine, promoting apoptosis mediated by A_1 receptor-stimulated caspase-9/-3 activation [18]. In addition, the selective agonism of the A_1 adenosine receptor reduced cell proliferation in different tumor human cell lines [20]. Given these data, and knowing that the overexpression of ecto-5'-NT promotes an increase in active caspase-3 immunolabeling in MB cell lines (Fig 7), we suggest that this enzyme can induce apoptosis in MB cell lines to activate the A_1 adenosine receptor to produce adenosine in the extracellular medium.

In addition, D283hCD73 generated a tumor with more differentiated cells, as demonstrated by the enhanced synaptophysin immunoreactivity (Fig 6). In agreement with the results reported here, the samples of patients with prostate carcinoma revealed less ecto-5'-NT expression in tumor tissues compared to normal differentiated prostatic tissue [21]. Furthermore, enhanced ecto-5'-NT expression levels may be related to good prognosis, as suggested by the increased survival rates of these patients. Furthermore, ovarian cancer patients with good prognosis also presented high tumor differentiation levels and ecto-5'-NT expression [22]. Together, these data suggest that the overexpression of ecto-5'-NT in the D283 MB cell line makes it less aggressive and favors the reduction of tumor growth. In agreement with the increased number of differentiated cells, D283hCD73 showed a lower proliferative index, corroborated by a reduced mitotic index and the lowest frequency of Ki67 labeling, as well increased active caspase-3 immunolabeling, which is indicative of apoptosis. This tumor also presented less CD31 immunoreactivity than did D283ev, indicating decreased tumor mass vascularization, which is associated with reduced tumor growth. Thus, taken together, these results could justify the reduced tumor growth presented by the D283hCD73 MB cell line.

All MB types are considered by the WHO (World Health Organization) to be highly malignant tumors [13]. However, we suggested that MB tumor progression depends on ecto-5'-NT expression levels. Animals that were injected with Daoy MB cells generated a large tumor only after four months of growth (S4 Fig), in contrast to the tumors generated by the D283ev cell line, which achieved their maximum size following 45 days of inoculation. Because the Daoy MB cell line was also able to generate a malignant tumor (S2 Table), the results presented here suggest that ecto-5'-NT expression may not be related to the degree of malignancy but rather to decreased tumor growth, making it slower and thus increasing the survival time. Ecto-5'-NT

expression is supposedly regulated by the Wnt/β-catenin canonic pathway, which has been considered a good prognostic marker for MB patients [9, 11]. Thus, although all MB are malignant, these data suggest that the expression of ecto-5'-NT by this type of childhood tumor can be associated with good prognosis, where these tumors present a slower growth and become susceptible to therapy.

In view of the data presented here and the literature review, we suggest that the reduction of the tumor growth after ecto-5'-NT overexpression can be attributed to two possible events: 1) an increase of differentiated cells in the tumor mass generated by D283hCD73 that makes the tumor cells less proliferative and 2) the activation of A_1 adenosine receptors expressed by D283hCD73 cells, which can promote apoptosis and slow tumor growth.

Finally, ecto-5'-NT positively affects the cancer progression of different types of adult tumors (breast and bladder cancer, melanoma and glioma) [1]. In specific childhood tumors, such as MB, this enzyme is involved with a subtype that presents a profile with good prognosis [9]. Adult tumors can present behaviors different from those of childhood tumors, and the literature emphasizes that a comparison between these behaviors cannot be routinely performed [23, 24]. This factor could also be considered important in explaining why ecto-5'-NT expression in MB is involved in a reduction of tumor growth. In conclusion, these data suggest ecto-5'-NT is an important target for controlling MB progression, suggesting a novel diagnostic tool and a target for therapeutic intervention. Additional studies are necessary to demonstrate the relationship between ecto-5'-NT and P1 adenosine receptors in MB progression.

Supporting Information

S1 Fig. Ecto-5'-NT expression reduces tumor growth in the D283 MB cell line. Following transplantation and maintenance of animals for tumor growth, as stated in Materials & Methods, all animals were euthanized and the following analyses were performed: measurement of animal body weight **(A)**, the final tumor weight **(B)** and tumor size **(C)**. The values represent the mean ± SD with n = 10 for each group analyzed where (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$, indicating a statistical difference in relation to the Daoy cell line and (#) $p < 0.05$; (##) $p < 0.01$; (###) $p < 0.001$, indicating a statistical difference in relation to the D283ev cell line.
(DOCX)

S2 Fig. Differences in tumor growth after finalization of the *in vivo* experiment.
(DOCX)

S3 Fig. Quantification of Ki67 and CD31 immunolabeling. Percentages of Ki67- and CD31-positive cells were quantified by immunohistochemistry in MB tumor samples. Five images (x 400) were captured per sample in a random manner using the Carl Zeiss-Imager.M2 microscope and quantified with the ImageJ Software.
(DOCX)

S4 Fig. Determination of tumor growth after Daoy cell engraftment. To determine human MB tumor growth in a nude mice *in vivo* model 1 x 10^6Daoy cells were implanted by subcutaneous injection in the dorsal region of nude mice. During the tumor growth the following data were obtained: **(A)** Measurements of the maximum and minimum diameters of the tumor mass, which determines tumor growth (mm^3). **(B)** Following finalization of the experiment, all animals were euthanized and the final tumor weight was determined. The values represent mean values ± SD (n = 6) for each analyzed cell group, where (*) $p < 0.05$ and (***) $p<0.001$.
(DOCX)

S1 Table. Ecto-5'-NT and adenosine receptor primer sequences.
(DOCX)

S2 Table. Histopathological characteristics of implanted Daoy MB, four months after implantation.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ARC MMP HU AMOB. Performed the experiments: ARC MMP HDNS FD FHO FF. Analyzed the data: ARC FF FHO HU AMOB. Contributed reagents/materials/analysis tools: ALA RR JL JS HU AMOB. Wrote the paper: ARC MMP FD ALA RR JS HU AMOB.

References

1. Gao Z, Dong K, Zhang H (2014) The Roles of CD73 in Cancer. Bio Med Res Inter; doi: 10.1155/2014/460654.
2. Yegutkin GG, Marttila-Ichihara F, Karikoski M, Niemelä J, Laurila JP, Elima K, et al (2011) Altered purinergic signaling in CD73-deficient mice inhibits tumor progression. Eur J Immunol; 41:1231–1241. doi: 10.1002/eji.201041292 PMID: 21469131
3. Sadej R, Spychala J, Skladanowski AC (2006) Expression of ecto-5'-nucleotidase (CD73) in cell lines from various stages of human melanoma. Melanoma Res; 16(3):213–222. PMID: 16718268
4. Wang L, Zhou X, Zhou T, Ma D, Chen S, Zhi X, et al. (2008) Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. J Cancer Res Clin Oncol; 134:365–372. PMID: 17671792
5. Wink MR, Lenz G, Braganhol E, Tamajusuku ASK, Schwartsmann G, Sarkis JJF, et al. (2003) Altered Extracellular ATP, ADP and AMP Catabolism in Glioma Cell Lines. Cancer Lett; 198:211–218. PMID: 12957360
6. Bavaresco L, Bernardi A, Braganhol E, Cappellari AR, Rockenbach L, Farias PF, et al. (2008) The Role of Ecto-5'-nucleotidase/CD73 in Glioma Cell line Proliferation. Mol Cell Biochem; 319(1–2):61–68. doi: 10.1007/s11010-008-9877-3 PMID: 18636315
7. Cappellari AR, Vasques GJ, Bavaresco L, Braganhol E, Battastini AMO (2011) Involvement of Ecto-5'-nucleotidase/CD73 in U138MG Glioma Cell Adhesion. Mol Cell Biochem; 359(1–2):315–322. doi: 10.1007/s11010-011-1025-9 PMID: 21858682
8. Supernat A, Markiewicz A, Welnicka-Jaskiewicz M, Seroczynska B, Skokowski J, Sejda A, et al. (2012) CD73 expression as a Potential Marker of Good Prognosis in Breast Carcinoma. Appl immunohistochem Mol Morphol; 20(2):103–107. PMID: 22553809
9. Cappellari AR, Rockenbach L, Dietrich F, Clarimundo V, Glaser T, Braganhol E, et al. (2012) Characterization of Ectonucleotidases in Human Medulloblastoma Cell lines: ecto-5'NT/CD73 in Metastasis as Potential Prognostic Factor. PLoSOne; 7(10):e47468.
10. Spychala J, Kitajewski J (2004) Wnt and beta-catenin signaling target the expression of ecto-5'-nucleotidase and increase extracellular adenosine generation. Exp Cell Res; 296: 99–108. PMID: 15149841
11. Ellison DW, Onilude OE, Lindsey JC, Lusher ME, Weston CL, Taylor RE, et al. (2005) beta-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. J Clin Oncol; 23: 7951–7957. PMID: 16258095
12. Northcott PA, Korshunov A, Pfister SM, Taylor MD (2012) The clinical implications of medulloblastoma subgroups. Nat Rev Neurol; 8: 340–351. doi: 10.1038/nrneurol.2012.78 PMID: 22565209

13. Louis DN, Ohgaki H, Wiestler Od, Cavenee WK, Burger PC, Jouvet A, et al. (2007) The 2007 WHO Classification of tumors of the Central Nervous System. Acta Neuropathol; 114(2):97–109. PMID: 17618441

14. Lecka J, Singh M, Sevigny J. (2010) Inhibition of vascular ectoncleotidase activities by the pro-drgs ticlopidine and clopidogrel favours platelet aggregation. Br J Pharmcol; 161:1150–1160.

15. Bavaresco L, Bernardi A, Braganhol E, Wink MR, Battastini AMO (2007) Dexamethasone inhibits proliferation and stimulates ecto-5'-nucleotidase/CD73 activity in C6 rat glioma cel line. J Neurooncol; 84:1–8. PMID: 17453149

16. Bernardi A, Bavaresco L, Wink MR, Jacques-Silva MC, Delgado-Cañedo A, Lenz G, et al. (2007) Indomethacin stimulates activity and expression of ecto-5'-nucleotidase/CD73 in glioma cell lines. Europ J Pharmacol; 569:8–15.

17. Yang D, Yaguchi T, Yamamoto H, Nishizaki T (2007) Intracellularly transported adenosine induces apoptosis in HuH-7 human hepatoma cells by downregulating c-FLIP expression causing caspase-3/-8 activation. Biochemical Pharmacology; 73:1665–1675. PMID: 17303086

18. Sai KH, Yang D, Yamamoto H, Fujikawa H, Yamamoto S, Nagata T, et al. (2006) A1 adenosine receptor signal and AMPK involving caspase-9/-3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. Neuro Toxicology; 27:458–467.

19. Saito M, Yaguchi T, Yasuda Y, Nakano T, Nishizaki T. (2010) Adenosine suppresses CW2 human colonic cancer growth by inducing apoptosis via A1 adenosine receptors. Cancer Lett; 290:211–215. doi: 10.1016/j.canlet.2009.09.011 PMID: 19822392

20. Hosseinzadeh H, Jaafari MR, Shamsara J (2008) Selective inhibitory effect of adenosine A1 receptor agonists on the proliferation of human tumor cell lines. Iran Biomed J; 12(4):203–208. PMID: 19079533

21. Rackley RR, Lewis TJ, Preston EM, Delmoro CM, Bradley EL, Resnik MI, et al. (1989) 5`-Nucleotidase Activity in Prostatic Carcinoma and Benign Prostatic Hyperplasia. Cancer Res; 49:3702–3707. PMID: 2471588

22. Oh HK, Sin JI, Choi J, Park SH, Lee TS, Choi YS. (2012) Overexpression of CD73 in epithelial ovarian carcinoma is associated with better prognosis, lower stage, better differentiation and lower regulatory T cell infiltration. J Gynecol Oncol; 23(4):274–281. doi: 10.3802/jgo.2012.23.4.274 PMID: 23094131

23. Paugh BS, Qu C, Jones C, Liu Z, Adamowicz-Brice M, Zhang J, et al. (2010) Integrated Molecular Genetic Profiling of Pediatric High-Grade Gliomas Reveals Key Differences With the Adult Disease. J Clinl Oncol; 28:3061–3068.

24. Puget S, Philippe C, Bax DA, Job B, Varlet P, Junier MP, et al. (2011) Mesenchymal Transition and PDGFRA Amplification/Mutation Are Key Distinct Oncogenic Events in Pediatric Diffuse Intrinsic Pontine Gliomas. PLoSOne; 7(2):e30313.

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Brilliant Blue G, But Not Fenofibrate, Treatment Reverts Hemiparkinsonian Behavior and Restores Dopamine Levels in an Animal Model of Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the substantia nigra and their projections to the striatum. Several processes have been described as potential inducers of the dopaminergic neuron death, such as inflammation, oxidative stress, and mitochondrial dysfunction. However, the death of dopaminergic neurons seems to be multifactorial, and its cause remains unclear. ATP-activating purinergic receptors influence various physiological functions in the CNS, including neurotransmission. Purinergic signaling is also involved in pathological scenarios, where ATP is extensively released and promotes sustained purinergic P2X7 receptor (P2X7R) activation and consequent induction of cell death. This effect occurs, among other factors, by oxidative stress and during the inflammatory response. On the other hand, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is involved in energy metabolism and mitochondrial biogenesis. Expression and activity upregulation of this protein has been related with reduction of oxidative stress and neuroprotection. Therefore, P2X7R and PGC-1α are potential targets in the treatment of PD. Here hemiparkinsonism was induced by unilateral stereotactic injection of 6-OHDA in a rat model. After 7 days, the establishment of PD was confirmed and followed by treatment with the P2X7R antagonist Brilliant Blue G (BBG) or PGC-1α agonist fenofibrate. BBG, but not fenofibrate, reverted hemiparkinsonian behavior accompanied by an increase in tyrosine hydroxylase immunoreactivity in the substantia nigra. Our results suggest that the P2X7R may be a therapeutic target in Parkinson's disease.

Key words: Parkinson's disease (PD); P2X7 receptor; Brilliant Blue G (BBG); PGC-1α; Fenofibrate

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and their projections to the striatum, resulting in motor deficits[1]. Therapies with dopaminergic drugs are widely used; however, lately, strategies for dopaminergic cell therapy and endogenous neuroprotection are being explored. Purinergic receptors are divided into P1 and P2 receptors, the latter classified into two subtypes, the ionotropic P2X and metabotropic P2Y receptors. While P2X receptors consist of mainly nonselective ion channels activated by 5′-adenosine triphosphate (ATP) and its derivatives, P2Y receptors are coupled to G proteins and are activated by a range of agonists such as ATP, ADP, UTP, and UDP and derivatives of these nucleotides[2].

Purinergic receptors also play a role under pathophysiological conditions. When the brain is injured or neurodegeneration occurs, ATP is extensively released into the extracellular matrix, triggering apoptosis or cell survival pathways, depending on the activation of specific P2 receptor subtypes[3]. The P2X7 subtype (P2X7R) expressed by a range of cells, including neural cells, is activated by ATP and its derived molecules, such as α,β-meATP and BzATP. ATP release occurring due to cell death in a neurodegenerative state promotes sustained

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P2X7R activation, inducing the formation of large pores in the plasmatic membrane, disrupting ion balance, and triggering cell death[4].

One of the mechanisms involved in P2X7R cell death induction is stimulation of oxidative stress. The peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC-1α) is a protein that interacts with nuclear receptors, such as PPARγ, and negatively regulates transcription of NF-κB, inhibiting inflammatory responses. PGC-1α acts by mediating interactions between transcription factors and RNA polymerases, altering the expression of mitochondrial proteins. The modulation of PGC-1α occurs by phosphorylation of different sites through a range of phosphokinases, including p38 mitogen-activated kinase (MAPK) and Akt, whose combination can stimulate or inhibit PGC-1α expression[5]. A study showed that the use of the P2X7R antagonist Brilliant Blue G (BBG) attenuated apoptosis of neuronal cells in subarachnoid hemorrhage through MAPK inhibition[6], indicating modulation of the MAPK pathway and a possible interference in PGC-1α activity. Moreover, Nagasawa and colleagues indicated that nuclear receptors for PPARγ may control P2X7R activity in astrocyte culture[7]. Therefore, activation of PGC-1α could be a novel therapeutic target in the treatment of diseases related to changes in mitochondrial functions. For instance, the genes expressed in response to PGC-1α stimulation in PD are less abundant, indicating a decrease in cytoprotective pathway activation. Recently, it was demonstrated that PGC-1α activation prevents the loss of dopaminergic neurons in cellular models of PD, indicating that this protein could be a potential target in PD treatment[8].

The activation of P2X7R plays a pivotal role in PD. P2X7R activity and consequent ion influx lead to the death of nigrostriatal dopaminergic neurons by IL-1β release, contributing to worsening of the degenerative state[9]. It was demonstrated that the P2X7R antagonist A-438079 prevented dopamine store depletion in the 2,4,5-trihydroxyphenethylamine (6-OHDA) animal model of PD without counteracting dopaminergic cell loss[10]. A recent study using Wistar rats lesioned with 6-OHDA striatal injections that received intraperitonial (IP) BBG administration on the same day, that is, before lesion establishment and therefore indicating a preventive characteristic, resulted in a neuroprotective effect by partial prevention of dopaminergic loss in the striatum and substantia nigra[11]. Altogether, these data indicate that P2X7R inhibition could improve PD injury. Altogether, studies of P2X7R activity control in PD shall provide a key piece for understanding regeneration mechanisms in this neurodegenerative disease.

Based on these data, the aim of this study was to evaluate the neuroprotective/regenerative effects triggered through blocking P2X7R activity by BBG or through activation of PGC-1α by fenofibrate in an animal model of PD.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats that were 8 weeks old at the beginning of treatment were provided by the animal facility of the Institute of Chemistry, University of Sao Paulo, and housed there with free access to food and water and on a light/dark cycle of 12:12 h. All procedures were previously approved by the local ethics committee and complied with the Guidelines of the Brazilian College of Animal Experimentation (COBEA) and National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

Model Induction

For induction of unilateral lesion of the nigrostriatal pathway, the animals were anesthetized with ketamine (90 mg/kg, IP; Ceva, Paulínia, Brazil) and xylazine (13 mg/kg, IP; Ceva) and placed in the stereotaxic frame (KOPF, Los Angeles, CA, USA). The skull was perforated with a drill (1 mm in diameter), and 3 μl of freshly prepared 6-OHDA (7 μg/μl; Sigma-Aldrich, St. Louis, MO, USA), calculated as free base, dissolved in 0.9% saline solution containing 0.02% ascorbic acid (Sigma-Aldrich), was injected into the right hemisphere of the animal brains[12], following stereotactic coordinates from the bregma: AP, −4.4; ML, −1.2; DV, −8.2[13]. The needle was kept at the injection site for 3 min before and after injection[14]. After surgery, the animals received 1 ml of glycosylated saline (Merck-Millipore, Darmstadt, Germany) and 200 μl of veterinary pentabiotic (Fort Dodge, Campinas, Brazil) by subcutaneous and intramuscular injection, respectively.

Brilliant Blue G (BBG) and Fenofibrate Administration

Both treatments were initiated following 7 days of injury induction, schemed as in Figure 1. Treatment with BBG (100 mg/ml dissolved in 0.9% saline; Sigma-Aldrich) was performed by IP administration for 7 days at a dose of 50 mg/kg daily[15–18]. Saline injections were performed for control experiments. Fenofibrate (0.2%) was added to the food of the animals during manufacturing by the Rhoster company (Araçoiaba da Serra, Brazil). An identical diet, but without fenofibrate, was used for the control group. The animals were kept in individual boxes for 14 days. The amount in grams of food consumed was measured daily, allowing the monitoring of the quantity of fenofibrate that each animal received. The effectiveness of treatment with fenofibrate at a concentration of 0.2% in the diet has already been described[19].

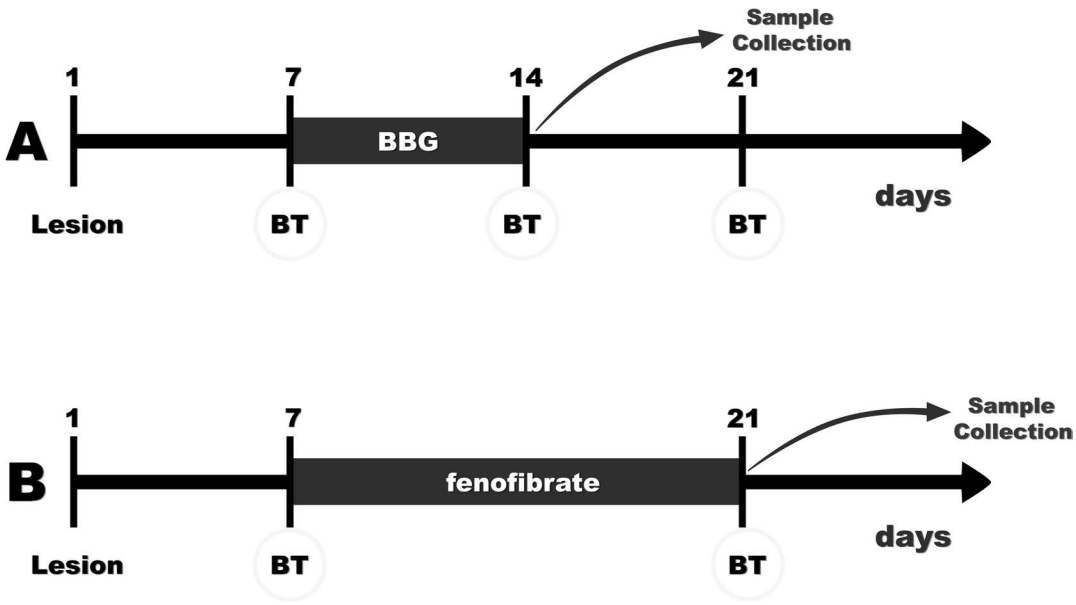

Figure 1. Evaluation and treatment schedules. The black arrow indicates the course of the experiments (time unit: day). On day 1, animals were lesioned by unilateral 2,4,5-trihydroxyphenethylamine (6-OHDA) injection. Seven days later, animals underwent rotational tests to confirm hemiparkinsonism, and treatments were started. The animals were treated for 7 days with BBG and then submitted to rotational testing on days 14 and 21 (A). Animals treated with fenofibrate for 14 days were submitted to a second round of rotational testing on day 21 (B). For immunohistochemistry or Western blotting analysis, animals were sacrificed, and samples were collected on the last day of treatment. BT, behavioral test; BBG, Brilliant Blue G.

Behavioral Test: Rotational Behavior Induced by Apomorphine

Animals underwent rotational tests 7 days after the lesion of the nigrostriatal pathway with 6-OHDA, and then 7 and 14 days following treatment with BBG and 14 days after fenofibrate administration. For rotational tests, animals were weighed and injected with apomorphine hydrochloride (Sigma-Aldrich) at a dose of 0.5 mg/kg (10 mg/ml, IP) in 0.9% sterile saline supplemented with 0.02% ascorbic acid. The number of rotations in a counterclockwise direction, that is, to the side opposite to the lesion, was recorded for 30 min and shown as rotations/min. This test was used to confirm the lesion and analyze treatment effects[19]. Only animals showing rotational behavior 7 days following 6-OHDA were included in the study.

Immunohistochemistry

Immediately after the second rotational test, animals were deeply anesthetized with a mix of ketamine/xylazine and perfused with a peristaltic pump (World Precision Instruments, Sarasota, FL, USA) with 200 ml of phosphate-buffered saline (PBS; Synth, Diadema, Brazil) per animal to remove all blood. Subsequently, tissue fixation with a 250-ml solution of PBS/4% paraformaldehyde (PFA; Sigma-Aldrich) at 4°C was performed. At the end of perfusion, brains were removed and placed in a solution of PBS/30% sucrose (Synth) for 48 h at 4°C. Coronal sections were made in a cryostat (HM 500 OM; MICROM International GmbH, Walldorf, Germany) to obtain sequential sections of 30 μm. Slices were then treated with 0.3% H_2O_2 (Synth) to eliminate endogenous peroxidase activity and incubated overnight with anti-tyrosine hydroxylase (TH; for 24 h; 1:500; Sigma-Aldrich) and anti-P2X7R (for 48 h; 1:500; Sigma-Aldrich) polyclonal antibodies in PBS containing 0.1% Triton X-100 (Sigma-Aldrich). The slices were washed three times with PBS, followed by 2 h of incubation with peroxidase-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, ME, USA) at a 1:500 dilution. After washing with PBS, the reaction was developed using SIGMAFAST™ 3,3′-diaminobenzidine tablets (Sigma-Aldrich).

Images were obtained using the EVOS XL Cell Imaging System (Thermo-Fisher, Waltham, MA, USA). Optical density measurements of photomicrographs of TH-immunostained slices were performed to determine the density of the TH^+ fibers in the striatum. The measurement was made using grayscale in ImageJ software (US NIH, Bethesda, MD, USA). The values were normalized using the hemisphere control as 100% to eliminate any difference in the background of each slice. In the substantia nigra, the number of TH^+ cells was detected and

quantified by automated analysis using the StrataQuest software (TissueGnostics GmbH, Vienna, Austria). Nine slices obtained for each animal were subjected to automated counting of TH^+ cells followed by determination of mean values ± standard error (SE) of injured and their respective control hemispheres.

Western Blotting

Animals were anesthetized with ketamine (90 mg/kg, IP) and xylazine (13 mg/kg, IP) and decapitated using a guillotine for removal of the striatum for subsequent protein analysis. The tissue was homogenized in lysis buffer containing 20 mM tris(hydroxymethyl) aminomethane (LGC, Cotia, Brazil), 1 mM ethylenediaminetetraacetic acid (EDTA; Synth), 20% glycerol (Sigma-Aldrich), 0.5% Igepal® (Sigma-Aldrich), and protease inhibitor cocktail (Amresco, Solon, OH, USA), pH 7.5. Total protein contents were quantified using the Bradford reagent (Thermo-Fisher), and 30 μg was separated with the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique using the Owl Dual-Gel Vertical Electrophoresis System (Thermo-Fisher) with a 10% SDS-polyacrylamide gel-containing acrylamide, bis-acrylamide (Sigma-Aldrich), tris(hydroxymethyl)aminomethane (LGC), and SDS (Synth); transferred to polyvinylidene difluoride (PVDF) membranes (Merck-Millipore); and incubated for 16 h with anti-β-actin (1:100; Sigma-Aldrich), anti-TH (1:500; Sigma-Aldrich), and anti-P2X7R (1:500; Sigma-Aldrich) primary antibodies at 4°C. Then membranes were incubated for 2 h at room temperature with a secondary antibody conjugated to Alexa Fluor 555 (1:1,000; Thermo-Fisher) for β-actin and to Alexa Fluor 647 (1:1,000; Thermo-Fisher) for TH and P2X7R visualization. Membranes were scanned with the Typhoon phosphor imager (GE Healthcare, Pittsburgh, PA, USA), and detected bands were subjected to densitometric analysis with the ImageJ software.

Statistical Analysis

Statistical analyses were performed using Prism software (version 288 5.01; GraphPad Software, San Diego, CA, USA). Behavioral rotational immunohistochemistry and Western blotting data were analyzed with two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A significance level of $p < 0.05$ was assumed. The results shown are mean values of at least three independent experiments. Error bars represent the mean ± standard error of the mean (SEM).

RESULTS

The effects of P2X7R inhibition and PGC-1α activation were studied in an animal model of hemiparkinsonism. Seven days after the induction of the 6-OHDA lesion, animals presented a rotational behavior after the administration of apomorphine, characteristic for this animal model of PD, confirming lesion establishment. After 7 days of daily treatment with BBG (50 mg/kg, IP) or saline, animals were resubmitted twice to the behavioral test (on days 14 and 21). While saline-treated animals presented a rotational behavior, measured as rotations/min similar to the first trial (12 ± 5.2, first trial; 12.6 ± 6.3, second trial; 14 ± 3.6, third trial), BBG treatment reduced these motor deficits of hemiparkinsonism (13.2 ± 5.1, first trial; 5.1 ± 2.2, second trial; 6.1 ± 1.9, third trial). This difference is significant ($p < 0.05$) when compared to the first tests (prior to BBG treatment) and when compared

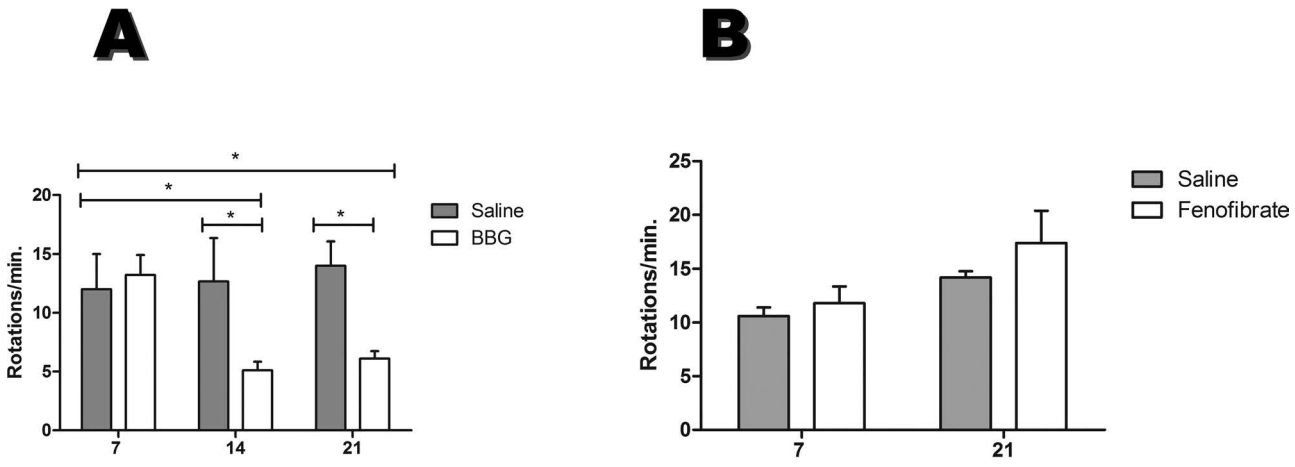

Figure 2. Reversion of rotation behavior following BBG treatment. The bar plots show the comparison between numbers of rotations (A) before and after 7 days of BBG ($n = 12$) or saline ($n = 6$) treatment. After the treatment, the animals were submitted to two trials of behavioral test following 7 and 14 days of treatment with BBG (B) before and after 14 days of food without ($n = 6$) or with fenofibrate ($n = 5$). Data are shown as mean values ± SEM. *$p < 0.05$ in relation to the BBG-treated animal group before treatment.

to the saline group (Fig. 2A). On the other hand, therapy for 14 days of diet supplemented with 0.2% PGC-1α activator (fenofibrate), which was stably consumed by the rats (data not shown), had no effect on apomorphine-induced rotational profile in this animal model of PD (10.6±0.8, first trial; 14.6±0.5 rotations/min, second trial) (Fig. 2B).

