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Stefania Giotti Cioato

EFEITOS ANTINOCICEPTIVOS E NEUROQUIMICOS DE AGONISTA DE RECEPTOR DE ADENOSINA A_3 EM MODELOS ANIMAIS DE DOR

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Stefania Giotti Cioato

EFEITOS ANTINOCICEPTIVOS E NEUROQUIMICOS DE AGONISTA DE RECEPTOR DE ADENOSINA A_3 EM MODELOS ANIMAIS DE DOR

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

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Aos meus pais

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RESUMO

Introdução: dor crônica representa um problema de saúde pública que acomete a qualidade de vida dos indivíduos e despende alto custo socioeconômico. E, as terapias farmacológicas, atualmente disponíveis, ainda resultam no uso descontinuado devido seus efeitos colaterais ou alívio inadequado da dor. Desta forma, a busca por novas modalidades de tratamento que permitam uma melhor abordagem terapêutica dos quadros dor, com menos efeitos adversos e baixo custo torna-se de fundamental importância, sobretudo nas condições crônicas. Neste contexto, o uso de agonistas seletivos de receptor adenosinérgico A_3 (A_3AR) parece representar uma opção terapêutica viável para o tratamento da dor crônica de diferentes etiologias.
Objetivo: contudo, o objetivo deste trabalho foi investigar o efeito antinociceptivo de tratamento agudo com agonista A_3AR (IB-MECA) em modelo animal de dor crônica inflamatória ou dor crônica neuropática, por meio de respostas comportamentais nociceptivas. Adicionalmente, correlacionar este efeito com níveis centrais de neurotrofinas e citocinas.
Metodologia: este estudo experimental foi desenvolvido na Unidade de Experimentação Animal do Hospital de Clínicas de Porto Alegre (sob CEUA n° 2015-0530, 2018-0377), e foram utilizado ratos Wistar adultos (50-60 dias), em condições ideais de biotério. Os ratos foram randomizados por peso corporal (g) em diferentes grupos dentro de cada modelo de dor (controle, controle+veículo, controle+droga padrão, controle+IB-MECA, dor, dor+veículo, dor+ droga padrão, dor+IB-MECA). Testes nociceptivos realizados foram: placa quente, von frey e Randal Selitto. Medias bioquímicas foram: níveis centrais de citocinas (interleucinas 1β e 10) neurotrofinas (fator neurotróficos derivado do encéfalo- BDNF e fator de crescimento neuronal –NGF) por técnica de ELISA. 1° protocolo experimental: modelo de dor crônica inflamatória por meio de injeção na pata traseira de Adjuvante Completo de Freund (CFA,100µL). 2° protocolo experimental: modelo de dor crônica neuropática por meio da constrição crônica do isquiático. Dados foram analisados pelo SPSS versão 20.0 e $P<0.05$ foi considerado significativamente diferente.
Resultados: IB-MECA reverteu o comportamento hipernociceptivo apresentado em ambos modelos de dor crônica. E não foi encontrado correlação entre este efeito e níveis centrais de citocinas (IL-1β e IL-10) e neurotrofinas (BDNF e NGF).
Conclusão: administração aguda de IB-MECA é capaz de desencadear efeito antinociceptivo em modelos de dor crônica inflamatória e neuropática; não estando diretamente correlacionado com alterações nos níveis de citocinas e neurotrofinas, pelo menos, nas condições experimentais do presente trabalho, como marcadores analisados e administração aguda de IB-MECA. Adicionalmente, estudos experimentais utilizando modelos animais que permitam a investigação dos quadros de dor crônica, assim como de novas terapias farmacológicas, avaliando o seu impacto nas respostas nociceptivas, seus efeitos neuroquímicos e moleculares devem ser encorajados, principalmente, relacionados ao receptor A_3AR e seus efeitos antinociceptivos, onde ainda há uma escassez no entendimentos dos mecanismos intracelulares envolvidos com seu efeito analgésico.

