UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

AVALIAÇÃO DO PAPEL DO RECEPTOR PURINÉRGICO P2X7 EM MODELO DE HIPERATIVIDADE INDUZIDA POR D-ANFETAMINA EM CAMUNDONGOS

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Mestre em Bioquímica.

Porto Alegre, fevereiro de 2014.

CIP - Catalogação na Publicação

Gubert, Carolina de Moura
Avaliação do papel do receptor purinérgico P2X7 em modelo de hiperatividade induzida por D-anfetamina em camundongos / Carolina de Moura Gubert. -- 2014.
110 f.

Orientadora: Ana Maria Oliveira Battastini.
Coorientador: Flavio Pereira Kapczinski.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2014.

1. Transtorno bipolar. 2. Sistema purinérgico. 3. receptor P2X7. I. Battastini, Ana Maria Oliveira , orient. II. Kapczinski, Flavio Pereira, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

"*La ciencia es un cuadrúpedo que camina sobre la pata del empirismo hecho de datos, experimentación y observación; la pata de la racionalidad hecha de teorías lógicas; la pata de la verificación, y la pata de la imaginación*".

(Edgar Morin)

AGRADECIMENTOS

À minha orientadora, profª Ana Maria Oliveira Battastini, pela orientação, interesse, confiança, ensinamentos e dedicação a esta dissertação e à minha formação;

Ao meu co-orientador, profº Flávio Kapczinski, pelo incentivo, pela referência como pesquisador e pela oportunidade de aprendizado e crescimento profissional;

À Professora Fernanda Bueno Morrone, pela atenção, confiança, generosidade, oportunidade e incentivo;

Aos autores dos artigos que compõe esta dissertação, principalmente aos colegas e amigos Bianca Pfaffenseller, Pâmela Ferrari, Gabriel Fries e Bianca Aguiar, por todo o auxílio, interesse e carinho dedicado a mim e ao trabalho;

À bancada do Laboratório de Psiquiatria Molecular, meus amigos queridos, Bruna Panizzutti, Bruna Maria Ascoli, Bianca Pfaffenseller, Bianca Aguiar, Gabriela Colpo, Laura Stertz, Pâmela Ferrari, Emily Galvão, Giovana Bristot, Maurício Barth, André Contri e Thaís Martini, pela parceria, amizade, descontração, apoio e auxílio. Em especial ao Gabriel Fries, que mesmo à distância continuou sendo o amigo próximo, incentivador e inspirador de sempre;

Aos Professores do Laboratório de Psiquiatria Molecular, pelas discussões, exemplos, incentivos e preocupação com o crescimento científico e profissional dos alunos;

Aos novos amigos do Laboratório 22, que esta dissertação me proporcionou, Fabrício Figueiró, Franciane Mendes, Fabrícia Dietrich, Letícia Bergamin, Liliana Rockembach, Angélica Cappellari, Elisa Jandrey, Fabiana Manica e Mariana Quevedo;

Aos colegas do Laboratório de Farmacologia Aplicada, pela ajuda, atenção e companheirismo;

Aos alicerces de tudo, minha família;

À Julia Wunsch, Isadora Librenza e Helena Fantinelli, por estarem na minha vida sempre;

Ao meu namorado Leandro Todeschini, principal incentivador e apoiador da minha vida, incluindo a científica. Obrigada pelo carinho, paciência e confiança.

Sumário

PARTE I

Resumo

O sistema purinérgico tem sido amplamente implicado em condições médicas, entre elas, o transtorno bipolar (TB). Até onde sabemos, nenhum estudo foi realizado com o objetivo de esclarecer a possível contribuição do receptor P2X7 (P2X7R) na patofisiologia do TB ou mesmo com o objetivo de revisar o que já fora previamente publicado sobre este assunto. Nos presentes trabalhos, nosso objetivo foi em primeiro lugar explorar a relação entre o receptor e esse transtorno e identificar os mecanismos pelos quais o P2X7R desempenha um papel no TB e por fim revisar a literatura, focando na sinalização purinérgica no TB, com ênfase na sinalização por ATP e adenosina, destacando o potencial papel do P2X7R na modulação da inflamação e ativação microglial em pacientes bipolares. Para o primeiro objetivo, analisamos o efeito de moduladores do P2X7R (BzATP, BBG e A438079) sobre o comportamento (atividade locomotora) e sobre marcadores de neuroinflamação (IL-1β, TNF-α e IL-6), excitotoxicidade (GFAP, TBARS) e neuroplasticidade (BDNF) em um modelo farmacológico de mania induzido pelo tratamento agudo e crônico com D – anfetamina (AMPH) (2mg/kg). Demonstramos na atividade locomotora uma aparente falta de capacidade de resposta à AMPH por animais com P2X7R bloqueado ou ausente ($P2X7R^{-/-}$). Da mesma forma, observamos que o P2X7R participa do aumento do ambiente pró-inflamatório e excitotóxico induzido pela AMPH, demonstrado pelo papel de reversão do bloqueio do P2X7R ou pela perda de resposta dos animais $P2X7R^{-/-}$. Em conclusão, nossos resultados suportam a hipótese de que o P2X7R possui um papel na patofisiologia do TB, como sugerido pelo nosso modelo animal, principalmente por mediar a neuroinflamação e a excitotoxicidade e finalmente levando a mudanças comportamentais. Assim, acreditamos que o P2X7R possui potencial para se tornar um alvo terapêutico do TB. Baseado neste cenário, estudos mais detalhados do papel do P2X7R no TB são necessários, especialmente devido à sua capacidade de agir sobre a microglia e modular a neuroinflamação e a excitotoxicidade.

Abstract

The purinergic system has been increasingly implicated in medical conditions, among them bipolar disorder (BD). Up to date, no study has been conducted in order to clarify the possible contribution of the P2X7 receptor (P2X7R) to BD pathophysiology or even to review the previously data about this subject. In the presents works, we aim firstly to explore the association between the receptor and the disorder and identify the mechanisms by which the P2X7R plays a role in BD and lastly to review the literature focused on purinergic signaling in BD, with an emphasis on ATP and adenosine signaling, highlighting the potential role of P2X7R in modulating inflammation and microglia activation in bipolar patients. For the first aim, we analyzed the P2X7R modulatory effect (BzATP, BBG and A438079) on behavior (locomotor activity) and on markers of neuroinflammation (IL-1β, TNF-α and IL-6), excitotoxicity (GFAP, TBARS), and neuroplasticity (BDNF) in a pharmacological model of mania induced by acute and chronic D-amphetamine (AMPH) treatment (2 mg/kg). We demonstrate in the locomotor activity an apparent lack of responsiveness to AMPH by animals with blocked or absent P2X7R ($P2X7R^{-/-}$). Likewise, we observed that the P2X7R participates in the AMPH-induced increase of proinflammatory and excitotoxicity environment, demonstrated by the reversal role of P2X7R blockage or the lack of response by $P2X7R^{-/-}$ mice. In conclusion, our results provide support for the hypothesis that P2X7R plays a role in BD pathophysiology, as suggested by our animal model, mainly by mediating neuroinflammation and excitotoxicity and ultimately leading to behavioral changes. Thus, we believe that P2X7R has the potential to become a therapeutic target of BD. Based on this scenario, a more detailed study of the role of P2X7R in BD is warranted, especially due to its ability to act on microglia and modulate neuroinflammation and excitotoxicity.

LISTA DE ABREVIATURAS

TB	Transtorno Bipolar
DSM-IV	Manual Diagnóstico e Estatístico de Transtornos Mentais
AMPH	D - anfetamina
BDNF	Fator neurotrófico derivado do cérebro
SNC	Sistema nervoso central
GABA	Ácido gama-aminobutírico
EROS	Espécies reativas de oxigênio
EO	Estresse oxidativo
GFAP	Proteína glial fibrilar ácida
CPF	Córtex pré-frontal
IL–1β	Interleucina-1β
TNFα	Fator de necrose tumoral α
IL-6	Interleucina - 6
NF-κB	Fator nuclear - κB
C3	Complemento 3
C4	Complemento 4
iNOS	Óxido nítrico sintase induzível
P2X7R	Receptor P2X7
ATP	Adenosina trifosfato
BzATP	2',3'-O-(4-benzoylbenzoyl)-ATP
A438079	(3-[[5-(2,3 dichlorophenyl)-1H-tetrazol-1- yl]methyl]pyridine hydrochloride)
NFAT	Fator nuclear de células T ativadas
PLA2	Fosfolipase A2
PLD	Fosfolipase D
P-Tyr	Tirosina fosforilada
MAPK	Proteína cinase ativada por mitógenos
ERK 1/2	Cinase regulada por sinal extracelular 1/2
CREB	Proteína ligante ao elemento de resposta ao AMPc
AP-1	Ativador da proteína-1
COX-2	Ciclooxigenase 2
DM	Depressão maior
P2X7R$^{-/-}$	Camundongos "knockout" para o P2X7R
P2X7R$^{+/+}$	Camundongos C57BL/6 selvagens
DAT	Receptor de dopamina

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1. INTRODUÇÃO

1.1. Transtorno Bipolar

O Transtorno Bipolar (TB) é uma doença crônica e grave que apresenta curso episódico. Possui potencial para alto grau de severidade, recorrência e intensidade (Angst, Gamma e Lewinsohn, 2002). Estima-se que sua prevalência seja de 2,4% da população mundial (Merikangas *et al.*, 2007). A Organização Mundial da Saúde já considerou o TB como uma das dez principais causas de incapacitação mundial, além de ser um problema de saúde pública, devido à associação a um alto índice de suicídio e desemprego (Belmaker, 2004; Kupfer, 2005)

A ocorrência de pelo menos um episódio maníaco ou hipomaníaco é suficiente para o diagnóstico de TB, do tipo I ou do tipo II, respectivo ao episódio (Belmaker, 2004). O TB do tipo I é caracterizado por episódios maníacos ou mistos (sintomas maníacos e depressivos concomitantemente), geralmente intercalados por episódios depressivos, enquanto que no TB do tipo II não ocorrem episódios maníacos, ocorrem episódios hipomaníacos (Belmaker, 2004).

O episódio maníaco é definido pelo Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-IV) por um período distinto de humor anormal e persistentemente elevado, expansivo ou irritável, com duração de no mínimo uma semana. Pode ser acompanhado de, entre outros sintomas, grandiosidade, diminuição da necessidade de sono, agitação psicomotora, aceleração do pensamento, comportamento direcionado a atividades prazerosas, potencialmente imprudentes e perigosas (Belmaker, 2004). O episódio hipomaníaco assemelha-se ao episódio maníaco pelos sintomas e critérios do humor elevado, mas caracteriza-se como um episódio sem sintomas psicóticos ou sintomas que possam causar dano ou colocar em perigo a vida de um indivíduo ou a do próprio paciente; este apresenta uma duração mínima de 4 dias (Belmaker, 2004).

Comumente, os pacientes apresentam períodos de exacerbação dos sintomas (episódios agudos), intercalados por períodos sub-sindrômicos e de remissão (eutimia). Entretanto, o curso da doença é bastante heterogêneo; alguns pacientes permanecem estáveis por longos períodos e, outros, experimentam episódios frequentes, predominantemente de estado depressivo. Tais alterações de humor são suficientemente graves a ponto de causar prejuízos tanto no âmbito ocupacional quanto social do indivíduo (Belmaker, 2004).

Post e colaboradores (Post *et al.*, 2003) observaram que o prejuízo causado pela doença parece estar mais relacionado com a recorrência dos episódios do que com a gravidade de um dado episódio. Dessa maneira, a demora no diagnóstico e o número maior de crises refletem em uma piora cognitiva e clínica geral dos pacientes bipolares, que normalmente já apresentam altas taxas de déficit cognitivo e funcional, mesmo em períodos de remissão, o que contribui para a disfunção psicossocial associada ao transtorno (Zarate *et al.*, 2000).

Em virtude de seu curso crônico e a frequente reincidência e gravidade dos sintomas de humor, o tratamento do TB que é basicamente farmacológico (ácido valpróico, lítio, carbamazepina, antipsicótico atípicos, entre outros fármacos) é baseado no manejo dos episódios agudos e no tratamento de manutenção para prevenir a ocorrência de novos episódios (Yatham *et al.*, 2005). Entretanto, subtipos de TB, como os cicladores rápidos e os pacientes que possuem episódios mistos, geralmente, não respondem bem ao tratamento, sendo refratários. Ainda, o TB está associado a um aumento da morbidade e mortalidade por condições médicas gerais, como doenças cardiovasculares, obesidade e diabetes *mellitus* (Kupfer, 2005).

Devido à gravidade do transtorno, a baixa eficiência e abrangência do tratamento utilizado e as comorbidades associadas, existe a necessidade do melhor entendimento da patofisiologia da doença, na esperança do desenvolvimento de novos tratamentos mais efetivos e abrangentes que possam auxiliar na melhora na qualidade de vida desses pacientes.

1.2. Neurobiologia do Transtorno Bipolar

Apesar dos estudos e avanços recentes sobre o TB que permitiram um melhor entendimento da patofisiologia envolvida neste transtorno, a neurobiologia ainda está longe de ser completamente compreendida (Kapczinski, Vieta*, et al.*, 2008). Conceitualmente, tem sido proposto que o TB é uma patologia complexa e multifatorial, sabendo-se que vários fatores podem estar envolvidos no mecanismo da doença, incluindo fatores genéticos e epigenéticos, sinalização através de fatores neurotróficos, inflamatórios, estresse oxidativo, entre outros (Berk *et al.*, 2011; Rosenblat *et al.*, 2014).

Por ser um transtorno multifatorial, o TB pode ser resultante da interação entre fatores genéticos que causam susceptibilidade e fatores ambientais, como estresse e eventos traumáticos (Caspi e Moffitt, 2006; Barnett e Smoller, 2009). Um dos achados mais bem estabelecidos é o papel de fatores genéticos no TB, como evidenciado por estudos com gêmeos. Todos os estudos publicados a respeito mostraram uma maior concordância para o TB em gêmeos monozigóticos em comparação a gêmeos dizigóticos (revisado em (Barnett e Smoller, 2009)). Nas últimas duas décadas uma série de estudos de ligação e de associação foram realizados com o objetivo de buscar a base genética do transtorno, porém genes causais ou fatores de risco genéticos ainda não foram identificados.

Estudos de neuroquímica evidenciam a importância do neurotransmissor dopamina nos episódios de mania (Berk *et al.*, 2007). Isso é evidenciado pelo fato de que antipsicóticos com eficácia anti-maníaca são capazes de bloquear a neurotransmissão dopaminérgica (Yatham *et al.*, 2002), além de que psicoestimulantes que aumentam os níveis de dopamina, como a D-anfetamina (AMPH), podem causar efeitos psicológicos semelhantes aos sintomas maníacos em indivíduos saudáveis (Mamelak, 1978). Considerando ainda que o episódio maníaco está associado a um aumento nos níveis de dopamina (Berk *et al.*, 2007), a administração de

levodopa em pacientes com TB demonstrou induzir episódios hipomaníacos nestes indivíduos (Peet e Peters, 1995).

Estudos neuroanatômicos indicam a presença de alterações no volume de regiões cerebrais específicas acompanhadas de atrofia ou perda celular no TB, o que aponta para um padrão neurodegenerativo da doença, com a presença de neuroinflamação, excitotoxicidade e prejuízo da neuroplasticidade (Hajek, Carrey e Alda, 2005; Kim *et al.*, 2007a; Rao *et al.*, 2010). Estudos de imagem estrutural demonstraram volumes reduzidos de substância cinzenta em áreas do córtex orbital e pré-frontal medial, estriado ventral e hipocampo, assim como um alargamento significativo do terceiro ventrículo em comparação com controles saudáveis (Beyer e Krishnan, 2002). Estudos neuropatológicos post-mortem complementares mostraram ainda, reduções anormais no volume cortical, redução na contagem de células gliais e/ou no tamanho dos neurônios do córtex pré-frontal subgenual, córtex orbital, córtex pré-frontal ântero-lateral e amígdala (Deep-Soboslay *et al.*, 2008). Algumas dessas mudanças parecem ser reversíveis com o tratamento com estabilizadores de humor (Sassi *et al.*, 2002).

A comunidade científica tem se esmerado em compreender a patofisiologia do TB, com alguns achados absolutamente relevantes em diferentes vias celulares. Acreditamos que este padrão reportado de neuroinflamação, excitotoxicidade e prejuízo da neuroplasticidade, sejam um campo ainda pouco explorado que possui grande potencial para novas possibilidades de abordagem para tratamento do TB.

1.2.1. Transtorno Bipolar e Neuroplasticidade

1.2.1.1. Transtorno Bipolar e BDNF

Plasticidade é encontrada em todo o sistema nervoso. Entende-se que esta condição seja a base de muitos aspectos-chave do desenvolvimento, assim como da aprendizagem, da memória e de mecanismos de reparo. Processos de neuroplasticidade incluem plasticidade sináptica,

crescimento e remodelação celular e neurogênese (Pittenger, 2013). A desregulação destes processos pode contribuir para uma variedade de doenças neuropsiquiátricas, incluindo o TB, onde os déficits cognitivos apresentados no transtorno são o principal indicativo de comprometimento dos processos de neuroplasticidade (Zarate *et al.*, 2000). É bem estabelecido o papel da família das neurotrofinas, principalmente o fator neurotrófico derivado do cérebro (BDNF), em várias formas de plasticidade sináptica, sendo consideradas poderosas mediadoras moleculares da neuroplasticidade (Gómez-Palacio-Schjetnan e Escobar, 2013).

A família das neurotrofinas é composta por fatores regulatórios que medeiam a diferenciação e a sobrevivência de neurônios e modulam a transmissão e plasticidade sináptica (Bibel e Barde, 2000). Dentre as neurotrofinas, o BDNF é a mais abundante no sistema nervoso central (SNC) e parece induzir efeitos neurotróficos e neuroprotetores de longo prazo (Murer, Yan e Raisman-Vozari, 2001). O BDNF além de sua ação sobre a plasticidade sináptica possui uma importante função na liberação de neurotransmissores, facilitando a liberação de glutamato, ácido gama-aminobutírico (GABA), dopamina e serotonina (Tyler, Perrett e Pozzo-Miller, 2002; Yoshii e Constantine-Paton, 2010). Já foi demonstrado que o estresse crônico diminui os níveis de BDNF no SNC de ratos, e a sua expressão é aumentada em diferentes regiões cerebrais após tratamento crônico com fármacos antidepressivos e estabilizadores de humor (Frey, Andreazza, Ceresér, Martins, Valvassori*, et al.*, 2006).

Níveis séricos de BDNF estão diminuídos em pacientes com TB em episódios maníacos e depressivos (Cunha *et al.*, 2006). Além disso, Kauer-Sant'Anna e colaboradores (Kauer-Sant'anna *et al.*, 2009) compararam pacientes com TB que haviam tido apenas um episódio de humor e pacientes com múltiplos episódios, e mostraram que os níveis de BDNF diminuem com o número de episódios. Esses dados levaram à hipótese de que as mudanças relacionadas ao aumento no número de episódios devem explicar, pelo menos em parte, algumas das mudanças estruturais observadas em pacientes com TB. Os níveis de BDNF também foram correlacionados negativamente com a duração da doença (Kauer-Sant'anna *et al.*, 2009).

De uma forma geral, esses resultados sugerem um envolvimento significativo do BDNF na patofisiologia do TB (Kapczinski, Frey*, et al.*, 2008) marcando a presença de disfunção na neuroplasticidade no transtorno.