Percentages of TH^+ cells measured by immunohistochemical analysis following BBG treatment for 7 days indicated recovery in the 6-OHDA-lesioned hemisphere (86.7% in relation to the unlesioned hemisphere), when compared to the control group treated with saline (19.9% in relation to the unlesioned hemisphere) (Fig. 3A–C). Immunohistochemical analysis of the striatum did not show any significant differences between groups (62.9% in the saline group; 61.5% in the BBG-treated group, in relation to unlesioned hemispheres; images not shown). Western blotting analysis confirmed these data in the substantia nigra (Fig. 3D), although a slight intensity increase was observed in the TH band corresponding to the striatum of the 6-OHDA-lesioned hemisphere, which had been treated with BBG (densitometry results in A.U.: 0.74±0.46, BBG-unlesioned hemisphere; 0.64±0.24, BBG-lesioned hemisphere; 0.85±0.19, saline-unlesioned hemisphere; 0.58±0.23, saline-lesioned hemisphere).

Immunohistochemical analysis of patterns of the TH^+ fibers in the striatum (data not shown) and TH^+ cells in the substantia nigra of fenofibrate- (Fig. 4A) and saline-treated

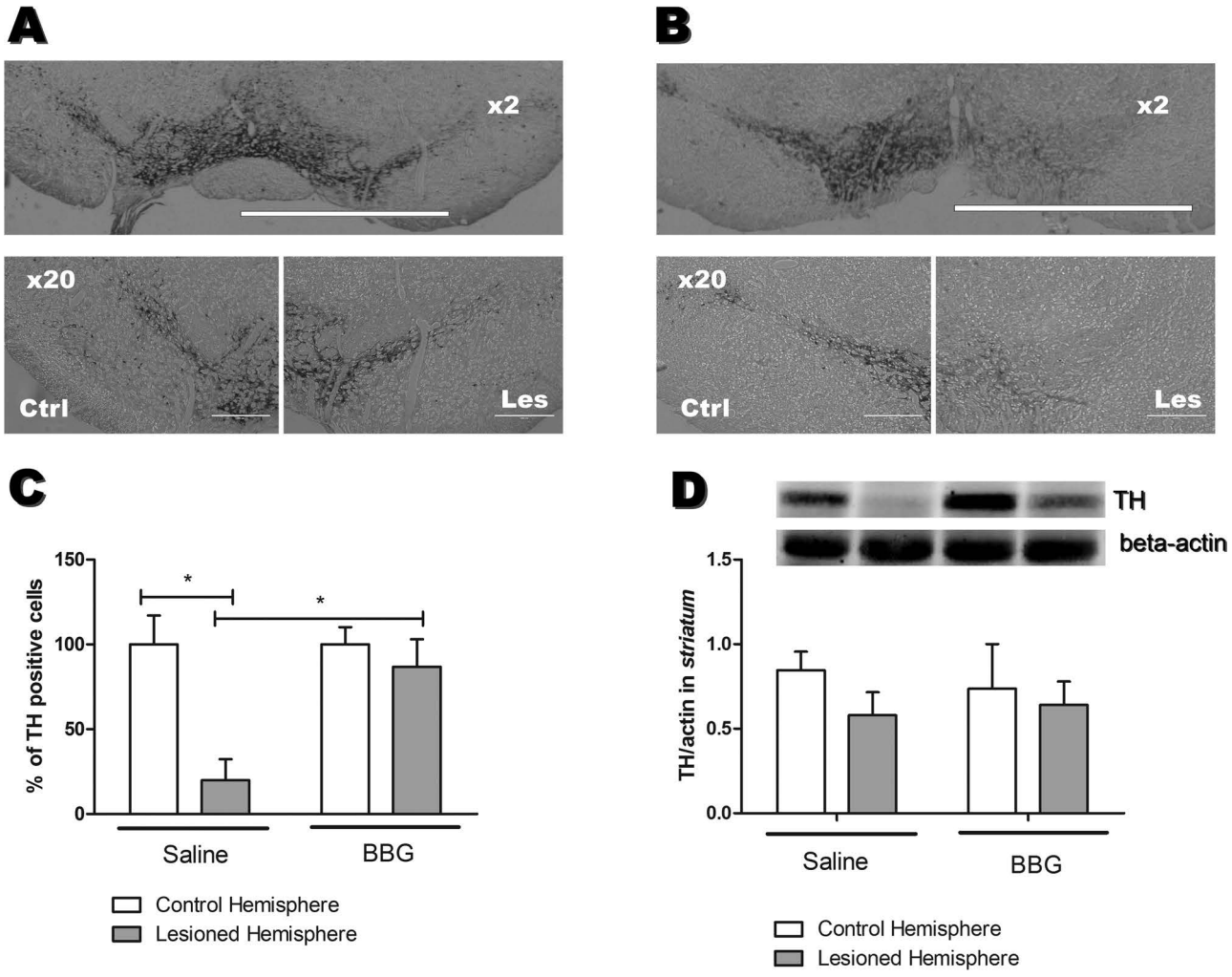

Figure 3. Tyrosine hydroxylase-positive (TH^+) cells and protein expression following 7 days of BBG treatment. TH^+ cells in the substantia nigra of (les) lesioned and (ctrl) unlesioned control hemispheres of (A) BBG- and (B) saline-treated animals. (C) Percentages of TH^+ cells in the substantia nigra ($n=6$). Cells were automatically counted using the StrataQuest Software (TissueGnostics). (D) Western blotting analysis of TH protein expression (60 kDa) in the striatum ($n=3$). For normalization of relative TH expression levels, β-actin (42 kDa) expression levels were measured, which did not change under the used experimental conditions. *$p<0.05$, when compared to the control hemisphere of saline-treated animals. Scale bars: 500 μm (2× amplified image), 50 μm (20× amplified image).

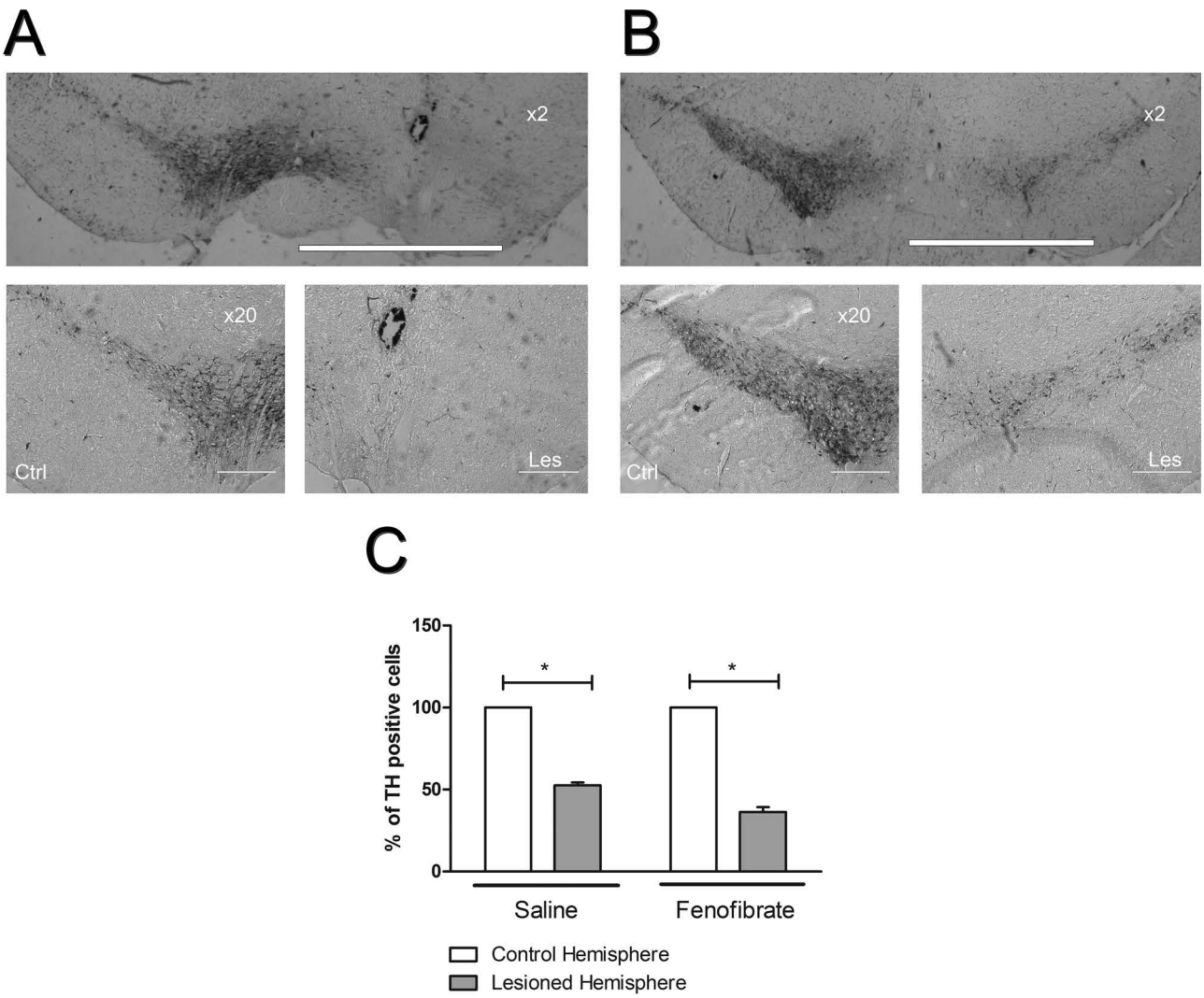

Figure 4. Effects of fenofibrate on the percentage of TH^+ cells in the substantia nigra. Representative slices of TH^+ cells in the substantia nigra of (les) lesioned and (ctrl) control hemispheres of hemiparkinsonian rats undergoing 14 days of (A) fenofibrate or (B) saline treatment. (C) Bar plot showing the comparison between percentages of TH^+ fibers in the substantia nigra of lesioned and unlesioned control hemispheres of animals receiving food without ($n=6$) or with fenofibrate ($n=5$). *$p<0.01$, when compared to the unlesioned control hemisphere of the respective treatment. Scale bars: 50 μm.

animals (Fig. 4B) did not reveal any statistically relevant differences (36.3% in the substantia nigra and 62.3% in the striatum, in relation to unlesioned hemispheres) compared to those animals that had received placebo (52.5% in the substantia nigra and 54.8% in the striatum, in relation to unlesioned hemispheres). Statistically, the numbers of dopaminergic cells (TH^+) in the injured hemisphere were different from those observed in the unlesioned control hemispheres in both groups, indicating no recovery (Fig. 4C).

Western blotting analysis of the striatum showed that animals treated with BBG presented a statistically significant decrease in P2X7R expression levels ($p<0.05$) in the lesioned hemispheres compared with the unlesioned hemispheres (densitometry results in A.U: 1.03 ± 0.03, BBG-unlesioned hemisphere; 0.4 ± 0.09, BBG-lesioned hemisphere) and with the saline-treated animals (0.8 ± 0.07, saline-unlesioned hemisphere; 0.98 ± 0.04, saline-lesioned hemisphere) (Fig. 5).

DISCUSSION

P2X7R activity has been implicated in various neurodegenerative diseases, including PD, Alzheimer's disease, Huntington's disease, and multiple sclerosis[20]. As underlying mechanisms, large concentrations of ATP are released from damaged cells, resulting in prolonged

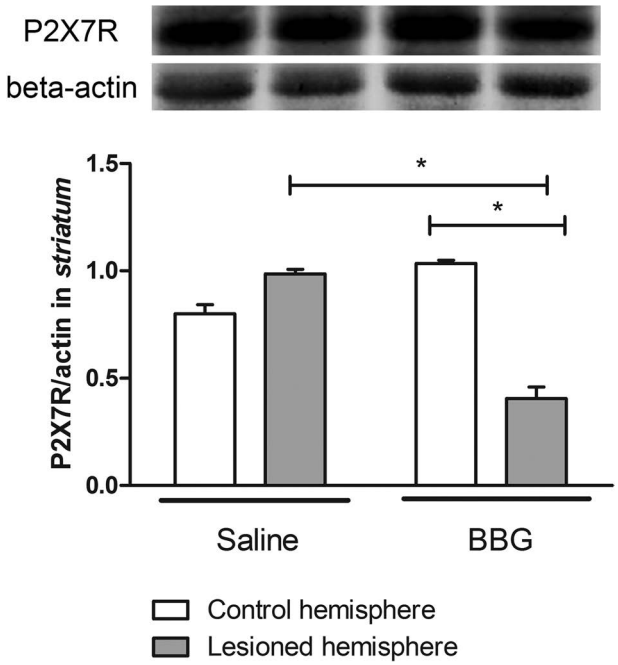

Figure 5. Western blotting analysis of P2X7R expression in the striatum of animals treated with BBG. Analysis of P2X7R expression (75 kDa) in the striatum of hemiparkinsonian rats undergoing 7 days of BBG or saline treatment. For normalization of relative P2X7R expression levels, β-actin (42 kDa) expression was measured, which did not change under the used experimental conditions ($n = 3$).

exposure of neighboring cells expressing P2X7R to elevated ATP, causing cytoskeletal changes, neuroinflammation, and apoptotic cell death due to overloading of the cytoplasm and mitochondria by uncontrolled calcium influx. BBG, a noncompetitive and blood–brain barrier-permeant antagonist of the P2X7R, blocks the rat receptor subtype at least 1,000-fold more potent than P2X4R. Furthermore, BBG inhibited YO-PRO1 uptake and blebbing of membranes, which are characteristics for P2X7R activity[17].

The results presented in this work provide evidence from behavorial and immunohistochemical studies that treatment with the P2X7R antagonist BBG reverses the depletion of dopaminergic neurons in an animal model of PD induced by 6-OHDA injection. 6-OHDA, a structural analog of dopamine, is a neurotoxin, which uses dopamine transporters to reach the cytosol of dopaminergic neurons. The intracellular increase in 6-OHDA concentration results in mitochondrial dysfunction, accumulation of reactive species of oxygen, and consequent cell death[18].

Several cell types have their signaling pathways associated with activation of P2X7R, including immune cells that release proinflammatory cytokines, leading to the formation of reactive oxygen species and nitrogen, formation of phagolysosomes, and apoptosis[22–24]. Moreover, P2X7R is the major physiological regulator of the secretion of IL-1[25]. This microenvironment activates phagocytic functions, which in turn release cytokines and interleukins activating astrocytes and microglia, regulating central nervous system immune responses, modulating survival and migration of neurons, and altering neuronal plasticity[26].

Our results show the efficacy of BBG treatment in an animal PD model induced by 6-OHDA injection. While BBG treatment increased the TH immunoreactivity in the lesioned substantia nigra, such regenerative effects were not observed in the striatum. The absence of regeneration in the striatum under our experimental conditions differs from the results observed by Carmo and colleagues, who noted a prevention of TH^+ neuron deficit when animals were treated with BBG concomitantly with the 6-OHDA injection[11]. This might be due to the preventive characteristic of the protocol of Carmo and colleagues[11], since the lesion was not established before BBG administration and differs from the protocol presented here, where the dopaminergic deficit was completed before BBG administration.

Dopamine deficit in the PD striatum is preceded by a degeneration of these neurons in the substantia nigra, the structure where dopaminergic neurons are located and cast their projections to the striatum. Our results indicate that BBG treatment was sufficient to reverse the loss of dopaminergic neurons in the substantia nigra but not to restore the ramifications that continue until the striatum. In view of that, the moment of BBG effect evaluation following lesion induction becomes important for the extent of regeneration of dopaminergic neurons. It is worthwhile to emphasize that the conditions chosen in our present work better reflect the reality of PD in comparison with those of Carmo and colleagues[11], as treatment starts following degeneration of a majority of dopaminergic neurons.

Even without noting the recovery from dopaminergic deficits in the striatum, a decrease in the number of rotations in the apomorphine-induced rotational test was obtained. Apomorphine injection induces rotations in animals that have at least 90% dopamine depletion in one of the hemispheres[27]. Therefore, a complete reversal of dopaminergic degeneration is not required to observe improvements in the rotational test. In our study, BBG treatment for 7 days (50 mg/kg, IP) reduced the number of apomorphine-induced rotations by almost 70%. Carmo and colleagues[11] showed that BBG treatment at a dose of 45 mg/kg every 48 h for 14 days presented around 50% less rotations than the lesioned group,

corroborating our behavioral findings, even though their treatment protocol was started prior to lesion establishment. One possible mechanism that requires further study for the P2X7 antagonist repair of dopaminergic neurons may be based on mobilization of neural stem and progenitor cells from the nearby subventricular zone[28]. In agreement, pharmacological inhibition or gene expression silencing of P2X7R-promoted neurogenesis of stem cell lines in vitro[29,30] and functional P2X7R are expressed by adult NPCs in the subventricular zone[31]. Support for dopaminergic neurogenesis comes from the work of Albright et al.[32], who used a nestin$^+$ lineage tracing approach for proving adult neurogenesis for generating new dopaminergic neurons. Future studies may explore this hypothesis.

PGC-1α, a transcriptional coactivator of PPARγ, exerts neuroprotective functions by its anti-inflammatory and antioxidative characteristics. Thus, this protein might be an excellent target for PD treatment, which has in its pathophysiology these two components. However, our data show that the 0.2% fenofibrate-supplemented diet for 14 days was neither able to reverse hemiparkinsonian behavior nor restore dopaminergic levels in either the striatum or the substantia nigra. These data suggest that fenofibrate acts through anti-inflammatory effects rather than antioxidative, since fenofibrate and PPARγ agonists were discovered to be effective in the animal PD model using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which presents a more accentuated inflammatory element than with a 6-OHDA lesion, whose mechanism of action occurs mainly through energy imbalance[19,33]. Although fenofibrate per se does not affect all cell death pathways involved in PD, this compound could be studied for cotreatment with other antioxidant agents, thus covering these two mechanisms of cell death.

In summary, P2X7R inhibition following establishment of PD in an animal model partially reversed behavioral deficits, such as apomorphine-induced rotation, foreseeing possible therapeutic applications. Blood–brain barrier-permeable compounds, such as BBG, are especially suitable for such approaches.

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REFERENCES

1. Schwindt TT, Ferrazoli EG, Ulrich H. Parkinson's Disease: Genetics, mechanisms and diagnosis. In: Hajj GNM, editor. Young perspectives for old diseases. Sharjah (UAE): Bentham Science Publishers Ltd.; 2014. p. 155–76.
2. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: An overview. Trends Neurosci. 2009;32:19–29.
3. Rathbone MP, Middlemiss PJ, Gysbers JW, Andrew C, Herman MA Reed JK, Ciccarelli R, Di Iorio P, Caciagli F. Trophic effects of purines in neurons and glial cells. Prog Neurobiol. 1999;59:663–90.
4. Volonté C, Apolloni S, Skaper SD, Burnstock G. P2X7 receptors: Channels, pores and more. CNS Neurol Disord Drug Targets 2012;11:705–21.
5. Róna-Vörös K, Weydt P. The role of PGC-1α in the pathogenesis of neurodegenerative disorders. Curr Drug Targets 2010;11:1262–9.
6. Chen S, Ma Q, Krafft PR, Chen Y, Tang J, Zhang J, Zhang JH. P2X7 receptor antagonism inhibits p38 mitogen-activated protein kinase activation and ameliorates neuronal apoptosis after subarachnoid hemorrhage in rats. Crit Care Med. 2012;41:e466–74.
7. Nagasawa K, Miyaki J, Kido Y, Higashi Y, Nishida K, Fujimoto S. Possible involvement of PPAR gamma in the regulation of basal channel opening of P2X7 receptor in cultured mouse astrocytes. Life Sci. 2009;84(23–24):825–31.
8. Zheng B, Liao Z, Locascio JJ, Lesniak KA, Roderick SS, Watt ML, Eklund AC, Zhang-James Y, Kim PD, Hauser MA, Grünblatt E, Moran LB, Mandel SA, Riederer P, Miller RM, Federoff HJ, Wüllner U, Papapetropoulos S, Youdim MB, Cantuti-Castelvetri I, Young AB, Vance JM, Davis RL, Hedreen JC, Adler CH Beach TG, Graeber MB, Middleton FA, Rochet JC, Scherzer CR. PGC-1α, a potential therapeutic target for early intervention in Parkinson's disease. Sci Transl Med. 2010;2:52ra73.
9. Fuxe K, Manger P, Genedani S, Agnati L. The nigrostriatal DA pathway and Parkinson's disease. J Neural Transm. 2006;Suppl. 70:71–83.
10. Marcellino D, Suárez-Boomgaard D, Sánchez-Reina MD, Aguirre JA, Yoshitake T, Yoshitake S, Hagman B, Kehr J, Agnati LF, Fuxe K, Rivera A. On the role of P2X(7) receptors in dopamine nerve cell degeneration in a rat model of Parkinson's disease: Studies with the P2X(7) receptor antagonist A-438079. J Neural Transm. 2010;117:681–7.
11. Carmo MR, Menezes AP, Nunes AC, Pliássova A, Rolo AP, Palmeira CM, Cunha RA, Canas PM, Andrade GM. The P2X7 receptor antagonist Brilliant Blue G attenuates contralateral rotations in a rat model of Parkinsonism through a combined control of synaptotoxicity, neurotoxicity and gliosis. Neuropharmacology 2014;81:142–52.
12. Annett LE, Rogers DC, Hernandez TD, Dunnett SB. Behavioural analysis of unilateral monoamine depletion in the marmoset. Brain 1992;115:825–56.
13. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 7th ed. San Diego (CA): Academic Press; 2014.
14. Ebert AD, McMillan EL, Svendsen CN. Isolating, Expanding, and infecting human and rodent fetal neural progenitor cells. Curr Protoc Stem Cell Biol. 2008;6:2D.2.1–2D.2.16.
15. Arbeloa J, Pérez-Samartín A, Gottlieb M, Matute C. P2X7 receptor blockade prevents ATP excitotoxicity in neurons and reduces brain damage after ischemia. Neurobiol Dis. 2012;45:954–61.

16. Diaz-Hernandez M, Diez-Zaera M, Sanchez-Nogueiro J, Gomez-Villafuertes R, Canals JM, Alberch J, Miras-Portugal MT, Lucas JJ. Altered P2X7-receptor level and function in mouse models of Huntington's disease and therapeutic efficacy of antagonist administration. FASEB J. 2009;23:1893–906.
17. Jiang LH, Mackenzie AB, North RA, Surprenant A. Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors. Mol Pharmacol. 2000;58(1):82–8.
18. Ryu JK, McLarnon JG. Block of purinergic P2X(7) receptor is neuroprotective in an animal model of Alzheimer's disease. Neuroreport 2008;19:1715–9.
19. Kreisler A, Duhamel A, Vanbesien-Mailliot C, Destée A, Bordet R. Differing short-term neuroprotective effects of the fibrates fenofibrate and bezafibrate in MPTP and 6-OHDA experimental models of Parkinson's disease. Behav Pharmacol. 2010;21(3):194–205.
20. Tewari M, Seth P. Emerging role of P2X7 receptors in CNS health and disease. Ageing Res Rev. 2015;24(Pt B):328–42.
21. Simola N, Morelli M, Carta AR. The 6-hydroxydopamine model of Parkinson's disease. Neurotox Res. 2007;11(3–4): 151–67.
22. Di Virgilio F. The therapeutic potential of modifying inflammasomes and NOD-like receptors. Pharmacol Rev. 2013;65:872–905.
23. Hewinson J, Mackenzie AB. P2X(7) receptor-mediated reactive oxygen and nitrogen species formation: From receptor to generators. Biochem Soc Trans. 2007;35(Pt 5):1168–70.
24. Miller CM, Boulter NR, Fuller SJ, Zakrzewski AM, Lees MP, Saunders BM, Wiley JS, Smith NC. The role of the $P2X_7$ receptor in infectious diseases. PLoS Pathog. 2011;7(11):e1002212.
25. Muñoz-Planillo R, Kuffa P, Martínez-Colón G, Smith BL, Rajendiran TM, Núñez G. K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 2013;38:1142–53.
26. Hanisch UK. Microglia as a source and target of cytokines. Glia 2002;40:140–55.
27. Ungerstedt U. Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. Acta Physiol Scand. 1971;Suppl. 367: 69–93.
28. van den Berge SA, van Strien ME, Hol EM. Resident adult neural stem cells in Parkinson's disease—The brain's own repair system? Eur J Pharmacol. 2013;719:117–27.
29. Glaser T, de Oliveira SL, Cheffer A, Beco R, Martins P, Fornazari M, Lameu C, Junior HM, Coutinho-Silva R, Ulrich H. Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. PLoS One 2014; 9(5):e96281.
30. Yuahasi KK, Demasi MA, Tamajusuku AS, Lenz G, Sogayar MC, Fornazari M, Lameu C, Nascimento IC, Glaser T, Schwindt TT, Negraes PD, Ulrich H. Regulation of neurogenesis and gliogenesis of retinoic acid-induced P19 embryonal carcinoma cells by P2X2 and P2X7 receptors studied by RNA interference. Int J Dev Neurosci. 2012;30:91–7.
31. Messemer N, Kunert C, Grohmann M, Sobottka H, Nieber K, Zimmermann H, Franke H, Nörenberg W, Straub I, Schaefer M, Riedel T, Illes P, Rubini P. P2X7 receptors at adult neural progenitor cells of the mouse subventricular zone. Neuropharmacology 2013;73:122–37.
32. Albright JE, Stojkovska I, Rahman AA, Brown CJ, Morrison BE. Nestin-positive/SOX2-negative cells mediate adult neurogenesis of nigral dopaminergic neurons in mice. Neurosci Lett. 2016;615:50–4.
33. Lecca D, Nevin DK, Mulas G, Casu MA, Diana A, Rossi D, Sacchetti G, Carta AR. Neuroprotective and anti-inflammatory properties of a novel non-thiazolidinedione PPARγ agonist *in vitro* and in MPTP-treated mice. Neurosci. 2015;302: 23–35.

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REVIEW
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Purinergic Receptors in Neurological Diseases With Motor Symptoms: Targets for Therapy

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Since proving adenosine triphosphate (ATP) functions as a neurotransmitter in neuron/glia interactions, the purinergic system has been more intensely studied within the scope of the central nervous system. In neurological disorders with associated motor symptoms, including Parkinson's disease (PD), motor neuron diseases (MND), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Huntington's Disease (HD), restless leg syndrome (RLS), and ataxias, alterations in purinergic receptor expression and activity have been noted, indicating a potential role for this system in disease etiology and progression. In neurodegenerative conditions, neural cell death provokes extensive ATP release and alters calcium signaling through purinergic receptor modulation. Consequently, neuroinflammatory responses, excitotoxicity and apoptosis are directly or indirectly induced. This review analyzes currently available data, which suggests involvement of the purinergic system in neuro-associated motor dysfunctions and underlying mechanisms. Possible targets for pharmacological interventions are also discussed.

Keywords: Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, neurodegeneration, ataxia, Huntington's disease, restless leg syndrome, purinergic receptors

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INTRODUCTION

The unexpected discovery and description of non-adrenergic and non-cholinergic inhibitory nerves working through adenosine triphosphate (ATP) and its metabolites gave rise to the introduction of the purinergic system concept in the early 70's (Burnstock et al., 1970; Burnstock, 1972). Later, purines were also described as important co-transmitters in both central (CNS) and peripheral nervous systems, as they are able to modulate and be modulated by many other neurotransmission systems and signaling pathways (Burnstock, 1997, 2009; Abbracchio et al., 2009).

After proposal of purinergic neurotransmission, the following decades were dedicated to the isolation and characterization of the two families of purinergic receptors, which are distinguished by their main agonists: P1 receptors, a family of protein G-coupled metabotropic adenosine (A_1, $_{2A}A_{2A}$, A_{2B}, A_3) receptors, and P2 receptors. P2 receptors are sub-divided into P2X(1–7) channels, activated by ATP, and G protein-coupled metabotropic P2Y(1–12) receptors, which show sensitivity to ATP, adenosine diphosphate (ADP), uridine di- and triphosphate (UDP and UTP, respectively), or UDP-glucose depending on the receptor subtype. Beyond

receptors, membrane nucleotide/nucleoside transporters and channels (e.g., pannexins) as well as ectonucleotidases play important roles in purinergic signaling. These are responsible for the exchange of purines between intracellular and extracellular environments and their enzymatic extracellular conversion, respectively (Zimmermann et al., 1998; Zimmermann, 2006; Scemes et al., 2007; Abbracchio et al., 2009; Lapato and Tiwari-Woodruff, 2017).

P2X receptors are ion channels that promote a non-selective exchange of cations, mainly Ca^{2+}, Na^{+}, Mg^{2+}, and K^{+}. ATP-activation of P2X receptors is especially important for Ca^{2+}-induced intracellular signaling pathways (Surprenant and North, 2009; Puchałowicz et al., 2015). P2Y and adenosine receptors are coupled to Gq/Gi/Gs proteins, depending on the receptor subtype (Puchałowicz et al., 2015). The activation of Gq proteins triggers a signaling cascade through phospholipase C/inositol-1,4,5-triphosphate (PLC/IP3), resulting in the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm. Gs/Gi protein activation, however, will work through the stimulation/inhibition of adenylate cyclase, respectively, with subsequent up- or down-regulation of cyclic AMP (cAMP) production. Final effects of purinergic receptor-promoted signaling will depend on the cell type and other intra-/inter-cellular conditions, as i.e., in physiological embryonic and adult neurogenesis (Oliveira et al., 2016), and in various pathological scenarios, such as inflammatory (Beamer et al., 2016; Madeira et al., 2017; Przybyła et al., 2018), oncological (Allard et al., 2016; Vijayan et al., 2017; Whiteside, 2017; Kazemi et al., 2018), neurological (Burnstock et al., 2011; Stockwell et al., 2017), metabolic (Lindberg et al., 2015; Csóka et al., 2017; Parpura et al., 2017; Tozzi and Novak, 2017; Labazi et al., 2018), psychiatric (Cunha, 2008; Lindberg et al., 2015; Ortiz et al., 2015; Krügel, 2016; Cheffer et al., 2017; Oliveros et al., 2017), cognitive (Illes and Verkhratsky, 2016), and peripheral neuromuscular and/or neuromotor diseases (Robitaille, 1995; Kalmar, 2005; Burnstock et al., 2013; Jiménez et al., 2014; Bogacheva and Balezina, 2015; Puchałowicz et al., 2015; Safarzadeh et al., 2016).

In the CNS, extracellular nucleotides also participate as messengers for communication between neuronal and non-neuronal cells. As key players in neuron-glia interactions and microglial activation (Fields and Burnstock, 2006; Cunha, 2008, 2016; Färber et al., 2008; Boison et al., 2010; Lecca et al., 2012; Tsuda and Inoue, 2016; Inoue, 2017; Tsuda, 2017), both adenosine and ATP are essential modulators of neuroinflammatory responses, excitotoxicity, oxidative stress and cell death, especially via A_{2A} and P2X7 receptors activity, respectively (Cunha, 2016; Borea et al., 2017; Faas et al., 2017; Faria et al., 2017; He et al., 2017; Lu et al., 2017; Miras-Portugal et al., 2017; Vuorimaa et al., 2017). Differently from other P2X receptors, the P2X7 receptor subtype needs higher ATP concentrations for channel opening and Ca^{2+} influx and remains longer activated, recruiting pannexin pores (Volont et al., 2012; Sun et al., 2013). Through pannexin pores, large amounts of ATP are released into the extracellular environment, stimulating other purinergic receptors, and signaling cascades widely associated with pathological conditions (Bartlett et al., 2014), such as the A_{2A} receptor, which is activated by adenosine released from damaged cells or produced from ATP hydrolysis (Cunha, 2016).

Here, we explore the importance of purinergic signaling in neurological diseases with motor symptoms, including Parkinson's disease (PD), motor neuron diseases (MND), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Huntington's Disease (HD), restless leg syndrome (RLS), and ataxias. We discuss common mechanisms already known to be involved in these conditions (**Table 1**), revise the role of the purinergic system in demyelination processes (**Figure 1**), and discuss new insights for further neural pathologies that might have motor impairments to identify potential targets for pharmacological therapies to decelerate disease progression and improve motor activity.

MOTOR NEURON DISEASES

Motor neurons (MNs) are classified in different categories according to their soma location, electrical speed transmission, and other cellular and physiological characteristics. Regarding the location of their somas, MNs can be classified as upper or lower MNs. Lower MNs have their soma located either in the brainstem—where they control head and neck muscle contraction through cranial nerves—and in the anterior horn of the spinal cord—where their axons innervate and control skeletal muscle contraction through spinal nerves. Upper MNs have somas located in the primary motor cortex and axons that project either to the brainstem or to the spinal cord through corticobulbar and corticospinal tracts, respectively. Upper MNs axons in the brainstem interact with lower MNs, regulating their control of head and neck contraction, while upper MNs project to the spinal cord synapse with lower MNs that innervate skeletal muscles, controlling their contraction (Rezania and Roos, 2013; Verschueren, 2017).

MND are neurodegenerative conditions that affect MNs and result in motor dysfunctions without compromising sensorial neurons. MND are classified according to the damage location in relation to the spinal cord. Diseases affecting lower MNs and upper MNs are known as lower MNDs and upper MNDs, respectively. Lower MNDs include progressive muscular atrophy, spinal muscular atrophy (SMA), spinal and bulbar muscular atrophy, and monomelic amyotrophy (Hirayama disease). However, the most prevalent subtype of MND is ALS,

Abbreviations: 6-OHDA, 6-hydroxydopamine; ALS, amyotrophic lateral sclerosis; ATP, ADP, AMP, adenosine tri-, di-, monophosphate; BBG, Brilliant Blue G; BDNF, brain-derived neurotrophic factor; BzATP, 2′-3′-O-(benzoyl-benzoyl) ATP; cAMP, cyclic AMP; CNS, central nervous system; COX2, cyclooxigenase 2; EAE, encephalomyelitis; HD, Huntington Disease; Htt, huntingtin protein; IL-1β/2/6/17A/23, Interleukin 1β/2/6/17A/23; iPSC, induced pluripotent stem cells; L-DOPA, L-3,4-dihydroxyphenylalanine; LPS, lipopolysaccharide; MAPK/ERK, mitogen activated protein kinases/extracellular signal-regulated kinases signaling induction pathway; MNs, motor neurons; MND, motor neuron diseases; MPP+, 1-methyl-4-phenylpyridinium; MS, multiple sclerosis; NOX2, nicotinamide adenine dinucleotide phosphate-oxidase 2; PBMC, peripheral blood mononuclear cells; PD, Parkinson's disease; QA, quinolinic acid; ROS, Reactive oxygen species; RLS, Restless leg syndrome; SCA, Spinocerebellar ataxia; SMA, spinal muscular atrophy; SNc, substantia nigra pars compacta; SOD1 (G93A), superoxide dismutase 1 (glycin-93 to serine)-mutant mouse; TNF-α, tumor necrosis factor alpha; TrkB, tropomyosin kinase receptor b; UTP, UDP, uridine tri-, diphosphate.

TABLE 1 | Evidence of purinergic receptors involvement in neurological diseases with major motor dysfunctions.

Disease	Purinergic involvement	Model/Sample	Drug	Effects	References
ALS	P2X4 receptor positive activity modulation	MNs culture/SOD1 (G93A) mice	Preincubation with Ivermectin (10 mM); Ivermectin 12 mg per liter of water during 70 days	Neuroprotective against glutamate-induced excitotoxicity; Improves lifespan and increases ventral horn MNs numbers	Andries et al., 2007
	P2X4 receptor	SOD1 (G93A) rats	–	Strong immunoreactivity in the ventral horns	Casanovas et al., 2008
	P2X7 receptor activation	Microglial cells derived from transgenic SOD1 (G93A) mice;	BzATP (10 and 100 μM)	Increase in NOX2 activity and ROS synthesis	Apolloni et al., 2013b
			BzATP (10 μM)	Transition from microglial M2 to M1 activated phenotype, increased TNF-α production and COX2 activation	D'Ambrosi et al., 2009
		Microglia/Neuron co-culture	BzATP (10 μM)	Cell death due to ROS and NOS production	Skaper et al., 2006; D'Ambrosi et al., 2009
		Cultured rat spinal cord MNs	ATP (1–100 μM)	MNs cell death through peroxinitrite/Fas death pathway	Gandelman et al., 2013
		MNs co-cultured with SOD1G93 astrocytes	ATP (100 μM, 5 days) or BzATP (10 μM, 48 h)	Astrocytes become neurotoxic for MNs through increased oxidative stress	Gandelman et al., 2010
	P2X7 receptor deletion	$P2X7^{(-/-)}$/SOD1-G93A mice	–	Accelerates disease onset and progression, increased pro-inflammatory markers as well as astrogliosis, microgliosis, and MNs cell death	Apolloni et al., 2013a
	A_{2A} receptor antagonism	SOD1 (G93A) mice	Caffeine, 1.5 mg/day for 70 days, in drinking water	Shortened mice survival	Potenza et al., 2013
		Rat spinal cord cells culture	Chronic enprofylline treatment	Decreased MNs susceptibility to excitotoxic environment through inhibition of BDNF-promoted death pathway	Mojsilovic-Petrovic et al., 2006
	A_{2A} receptor expression or levels	SOD1 G93A mice	–	Decreased expression in spinal cord	Potenza et al., 2013
		SOD1 G93A mice and end-stage humans with ALS	–	Increased expression in spinal cord of symptomatic mice and patients	Ng et al., 2015
		ALS patient lymphocytes	–	Increased density in lymphocytes, positively related with clinical status of patients	Vincenzi et al., 2013
	A_{2A} receptor activation	SOD1 G93A mice	CGS21680, 5 mg/kg, i.p., during 4 weeks	Delays ALS onset possibly by stimulating non-truncated forms of the TrkB receptor	Yanpallewar et al., 2012
		NSC34 cells	T1–11 (30 μM)	Normalized abnormal cellular redistribution of human antigen R, found in MNs of ALS patients	Liu et al., 2015
	A_1 and A_{2A} receptors	SOD1 G93A mice presymptomatic (4–6 weeks old) symptomatic (12–14 weeks old)	N(6)-cyclopentyladenosine (50 nM); CGS 21680 (5 nM)	Impaired cross-talk between receptors in presymptomatic mice, increased A_1 receptor activation in symptomatic mice	Nascimento et al., 2015

(Continued)

TABLE 1 | Continued

Disease	Purinergic involvement	Model/Sample	Drug	Effects	References
Spinal muscular atrophy	ATP response	iPSC-derived astrocyte culture from SMA patients	ATP (10 μM)	Increased basal intracellular calcium levels accompanied by a reduced calcium response to ATP application	McGivern et al., 2013
Multiple sclerosis	P2X7 receptor expression and protein levels	Cultured PBMC from MS patients	Glatiramer acetate (50 μg/ml, 48 h) BzATP (300 μM, 30 min)	Glatiramer acetate, used to treat MS patients, reduced P2X7 receptor expression in BzATP-stimulated cells	Caragnano et al., 2012
		MS patients' spinal cords	–	Increased P2X7 receptor protein levels in microglia	Yiangou et al., 2006
		EAE rat brains	–	Increased P2X7 receptor expression related to synaptosomal fraction in the symptomatic phase and to the glial fraction in recovered rat brains	Grygorowicz et al., 2011
	P2X7 receptor polymorphisms	MS patients	–	Patients with T allele of rs17525809 polymorphism present a more prominent activity, which may contribute to MS development	Oyanguren-Desez et al., 2011
	P2X7 receptor deletion	P2X7R$^{-/-}$ EAE mice model	–	Enhanced mouse susceptibility to EAE	Chen and Brosnan, 2006
				Suppressed clinical symptoms in EAE mice	Sharp et al., 2008
	P2X7 receptor antagonism	EAE mouse model	BBG (10 mg/kg daily, delivered from pellets, during 20 days)	Antagonism improved symptoms and promoted remyelination	Matute et al., 2007
	P2Y12 receptor levels	MS patients cortical tissue	–	Reduced protein levels near demyelination areas	Amadio et al., 2010
	P2Y12 receptor deletion	P2Y12 knockout EAE mice	–	Mice developed more severe EAE related to higher release of IL-23 cytokines and imbalanced Th-cell subtype frequencies	Zhang et al., 2017
	A_{2A} receptor antagonism	MS patients	Coffee consumption exceeding 900 mL daily	Reduced MS risk in comparison to control group	Hedström et al., 2016
	A_1 receptor deletion	A1AR$^{-/-}$ EAE mice		Induced severe EAE, with more prominent demyelination, axonal injury, and microglia activation	Tsutsui et al., 2004
	A_1 receptor activation	A1AR$^{-/-}$ EAE mice	Caffeine (2 mg/kg) + adenosine amine congener (10 μg/kg), subcutaneous pump, during 25 days	Reduced EAE severity induced by A_1 receptor expression deletion	Tsutsui et al., 2004
		Cultured PBMC from MS patients	R-phenylisopropyl-adenosine (1 mM)	Inhibited IL-6 production	Mayne et al., 1999
Parkinson's disease	P2X1 receptor antagonism	H4 cells overexpressing α-synuclein	Pre-treatment with NF449 (1–5 μM) followed by 48 h treatment with ATP (3 mM, every hour)	Prevented ATP-induced α-synuclein aggregation in a dose dependent manner	Gan et al., 2015
	P2X7 receptor antagonism	6-OHDA lesioned rats	A-438079 (30 mg/kg, i.p., before lesion establishment)	Prevented depletion of dopamine in striatum without reducing dopaminergic neuron cell death	Marcellino et al., 2010

(Continued)

TABLE 1 | Continued

Disease	Purinergic involvement	Model/Sample	Drug	Effects	References
			BBG (45 mg/kg, i.p., every 48 h during 2 weeks, before lesion establishment)	Prevented loss of tyrosine-hydroxylase immunoreactivity and attenuated rotational behavior and memory deficit	Carmo et al., 2014
			BBG (50 mg/kg, i.p., daily, during 1 week, after lesion establishment)	Reverted dopaminergic neurons loss in substantia nigra and rotational behavior	Ferrazoli et al., 2017
		BV2 microglia cells	Pretreatment with BBG (1 μM)	Antagonism and/or deletion of P2X7 receptor blocked the interaction between α-synuclein and P2X7 receptors and decreased ROS production induced by α-synuclein	Jiang et al., 2015
		SH-SY5Y cells	Pretreatment with PPADS (100 μM) or AZ 11645373 (10 μM)	Prevented abnormal calcium influx induced by α-synuclein	Wilkaniec et al., 2017
	P2X7 receptor polymorphism	PBMC from PD patients	–	1513A>C (rs3751143) polymorphism increased PD risk by facilitating pore formation and cell death	Liu et al., 2013
	P2Y6 receptor antagonism	SH-SY5Y cells	Pretreatment with MRS2578 (1.0 μM)	Decreased ROS production and other inflammatory markers induced by MPP+	Qian et al., 2017
	A_{2A} receptor antagonism	6-OHDA lesioned rats	8-ethoxy-9-ethyladenine (8 mg/kg, daily, during 28 days, minipumps)	Enhanced effect of low doses of L-DOPA without increased dyskinesia	Fuzzati-Armentero et al., 2015
		MPTP treated monkeys	KW-6002 (10.0 mg/kg, orally)	Increased effect of D2 receptor agonist quinpirole, D1 receptor agonist SKF80723 and low doses of L-DOPA without increased dyskinesia	Kanda et al., 2000
		PD patients with PD gene risk variant *LRRK2* R1628P	Caffeine intake through coffee and tea consumption	Decreased PD risk in subjects with LRRK2 variant R1628P	Kumar et al., 2015
		PD patients with GRIN2A variant rs4998386-T allele	Caffeine intake through coffee consumption	Increased protective effect of GRIN2A variant rs4998386-T allele	Hamza et al., 2011; Yamada-Fowler et al., 2014
		A_{2A} receptor knockout mice, SH-SY5Y cells	SCH 58261, ZM 241385	Decreased α-synuclein aggregation, prevent neuronal death induced by extracellular α-synuclein and restrain overactivation of NMDA receptors	Ferreira et al., 2015
		Brain slices from mice treated with MPTP	Preladenant (5 μM)	Facilitated beneficial microglial responses to injury	Gyoneva et al., 2014
		Rats treated with LPS	Caffeine 10 and 20 mg/kg; KW6002 1.5 and 3 mg/kg; i.p. for 6 days	Prevented striatal dopaminergic deficit and hydroxyl radicals release	Gołembiowska et al., 2013
	A_{2A} receptor number	Mice injected with α-Syn fibrils	–	Hippocampal A_{2A} receptors number increased after injections of α-synuclein in mice	Hu et al., 2016
	A_{2A} receptor polymorphisms	PD patients	–	rs3032740 and rs5996696 polymorphisms are inversely linked to PD risk	Popat et al., 2011

(Continued)

TABLE 1 | Continued

Disease	Purinergic involvement	Model/Sample	Drug	Effects	References
Huntington's disease	P2X7 receptor antagonist	Tet/HD94 and R6/1	BBG (45.5 mg/kg, i.p., every 48 h during 28 days)	Reduce body weight loss, improve motor functions, and prevent neuronal loss	Diaz-Hernandez et al., 2009
	A_1 receptor agonist	3-NPA mouse and rat model	Pre-treatment of R-PIA (1.75 mg/kg, i.p.) 15 min prior 3-NPA application	Reduction of seizure but not prevention of neuronal loss	Zuchora and Urbańska, 2001
		3-NPA rat model	ADAC (100 μg/kg, i.p., daily for 2 days) 3 days after 3-NPA	Reduction in striatal lesion and degeneration, improvement of motor functions	Blum et al., 2002
	A_1 receptor antagonist	Intracraneal application malonate 6 μmol in Swiss-Webster mice and 3 μmol Sprague Dawley rats	Pre-treatment with CPX 1 mg/kg, i.p.	Stimulate DAergic and GABAergic neuron death	Alfinito et al., 2003
	A_{2A} receptor polymorphisms	1876 C/T		Silent mutation in A_{2A} receptor	Dhaenens et al., 2009
		1876 T/T		Accelerates HD onset by 3.5 years	
		rs2298383		Early onset of HD	Taherzadeh-Fard et al., 2010
	A_{2A} receptor antagonist	Intracranial application malonate 6 μmol in Swiss-Webster mice and 3 μmol Sprague Dawley rats	Pre-treatment with DMPX 5 mg/kg, i.p.	Provided protection to DAergic and GABAergic cells against malonate	Alfinito et al., 2003
		Human	<190 mg/day caffeine	Accelerates HD onset.	Simonin et al., 2013
		3-NPA mouse model	8-(3-chlorostyryl) caffeine (5 mg/kg and 20 mg/kg, i.p.) 2x day for 5 days prior 3-NPA application	Reduction in striatal damage	Fink et al., 2004
		R6/2 mice	SCH58261 (0.01 mg/kg, i.p.)	Reduction in striatal BDNF levels at earlier HD stage	Potenza et al., 2007
			SCH58261 (50 nM): microdialysis application in striatum)	Reduction of glutamate and adenosine level	Gianfriddo et al., 2004
			Application of SCH58261 (0.01 mg/kg, i.p.) daily for 7 days at age of 5 weeks	Reduced NMDA-induced toxicity and emotional responses	Domenici et al., 2007
		Corticostriatal slices from R6/2 mice	ZM241385 (100 nM)	Prevention of BDNF positive effect on NMDA toxicity	Martire et al., 2010
		ST14/SQ120 cells			
		Primary rat striatal culture	Pre-treatment with SCH 58261 (30 nM) prior bath application QA 900 μM	Enhanced QA-induced increase in intracellular calcium concentration	Popoli et al., 2002
		QA rat model	Pre-treatment with SCH 58261 (0.01 mg/kg, i.p.) prior to QA application	Blocked the effect of QA on striatal gliosis, EEG changes, motor activity and glutamate levels	Popoli et al., 2002
			DMPX (0.2 μg, i.p.) application 5 min after QA application	Blocked QA-induced EEG abnormalities in frontal cortex	Reggio et al., 1999

(Continued)

TABLE 1 | Continued

Disease	Purinergic involvement	Model/Sample	Drug	Effects	References
			Pre-treatment with SCH58261 (0.01 mg/kg, i.p.) 20 min before QA application	Reduction in rearing behavior and anxiety levels	Scattoni et al., 2007
			SCH58261 (0.01 and 1 mg/kg, i.p.) daily for 1 or 3 weeks	Reduction in striatal BDNF levels	Potenza et al., 2007
		Transgenic HD rat model	KW-6002 (1 and 3 mg/kg, i.p.)	No beneficial locomotor activity at 6 and 12 month age	Orrú et al., 2011
			SCH 442416 (0.3 and 1 mg/kg, i.p.)	No significant effect in reducing electromyography responses	
	A_{2A} receptor agonist	Primary rat striatal culture	Pre-treatment with CGS21680 (100 nM.) prior bath application QA 900 μM	Reduced QA-induced increase in intracellular calcium concentration	Popoli et al., 2002
		Corticostriatal slices from R6/2 mice	CGS21680 (30 nM)	Beneficial effect against NDMA-induced toxicity	Ferrante et al., 2010
		R6/2 mice	CGS21680 (5 μg/kg, i.p.) daily for 2 weeks	Delay decline in motor performance and inhibit reduction in brain weight	Chou et al., 2005
			CGS21680 (0.5 mg/kg, i.p.) daily for 3 weeks	Brain region dependent alteration in NMDA glutamate receptor subunits density	Ferrante et al., 2010
			CGS21680 (0.5 mg/kg, i.p.)	No changes in behavior compared to wild type	Martire et al., 2007
		Corticostriatal slices from R6/2 mice	CGS21680 (5 μg/kg, i.p.) daily for 2 weeks	Brain region dependent alteration in NMDA subunits	Ferrante et al., 2010
	A_{2A} receptor knockout	N171-82Q mouse model	–	Aggravate survival and motor functions and decrease in specific markers for sub-population medium spiny neurons	Mievis et al., 2011
		3-NPA mouse model	A_{2A} receptor knockout mice treated with 3-NPA	Reduction in striatal damage	Fink et al., 2004
Ataxia	A_{2A} receptor antagonism	SCA3 mice model	Caffeine (1 g/L, drinking water during 2 weeks)	Decreased synaptotoxicity and reactive gliosis	Gonçalves et al., 2013
		(TgMJD) mice	Caffeine (1 g/L, drinking water during 2 weeks)	Prevented motor symptoms and cognitive impairment	Gonçalves et al., 2013
	P2X receptors	CHO-K1 cells with mutant PKCγ	ATP (1 mM)	Increased damaging aggregation of mutant PKCγ	Seki et al., 2005
Restless leg syndrome	A_{2A} receptor	Iron deficient mice	–	Increased in striatal presynaptic neurons	Gulyani et al., 2009
	A_1 and A_{2A} receptors	Iron deficient mice	–	Decreased A_1 and D2 receptor density in animals with mild, moderate and severe deficiency; increased pre-synaptic A_{2A} receptor density in the latter	Quiroz et al., 2016

in which both upper and lower MNs are affected and where non-neuronal cells as microglia and astrocytes play a central role in its pathogenesis and progression (Rezania and Roos, 2013; Verschueren, 2017).

Amyotrophic Lateral Sclerosis

ALS is the main motor disorder in adulthood. It is characterized by a progressive loss of MNs from the motor cortex, brainstem, and spinal cord (Kiernan et al., 2011). As a result of this neuronal

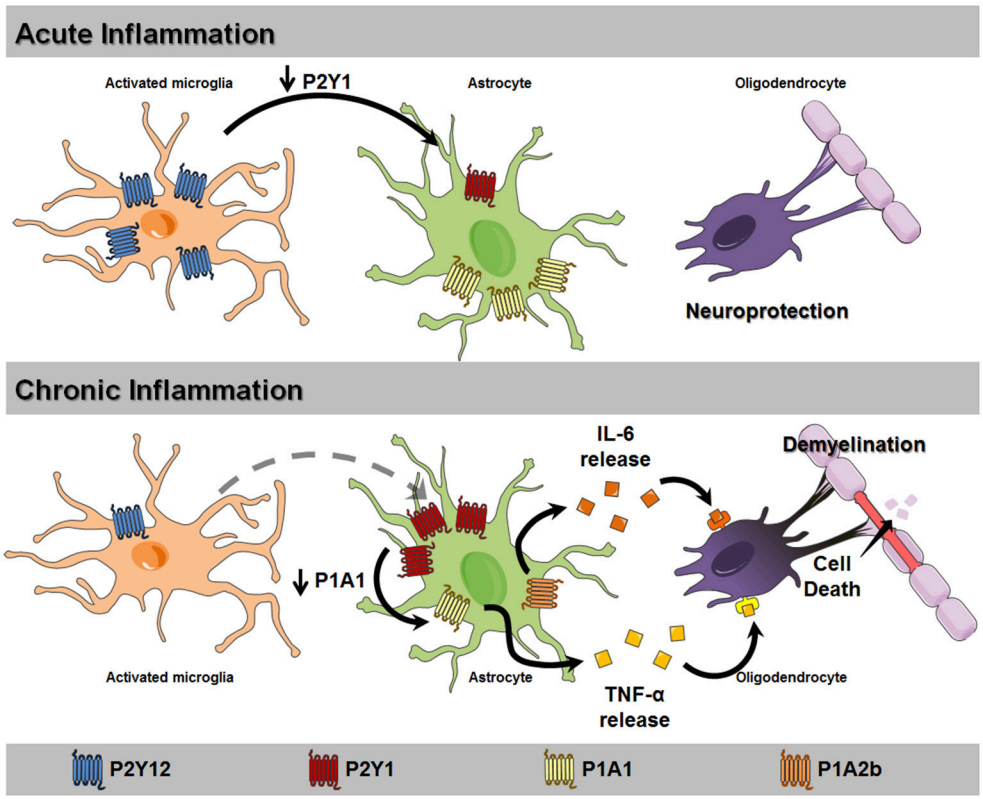

FIGURE 1 | Proposed mechanism for glial purinergic dysfunction leading to loss of myelination and cell death. In acute inflammation scenarios, microglial activation upregulates P2Y12 receptor expression and activity (blue), stimulating microglial motility to the injury site. The activation of these receptors reduces P2Y1 receptor (red) expression in astrocytes, increasing reactive astrogliosis and promoting neuroprotection. Chronic inflammation, as observed in motor neuron diseases (MND), unable of upregulating microglial P2Y12 receptor expression results in constant astrocytic P2Y1 receptor activation and reduction of A_1 receptor expression (yellow). These events result in stimulating tumor necrosis factor α (TNF-α) release, which in turn induces A_{2B} receptor activation (orange) and release of IL-6. These detrimental factors induce oligodendrocyte death and neuron demyelination, aggravating the pathological scenario.

loss, muscle weakness, spasticity, and muscle atrophy occur, inducing progressive paralysis. ALS is a very aggressive pathology that usually evolves in a fast-progressive way. Patients have a lifespan of 2–5 years after diagnosis. Death is frequently due to breathing failure. Most of ALS cases (90%) are sporadic, while a small proportion (10%) is linked to genetic mutations that usually follow an autosomal dominant transmission (Harms and Baloh, 2013; Renton et al., 2013). Cognitive impairment is also associated with ALS. In fact, 30% of ALS cases develop frontotemporal dementia (Lomen-Hoerth, 2011). The C9ORF72 mutation is responsible for the main part of ALS and frontotemporal dementia inherited cases (DeJesus-Hernandez et al., 2011).

The cause of and reasons for MNs death are still unknown. Particularly, it is still not known why this specific neuronal population is affected. However, intense research performed throughout the last two decades has uncovered several hallmarks and molecular mechanisms involved in ALS neurodegeneration. Among them, neuroinflammation, which is understood to be a maintained immune system response in the CNS, plays a central role in the pathogenesis of ALS. This response includes astrocytic and microglial activation and lymphocyte infiltration (Barbeito et al., 2010). There is strong evidence for a compromised energetic metabolism in ALS. Several genes involved in the mitochondrial electron transport chain are altered in their mRNA expression levels (Ferraiuolo et al., 2007, 2011; Lederer et al., 2007; Raman et al., 2015). Further, numerous studies reported structural and functional abnormalities in mitochondria, resulting in increased reactive oxygen species (ROS) and decreased ATP production (Jung et al., 2002; Mattiazzi et al., 2002; Menzies et al., 2002; Wiedemann et al., 2002; Browne et al., 2006), with supposed impacts on purinergic signaling. Here, we will discuss the contribution of purinergic signaling in ALS etiology.

Purinergic Involvement in ALS

P2X receptors

ATP mediates intercellular communication by acting as a messenger between neurons and glia via activation of several purinergic P2 receptors. The involvement of purinergic receptors in ALS has been documented, such as the P2X4 receptor subtype, which is implicated in neuroprotection (Andries et al., 2007) and microglial activation (Tsuda et al., 2003). Positive allosteric modulation of P2X4 receptor activity with Ivermectin

and pre-incubation with low ATP concentration has shown to induce neuroprotection against glutamate-induced excitotoxicity in MNs cultures, a phenotype observed in several ALS models. Allosteric P2X4 receptor activation also improved the lifespan of superoxide dismutase 1 (SOD1) transgenic mice harboring the G92A mutation (Gly-93 to Ala)—a conventional animal model of ALS—by 10% and increased the number of ventral horn MNs in the spinal cord (Andries et al., 2007). Ventral horns are the main neurodegenerative regions affected in ALS. These findings indicate that purinergic receptors modulate excitability, exerting neuroprotection in ALS (Miles et al., 2002). However, it has been described that the allosteric P2X4 receptor activator Ivermectin acts on AMPA receptors inhibiting glutamate excitotoxicity, which could be also responsible for these observed beneficial effects.

Interestingly, the P2X4 receptor has been suggested as a novel marker for non-typical apoptotic and degenerating MNs both in the spinal cord and in other degenerated areas, which had not been previously linked to ALS. P2X4 receptor-immunoreactivity was enhanced in the ventral horns of SOD1 (G93A) transgenic rats. These P2X4 receptor-positive cells were surrounded by microglia with a neuronophagic phenotype (Casanovas et al., 2008). Moreover, Tsuda et al. proved that the P2X4 receptor is expressed selectively in activated microglia after neural injury in the spinal cord and that this expression is required for neuropathic pain (Tsuda et al., 2003). The same study showed that pharmacological inhibition of P2X4 receptors induced a reduction in neuropathic pain, indicating a direct relationship between P2X4 receptor activation and microglial reactivity (Tsuda et al., 2003). Further studies regarding the role of P2X4 receptors in activated and resting microglia are needed for elucidating the participation of the P2X4 receptor in ALS etiology and progression.

The P2X7 receptor is expressed in microglia (Ferrari et al., 1996), spinal cord neurons (Deuchars et al., 2001; Wang et al., 2004), astrocytes (Ballerini et al., 1996), and oligodendrocytes (Matute et al., 2007). Activated microglia from the dorsolateral white matter in the spinal cord of sporadic ALS patients presented increased P2X7 receptor immunoreactivity (Yiangou et al., 2006). This receptor has been tightly linked to neuroinflammation. *In vitro* studies also showed increased densities of P2X7 and P2X4 receptors, upregulation of P2Y6 receptor expression, and decreased ectonucleotidase CD39 hydrolytic activity in transgenic mice SOD1 (G93A)-derived microglia, all indicating a potentiation of the purinergic system in ALS. In fact, SOD1 (G93A) microglia treated with ATP or 2′-3′-O-(benzoyl-benzoyl) ATP (BzATP), a potent P2X7 receptor agonist, presented a prominent transition from the microglial M2 to the M1 activated phenotype, accompanied by augmented production of tumor necrosis factor alpha (TNF-α) and cycloxygenase 2 (COX2) (D'Ambrosi et al., 2009). BzATP treatment of SOD1 (G93A) microglia also increased the presence of inflammatory markers, such as nicotinamide adenine dinucleotide phosphate-oxidase 2 (NOX2) activity and ROS production, indicating damaging effects resulting from P2X7 receptor activation (Apolloni et al., 2013b). As expected, P2X7 receptor activation in microglia-neuronal co-culture induced cell death by ROS and reactive nitrogen species generation (Skaper et al., 2006; D'Ambrosi et al., 2009). Complementary to the involvement of P2X7 receptors, Parisi et al. (2013, 2016) reported an overproduction of several microRNAs in neuroinflammation. In agreement, expression rates of these microRNAs were upregulated in ALS models upon P2X7 receptor stimulation (Parisi et al., 2016).