1.2.2. Transtorno Bipolar e Excitotoxicidade

1.2.2.1. Transtorno Bipolar e Estresse Oxidativo

Declínio cognitivo, piora dos sintomas e atrofia cerebral são condições amplamente descritas no TB e sugerem que a doença progride ao longo do tempo. Devido a estes fatores, tem sido descrita a presença de excitotoxicidade no transtorno (Mueller e Meador-Woodruff, 2004; Hashimoto, Sawa e Iyo, 2007a), elemento que poderia explicar a evolução destes quadros (Rao *et al.*, 2010).

A produção excessiva de espécies reativas de oxigênio (EROS) pode ser consequência de uma já estabelecida excitotoxicidade celular (Cornelius *et al.*, 2013). Em condições fisiológicas há um equilíbrio entre os sistemas oxidativo e antioxidante no organismo. O estresse oxidativo (EO) representa o desequilíbrio entre esses sistemas em favor do primeiro e tem sido cada vez mais implicado na patogênese de diversas doenças, incluindo em doenças neurológicas e transtornos psiquiátricos, como os transtornos de humor (Andreazza *et al.*, 2007; Wang *et al.*, 2009).

Aumento nos níveis de EO neuronal demonstrou causar disfunção na transdução de sinal, plasticidade e resistência celular, devido principalmente a dano lipídico por indução de peroxidação de membranas celulares (Mahadik *et al.*, 1998; Mahadik, Evans e Lal, 2001). O acúmulo de dano oxidativo pode ainda levar à morte celular neuronal por apoptose ou como consequência da agregação de proteínas oxidadas, o que pode resultar na insuficiência de mecanismos estabilizadores do humor (Mahadik, Evans e Lal, 2001).

A participação do EO na patofisiologia do TB tem sido amplamente reportada. Uma metanálise realizada por Andreazza AC e colaboradores (Andreazza *et al.*, 2008) demonstrou que pacientes com TB apresentam elevados níveis de EO, através de um aumento na peroxidação lipídica e de conteúdo de óxido nítrico (Andreazza *et al.*, 2008). Da mesma forma, aumento dos níveis de peroxidação lipídica foram encontrados no córtex cingulado de tecido post-mortem de pacientes com TB (Wang *et al.*, 2009). Outros estudos que corroboraram com o envolvimento do EO na patogênese do TB apresentaram alterações em hemácias (Kuloglu *et al.*, 2002; Ranjekar *et al.*, 2003; Ozcan *et al.*, 2004) e no soro (Andreazza *et al.*, 2007; Frey *et al.*, 2007; Kodydková *et al.*, 2009) de atividade das enzimas antioxidantes: superóxido dismutase, catalase e glutationa peroxidase; assim como uma alta taxa de peroxidação lipídica e de carbonilação de proteínas (Kuloglu *et al.*, 2002; Ranjekar *et al.*, 2003; Ozcan *et al.*, 2004).

Ainda, suportando os resultados anteriores, Shao L. e colaboradores (Shao, Young e Wang, 2005) em cultura primária de células corticais de modelo animal demonstraram que o tratamento crônico com estabilizadores de humor (lítio e valproato) inibe danos oxidativos a proteínas e por sua vez produz um efeito neuroprotetor contra a excitotoxicidade e neuroinflamação (Shao, Young e Wang, 2005).

1.2.2.2. Transtorno Bipolar e GFAP

Os astrócitos são células multifuncionais, que além de possuir um papel trófico e estrutural, são considerados componentes dinâmicos de conectividade e função cerebral. Uma ação fundamental exercida pelos astrócitos é a participação na resposta cerebral a insultos tóxicos e traumáticos, através de um processo complexo, denominado astrogliose (Colangelo, Alberghina e Papa, 2014). A astrogliose envolve mudanças morfológicas e funcionais, incluindo hipertrofia, aumento da produção de filamentos intermediários como a proteína glial fibrilar ácida (GFAP) e aumento de proliferação (Sofroniew e Vinters, 2010; Parpura *et al.*, 2012).

A astrogliose é um indicativo de dano na comunicação neurônio-glia, o que pode levar ao estabelecimento de excitotoxicidade, diminuição do metabolismo neuronal e antioxidante, alteração no metabolismo neurotrófico e consequente diminuição na neuroplasticidade (Colangelo, Alberghina e Papa, 2014). GFAP é então a principal proteína de filamentos intermediários astrocíticos e a sua *up*-regulação é considerada um marcador de astrogliose e excitotoxicidade (Eng, Ghirnikar e Lee, 2000; Fatemi *et al.*, 2004). Os astrócitos modulam a eficácia sináptica no SNC e o GFAP é um elemento importante para as interações neurônio-astrócito (Eng, Ghirnikar e Lee, 2000).

Astrogliose está presente em uma série de doenças que afetam o SNC, incluindo doenças neurodegenerativas e transtornos psiquiátricos, indicando que uma melhor compreensão dos mecanismos de ativação astroglial contribuiria para uma série de diferentes condições (Webster *et al.*, 2005; Rinaldi e Caldwell, 2013). Em um estudo post-mortem, Muller e colaboradores (Müller *et al.*, 2001) demonstraram um aumento no imunoconteúdo de GFAP em áreas CA1 e CA2 em pacientes com transtornos afetivos. Da mesma forma, recentemente foi descrito um aumento na expressão de GFAP em córtex pré-frontal (CPF) post-mortem de indivíduos com TB e esquizofrenia que apresentavam psicose (Feresten *et al.*, 2013).

Foi demonstrado também, aumento dos níveis de GFAP por imunoconteúdo, expressão gênica e imunohistoquímica em CPF post-mortem de pacientes com TB em comparação a controles (Rao *et al.*, 2010). Similarmente foi descrito um aumento nos níveis de GFAP em hipocampo de ratos submetidos ao modelo animal de mania induzido por AMPH crônica (Frey, Andreazza, Ceresér, Martins, Petronilho*, et al.*, 2006). Estes resultados indicam uma resposta astroglial tanto em pacientes quanto em modelo animal de TB, que certamente merece ser melhor estudada e compreendida.

1.2.3. Transtorno Bipolar e neuroinflamação

1.2.3.1. Transtorno Bipolar e citocinas pró-inflamatórias

Existem muitas evidências de que o TB está associado à neuroinflamação (Mundo *et al.*, 2003; Mueller e Meador-Woodruff, 2004; Hashimoto, Sawa e Iyo, 2007b; Kim *et al.*, 2007b; Rao *et al.*, 2007), assim como muitos estudos têm demonstrado que ambos os processos são associados com o aumento dos níveis de citocinas pró-inflamatórias, consideradas marcadores de neuroinflamação (Huang *et al.*, 1997; Rao *et al.*; Chang *et al.*, 2008).

As principais citocinas pró-inflamatórias envolvidas com o TB são Interleucina-1β (IL–1 β), fator de necrose tumoral-α (TNF-α) e Interleucina 6 (IL – 6) (Chang *et al.*, 2008). Goldstein e colaboradores (Goldstein *et al.*, 2009) revisaram a literatura e encontraram 27 artigos relativos à inflamação e TB, relatando alterações de níveis de citocinas tanto durante estados de humor do TB (mania e depressão), quanto na fase de remissão. Da mesma maneira, foi descrito um aumento dos níveis plasmáticos das citocinas pró-inflamatórias, IL-6, TNF-α, IL–1 β assim como, um aumento do fator nuclear-κB (NFκB) (Maes *et al.*, 1995; Ortiz-Domínguez *et al.*, 2007; Drexhage *et al.*, 2010). Também, o aumento dos níveis de proteínas de fase aguda, incluindo haptoglobina e proteína C reativa (PCR) (Maes *et al.*, 1997; Dickerson *et al.*, 2007), e altas concentrações plasmáticas de complemento 3 (C3) ou complemento 4 (C4) em pacientes com TB (Maes *et al.*, 1997; Wadee *et al.*, 2002).

O estudo de Rao e colaboradores (Rao *et al.*, 2010), já anteriormente citado, analisou marcadores de excitotoxicidade e neuroinflamação em CPF post-mortem de indivíduos com TB em comparação com indivíduos sem o diagnóstico, demonstrando um aumento significativo de níveis de mRNA de c-Fos e de óxido nítrico sintase induzível (iNOS), ambos marcadores de excitotoxicidade. Este mesmo estudo também demonstrou um aumento dos níveis proteicos e de mRNA de IL-1β, receptor de IL-1 β e subunidades de NF-κB. Os autores concluíram que marcadores de excitotoxicidade e neuroinflamação estão significantemente mais expressos em

CPF post-mortem de indivíduos com TB, em comparação a controles, sugerindo ainda que o aumento de expressão talvez esteja associada à morte celular, à atrofia cerebral e ao declínio cognitivo, todos descritos em pacientes com TB. Dessa forma, é discutido que a presença de excitotoxicidade e neuroinflamação possa ser um marco na patofisiologia do TB.

A relação entre TB e neuroinflamação/excitotoxicidade abre um amplo campo de foco para o entendimento da neurobiologia do TB, em virtude disso, muitas linhas de pesquisa que buscam novos alvos terapêuticos têm se estabelecido, incluindo o estudo do sistema purinérgico, com um maior enfoque no receptor P2X7 (P2X7R), pertencente a essa grande família.

1.3. Receptor Purinérgico P2X7

Purinas extracelulares (adenosina, ADP, e ATP) e pirimidinas (UDP e UTP) são moléculas sinalizadoras importantes que medeiam diversos efeitos biológicos, através da ativação de receptores purinérgicos (Ralevic e Burnstock, 1998). Existem duas famílias principais de receptores purinérgicos: receptores de adenosina ou P1 e, os receptores P2, que reconhecem principalmente ATP, ADP, UTP e UDP. Os receptores P1/Adenosina são acoplados à proteína G e se subdividem em 4 subtipos, A1, A2A, A2B, e A3, de acordo com a estrutura molecular, bioquímica e caracterização farmacológica. Baseado em diferentes estruturas moleculares e mecanismo de transdução de sinal, os receptores P2 se dividem em duas famílias de receptores: receptores P2X ionotrópicos e P2Y metabotrópicos. (Ralevic e Burnstock, 1998; Burnstock, 2006). Atualmente, sete subtipos de receptores P2X (P2X1 à P2X7) e oito subtipos de receptores P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) já foram identificados (Burnstock, 2006).

Durante o processo inflamatório, ATP e adenosina são liberados no sítio de inflamação como resultado do dano celular. Plaquetas ativadas e células endoteliais secretam ATP e ADP

sob condições de estresse fisiológico. Além disto, nucleotídeos podem ser transportados ativamente ao meio extracelular sob ativação celular (Luttikhuizen *et al.*, 2004; Burnstock, 2006). O ATP está envolvido no desenvolvimento da inflamação através de uma combinação de ações, que são: liberação de histamina dos mastócitos, provocando a produção de prostaglandinas; e a produção e liberação de citocinas das células imunes. Ao contrário do ATP, a adenosina possui uma ação anti-inflamatória. (Burnstock, 2006).

Praticamente, todos os tipos celulares expressam na membrana plasmática receptores para nucleotídeos extracelulares denominados receptores P2, que por sua vez são divididos nas duas subfamílias: acoplados à proteína G – receptores P2Y e canais iônicos seletivos permeáveis a Na^+, K^+ e Ca^{2+} – receptores P2X (North, 2002; Burnstock, 2004; Skaper, Debetto e Giusti, 2010b). Como já citado, existem sete isoformas do receptor P2X, que compartilham uma estrutura similar, compreendendo dois domínios transmembrana (Skaper, Debetto e Giusti, 2010b).

O trifosfato de adenosina (ATP) pode atuar na presença de receptores purinérgicos do tipo P2X7 em células do sistema imunológico, sugerindo uma relação com a regulação da função imune e de respostas inflamatórias. A ativação do P2X7R também possui propriedades citotóxicas marcantes, sendo capaz de levar à morte celular em resposta a insultos patológicos (Skaper, Debetto e Giusti, 2010b). O receptor P2X7 se destaca com relação aos demais membros da família dos receptores P2X, pois apresenta uma afinidade a altas concentrações (acima de 100mM) de ATP, sendo capaz de formar um poro permeável a solutos hidrofílicos de até 900 Da, sob ativação sustentada (Skaper, Debetto e Giusti, 2010b).

O ATP é o ativador fisiológico conhecido do P2X7R (Ferrari *et al.*, 1997; Chakfe *et al.*, 2002). Em condições normais, o ATP extracelular está presente em baixas concentrações, que aumentam significativamente sob quadros inflamatórios, *in vivo* (Lazarowski, Boucher e Harden, 2000) e, em resposta a trauma (isquemia/hipóxia) (Nieber, Eschke e Brand, 1999). Citocinas pró-inflamatórias aumentam a expressão de P2X7R e a sensibilidade ao ATP

extracelular (Humphreys e Dubyak, 1998; Narcisse *et al.*, 2005). Dentro do SNC, P2X7R são encontrados principalmente na microglia, células de Schawann e astrócitos (Ferrari *et al.*, 1996; Collo *et al.*, 1997; Sim *et al.*, 2004). O receptor P2X7 ativa uma gama diversificada de respostas celulares, sendo a atividade melhor caracterizada do P2X7R sua ação sob as citocinas, principalmente a IL-1β, mediação da ativação microglial e consequente excitotoxicidade (Ferrari *et al.*, 1997).

Os P2X7R são ativados por altas concentrações de ATP e com maior potência pelo agonista 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP). Da mesma forma, vários antagonistas dos receptores P2X7 têm sido identificados a fim de avaliar as funções deste receptor, um dos melhores descritos é o (3-[[5-(2,3 dichlorophenyl)-1H-tetrazol-1- yl]methyl]pyridine hydrochloride) (A438079), que é um potente antagonista competitivo ortostérico deste receptor (Yan *et al.*, 2011).

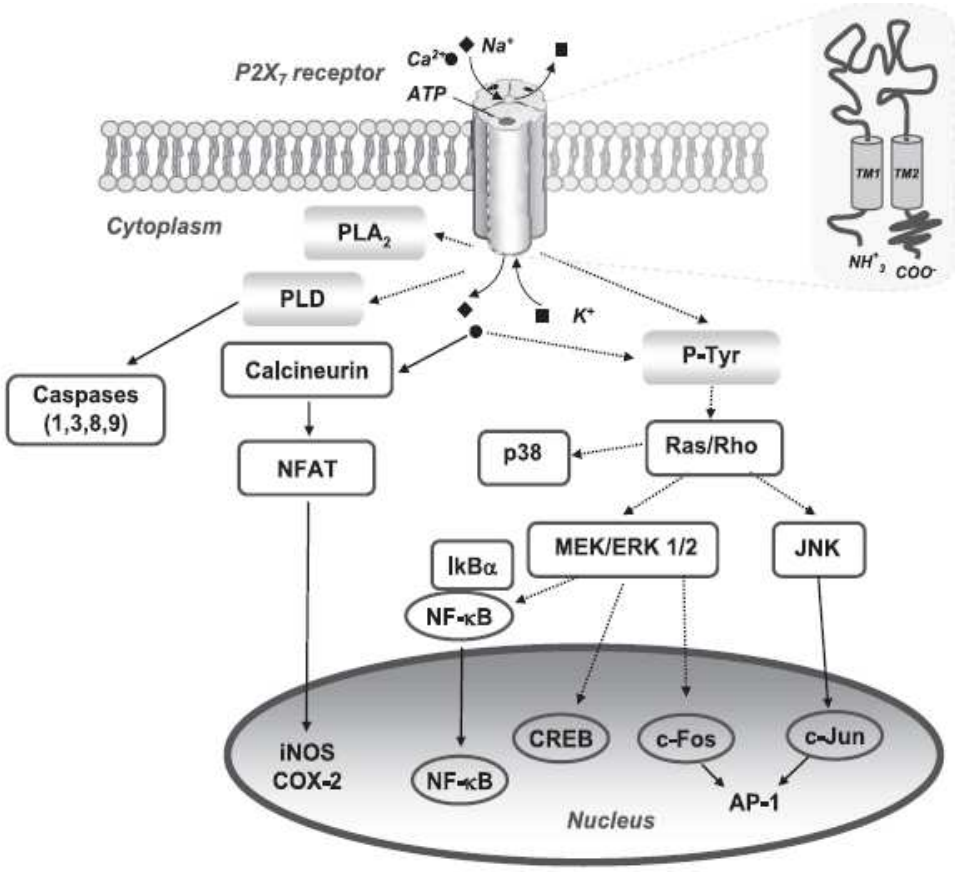

Figura 1. Representação de eventos de transdução de sinal após ativação do receptor P2X7 na microglia (retirado de (Skaper, Debetto e Giusti, 2010b).

Cada P2X7R funcional é um trímero, com as três subunidades de proteínas dispostas em torno de um poro permeável a cátions. As subunidades possuem topologia comum, dois domínios (TM1 e TM2), um longo laço extracelular contendo 10 cisteínas com espaço similar e sítios de glicosilação. O terminal amino e carboxi são intracelulares. A ativação breve com ATP (<10s) sob P2X7R, resulta em uma abertura no canal rápida e reversível, que é permeável a Na^+, K^+ e Ca^{2+}. O P2X7R ativado induz a liberação de IL-1β, que é mediada pela ativação da enzima conversora de IL-1(caspase-1). O efluxo de K^+ ativa a enzima conversora de IL-1β, que cliva a pro-IL-1β a IL-1β ativa, que por sua vez é liberada da célula. O influxo de Ca^{2+} desencadeia a ativação da calcineurina e a defosforilação/ativação do fator nuclear de células T ativadas (NFAT). A ativação do receptor P2X7 também resulta na ativação das fosfolipases A_2 e D (PLA_2, PLD), bem como na fosforilação da tirosina (P-Tyr) e ativação de cinases da família das proteínas cinases ativadas por mitógenos (MAPK), incluindo a cinase regulada por sinal extracelular 1/2 (ERK 1/2). Estas podem influenciar a atividade de fatores de transcrição como NF-κB, a proteína reguladora da expressão gênica, proteína ligante ao elemento de resposta do AMPc (CREB) e o ativador da proteína-1 (AP-1), que levam à superexpressão de genes pró-inflamatórios, tais como a Ciclooxigenase-2 (COX-2) e a iNOS, capaz de contribuir para o estabelecimento de excitotoxicidade (Ferrari, Stroh e Schulze-Osthoff, 1999; Potucek, Crain e Watters, 2006). A ativação de receptores P2X7 também leva à estimulação da MAPK p38, com consequente fosforilação/ativação da CREB (Skaper, Debetto e Giusti, 2010b).

O P2X7R possui um papel importante na modulação da neurotransmissão glutamatérgica, na liberação de citocinas pró-inflamatórias/fatores de excitotoxicidade, estabelecimento e manutenção da ativação microglial e no dano/morte neuronal (Sun, 2010). Considerando que todos esses fatores modulados pelo P2X7R são descritos na patofisiologia do TB, é de se pensar que este receptor possa estar envolvido com o transtorno.

1.3.1. P2X7R e Transtorno Bipolar

O gene do P2X7 que codifica o receptor está localizado no cromossomo 12q24. Esta região tem sido implicada em estudos de ligação a vários transtornos psiquiátricos, incluindo depressão maior (DM) (Abkevich *et al.*, 2003; Zubenko *et al.*, 2003; Mcguffin *et al.*, 2005; Lucae *et al.*, 2006) e TB (Dawson *et al.*, 1995; Ewald *et al.*, 1998; Morissette *et al.*, 1999; Degn *et al.*, 2001; Maziade *et al.*, 2001; Curtis *et al.*, 2003; Shink *et al.*, 2005).

Analisando em conjunto os resultados de associação alélica e genotípica, bem como de haplótipos de base familiar, pode-se dizer que esta região cromossômica, onde está o gene que codifica o P2X7R, está em um lócus de suceptibilidade a DM e TB (Barden *et al.*, 2006; Lucae *et al.*, 2006), sugerindo que polimorfismos do P2X7R podem desempenhar um papel importante no desenvolvimento destes transtornos.

Em camundongos "knockout" para o P2X7R ($P2X7R^{-/-}$), analisados em modelos de depressão e ansiedade, foi encontrado um chamado fenótipo "antidepressivo-like", um fenótipo de resistência à DM, somado a uma maior capacidade de resposta a uma dose subterapêutica de imipramina, o antidepressivo mais utilizado no tratamento farmacológico da DM (Basso *et al.*, 2009; Skaper, Debetto e Giusti, 2010b). Dessa maneira, os autores especulam a possibilidade de que, futuramente, antagonistas de P2X7R possam constituir um novo alvo terapêutico para a DM.

Embora ambos os transtornos, TB e DM sejam da mesma forma relacionados ao P2X7R, até onde se sabe, há somente estudos bioquímicos, comportamentais e farmacológicos, que relacionam P2X7R à DM, nenhum ainda reportado em relação ao TB.

1.4. Modelo Animal de Mania

O conhecimento limitado acerca da neurobiologia do TB dificulta o desenvolvimento de modelos animais para o transtorno, uma vez que este apresenta-se de forma muito heterogênea na população e não há alvos bioquímicos ou genéticos específicos consolidados que possam ser manipulados para o desenvolvimento dos modelos. No entanto, a tarefa é necessária – praticamente todas as medicações existentes foram descobertas ao acaso ou resultaram de testes de medicações aprovadas para outros usos em modelos animais existentes (no caso, antipsicóticos e anticonvulsivantes).

No caso do TB, o desenvolvimento de modelos animais adequados é particularmente difícil pelo fato do transtorno ser cíclico e apresentar uma grande heterogeneidade clínica. Os conhecimentos acerca de sua patofisiologia são ainda limitados e existem poucos endofenótipos clínicos bem validados. Os mecanismos de ação das medicações estabilizadoras de humor e os dados a respeito de genes que conferem susceptibilidade também são insuficientes. A maioria dos modelos animais utilizados é, portanto, específica para o episódio de mania ou para o episódio de depressão e poucos se aventuram em mimetizar a ciclicidade e a recorrência típica do TB em animais (Post, 2007a).

Existem diferentes abordagens para o desenvolvimento de modelos animais: eles podem basear-se em sintomas, endofenótipos/patofisiologia ou na resposta a medicamentos. Um dos modelos animais de mania mais bem consolidados na literatura é o modelo induzido pela administração de AMPH em ratos ou camundongos (Frey, Valvassori*, et al.*, 2006; Yates *et al.*, 2007), baseando-se no fato que há um aumento nos níveis de dopamina em pacientes durante o episódio de mania. O teste comportamental comumente utilizado é o campo aberto, possibilitando a avaliação de parâmetros exploratórios e locomotores. As validades de constructo e preditiva do modelo tem sido comprovadas (Frey, Andreazza, Ceresér, Martins, Valvassori*, et al.*, 2006; Walz *et al.*, 2008; Valvassori *et al.*, 2010), correlacionando achados nos animais com dados clínicos e bioquímicos observados em pacientes.

JUSTIFICATIVA DA PESQUISA

O presente trabalho procura acrescentar à literatura e ao conhecimento científico, achados sobre a patofisiologia do TB, em virtude desta ser parcialmente conhecida. Nossa preocupação e motivação em estudar o assunto estão relacionadas à alta prevalência deste transtorno na população e seu prejuízo na funcionalidade de vida do paciente, bem como os gastos gerados ao sistema de saúde. Entendemos que mais estudos são necessários para o esclarecimento dos mecanismos responsáveis pela doença, com o objetivo de fornecer ao paciente um tratamento adequado e que de alguma maneira minimize os danos que o TB provoca nestes indivíduos.

Acreditamos que o receptor purinérgico P2X7 possa vir a se tornar um importante alvo terapêutico para o TB, principalmente baseado no fato de que as vias de ação conhecidas que a ativação deste receptor está envolvida são basicamente as mesmas já bem estabelecidas no TB, a neuroinflamação e a excitotoxicidade. Dessa forma, pretendemos verificar se existe de fato esta relação, através da modulação deste receptor em um modelo animal estabelecido de mania, a partir da análise comportamental e da utilização de marcadores de neuroinflamação, excitotoxicidade e neuroplasticidade.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar se há envolvimento do receptor P2X7 na patofisiologia do TB, avaliando as consequências de sua modulação em um modelo animal de mania, sobre parâmetros comportamentais, e marcadores de neuroinflamação, de excitotoxicidade e de neuroplasticidade.

2.2. Objetivos específicos

i. Verificar a partir do teste de campo aberto a resposta comportamental do modelo animal de mania, diante da modulação do P2X7R;
ii. Analisar os níveis protéicos de citocinas pró-inflamatórias IL-1β, IL-6, e TNF-α em córtex pré-frontal, hipocampo e estriado de camundongos C57BL/6 selvagens ($P2X7R^{+/+}$) e camundongos $P2X7R^{-/-}$;
iii. Analisar os níveis de BDNF, GFAP e equivalente a danos por estresse oxidativo a lipídeos, nos mesmos tecidos cerebrais.
iv. Revisar a literatura com o objetivo de sistematizar e esclarecer o que já fora produzido a respeito do sistema purinérgico e mais especificamente do P2X7R em relação ao TB;

PARTE II

3. CAPÍTULOS

Capítulo I

The role of P2X7R in an animal model of mania induced by D-amphetamine

A ser submetido à revista: "Bipolar Disorders"

Capítulo II

The P2X7 purinergic receptor as a molecular target in bipolar disorder

Publicação: "Neuropsychiatria i Neuropsychologia 2013; 8, 1: 1–7

3.1. Capítulo I

The role of P2X7R in an animal model of mania induced by D-amphetamine

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A ser submetido à revista: "Bipolar Disorders"

Title: Role of P2X7R in an animal model of mania induced by D-amphetamine

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Word count of the Abstract: 194

Word count of the body of the manuscript: 7496

Abstract

Objectives: Up to date, no study has been conducted in order to clarify the possible contribution of P2X7R to BD pathophysiology. In the present work, we aim to explore the association between the receptor and the disorder and identify the mechanisms by which the P2X7R plays a role in BD.

Methods: We analyzed the P2X7R modulatory effect (BzATP, BBG and A438079) on behavior (locomotor activity) and on markers of neuroinflammation (IL-1β, TNF-α and IL-6), excitotoxicity (GFAP, TBARS), and neuroplasticity (BDNF) in a pharmacological model of mania induced by acute and chronic AMPH treatment (2 mg/kg).

Results: We demonstrate in the locomotor activity an apparent lack of responsiveness to AMPH by animals with blocked or absent P2X7R. Likewise, we observed that the P2X7R participates in the AMPH-induced increase of proinflammatory and excitotoxicity environment, demonstrated by the reversal role of P2X7R blockage or the lack of response by $P2X7R^{-/-}$ mice.

Conclusions: Our results provide support for the hypothesis that P2X7R plays a role in BD pathophysiology, as suggested by our animal model, mainly by mediating neuroinflammation and excitotoxicity and ultimately leading to behavioral changes. Thus, P2X7R has the potential to become a therapeutic target of BD.

Key words: bipolar disorder, P2X7R, D-amphetamine

Introduction

Bipolar disorder (BD) is a chronic psychiatric illness characterized by recurrent episodes of mania and depression intercalated with euthymic phases (1), affecting about 2.4% of the world population (2). This disorder has been associated with increased morbidity and mortality, mainly due to high suicide rates (3) and medical comorbidities (4). The development of animal models has been an important tool in the investigation of BD neurobiology and of new drugs for its treatment. In this sense, both acute and chronic uses of psychostimulants such as amphetamine (AMPH) have been widely used as animal models of mania (5, 6).

The precise mechanisms underlying BD pathophysiology remains unknown. Reports of cognitive decline and progressive brain atrophy suggest that this disorder presents a progressive presentation and possibly a neurodegenerative compound, with the involvement of excitotoxicity, neuroinflammation and impaired neuroplasticity (7, 8). For instance, extensive evidence points to increased levels of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β and nuclear factor-κ B (NF-κB) in plasma levels and in postmortem frontal cortex from BD patients compared with control subjects (8-10). In addition, the presence of excitotoxicity was demonstrated in postmortem frontal cortex from BD patients in comparison with control individuals, such as an increase in gliosis markers (glial fibrillary acidic protein (GFAP) and CD11b) (8). Similarly, several data suggest that increased oxidative stress and lower levels of brain-derived neurotrophic factor (BDNF) (11, 12) play a prominent role in the excitotoxicity and reduction of neuroplasticity in BD, respectively (13).

The purinergic system has been increasingly implicated in the pathophysiology of medical conditions of central nervous system (CNS), primarily the P2X7R (14). This particular receptor is an adenosine 5'-triphosphate (ATP)-binding ligand-gated ion channel beeing activated only by high concentrations of extracellular ATP (15) and plays a key role in the modulation of the inflammatory response and of the pathological activation of glial cells. Moreover, it also has the ability to mediate cell death, which assigns an important contribution in mediating neuroinflammation and

excitotoxicity (16). These functions could potentially underlie a role for P2X7R on BD and neurodegenerative diseases.

It has been shown that the gene coding for the purinergic P2X7 receptor (P2X7R) is located on a susceptibility locus associated with BD (17), however,up to date, no study has been conducted in order to clarify the possible contribution of P2X7R to BD pathophysiology. In the present work, we aim to explore the association between the receptor and the disorder and identify the mechanisms by which the P2X7R plays a role in BD. In order to do that, we analyzed the P2X7R modulatory effect on behavior and on markers of neuroinflammation, excitotoxicity, and neuroplasticity in a pharmacological model of mania induced by acute and chronic AMPH treatment.

Materials and methods

Animals

Male wild-type C57BL/6 - $P2X7R^{+/+}$ (WT) and P2X7R *knockout* ($P2X7R^{-/-}$) mice (age: 6-8 weeks; weight: 18-25 g) were used throughout this study. C57BL/6 mice were obtained from Universidade Federal de Pelotas (UFPEL, Pelotas, RS, Brazil) and $P2X7R^{-/-}$ mice were donated by Dr Robson Coutinho-Silva, from Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil). The $P2X7R^{-/-}$ mice were generated by the method developed by Dr James Mobley (PGRD, Pfizer Inc, Groton, CT, USA), whereas the $P2X7R^{-/-}$ mice were C57BL/6 inbred. The animals were housed in groups of four per cage and maintained in controlled temperature (22 ± 2 ºC) and humidity (60–70%), under a 12 h light–dark cycle, with food and water *ad libitum*. Animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. All of the tests were performed between 7 am and 7 pm. The experimental procedures reported in this manuscript followed the National Institute of Health Guide for the Care and Use of

Laboratory Animals (18) and the Brazilian College of Animal Experimentation and were approved by the Institutional Animal Ethics Committee (protocol number: 10/00206).

Drugs and treatment

Mice received intraperitoneal (i.p.) injections of D-amphetamine sulfate salt (AMPH) (2 mg/kg) or vehicle. AMPH and all other drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise. Agonists and antagonists of P2X7R were delivered by intracerebroventricular (i.c.v.) microinjection in 2µl volume in the following concentrations: selective P2X7R agonist, 3'-O-(4-benzoyl) benzoyl-adenosine 5'-triphosphate (BzATP), 10.5 nmol; non-selective P2X7R antagonist brilliant blue G (BBG), 20 nmol; and selective P2X7R antagonist, A438079 (3-[[5-(2,3 dichlorophenyl)-1H-tetrazol-1- yl]methyl]pyridine hydrochloride), 1.75 nmol. A438079 was obtained from Tocris Biosciences (Ellisville, MO, USA). Treatment protocols were performed as follows: in the acute treatment with AMPH, the animals received the i.c.v. microinjection of BzATP, A438079 or vehicle, followed by a single i.p. injection of AMPH or vehicle and the behavioral test. The time interval between administrations of the drugs was 15 minutes. In the chronic treatment with AMPH, mice received the i.p. injection of either AMPH or vehicle once a day for a period of 7 days. No behavioral assessment was performed between days 1 and 6. On the 7th day of treatment, the animals received a single i.c.v. microinjection of BzATP, BBG, A438079 or vehicle. In all cases, animals were subjected to the behavioral test (open-field) immediately after last injection of AMPH or vehicle was administered. Again, the time interval between drugs administrations was 15 minutes. Saline (0.9 % NaCl) was used as vehicle in all cases. BBG treatment was not included in the acute AMPH model due to technical problems.

Locomotor activity

Locomotor activity was assessed by the open field test. The experiments were conducted in a sound-attenuated room under low-intensity light. Each animal was individually placed in the periphery of the arena of an acrylic box (40 x 60 x 50 cm) and was left free for 60 minutes. The behavior was recorded and analyzed using the ANY-Maze video-tracking system (Stoelting, CO). The overall distance that animals traveled during the 60 minutes of observation was quantified. The apparatus was cleaned with ethanol 70% between each trial.

Preparation of samples

Immediately after the behavioral test, animals were euthanized and different brain structures were isolated striatum (STR), prefrontal cortex (PFC) and hippocampus (HPC). Brain tissue samples were homogenized (w/v, 1:10) with ice-cold 0.1 M phosphate buffer (pH 7.4) with the addition of protease inhibitor cocktail (Sigma-Aldrich, USA). The homogenates were centrifuged at 5,000 rpm for 5 minutes, and aliquots of supernatants were separated and stored at -80ºC until further analyses.

Biochemical determinations

Determination of proinflammatory cytokines levels

The concentration of cytokines was determined by flow cytometry using the BD™ Cytometric Bead Array (CBA) Mouse TNF-α, IL-6 and IL1-β Enhanced Sensitivity Flex Set (BD Biosciences, San Diego, CA). Sample processing and data analyses were performed according to the manufacturer's guidelines. Briefly, homogenate samples and standard curve ranging from 274 to 200,000 fg/mL of each cytokine were incubated with the three cytokine capture beads for 1 h, then washed and incubated for another 1 h with PE-conjugated detection antibodies. Afterwards, samples were washed and sample data were acquired using a FACSCalibur flow cytometer (BD

Biosciences, San Diego, CA). Results were generated in graphical and tabular format using the BD CBA Analysis Software FCAP Array™ (BD Biosciences, San Diego, CA) and were expressed as fg/mL.

Determination of GFAP levels

GFAP levels were measured using an anti-GFAP ELISA, according to manufacturer instructions (Millipore, USA). Briefly, microtiter plates (96-well anti-GFAP coated plate) were incubated for 2 h with the samples diluted 1:3 in sample diluent, and the standard curve ranged from 1.5 to 100 ng/ml of GFAP. The plates were then washed four times with wash buffer, and a biotinylated anti-GFAP detection antibody was added to each well and incubated for 1 h at room temperature. After washing, an enzyme solution was added to each well and incubated at room temperature for 30 minutes. After the addition of substrate and stop solution, the amount of GFAP was determined by absorbance at 450 nm. Total protein content was measured using the Bradford method (19) and results were expressed as pg/μg of protein.

Determination of BDNF levels

Tissue concentrations of BDNF were measured by sandwich-ELISA assay using monoclonal antibodies specific for BDNF from R&D Systems (Minneapolis, Minnesota, USA). Briefly, microtiter plates (96-well flat-bottom) were coated overnight at room temperature with the monoclonal anti-BDNF antibody (clone 37129) at 4 μg/mL in PBS. Then, plates were washed with wash buffer (PBS, pH 7.4, with 0.05% Tween 20) and were blocked for 1 hour at room temperature with PBS containing 5% nonfat milk powder. After washing, plates were coated for 2 hours with the samples diluted 1:3 in sample diluent and a standard curve ranging from 7.8 to 500 pg/mL of BDNF. Plates were washed and a biotinylated anti-BDNF antibody (clone 37141) was added at 0.2 ug/mL, followed by an incubation for 2 hours. After washing, an incubation with streptavidin-

peroxidase conjugate (diluted 1:200 in sample diluent) for 20 min at room temperature was performed. Afterwards, plates were washed and incubated with a substrate solution, followed by the addition of a stop solution (H_2SO_4 1 M). After 20 min of incubation, the amount of BDNF was determined by the absorbance at 450 nm with correction at 540 nm. Total protein content was measured using the Bradford method (19) and results were expressed as pg/μg of protein.

Determination of TBARS levels

Lipid peroxidation levels were measured using the commercial thiobarbituric acid reactive substances (TBARS) assay kit according to manufacturer's instructions (Cayman, Ann Arbor, USA). Lipid peroxidation was determined spectrophotometrically at 535 nm. Results were expressed in mM of MDA and have been taken as an index of reactive oxygen species (ROS) production.

Statistical analysis

All data were expressed as mean ± S.E.M and were analyzed by One-way ANOVA followed by the Tukey's Multiple Comparison post hoc test for unequal sample. $P < 0.05$ was considered statistically significant. The statistical program used was GraphPad Prism 5.0 Version for Windows, GraphPad Software (San Diego, CA, USA).

Results

P2X7R modulatory effect on mice behavior in the model of mania induced by acute and chronic AMPH

This first experimental observation was designed to identify a possible relationship between the P2X7R and the animal model of mania by means of the receptor's modulation. **Fig 1a** represents the pharmacological modulation of P2X7R in the acute and chronic AMPH treatment. Acute and chronic AMPH treatments significantly increased the locomotor activity compared to

control animals (p = 0.03 and p = 0.02, respectively), even did AMPH along with the administration of the BzATP (p = 0.01 and p = 0.03, respectively). On the other hand, acute AMPH had no significant effect on locomotor activity when administered along with the A438079 (p = 0.08), indicating that the administration of the selective P2X7R antagonist decreases the expected response of the animals to acute AMPH. Similarly, the treatment with BBG and A438079 blocked the action of AMPH, being able to return the locomotor activity to control level (p = 0.66 and p.99 in comparison with vehicle/vehicle group, respectively) In addition, the treatment with the A438079 significantly reduced the locomotor activity when compared to the vehicle/AMPH and BzATP/AMPH groups (p = 0.03 and p = 0.04, respectively), pointing to a reversal role of the selective P2X7R antagonist on the AMPH-induced hyperactivity.

Fig 1b shows the acute and chronic AMPH responses in $P2X7R^{-/-}$ animals. As expected, AMPH increased the locomotor activity in WT animals in comparison to the WT/vehicle control groups, both in the acute (p = 0.002) and chronic (p = 0.001) AMPH treatments. As suggested by the results with the P2X7R antagonists, AMPH had no effect in the $P2X7R^{-/-}$ animals compared with the $P2X7R^{-/-}$/vehicle control groups, both in the acute (p = 0.66) and chronic (p = 0.62) AMPH groups. Moreover, the WT/AMPH group showed a significant increase in the locomotor activity compared to the $P2X7R^{-/-}$/vehicle groups in acute (p = 0.001) and chronic (p = 0.001) AMPH treatment, which was also the case when compared to the $P2X7R^{-/-}$/AMPH groups, both in acute (p = 0.018) and chronic (p = 0.009) AMPH treatments. All behavioral results suggest a lack of responsiveness to AMPH in animal where P2X7R is blocked or absent.

P2X7R modulatory effects on IL-1β, TNF-α, IL-6, GFAP, TBARS and BDNF levels in the model induced by acute AMPH.

As a second approach, we evaluated a serie of molecules previously shown to be involved in the BD pathophysiology in order to identify the pathways by which P2X7R may be acting on the

acute AMPH-induced behavior. As shown in **Table 1**, there was a significant increase in the levels of IL-1β in striatum ($p = 0.025$) after acute AMPH injection when compared to control. TNF-α striatum levels were decreased in the A438079/AMPH group in comparison with vehicle/AMPH group ($p = 0.026$), indicating a reversal role of the AMPH-induced proinflammatory environment by the blockage of P2X7R in striatum. Likewise, in the hippocampus, the GFAP content was decreased in the A438079/AMPH group compared with vehicle/AMPH group ($p = 0.022$), which suggests that the blockage of P2X7R in the hippocampus decreased the AMPH-induced astrogliosis.