Astrocytes, the most abundant cell type in the CNS, show low expression of P2X7 receptor under physiological conditions. However, this potential cytotoxic receptor presents upregulated expression and increased activity following injury or under pro-inflammatory conditions (Franke et al., 2004; Narcisse et al., 2005; Lovatt et al., 2007). SOD1 (G93A) mice-derived astrocytes showed increased extracellular ATP-induced signaling as well as increased ATP hydrolysis (Gandelman et al., 2010). As previously reported for microglia, P2X7 receptor activation resulted in astrocyte cytotoxicity accompanied by production of reactive oxygen and nitrogen species that are harmful to MNs (D'Ambrosi et al., 2009).

In vitro studies presented consistent data regarding P2X7 receptor function in inflammation through microglia and astrocytes, which are detrimental for MNs survival. Low doses of ATP or BzATP induced spinal MNs death through the peroxinitrite/Fas pathway (Gandelman et al., 2013). However, *in vitro* studies fail to mimic the biological interplay between neuronal and glial cell types. Activation of the Fas pathway, or "Fas-death pathway," is required for inducing death of MNs in trophic factor deprivation environment (Raoul et al., 1999; Barthélémy et al., 2004). Fas can trigger two different signaling pathways: (1) activation of Fas-associated death domain (FADD) and caspase 8, inducing mitochondrial cytochrome c release, or (2) activation of FADD-associated protein 6 (Daxx), activating Ask1 and p38, ultimately increasing production of nitric oxide and peroxynitrite through NOS1 (Estévez et al., 1998, 2000; Raoul et al., 1999, 2002). Although the latter pathway has been described in MNs, it is not restricted to this neuronal population alone.

Though studies have linked the P2X7 receptor to neuroinflammation, surprising results have been found in ALS murine models lacking P2X7 receptor. The genetic deletion of P2X7 receptor expression ($P2X7^{-/-}$) accelerated disease onset and progression, induced neuroinflammatory responses, and produced MNs depletion at end stages of the disease in comparison with $P2X7^{+/+}$/SOD1 (G93A) animals (Apolloni et al., 2013a). The heterozygous SOD1 (G93A) P2X7 $receptor^{+/-}$ animal model did not present any significant differences in body weight, disease onset or motor performance.

While the heterozygous SOD1 (G93A) P2X7 $receptor^{+/-}$ animal model did not present any significant differences in body weight, disease onset, or motor performance, the genetic deletion of P2X7 receptor expression ($P2X7^{-/-}$), instead of improving ALS disease conditions, accelerated disease onset and progression, induced neuroinflammatory responses, and produced MNs depletion at end stages of the disease in comparison with $P2X7^{+/+}$/SOD1 (G93A) animals (Apolloni et al., 2013a). These detrimental effects on P2X7 receptor-knockout SOD1 (G93A) mice shed light on possible dual

effects of the P2X7 receptor in maintaining normal glial activation/trophic phenotypes at early stages of ALS and promoting a pronounced immunoinflammatory response in advanced stages of the disease. Moreover, P2X7 as well as P2X4 receptor expression levels were upregulated in neurons of asymptomatic SOD1 (G93A) mouse peripheral nervous system; however, more information about the mechanisms of action of these receptors in ALS is required (Volont et al., 2016).

The P2X7 receptor has been implicated in detrimental processes other than neuroinflammation. For instance, heat shock proteins that are elements involved in the unfolded protein response are also a neuroprotective mechanism against unfolded proteins that accumulate in the endoplasmic reticulum in response to stress, a phenotype associated with several ALS models. Specifically, the heat shock protein 90 (Hsp90) expression is upregulated in SOD1 (G93A) animal models as well as in ALS patients. However, it is not clear whether this upregulation is beneficial or prejudicial as *in vitro* studies reported that Hsp90 is able to induce MNs cell death through P2X7 receptor and FAS signaling (Franco et al., 2013). On the other hand, two chaperones, HSP90α and HSP70-1A, interact with A_{2A} purinergic receptors. By this interaction, they retain the receptor in the endoplasmic reticulum prior to exportation, ensuring its correct folding and acting as a protein quality control system (Bergmayr et al., 2013).

P2Y receptors

The metabotropic P2Y12 receptor has been proposed as a marker for ALS progression. It is co-expressed with CD11b in microglia and is also functional in oligodendrocytes. Its immunoreactivity is gradually lost in the dorsal and ventral horns of the spinal cord during ALS disease in the SOD1 (G93A) model, while CD68 immunoreactivity increases, indicating that P2Y12 receptor expression as marker for M2 microglia (Amadio et al., 2014). However, no specific function of this receptor has yet been described in association with either microglia or oligodendrocytes.

Adenosine receptors

Among the four adenosine receptors, the A_{2A} receptor subtype has been mostly described to be involved in ALS. *In vivo* and *in vitro* studies suggest a role of A_{2A} receptor associated with both improvement and attenuation of ALS progression, which could suggest a stage-dependent role of this receptor.

The A_{2A} receptor has been reported as the main target for caffeine, a non-selective adenosine antagonist (Fredholm et al., 1999; Karcz-Kubicha et al., 2003). The first investigation of the possible neuroprotective effect of caffeine intake and ALS development was performed in an epidemiological study, showing a reduced ALS risk in 377 European patients (Beghi et al., 2011). However, a longitudinal analysis based on over one million individuals from five cohort studies failed to demonstrate this association (Fondell et al., 2015). Similarly, an Italian case-control study found no association with caffeine intake (Pupillo et al., 2017). In the SOD1 (G93A) ALS mouse model, A_{2A} receptor blockade by chronic consumption of caffeine shortened survival and decreased motor performance (Potenza et al., 2013). An interesting finding of this study was the decrease in A_{2A} receptor protein levels only in the spinal cord from the SOD1 (G93A) control group and not in caffeine-treated animals. Whether this downregulation of receptor protein expression is due to the heterogeneity of analyzed cell types (MNs, astrocytes and microglia) or a true outcome of the disease must be determined. In fact, another study showed an increased expression of A_{2A}, but not A_1 receptors, in the spinal cords of symptomatic SOD1 (G93A) mice and in spinal cords of human end-stage ALS patients (Ng et al., 2015).

In the pathophysiology of ALS, one described mechanism that is associated with susceptibility of MNs to excitotoxic insults is activation of the receptor tropomyosin kinase receptor B (TrkB) by brain-derived neurotrophic factor (BDNF) (Fryer et al., 2001; Hu and Kalb, 2003). In this pro-death pathway, BDNF (Koh et al., 1995; Ishikawa et al., 2000; Kim, 2003) agonist stimulation of A_{2A} receptors leads to the damaging transactivation of TrkB (Lee and Chao, 2001; Rajagopal et al., 2004). This neurotoxic pathway is diminished by blockade of A_{2A} receptor in rat MNs *in vitro* injured by the levels of ALS-related mutated proteins, such as SOD1 (G85R) and p150glued (G59S) (Mojsilovic-Petrovic et al., 2006). A physical interaction between TrkB and A_{2A} receptor was demonstrated, in which their disruption by cholesterol depletion blocks the detrimental effect of BDNF to render MNs vulnerable to insult in a similar way observed by *in vitro* A_{2A} receptor blockade (Mojsilovic-Petrovic et al., 2006). In addition to pharmacological inhibition, partial genetic ablation of A_{2A} receptors in SOD1 (G93A) mice protected MNs from astrocyte-induced cell death and delayed disease progression in the mouse model (Ng et al., 2015).

During neuromuscular transmission, adenosine is an important modulator of acetylcholine release by acting on both inhibitory A_1 and excitatory A_{2A} receptors (Correia-de-Sá et al., 1991). In pre-symptomatic SOD1 (G93A) mice, a loss of functional cross-talk between A_1 and A_{2A} receptors was reported, suggesting adenosine signaling dysfunction prior to ALS onset (Nascimento et al., 2015). In the early asymptomatic ALS phase, activation of A_{2A} receptors by the agonist CGS21680 enhanced acetylcholine-evoked release, whereas this excitatory effect was no longer observed during the symptomatic phase (Nascimento et al., 2015). Intracellular Ca^{2+} homeostasis was also dysfunctional in MNs from SOD1 (G93A) mice (Fuchs et al., 2013). A_{2A} receptor activation increased the levels of cytosolic Ca^{2+} (Kobayashi et al., 1998; Palma et al., 2011), while the opposite effect was observed after A_{2A} receptor blockade (Li and Wong, 2000; Correia-de-Sá et al., 2002) and after A_1 receptor activation (De Lorenzo et al., 2004). The described loss of a functional equilibrium between A_1 and A_{2A} receptor actions in presymptomatic ALS mice could induce a hyperexcitable adenosinergic tonus in neuromuscular transmission, contributing to the Ca^{2+}-mediated excitotoxicity at initial stages of the disease (Nascimento et al., 2015). According to this hypothesis, A_{2A} receptors could act in an excitatory context during the pre-symptomatic phase, whereas A_{2A} receptor excitatory action disappears during the symptomatic phase (Nascimento et al., 2015). This stage-dependent effect of A_{2A} receptors could explain the

different effects on modulation of this receptor in ALS models. Nevertheless, further investigation of this receptor through ALS progression is needed.

Outside the neuromuscular context, only A_{2A} receptor density was up-regulated in lymphocytes from ALS patients, while A_1, A_{2B}, and A_3 receptors densities and affinities did not change compared to age-matched healthy subjects. Surprisingly, A_{2A} receptor density was positively correlated with improved clinical and functional status according to the revised ALS Functional Rating Scale (Vincenzi et al., 2013). Furthermore, cAMP production in ALS lymphocytes was increased by pharmacological stimulation of the A_{2A} receptor by its agonist CGS21680. Within the immune system, higher levels of cAMP reduce the production of pro-inflammatory mediators and increase the production of anti-inflammatory factors (Raker et al., 2016). Therefore, in addition to its described anti-inflammatory function (Sitkovsky, 2003; Haskó, 2004), these findings indicate a possible protective role for the A_{2A} receptor, specifically in the peripheral immune system.

In MNs, aberrant RNA metabolism—due to mislocalization and/or dysfunction of RNA-binding proteins—has been implicated in ALS (Strong, 2010). Human antigen R, a RNA-binding protein that translocates from nucleus to the cytoplasm, could be associated with pathogenic pathways of ALS (Liu et al., 2015). Stimulation of A_{2A} receptors with the agonist T1–11 normalized the cellular redistribution of human antigen R in the MNs cell line NSC-34, providing potential therapeutic interventions for improving the sustainability of MNs against stress and delaying ALS progression.

Conclusion

ALS is a multifactorial disease with a marked loss of MNs and an important contribution of non-neuronal cells to its pathogenesis and progression. Several works reported the involvement of diverse elements from the purinergic system in ALS, with a critical contribution to neuroinflammation through microglia and astrocyte activation. Two elements play a crucial role in ALS pathogenesis regarding the purinergic system. P2X4 and P2X7 receptors participate in microglia reactivity and astrogliosis, which both produce detrimental effects on MNs maintenance and survival. However, their involvement in ALS progression is more complex as shown *in vivo* models. There is a specific time window, at late pre-symptomatic stages of the disease, where antagonism of the purinergic P2X7 receptor may be beneficial. However, P2X7 receptor inhibition after this point produces negative effects on cell survival. During the early phase of ALS, the A_{2A} receptor mediates excitotoxicity effects on neuromuscular junction, whereas this effect is no longer observed with the progression of the disease, at the symptomatic phase. These observations indicate a possible change of function of this receptor depending on disease state. In terms of the variety of extracellular nucleotide-degrading enzymes and purinergic receptors, which assemble as homo- or heterocomplexes and vary in composition in different CNS cell types, more intense research has to be performed to clarify short- and long-term implications of purinergic signaling in ALS.

Other Motor Neuron Diseases

Spinal Muscular Atrophy (SMA) is a MND that affects MNs in the spinal cord and brainstem. Patients share manifestations similar to ALS, such as weakness, muscle atrophy/paralysis, and respiratory impairment that can lead to death (Crawford and Pardo, 1996; Lefebvre et al., 1997). The most frequent type of SMA is caused by deletions in the survival motor neuron 1 (SMN1) gene, which is involved in biosynthesis of RNA and proteins (Burghes et al., 1994; Lefebvre et al., 1995; Jablonka et al., 2000; Gabanella et al., 2007; Bebee et al., 2010; Lotti et al., 2012). High SMN1 expression in neurons and glia in a SMA transgenic mice model rescued MNs survival, indicating a non-autonomous cellular contribution in SMA (Gavrilina et al., 2008).

Currently, the only study shedding light on the involvement of the purinergic system in SMA used human induced pluripotent stem cell (iPSC)-derived astrocytes (McGivern et al., 2013). Authors reported that this population presented increased basal cytosolic Ca^{2+} concentrations and reduced responses to ATP application, suggesting a possible impairment of the purinergic system in the disease. For instance, P2Y2 receptor activation triggered intracellular Ca^{2+} mobilization in control cells, which was not observed in iPSC-derived astrocytes (Zhu and Kimelberg, 2001; Verkhratsky et al., 2012). The cause for this dysfunction, either because of altered kinetics or compromise of downstream signaling elements, still needs to be clarified. Further, this work was restricted to P2Y2 receptors, while other purinergic receptor subtypes may be involved in the same pathology. The role of the purinergic system in SMA disease should also be studied in microglia and neurons for postulating mechanisms of purinergic signaling in this MNs disorder.

Although no direct experimental evidence links the purinergic system to other previously mentioned MND, the participation of P2 and A_{2A} receptors in the physiology of peripheral nervous system is well-established. Schwann cells—peripheral glial cells responsible for myelin maintenance and injury—destroy their own myelin after peripheral nerve injury and remove myelin and cell debris (Band et al., 1986). Since demyelination is a secondary process present in MND pathophysiology, Schwann cell activity may contribute to disease progression. In fact, injured sciatic nerve induces Schwann cell proliferation via ATP activation of P2X7 receptor while A_{2A} receptors activation inhibits it, both through MAPK/ERK pathways (Stevens and Fields, 2000; Song et al., 2015).

Schwann cells located near the amphibian neuromuscular junction are activated by synaptic ATP release (Robitaille, 1998), and purinergic signaling has key roles in presynaptic modulation (Todd and Robitaille, 2006). However, as suggested by the use of suramin (a non-selective P2 receptor antagonist), activation of Schwann cells by local applications of ATP did not depend on P2 receptors, indicating a possible involvement of A_1 receptor activation by ATP metabolites (Rochon et al., 2001). An *in vitro* model of neuromuscular junction injury showed that ATP was an activating signal for Schwann cells in response to nerve function impairment, triggering purinergic signaling (Rodella et al., 2017). Xu et al. (2013) brought evidence for the involvement of purinergic signaling pathway in glia-derived neurotrophic factor (GDNF) release by Schwann cells

in nerve injure. ALS patients presented increased GDNF levels in the cerebrospinal fluid in comparison to control groups as a protective response to nerve injury (Grundström et al., 2000). Moreover, ATP and ADP released by injured nerves activated purinergic receptors that stimulate protein kinase-C and -D pathways (Xu et al., 2013). Furthermore, purinergic receptors promoted myelination processes in oligodendrocytes and inhibited them in Schwann cells. The importance of the purinergic system involvement in demyelination process in MND is clear, but a better understanding of the degenerative process is necessary for developing therapies (Xu et al., 2013).

MULTIPLE SCLEROSIS

MS is an autoimmune disease of the CNS. It is estimated to affect ∼2.5 million people worldwide and is highly incapacitating; 50% of patients will need to use a wheelchair in the years following disease onset between 25 and 45 years of age. The symptomatology of MS is heterogeneous and includes motor impairment, cognitive, visual, and sensory deficits, fatigue, and pain (Compston and Coles, 2008). The etiology of MS is unknown. However, it is speculated that environmental and genetic factors play a role in disease development (Dendrou et al., 2015).

The pathophysiology of MS is characterized by chronic inflammation, in which T cells become responsive for different myelin epitopes, triggering a cascade of events resulting in axonal demyelination and neuronal transmission impairment (Sun et al., 1991; Koehler et al., 2002). The hallmarks of MS are axonal loss, astrogliosis/microgliosis, oligodendrocytes damage, inflammatory focal lesions and T-cell activation (Goldenberg, 2012; Luo et al., 2017). There is a range of immune modulatory drugs used to alleviate MS symptoms. However, these drugs induce troubling side effects including development of other autoimmune disorders and fatal opportunistic infections (Dendrou et al., 2015). Thus, better understanding of the disease in order to develop more effective and safe treatments is needed.

Purinergic Involvement in MS

The first report of the involvement of purinergic signaling in MS came from Mayne et al. (1999), when it was found increased plasma and serum TNF-α levels in MS patients correlated with low levels of adenosine. The induced experimental autoimmune encephalomyelitis (EAE) mouse model, established by myelin oligodendrocyte glycoprotein or myelin basic protein peptide inoculation and immunization, provides some clues on the mechanical role of purinergic signaling in early-stage MS. The EAE model shows similar features as seen in the CNS of MS patients, such as infiltrating T-cells and presence of IgG antibodies as well as hind limb paralysis (Lassmann, 1983; Miller and Karpus, 1994; Eng et al., 1996; Constantinescu et al., 2011). The four purinergic receptors especially known to be involved in MS are P2X7, P2Y12, A_1, and A_{2A} receptors.

P2X receptors

The participation of P2X receptors in MS has been proposed, since they modulate astrocytes and axon-oligodendrocyte communication, which is necessary for myelination formation and repair (Butt, 2006). *Post-mortem* tissue from MS patients exhibited increased P2X7 receptor expression in microglia from spinal cord and brain white matter (Yiangou et al., 2006) in astrocytes localized in active brain lesions (Narcisse et al., 2005) and in oligodendrocytes from optic nerve samples (Matute et al., 2007). Immunohistochemistry analysis of brain sections from the frontal cortex of MS patients showed immunostaining for P2X1, P2X2, P2X3, P2X4, and P2X7 receptors, while P2X6 receptor subunits could not be detected (Amadio et al., 2010). Analysis of blood monocytes from MS patients did not show any differences in P2X7 receptor expression in comparison to healthy controls (Caragnano et al., 2012). However, monocytes from MS patients undergoing treatment with glatiramer acetate—which acts displacing myelin basic protein from the binding site on MHC-II molecules, preventing the activation of myelin-specific T cells—exhibit reduced P2X7 receptor and interleukin (IL)-1β expression, indicating that this treatment may act by decreasing P2X7 receptor pro-inflammatory effects (Caragnano et al., 2012).

Alterations in the P2X7 receptor gene have been identified in MS, leading to gain-of-function of this protein (Oyanguren-Desez et al., 2011). A polymorphism in P2X7 receptor T-allele, resulting in an Ala-76 to Val transition (A76V), induced an increase in Ca^{2+} permeability, ethidium bromide uptake, and electrophysiological responses. Also, P2X7 receptor A-allele substitution of His-155 to Tyr-382, increased Ca^{2+} influx (Oyanguren-Desez et al., 2011). Similar findings in P2X7 receptor gain-of-function due to His-155 to Tyr substitution (H155T) was previously described for leukemic lymphocytes (Cabrini et al., 2005) and suggested to be essential for ATP-dependent P2X7 receptor activation, since the residue 155 is important for P2X7 receptor protein folding (Bradley et al., 2011). On the other hand, a genetic study demonstrated that the presence of P2X7 receptor loss-of-function due to an Arg-307 to Gln (N307Q) polymorphism provided a two-fold protective effect against MS outcome (Gu et al., 2015). Thus, human P2X7 receptor variants are associated with a reduced or increased risk of MS development (Oyanguren-Desez et al., 2011; Gu et al., 2015).

In vivo studies with the EAE mouse model demonstrated that absence of P2X7 receptors resulted in a severe disease phenotype. In addition, microglia and invading brain macrophages were positive for P2X7 receptor immunostaining (Witting et al., 2006). P2X7 receptor knockout mice ($P2X7^{-/-}$), where EAE was experimentally induced, showed lower number of apoptotic lymphocytes in the CNS and increased expression of interferon γ in the spinal cord, with no alterations in TNF-α and IL-2 protein levels (Chen and Brosnan, 2006). Furthermore, P2X7 $receptor^{-/-}$ EAE mice have lower production of endocannabinoids and reduced axonal damage in comparison to wild type animals (Witting et al., 2006). The administration of a P2X7 receptor antagonist during the chronic phase of EAE in mice attenuated symptoms and tissue damage, including remyelination, by improving axonal conductivity and neurological latency (Matute et al., 2007). These results suggest that the P2X7 receptor plays a detrimental role in the development and chronic phase of MS.

A study by Sharp et al. (2008) demonstrated that the absence of P2X7 receptor results in lower frequency of EAE development, including reduced astrocyte activation with no changes in microglia, antigen responsive T-cell population, or cytokine production by splenic-T cells. These results differ from previous available data on P2X7 receptor $^{-/-}$ and EAE mice. In the former two studies, deletion of exon 5 in P2X7 receptor was used to derive the knockout mice (Chen and Brosnan, 2006; Witting et al., 2006), while in Sharp et al. (2008) exon 1 was deleted, resulting in macrophages inability to produce IL-1β. Based on these data, regulation of P2X7 receptor activation status can provide beneficial advantages for MS.

Activation of P2X7 receptors in astrocytes induces the release of purines (Ballerini et al., 1996) and limits glutamate removal from the extracellular compartment (Lo et al., 2008), eventually culminating in neuronal/oligodendrocyte excitotoxicity (Pitt et al., 2000; Matute, 2011). Upon stimulation with IL-1β, astrocytes showed P2X7 receptor expression upregulation, indicating that the P2X7 receptor expression depends on the presence of pro-inflammatory cytokines (Narcisse et al., 2005). Furthermore, a hyperactivation of P2X7 receptors in oligodendrocytes causes excitotoxicity by cytosolic Ca^{2+} overload and consequent tissue damage (Matute et al., 2007).

Yiangou et al. (2006) proposed a mechanism for the involvement of P2X7 receptor in MS: increased extracellular ATP levels caused by cell death activate P2X7 receptors in microglia and macrophages, consequently stimulate IL-1β production and release. IL-1β will induce COX2, an enzyme known to be detrimental during inflammation (Minghetti, 2004). This induction will intensify cell death and production of pro-inflammatory cytokines. In addition, in the EAE rat model, protein levels of P2X7 receptor were analyzed at symptomatic manifestation and after recovery (Grygorowicz et al., 2011). During symptom onset, P2X7 receptor was found to be overproduced in synaptosomes and in glial cells homogenates. The elevated protein level of P2X7 receptor was stable at the recovery phase mainly in the glial fraction, suggesting sustained astrogliosis. The use of P2X7 receptor antagonists, such as periodate-oxidized ATP and Brilliant Blue G (BBG), for the treatment of the neurodegenerative phase of MS has been patented (EP1655032 B1), providing novel tools for clinical and research purposes.

P2Y receptors

During inflammatory responses, P2Y receptors are up regulated in microglia to promote phagocytosis and migration, preventing oxidative stress followed by apoptosis and controlling the expression of pro-inflammatory cytokines (Förster and Reiser, 2015). In MS, the P2Y12 receptor was found in oligodendrocytes from *post-mortem* brain samples and its expression was decreased in areas corresponding to demyelination in gray and subcortical white matter (Amadio et al., 2010). Immunohistochemistry studies revealed expression of P2Y12, P2Y11, and P2Y14 receptors in the frontal cortex of MS patients (Amadio et al., 2010). However, the functions of P2Y11 and P2Y14 receptors are not known. Therefore, we will further focus on the P2Y12 receptor.

Microglia, macrophages and neuronal cells did not show any expression of P2Y12 receptors, while receptor-positive staining was found to be co-localized with myelin-binding proteins and astrocytes. In the white matter of MS patients, microglia expressing the major histocompatibility complex class II, revealed immunostaining for P2Y12 receptors, indicating that microglia possibly phagocytized myelin-bearing P2Y12 receptors (Amadio et al., 2010). Presence of P2Y12 receptors in astrocytes and oligodendrocytes suggests that signaling of this receptor is involved in remyelination.

To determine the effect of P2Y12 receptor in MS, knockout models of this receptor resulted in an enhanced EAE phenotype in mice (Zhang et al., 2017). The EAE pathology was characterized by an increase in IL-17A cytokine levels in serum, higher number of T-helper cell subset (Th17) in spleen and CNS, as well as the presence of granulocyte-macrophage colony-stimulating factor (Zhang et al., 2017). Bone marrow-derived dendritic cells from P2Y12$^{-/-}$ mice challenged to model EAE have increased release of IL-23, which is an essential factor to promote differentiation of CD4+ T cells toward the Th17 cell subtype (Zhang et al., 2017). The authors concluded that P2Y12 receptors are important for balancing Th-cell populations, and receptor function dysregulation leads to altered cytokine profiles, contributing to EAE.

Adenosine receptors

Analysis of peripheral blood mononuclear cells (PBMC) from MS patients showed that A_1 receptor protein level was significantly reduced (Mayne et al., 1999; Johnston et al., 2001), but gene expression was unaltered (Mayne et al., 1999). In healthy controls, activation of A_1 receptors in PBMCs resulted in inhibition of TNF-α while in MS patients IL-6 was inhibited and had no effect on TNF-α protein level (Mayne et al., 1999). It has been previously demonstrated that constant presence of high TNF-α levels can induce demyelination in a similar way as observed in MS patients, indicating that dysregulation of TNF-α by A_1 receptors can be an initiating factor for MS pathology (Probert et al., 1995).

Histological analyses of *post-mortem* brain tissues showed lower expression of A_1 receptor in the glial population, specifically the A_1-β receptor spliced variant (Johnston et al., 2001). Induction of the EAE model in $A_1^{-/-}$ mice caused a severe progressive-relapsing form of MS with myelin and axonal loss (Tsutsui et al., 2004). Macrophages produced IL-1β and metalloproteinase 12, as well as soluble factors that damaged oligodendrocytes. Analysis of spinal cord of $A_1^{-/-}$ EAE mice showed increased release of pro-inflammatory cytokines. In contrast, $A_1^{+/+}$ EAE mice had diminished A_1 receptor expression in microglia, corresponding to inflammation. Chronic caffeine administration upregulated A_1 receptor expression in microglia, and when treated concomitantly with A_1 receptor agonist alleviated EAE pathology in the $A_1^{+/+}$ EAE mice model (Tsutsui et al., 2004). Furthermore, coffee consumption and MS risk were recently investigated. In individuals, who reported high coffee consumption and in animal models of MS, caffeine decreased the risk of developing neuroinflammation and had neuroprotective

and anti-inflammatory properties (Hedström et al., 2016; Olsson et al., 2017).

Current therapeutic recommendations in MS include interferon-β and *glatiramer* (Wiendl et al., 2008). Worthwhile of mentioning, interferon-β treatment increases expression of CD73, responsible for the conversion of AMP into adenosine, in endothelial cells (Airas et al., 2007). Similarly, in an induced-demyelinated rat model, interferon-β treatment also enhanced CD73 activity in synaptosomes from cerebral cortex (Spanevello et al., 2006). CD73 activity is also required for lymphocyte infiltration into the CNS during EAE development (Mills et al., 2008). Thus, interference with levels of purines could be an additional factor, by which interferon-β benefits MS patients.

While the A_1 receptor subtype is an unequivocal negative modulator of MS and EAE (Tsutsui et al., 2004), the A_{2A} receptor subtype presents a complex role in this disease. Interestingly, A_{2A} receptor expression is increased in the brain of patients with secondary progressive MS, evidenced by positron emission tomography (PET) imaging of radioligand binding to the A_{2A} receptor (Rissanen et al., 2013). This receptor is both highly expressed by lymphocytes and the main mediator of anti-inflammatory effects of adenosine (Blackburn et al., 2009). In the EAE model, A_{2A} receptor-selective antagonist SCH58261 treatment protected mice from EAE induction and CNS lymphocyte infiltration (Mills et al., 2008, 2012). On the other hand, A_{2A} receptor-deficient ($A_{2A}^{-/-}$) mice developed a more severe paralysis after EAE induction, characterized by increased numbers of lymphocytes and activated macrophages/microglia in the CNS (Mills et al., 2012), severe demyelinated phenotype, axonal injury in spinal cord and cerebral cortex and pro-inflammatory cytokine profile in the CNS, blood, and spleen (Yao et al., 2012). Mechanisms of these opposite effects following genetic (knockout animal) or pharmacological (antagonists) blockade of A_{2A} receptors were revealed by assays with bone marrow chimeric mice (subjected to radiation and replacement of immune cells by bone marrow from donor animals) (Mills et al., 2012). This model also reveals the contribution of A_{2A} receptor signaling in immune and non-immune cells during EAE. In fact, $A_{2A}^{-/-}$ donor hematopoietic cells induced severe EAE, whereas the absence of A_{2A} receptor in non-immune cells protected mice from disease development. Taken together, these data demonstrate that expression of A_{2A} receptors in lymphocytes is crucial for limiting the severity of inflammation, while the A_{2A} receptor on nonimmune cells is necessary for disease development. Moreover, without A_{2A} receptor expression by blood brain barrier cells (and other non-immune cells), immune cells fail to infiltrate the CNS, protecting mice from disease development (Mills et al., 2012), similarly to the effects of pharmacological blockade of A_{2A} receptors (Mills et al., 2008, 2012).

A_3 receptor signaling is associated with degranulation of mast cells. Activation of A_3 receptor inhibits adenylate cyclase, stimulates phospholipase C and B, and induces calcium release from intracellular stores. It has been suggested that A_3 receptors may also inhibit binding of neutrophils to endothelial cells. A_3 receptor is expressed in whole brain (Safarzadeh et al., 2016). Though it is still unclear whether the A_3 receptor is involved in MS, this receptor has been demonstrated to mediate the inhibition of TNF-α production by adenosine (Lee et al., 2006; Levy et al., 2006). Therefore, this receptor may play important roles in the pathophysiology of MS and, such as for the A_{2A} receptor, A_3 receptor inhibition may be a potential therapeutic approach.

A_{2B} receptor signaling also modulates the pathogenesis of EAE phenotype. This receptor is upregulated in peripheral leukocytes of MS patients and in the mouse model. Activity inhibition of A_{2B} receptor with the selective antagonist CVT-6883 or its genetic deletion attenuated adenosine-mediated IL-6 production, infiltration of peripheral leukocytes and clinical symptoms in the EAE model (Wei et al., 2013). The presented studies suggest that adenosinergic activation of A_1 receptor regulating inflammatory cytokine TNF-α and IL-6 production is altered in MS, probably due to alterations at transcriptional levels of A_1 receptor and/or to adenosine availability (Mayne et al., 1999; Johnston et al., 2001).