Acute AMPH also increased the index of ROS production (TBARS) in the striatum when compared with the vehicle/vehicle control group ($p < 0.001$), which was reversed with the administration of the A438079 ($p = 0.005$), pointing to a possible decrease of AMPH-induced oxidative stress by P2X7R blocking. Similarly, after acute AMPH treatment, TBARS levels were increased in the prefrontal cortex and striatum in WT animals when compared with the control WT/vehicle group ($p = 0.007$ and $p = 0.009$, respectively). Moreover, the WT/AMPH group showed a significant increase compared to the $P2X7R^{-/-}$/vehicle group in the prefrontal cortex ($p < 0.001$) and striatum ($p = 0.045$), which was also seen in comparison with the $P2X7R^{-/-}$/AMPH group in prefrontal cortex ($p = 0.036$). No significant effects of AMPH were seen in $P2X7R^{-/-}$ animals, which complements our previous results and indicates a lack of responsiveness to AMPH in animal where P2X7R is blocked or absent. We have also evaluated the levels of IL-6 and BDNF in the acute AMPH model, but no significant differences were found (data not shown).

P2X7R modulation of the proinflammatory cytokines response in the model of mania induced by chronic AMPH.

There was a significant increase in the levels of IL-1β in striatum ($p = 0.003$, Figure 2; **Supplementary table 2**) after chronic AMPH injection when compared to the control group, which

was reversed by the treatment with the BBG and the A438079 ($p = 0.005$ and $p = 0.005$, respectively) (**Fig. 2a**). In the hippocampus, IL-1β levels were increased in the BzATP/AMPH group compared to vehicle/vehicle control group ($p = 0.025$), which was reversed by the treatment with the A438079 ($p = 0.023$) (**Fig. 2a**). Chronic AMPH also increased IL-1β levels in WT animals in comparison to the WT/vehicle control groups, both in the striatum ($p = 0.015$) and in the hippocampus ($p = 0.017$) (**Fig. 2b**). As seen in the groups treated with the P2X7R antagonists, chronic AMPH had no effect in the $P2X7R^{-/-}$ animals compared with the $P2X7R^{-/-}$/vehicle control groups, both in the striatum ($p = 0.79$) and in the hippocampus ($p = 0.99$) (Fig 2c). Moreover, the WT/AMPH group showed a significant increase in IL-1β levels compared to the $P2X7R^{-/-}$/vehicle group in striatum ($p = 0.009$) and hippocampus ($p = 0.018$), which was also found when compared with the $P2X7R^{-/-}$/AMPH group, both in striatum ($p = 0.04$) and hippocampus ($p = 0.012$). Similarly, there was a significant increase in the levels of TNF-α ($p = 0.018$) in hippocampus in the BzATP/AMPH group when compared to the control group, which was reversed by the treatment with the A438079 ($p = 0.044$) (**Fig. 2c**).

Chronic AMPH increased TNF-α levels in WT animals when compared with the WT/vehicle control group in the striatum ($p = 0.011$) and hippocampus ($p = 0.016$) (**Fig. 2d**). Moreover, AMPH had no effect in the $P2X7R^{-/-}$ animals compared with the $P2X7R^{-/-}$/vehicle control group in the striatum ($p = 0.19$) and in the hippocampus ($p = 0.08$). Furthermore, the WT/AMPH group showed a significant increase in TNF-α levels compared to the $P2X7R^{-/-}$/vehicle group in striatum ($p = 0.017$) and hippocampus ($p = 0.012$). The proinflammatory cytokines results suggest that P2X7R participates in the increase of IL-1β and TNF-α in response to the chronic treatment of AMPH. We have also evaluated the levels of IL-6, but no significant difference was found between groups (data not shown).

Assessment of the P2X7R modulatory effects on a marker of astrogliosis in the model of mania induced by chronic AMPH.

There was a significant increase in the levels of GFAP in the prefrontal cortex (**Fig. 3a; Supplementary table 2**) in the vehicle/AMPH ($p < 0.001$), BzATP/AMPH ($p = 0.03$) and A438079/AMPH ($p = 0.005$) groups when compared to the vehicle/vehicle control group. When compared with the vehicle/AMPH group, GFAP levels were decreased in BzATP/AMPH ($p = 0.001$), BBG/AMPH ($p = 0.001$) and A438079/AMPH ($p = 0.032$) groups. In the striatum (**Fig. 3a**), there was a significant increase in the levels of GFAP in the vehicle/AMPH ($p = 0.044$), BzATP/AMPH ($p < 0.001$) and A438079/AMPH ($p = 0.005$) groups when compared to the control group. Even in the hippocampus, chronic AMPH treatment also increased the GFAP levels ($p = 0.32$) compared to the control group, and the treatment with the BBG demonstrated a tendency to reverse this AMPH-induced increase ($p = 0.052$). These results suggest a participation of a P2XR in the chronic AMPH-induced astrogliosis.

P2X7R modulation effects on BDNF levels in the model of mania induced by chronic AMPH.

There was a significant decrease in the levels of BDNF in the prefrontal cortex of animals treated with chronic AMPH compared with vehicle/vehicle control group ($p = 0.037$) (**Fig. 4a; Supplementary table 2**). The modulation of P2X7R did not result in any changes, indicating that this may not be the target by which P2X7R modulation led to the previously reported behavioral changes.

P2X7R modulation effects on TBARS in the model of mania induced by chronic AMPH.

The last assessment was the TBARS levels, which were taken as an indicator of oxidative stress (**Fig. 5; Supplementary table 2**). There was an increase in TBARS levels in the prefrontal cortex in the BzATP/AMPH group ($p = 0.037$) when compared to the control group, which was reversed by the treatment with the A438079 ($p = 0.01$) (**Fig. 5a**). In the hippocampus, chronic

AMPH treatment increased TBARS levels ($p = 0.022$) when compared to control group, which was reversed by the treatment with the BBG and the A438079 ($p = 0.016$ and $p = 0.016$, respectively) (**Fig. 5a**). In this same vein, these results suggest the involvement of P2X7R in the chronic AMPH-induced increase of oxidative stress.

Discussion

Data emerging from the present study provides evidence for a relationship between the P2X7R and the AMPH-induced hyperactivity, as demonstrated by an apparent lack of responsiveness to AMPH by animals with blocked or absent P2X7R. Taken together, out results suggest the involvement of P2X7R in the animal model of mania, suggesting a role for this receptor in the pathophysiology of BD.

To our knowledge, this is the first study dedicated to verify and explore with behavioral and biochemical parameters the involvement of the P2X7R in BD. However, this relationship has already been suggested mainly by linkage and association genetic studies. Of note, the *P2X7R* gene has been described to be located in the 12q23-24 chromosome region, which has been suggested as a susceptible locus for BD (20). Further studies have reported an association between the *P2X7R* gene and BD, especially with the non-synonymous single nucleotide polymorphism (SNP) rs2230912, Gln460Arg (21). In addition, other polymorphisms in this gene, such as the SNPs rs1718119 and rs1621388, have also been associated with BD manic symptoms (22).

Besides these data, a study showed that $P2X7R^{-/-}$ mice demonstrated an antidepressant like phenotype and had an augmented response compared with WT mice when treated with imipramine (14). In this sense, there is considerable evidence suggesting the involvement of the P2X7R in the pathophysiology of major depression (23, 24). Interestingly, acute (*in vitro*) and chronic (*in vivo*) treatment with known mood stabilizers (lithium and valproate) prevented ATP-induced cell death (25), probably via P2X7R. Moreover, chronic treatment of rats with lithium induced an increase in

ATP and AMP hydrolysis in hippocampal synaptosomes (26), suggesting that mood stabilizers may act also by modulating ectonucleotidases and directly interfering with the purinergic system.

Animal models for BD have always been a challenge due to the cyclical nature of the disorder, so that the majority of models employed are therefore specific to the acute episodes (27). Both acute and chronic treatments with AMPH have been widely used as an animal model of mania, being based on the observation of hyperlocomotion (6, 28) and analysis of different brain regions (29, 30). This animal model mimics several behavioral (e.g., hyperactivity) and neurochemical alterations (e.g., the postulated dopamine dysregulation) observed in manic-like symptoms of BD individuals (31-33).

The results regarding the proinflammatory cytokines suggest that the P2X7R participates in the AMPH-induced increase of proinflammatory environment, demonstrated by the reversal role of P2X7R blockage or the lack of response by $P2X7R^{-/-}$ mice. Of note, treatment with methamphetamine, a psychostimulant that shares a nearly identical chemical structure with AMPH (34), has been associated with increasing levels of proinflammatory cytokines in brains of animals (35). We demonstrated the same proinflammatory response after chronic treatment with AMPH and to a lesser extent after acute AMPH. As elevated levels of proinflammatory cytokines have been repeatedly demonstrated in BD patients (recently reviewed by (36), this response to AMPH contributes to the legitimacy of using this model as a model of mania. It is well known that the P2X7R has a central role in neuroinflammation (37), mainly due to increased production and release of IL-1β, TNF-α and IL-6 (38, 39) and due its crucial role in the processing and release of IL-1β (40). Indeed, mice deficient in P2X7R demonstrated decreased inflammatory responses (41), confirming the relationship between neuroinflammation and P2X7R. Based on our results of blocked or absent P2X7R, it is logical to conclude that the P2X7R is directly involved in this inflammatory response.

Lipid peroxidation is one of the major consequences of free radical-mediated brain injury (42). In the present study, we suggest the involvement of P2X7R on the increase of TBARS levels

induced by acute and chronic AMPH administrations. Accordingly, previous studies have reported an increase in lipid peroxidation in the AMPH-induced model of mania (29). Similarly, several lines of evidence suggest that increased oxidative stress plays a prominent role in the progressive brain changes observed in BD patients (13, 43). The P2X7R activation has also been related with enhanced oxidative stress (44, 45), justifying the observed responses by blocked or absent P2X7R. This receptor might be involved in the AMPH-induced production of oxidative stress/excitotoxicity. We also sought to determine whether the treatments could result in same alterations in BDNF levels, based on available evidence documenting decreased BDNF levels in individuals with BD (46) and in the animal model of AMPH (47). Our results confirm previous studies that describe a decrease in BDNF levels after chronic AMPH treatment. Of note, the modulation of P2X7R did not result in any changes, indicating that this may not be the target by which P2X7R modulation led to the previously reported behavioral changes.

According to the results of GFAP content, especially in the chronic treatment of AMPH, we can clearly observe the action of AMPH on astrogliosis and the involvement of a P2X receptor in this response, although probably not P2X7R. Repeated exposure to AMPH has already been shown to lead to an astroglial response in the HPC (28) and dorsal/ventral caudate–putamen of rats (48). Our results confirm these findings at the HPC and add the PFC and the STR response. Previous studies reported that this astroglial response could be associated to a mild neurotoxicity (48), suggesting an excitotoxicity action of AMPH, which has already been demonstrated in postmortem frontal cortex from BD patients (8). The fact that treatment with the BBG and not A438079 reversed chronic AMPH-induced astrogliosis indicates that the P2X7R has no effect on this response, suggesting that others receptors antagonized by the BBG, i.e. the P2X4 and/or the P2X5 receptors, might be of relevance for this effect (49). More studies are needed to determine the pathways by which the purinergic modulation reverses astrogliosis induced by AMPH.

Overall, we report that AMPH treatment is inducing neuroinflammation and excitotoxicity, and that these effects are being mediated, at least in part, by the P2X7R. Some of these responses

are partially explained by astrocytic activation, mainly because this effect was not reversed by blocking P2X7R. In addition, it has been suggested that astrogliosis might follow abnormal neuronal functioning even without neuronal death or degeneration (50, 51). On the other hand, microglial activation seems to suitably explain the integration of the neuroinflammatory/excitotoxicity results and the P2X7R. It has been well recognized that microglial activation leads to the synthesis of proinflammatory and excitotoxity mediators, including IL-1β and TNF-α, chemokines and reactive oxygen species, triggering tissue impairment (37, 52), ultimately leading to the association of neurodegenerative diseases (52) and mental illnesses (8, 53, 54). Furthermore, a recent review suggested a key role for microglial activation in BD (55). The P2X7R, in turn, has been put forward as an essential component of the induction of microglial activation (37). Future studies should investigate the involvement of microglial activation in the AMPH-induced hyperactivity and if this involvement is subject to modulation by P2X7R.

In some of our experiments, only the co-administration of AMPH with BzATP led to a significant response of the corresponding biochemical parameter. However, this action was not homogeneous, not allowing us to conclude that P2X7R agonism could increase the response to AMPH. Also, we could see that the chronic administration of AMPH showed more significant results compared to the acute AMPH treatment, as previously shown (28). We hypothesize that our results are not simply a consequence of the acute pharmacological action of AMPH, but rather a consequence of a more prolonged exposure to the drug. This actually increases the construct validity of our animal model of mania. Lastly, these putative brain areas were selected due to the fact that they are the main areas involved in the pathophysiology of BD (29, 30).

The interpretation of the present results should respect certain limitations. Firstly, we have to consider that animal models for psychiatric disorders have some limitations in their face and construct validities (56, 57). However, the animal model of mania induced by AMPH has a well-defined predictive validity (47, 58). Moreover, our sample size was restricted, which may have

masked some results; however, we had enough statistical power in the behavioral test, which prompted us to keep the sample as it was for the further analyses. Also, we were not able to establish the specific mechanisms mediating the P2X7R participation. Our results suggest some pathways of relevance, but certainly more studies are needed to well characterize the P2X7R involvement in BD.

In conclusion, our findings suggest that P2X7R has the potential to become a therapeutic target of BD. Furthermore, our results provide support for the hypothesis that P2X7R plays a role in BD pathophysiology, as suggested by our animal model, mainly by mediating neuroinflammation and excitotoxicity and ultimately leading to behavioral changes. More studies are required to clarify the microglial role suggested from our results, as well as to better characterize the involvement of P2X7R in the pathophysiology of BD.

Acknowledgements

CG, GRF, PF are recipients of scholarships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). BP are recipients of scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This study was supported by National Institute for Translational Medicine, funded by CNPq, Brazil, and Fundo de Incentivo a Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE-HCPA).

Disclosures

CG, GRF, BP, PF, RCS, FBM and AMOB declare no possible conflict of interest, financial or otherwise, as well as grants or other forms of financial support. FK has received grant/research support from Astra-Zeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD and Stanley Medical Research Institute; has been a member of the speakers' boards for Astra-Zeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier.

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Table 1. P2X7R modulation effects on IL-1β, TNF-α, GFAP and TBARS levels in the model of mania induced by acute amphetamine.

Treatment	IL-1β[a]			TNF-α[a]			GFAP[b]			TBARS[c]		
	PFC	STR	HPC	PFC	STR	HPC	PFC	STR	HPC	PFC	STR	HPC
Vehicle/Vehicle	271.6 ± 57.61	137.1 ± 30.05	161.7 ± 30.79	2770.0 ± 484.0	1964.0 ± 710.1	1579.0 ± 565.0	169.3 ± 44.48	227.6 ± 64.74	288.7 ± 64.71	7.98 ± 0.45	2.34 ± 0.31	4.97 ± 0.25
Vehicle/AMPH	223.8 ± 48.92	432.0 ± 86.39*	218.6 ± 43.73	2054.0 ± 693.3	3117.0 ± 400.6	1604.0 ± 390.3	226.6 ± 49.06	175.7 ± 107.4	443.9 ± 51.5	10.54 ± 2.24	9.56 ± 1.60***	4.37 ± 0.14
BzATP/AMPH	390.8 ± 45.23	202.8 ± 51.79	223 ± 32.23	1835.0 ± 311.8	2384.0 ± 445.3	2344.0 ± 386.5	223.5 ± 46.61	199.6 ± 66.21	412.3 ± 101.5	7.57 ± 1.72	5.66 ± 0.93	6.35 ± 0.82
A438079/AMPH	267.2 ± 36.68	226.3 ± 71.01	187.7 ± 64.69	2481.0 ± 150.6	1104.0 ± 213.0#	2139.0 ± 739.6	213.3 ± 54.93	204.7 ± 67.81	129.0 ± 62.98#	11.19 ± 2.44	4.12 ± 0.85##	5.69 ± 0.64
WT/Vehicle	208.6 ± 34.53	313.9 ± 57.87	262.4 ±53.19	1202.0 ± 434.2	1244.0 ± 271.3	1131.0 ± 217.2	82.39 ± 26.94	34.38 ± 13.59	101.1 ± 43.46	20.02 ± 0.57	16.41 ± 0.73	14.33 ± 2.28
WT/AMPH	194.5 ± 25.99	365.4 ± 94.57	206.8 ± 53.57	1679.0 ± 157.7	1919.0 ± 420.8	1389.0 ± 563.3	116.0 ± 24.95	70.1 ± 26.99	215.0 ± 70.91	30.8 ± 2.9**	26.37 ± 2.25**	21.22 ± 2.53
P2X7R(-/-)/Vehicle	316.6 ± 56.94	268.0 ± 53.09	185.4 ± 38.50	1347.0 ± 157.0	2406.0 ± 396.3	1674.0 ± 306.3	60.3 ± 36.79	87.63 ± 29.64	218.2 ± 62.40	16.34 ± 1.18###	18.75 ± 1.47#	21.22 ± 4.51
P2X7R(-/-)/AMPH	317.1 ± 87.78	217.4 ± 59.73	331.7 ± 25.5	1398.0 ± 38.87	1804.0 ± 204.6	1566.0 ± 163.4	149.5 ± 18.32	96.37 ± 24.30	110.1 ± 55.37	21.70 ± 1.4#	23.96 ± 2.63	19.19 ± 1.73

Values represent means ± SEM of 5-6 animals per group. AMPH, amphetamine; BzATP, selective P2X7R agonist; A438079, selective P2X7R antagonist; WT, wild-tipe; $P2X7R^{-/-}$, P2X7R *knockout* mice; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; GFAP, glial fibrillary acidic protein; TBARS, thiobarbituric acid reactive substances; PFC, prefrontal cortex; STR, striatum and HPC, hippocampus. ANOVA followed by Tukey's Multiple Comparison post hoc test:
*p<0.05; **p<0.01; ***p<0.001 vs respective vehicle group
#p<0.05; ##p<0.01, ###p<0.001 vs vehicle/AMPH or WT/AMPH
[a] fg/mL
[b] pg/ug of protein
[c] μM of MDA

Supplementary table 1. Summary of P2X7R modulation effects in the model of mania induced by chronic amphetamine.

	CHRONIC AMPH						
Parameters	**Vehicle/AMPH**	**BzATP/AMPH**	**BBG/AMPH**	**A438079/AMPH**	**WT/AMPH**	**P2X7R$^{(-/-)}$/Vehicle**	**P2X7R$^{(-/-)}$/AMPH**
Locomotor activity	↑[a]	↑[a]	-	↓[b] ↓[c]	↑[d]	↓[e]	↓[e]
IL-1β							
PFC	-	-	-	-	-	-	-
STR	↑[a]	-	↓[b]	↓[b]	↑[d]	↓[e]	↓[e]
HPC	-	↑[a]	-	↓[c]	↑[d]	↓[e]	↓[e]
TNF-α							
PFC	-	-	-	-	-	-	-
STR	-	-	-	-	↑[d]	↓[e]	-
HPC	-	↑[a]	-	↓[c]	↑[d]	↓[e]	-
IL-6							
PFC	-	-	-	-	-	-	-
STR	-	-	-	-	-	-	-
HPC	-	-	-	-	-	-	-
GFAP							
PFC	↑[a]	↑[a] ↓[b]	↓[b]	↑[a] ↓[b]	-	-	-
STR	↑[a]	↑[a]	-	↑[a]	-	-	-
HPC	↑[a]	-	-	-	-	-	-
BDNF							
PFC	↓[a]	-	-	-	-	-	-
STR	-	-	-	-	-	-	-
HPC	-	-	-	-	-	-	-
TBARS							
PFC	-	↑[a]	-	↓[c]	-	-	-
STR	-	-	-	-	-	-	-
HPC	↑[a]	-	↓[b]	↓[b]	-	-	-

↑, ↓: Statistically significant increase or decrease compared to [a]vehicle/vehicle, [b]vehicle/AMPH, [c]BzATP/AMPH, [d]WT/vehicle and [e]WT/AMPH treatment group. AMPH, amphetamine; BzATP, selective P2X7R agonist; A438079, selective P2X7R antagonist; WT, wild-type; P2X7R$^{-/-}$, P2X7R *knockout* mice; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; GFAP, glial fibrillary acidic protein; TBARS, thiobarbituric acid reactive substances; PFC, prefrontal cortex; STR, striatum and HPC, hippocampus.

Figure Legends

Figure 1. Analysis of the P2X7R modulatory effect on behavior in the model of mania induced by acute and chronic AMPH.

Effect of single i.c.v administration of the selective P2X7 agonist, BzATP (10.5 nmol), the non-selective P2X7R antagonist, BBG (20 mmol), and the selective P2X7R antagonist, A438079 (1.75 nmol), on locomotor activity, demonstrated as distance traveled (m) in a model of mania induced by acute (2 mg/kg, i.p., single injection) and chronic (2 mg/kg, i.p., once a day, 7 days) amphetamine (AMPH) in mice **(a)**. Behavioral changes of $P2X7R^{-/-}$ and WT mice in the model of mania induced by acute (2 mg/kg, i.p., single injection) and chronic (2 mg/kg, i.p., once a day, 7 days) AMPH **(b)**. ANOVA followed by Tukey's Multiple Comparison post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are expressed as the means ± S.E.M of 5-6 animals per group.

Figure 2. Determination of the P2X7R modulatory effect on the proinflammatory cytokines response in the model of mania induced by chronic AMPH.

Effect of single i.c.v administration of the selective P2X7 agonist, BzATP (10.5nmol), the non-selective P2X7R antagonist, BBG (20mmol), and the selective P2X7R antagonist, A438079 (1.75nmol), on IL-1β **(a)** and TNF-α **(c)** levels in prefrontal cortex, striatum and hippocampus in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) amphetamine (AMPH) in mice. Assessment of IL-1β **(b)** and TNF-α **(d)** levels in prefrontal cortex, striatum and hippocampus of the $P2X7R^{-/-}$ and WT mice in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) AMPH. ANOVA followed by Tukey's Multiple Comparison post hoc test: *$p < 0.05$, **$p < 0.01$. Data are expressed as the means ± S.E.M of 5-6 animals per group.

Figure 3. Assessment of the P2X7R modulation effects on GFAP in the model of mania induced by chronic AMPH.

Effect of single i.c.v administration of the selective P2X7 agonist, BzATP (10.5nmol), the non-selective P2X7R antagonist, BBG (20mmol), and the selective P2X7R antagonist, A438079 (1.75nmol), on the GFAP levels in prefrontal cortex, striatum and hippocampus in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) amphetamine (AMPH) in mice **(a)**. GFAP levels in prefrontal cortex, striatum and hippocampus of the $P2X7R^{-/-}$ and WT mice in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) AMPH **(b)**. ANOVA followed by Tukey's Multiple Comparison post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are expressed as the means ± S.E.M of 5-6 animals per group.

Figure 4. Evaluation of the P2X7R modulatory effects on BDNF levels in the model of mania induced by chronic AMPH.

Effect of single i.c.v administration of the selective P2X7 agonist, BzATP (10.5nmol), the non-selective P2X7R antagonist, BBG (20mmol), and the selective P2X7R antagonist, A438079 (1.75nmol), on the BDNF levels in prefrontal cortex, striatum and hippocampus in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) amphetamine (AMPH) in mice **(a)**. BDNF levels in prefrontal cortex, striatum and hippocampus of the $P2X7R^{-/-}$ and WT mice in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) AMPH **(b)**. ANOVA followed by Tukey's Multiple Comparison post hoc test: *$p < 0.05$. Data are expressed as the means ± S.E.M of 5-6 animals per group.

Figure 5. P2X7R modulatory effects on TBARS in the model of mania induced by chronic AMPH.

Effect of single i.c.v administration of the selective P2X7 agonist, BzATP (10.5nmol), the non-selective P2X7R antagonist, BBG (20mmol), and the selective P2X7R antagonist, A438079 (1.75nmol), on the lipid peroxidation levels in prefrontal cortex, striatum and hippocampus in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) amphetamine (AMPH) in mice **(a)**. Lipid peroxidation levels in prefrontal cortex, striatum and hippocampus of the P2X7R−/− and WT mice in the model of mania induced by chronic (2 mg/kg, i.p., once a day, 7 days) AMPH **(b)**. ANOVA followed by Tukey's Multiple Comparison post hoc test: *$p < 0.05$. Data are expressed as the means ± S.E.M of 5-6 animals per group.

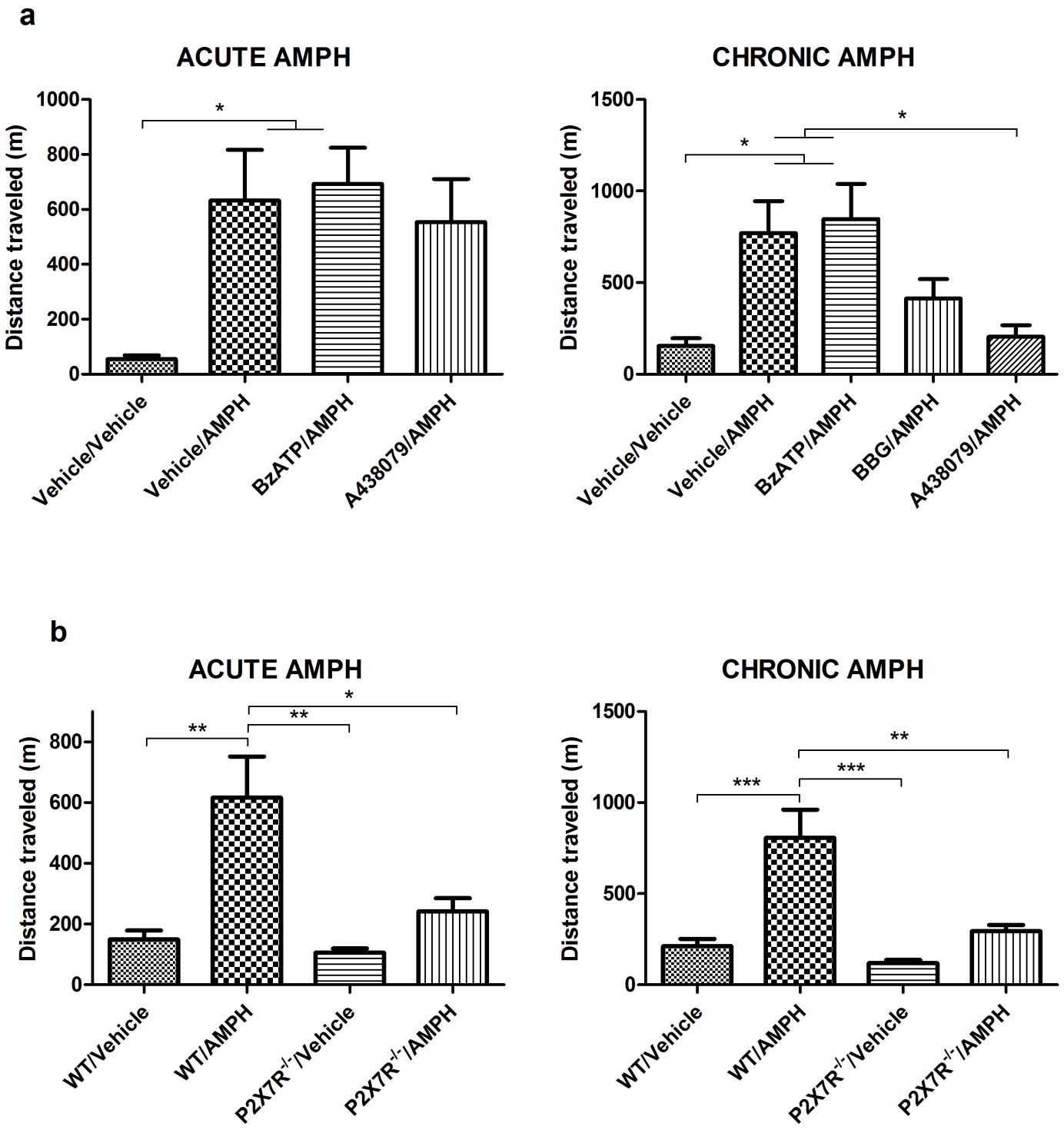
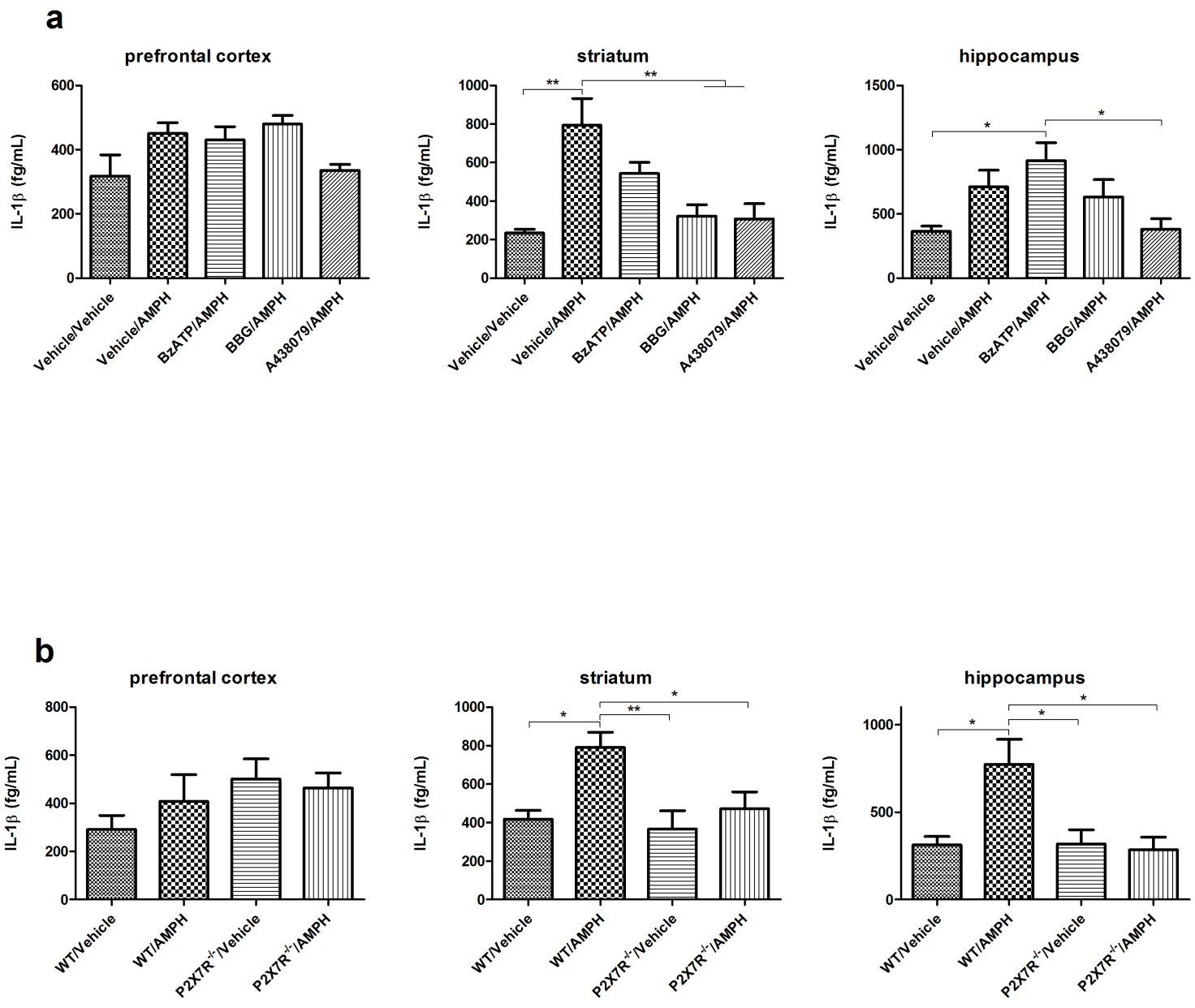


Figure 1. Analysis of the P2X7R modulatory effect on behavior in the model of mania induced by acute and chronic AMPH.


a
prefrontal cortex
striatum
hippocampus
IL-1β (fg/mL)
Vehicle/Vehicle
Vehicle/AMPH
BzATP/AMPH
BBG/AMPH
A438079/AMPH
b
WT/Vehicle
WT/AMPH
P2X7R-/-/Vehicle
P2X7R-/-/AMPH

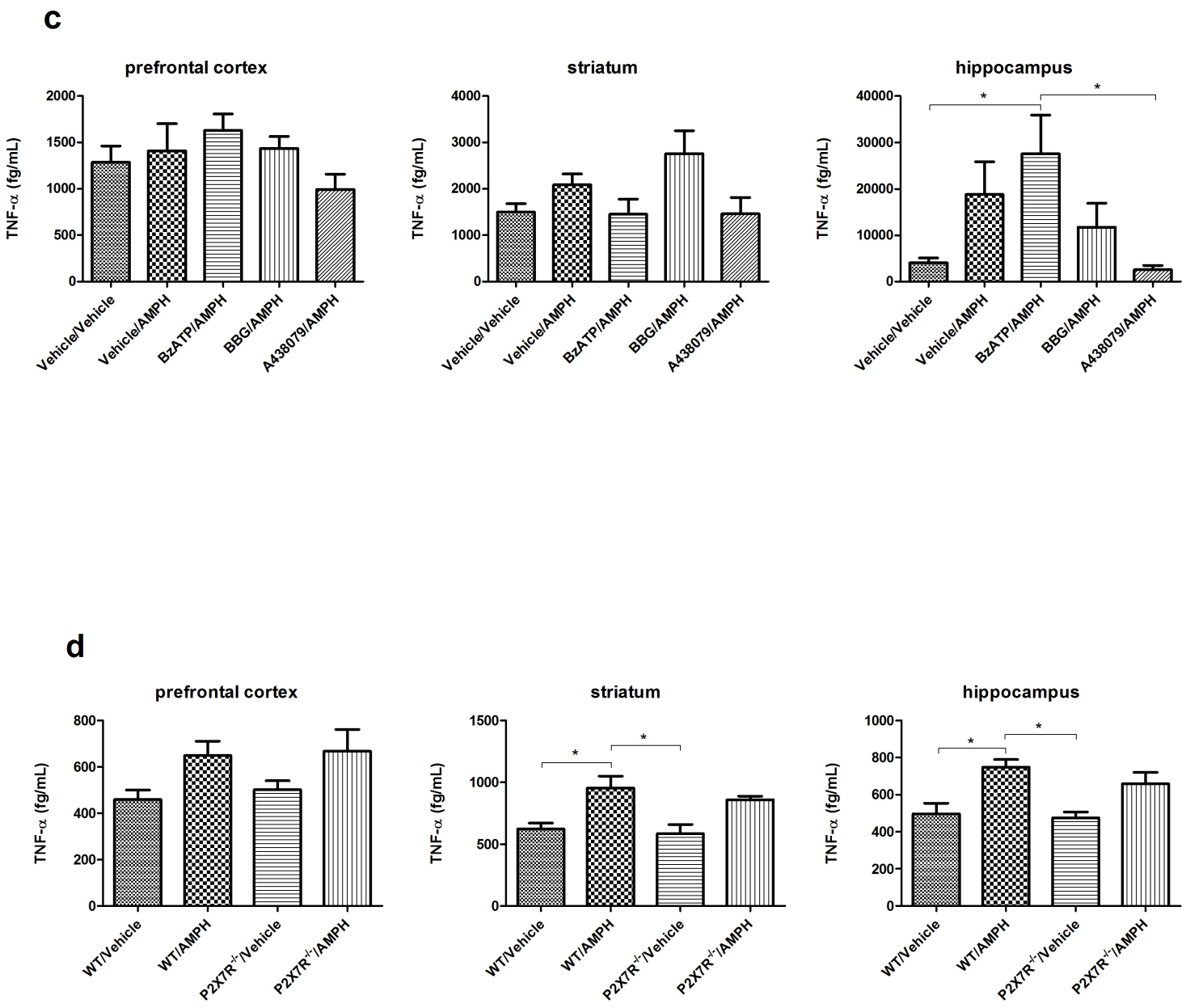


Figure 2. Determination of the P2X7R modulatory effect on the proinflammatory cytokines response in the model of mania induced by chronic AMPH.

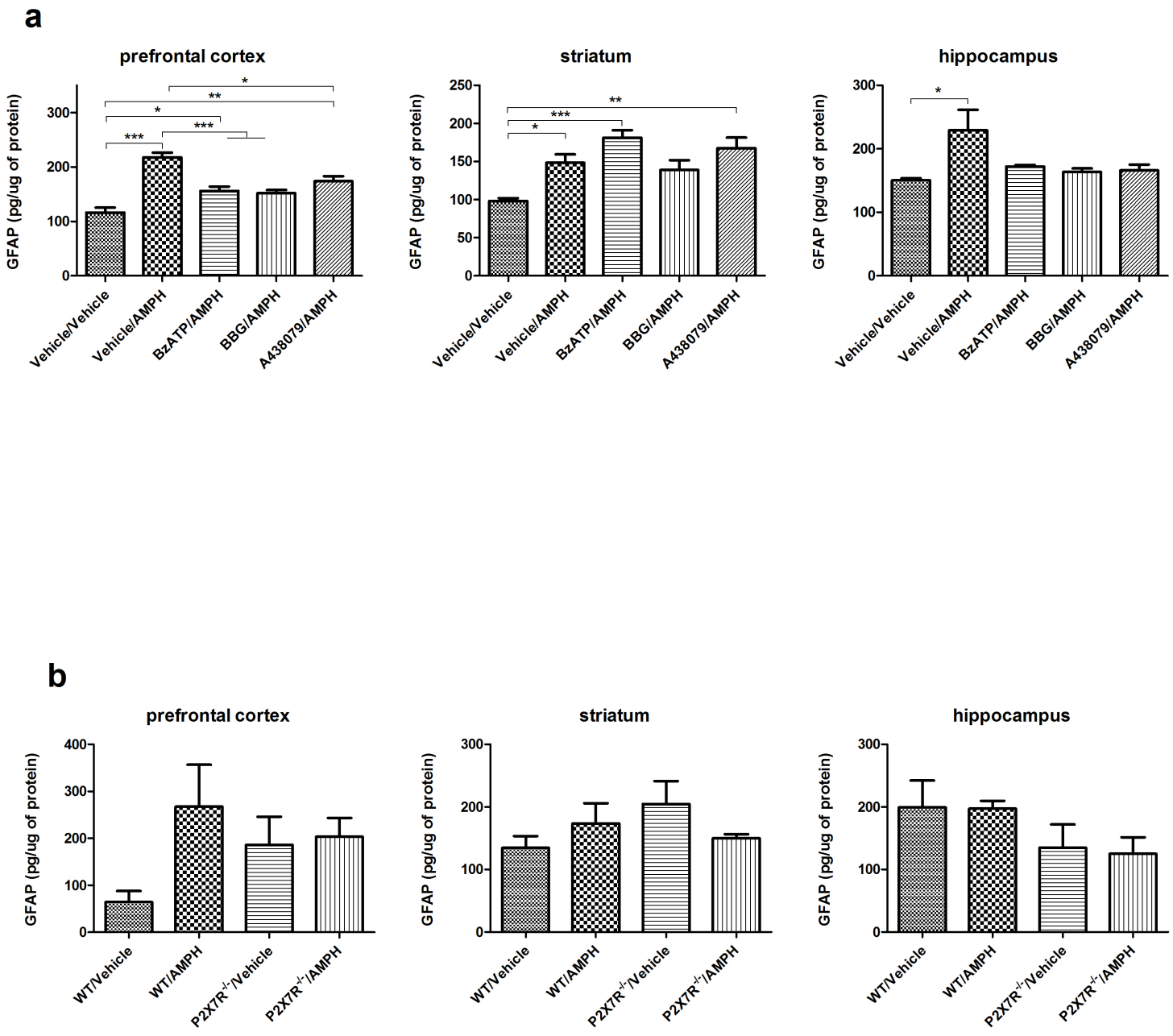


Figure 3. Assessment of the P2X7R modulation effects on GFAP in the model of mania induced by chronic AMPH.