Conclusion

Since current therapeutic recommendations for MS have partial efficacy on clinical outcomes and disease progression, the search for new therapeutic tools is necessary. In this context, *post-mortem* analysis of brain tissue from both MS patients and EAE mouse/rat models elicited potential therapeutic targets through: (1) blockade of P2X7 receptor and stimulation of A_1 receptor, inhibiting inflammation; (2) P2Y12 receptor stimulation, favoring remyelination; and (3) blockade of A_{2B} receptors and CD73, inhibiting the infiltration of leukocytes into the CNS.

PARKINSON'S DISEASE

Parkinson's Disease (PD) is the second most common neurodegenerative disease. Its incidence increases with age reaching over 4% of the population over 80 years old (de Lau and Breteler, 2006). PD is considered a motor disease as a reflex of its clinical symptoms, as resting tremors of extremities, muscular rigidity, postural imbalance, and bradykinesia (Braak et al., 2013). The pathology of PD is characterized by progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc) and their projections to the striatum, structures associated to voluntary motor movements' control. Besides dopaminergic neuronal degeneration, the presence of protein aggregates (known as Lewy bodies) due to misfolding of α-synuclein occurs in the SNc, *locus ceruleus*, amygdala, and the CA2 area of the hippocampus (Jellinger, 2011). The mechanisms underlying these events have yet to be clarified, although a genetic predisposition associated with insults as traumatic brain injury and ischemia seems to induce α-synuclein aggregation (Shahaduzzaman et al., 2013; Kim and Vemuganti, 2017). The majority of genes linked to familial PD development, such as α-synuclein and leucine-rich repeat kinase-2 (LRRK2) apparently follow a non-Mendelian genetic inheritance pattern. Even so, people who have first-degree relatives affected by sporadic PD have increased chances of developing PD (Elbaz et al., 1999).

Mitochondrial dysfunction and purinergic receptor signaling are also involved in the mechanism of the disorder (Takenouchi et al., 2010; Hoang, 2014).

Currently, dopamine agonists as L-3,4-dihydroxyphenylalanine (L-DOPA) are the most common agents used in therapy. L-DOPA is a precursor of catecholamines such as dopamine and is able to cross the blood-brain barrier. However, long term use of L-DOPA loses efficacy and dose adjustments are needed, triggering side effects such as dyskinesias in 50% of patients after 5 years of continuous treatments (Lang, 2009; Olanow et al., 2009). Present studies on molecular aspects of PD, together with the development of new drugs and tests for improving diagnosis accuracy, will bring new therapeutics perspectives for the disease.

Purinergic Involvement in PD

P2X receptors

Although immunohistochemistry analysis did not reveal any difference between intact and lesioned striatum and SNc (Amadio et al., 2007) for P2X7 receptors, antagonism of this receptor has been shown to prevent or reverse hemiparkinsonian behavior in animals lesioned with 6-hydroxydopamine (6-OHDA), a neurotoxin that mimics PD's pathology. Acute SNc injections of the P2X7 receptor antagonist A-438059, 60 min before and 60 min after rat 6-OHDA lesion, prevented dopamine striatal deficit in comparison to the intact hemisphere, with the P2X7 receptor localized in glial cells (Marcellino et al., 2010). BBG administered in a dose of 45 mg/kg daily after 6-OHDA lesion prevented hemiparkinsonian behavior, short-term memory impairment and dopamine deficit in the striatum and SNc (Carmo et al., 2014). While these studies showed only a preventive effect of P2X7 receptor antagonism, BBG at a dose of 50 mg/kg reversed 6-OHDA lesion in striatum and SNc. In this work, BBG treatment started 1 week after 6-OHDA injection, a period of time sufficient for the lesion to settle, thus proving the reversal effect (Ferrazoli et al., 2017).

Neuronal death seems to aggravate protein aggregation observed in PD. Intense ATP release and consequent purinergic receptors activation were considered to be a key trigger. In fact, P2X1 receptor antagonism or genetic deletion reduced α-synuclein aggregation induced by ATP released by dying cells *in vitro* (Gan et al., 2015). Moreover, P2X1 receptor activation induced lysosomal dysfunction that seems to be involved in α-synuclein aggregation, since it delayed protein turnover and led to its accumulation (Gan et al., 2015). Although P2X7 receptor blockade did not result in reduction of α-synuclein aggregation in this study, ATP release triggered by α-synuclein *in vitro* activated the P2X7 receptor and mobilized the release of intracellular Ca^{2+}, showing that P2X7 receptor activation is a consequence of α-synuclein aggregation (Wilkaniec et al., 2017). Additionally, another study showed that microglial cells challenged with α-synuclein presented increased ROS production through P2X7 receptor activation, which was prevented in the presence of a receptor antagonist (Jiang et al., 2015). Thus, it seems that P2X1 receptor activation contributes to α-synuclein aggregation, which in turn modulates P2X7 receptor activity, ROS production and, finally, ATP release.

Taking into account that few studies directly link purinergic receptors with genetic predisposition to PD, the P2X7R 1513A>C polymorphism that facilitates pore formation by P2X7 receptor activation and leads to cell death (Gu et al., 2001) was shown to be a risk factor in sporadic PD in a Han Chinese population (Liu et al., 2013)

P2Y receptors

There is little data directly connecting P2Y receptors to PD. Recent studies are drawing attention to the role of P2Y6 receptors in PD development and progression. An *in vitro* study showed that P2Y6 receptor gene expression is increased in SH-SY5Y cells—a human neuroblastoma lineage that when differentiated presents markers for dopaminergic neurons—when challenged with neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) (Qian et al., 2017). Thus, its antagonism or deletion decreased MPP^+ effects in cell death through reduced ROS production (Yang et al., 2017). In the CNS, UDP released by damaged cells induces expression of cytokines CCL2 and CCL3 in microglia and phagocytic activity through activation of P2Y6 receptors, indicating that this receptor subtype may be involved in inflammatory response in neurodegenerative diseases (Kim et al., 2011).

Recently, P2Y6 receptor levels were found to be increased in PBMC of PD patients younger than 80 years. To elucidate the involvement of P2Y6 receptor in these patients, the authors used an *in vitro* model of microglia challenged with lipopolysaccharide (LPS) and found increased P2Y6 receptor expression, supporting the hypothesized neuroinflammatory effect of microglia (Yang et al., 2017). Taking into account that P2Y6 receptor selective inhibition by MRS2578 is able to prevent microglial phagoptosis in a mixed neuronal/glial culture in inflammatory conditions (Neher et al., 2014), P2Y6 receptor antagonism seems to be a promising tool to attenuate neuronal death in PD by preventing lesion worsening due to phagocytosis of viable neurons.

Adenosine receptors

It is known that A_{2A} receptors are enriched in dopaminergic brain areas and that their activity modulation affects dopamine receptors (Burnstock et al., 2011). In fact, A_{2A} receptors form heterodimers with dopaminergic D2 and A_1 receptors in glutamatergic synapses, modulating the balance between excitatory and inhibitory impulses that may aggravate PD symptomatology (reviewed by Schiffmann et al., 2007). In animals, a range of A_{2A} receptor antagonists have being shown to potentiate therapeutic effect of low doses of L-DOPA in MPP^+ lesioned monkeys and marmosets and in 6-OHDA lesioned rodents (Kanda et al., 2000; Fuzzati-Armentero et al., 2015) In fact *istradefylline,* a A_{2A} receptor antagonist, was recently approved in Japan to be used concomitantly with L-DOPA treatment, once the compound enhances antiparkinsonian effect of L-DOPA and allow the usage of lower doses of L-DOPA with less long-term side effects (Zhu et al., 2014).

A_{2A} receptors are supposedly involved in synucleinopathy process. A_{2A} receptor-knock out mice presented resistance in preventing dopaminergic deficits upon α-synuclein-induced insults (Kachroo and Schwarzschild, 2012). Attempting to

clarify involved mechanisms, Ferreira et al. found that A_{2A} receptor antagonism decreased α-synuclein aggregation, prevented neuronal death induced by extracellular α-synuclein and restrained hyperactivation of NMDA-glutamate receptors (Ferreira et al., 2015). A_{2A} receptor protein expression levels are increased upon hippocampal injections of α-synuclein in mice and closely co-localized with aggregates, suggesting a pathogenic role of this receptor in synucleinopathy (Hu et al., 2016).

Moreover, A_{2A} receptor antagonism may facilitate microglial response to injury. Microglial delayed containment of debris resulted from cell death can be associated with expansion of the lesion (Gyoneva et al., 2014). Further, both caffeine and selective A_{2A} receptor antagonist KW60002 prevented rat striatal dopaminergic deficit and hydroxyl radical release in LPS-induced inflammation (Gołembiowska et al., 2013). These data suggest inflammatory modulation by A_{2A} receptor antagonism in PD models.

Two polymorphisms of A_{2A} receptor (rs71651683 or rs5996696) were inversely associated with genetic PD risk, wherein caffeine intake intensified the inverse association. Moreover, two polymorphisms in CYP1A2a (rs762551 or rs2470890), an enzyme responsible for caffeine metabolism, in homozygous caffeine consumers showed a prominent reduction in the risk of developing PD (Popat et al., 2011).

Caffeine intake interferes with other genetic risk factors for PD. Subjects with LRRK2 risk variant R1628P showed 15 times increased risk of developing PD than not caffeine consumers (Kumar et al., 2015). GRIN2A rs4998386-T allele encodes a subtype of NMDA receptor, whose activity is enhanced by A_{2A} receptor activation and leads to glutamatergic excitotoxicity. A polymorphism in the GRIN2A rs4998386-T is considered protective for PD development *per se,* but in association with caffeine consumption, it can beneficially impact PD risk in a greater magnitude (Hamza et al., 2011; Yamada-Fowler et al., 2014). However, creatine consumption that increases ATP storage accelerated PD progression in GRIN2A caffeine consumers, possibly due to ATP conversion to adenosine and later A_{2A} receptor activation (Simon et al., 2017).

Conclusion

Taken together, evidence indicates that modulation of purinergic receptor expression and activity could be useful in PD treatment in several ways: (1) reducing microglia activation by damaged cells and α-synuclein aggregation through P2X7 and P2Y6 receptors antagonism; (2) preventing α-synuclein aggregation through P2X1 and A_{2A} receptors antagonism; (3) modulating inflammatory scenario through A_{2A} receptors antagonism; or (4) preventing dyskinesia induced by L-DOPA long-term use through combined treatment with A_{2A} receptor antagonists.

OTHER NEUROLOGICAL CONDITIONS WITH MOTOR DYSFUNCTIONS

Huntington's Disease

HD is an inherited neurological disorder caused by a mutation in IT15 gene that encodes huntingtin protein (Htt) predominantly found in neurons. This mutation results in abnormal (CAG)n repeats localized in 5′ coding sequence. HD is characterized by neurodegeneration of neuronal cells located in striatum and cerebral cortex, ultimately causing neuronal dysfunction and striatal death (Vonsattel and DiFiglia, 1998; Ross and Tabrizi, 2011).

Purinergic Involvement in HD

Adenosine receptor

Adenosinergic pathway plays an essential role in HD etiology and progression, especially through the A_{2A} receptor, as observed in patients and animal models (Popoli et al., 2007). The A_{2A} receptor is highly expressed in striatum (Schiffmann et al., 1991; Fink et al., 1992) especially in GABAergic/enkephalinergic neurons (Taherzadeh-Fard et al., 2010) and in post-synaptic striatopallidal GABAergic neurons (Martinez-Mir et al., 1991; Hettinger et al., 2001), antagonizing dopamine D2 receptors (Schiffmann et al., 2007), while presynaptic A_{2A} receptor activity promotes glutamate release (Shen et al., 2013). Further, presynaptic A_{2A} receptors in glutamatergic terminals impinging into medium spiny neurons play an essential role in the initial maladaptive plasticity in animal models of HD (Li et al., 2015), suggesting its involvement in the degeneration of striatal neurons. Reduction of A_{2A} receptor expression is based on the overexpression of mutant Htt protein showing expanded poly (Q), which affects CREB binding to its promoter region in the A_{2A} receptor gene. Under stimulation, A_{2A} receptor is able to promote its own gene expression via activation of PKC/CREB signaling as well as reduce Htt aggregations (Chiang et al., 2005). Striatal cells expressing mutant Htt showed increased A_{2A} receptor density and cAMP activity due to A_{2A} receptor activation (Varani et al., 2001). As expected, transgenic HD mice showed reduced A_{2A} receptor expression (Cha et al., 1999; Glass et al., 2000; Luthi-Carter et al., 2000), while exhibiting transient increases in A_{2A} receptor density and A_{2A} receptor-dependent activation of cAMP signaling at the earlier pre-symptomatic stage (Tarditi et al., 2006).

It has been proposed that modulation of A_{2A} receptor activity either by agonists or antagonists may prove to be beneficial for HD treatment. However, available data indicate that the beneficial effect observed after stimulation or inhibition of A_{2A} receptor activity depends on the disease stage. At earlier stages of HD, the use of SCH58261 (an A_{2A} receptor antagonist) in quinolinic acid (QA)-induced HD rats and R6/2 transgenic mice reduced striatal BDNF expression, precluding BDNF control of NMDA toxicity (Potenza et al., 2007; Tebano et al., 2010). In later stages, no effect on BDNF expression was observed (Martire et al., 2010; Tebano et al., 2010). QA-induced rats reproduced neurochemical changes of NMDA receptor from HD, e.g., increased glutamate outflow, reduced adenosine levels and degeneration of A_{2A} and dopamine receptors (Beal et al., 1991; Ishiwata et al., 2002; Gianfriddo et al., 2003). Treatment with SCH58261 2-3 weeks after QA injection increased striatal glutamate release, acting as damaging factor (Gianfriddo et al., 2003).

Preventive treatment with SCH58261 before QA induction in rats minimizes the effect of QA on motor activity, striatal gliosis, electroencephalographic (EEG) changes, and glutamate levels

(Popoli et al., 2002). However, cyclooxygenase-2 (COX-2) is inhibited in microglia but increased in cortical neurons, probably as a consequence of NMDA receptors activation, leading to neurotoxicity (Minghetti, 2004). Pretreated QA-induced rats also showed less rearing behavior and no changes in baseline motor activity after 2 weeks of induction; 6 months later, rats showed reduced anxiety but no changes in learning task when compared to QA-induced rats not pre-treated with SCH58261 (Scattoni et al., 2007). These findings suggest that SCH58261 acts on damaged striatum and not on damaged hippocampus, and that different populations of striatal neurons are responsive to SCH58261 (Scattoni et al., 2007). As reviewed by Cunha (Cunha, 2016), the blockage of A_{2A} receptor improves memory and motor functions indicating hippocampal activity, contradicting the findings of Scattoni et al.

On the other hand, in primary striatal cultures treated with QA, an increase in intracellular calcium concentration was observed which enhanced in presence of SCH58261, but reduced in presence of A_{2A} receptor agonist CGS21680 (Popoli et al., 2002). Another A_{2A} receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), completely blocked encephalographic changes in prefrontal cortex in QA-induced rats (Reggio et al., 1999). The beneficial effect can be due to dopamine receptor activation that provides neuroprotection as a result of abolishment of A_{2A} receptor function, since D2 dopaminergic receptors are downregulated by A_{2A} receptors in D2/A_{2A} receptor heteromers (Reggio et al., 1999).

In a transgenic rat model of HD showing 51 repeated CAG sequences, the presence of post-synaptic A_{2A} receptor antagonist KW-6002, a known stimulant of locomotion, didn't alter the locomotion pattern between 3 and 6 months old. This indicated that the animals become indifferent to A_{2A} receptor modulation during that period (Orr,ú et al., 2011). Furthermore, the presynaptic A_{2A} receptor antagonist SCH-442416 did not reduce electromyography responses (Orr,ú et al., 2011).

The function of A_{2A} receptors has been studied in HD transgenic mouse models (R6/1 with later symptoms and R6/2 with earlier symptoms), which contain the first exon of human Htt gene and 115-150 CAG repeats (Li et al., 2005). During R6/2 mouse development, A_{2A} receptor protein density and A_{2A} receptor-dependent production of cAMP slightly increased at post-natal days 7–14, before the onset of motor symptoms (Tarditi et al., 2006). On the 21st day, changes are normalized to control (Tarditi et al., 2006). A_{2A} receptor expression, but not protein density, starts decreasing, indicating that protein turnover is altered in HD (Cha et al., 1999; Tarditi et al., 2006). Reduction of A_{2A} receptor coding mRNA can be explained by regulation of A_{2A} receptor gene methylation patterns, once R6/1 mice has less hydroxymethylcytosine and higher methylcytosine levels in 5′-UTR regions of the A_{2A} receptor gene (Villar-Menéndez et al., 2013).

Since turnover of A_{2A} receptor protein is altered in HD, inhibition of this receptor function is an advisable therapeutic approach. Starting at 5 weeks, the use of the A_{2A} receptor antagonist SCH58261 in R6/2 mice ameliorated NMDA-induced toxicity and emotional/anxiety response (Domenici et al., 2007). After week 8, administration of SCH58261 leads to NMDA receptors remodeling (NR_1 and NR_{2A} receptor /NR_{2B} ratio) in striatum (Martire et al., 2010). R6/2 mice at age of 10–11 weeks old showed increased adenosine levels correlated with the presence of p38 MAPK in striatal neurons, resulting in striatal damage (Gianfriddo et al., 2004). The usage of SCH58261 greatly reduced striatal adenosine levels and glutamate outflow, suggesting that SCH58261 was acting on A_{2A} receptors located in corticostriatal glutamatergic terminals (Gianfriddo et al., 2004). When treated with SCH58261, rearing and grooming behaviors were reduced in R6/2 mice, but increased in wild type mice, suggesting that A_{2A} receptor antagonism effects on behavior depended on the presence of mutant Htt (Domenici et al., 2007). However, there are contradictory findings regarding the effect of SCH58261. While this compound has shown beneficial effect by reducing NMDA toxicity in striatum *in vivo*, it did not prevent NMDA toxicity from *in vitro* culture of corticostriatal slices obtained from R6/2 mice (Martire et al., 2010).

In order to determine whether the A_{2A} receptor is involved in HD etiology, A_{2A} receptor knockout mice were induced with mitochondrial toxin 3-nitropropionic acid (3-NPA) which blocks succinate dehydrogenase, inducing HD phenotype. Only 1 out of 8 showed striatal lesion after 3-NPA induction, indicating that the absence of A_{2A} receptor has protective effect against HD development (Fink et al., 2004). To confirm this finding, wild type mice were pre-treated with the A_{2A} receptor antagonist 8-(3-chlorostypyl)-caffeine. The animals did not show any striatal lesions after 3-NPA treatment (Fink et al., 2004). On the other hand, ablation of A_{2A} receptors in HD N171-82Q transgenic mouse model completely aggravated motor performance and survival, reducing the expression of striatal encephalin (Mievis et al., 2011). This observation suggests that early and chronic blockade of A_{2A} receptor is not favorable for HD development (Mievis et al., 2011), but memory improvement was observed in R6/2 mice with complete genetic A_{2A} receptor ablation (Li et al., 2015).

In symptomatic R6/2 mice, activation of A_{2A} receptors by CGS21680 delayed the deterioration of motor conditions, prevented reduction in brain weight, diminished the levels of choline, normalized glucose levels, and altered NMDA receptor subunit composition and basal synaptic transmission, without changing its expression (Chou et al., 2005; Martire et al., 2007; Potenza et al., 2007; Ferrante et al., 2010; Tebano et al., 2010). Cultivation of corticostriatal slices from R6/2 mice in presence of CGS21680 also showed reduced NMDA toxicity, suggesting a crosstalk between A_{2A} receptor and BDNF (Tebano et al., 2010). Treatment of striatum slices from R6/2 with CGS21680 resulted in an increase in extracellular field potential, while the opposite effect was observed in wild type slices, where the use of an A_{2A} receptor agonist potentiated toxicity via NMDA receptor activation (Martire et al., 2007).

Single nucleotide polymorphisms (SNPs) in the $ADORA_{2A}$ gene have been identified in HD patients. A C>T genotype (1876 C/T; rs5751876) SNP results in a silent mutation with unknown function and influences the age of onset of HD, while the T/T genotype increases the age of onset of HD by 3.8 years when compared to the C/C genotype (Dhaenens et al., 2009). A SNP in intron 1 (rs2298383) is linked to early onset of HD

(Taherzadeh-Fard et al., 2010). Analysis of HD patient peripheral blood cells led to increased aberrant A_{2A} receptor signaling, which correlates with the age of the patient, numbers of expanded CAG repeats and number of A_{2A} receptor ligand-binding sites (Maglione et al., 2005a,b). The linear correlation is more evident in patients suffering from chorea—an early disruption of the striatum in HD. Neutrophils from HD patients have higher A_{2A} receptor dysfunction in homozygous vs. heterozygous HD patients while no changes in A_1 or A_3 receptors are observed in peripheral blood cells (Varani et al., 2003). A_{2A}-cannabinoid CB1 receptor heterodimers exert crucial function by controlling neuronal excitability (Moreno et al., 2017), while activation of striatal A_{2A} receptors may inhibit CB1 function independent from heterodimer formation (Ferreira et al., 2015). Patients harboring high-grade HD do not possess A_{2A}-CB1 receptor heterodimers in the caudate-putamen region due to the lack of CB1 receptors (Moreno et al., 2017). Recent evidence suggests that consuming more than 190 mg/day of caffeine may accelerate HD onset (Simonin et al., 2013), contradicting findings in animal models that point toward beneficial effects of A_{2A} receptor antagonism in HD.

HD is also characterized by oxidative stress resulting from mitochondrial dysfunction, leading to GABAergic neuronal loss and proneness to DNA damage (Chiu et al., 2015). GABAergic neurons derived from HD-iPSC showed an increase in DNA damage and oxidative stress, which can be dramatically reduced by A_{2A} receptor activation (Chiu et al., 2015). Stimulation of A_{2A} receptors minimizes oxidative stress-induced apoptosis by activation of the cAMP/PKA signaling pathway (Chiu et al., 2015), which is essential for reversing the effect of reduced A_{2A} receptor activity via CREB transcription factor activation (Chiang et al., 2005). However, findings *in vivo* contradict the beneficial effect of A_{2A} receptor agonism on PKA signaling. In R6/1 mice, dopamine D1 and A_{2A} receptors are hyperactive showing greater cAMP/PKA signaling (Tyebji et al., 2015). Chronic administration of antagonists of dopamine D1 and A_{2A} receptors normalized PKA levels and improved cognitive dysfunction and synaptic plasticity. Pre-treatment of rats and mice with either 8-cyclopentyl-1,3-dipropylxanthine (CPX; A_1 receptor antagonist) or DMPX prior application of manolate (an inhibitor of mitochondria acting in striatum) showed that DMPX prevented GABAergic cell loss while CPX promotes cell death (Alfinito et al., 2003). The A_1 receptor agonist R-PIA prevented seizures but not neurodegeneration in the 3-nitropropionic acid (3-NPA) model of neurotoxicity (Zuchora and Urbańska, 2001), while the A_1 receptor agonist adenosine amine congener (ADAC) protects against excitotoxicity, delays degeneration and improves motor functions in the same model (Blum et al., 2002). In view of that, the effect of A_1 receptors depended on the respective used antagonist.

P2 receptors

The role of P2X signaling in HD has not yet been studied in detail. Evidence exists that signaling via ATP induced cell death in HD models while blockade of ATP production reduces cell loss (Varma et al., 2007). At the present, the only evidence available is the role of P2X7 receptor in HD pathogenesis. In two HD mice model, Tet/HD94 and R6/1, P2X7 receptor expression is increased, as well as P2X7 receptor-induced Ca^{2+} permeability (Diaz-Hernandez et al., 2009). Treatment with the P2X7 receptor antagonist BBG ameliorates motor coordination deficits and body weight loss while inhibiting neuronal loss. *In vitro*, neurons expressing mutant Htt are prone to cell death induction by apoptosis after P2X7 receptor stimulation (Diaz-Hernandez et al., 2009).

Conclusion

The available data on the involvement of A_{2A} receptors in HD progression is evident, suggesting that the prevention of its activation could delay disease progression. Taken together, it can be proposed that a combination of A_1 receptor agonist and A_{2A} receptor antagonist might be a good therapeutic approach for HD. It must be taken in consideration that the effect of A_{2A} receptor antagonism depends on age, doses, and length of treatment. Although antagonism of P2X7 receptor may be promising, the involvement of other P2 receptors remains unclear and needs to be investigated.

Ataxias

Ataxia, or dysfunction in motor coordination, is a major consequence of cerebellar and spinocerebellar tract dysfunction that can be induced by several factors, including genetic and sporadic forms, commonly related to immune system mechanisms (Mariotti et al., 2005). Spinocerebellar ataxia (SCA), a genetic-related form of progressive ataxia resulted by cerebellar degeneration, is classified according to mode of inheritance and gene/chromosome locus affected (Matilla-Dueñas et al., 2012). The most prevalent and severe forms of SCA are caused by an increase in CAG sequence repeats in genes that encode proteins related to disease development (Paulson et al., 2017). For example, the expansion in polyglutamine affecting ataxin-2 protein can be observed in SCA type 2, while ataxin-3 related expansion occurs in SCA type 3. Other forms of SCA can be characterized by other genetic mutations, such as the type 14, in which mutations in the protein kinase C-γ gene induce cerebellar degeneration (Seki et al., 2005).

Purinergic Involvement in Ataxias

Attempting to identify survival characteristics of some cell in SCA type 2, wild type ataxin-2 positive neurons showed resistance in cell-death induced by axotomy (Viscomi et al., 2005) and, although this lesion up-regulated P2X1 and P2X2 receptors in precerebellar nuclei (Florenzano et al., 2002) and induced P2X1 receptor in ataxin-2 positive neurons, the percentage of cells expressing P2X1 receptor was not altered (Viscomi et al., 2005). Viscomi and co-workers suggested that these purinergic receptors could influence resistance against cell death without being essential for cell survival, since there are several pathways involved in neuronal death. The elucidation of purinergic receptor involvement in SCA type 3 is focused on adenosine receptors. The blockade of A_{2A} receptors through caffeine ingestion reduced damaging morphological changes induced by mutant ataxin-3 injection. Moreover, these damaging effects were abolished in knockout mice for A_{2A} receptors (Gonçalves et al., 2013). Behavioral improvements were also observed in transgenic c57Bl6 mice expressing truncated polyglutamine

ataxin-3 with severe ataxia, reinforcing the protective effect of A_{2A} receptor antagonism in the SCA type 3 (Gonçalves et al., 2017). In the SCA type 14 *in vitro* model, stimulation of purinergic receptors with ATP transiently increased translocation of mutant protein kinase C-γ to the plasma membrane and subsequent increased damaging aggregation in the cytoplasm (Seki et al., 2005).

Restless Leg Syndrome

Restless leg syndrome (RLS) is a neurological condition characterized by an urge to move legs during rest, following a circadian cycle with worsening during night and even during sleep (named periodic limb movements of sleep). Pathophysiological mechanisms have not been fully elucidated, and conflicting results are reported in the literature. Dopaminergic transmission seems to be involved, since the use of dopaminergic-inducing drugs improved symptoms (Garcia-Borreguero and Cano-Pumarega, 2017). Due to the high affinity of the agonists with best responsiveness to RLS for D3 dopaminergic receptors, it is postulated that the D3 receptor subtype has major responsibility for RLS improvement (Ferré et al., 2018). However, the risk of symptoms worsening after long-term use of these drugs stimulated the search for alternative therapies, based on glutamatergic ligands and reversal of iron deficiency (Ferré et al., 2017). Striatal glutamatergic terminals are found to be hypersensitive in an animal model of RLS with increased glutamate and dopamine release. It is known that, besides increased dopamine release, there is a decreased synaptic D2 receptor density in this animal model (Ferré et al., 2018).

Purinergic Involvement in RLS

Recently, Ferré's group pointed at a relation between adenosine receptors and brain iron deficiency in RLS. Using an animal model for RLS, in which mice and rats adhered to an iron deficient diet, striatal presynaptic A_{2A} receptor density was upregulated (Gulyani et al., 2009). In the same animal model, A_1 receptor density was found decreased in animals with mild, moderate and severe deficiency accompanied by dopaminergic D2 receptor downregulation, and increased pre-synaptic A_{2A} receptor density in animals submitted to a more iron deficient diet (Quiroz et al., 2016). Thus, the post-synaptic A_1 receptor, which can be found as heteromers with D1 dopaminergic receptors and antagonizes their activity, as well as presynaptic A_{2A} receptors forming heteromers with D2 receptors whose activation decreases D2 receptor affinity for agonists, could be targets to improve movement impairment (Ferré et al., 1994; Ferre et al., 1996; Ferré et al., 2007, 2018). Finally, A_1/A_{2A} receptor heteromers found in the striatal glutamatergic terminals, activated by different adenosine concentrations, decreased glutamate release, a condition found in brain iron deficiency animals (Ciruela et al., 2006; Ferré, 2010).

OUTCOMES FOR HYPOTHESES ON P2 PURINERGIC SIGNALING

Although P2X7 receptor expression and levels in neurons is controversial, the involvement of this receptor in neurodegeneration is well-stated (Illes et al., 2017). Of all purinergic receptors, the P2X7 receptor has the lowest affinity for ATP, and only high concentrations of this nucleotide induce channel formation (North, 2002; Khakh and Alan North, 2006). Of the neurological diseases presented here, immune system responses and neural cell death correspond with the release of elevated levels of ATP into the extracellular space. In these scenarios, ATP in excess acts as a toxin that can directly induce oligodendrocyte death by activating P2X7 receptors, resulting in progressive neural damage (Matute et al., 2007; Domercq et al., 2009). Corroborating this idea, it is well-known that the P2X7 receptor triggers pro-inflammatory effects (Lister et al., 2007), and its antagonism can counteract chronic inflammation observed in these diseases (**Figure 2**). Thus, beneficial effects of P2X7 receptor antagonism are of interest for future therapeutic approaches.

As further investigations are necessary to better understand the real role of purinergic signaling in the diseases here presented, we also propose a novel mechanistic perspective, in which purinergic receptors from glial cells are key initiators of motor dysfunction in PD, MS, ALS and other MND. Although there is no data regarding P2Y1 receptor functions in these pathological conditions, it is known that this receptor plays a crucial role in astrocyte responses accompanied by P2Y12 and adenosine receptor activity modulation (Mamedova et al., 2006).