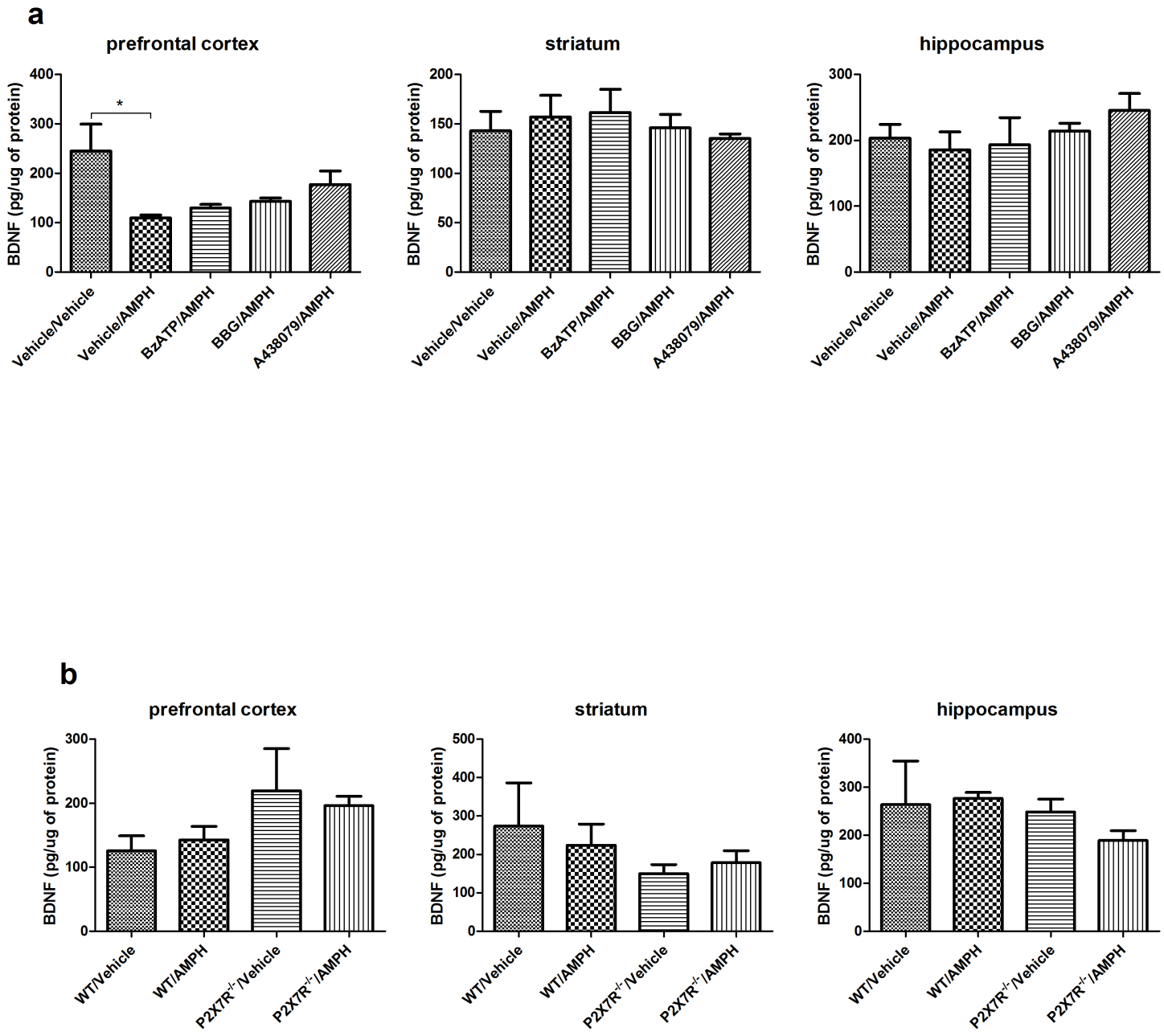


Figure 4. Evaluation of the P2X7R modulatory effects on BDNF levels in the model of mania induced by chronic AMPH.

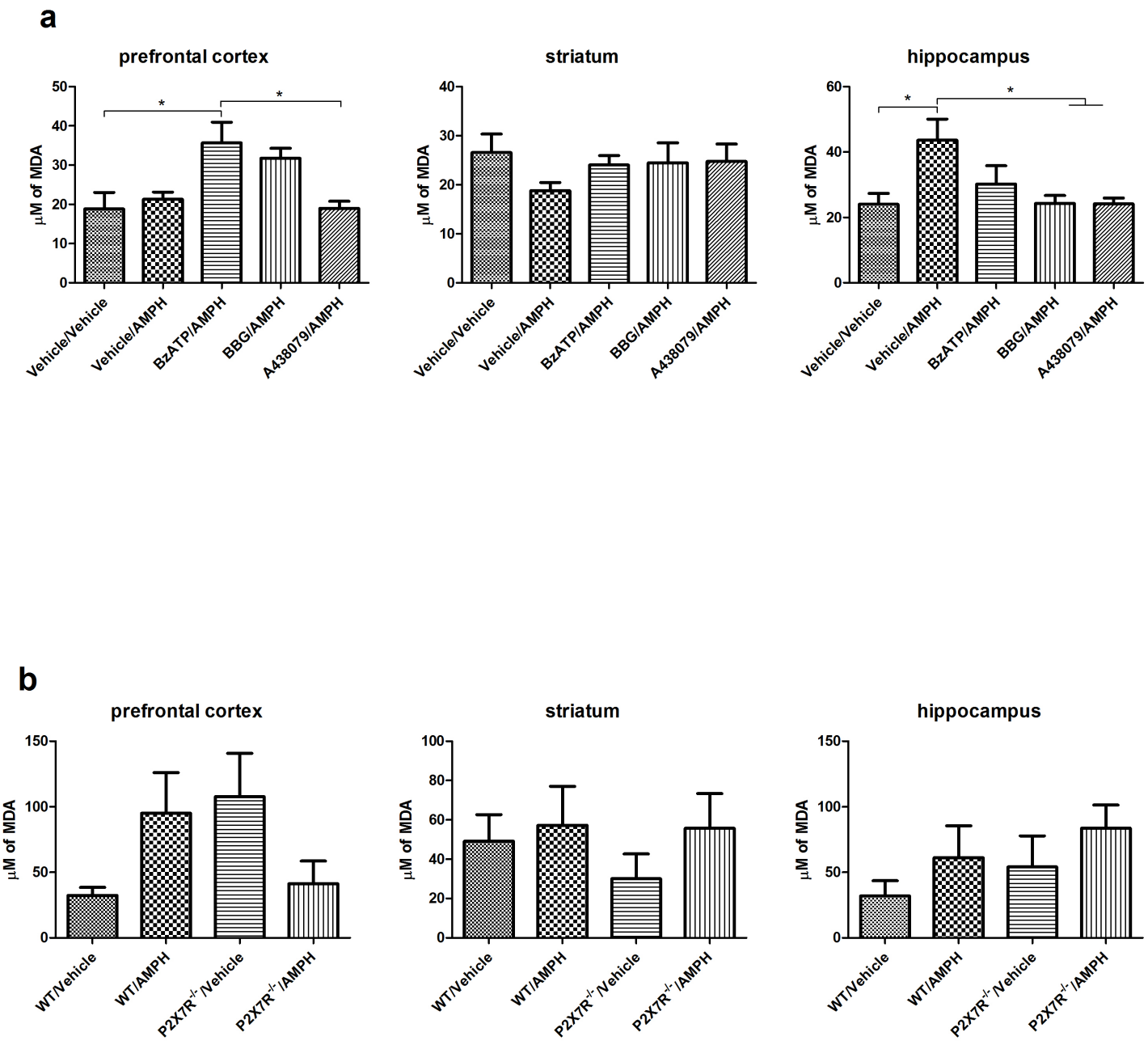


Figure 5. P2X7R modulatory effects on TBARS in the model of mania induced by chronic AMPH.

3.2. Capítulo II

The P2X7 purinergic receptor as a molecular target in bipolar disorder

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Publicação: "Neuropsychiatria i Neuropsychologia 2013; 8, 1: 1–7"

Review article

The P2X7 purinergic receptor as a molecular target in bipolar disorder

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Neuropsychiatria i Neuropsychologia 2013; 8, 1: 1–7

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Abstract

The purinergic system has been increasingly implicated in medical conditions, among them bipolar disorder (BD). This is based primarily on the role of extracellular adenosine triphosphate (ATP) and purinergic receptors in cytokine regulation and the pathological activation of glial cells, leading to neuroinflammation. In addition, adenosine metabolism is directly related to the pathophysiology of BD. Among purinergic receptors, P2X7 was associated with BD in several genetic studies. This particular receptor has a key role in the modulation of the inflammatory response, acting as a sensor of harm and responding to ATP released from injured or stressed cells in the central nervous system, ultimately driving microglial cells from their resting into the activated form. Of note, markers of excitotoxicity and neuroinflammation are significantly upregulated in frontal cortex from BD patients compared with controls, justifying the need for further research. The present review focuses on purinergic signaling in BD, with an emphasis on ATP and adenosine signaling, highlighting the potential role of P2X7R in modulating inflammation and microglia activation in bipolar patients. Due to its ability to act on microglia and modulate neuroinflammation, we believe that more detailed studies of the role of P2X7R in BD are warranted.

Key words: bipolar disorder, purinergic system, P2X7R, microglia, inflammation.

Introduction

The purinergic system includes nucleotides (most notably, adenosine triphosphate – ATP), nucleosides, as well as a large family of ectonucleotidases (Baroja-Mazo *et al*. 2013). There are two main purinoceptor classes: P1 receptors, which are activated by adenosine, and the P2 family, which is subdivided into P2Y and P2X, activated by different nucleotides and ATP, respectively (Baroja-Mazo *et al*. 2013). Purinergic signaling has been identified in virtually all cells and it is implicated in many neuronal and non-neuronal mechanisms, in physiological as well as pathological conditions, including secretion, immune responses, cell proliferation, cell death, pain and inflammation (Baroja-Mazo *et al*. 2013). Several purinergic receptor subtypes have been shown to be widely distributed throughout the CNS, in neurons and glia (Weisman *et al*. 2012).

Recently, purinergic pathophysiology has been emphasized in numerous medical conditions, especially neurodegenerative and psychiatric disorders, including bipolar disorder (BD)

(McQuillin *et al*. 2009; Lopes *et al*. 2011). This expanding area of research is based primarily on the role of extracellular ATP and purinergic receptors in cytokine regulation and pathological activation of glial cells, leading to neuroinflammation (Rao *et al*. 2010). Likewise, adenosine metabolism has been directly related to the pathophysiology of BD (Salvadore *et al*. 2010). Kraeplin proposed a long time ago an association between manic symptoms and purinergic system dysfunction when he related the former to hyperuricemia, uric acid excretion and gout (Kraeplin 1921). Current evidence points to purinergic system impairment in BD patients, mainly in adenosine P1 and P2X receptors. Recently, genetic studies demonstrated a potential role for purinergic system dysfunction in the pathophysiology of BD, primarily in the P2X7 receptor (P2X7R) (Backlund *et al*. 2011).

The present review focuses on purinergic signaling in BD, with an emphasis on ATP and adenosine signaling, highlighting the potential role of P2X7R in modulating inflammation and microglial activation in bipolar patients.

P1 receptors

Extracellular adenosine is present in organisms as an intermediate metabolite of ATP catabolism (Fredholm *et al*. 2001). It acts by regulating several physiological processes (Ferré 1997). Adenosine receptors are present in the CNS, among other tissues, and are divided into four types of receptors: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al*. 2001). The A_1 receptor is responsible for many of the inhibitory effects of adenosine in the CNS, and has widespread distribution in different brain areas such as the cortex, hippocampus, cerebellum and thalamus, where it is found in higher concentrations (Ribeiro *et al*. 2002; Stone *et al*. 2009). Its activation causes a decrease in neuronal excitability, reduction of uric acid levels, and inhibition of Ca^{2+}-dependent excitatory neurotransmitter release, thus being responsible for the modulation of neurotransmitter release (Lopes *et al*. 2011).

Increased levels of uric acid have been implicated in the pathophysiology of BD (Salvadore *et al*. 2010). Uric acid is the nitrogenous end product of purine metabolism and is generated by the enzyme xanthine oxidase from xanthine and hypoxanthine (for review, see Moriwaki *et al*. 1999). Studies suggest that the purinergic modulator allopurinol acts by inhibiting the enzyme xanthine oxidase, reducing uric acid levels as a consequence. Patients in their first manic episode showed elevated plasma levels of uric acid compared to controls, indicating that a purinergic system dysfunction could be present early in the course of the disorder (Salvadore *et al*. 2010).

A randomized, double blind, placebo-controlled trial in patients with moderate to severe mania found that the use of allopurinol with lithium and haloperidol for 8 weeks compared to haloperidol lithium and placebo resulted in reduction of agitation and manic symptoms, as assessed by the Young Mania Rating Scale (YMRS) (Akhondzadeh *et al*. 2006). Based on this evidence, allopurinol has been proposed as an adjunctive drug to lithium for the treatment of manic episodes in patients with BD.

Since refractoriness rates among bipolar patients are still very high and a significant number of manic patients are not responsive to conventional mood stabilizers, often requiring different combinations of drugs, the purinergic pathways may provide future therapeutic targets for BD.

P2 receptors

Most of the evidence pointing to an association between BD and purinergic signaling relies on the P2 family of receptors, which are preferentially activated by extracellular ATP (Abbracchio and Burnstock 1998). These receptors are divided into two distinct families: the P2X ligand-gated ionotropic channel receptors, and the P2Y, metabotropic G-protein coupled receptors. Because they act as ion channels, P2X receptors respond more rapidly than P2Y and are mainly involved in rapid excitatory neurotransmission (Burnstock and Williams 2000). These receptors act as ion channels with high calcium permeability that open upon binding of extracellular ATP (North 2002). Of note, the activation of P2X7R, which presents a very low affinity for ATP, requires near millimolar concentrations of ATP (Skaper *et al*. 2009). Massive ATP release into the extracellular milieu can take place after acute cell injury or death, and also under inflammatory conditions and in response to tissue trauma (Skaper *et al*. 2009). Once activated by high ATP levels, P2X7R can act as a nonselective ion pore; however, continuous stimulation results in the formation of a larger pore, which facilitates the uptake of cationic molecules up to 900 Da, possibly triggering the activation of apoptosis or cell lysis (Skaper *et al*. 2009).

P2X7R mediates cellular processes such as apoptosis, as well as cell proliferation and pro-

inflammatory cytokine release (Apolloni *et al*. 2009). It acts on neurotransmission (supposedly influencing dopamine release), neuromodulation and neurotrophic mechanisms (Backlund *et al*. 2011). The expression of P2X7R is not restricted to immune-related cells, but is also present in microglia and astrocytes in the brain (Chakfe *et al*. 2002; Walter *et al*. 2004). In the CNS, it is found in the cerebral cortex, hippocampus, brainstem, nucleus accumbens, and spinal cord (Weisman *et al*. 2012).

The role of P2X7R in BD has been suggested mainly by linkage and association genetic studies. The P2X7R gene is located in the 12q23-24 chromosome region, which has been described as a susceptible locus for BD (Abkevich *et al*. 2003). Further studies have reported an association between the P2X7R gene and BD, especially with the non-synonymous single nucleotide polymorphism (SNP) rs2230912, Gln460Arg (McQuillin *et al*. 2009). In addition, other polymorphisms in this gene, such as the SNPs rs1718119 and rs1621388, have also been associated with BD manic symptoms (Backlund *et al*. 2011). This association may be mediated by the role of P2X7R in pro-inflammatory cytokine release, given that mania has been associated with increased cytokine levels (Stertz *et al*. 2013). Of note, some manic symptoms may be particularly vulnerable to such pro-inflammatory action (Backlund *et al*. 2011). In addition, it has been proposed that neuroticism, a personality trait reflecting individual differences in emotional stability and vulnerability to stress and anxiety, mediates the effect of another P2X7R polymorphism (rs208294) on medium-term outcome in major depressive disorder and BD (Mantere *et al*. 2012). This same SNP, together with rs2230912, was also associated with increased risk for a familial mood disorder in three independent cohorts, in which carriers of the risk alleles were ill for longer periods of time (Soronen *et al*. 2011).

A few animal studies have strengthened the association between P2X7R and BD. The data are still inconclusive, showing that P2X7R knockout mice demonstrated an antidepressant-like phenotype and when treated with imipramine, a tricyclic antidepressant, had an augmented response compared with wild type (Basso *et al*. 2009). However, other authors described an impaired adaptive coping response to repeated stress, as well as greater anxiety behavior (Boucher *et al*. 2011). In addition, a recent study employing a novel animal model of mania found that P2X7R expression is downregulated in the hippocampus, possibly contributing to the manic-like behaviors reported for the mice (Saul *et al*. 2012). This discrepancy may account for a different role of the receptor in manic and depressive episodes. Interestingly, acute (*in vitro*) and chronic (*in vivo*) treatment with known mood stabilizers (lithium and valproate) prevented ATP-induced cell death (Wilot *et al*. 2007). Moreover, chronic treatment of rats with lithium induced an increase in ATP and AMP hydrolysis in hippocampal synaptosomes (Wilot *et al*. 2004), suggesting that mood stabilizers may act by modulating ectonucleotidases and interfering with the purinergic system. Altogether, these studies strongly support the hypothesis that P2 receptors, mostly P2X7R, play a role in the pathophysiology of BD and thus deserve further studies.

Role of P2X7 receptor in inflammation

The involvement of the purinergic system with BD may be related to its potential role in modulating inflammation and microglial activation. Microglia are immunocompetent cells of the CNS (Pessac *et al*. 2001), producing either a neuroprotective or an inflammatory response (Stertz *et al*. 2013). Under normal conditions microglia exhibit a resting state, producing and releasing anti-inflammatory cytokines and neurotrophic factors, removing cellular debris and neutralizing pathogens (Ekdahl 2012; Nimmerjahn *et al*. 2005; Monif *et al*. 2010). In pathological conditions, including response to tissue injury, trauma or toxins, microglia become activated and assume markedly different biochemical and morphological states (Monif *et al*. 2010). Microglial activation leads to the synthesis of proinflammatory mediators, triggering tissue impairment (Weitz and Town 2012). Excessive activity of microglia exposes the CNS to proinflammatory cytokines, including interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), chemokines, reactive oxygen species, and proteases, all of which could have severe deleterious consequences in excessive amounts (Monif *et al*. 2010; Suzuki *et al*. 2004). This condition promotes neuroinflammation and contributes to a variety of pathological conditions, mainly neurodegenerative diseases like Alzheimer's (Weitz and Town 2012) and mental illnesses, such as depression, schizophrenia and BD (Rao *et al*. 2010; Bayer *et al*. 1999, Morgan *et al*. 2010).