During an initial inflammatory response, microglial activation induces P2Y12 receptor expression level and/or activity upregulation, stimulating motility toward the injury site and resulting in reduced P2Y1 receptor expression in astrocytes. This downregulation in P2Y1 receptor expression stimulates an increase in reactive astrogliosis and a phenotypical change in order to promote neuroprotection (Haynes et al., 2006; Mamedova et al., 2006; Shinozaki et al., 2017). However, constant inflammatory responses disable microglia of stimulating P2Y12 receptor expression and activity, resulting in a permanent activation of P2Y1 receptors in astrocytes and extended ROS production (Rodrigues et al., 2015). This condition will lead to a downregulation of A_1 receptor expression, stimulating TNF-α production and release, thereby promoting IL-6 secretion through A_{2B} receptor activation. As a result, TNF-α and IL-6 accumulation damages oligodendrocytes and provoke demyelination. A scheme of this mechanism is proposed in **Figure 1**.

OVERALL CONCLUSION

The purinergic signaling system has risen in the past years as a meaningful research object for understanding and treating several pathologies, as reviewed by Burnstock (2017). Purinergic receptors and enzymes are in the spotlight for new therapeutic interventions as key regulators of neuron-glia communication, as well as modulators of many signaling pathways associated to neuroprotection, neurodegeneration, and neuroregeneration (Burnstock, 2016; Ribeiro et al., 2016). In this review, we highlighted available data linking purinergic signaling pathways to neurological diseases, such as PD, MS, ALS, and other MND, putting together published knowledge with novel hypotheses for overcoming motor dysfunctions. The evidence is summarized in **Table 1**. A common mechanism supporting P2X7 receptor

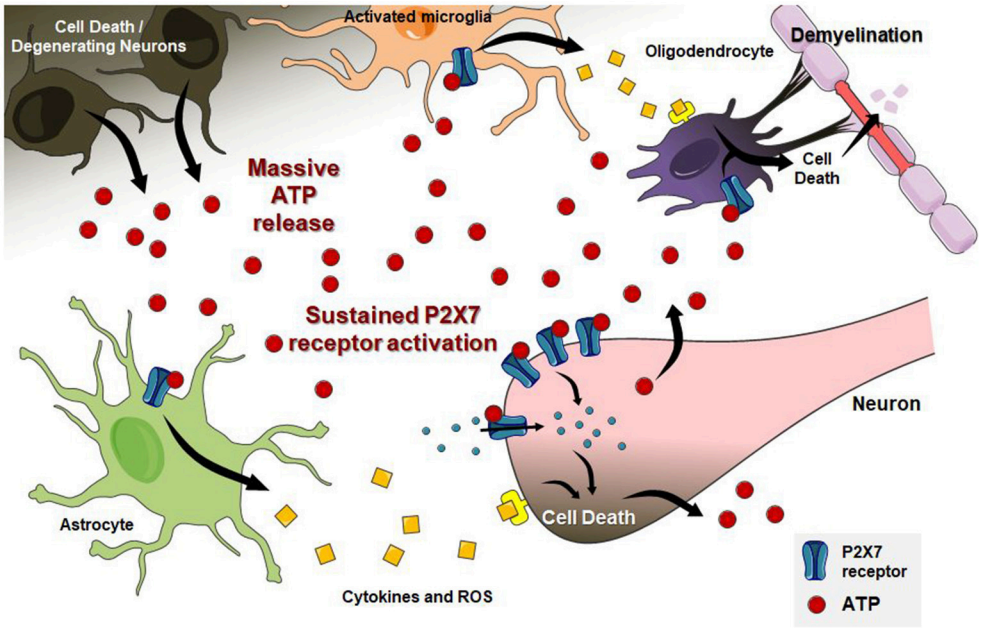

FIGURE 2 | Common mechanism involving P2X7 receptor-mediated cell death in the central nervous system under neurological diseases affecting motor functions. Degenerating neurons release large amounts of ATP, leading to sustained P2X7 receptor activation in: a. Astrocytes, inducing the release of cytokines and reactive oxygen species (ROS); b. Microglia, inducing an activated state and release of cytokines and ROS; c. Oligodendrocytes, inducing cell death and neuron demyelination; d. Neurons (in spite of the controversial discussion on expression of P2X7 receptors in this cell type), inducing pore formation, ion influx and cell death, releasing more ATP into the extracellular space. Moreover, cytokines and ROS released by astrocytes and microglia act on other neural cells, culminating in apoptotic pathway activation.

hyperactivation through high levels of ATP release during disease development is illustrated in **Figure 2**. Moreover, a novel mechanism based on purinergic modulation of glial cells is proposed in **Figure 1**. These approaches suggest novel possible research targets to understand the here presented motor dysfunctions and other factors associated to neurological impairment that have not been studied yet. Applied research will need to be conducted for the development of novel pharmacological treatments to improve patients' lifespan and quality.

AUTHOR CONTRIBUTIONS

ÁO-G: Contribution to idea proposal, organization of sections, writing PD, other MND, RLS, and Ataxia sections, figures, table. YN: Writing MS, HD, and mechanism hypothesis sections. LS-A: Writing ALS and SMA sections. MG: Writing introduction, HD, and conclusion sections. JC-V: Writing ALS section. MP: Writing MS section. HdS: Writing PD section. HU: Conceptualization of the manuscript, supervision of manuscript elaboration, editing and revision, and critical overview.

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REFERENCES

Abbracchio, M. P., Burnstock, G., Verkhratsky, A., and Zimmermann, H. (2009). Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* 32, 19–29. doi: 10.1016/j.tins.2008.10.001

Airas, L., Niemela, J., Yegutkin, G., and Jalkanen, S. (2007). Mechanism of action of IFN-beta in the treatment of multiple sclerosis: a special reference to CD73 and adenosine. *Ann. N. Y. Acad. Sci.* 1110, 641–648. doi: 10.1196/annals.1423.067

Alfinito, P. D., Wang, S.-P., Manzino, L., Rijhsinghani, S., Zeevalk, G. D., and Sonsalla, P. K. (2003). Adenosinergic protection of dopaminergic and GABAergic neurons against mitochondrial inhibition through receptors located in the substantia nigra and striatum, respectively. *J. Neurosci.* 23, 10982–10987.

Allard, B., Beavis, P. A., Darcy, P. K., and Stagg, J. (2016). Immunosuppressive activities of adenosine in cancer. *Curr. Opin. Pharmacol.* 29, 7–16. doi: 10.1016/j.coph.2016.04.001

Amadio, S., Montilli, C., Magliozzi, R., Bernardi, G., Reynolds, R., and Volonté, C. (2010). P2Y12 receptor protein in cortical gray matter lesions in multiple sclerosis. *Cereb. Cortex* 20, 1263–1273. doi: 10.1093/cercor/bhp193

Amadio, S., Montilli, C., Picconi, B., Calabresi, P., and Volonté, C. (2007). Mapping P2X and P2Y receptor proteins in striatum and substantia nigra: an immunohistological study. *Purinergic Signal.* 3, 389–398. doi: 10.1007/s11302-007-9069-8

Amadio, S., Parisi, C., Montilli, C., Carrubba, A. S., Apolloni, S., and Volonté, C. (2014). P2Y 12 receptor on the verge of a neuroinflammatory breakdown. *Mediators Inflamm.* 2014:975849. doi: 10.1155/2014/975849

Andries, M., Van Damme, P., Robberecht, W., and Van Den Bosch, L. (2007). Ivermectin inhibits AMPA receptor-mediated excitotoxicity in cultured motor neurons and extends the life span of a transgenic mouse model of amyotrophic lateral sclerosis. *Neurobiol. Dis.* 25, 8–16. doi: 10.1016/j.nbd.2006.08.018

Apolloni, S., Amadio, S., Montilli, C., Volonté, C., and D'Ambrosi, N. (2013a). Ablation of P2X7 receptor exacerbates gliosis and motoneuron death in the SOD1-G93A mouse model of amyotrophic lateral sclerosis. *Hum. Mol. Genet.* 22, 4102–4116. doi: 10.1093/hmg/ddt259

Apolloni, S., Parisi, C., Pesaresi, M. G., Rossi, S., Carrì, M. T., Cozzolino, M., et al. (2013b). The NADPH oxidase pathway is dysregulated by the P2X7 receptor in the SOD1-G93A microglia model of amyotrophic lateral sclerosis. *J. Immunol.* 190, 5187–5195. doi: 10.4049/jimmunol.1203262

Ballerini, P., Rathbone, M. P., Di Iorio, P., Renzetti, A., Giuliani, P., D'Alimonte, I., et al. (1996). Rat astroglial P2Z (P2X7) receptors regulate intracellular calcium and purine release. *Neuroreport* 7, 2533–2537.

Band, H., Bhattacharya, A., and Talwar, G. P. (1986). Mechanism of phagocytosis by Schwann cells. *J. Neurol. Sci.* 75, 113–119. doi: 10.1016/0022-510X(86)90054-7

Barbeito, A. G., Mesci, P., and Boillée, S. (2010). Motor neuron–immune interactions: the vicious circle of ALS. *J. Neural Transm.* 117, 981–1000. doi: 10.1007/s00702-010-0429-0

Barthélémy, C., Henderson, C. E., and Pettmann, B. (2004). Foxo3a induces motoneuron death through the Fas pathway in cooperation with JNK. *BMC Neurosci.* 5:48. doi: 10.1186/1471-2202-5-48

Bartlett, R., Stokes, L., and Sluyter, R. (2014). The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. *Pharmacol. Rev.* 66, 638–675. doi: 10.1124/pr.113.008003

Beal, M. F., Ferrante, R. J., Swartz, K. J., and Kowall, N. W. (1991). Chronic quinolinic acid lesions in rats closely resemble Huntington's disease. *J. Neurosci.* 11, 1649–659.

Beamer, E., Gölöncsér, F., Horváth, G., Beko, K., Otrokocsi, L., Koványi, B., et al. (2016). Purinergic mechanisms in neuroinflammation: an update from molecules to behavior. *Neuropharmacology* 104, 94–104. doi: 10.1016/j.neuropharm.2015.09.019

Bebee, T. W., Gladman, J. T., and Chandler, D. S. (2010). Splicing of the survival motor neuron genes and implications for treatment of SMA. *Front. Biosci. (Landmark Ed).* 15, 1191–1204. doi: 10.2741/3670

Beghi, E., Pupillo, E., Messina, P., Giussani, G., Chiò, A., Zoccolella, S., et al. (2011). Coffee and amyotrophic lateral sclerosis: a possible preventive role. *Am. J. Epidemiol.* 174, 1002–1008. doi: 10.1093/aje/kwr229

Bergmayr, C., Thurner, P., Keuerleber, S., Kudlacek, O., Nanoff, C., Freissmuth, M., et al. (2013). Recruitment of a cytoplasmic chaperone relay by the A $_{2A}$ adenosine receptor. *J. Biol. Chem.* 288, 28831–28844. doi: 10.1074/jbc.M113.464776

Blackburn, M. R., Vance, C. O., Morschl, E., and Wilson, C. N. (2009). Adenosine receptors and inflammation. *Handb. Exp. Pharmacol.* 193, 215–269. doi: 10.1007/978-3-540-89615-9_8

Blum, D., Gall, D., Galas, M.-C., D'alcantara, P., Bantubungi, K., and Schiffmann, S. N. (2002). The adenosine A 1 receptor agonist adenosine amine congener exerts a neuroprotective effect against the development of striatal lesions and motor impairments in the 3-nitropropionic acid model of neurotoxicity. *J. Neurosci.* 22, 9122–9133.

Bogacheva, P. O., and Balezina, O. P. (2015). Postsynaptic potentiation in mouse motor synapses induced by ATP accumulation in synaptic cleft. *Bull. Exp. Biol. Med.* 159, 583–587. doi: 10.1007/s10517-015-3018-2

Boison, D., Chen, J.-F., and Fredholm, B. B. (2010). Adenosine signaling and function in glial cells. *Cell Death Differ.* 17, 1071–1082. doi: 10.1038/cdd.2009.131

Borea, P. A., Gessi, S., Merighi, S., Vincenzi, F., and Varani, K. (2017). Pathological overproduction: the bad side of adenosine. *Br. J. Pharmacol.* 174, 1945–1960. doi: 10.1111/bph.13763

Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A., Jansen Steur, E. N., and Braak, E. (2013). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* 24, 197–211. doi: 10.1016/S0197-4580(02)00065-9

Bradley, H. J., Baldwin, J. M., Goli, G. R., Johnson, B., Zou, J., Sivaprasadarao, A., et al. (2011). Residues 155 and 348 contribute to the determination of P2X7 receptor function via distinct mechanisms revealed by single-nucleotide polymorphisms. *J. Biol. Chem.* 286, 8176–8187. doi: 10.1074/jbc.M110.211284

Browne, S. E., Yang, L., DiMauro, J.-P., Fuller, S. W., Licata, S. C., and Beal, M. F. (2006). Bioenergetic abnormalities in discrete cerebral motor pathways presage spinal cord pathology in the G93A SOD1 mouse model of ALS. *Neurobiol. Dis.* 22, 599–610. doi: 10.1016/j.nbd.2006.01.001

Burghes, A. H., Ingraham, S. E., Kóte-Jarai, Z., Rosenfeld, S., Herta, N., Nadkarni, N., et al. (1994). Linkage mapping of the spinal muscular atrophy gene. *Hum. Genet.* 93, 305–312. doi: 10.1007/BF00212028

Burnstock, G. (1972). Purinergic nerves. *Pharmacol. Rev.* 24, 509–581.

Burnstock, G. (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* 36, 1127–1139. doi: 10.1016/S0028-3908(97)00125-1

Burnstock, G. (2009). Purinergic cotransmission. *Exp. Physiol.* 94, 20–24. doi: 10.1113/expphysiol.2008.043620

Burnstock, G. (2016). An introduction to the roles of purinergic signalling in neurodegeneration, neuroprotection and neuroregeneration. *Neuropharmacology* 104, 4–17. doi: 10.1016/j.neuropharm.2015.05.031

Burnstock, G. (2017). The therapeutic potential of purinergic signalling. *Biochem. Pharmacol.* doi: 10.1016/j.bcp.2017.07.016. [Epub ahead of print].

Burnstock, G., Arnett, T. R., and Orriss, I. R. (2013). Purinergic signalling in the musculoskeletal system. *Purinergic Signal.* 9, 541–572. doi: 10.1007/s11302-013-9381-4

Burnstock, G., Fredholm, B. B., and Verkhratsky, A. (2011). Adenosine and ATP receptors in the brain. *Curr. Top. Med. Chem.* 11, 973–1011. doi: 10.2174/156802611795347627

Burnstock, G., Campbell, G., Satchell, D., and Smythe, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.* 40, 668–688. doi: 10.1111/j.1476-5381.1970.tb10646.x

Butt, A. M. (2006). Neurotransmitter-mediated calcium signalling in oligodendrocyte physiology and pathology. *Glia* 54, 666–675. doi: 10.1002/glia.20424

Cabrini, G., Falzoni, S., Forchap, S. L., Pellegatti, P., Balboni, A., Agostini, P., et al. (2005). A His-155 to Tyr polymorphism confers gain-of-function to the human P2X7 receptor of human leukemic lymphocytes. *J. Immunol.* 175, 82–89. doi: 10.4049/jimmunol.175.1.82

Caragnano, M., Tortorella, P., Bergami, A., Ruggieri, M., Livrea, P., Specchio, L. M., et al. (2012). Monocytes P2X7 purinergic receptor is modulated by glatiramer acetate in multiple sclerosis. *J. Neuroimmunol.* 245, 93–97. doi: 10.1016/j.jneuroim.2012.02.002

Carmo, M. R., Menezes, A. P., Nunes, A. C., Pliássova, A., Rolo, A. P., Palmeira, C. M., et al. (2014). The P2X7 receptor antagonist Brilliant Blue G attenuates contralateral rotations in a rat model of Parkinsonism through a combined control of synaptotoxicity, neurotoxicity and gliosis. *Neuropharmacology* 81, 142–152. doi: 10.1016/j.neuropharm.2014.01.045

Casanovas, A., Hernández, S., Tarabal, O., Rosselló, J., and Esquerda, J. E. (2008). Strong P2X4 purinergic receptor-like immunoreactivity is selectively associated with degenerating neurons in transgenic rodent models of amyotrophic lateral sclerosis. *J. Comp. Neurol.* 506, 75–92. doi: 10.1002/cne.21527

Cha, J. H., Frey, A. S., Alsdorf, S. A., Kerner, J. A., Kosinski, C. M., Mangiarini, L., et al. (1999). Altered neurotransmitter receptor expression in transgenic mouse models of Huntington's disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354, 981–989.

Cheffer, A., Castillo, A. R., Corrêa-Velloso, J. C., Gonçalves, M. C. B., Naaldijk, Y., Nascimento, I. C., et al. (2017). Purinergic system in psychiatric diseases. *Mol. Psychiatry* 23, 94–106. doi: 10.1038/mp.2017.188

Chen, L., and Brosnan, C. F. (2006). Exacerbation of experimental autoimmune encephalomyelitis in P2X7R-/- mice: evidence for loss of apoptotic activity in lymphocytes. *J. Immunol.* 176, 3115–3126. doi: 10.4049/jimmunol.176.5.3115

Chiang, M. C., Lee, Y. C., Huang, C. L., and Chern, Y. (2005). cAMP-response element-binding protein contributes to suppression of the A2A adenosine receptor promoter by mutant huntingtin with expanded polyglutamine residues. *J. Biol. Chem.* 280, 14331–14340. doi: 10.1074/jbc.M413279200

Chiu, F. L., Lin, J. T., Chuang, C. Y., Chien, T., Chen, C. M., Chen, K. H., et al. (2015). Elucidating the role of the A2Aadenosine receptor in neurodegeneration using neurons derived from Huntington's disease iPSCs. *Hum. Mol. Genet.* 24, 6066–6079. doi: 10.1093/hmg/ddv318

Chou, S.-Y., Lee, Y.-C., Chen, H.-M., Chiang, M.-C., Lai, H.-L., Chang, H.-H., et al. (2005). CGS21680 attenuates symptoms of Huntington's disease in a transgenic mouse model. *J. Neurochem.* 93, 310–320. doi: 10.1111/j.1471-4159.2005.03029.x

Ciruela, F., Casadó, V., Rodrigues, R. J., Luján, R., Burgueño, J., Canals, M., et al. (2006). Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. *J. Neurosci.* 26, 2080–2087. doi: 10.1523/JNEUROSCI.3574-05.2006

Compston, A., and Coles, A. (2008). Multiple sclerosis. *Lancet* 372, 1502–1517. doi: 10.1016/S0140-6736(08)61620-7

Constantinescu, C. S., Farooqi, N., O'Brien, K., and Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br. J. Pharmacol.* 164, 1079–1106. doi: 10.1111/j.1476-5381.2011.01302.x

Correia-de-Sá, P., Sebastião, A. M., and Ribeiro, J. A. (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat. *Br. J. Pharmacol.* 103, 1614–1620. doi: 10.1111/j.1476-5381.1991.tb09836.x

Correia-de-Sá, P., Timóteo, M. A., and Ribeiro, J. A. (2002). A2A adenosine receptor facilitation of neuromuscular transmission. *J. Neurochem.* 74, 2462–2469. doi: 10.1046/j.1471-4159.2000.0742462.x

Crawford, T. O., and Pardo, C. A. (1996). The neurobiology of childhood spinal muscular atrophy. *Neurobiol. Dis.* 3, 97–110. doi: 10.1006/nbdi.1996.0010

Csóka, B., Töro, G., Vindeirinho, J., Varga, Z. V., Koscsó, B., Németh, Z. H., et al. (2017). A2A adenosine receptors control pancreatic dysfunction in high-fat-diet-induced obesity. *FASEB J.* 31, 4985–4997. doi: 10.1096/fj.201700398R

Cunha, R. A. (2008). Different cellular sources and different roles of adenosine: A1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A2A receptor-mediated facilitation of plasticity. *Neurochem. Int.* 52, 65–72. doi: 10.1016/j.neuint.2007.06.026

Cunha, R. A. (2016). How does adenosine control neuronal dysfunction and neurodegeneration? *J. Neurochem.* 139, 1019–1055. doi: 10.1111/jnc.13724

D'Ambrosi, N., Finocchi, P., Apolloni, S., Cozzolino, M., Ferri, A., Padovano, V., et al. (2009). The proinflammatory action of microglial P2 receptors is enhanced in SOD1 models for amyotrophic lateral sclerosis. *J. Immunol.* 183, 4648–4656. doi: 10.4049/jimmunol.0901212

DeJesus-Hernandez, M., Mackenzie, I. R., Boeve, B. F., Boxer, A. L., Baker, M., Rutherford, N. J., et al. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* 72, 245–256. doi: 10.1016/j.neuron.2011.09.011

de Lau, L. M., and Breteler, M. M. (2006). Epidemiology of Parkinson's disease. *Lancet Neurol.* 5, 525–535. doi: 10.1016/S1474-4422(06)70471-9

Dendrou, C. A., Fugger, L., and Friese, M. A. (2015). Immunopathology of multiple sclerosis. *Nat. Rev. Immunol.* 15, 545–558. doi: 10.1038/nri3871

De Lorenzo, S., Veggetti, M., Muchnik, S., and Losavio, A. (2004). Presynaptic inhibition of spontaneous acetylcholine release induced by adenosine at the mouse neuromuscular junction. *Br. J. Pharmacol.* 142, 113–124. doi: 10.1038/sj.bjp.0705656

Deuchars, S. A., Atkinson, L., Brooke, R. E., Musa, H., Milligan, C. J., Batten, T. F., et al. (2001). Neuronal P2X7 receptors are targeted to presynaptic terminals in the central and peripheral nervous systems. *J. Neurosci.* 21, 7143–7152.

Dhaenens, C. M., Burnouf, S., Simonin, C., Van Brussel, E., Duhamel, A., Defebvre, L., et al. (2009). A genetic variation in the ADORA2A gene modifies age at onset in Huntington's disease. *Neurobiol. Dis.* 35, 474–476. doi: 10.1016/j.nbd.2009.06.009

Díaz-Hernández, M., Díez-Zaera, M., Sánchez-Nogueiro, J., Gómez-Villafuertes, R., Canals, J. M., Alberch, J., et al. (2009). Altered P2X7-receptor level and function in mouse models of Huntington's disease and therapeutic efficacy of antagonist administration. *FASEB J.* 23, 1893–906. doi: 10.1096/fj.08-122275

Domenici, M. R., Scattoni, M. L., Martire, A., Lastoria, G., Potenza, R. L., Borioni, A., et al. (2007). Behavioral and electrophysiological effects of the adenosine A 2A receptor antagonist SCH 58261 in R6/2 Huntington's disease mice. *Neurobiol. Dis.* 28, 197–205. doi: 10.1016/j.nbd.2007.07.009

Domercq, M., Perez-Samartin, A., Aparicio, D., Alberdi, E., Pampliega, O., and Matute, C. (2009). P2X7 receptors mediate ischemic damage to oligodendrocytes. *Glia* 58, 730–740. doi: 10.1002/glia.20958

Elbaz, A., Grigoletto, F., Baldereschi, M., Breteler, M. M., Manubens-Bertran, J. M., Lopez-Pousa, S., et al. (1999). Familial aggregation of Parkinson's disease: a population-based case-control study in Europe. EUROPARKINSON study group. *Neurology* 52, 1876–1882.

Eng, L. F., Ghirnikar, R. S., and Lee, Y. L. (1996). Inflammation in EAE: role of chemokine/cytokine expression by resident and infiltrating cells. *Neurochem. Res.* 21, 511–525. doi: 10.1007/BF02527717

Estévez, A. G., Sampson, J. B., Zhuang, Y. X., Spear, N., Richardson, G. J., Crow, J. P., et al. (2000). Liposome-delivered superoxide dismutase prevents nitric oxide-dependent motor neuron death induced by trophic factor withdrawal. *Free Radic. Biol. Med.* 28, 437–446. doi: 10.1016/S0891-5849(99)00261-0

Estévez, A. G., Spear, N., Manuel, S. M., Barbeito, L., Radi, R., and Beckman, J. S. (1998). Role of endogenous nitric oxide and peroxynitrite formation in the survival and death of motor neurons in culture. *Prog. Brain Res.* 118, 269–280. doi: 10.1016/S0079-6123(08)63214-8

Faas, M. M., Sáez, T., and de Vos, P. (2017). Extracellular ATP and adenosine: the Yin and Yang in immune responses? *Mol. Aspects Med.* 55, 9–19. doi: 10.1016/j.mam.2017.01.002

Färber, K., Markworth, S., Pannasch, U., Nolte, C., Prinz, V., Kronenberg, G., et al. (2008). The ectonucleotidase *cd39* /ENTPDase1 modulates purinergic-mediated microglial migration. *Glia* 56, 331–341. doi: 10.1002/glia.20606

Faria, R. X., Freitas, H. R., and Reis, R. A. M. (2017). P2X7 receptor large pore signaling in avian Müller glial cells. *J. Bioenerg. Biomembr.* 49, 215–229. doi: 10.1007/s10863-017-9717-9

Ferré, S. (2010). Role of the central ascending neurotransmitter systems in the psychostimulant effects of caffeine. *J. Alzheimers Dis.* 20, S35–S49. doi: 10.3233/JAD-2010-1400

Ferré, S., Agnati, L. F., Ciruela, F., Lluis, C., Woods, A. S., Fuxe, K., et al. (2007). Neurotransmitter receptor heteromers and their integrative role in "local modules": the striatal spine module. *Brain Res. Rev.* 55, 55–67. doi: 10.1016/j.brainresrev.2007.01.007

Ferré, S., Earley, C., Gulyani, S., and Garcia-Borreguero, D. (2017). In search of alternatives to dopaminergic ligands for the treatment of restless legs syndrome: iron, glutamate, and adenosine. *Sleep Med.* 31, 86–92. doi: 10.1016/j.sleep.2016.08.019

Ferré, S., O'Connor, W. T., Snaprud, P., Ungerstedt, U., and Fuxe, K. (1994). Antagonistic interaction between adenosine A2A receptors and dopamine D2 receptors in the ventral striopallidal system. Implications for the treatment of schizophrenia. *Neuroscience* 63, 765–773.

Ferre, S., O'Connor, W. T., Svenningsson, P., Bjorklund, L., Lindberg, J., Tinner, B., et al. (1996). Dopamine D1 receptor-mediated facilitation of GABAergic neurotransmission in the rat strioentopenduncular pathway and its modulation by adenosine A1 receptor-mediated mechanisms. *Eur. J. Neurosci.* 8, 1545–1553.

Ferré, S., Quiroz, C., Guitart, X., Rea, W., Seyedian, A., Moreno, E., et al. (2018). Pivotal role of adenosine neurotransmission in restless legs syndrome. *Front. Neurosci.* 11:722. doi: 10.3389/fnins.2017.00722

Ferraiuolo, L., Heath, P. R., Holden, H., Kasher, P., Kirby, J., and Shaw, P. J. (2007). Microarray analysis of the cellular pathways involved in the adaptation to and progression of motor neuron injury in the SOD1 G93A mouse model of familial ALS. *J. Neurosci.* 27, 9201–9219. doi: 10.1523/JNEUROSCI.1470-07.2007

Ferraiuolo, L., Higginbottom, A., Heath, P. R., Barber, S., Greenald, D., Kirby, J., et al. (2011). Dysregulation of astrocyte-motoneuron cross-talk in mutant superoxide dismutase 1-related amyotrophic lateral sclerosis. *Brain* 134, 2627–2641. doi: 10.1093/brain/awr193

Ferrante, A., Martire, A., Armida, M., Chiodi, V., Pézzola, A., Potenza, R. L., et al. (2010). Influence of CGS 21680, a selective adenosine A 2A receptor agonist, on NMDA receptor function and expression in the brain of Huntington's disease mice. *Brain Res.* 1323, 184–191. doi: 10.1016/j.brainres.2010.01.080

Ferrari, D., Villalba, M., Chiozzi, P., Falzoni, S., Ricciardi-Castagnoli, P., and Di Virgilio, F. (1996). Mouse microglial cells express a plasma membrane pore gated by extracellular ATP. *J. Immunol.* 156, 1531–1539.

Ferrazoli, E. G., de Souza, H. D. N., Nascimento, I. C., Oliveira-Giacomelli, Á., Schwindt, T. T., Britto, L. R., et al. (2017). Brilliant blue G, but not fenofibrate, treatment reverts hemiparkinsonian behavior and restores dopamine levels in an animal model of Parkinson's disease. *Cell Transplant.* 26, 669–677. doi: 10.3727/096368917X695227

Ferreira, D. G., Batalha, V. L., Vicente Miranda, H., Coelho, J. E., Gomes, R., Gonçalves, F. Q., et al. (2015). Adenosine A $_{2A}$ receptors modulate α-synuclein aggregation and toxicity. *Cereb. Cortex* 27:bhv268. doi: 10.1093/cercor/bhv268

Fields, R. D., and Burnstock, G. (2006). Purinergic signalling in neuron-glia interactions. *Nat. Rev. Neurosci.* 7, 423–436. doi: 10.1038/nrn1928

Fink, J. S., Kalda, A., Ryu, H., Stack, E. C., Schwarzschild, M. A., Chen, J. F., et al. (2004). Genetic and pharmacological inactivation of the adenosine A2Areceptor attenuates 3-nitropropionic acid-induced striatal damage. *J. Neurochem.* 88, 538–544. doi: 10.1046/j.1471-4159.2003.02145.x

Fink, J. S., Weaver, D. R., Rivkees, S. A., Peterfreund, R. A., Pollack, A. E., Adler, E. M., et al. (1992). Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res. Mol. Brain Res.* 14, 186–195. doi: 10.1016/0169-328X(92)90173-9

Florenzano, F., Viscomi, M. T., Cavaliere, F., Volonté, C., and Molinari, M. (2002). Cerebellar lesion up-regulates P2X1 and P2X2 purinergic receptors in precerebellar nuclei. *Neuroscience* 115, 425–434. doi: 10.1016/S0306-4522(02)00397-4

Fondell, E., O'Reilly, É. I., Fitzgerald, K. C., Falcone, G. J., Kolonel, L. N., Park, Y., et al. (2015). Intakes of caffeine, coffee and tea and risk of amyotrophic lateral sclerosis: Results from five cohort studies. *Amyotroph. Lateral Scler. Front. Degener.* 16, 366–371. doi: 10.3109/21678421.2015.1020813

Förster, D., and Reiser, G. (2015). Supportive or detrimental roles of P2Y receptors in brain pathology?–The two faces of P2Y receptors in stroke and neurodegeneration detected in neural cell and in animal model studies. *Purinergic Signal.* 11, 441–454. doi: 10.1007/s11302-015-9471-6

Franco, M. C., Ye, Y., Refakis, C. A., Feldman, J. L., Stokes, A. L., Basso, M., et al. (2013). Nitration of Hsp90 induces cell death. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1102–E1111. doi: 10.1073/pnas.1215177110

Franke, H., Günther, A., Grosche, J., Schmidt, R., Rossner, S., Reinhardt, R., et al. (2004). P2X7 receptor expression after ischemia in the cerebral cortex of rats. *J. Neuropathol. Exp. Neurol.* 63, 686–699. doi: 10.1093/jnen/63.7.686

Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A., and Zvartau, E. E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* 51, 83–133.