Several studies have shown a significant relation between increased morbidity and heightened inflammatory levels in cardiovascular and metabolic syndromes and the same was observed in BD patients (Leboyer *et al*. 2012). These findings suggest the involvement of a proinflammatory state in the pathophysiology of BD (Berk *et al*. 2011). Multiple lines of evidence indicate that BD is a "multi-systemic inflammatory disease", with biochemical alterations occurring in and beyond the CNS (Munkholm *et al*. 2013). Progressive impairment of cognitive functions and brain atrophy have been consistently described in BD and suggest that the disease is progressive with important components of excitotoxicity and neuroinflammation (Lewandowski *et al*. 2011; Rao *et al*. 2010).

In animal models it has been demonstrated that both excitotoxicity and neuroinflammation are associated with increased brain levels of reactive oxygen species, nitric oxide and, most notably, proinflammatory cytokines (Chang *et al*. 2008). In a model of excitotoxicity, the chronic administration of subconvulsive doses of N-methyl-D-aspartate (NMDA) upregulated rat brain protein and mRNA levels of neuroinflammatory markers IL-1β, glial fibrillary acidic protein (GFAP), inducible nitric oxide synthase (iNOS) and TNF-β (Chang *et al*. 2008). A recent postmortem study demonstrated that markers of excitotoxicity and neuroinflammation are significantly upregulated in frontal cortex from BD patients compared with controls (Rao *et al*. 2010). Increased levels of c-Fos and iNOS mRNA have also been described in the same samples (Rao *et al*. 2010), as well as increased protein and mRNA levels of IL-1β, IL-1 receptor (IL-1R), and transcription factor nuclear factor-kappa B (NF-κB) subunits (p50 and p65), ultimately contributing to upregulation of proinflammatory gene products (Weisman *et al*. 2012). Likewise, there was a significant increase in GFAP expression and in the levels of CD11b mRNA (a marker of astrocyte and microglial activation) (Rao *et al*. 2010). These results indicate an important role of the cascade activation of the IL-1R on microglial activation. The authors suggest that this upregulation might result in cell death with subsequent brain atrophy and cognitive impairment that have been reported in BD patients (Rao *et al*. 2010).

Other studies have shown significantly higher levels of IL-1β in cerebrospinal fluid of patients with one or more recent manic/hypomanic episodes compared with patients without recent episodes. These findings indicate a relationship between the presence of acute episodes and activation of the IL-1R cascade (Söderlund *et al*. 2011). Furthermore, a recent review discussed the relevance of microglial activation on BD (Stertz *et al*. 2013). Researchers have suggested activation of microglia by damage-associated molecules in the first acute episode and a state of constant activation after several episodes, resulting in excessive production of proinflammatory cytokines, mainly IL-1β and TNF-α (Weitz and Town 2012). This condition leads to inhibition of neurogenesis, damage and neuronal death, potentially perpetuating systemic toxicity (Stertz *et al*. 2013).

An important damage-associated molecule candidate that would trigger microglial activation could be ATP by means of P2X7R. ATP has already been included in the limited family of those factors that signal danger to the immune system (i.e. damage-associated molecular patterns [DAMPs]) (Di Virgilio *et al*. 2009). The same authors suggest that P2X7R acts as a "sensor of danger", responding to the so-called "danger signal" ATP, which is released from injured or stressed cells in the CNS and drives resting microglial cells into their activated form (Weisman *et al*. 2012). There are several studies showing microglia activation induced by P2X7R stimulation (Suzuki *et al*. 2004; Monif *et al*. 2010), suggesting P2X7R as a key component of neuroinflammation (Monif *et al*. 2010). Moreover, P2X7R has been put forward as an essential component for the induction of microglial activation (Monif *et al*. 2010). When the P2X7R nucleotide binding site was blocked with oxidized ATP (oxATP), microglial activation was significantly attenuated, indicating that receptor occupancy is essential for microglial activation (Monif *et al*. 2010). In this same line of thought, P2X7R activation promotes neuroinflammation by inducing the release of proinflammatory cytokines, such as IL-1β and TNF-α (Di Virgilio 2007; Tschopp and Schroder 2010), and activation of NF-κB, resulting in upregulation of proinflammatory gene products (Skaper *et al*. 2010). Indeed, mice deficient in P2X7R demonstrated decreased inflammatory responses (Lucattelli *et al*. 2011), confirming the relationship between neuroinflammation and P2X7R.

Considering that the pathophysiology of BD includes a proinflammatory state, with a potential key role of microglia activation and neuroinflammation, our current understanding is

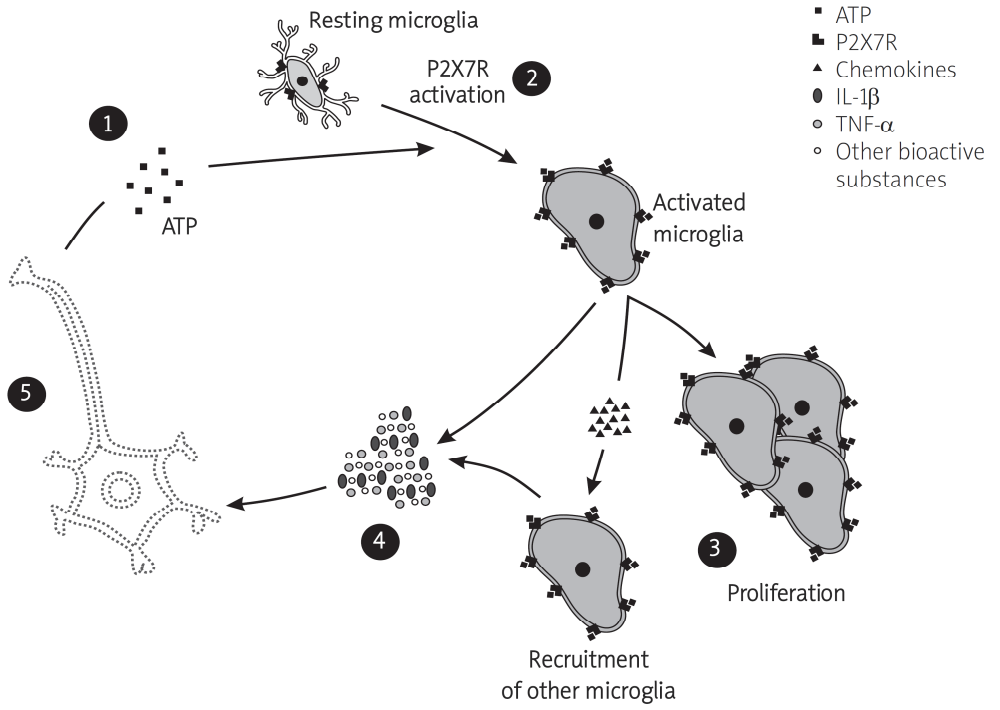

Fig. 1. The hypothetical role of P2X7R activation in the pathophysiology of bipolar disorder. 1) Acute episodes lead to neuronal injury that causes the release of damage-associated molecular patterns (DAMPs), such as ATP. 2) Released ATP may ultimately drive microglial cells from their resting state into their activated form through the activation of P2X7R. 3) Once activated by signaling cascades involving the activation of P2X7R, microglial cells induce proliferation and recruitment of other microglia through the release of chemokines. 4) Activated microglia release proinflammatory molecules (IL-1β, TNF-α) and other bioactive substances, such as reactive oxygen species and proteases, which can induce neuronal damage. 5) Therefore, in the continuous presence of ATP, microglial activation and excess of proinflammatory cytokines can form a self-perpetuating neuroinflammatory cycle.

reflected in a proposed scheme of the hypothetical role of P2X7R on microglial activation, which consists in elevated ATP release from injured or stressed cells, stimulating P2X7R and thus activating resting microglia as described in detail in Figure 1. Once activated, and by means of signaling cascades involving activation of P2X7R and upregulation of proinflammatory gene products, microglia release proinflammatory substances (IL-1β, TNF-α), which in turn are capable of promoting further microglial activation, in an autocrine manner. In the same way, the release of chemokines can also recruit other microglia. Therefore, in the continued presence of ATP, with increasing proinflammatory cytokines and other bioactive substances, a self-propagating cycle of neuroinflammation may be formed.

Conclusions

In summary, several lines of evidence suggest that the purinergic system is associated with the pathophysiology of BD, including the P1 adenosine receptors and P2 receptors. Specifically, genetic studies have been pointing to the P2X7R gene as a susceptibility gene for BD, and its role in the disorder has been increasingly acknowledged. Based on this scenario, a more detailed study of the role of P2X7R in BD is warranted, especially due to its ability to act on microglia and modulate neuroinflammation. A better understanding of the relevance of such alterations in the mechanisms of action of known mood stabilizers may further indicate the pathways through which the purinergic system, mainly the P2X7 receptor, is involved in BD pathophysiology.

Acknowledgments

This work was supported by grants from INCT Translational Medicine, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). CG and AR are recipients of scholarships from CNPq. GRF and BWA are recipi-

ents of scholarships from CAPES. The authors also thank Guilherme Gagliardi for his support in designing Figure 1.

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PARTE III

4. DISCUSSÃO

Relatos de declínio cognitivo e progressiva atrofia cerebral sugerem que o TB apresenta um padrão progressivo e possivelmente neurodegenerativo, com o já descrito envolvimento de excitotoxicidade, neuroinflamação e prejuízo na neuroplasticidade (Kim *et al.*, 2007a; Rao *et al.*, 2010). Considerando que o P2X7R possui a capacidade de mediar morte celular, neuroinflamação e excitotoxicidade (Sun, 2010), acreditamos que seja razoável considerar a participação deste receptor na patofisiologia do transtorno. Nesta dissertação avaliamos a associação entre o P2X7R e o TB, a fim de determinar esta relação até então sugerida por estudos genéticos e identificar os principais mecanismos pelos quais este receptor desempenha um papel neste transtorno psiquiátrico. Em um segundo momento, a fim de compreender e sugerir uma hipótese para este envolvimento realizamos uma revisão da literatura focando na sinalização purinérgica no TB, onde encontramos um bom aporte de trabalhos que descreve diferentes maneiras em que o sistema purinérgico e o TB podem ser relacionados. Entretanto, destacamos o potencial papel do P2X7R na modulação da inflamação e ativação microglial em pacientes bipolares, o qual imaginamos ser a principal via de conexão entre este receptor e o transtorno.

O tratamento com AMPH, agudo e crônico, tem sido amplamente utilizado como modelo animal de mania, baseado na observação da hiperlocomoção (Frey, Andreazza, Ceresér, Martins, Petronilho*, et al.*, 2006; Macêdo *et al.*, 2012). Altos níveis de dopamina são os principais responsáveis pelos efeitos farmacológicos da AMPH. A AMPH aumenta os níveis de dopamina na fenda sináptica, possuindo ações vesiculares (aumentando a concentração do neurotransmissor no citosol do neurônio pré-sináptico) e interagindo com o transportador de dopamina (DAT) (induzindo, assim, um transporte reverso de dopamina do neurônio pré-sináptico para a fenda). Além disso, a AMPH ainda é capaz de ligar-se de forma reversível ao DAT e bloquear a habilidade do transportador de recaptar dopamina da fenda (Sulzer *et al.*, 2005). A AMPH é muito ativa nas vias de recompensa mesolímbica e mesocortical, sendo o estriado, o núcleo *accumbens* e o estriado ventral seus sítios primários de ação (Del Arco *et al.*, 1999; Drevets *et al.*, 2001), os neurônios

dopaminérgicos são ainda projetados da área tegmental ventral e da substância nigra compacta, para quase todas as áreas do cérebro, incluindo o córtex pré-frontal (CPF), o lobo temporal medial e o hipocampo, podendo explicar as disfunções observadas em pacientes bipolares (Li *et al.*, 2010). Este modelo animal é bem aceito devido principalmente ao fato de serem observados alterações neuroquímicas, como uma desregulação dopaminérgica, semelhante a induzida por AMPH em pacientes em estado maníaco (Strakowski e Sax, 1998; Asghar *et al.*, 2003; Berk *et al.*, 2007).

Dados resultantes do presente trabalho (Capítulo I) fornecem evidências de uma relação entre o P2X7R e a hiperatividade induzida por AMPH, como demonstrado pelo experimento comportamental, onde pudemos observar nos animais com o P2X7R bloqueado ou ausente uma aparente falta de capacidade de resposta à AMPH. O tratamento agudo e crônico com AMPH foi capaz de aumentar significativamente a locomoção dos animais, o que não ocorreu com os animais co-tratados com antagonista tanto seletivo quanto não seletivo do P2X7R. Além disso, este comportamento de hiperlocomoção induzido por AMPH foi significativamente revertido pelo tratamento com o antagonista seletivo do P2X7R. Da mesma forma, a AMPH não teve nenhum efeito sobre o comportamento de locomoção dos animais $P2X7R^{-/-}$. Em conjunto, os resultados apontam para o envolvimento do P2X7R no modelo animal de mania, sugerindo um papel para este receptor na patofisiologia do TB.

Para o nosso conhecimento, este é o primeiro estudo dedicado a verificar e explorar a partir de parâmetros comportamentais e bioquímicos o envolvimento do P2X7R no TB, embora estudos genéticos já tenham descrito esta relação e afirmado que o gene do P2X7R está situado em uma região cromossômica que configura um lócus de susceptibilidade ao transtorno (Abkevich *et al.*, 2003). Da mesma forma a depressão maior é relacionada ao P2X7R, com um número maior de estudos que sugerem o antagonismo do P2X7R como uma nova proposta terapêutica à DM (Sperlagh *et al.*, 2012; Halmai *et al.*, 2013). Basso e colaboradores (Basso *et al.*, 2009) inclusive demonstraram que animais $P2X7R^{-/-}$ apresentam um fenótipo tipo-antidepressivo e possuem uma

resposta aumentada à imipramina, um clássico fármaco antidepressivo, quando comparado com animais $P2X7R^{+/+}$.

O segundo resultado importante mostrado no Capítulo I foi uma provável participação do P2X7R no aumento do ambiente pró-inflamatório induzido pela AMPH, demonstrado principalmente pelo papel de reversão que o bloqueio do P2X7R apresentou nessa resposta, assim como pela falta de resposta inflamatória observada nos camundongos $P2X7R^{-/-}$. Metanfetamina, um estimulante que compartilha a mesma estrutura química da AMPH (Melega *et al.*, 1995), tem sido associado a níveis crescentes de citocinas pró-inflamatórias em cérebro de animais (Gonçalves *et al.*, 2008), o que corrobora com os nossos achados referentes à AMPH. Como os níveis elevados de citocinas pró-inflamatórias têm sido repetidamente demonstrados em pacientes com TB (recentemente revisada pelo (Rosenblat *et al.*, 2014)), esta resposta à AMPH demonstrado no nosso trabalho contribui em primeiro lugar, para a legitimidade de uso deste modelo animal como um modelo de mania, principalmente porque esta resposta ainda não havia sido reportada.

Sabe-se ainda que o P2X7R possui um papel central na neuroinflamação (Monif, Burnstock e Williams, 2010), principalmente devido ao fato de que sua ativação leva ao aumento da translocação de fatores de transcrição que resultarão no aumento da produção e da liberação das citocinas pró-inflamatórias, principalmente: IL-1β, TNF-α e IL-6 (Di Virgilio, 2007; Skaper, Debetto e Giusti, 2010a). Outra função desempenhada pelo P2X7R crucial para o estabelecimento da neuroinflamação é o seu papel no processamento e liberação de IL-1β, onde a ativação do P2X7R causa o efluxo de K^+, fundamental para a ativação da enzima conversora de IL-1β ou caspase-1, que por sua vez cliva a pro-IL-1β a IL-1β ativa, permitindo dessa maneira que esta seja liberada da célula. (Ferrari *et al.*, 2006). De fato, camundongos $P2X7R^{-/-}$ demonstraram diminuída resposta inflamatória (Lucattelli *et al.*, 2011), confirmando a relação entre neuroinflamação e o P2X7R. Com base em nossos resultados e no que já fora demonstrado, é lógico pensar que o P2X7R medeia diretamente o processo inflamatório induzido pela AMPH.

Nossos resultados relativos ao estresse oxidativo (EO) demostraram que da mesma forma, a AMPH aumenta estes níveis, tanto no tratamento agudo quanto no crônico de AMPH, e aparentemente o P2X7R também participa deste aumento. Outros estudos já demonstraram aumento de EO em cérebro de paciente bipolares (Steckert *et al.*, 2010; Berk *et al.*, 2011), assim como outros estudos já demonstraram o envolvimento do P2X7R no estabelecimento do EO (Apolloni *et al.*, 2009; Martel-Gallegos *et al.*, 2013), nos levando a sugerir a participação direta deste receptor nesta resposta excitotóxica induzida pela AMPH. Entretanto, outros estudos são necessários a fim de caracterizar esta resposta, com outros equivalentes de EO complementares, como equivalente de EO à proteínas, ou mesmo total, também o acesso das defesas antioxidantes, a fim de configurar de fato um imbalanço pró-oxidativo.

No capítulo I avaliamos também os níveis de BDNF nas mesmas estruturas analisadas, devido ao envolvimento deste fator neurotrófico no TB, tanto em pacientes (Post, 2007b) quanto em modelo animal com AMPH (Frey, Andreazza, Ceresér, Martins, Valvassori*, et al.*, 2006). Nossos resultados confirmam resultados anteriores que descreveram uma diminuição de BDNF induzido pela AMPH, entretanto a modulação do P2X7R não surtiu nenhum efeito sobre esses níveis, indicando que esta via pode não estar envolvida no papel do P2X7R sobre a hiperatividade induzida por AMPH. Por outro lado, não podemos descartar a influência do tempo de exposição aos fármacos moduladores do receptor, que no nosso modelo experimental foi de uma hora, talvez este tempo não tenha sido suficiente para resultar em alguma resposta à nível neurotrófico, que geralmente configura uma resposta mais demorada. Talvez maiores estudos com modelos experimentais diferentes possam responder esta questão que nos fica em aberto.

Finalmente, com o tratamento crônico com AMPH, nós pudemos claramente observar a ação da AMPH sobre o imunoconteúdo de GFAP, um indicador de astrogliose, e o envolvimento de algum receptor P2X, que provavelmente não o P2X7R, nesta resposta. A exposição repetida à AMPH já foi descrita como indutora de astrogliose em hipocampo de ratos (Frey, Andreazza, Ceresér, Martins, Petronilho*, et al.*, 2006). Nossos resultados confirmam este achado e acrescentam

esta mesma resposta em CPF e em estriado. Estudos anteriores reportaram que essa resposta astroglial poderia estar associada à neutotoxicidade (Armstrong *et al.*, 2004), sugerindo uma ação de excitotoxicidade da AMPH, que já fora demonstrado em córtex frontal post-mortem de pacientes com TB (Rao *et al.*, 2010). O fato de o tratamento com BBG (um antagonista não específico de P2X7R) e não com A438079 (um antagonista específico do P2X7R) ter revertido a astrogliose induzida por AMPH crônica indica que o P2X7R não possui efeito nesta resposta, sugerindo a participação de outros receptores que são antagonizados pelo BBG, como os receptores P2X4 e/ou P2X5 (Burnstock, 2007). Mais estudos são necessários para determinar as vias pelas quais a modulação purinérgica reverte a astrogliose induzida pela AMPH.