Fryer, H. J., Wolf, D. H., Knox, R. J., Strittmatter, S. M., Pennica, D., O'Leary, R. M., et al. (2001). Brain-derived neurotrophic factor induces excitotoxic sensitivity in cultured embryonic rat spinal motor neurons through activation of the phosphatidylinositol 3-kinase pathway. *J. Neurochem.* 74, 582–595. doi: 10.1046/j.1471-4159.2000.740582.x

Fuchs, A., Kutterer, S., Mühling, T., Duda, J., Schütz, B., Liss, B., et al. (2013). Selective mitochondrial Ca $^{2+}$ uptake deficit in disease endstage vulnerable motoneurons of the SOD1 ^{G93A} mouse model of amyotrophic lateral sclerosis. *J. Physiol. (Lond).* 591, 2723–2745. doi: 10.1113/jphysiol.2012.247981

Fuzzati-Armentero, M.-T., Cerri, S., Levandis, G., Ambrosi, G., Montepeloso, E., Antoninetti, G., et al. (2015). Dual target strategy: combining distinct non-dopaminergic treatments reduces neuronal cell loss and synergistically modulates l -DOPA-induced rotational behavior in a rodent model of Parkinson's disease. *J. Neurochem.* 134, 740–747. doi: 10.1111/jnc.13162

Gabanella, F., Butchbach, M. E., Saieva, L., Carissimi, C., Burghes, A. H., and Pellizzoni, L. (2007). Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. *PLoS ONE* 2:e921. doi: 10.1371/journal.pone.0000921

Gan, M., Moussaud, S., Jiang, P., and McLean, P. J. (2015). Extracellular ATP induces intracellular alpha-synuclein accumulation via P2X1 receptor-mediated lysosomal dysfunction. *Neurobiol. Aging* 36, 1209–1220. doi: 10.1016/j.neurobiolaging.2014.10.037

Gandelman, M., Levy, M., Cassina, P., Barbeito, L., and Beckman, J. S. (2013). P2X7 receptor-induced death of motor neurons by a peroxynitrite/FAS-dependent pathway. *J. Neurochem.* 126, 382–388. doi: 10.1111/jnc.12286

Gandelman, M., Peluffo, H., Beckman, J. S., Cassina, P., and Barbeito, L. (2010). Extracellular ATP and the P2X7 receptor in astrocyte-mediated motor neuron death: implications for amyotrophic lateral sclerosis. *J. Neuroinflammation* 7:33. doi: 10.1186/1742-2094-7-33

Garcia-Borreguero, D., and Cano-Pumarega, I. (2017). New concepts in the management of restless legs syndrome. *BMJ* 356:j104. doi: 10.1136/bmj.j104

Gavrilina, T. O., McGovern, V. L., Workman, E., Crawford, T. O., Gogliotti, R. G., DiDonato, C. J., et al. (2008). Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Hum. Mol. Genet.* 17, 1063–1075. doi: 10.1093/hmg/ddm379

Gianfriddo, M., Corsi, C., Melani, A., Pèzzola, A., Reggio, R., Popoli, P., et al. (2003). A denosine A antagonism increases striatal glutamate outflow in the 2A quinolinic acid rat model of Huntington's disease. *Brain Res.* 979, 225–229. doi: 10.1016/S0006-8993(03)02942-1

Gianfriddo, M., Melani, A., Turchi, D., Giovannini, M. G., and Pedata, F. (2004). Adenosine and glutamate extracellular concentrations and mitogen-activated protein kinases in the striatum of Huntington transgenic mice. Selective antagonism of adenosine A_{2A} receptors reduces transmitter outflow. *Neurobiol. Dis.* 17, 77–88. doi: 10.1016/j.nbd.2004.05.008

Glass, M., Dragunow, M., and Faull, R. L. (2000). The pattern of neurodegeneration in Huntington's disease: a comparative study of cannabinoid, dopamine, adenosine and GABA(A) receptor alterations in the human basal ganglia in Huntington's disease. *Neuroscience* 97, 505–519. doi: 10.1016/S0306-4522(00)00008-7

Goldenberg, M. M. (2012). Multiple sclerosis review. *P T* 37, 175–184.

Gołembiowska, K., Wardas, J., Noworyta-Sokołowska, K., Kaminska, K., and Górska, A. (2013). Effects of adenosine receptor antagonists on the *in vivo* LPS-induced inflammation model of Parkinson's disease. *Neurotox. Res.* 24, 29–40. doi: 10.1007/s12640-012-9372-1

Gonçalves, N., Simões, A. T., Cunha, R. A., and de Almeida, L. P. (2013). Caffeine and adenosine A_{2A} receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado-Joseph disease. *Ann. Neurol.* 73, 655–666. doi: 10.1002/ana.23866

Gonçalves, N., Simões, A. T., Prediger, R. D., Hirai, H., Cunha, R. A., and Pereira de Almeida, L. (2017). Caffeine alleviates progressive motor deficits in a transgenic mouse model of spinocerebellar ataxia. *Ann. Neurol.* 81, 407–418. doi: 10.1002/ana.24867

Grundström, E., Lindholm, D., Johansson, A., Blennow, K., and Askmark, H. (2000). GDNF but not BDNF is increased in cerebrospinal fluid in amyotrophic lateral sclerosis. *Neuroreport* 11, 1781–1783. doi: 10.1097/00001756-200006050-00037

Grygorowicz, T., Sulejczak, D., and Struzynska, L. (2011). Expression of purinergic P2X7 receptor in rat brain during the symptomatic phase of experimental autoimmune encephalomyelitis and after recovery of neurological deficits. *Acta Neurobiol. Exp.* 71, 65–73.

Gu, B. J., Field, J., Dutertre, S., Ou, A., Kilpatrick, T. J., Lechner-Scott, J., et al. (2015). A rare P2X7 variant Arg307Gln with absent pore formation function protects against neuroinflammation in multiple sclerosis. *Hum. Mol. Genet.* 24, 5644–5654. doi: 10.1093/hmg/ddv278

Gu, B. J., Zhang, W., Worthington, R. A., Sluyter, R., Dao-Ung, P., Petrou, S., et al. (2001). A Glu-496 to Ala polymorphism leads to loss of function of the human P2X7 receptor. *J. Biol. Chem.* 276, 11135–11142. doi: 10.1074/jbc.M010353200

Gulyani, S., Earley, C. J., Camandola, S., Maudsley, S., Ferré, S., Mughal, M. R., et al. (2009). Diminished iron concentrations increase adenosine A_{2A} receptor levels in mouse striatum and cultured human neuroblastoma cells. *Exp. Neurol.* 215, 236–242. doi: 10.1016/j.expneurol.2008.10.007

Gyoneva, S., Shapiro, L., Lazo, C., Garnier-Amblard, E., Smith, Y., Miller, G. W., et al. (2014). Adenosine A_{2A} receptor antagonism reverses inflammation-induced impairment of microglial process extension in a model of Parkinson's disease. *Neurobiol. Dis.* 67, 191–202. doi: 10.1016/j.nbd.2014.03.004

Hamza, T. H., Chen, H., Hill-Burns, E. M., Rhodes, S. L., Montimurro, J., Kay, D. M., et al. (2011). Genome-wide gene-environment study identifies glutamate receptor gene GRIN2A as a Parkinson's disease modifier gene via interaction with coffee. *PLoS Genet.* 7:e1002237. doi: 10.1371/journal.pgen.1002237

Harms, M. B., and Baloh, R. H. (2013). Clinical neurogenetics: amyotrophic lateral sclerosis. *Neurol. Clin.* 31, 929–950. doi: 10.1016/j.ncl.2013.05.003

Haskó, G. (2004). Adenosine: an endogenous regulator of innate immunity. *Trends Immunol.* 25, 33–39. doi: 10.1016/j.it.2003.11.003

Haynes, S. E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M. E., Gan, W.-B., et al. (2006). The P2Y12 receptor regulates microglial activation

by extracellular nucleotides. *Nat. Neurosci.* 9, 1512–1519. doi: 10.1038/nn1805

He, Y., Taylor, N., Fourgeaud, L., and Bhattacharya, A. (2017). The role of microglial P2X7: modulation of cell death and cytokine release. *J. Neuroinflammation* 14:135. doi: 10.1186/s12974-017-0904-8

Hedström, A. K., Mowry, E. M., Gianfrancesco, M. A., Shao, X., Schaefer, C. A., Shen, L., et al. (2016). High consumption of coffee is associated with decreased multiple sclerosis risk; results from two independent studies. *J. Neurol. Neurosurg. Psychiatry* 87, 454–460. doi: 10.1136/jnnp-2015-312176

Hettinger, B. D., Lee, A., Linden, J., and Rosin, D. L. (2001). Ultrastructural localization of adenosine A2A receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *J. Comp. Neurol.* 431, 331–346. doi: 10.1002/1096-9861(20010312)431:3<331::AID-CNE1074>3.0.CO;2-W

Hoang, Q. Q. (2014). Pathway for Parkinson disease. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2402–2403. doi: 10.1073/pnas.1324284111

Hu, P., and Kalb, R. G. (2003). BDNF heightens the sensitivity of motor neurons to excitotoxic insults through activation of TrkB. *J. Neurochem.* 84, 1421–1430. doi: 10.1046/j.1471-4159.2003.01599.x

Hu, Q., Ren, X., Liu, Y., Li, Z., Zhang, L., Chen, X., et al. (2016). Aberrant adenosine A2A receptor signaling contributes to neurodegeneration and cognitive impairments in a mouse model of synucleinopathy. *Exp. Neurol.* 283, 213–223. doi: 10.1016/j.expneurol.2016.05.040

Illes, P., Khan, T. M., and Rubini, P. (2017). Neuronal P2X7 receptors revisited: do they really exist? *J. Neurosci.* 37, 7049–7062. doi: 10.1523/JNEUROSCI.3103-16.2017

Illes, P., and Verkhratsky, A. (2016). Purinergic neurone-glia signalling in cognitive-related pathologies. *Neuropharmacology* 104, 62–75. doi: 10.1016/j.neuropharm.2015.08.005

Inoue, K. (2017). Purinergic signaling in microglia in the pathogenesis of neuropathic pain. *Proc. Japan Acad. Ser. B* 93, 174–182. doi: 10.2183/pjab.93.011

Ishikawa, Y., Ikeuchi, T., and Hatanaka, H. (2000). Brain-derived neurotrophic factor accelerates nitric oxide donor-induced apoptosis of cultured cortical neurons. *J. Neurochem.* 75, 494–502. doi: 10.1046/j.1471-4159.2000.0750494.x

Ishiwata, K., Ogi, N., Hayakawa, N., Oda, K., Nagaoka, T., Toyama, H., et al. (2002). Adenosine A_{2A} receptor imaging with [^{11}C]KF18446 PET in the rat brain after quinolinic acid lesion: comparison with the dopamine receptor imaging. *Ann. Nucl. Med.* 16, 467–475. doi: 10.1007/BF02988643

Jablonka, S., Rossoll, W., Schrank, B., and Sendtner, M. (2000). The role of SMN in spinal muscular atrophy. *J. Neurol.* 247(Suppl. 1), I37–I42. doi: 10.1007/s004150050555

Jellinger, K. A. (2011). "Parkinson's disease," in *Neurodegeneration : The Molecular Pathology of Dementia and Movement Disorders*, eds D. W. Dickson and R. O. Welle (Bassel: Wiley-Blackwell), 477.

Jiang, T., Hoekstra, J., Heng, X., Kang, W., Ding, J., Liu, J., et al. (2015). P2X7 receptor is critical in α-synuclein–mediated microglial NADPH oxidase activation. *Neurobiol. Aging* 36, 2304–2318. doi: 10.1016/j.neurobiolaging.2015.03.015

Jiménez, M., Clavé, P., Accarino, A., and Gallego, D. (2014). Purinergic neuromuscular transmission in the gastrointestinal tract; functional basis for future clinical and pharmacological studies. *Br. J. Pharmacol.* 171, 4360–4375. doi: 10.1111/bph.12802

Johnston, J. B., Silva, C., Gonzalez, G., Holden, J., Warren, K. G., Metz, L. M., et al. (2001). Diminished adenosine A1 receptor expression on macrophages in brain and blood of patients with multiple sclerosis. *Ann. Neurol.* 49, 650–658. doi: 10.1002/ana.1007

Jung, C., Higgins, C. M., and Xu, Z. (2002). Mitochondrial electron transport chain complex dysfunction in a transgenic mouse model for amyotrophic lateral sclerosis. *J. Neurochem.* 83, 535–545. doi: 10.1046/j.1471-4159.2002.01112.x

Kachroo, A., and Schwarzschild, M. A. (2012). Adenosine A_{2A} receptor gene disruption protects in an α-synuclein model of Parkinson's disease. *Ann. Neurol.* 71, 278–282. doi: 10.1002/ana.22630

Kalmar, J. M. (2005). The influence of caffeine on voluntary muscle activation. *Med. Sci. Sports Exerc.* 37, 2113–2119. doi: 10.1249/01.mss.0000178219.18086.9e

Kanda, T., Jackson, M. J., Smith, L. A., Pearce, R. K., Nakamura, J., Kase, H., et al. (2000). Combined use of the adenosine A2A antagonist KW-6002 with l-DOPA or with selective D1 or D2 dopamine agonists increases antiparkinsonian activity but not dyskinesia in MPTP-treated monkeys. *Exp. Neurol.* 162, 321–327. doi: 10.1006/exnr.2000.7350

Karcz-Kubicha, M., Antoniou, K., Terasmaa, A., Quarta, D., Solinas, M., Justinova, Z., et al. (2003). Involvement of adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. *Neuropsychopharmacology* 28, 1281–1291. doi: 10.1038/sj.npp.1300167

Kazemi, M. H., Raoofi Mohseni, S., Hojjat-Farsangi, M., Anvari, E., Ghalamfarsa, G., Mohammadi, H., et al. (2018). Adenosine and adenosine receptors in the immunopathogenesis and treatment of cancer. *J. Cell. Physiol.* 233, 2032–2057. doi: 10.1002/jcp.25873

Khakh, B. S., and Alan North, R. (2006). P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 442, 527–532. doi: 10.1038/nature04886

Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., et al. (2011). Amyotrophic lateral sclerosis. *Lancet* 377, 942–955. doi: 10.1016/S0140-6736(10)61156-7

Kim, B., Jeong, H. K., Kim, J. H., Lee, S. Y., Jou, I., and Joe, E. H. (2011). Uridine 5′-diphosphate induces chemokine expression in microglia and astrocytes through activation of the P2Y6 receptor. *J. Immunol.* 186, 3701–3709. doi: 10.4049/jimmunol.1000212

Kim, H. (2003). TrkB mediates BDNF-induced potentiation of neuronal necrosis in cortical culture. *Neurobiol. Dis.* 14, 110–119. doi: 10.1016/S0969-9961(03)00103-7

Kim, T., and Vemuganti, R. (2017). Mechanisms of Parkinson's disease-related proteins in mediating secondary brain damage after cerebral ischemia. *J. Cereb. Blood Flow Metab.* 37, 1910–1926. doi: 10.1177/0271678X17694186

Kobayashi, S., Conforti, L., Pun, R. Y. K., and Millhorn, D. E. (1998). Adenosine modulates hypoxia-induced responses in rat PC12 cells via the A_{2A} receptor. *J. Physiol.* 508, 95–107. doi: 10.1111/j.1469-7793.1998.095br.x

Koehler, N. K., Genain, C. P., Giesser, B., and Hauser, S. L. (2002). The human T cell response to myelin oligodendrocyte glycoprotein: a multiple sclerosis family-based study. *J. Immunol.* 168, 5920–5927. doi: 10.4049/jimmunol.168.11.5920

Koh, J., Gwag, B. J., Lobner, D., and Choi, D. W. (1995). Potentiated necrosis of cultured cortical neurons by neurotrophins. *Science* 268, 573–575. doi: 10.1126/science.7725105

Krügel, U. (2016). Purinergic receptors in psychiatric disorders. *Neuropharmacology* 104, 212–225. doi: 10.1016/j.neuropharm.2015.10.032

Kumar, P. M., Paing, S. S., Li, H., Pavanni, R., Yuen, Y., Zhao, Y., et al. (2015). Differential effect of caffeine intake in subjects with genetic susceptibility to Parkinson's Disease. *Sci. Rep.* 5:15492. doi: 10.1038/srep15492

Labazi, H., Teng, B., and Mustafa, S. J. (2018). Functional changes in vascular reactivity to adenosine receptor activation in type I diabetic mice. *Eur. J. Pharmacol.* 820, 191–197. doi: 10.1016/j.ejphar.2017.12.034

Lang, A. E. (2009). When and how should treatment be started in Parkinson disease? *Neurology* 72, S39–S43. doi: 10.1212/WNL.0b013e318198e177

Lapato, A. S., and Tiwari-Woodruff, S. K. (2017). Connexins and pannexins: At the junction of neuro-glial homeostasis & disease. *J. Neurosci. Res.* 96, 31–44. doi: 10.1002/jnr.24088

Lassmann, H. (1983). Chronic relapsing experimental allergic encephalomyelitis: its value as an experimental model for multiple sclerosis. *J. Neurol.* 229, 207–220. doi: 10.1007/BF00313549

Lecca, D., Ceruti, S., Fumagalli, M., and Abbracchio, M. P. (2012). Purinergic trophic signalling in glial cells: functional effects and modulation of cell proliferation, differentiation, and death. *Purinergic Signal.* 8, 539–557. doi: 10.1007/s11302-012-9310-y

Lederer, C. W., Torrisi, A., Pantelidou, M., Santama, N., and Cavallaro, S. (2007). Pathways and genes differentially expressed in the motor cortex of patients with sporadic amyotrophic lateral sclerosis. *BMC Genomics* 8:26. doi: 10.1186/1471-2164-8-26

Lee, F. S., and Chao, M. V. (2001). Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3555–3560. doi: 10.1073/pnas.061020198

Lee, J. Y., Jhun, B. S., Oh, Y. T., Lee, J. H., Choe, W., Baik, H. H., et al. (2006). Activation of adenosine A3 receptor suppresses lipopolysaccharide-induced TNF-alpha production through inhibition of PI 3-kinase/Akt and NF-kappaB activation in murine BV2 microglial cells. *Neurosci. Lett.* 396, 1–6. doi: 10.1016/j.neulet.2005.11.004

Lefebvre, S., Bürglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., et al. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 80, 155–165. doi: 10.1016/0092-8674(95)90460-3

Lefebvre, S., Burlet, P., Liu, Q., Bertrandy, S., Clermont, O., Munnich, A., et al. (1997). Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.* 16, 265–269. doi: 10.1038/ng0797-265

Levy, O., Coughlin, M., Cronstein, B. N., Roy, R. M., Desai, A., and Wessels, M. R. (2006). The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. *J. Immunol.* 177, 1956–1966. doi: 10.4049/jimmunol.177.3.1956

Li, J. Y., Popovic, N., and Brundin, P. (2005). The use of the R6 transgenic mouse models of Huntington's disease in attempts to develop novel therapeutic strategies. *NeuroRx* 2, 447–464. doi: 10.1602/neurorx.2.3.447

Li, S. N., and Wong, P. T. (2000). The adenosine receptor agonist, APNEA, increases calcium influx into rat cortical synaptosomes through N-type channels associated with A2a receptors. *Neurochem. Res.* 25, 457–459. doi: 10.1023/A:1007503907823

Li, W., Silva, H. B., Real, J., Wang, Y.-M., Rial, D., Li, P., et al. (2015). Inactivation of adenosine A2A receptors reverses working memory deficits at early stages of Huntington's disease models. *Neurobiol. Dis.* 79, 70–80. doi: 10.1016/j.nbd.2015.03.030

Lindberg, D., Shan, D., Ayers-Ringler, J., Oliveros, A., Benitez, J., Prieto, M., et al. (2015). Purinergic signaling and energy homeostasis in psychiatric disorders. *Curr. Mol. Med.* 15, 275–295. doi: 10.2174/1566524015666150330163724

Lister, M. F., Sharkey, J., Sawatzky, D. A., Hodgkiss, J. P., Davidson, D. J., Rossi, A. G., et al. (2007). The role of the purinergic P2X7 receptor in inflammation. *J. Inflamm. (Lond).* 4:5. doi: 10.1186/1476-9255-4-5

Liu, H., Han, X., Li, Y., Zou, H., and Xie, A. (2013). Association of P2X7 receptor gene polymorphisms with sporadic Parkinson's disease in a Han Chinese population. *Neurosci. Lett.* 546, 42–45. doi: 10.1016/j.neulet.2013.04.049

Liu, Y.-J., Lee, L.-M., Lai, H.-L., and Chern, Y. (2015). Aberrant activation of AMP-activated protein kinase contributes to the abnormal distribution of HuR in amyotrophic lateral sclerosis. *FEBS Lett.* 589, 432–439. doi: 10.1016/j.febslet.2014.12.029

Lo, J.-C., Huang, W.-C., Chou, Y.-C., Tseng, C.-H., Lee, W.-L., and Sun, S. H. (2008). Activation of P2X $_7$ receptors decreases glutamate uptake and glutamine synthetase activity in RBA-2 astrocytes via distinct mechanisms. *J. Neurochem.* 105, 151–164. doi: 10.1111/j.1471-4159.2007.05119.x

Lomen-Hoerth, C. (2011). Clinical phenomenology and neuroimaging correlates in ALS-FTD. *J. Mol. Neurosci.* 45, 656–662. doi: 10.1007/s12031-011-9636-x

Lotti, F., Imlach, W. L., Saieva, L., Beck, E. S., Hao, L. T., Li, D. K., et al. (2012). An SMN-dependent U12 splicing event essential for motor circuit function. *Cell* 151, 440–454. doi: 10.1016/j.cell.2012.09.012

Lovatt, D., Sonnewald, U., Waagepetersen, H. S., Schousboe, A., He, W., Lin, J. H.-C., et al. (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *J. Neurosci.* 27, 12255–12266. doi: 10.1523/JNEUROSCI.3404-07.2007

Lu, W., Albalawi, F., Beckel, J. M., Lim, J. C., Laties, A. M., and Mitchell, C. H. (2017). The P2X7 receptor links mechanical strain to cytokine IL-6 up-regulation and release in neurons and astrocytes. *J. Neurochem.* 141, 436–448. doi: 10.1111/jnc.13998

Luo, C., Jian, C., Liao, Y., Huang, Q., Wu, Y., Liu, X., et al. (2017). The role of microglia in multiple sclerosis. *Neuropsychiatr. Dis. Treat.* 13, 1661–1667. doi: 10.2147/NDT.S140634

Luthi-Carter, R., Strand, A., Peters, N. L., Solano, S. M., Hollingsworth, Z. R., Menon, A. S., et al. (2000). Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. *Hum. Mol. Genet.* 9, 1259–1271. doi: 10.1093/hmg/9.9.1259

Madeira, M. H., Boia, R., Ambrósio, A. F., and Santiago, A. R. (2017). Having a coffee break: the impact of caffeine consumption on microglia-mediated inflammation in neurodegenerative diseases. *Mediators Inflamm.* 2017, 1–12. doi: 10.1155/2017/4761081

Maglione, V., Cannella, M., Martino, T., De Blasi, A., Frati, L., and Squitieri, F. (2005a). The platelet maximum number of A2A-receptor binding sites (Bmax) linearly correlates with age at onset and CAG repeat expansion in Huntington's disease patients with predominant chorea. *Neurosci. Lett.* 393, 27–30. doi: 10.1016/j.neulet.2005.09.037

Maglione, V., Giallonardo, P., Cannella, M., Martino, T., Frati, L., and Squitieri, F. (2005b). Adenosine A_{2A} receptor dysfunction correlates with age at onset anticipation in blood platelets of subjects with Huntington's disease. *Am. J. Med. Genet.* 139B, 101–105. doi: 10.1002/ajmg.b.30223

Mamedova, L. K., Gao, Z.-G., and Jacobson, K. A. (2006). Regulation of death and survival in astrocytes by ADP activating P2Y1 and P2Y12 receptors. *Biochem. Pharmacol.* 72, 1031–1041. doi: 10.1016/j.bcp.2006.07.017

Marcellino, D., Suárez-Boomgaard, D., Sánchez-Reina, M. D., Aguirre, J. A., Yoshitake, T., Yoshitake, S., et al. (2010). On the role of P2X7 receptors in dopamine nerve cell degeneration in a rat model of Parkinson's disease: studies with the P2X7 receptor antagonist A-438079. *J. Neural Transm.* 117, 681–687. doi: 10.1007/s00702-010-0400-0

Mariotti, C., Fancellu, R., and Donato, S. (2005). An overview of the patient with ataxia. *J. Neurol.* 252, 511–518. doi: 10.1007/s00415-005-0814-z

Martinez-Mir, M. I., Probst, A., and Palacios, J. M. (1991). Adenosine A2 receptors: Selective localization in the human basal ganglia and alterations with disease. *Neuroscience* 42, 697–706. doi: 10.1016/0306-4522(91)90038-P

Martire, A., Calamandrei, G., Felici, F., Scattoni, M. L., Lastoria, G., Domenici, M. R., et al. (2007). Opposite effects of the A_{2A} receptor agonist CGS21680 in the striatum of Huntington's disease versus wild-type mice.*Neurosci. Lett.* 417, 78–83. doi: 10.1016/j.neulet.2007.02.034

Martire, A., Ferrante, A., Potenza, R. L., Armida, M., Ferretti, R., Pézzola, A., et al. (2010). Remodeling of striatal NMDA receptors by chronic A2A receptor blockade in Huntington's disease mice. *Neurobiol. Dis.* 37, 99–105. doi: 10.1016/j.nbd.2009.09.012

Matilla-Dueñas, A., Corral-Juan, M., Volpini, V., and Sanchez, I. (2012). *The Spinocerebellar Ataxias: Clinical Aspects And Molecular Genetics*. New York, NY: Springer.

Mattiazzi, M., D'Aurelio, M., Gajewski, C. D., Martushova, K., Kiaei, M., Beal, M. F., et al. (2002). Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J. Biol. Chem.* 277, 29626–29633. doi: 10.1074/jbc.M203065200

Matute, C. (2011). Glutamate and ATP signalling in white matter pathology. *J. Anat.* 219, 53–64. doi: 10.1111/j.1469-7580.2010.01339.x

Matute, C., Torre, I., Perez-Cerda, F., Perez-Samartin, A., Alberdi, E., Etxebarria, E., et al. (2007). P2X7 Receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. *J. Neurosci.* 27, 9525–9533. doi: 10.1523/JNEUROSCI.0579-07.2007

Mayne, M., Shepel, P. N., Jiang, Y., Geiger, J. D., and Power, C. (1999). Dysregulation of Adenosine A 1 Receptor– Mediated Cytokine Expression in Peripheral Blood Mononuclear Cells from Multiple Sclerosis Patients. *Ann. Neurol.* 45, 633–639. doi: 10.1002/1531-8249(199905)45:5<633::AID-ANA12>3.0.CO;2-X

McGivern, J. V., Patitucci, T. N., Nord, J. A., Barabas, M. A., Stucky, C. L., and Ebert, A. D. (2013). Spinal muscular atrophy astrocytes exhibit abnormal calcium regulation and reduced growth factor production. *Glia* 61, 1418–1428. doi: 10.1002/glia.22522

Menzies, F. M., Cookson, M. R., Taylor, R. W., Turnbull, D. M., Chrzanowska-Lightowlers, Z. M., Dong, L., et al. (2002). Mitochondrial dysfunction in a cell culture model of familial amyotrophic lateral sclerosis. *Brain* 125, 1522–1533. doi: 10.1093/brain/awf167

Mievis, S., Blum, D., and Ledent, C. (2011). A_{2A} receptor knockout worsens survival and motor behaviour in a transgenic mouse model of Huntington's disease. *Neurobiol. Dis.* 41, 570–576. doi: 10.1016/j.nbd.2010.09.021

Miles, G. B., Parkis, M. A., Lipski, J., and Funk, G. D. (2002). Modulation of phrenic motoneuron excitability by ATP: consequences for respiratory-related output *in vitro*. *J. Appl. Physiol.* 92, 1899–1910. doi: 10.1152/japplphysiol.00475.2001

Miller, S. D., and Karpus, W. J. (1994). The immunopathogenesis and regulation of T-cell-mediated demyelinating diseases. *Immunol. Today* 15, 356–361. doi: 10.1016/0167-5699(94)90173-2

Mills, J. H., Kim, D.-G., Krenz, A., Chen, J.-F., and Bynoe, M. S. (2012). A2A adenosine receptor signaling in lymphocytes and the central nervous system regulates inflammation during experimental autoimmune encephalomyelitis. *J. Immunol.* 188, 5713–5722. doi: 10.4049/jimmunol.1200545

Mills, J. H., Thompson, L. F., Mueller, C., Waickman, A. T., Jalkanen, S., Niemela, J., et al. (2008). CD73 is required for efficient entry of lymphocytes into the

central nervous system during experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9325–9330. doi: 10.1073/pnas.0711175105

Minghetti, L. (2004). Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *J. Neuropathol. Exp. Neurol.* 63, 901–910. doi: 10.1093/jnen/63.9.901

Miras-Portugal, M. T., Sebastián-Serrano, Á., de Diego García, L., and Díaz-Hernández, M. (2017). Neuronal P2X7 Receptor: Involvement in Neuronal Physiology and Pathology. *J. Neurosci.* 37, 7063–7072. doi: 10.1523/JNEUROSCI.3104-16.2017

Mojsilovic-Petrovic, J., Jeong, G.-B., Crocker, A., Arneja, A., David, S., Russell, D., et al. (2006). protecting motor neurons from toxic insult by antagonism of adenosine A2a and Trk receptors. *J. Neurosci.* 26, 9250–9263. doi: 10.1523/JNEUROSCI.1856-06.2006

Moreno, E., Chiarlone, A., Medrano, M., Puigdellívol, M., Bibic, L., Howell, L. A., et al. (2017). Singular location and signaling profile of adenosine A 2A-Cannabinoid CB 1 receptor heteromers in the dorsal striatum. *Neuropsychopharmacology* 43, 964–977. doi: 10.1038/npp.2017.12

Narcisse, L., Scemes, E., Zhao, Y., Lee, S. C., and Brosnan, C. F. (2005). The cytokine IL-1beta transiently enhances P2X7 receptor expression and function in human astrocytes. *Glia* 49, 245–258. doi: 10.1002/glia.20110

Nascimento, F., Sebastião, A. M., and Ribeiro, J. A. (2015). Presymptomatic and symptomatic ALS SOD1(G93A) mice differ in adenosine A1 and A2A receptor-mediated tonic modulation of neuromuscular transmission. *Purinergic Signal.* 11, 471–480. doi: 10.1007/s11302-015-9465-4

Neher, J. J., Neniskyte, U., Hornik, T., and Brown, G. C. (2014). Inhibition of UDP/P2Y $_6$ purinergic signaling prevents phagocytosis of viable neurons by activated microglia *in vitro* and *in vivo*. *Glia* 62, 1463–1475. doi: 10.1002/glia.22693

Ng, S. K., kah, Higashimori, H., Tolman, M., and Yang, Y. (2015). Suppression of adenosine 2a receptor (A2aR)-mediated adenosine signaling improves disease phenotypes in a mouse model of amyotrophic lateral sclerosis. *Exp. Neurol.* 267, 115–122. doi: 10.1016/j.expneurol.2015.03.004

North, R. A. (2002). Molecular Physiology of P2X Receptors. *Physiol. Rev.* 82, 1013–1067. doi: 10.1152/physrev.00015.2002

Olanow, C. W., Stern, M. B., and Sethi, K. (2009). The scientific and clinical basis for the treatment of Parkinson disease (2009). *Neurology* 72, S1–S136. doi: 10.1212/WNL.0b013e3181a1d44c

Oliveira, A., Illes, P., and Ulrich, H. (2016). Purinergic receptors in embryonic and adult neurogenesis. *Neuropharmacology* 104, 272–281. doi: 10.1016/j.neuropharm.2015.10.008

Oliveros, A., Cho, C. H., Cui, A., Choi, S., Lindberg, D., Hinton, D., et al. (2017). Adenosine A2A receptor and ERK-driven impulsivity potentiates hippocampal neuroblast proliferation. *Transl. Psychiatry* 7, e1095. doi: 10.1038/tp.2017.64

Olsson, T., Barcellos, L. F., and Alfredsson, L. (2017). Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat. Rev. Neurol.* 13, 25–36. doi: 10.1038/nrneurol.2016.187

Orr,ú, M., Zanoveli, J., Quiroz, C., Nguyen, H. P., Guitart, X., and Ferr,é, S. (2011). Functional changes in postsynaptic adenosine A 2A receptors during early stages of a rat model of Huntington disease. *Exp. Neurol.* 232, 76–80. doi: 10.1016/j.expneurol.2011.08.005

Ortiz, R., Ulrich, H., Zarate, C. A., and Machado-Vieira, R. (2015). Purinergic system dysfunction in mood disorders: a key target for developing improved therapeutics. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 57, 117–131. doi: 10.1016/j.pnpbp.2014.10.016

Oyanguren-Desez, O., Rodríguez-Antigüedad, A., Villoslada, P., Domercq, M., Alberdi, E., and Matute, C. (2011). Gain-of-function of P2X7 receptor gene variants in multiple sclerosis. *Cell Calcium* 50, 468–472. doi: 10.1016/j.ceca.2011.08.002

Palma, A. G., Muchnik, S., and Losavio, A. S. (2011). Excitatory effect of the A2A adenosine receptor agonist CGS-21680 on spontaneous and K+-evoked acetylcholine release at the mouse neuromuscular junction. *Neuroscience* 172, 164–176. doi: 10.1016/j.neuroscience.2010.10.015

Parisi, C., Arisi, I., D'Ambrosi, N., Storti, A. E., Brandi, R., D'Onofrio, M., et al. (2013). Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation. *Cell Death Dis.* 4:e959. doi: 10.1038/cddis.2013.491

Parisi, C., Napoli, G., Amadio, S., Spalloni, A., Apolloni, S., Longone, P., et al. (2016). MicroRNA-125b regulates microglia activation and motor neuron death in ALS. *Cell Death Differ.* 23, 531–541. doi: 10.1038/cdd.2015.153

Parpura, V., Fisher, E. S., Lechleiter, J. D., Schousboe, A., Waagepetersen, H. S., Brunet, S., et al. (2017). Glutamate and ATP at the Interface Between Signaling and Metabolism in Astroglia: Examples from Pathology. *Neurochem. Res.* 42, 19–34. doi: 10.1007/s11064-016-1848-6

Paulson, H. L., Shakkottai, V. G., Clark, H. B., and Orr, H. T. (2017). Polyglutamine spinocerebellar ataxias — from genes to potential treatments. *Nat. Rev. Neurosci.* 18, 613–626. doi: 10.1038/nrn.2017.92

Pitt, D., Werner, P., and Rain, C. S. (2000). Glutamate excitotoxicity in a model of multiple sclerosis. *Nat. Med.* 6, 67–70. doi: 10.1038/71555

Popat, R. A., Van Den Eeden, S. K., Tanner, C. M., Kamel, F., Umbach, D. M., Marder, K., et al. (2011). Coffee, ADORA2A, and CYP1A2: the caffeine connection in Parkinson's disease. *Eur. J. Neurol.* 18, 756–765. doi: 10.1111/j.1468-1331.2011.03353.x

Popoli, P., Blum, D., Martire, A., Ledent, C., Ceruti, S., and Abbracchio, M. P. (2007). Functions, dysfunctions and possible therapeutic relevance of adenosine A2A receptors in Huntington's disease. *Prog. Neurobiol.* 81, 331–348. doi: 10.1016/j.pneurobio.2006.12.005

Popoli, P., Pintor, A., Domenici, M. R., Frank, C., Tebano, M. T., P.è, Zzola, A., et al. (2002). Blockade of Striatal Adenosine A 2A Receptor Reduces, through a Presynaptic Mechanism, Quinolinic Acid-Induced Excitotoxicity: Possible Relevance to Neuroprotective Interventions in Neurodegenerative Diseases of the Striatum. *J. Neurosci.* 22, 1967–1975.

Potenza, R. L., Armida, M., Ferrante, A., Pèzzola, A., Matteucci, A., Puopolo, M., et al. (2013). Effects of chronic caffeine intake in a mouse model of amyotrophic lateral sclerosis. *J. Neurosci. Res.* 91, 585–592. doi: 10.1002/jnr.23185

Potenza, R. L., Tebano, M. T., Martire, A., Domenici, M. R., Pepponi, R., Armida, M., et al. (2007). Adenosine A2A receptors modulate BDNF both in normal conditions and in experimental models of Huntington's disease. *Purinergic Signal.* 3, 333–338. doi: 10.1007/s11302-007-9066-y

Probert, L., Akassoglou, K., Pasparakis, M., Kontogeorgost, G., Kollias, G., and Levi-Montalcini, R. (1995). Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor a. *Neurobiology* 92, 11294–11298. doi: 10.1073/pnas.92.24.11294

Przybyła, T., Sakowicz-Burkiewicz, M., and Pawełczyk, T. (2018). Purinergic signaling in B cells. *Acta Biochim. Pol.* 65, 1–7. doi: 10.18388/abp.2017_1588

Puchałowicz, K., Baranowska-Bosiacka, I., Dziedziejko, V., and Chlubek, D. (2015). Purinergic signaling and the functioning of the nervous system cells. *Cell. Mol. Biol. Lett.* 20, 867–918. doi: 10.1515/cmble-2015-0050

Pupillo, E., Bianchi, E., Chi,ò, A., Casale, F., Zecca, C., Tortelli, R., et al. (2017). Amyotrophic lateral sclerosis and food intake. *Amyotroph. Lateral Scler. Front. Degener.* 19, 267–274. doi: 10.1080/21678421.2017.1418002

Qian, Y., Xu, S., Yang, X., and Xiao, Q. (2017). Purinergic receptor P2Y6 contributes to 1-methyl-4-phenylpyridinium-induced oxidative stress and cell death in neuronal SH-SY5Y cells. *J. Neurosci. Res.* 96, 253–264. doi: 10.1002/jnr.24119

Quiroz, C., Gulyani, S., Ruiqian, W., Bonaventura, J., Cutler, R., Pearson, V., et al. (2016). Adenosine receptors as markers of brain iron deficiency: Implications for Restless Legs Syndrome. *Neuropharmacology* 111, 160–168. doi: 10.1016/j.neuropharm.2016.09.002

Rajagopal, R., Chen, Z.-Y., Lee, F. S., and Chao, M. V. (2004). Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. *J. Neurosci.* 24, 6650–6658. doi: 10.1523/JNEUROSCI.0010-04.2004

Raker, V. K., Becker, C., and Steinbrink, K. (2016). The cAMP pathway as therapeutic target in autoimmune and inflammatory diseases. *Front. Immunol.* 7:123. doi: 10.3389/fimmu.2016.00123

Raman, R., Allen, S. P., Goodall, E. F., Kramer, S., Ponger, L.-L., Heath, P. R., et al. (2015). Gene expression signatures in motor neurone disease fibroblasts reveal dysregulation of metabolism, hypoxia-response and RNA processing functions. *Neuropathol. Appl. Neurobiol.* 41, 201–226. doi: 10.1111/nan.12147

Raoul, C., Estévez, A. G., Nishimune, H., Cleveland, D. W., deLapeyrière, O., Henderson, C. E., et al. (2002). Motoneuron death triggered by a specific pathway downstream of Fas. potentiation by ALS-linked SOD1 mutations. *Neuron* 35, 1067–1083. doi: 10.1016/S0896-6273(02)00905-4

Raoul, C., Henderson, C. E., and Pettmann, B. (1999). Programmed cell death of embryonic motoneurons triggered through the Fas death receptor. *J. Cell Biol.* 147, 1049–1062. doi: 10.1083/jcb.147.5.1049

Reggio, R., Pèzzola, A., and Popoli, P. (1999). The intrastratial injection of an adenosine A2 receptor antagonist prevents frontal cortex EEG abnormalities in a rat model of Huntington's disease. *Brain Res*. 83, 315–318. doi: 10.1016/S0006-8993(99)01489-4

Renton, A. E., Chi,ò, A., and Traynor, B. J. (2013). State of play in amyotrophic lateral sclerosis genetics. *Nat. Neurosci.* 17, 17–23. doi: 10.1038/nn.3584

Rezania, K., and Roos, R. P. (2013). Spinal Cord. *Neurol. Clin.* 31, 219–239. doi: 10.1016/j.ncl.2012.09.014

Ribeiro, F. F., Xapelli, S., Miranda-Lourenço, C., Tanqueiro, S. R., Fonseca-Gomes, J., Diógenes, M. J., et al. (2016). Purine nucleosides in neuroregeneration and neuroprotection. *Neuropharmacology* 104, 226–242. doi: 10.1016/j.neuropharm.2015.11.006

Rissanen, E., Virta, J. R., Paavilainen, T., Tuisku, J., Helin, S., Luoto, P., et al. (2013). Adenosine A2A receptors in secondary progressive multiple sclerosis: a [(11)C]TMSX brain PET study. *J. Cereb. Blood Flow Metab.* 33, 1394–1401. doi: 10.1038/jcbfm.2013.85

Robitaille, R. (1995). Purinergic receptors and their activation by endogenous purines at perisynaptic glial cells of the frog neuromuscular junction. *J. Neurosci.* 15, 7121–7131.

Robitaille, R. (1998). Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. *Neuron* 21, 847–855. doi: 10.1016/S0896-6273(00)80600-5

Rochon, D., Rousse, I., and Robitaille, R. (2001). Synapse-glia interactions at the mammalian neuromuscular junction. *J. Neurosci.* 21, 3819–3829.

Rodella, U., Negro, S., Scorzeto, M., Bergamin, E., Jalink, K., Montecucco, C., et al. (2017). Schwann cells are activated by ATP released from neurons in an *in vitro* cellular model of Miller Fisher syndrome. *Dis. Model. Mech.* 10, 597–603. doi: 10.1242/dmm.027870

Rodrigues, R. J., Tomé, A. R., and Cunha, R. A. (2015). ATP as a multi-target danger signal in the brain. *Front. Neurosci.* 9:148. doi: 10.3389/fnins.2015.00148

Ross, C. A., and Tabrizi, S. J. (2011). Huntington's disease: from molecular pathogenesis to clinical treatment. *Lancet Neurol.* 10, 83–98. doi: 10.1016/S1474-4422(10)70245-3

Safarzadeh, E., Jadidi-Niaragh, F., Motallebnezhad, M., and Yousefi, M. (2016). The role of adenosine and adenosine receptors in the immunopathogenesis of multiple sclerosis. *Inflamm. Res.* 65, 511–520. doi: 10.1007/s00011-016-0936-z

Scattoni, M. L., Valanzano, A., Pezzola, A., March, Z. D., De Fusco, F. R., Popoli, P., et al. (2007). Adenosine A2A receptor blockade before striatal excitotoxic lesions prevents long term behavioural disturbances in the quinolinic rat model of Huntington's disease. *Behav. Brain Res*. 176, 216–221. doi: 10.1016/j.bbr.2006.10.004

Scemes, E., Suadicani, S. O., Dahl, G., and Spray, D. C. (2007). Connexin and pannexin mediated cell–cell communication. *Neuron Glia Biol.* 3, 199–208. doi: 10.1017/S1740925X08000069

Schiffmann, S. N., Fisone, G., Moresco, R., Cunha, R. A., and Ferré, S. (2007). Adenosine A2Areceptors and basal ganglia physiology. *Prog. Neurobiol.* 83, 277–292. doi: 10.1016/j.pneurobio.2007.05.001

Schiffmann, S. N., Jacobs, O., and Vanderhaeghen, J. J. (1991). Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an *in situ* hybridization histochemistry study. *J. Neurochem.* 57, 1062–1067. doi: 10.1111/j.1471-4159.1991.tb08257.x

Seki, T., Adachi, N., Ono, Y., Mochizuki, H., Hiramoto, K., Amano, T., et al. (2005). Mutant protein kinase Cγ found in spinocerebellar ataxia type 14 is susceptible to aggregation and causes cell death. *J. Biol. Chem.* 280, 29096–29106. doi: 10.1074/jbc.M501716200

Shahaduzzaman, M., Acosta, S., Bickford, P. C., and Borlongan, C. V. (2013). α-Synuclein is a pathological link and therapeutic target for Parkinson's disease and traumatic brain injury. *Med. Hypotheses* 81, 675–680. doi: 10.1016/j.mehy.2013.07.025

Sharp, A. J., Polak, P. E., Simonini, V., Lin, S. X., Richardson, J. C., Bongarzone, E. R., et al. (2008). P2x7 deficiency suppresses development of experimental autoimmune encephalomyelitis. *J. Neuroinflammation* 5:33. doi: 10.1186/1742-2094-5-33

Shen, H.-Y., Canas, P. M., Garcia-Sanz, P., Lan, J.-Q., Boison, D., Moratalla, R., et al. (2013). Adenosine A2A receptors in striatal glutamatergic terminals and GABAergic neurons oppositely modulate psychostimulant action and DARPP-32 phosphorylation. *PLoS ONE* 8:e80902. doi: 10.1371/journal.pone.0080902

Shinozaki, Y., Shibata, K., Yoshida, K., Ikenaka, K., Tanaka, K. F., Correspondence, S. K., et al. (2017). Transformation of Astrocytes to a neuroprotective phenotype by microglia via P2Y 1 receptor downregulation. *Cell Rep.* 19, 1151–1164. doi: 10.1016/j.celrep.2017.04.047

Simon, D. K., Wu, C., Tilley, B. C., Lohmann, K., Klein, C., Payami, H., et al. (2017). Caffeine, creatine, GRIN2A and Parkinson's disease progression. *J. Neurol. Sci.* 375, 355–359. doi: 10.1016/j.jns.2017.02.032

Simonin, C., Duru, C., Salleron, J., Hincker, P., Charles, P., Delval, A., et al. (2013). Association between caffeine intake and age at onset in huntington's disease. *Neurobiol. Dis*. 58, 179–182. doi: 10.1016/j.nbd.2013.05.013

Sitkovsky, M., V (2003). Use of the A2A adenosine receptor as a physiological immunosuppressor and to engineer inflammation *in vivo*. *Biochem. Pharmacol.* 65, 493–501. doi: 10.1016/S0006-2952(02)01548-4

Skaper, S. D., Facci, L., Culbert, A. A., Evans, N. A., Chessell, I., Davis, J. B., et al. (2006). P2X7 receptors on microglial cells mediate injury to cortical neurons *in vitro*. *Glia* 54, 234–242. doi: 10.1002/glia.20379

Song, X. M., Xu, X. H., Zhu, J., Guo, Z., Li, J., He, C., et al. (2015). Up-regulation of P2X7 receptors mediating proliferation of Schwann cells after sciatic nerve injury. *Purinergic Signal.* 11, 203–213. doi: 10.1007/s11302-015-9445-8

Spanevello, R. M., Mazzanti, C. M., Kaizer, R., Zanin, R., Cargnelutti, D., Hannel, L., et al. (2006). Apyrase and 5'-nucleotidase activities in synaptosomes from the cerebral cortex of rats experimentally demyelinated with ethidium bromide and treated with interferon-beta. *Neurochem. Res.* 31, 455–462. doi: 10.1007/s11064-006-9039-5

Stevens, B., and Fields, R. D. (2000). Response of Schwann cells to action potentials in development. *Science* 287, 2267–2271. doi: 10.1126/science.287.5461.2267

Stockwell, J., Jakova, E., and Cayabyab, F. S. (2017). Adenosine A1 and A2A receptors in the brain: current research and their role in neurodegeneration. *Molecules* 22:676. doi: 10.3390/molecules22040676

Strong, M. J. (2010). The evidence for altered RNA metabolism in amyotrophic lateral sclerosis (ALS). *J. Neurol. Sci.* 288, 1–12. doi: 10.1016/j.jns.2009.09.029

Sun, C., Heid, M. E., Keyel, P. A., and Salter, R. D. (2013). The second transmembrane domain of P2X7 contributes to dilated pore formation. *PLoS ONE* 8:e61886. doi: 10.1371/journal.pone.0061886

Sun, J., Link, H., Olsson, T., Xiao, B. G., Andersson, G., Ekre, H. P., et al. (1991). T and B cell responses to myelin-oligodendrocyte glycoprotein in multiple sclerosis. *J. Immunol.* 146, 1490–1495.

Surprenant, A., and North, R. A. (2009). Signaling at Purinergic P2X Receptors. *Annu. Rev. Physiol.* 71, 333–359. doi: 10.1146/annurev.physiol.70.113006.100630

Taherzadeh-Fard, E., Saft, C., Wieczorek, S., Epplen, J. T., and Arning, L. (2010). Age at onset in Huntington's disease: replication study on the associations of ADORA2A, HAP1 and OGG1. *Neurogenetics* 11, 435–439. doi: 10.1007/s10048-010-0248-3

Takenouchi, T., Sekiyama, K., Sekigawa, A., Fujita, M., Waragai, M., Sugama, S., et al. (2010). P2X7 receptor signaling pathway as a therapeutic target for neurodegenerative diseases. *Arch. Immunol. Ther. Exp. (Warsz).* 58, 91–96. doi: 10.1007/s00005-010-0069-y

Tarditi, A., Camurri, A., Varani, K., Borea, P. A., Woodman, B., Bates, G., et al. (2006). Early and transient alteration of adenosine A2A receptor signaling in a mouse model of Huntington disease. *Neurobiol. Dis.* 23, 44–53. doi: 10.1016/j.nbd.2006.01.014

Tebano, M. T., Martire, A., Chiodi, V., Ferrante, A., and Popoli, P. (2010). Role of adenosine A 2A receptors in modulating synaptic functions and brain levels of BDNF: a possible key mechanism in the pathophysiology of Huntington's disease. *Rev. ScientificWorldJournal.* 10, 1768–1782. doi: 10.1100/tsw.2010.164

Todd, K. J., and Robitaille, R. (2006). Purinergic modulation of synaptic signalling at the neuromuscular junction. *Pflügers Arch. Eur. J. Physiol.* 452, 608–614. doi: 10.1007/s00424-006-0068-3

Tozzi, M., and Novak, I. (2017). Purinergic receptors in adipose tissue as potential targets in metabolic disorders. *Front. Pharmacol.* 8:878. doi: 10.3389/fphar.2017.00878

Tsuda, M. (2017). P2 receptors, microglial cytokines and chemokines, and neuropathic pain. *J. Neurosci. Res.* 95, 1319–1329. doi: 10.1002/jnr.23816

Tsuda, M., and Inoue, K. (2016). Neuron–microglia interaction by purinergic signaling in neuropathic pain following neurodegeneration. *Neuropharmacology* 104, 76–81. doi: 10.1016/j.neuropharm.2015.08.042

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W., et al. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424, 778–783. doi: 10.1038/nature01786

Tsutsui, S., Schnermann, J., Noorbakhsh, F., Henry, S., Yong, V. W., Winston, B. W., et al. (2004). Neurobiology of disease A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis. *J. Neurosci.* 24, 1521–1529. doi: 10.1523/JNEUROSCI.4271-03.2004

Tyebji, S., Saavedra, A., Canas, P. M., Pliassova, A., Delgado-García, J. M., Alberch, J., et al. (2015). Hyperactivation of D1 and A 2A receptors contributes to cognitive dysfunction in Huntington's disease. *Neurobiol. Dis.* 74, 41–57. doi: 10.1016/j.nbd.2014.11.004

Varani, K., Abbracchio, M. P., Cannella, M., Cislaghi, G., Giallonardo, P., Mariotti, C., et al. (2003). Aberrant A_{2A} receptor function in peripheral blood cells in Huntington's disease. *FASEB J.* 17, 2148–2150. doi: 10.1096/fj.03-0079fje

Varani, K., Rigamonti, D., Sipione, S., Camurri, A., Borea, P. A., Cattabeni, F., et al. (2001). Aberrant amplification of A 2A receptor signaling in striatal cells expressing mutant huntingtin. *FASEB J.* 15, 1245–1247. doi: 10.1096/fj.00-0730fje

Varma, H., Cheng, R., Voisine, C., Hart, A. C., and Stockwell, B. R. (2007). Inhibitors of metabolism rescue cell death in Huntington's disease models. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14525–14530. doi: 10.1073/pnas.0704482104

Verkhratsky, A., Rodríguez, J. J., and Parpura, V. (2012). Calcium signalling in astroglia. *Mol. Cell. Endocrinol.* 353, 45–56. doi: 10.1016/j.mce.2011.08.039

Verschueren, A. (2017). Motor neuropathies and lower motor neuron syndromes. *Rev. Neurol. (Paris).* 173, 320–325. doi: 10.1016/j.neurol.2017.03.018

Vijayan, D., Young, A., Teng, M. W. L., and Smyth, M. J. (2017). Targeting immunosuppressive adenosine in cancer. *Nat. Rev. Cancer* 17, 709–724. doi: 10.1038/nrc.2017.86

Villar-Menéndez, I., Blanch, M., Tyebji, S., Pereira-Veiga, T., Albasanz, J. L., Martín, M., et al. (2013). Increased 5-methylcytosine and decreased 5-hydroxymethylcytosine levels are associated with reduced striatal A2AR levels in Huntington's disease. *NeuroMolecular Med.* 15, 295–309. doi: 10.1007/s12017-013-8219-0

Vincenzi, F., Corciulo, C., Targa, M., Casetta, I., Gentile, M., Granieri, E., et al. (2013). A_{2A} adenosine receptors are up-regulated in lymphocytes from amyotrophic lateral sclerosis patients. *Amyotroph. Lateral Scler. Front. Degener.* 14, 406–413. doi: 10.3109/21678421.2013.793358

Viscomi, M. T., Florenzano, F., Amadio, S., Bernardi, G., and Molinari, M. (2005). Partial resistance of ataxin-2-containing olivary and pontine neurons to axotomy-induced degeneration. *Brain Res. Bull.* 66, 212–221. doi: 10.1016/j.brainresbull.2005.04.016

Volont,é, C., Apolloni, S., Parisi, C., and Amadio, S. (2016). Purinergic contribution to amyotrophic lateral sclerosis. *Neuropharmacology* 104, 180–193. doi: 10.1016/j.neuropharm.2015.10.026

Volont,é, C., Apolloni, S., Skaper, S. D., and Burnstock, G. (2012). P2X7 receptors: channels, pores and more. *CNS Neurol. Disord. Drug Targets* 11, 705–721. doi: 10.2174/187152712803581137

Vonsattel, J. P., and DiFiglia, M. (1998). Huntington disease. *J. Neuropathol. Exp. Neurol.* 57, 369–384. doi: 10.1097/00005072-199805000-00001

Vuorimaa, A., Rissanen, E., and Airas, L. (2017). *In vivo* PET Imaging of adenosine 2A receptors in neuroinflammatory and neurodegenerative disease. *Contrast Media Mol. Imaging* 2017:6975841. doi: 10.1155/2017/6975841

Wang, X., Arcuino, G., Takano, T., Lin, J., Peng, W. G., Wan, P., et al. (2004). P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat. Med.* 10, 821–827. doi: 10.1038/nm1082

Wei, W., Du, C., Lv, J., Zhao, G., Li, Z., Wu, Z., et al. (2013). Blocking A2B adenosine receptor alleviates pathogenesis of experimental autoimmune encephalomyelitis via inhibition of IL-6 production and Th17 differentiation. *J. Immunol.* 190, 138–146. doi: 10.4049/jimmunol.1103721

Whiteside, T. L. (2017). Targeting adenosine in cancer immunotherapy: a review of recent progress. *Expert Rev. Anticancer Ther.* 17, 527–535. doi: 10.1080/14737140.2017.1316197

Wiendl, H., Toyka, K. V., Rieckmann, P., Gold, R., Hartung, H.-P., Hohlfeld, R., et al. (2008). Basic and escalating immunomodulatory treatments in multiple sclerosis: Current therapeutic recommendations. *J. Neurol.* 255, 1449–1463. doi: 10.1007/s00415-008-0061-1

Wiedemann, F. R., Manfredi, G., Mawrin, C., Beal, M. F., and Schon, E. A. (2002). Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. *J. Neurochem.* 80, 616–625. doi: 10.1046/j.0022-3042.2001.00731.x

Wilkaniec, A., Gassowska, M., Czapski, G. A., Cieślik, M., Sulkowski, G., and Adamczyk, A. (2017). P2X7 receptor-pannexin 1 interaction mediates extracellular alpha-synuclein-induced ATP release in neuroblastoma SH-SY5Y cells. *Purinergic Signal.* 13, 347–361. doi: 10.1007/s11302-017-9567-2

Witting, A., Chen, L., Cudaback, E., Straiker, A., Walter, L., Rickman, B., et al. (2006). Experimental autoimmune encephalomyelitis disrupts endocannabinoid-mediated neuroprotection. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6362–6367. doi: 10.1073/pnas.0510418103

Xu, P., Rosen, K. M., Hedstrom, K., Rey, O., Guha, S., Hart, C., et al. (2013). Nerve injury induces glial cell line-derived neurotrophic factor (GDNF) expression in Schwann cells through purinergic signaling and the PKC-PKD pathway. *Glia* 61, 1029–1040. doi: 10.1002/glia.22491

Yamada-Fowler, N., Fredrikson, M., and Söderkvist, P. (2014). Caffeine interaction with glutamate receptor gene GRIN2A: Parkinson's disease in Swedish population. *PLoS ONE* 9:e99294. doi: 10.1371/journal.pone.0099294

Yang, X., Lou, Y., Liu, G., Wang, X., Qian, Y., Ding, J., et al. (2017). Microglia P2Y6 receptor is related to Parkinson's disease through neuroinflammatory process. *J. Neuroinflammation* 14:38. doi: 10.1186/s12974-017-0795-8

Yanpallewar, S. U., Barrick, C. A., Buckley, H., Becker, J., and Tessarollo, L. (2012). Deletion of the BDNF truncated receptor TrkB.T1 delays disease onset in a mouse model of amyotrophic lateral sclerosis. *PLoS ONE* 7:e39946. doi: 10.1371/journal.pone.0039946

Yao, S.-Q., Li, Z.-Z., Huang, Q.-Y., Li, F., Wang, Z.-W., Augusto, E., et al. (2012). Genetic inactivation of the adenosine A(2A) receptor exacerbates brain damage in mice with experimental autoimmune encephalomyelitis. *J. Neurochem.* 123, 100–112. doi: 10.1111/j.1471-4159.2012.07807.x

Yiangou, Y., Facer, P., Durrenberger, P., Chessell, I. P., Naylor, A., Bountra, C., et al. (2006). COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. *BMC Neurol.* 6:12. doi: 10.1186/1471-2377-6-12

Zhang, J., Li, Z., Hu, X., Su, Q., He, C., Liu, J., et al. (2017). Knockout of P2Y 12 aggravates experimental autoimmune encephalomyelitis in mice via increasing of IL-23 production and Th17 cell differentiation by dendritic cells. *Brain Behav Immun.* 62, 245–255. doi: 10.1016/j.bbi.2016.12.001

Zhu, C., Wang, G., Li, J., Chen, L., Wang, C., Wang, Y., et al. (2014). Adenosine A2A receptor antagonist istradefylline 20 versus 40 mg/day as augmentation for Parkinson's disease: a meta-analysis. *Neurol. Res.* 36, 1028–1034. doi: 10.1179/1743132814Y.0000000375

Zhu, Y., and Kimelberg, H. K. (2001). Developmental expression of metabotropic P2Y1 and P2Y2 receptors in freshly isolated astrocytes from rat hippocampus. *J. Neurochem.* 77, 530–541. doi: 10.1046/j.1471-4159.2001.00241.x

Zimmermann, H. (2006). Ectonucleotidases in the nervous system. *Novartis Found. Symp.* 276, 113–128; discussion 128–130, 233–237, 275–281. doi: 10.1002/9780470032244.ch10

Zimmermann, H., Braun, N., Kegel, B., and Heine, P. (1998). New insights into molecular structure and function of ectonucleotidases in the nervous system. *Neurochem. Int.* 32, 421–425. doi: 10.1016/S0197-0186(97)00126-5

Zuchora, B., and Urbańska, E. M. (2001). Effect of adenosine receptor agonists on neurodegenerative and convulsive activity of mitochondrial toxin, 3-nitropropionic acid. *Polish J. Pharmacol. Pol. J. Pharmacol* 53, 69–71.

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