No geral, os resultados do Capítulo I demonstram que o tratamento com AMPH é capaz de induzir neuroinflamação e excitotoxicidade, e que esses efeitos estão sendo mediados, pelo menos em parte, pelo P2X7R. Algumas dessas respostas são parcialmente explicadas pela ativação astrocitária, em função deste efeito não ser revertido pelo antagonismo do P2X7R. Além disso, tem sido sugerido que a astrogliose pode seguir seu funcionamento neuronal anormal mesmo sem a morte neuronal ou degeneração (Khurgel *et al.*, 1995; Girardi *et al.*, 2004), o que neste caso não teria a presença de neuroinflamação e excitotoxicidade, indicando a participação de outros fatores nos nossos resultados. Pensando em uma outra alternativa que pudesse acrescentar à esse ambiente estabelecido a explicação da integração entre os resultados neuroinflamatório/excitotóxico e o P2X7R, chegamos na ativação microglial.

O cérebro é rico em macrófagos residentes, chamados de células microgliais ou microglia, que são ativados principalmente em resposta a dano tecidual e infecções cerebrais, sendo os primeiros a detectar mudanças críticas à saúde e atividade neuronal (Ferré, 1997). As células microgliais participam do sistema imune, produzindo respostas predominantemente neuroprotetivas ou respostas predominantemente inflamatórias. Na primeira resposta, a microglia, em seu estado inativado, aumenta a produção e liberação de citocinas anti-inflamatórias, fatores neurotróficos e a produção de fatores bioativos envolvidos no reparo tecidual (Kraeplin, 1921). Na segunda resposta,

a ativação microglial leva à síntese e liberação de mediadores pró-inflamatórios, principalmente TNF-α e IL-1β. Quando esta liberação não é devidamente controlada, pode progredir para um quadro de dano ou morte neuronal (Leboyer *et al.*, 2012). Existe um *threshold* para a ativação microglial, onde neurônios saudáveis mantêm a microglia em seu estado inativado, via secreção de moléculas, incluindo CD200, CX3CL1, neurotransmissores e neurotrofinas (Ferré, 1997). Quando esse controle falha (como em situações de dano/morte neuronal ou perda de sinais regulatórios), a microglia se mantêm ativada e participa de uma forma crônica de neuroinflamação, que é bastante implicada na patofisiologia de doenças neurodegenerativas e, mais recentemente descritas, de transtornos psiquiátricos (Leboyer *et al.*, 2012).

Como já relatado anteriormente, Rao e colaboradores (Rao *et al.*, 2010) reportaram importantes marcadores de neuroinflamação e excitotoxicidade em CPF post-mortem de pacientes com TB. O mesmo trabalho também demonstrou a ativação de uma cascata de ativação microglial, assim como aumento de marcadores microgliose e astrogliose (GFAP, iNOS, c-fos e CD11b), indicadores de ativação microglial. Por outro lado, pacientes que experienciaram um ou mais episódios maníacos/hipomaníacos no período do último ano, demonstraram um aumento significativo dos níveis de IL-1β no líquido cefalorraquidiano comparado com pacientes que não haviam tido nenhum episódio recente, o que indica uma relação entre a presença de episódios e a ativação da cascata de ativação microglial (Lucattelli *et al.*, 2011). Os mecanismos citados acima sugerem que há ativação microglial no TB (**Figura 2**), entretanto, sabemos que o papel da ativação microglial no transtorno ainda não está completamente compreendido, sendo necessárias maiores investigações.

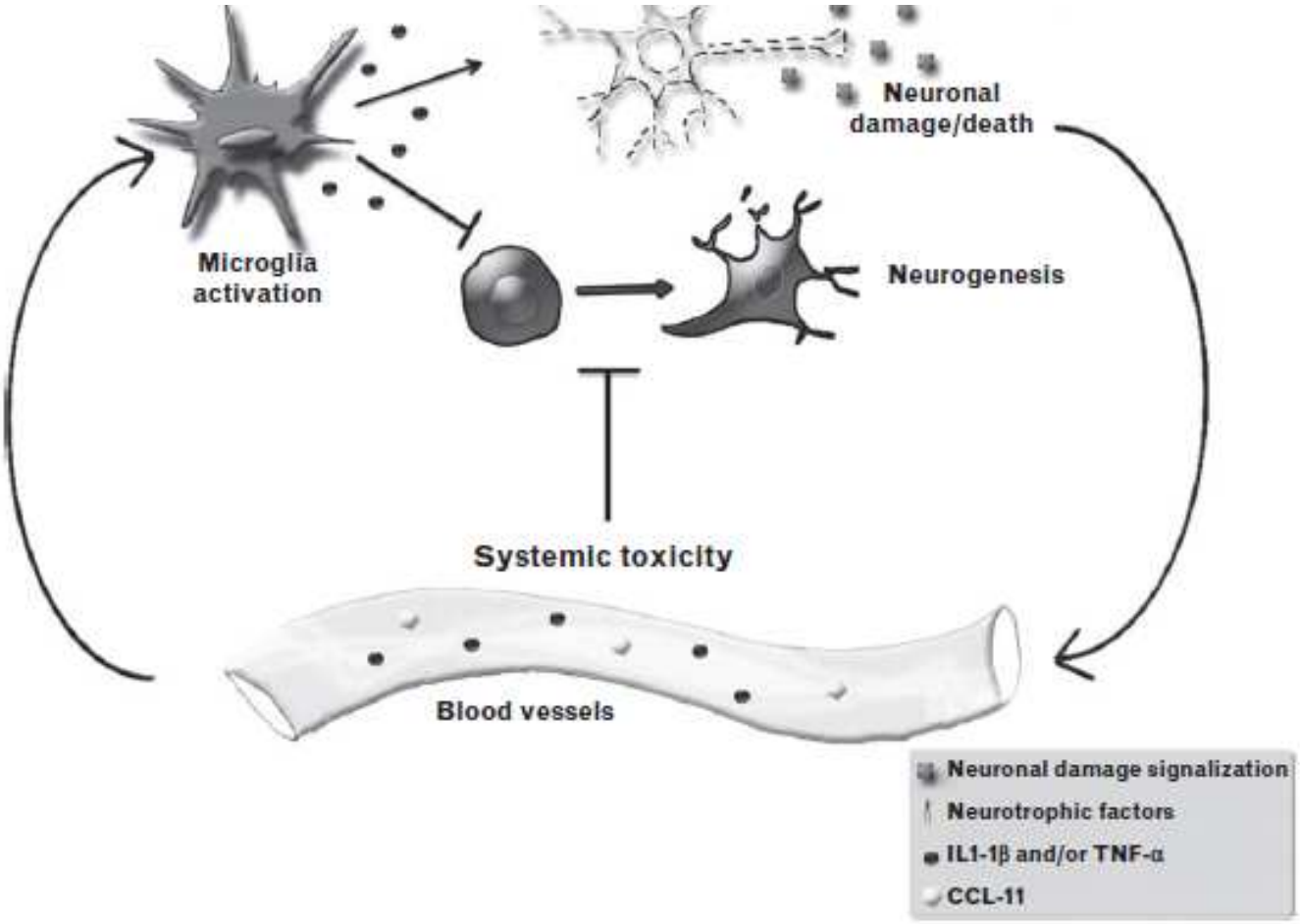

Figura 2. Papel hipotético da inflamação e ativação microglial na patofisiologia do TB. Após episódios, o excesso de produção de citocinas pró-inflamatóras, que excedem a capacidade de reparo do sistema, mantêm a microglia em um estado constantemente ativado. A constância da presença de TNF-α e IL-1β no meio extracelular causa inibição da neurogênese e dano neuronal. Moléculas associadas a dano/morte neuronal ou a falha no controle de inflamação por sua vez, perpetua a toxicidade sistêmica. Da mesma maneira, a CCL11 inibe a neurogênese e as citocinas liberadas perpetuam a ativação microglial, acarretando o estabelecimento de um ciclo inflamatório. Adaptado de (Stertz, Magalhães e Kapczinski, 2013).

Por sua vez, o P2X7R tem sido descrito como um componente essencial da indução de ativação microglial, como descrito na **Figura 3** (Skaper, Debetto e Giusti, 2009). Estudos futuros devem investigar o envolvimento da ativação microglial na hiperatividade induzida pela AMPH e esclarecer se esse envolvimento está sujeito a modulação pelo P2X7R.

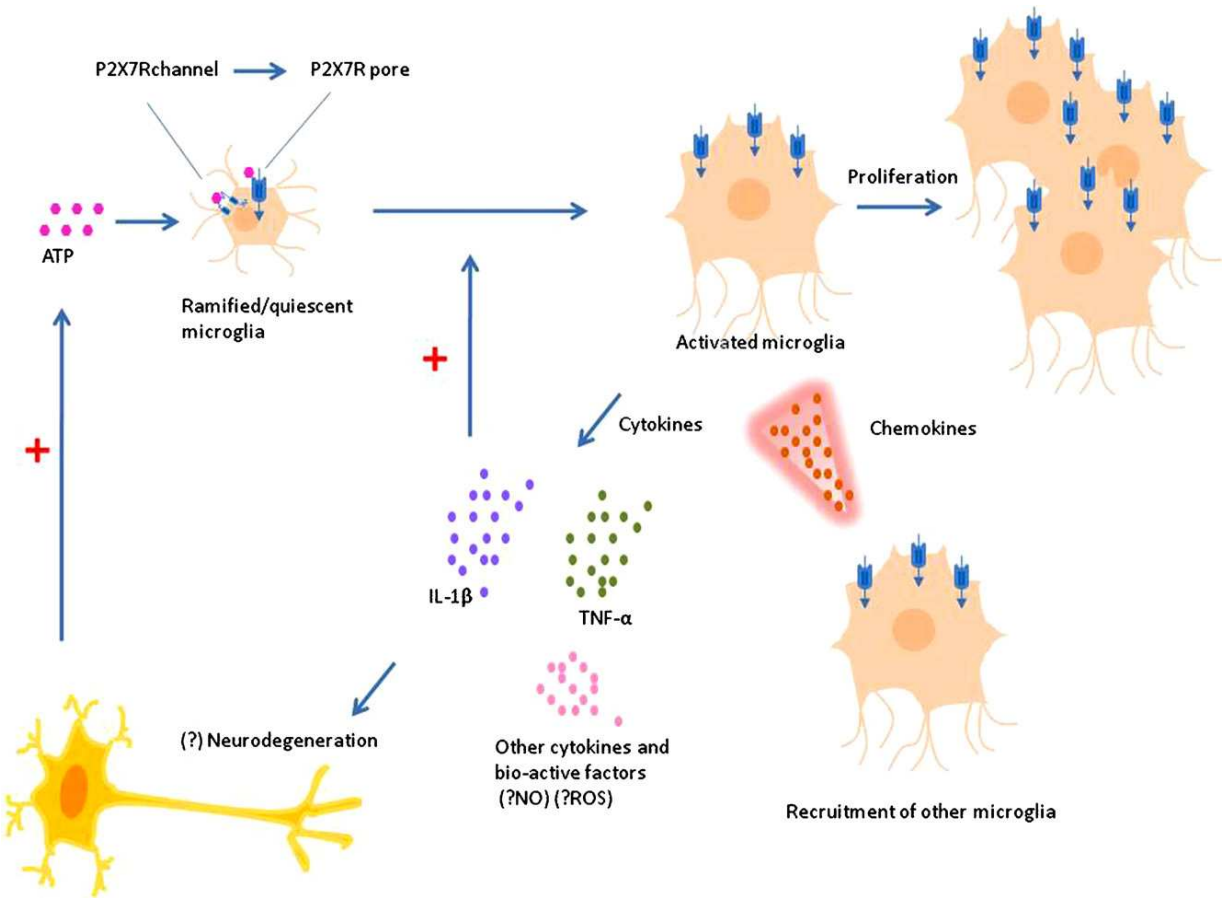

Figura 3. Diagrama esquemático da relação entre a ativação do P2X7R e a ativação microglial. ATP extracelular proveniente de dano/morte neuronal é capaz de ativar o P2X7R presente nas células microgliais, esta ação converte as células microgliais ao seu estado ativado. Microglias ativadas proliferam e através da liberação de quimiocinas recrutam novas microglias. Este pool microglial por sua vez, causa a liberação de citocinas pró-inflamatórias e outras substâncias bioativas, capazes de manter a ativação microglial e induzir o dano/morte neuronal, estabelecendo um ciclo de neurodegeneração e excitotoxicidade via ativação do P2X7R. (Adaptado de Monif M et al., 2010).

Os resultados obtidos neste trabalho, em conjunto com dados já discutidos nesta dissertação, nos levam a sugerir que o P2X7R possui um grande potencial de se tornar um alvo terapêutico para o TB. Além disso, nossos resultados fornecem suporte para a hipótese de que o P2X7R desempenha um papel na patofisiologia do TB, como sugerido pelo nosso modelo animal, principalmente por mediar a neuroinflamação e a excitotoxicidade e, finalmente, levando a mudanças comportamentais. Mais estudos são necessários para esclarecer o papel do P2X7R sobre a microglia e esta sobre o modelo animal de mania sugerido a partir de nossos resultados, bem como para melhor caracterizar o envolvimento do P2X7R na patofisiologia do TB.

5. CONCLUSÕES

- Existe uma relação entre o P2X7R e a hiperatividade induzida por AMPH, como demonstrado pelo experimento comportamental, onde pudemos observar nos animais com o P2X7R bloqueado ou ausente uma aparente falta de capacidade de resposta à AMPH;

- O P2X7R parece participar do aumento do ambiente pró-inflamatório induzido pela AMPH, principalmente pelo papel de reversão que o bloqueio do P2X7R apresentou nessa resposta, assim como pela falta de resposta inflamatória observada nos camundongos $P2X7R^{-/-}$;

- O P2X7R parece participar também do aumento de equivalente de EO induzido pela AMPH, pela mesma ação de reversão que o bloqueio do P2X7R apresentou e pela falta de resposta dos animais $P2X7R^{-/-}$;

- O P2X7R não parece estar envolvido na diminuição de BDNF induzida pela AMPH;

- A AMPH é capaz de induzir astrogliose nos animais com o envolvimento de algum receptor P2X, que aparentemente não o P2X7R, demonstrado pela ação de reversão que o BBG causou nesta resposta;

- É possível que a ativação microglial já descrita em pacientes bipolares e já demonstrada ser também mediada por P2X7R possa ser a via chave de integração do comportamento, neuroinflamação e excitotoxicidade demonstrado no modelo animal de mania;

- De qualquer maneira, o P2X7R parece ser um bom novo candidato à alvo terapêutico do TB.

PERSPECTIVAS

- Observar a ação microglial em córtex pré-frontal, hipocampo e estriado de animais tratados com AMPH crônica, para esclarecer a hipótese da relação entre o P2X7R, a ativação microglial e o comportamento;

- Descrever a via de ativação do P2X7R na já descrita modulação comportamental de camundongos tratados com AMPH crônica, nos mesmos tecidos cerebrais;

- Analisar o conteúdo de ATP, ativador do P2X7R, e de monoaminas nos mesmos tecidos de camundongos a fim de compreender se estes fatores sofrem ação da AMPH e se há uma possível modulação pelo agonista e/ou antagonista deste receptor;

- Verificar se estabilizadores de humor utilizados na clínica protegem neurônios dopaminérgicos humanos da já descritoa morte celular induzida por ATP e se o P2X7R está envolvido, contribuindo para o entendimento da via de ação desses fármacos;

- Descobrir se pacientes expressam de maneira diferenciada o P2X7R e se essa expressão difere também entre os estados do transtorno.

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7. ANEXOS

ANEXO 1 _ Aprovação do comitê de ética.

ANEXO 2_ Instruções para a submissão de manuscritos na revista *Bipolar Disorders*.

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 091/12 – CEUA

Porto Alegre, 12 de julho de 2012.

Senhora Pesquisadora:

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 12/00289, **"Avaliação do papel do receptor purinérgico P2x7 em modelos de hiperatividade induzida por D-Anfetamina em camundongos".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

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Coordenadora da CEUA/PUCRS

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7.2. ANEXO 2

Instruções para a submissão de manuscritos na revista Bipolar Disorders.

Author Guidelines

Bipolar Disorders - An International Journal of Psychiatry and Neurosciences will consider for publication full length research papers, brief reports, invited editorials, commentaries, review articles, case reports, and letters to the editors.

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Manuscripts with all tables and figures must be submitted online athttp://mc.manuscriptcentral.com/bdi and any written correspondence should be addressed to:

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PLEASE NOTE THAT from January 2013, all submissions to *Bipolar Disorders* must be submitted via an online submission system. For more information about the online submission system, please read the detailed instructions at http://mc.manuscriptcentral.com/bdi Author Centre or email the Editorial Assistant at kocandj@upmc.edu.

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Manuscripts with all tables and figures must be submitted online athttp://mc.manuscriptcentral.com/bdi. Submissions can be uploaded as a Word document or as Rich Text Format (rtf) files, plus separate figure files (gif, jpg, bmp, tif, pic) and table files (doc or xls). Pdf and ppt files are not accepted. The following rules are in general agreement with 'Uniform requirements for manuscripts submitted to biomedical journals' accepted by an International Steering Committee (see ref. 1 below).

The paper should be submitted in English. The main manuscript components (title page, abstract, text, acknowledgements, disclosures, references, legends, tables, and figures) should begin on a separate page. The pages of the manuscript - text, reference list, tables and legends to figures, in that order, should be numbered consecutively.

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The title page should contain: 1. a concise informative title; 2. author(s)'s names; 3. name of department(s)/ institution(s) to which the work is attributed for each individual author; 4. name, address, fax number, and e-mail address of the author to whom correspondence about the manuscript, and requests for offprints should be referred; 5. if the title exceeds 40 characters (letters and spaces), a running head of no more than 40 characters; 6. a word count for the whole paper.

Abstract and key words

The abstract should not exceed 250 words and should be arranged in a structured fashion to include **objectives, methods, results, and conclusions**. It should state the purpose of the study, basic procedures (study subject /patients / animals, and methods), main findings (specific data and statistical significance), and principal conclusions. For the News and Vies articles, a short (up to 100 words) non-structured abstract should be provided. Below the abstract, provide 3-10 key words that will assist indexers in cross-indexing your article. Use terms from the Medical Subject Headings list from Index Medicus, whenever possible.

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Present the background briefly, but do not review the subject extensively. Give only pertinent references. State the specific questions you want to answer.

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Present results in logical sequence in tables and illustrations. In the text, explain, emphasize, or summarize the most important observations. Units of measurement should be expressed in accordance with Système International d'Unite (SI Units).

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Acknowledgements

Acknowledge only persons who have made substantive contributions to the study. Authors are responsible for obtaining permission from everyone acknowledged by name because readers may infer their endorsement of the data and conclusions. Authors are expected to disclose any commercial or other relationships that could constitute a conflict of interest. All funding sources supporting the work should be acknowledged.

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Examples:

1. International Steering Committee. Uniform requirements for manuscripts submitted to biomedical journals. N Engl J Med 1997; 336: 309.

2. Gershon S, Soares JC. Current therapeutic profile of lithium. Arch Gen Psychiatry 1997; 54:16-20.

3. Soares JC, Mann JJ. The anatomy of mood disorders--review of structural neuroimaging studies. Biol Psychiatry 1997; 41: 86-106.

4. Post RM, Weiss SRB. Kindling and stress sensitization. In: Young LT, Joffe RT ed. Bipolar Disorder - Biological Models and Their Clinical Application. New York: Marcel Dekker, Inc., 1997: 93-126.

5. American Psychiatric Association. DSM-V Development. Available from: http://www.dsm5.org/.Last accessed August 11, 2011.

6. Malik A, Goodwin GM, Holmes EA. Contemporary approaches to frequent mood monitoring in bipolar disorder. J Exp Psychopathol 2011; doi:10.5127/jep.014311.

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References in Articles

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