Palavras-chave: dor neuropática, dor inflamatória, ratos, IB-MECA, testes nociceptivos

ABSTRACT

Introduction: chronic pain represents a public health problem that affects the quality of life of individuals and spends high socioeconomic cost. And, the currently available pharmacological therapies still result in discontinued use because of its side effects or inadequate pain relief. In this way, the search for new treatments that allow a better therapeutic approach of the pain frames, with fewer adverse effects and low cost, becomes essential, especially in chronic conditions. In this context, the use of selective A_3 adenosine receptor agonists (A_3AR) seems to represent a feasible therapeutical tool for treatment of chronic pain from different etiologies.
Objective: the main objective was to investigate the antinociceptive effect of acute treatment with A_3Ar agonist (IB-MECA) in an inflammatory and neuropathic chronic pain models, assessing nociceptive behavioural responses. In addition, to investigate a correlation between this effect and biomarkers central levels, such as neurotrophins and cytokines.
Methodology: this experimental study was developed at the Animal Experimentation Unit of the Hospital de Clínicas of Porto Alegre (CEUA n° 2015-0530, 2018-0377), and adult Wistar rats (50-60 days) were used under ideal conditions of biotery. The rats were randomized by body weight (g) in different groups within each pain model (control, control + vehicle, control + standard drug, control + IB-MECA, pain, pain + vehicle, pain + IB-MECA). Nociceptive tests performed were: hot plate, von frey and Randal Selitto. Biochemical measurements were: central levels of cytokines (interleukins 1β and 10) and neurotrophins (brain-derived neurotrophic factor-BDNF and neuronal growth factor -NGF) by ELISA. 1° protocol: chronic inflammatory pain model by injection of Complete Adjuvant of Freund (CFA, 100 µL) in the hindpaw. 2° protocol: chronic neuropathic pain model by the chronic constriction of the sciatic nerve. Data were analysed by SPSS version 20.0 and $P < 0.05$ was considered significantly different.
Results: IB-MECA reverted the hypernociceptive behaviour triggered by both chronic pain models; and no correlation was found between this effect and central levels of interleukins (IL-1β e IL-10) and neurotrophins (BDNF e NGF).
Conclusion: acute administration of IB-MECA is able to trigger the antinociceptive effect in the inflammatory and the neuropathic chronic pain models; however, this effect was not directly linkedd to changes in the central levels of cytokines and neurotrophins, at least, in the our experimental conditions, for example, the biomarkers analysed and acute administration of IB-MECA. In addition, experimental studies using animal models that allow the investigation of chronic pain, as well as, new pharmacological therapies, assessing nociceptive responses, and its neurochemical and molecular effects should be driven, mainly, to the A_3AR receptor and its effects antinociceptive, where there is still a lack of understanding of the intracellular mechanisms involved with its analgesic effect.

Keywords: CFA model; CCI model, rats; IB-MECA; nociceptive tests.

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PARTE I

Introdução, Revisão da Literatura, Objetivos e Justificativa

I. INTRODUÇAO

1. INTRODUÇAO

A dor crônica representa um problema de saúde pública relacionada à diminuição da qualidade de vida, limitações funcionais e absenteísmo despendendo alto custo socioeconômico (1), enquanto a terapêutica destas condições dolorosas está relacionada a elevados custos ao sistema de saúde direcionados a tratamentos farmacológicos, fisioterapêuticos e cirúrgicos. A fisiopatologia da dor é um processo complexo que depende de vários mecanismos em nível periférico e central. Lesões periféricas englobam mudanças na excitabilidade periférica e no gânglio da raiz dorsal, mudanças em neurônios da medula espinhal, sistemas descendentes inibitórios e mudanças plásticas corticais (2). Os processos periféricos descritos incluem descargas ectópicas e espontâneas, alterações na expressão de canais iônicos, surgimento colateral de neurônios aferentes primários, brotamento de neurônios simpáticos no gânglio da raiz dorsal e sensibilização do nociceptor. Os processos centrais são representados pela sensibilização central com alterações na expressão de neurotransmissores, neuromoduladores, receptores e canais iônicos (3). Nesse contexto, o sistema imune também tem um papel crítico no desenvolvimento e na manutenção da dor crônica, uma vez que várias citocinas pró-inflamatórias e anti-inflamatórias demonstraram funções no estabelecimento dessa condição dolorosa (4). Assim, as condições crônicas de dor são resultado de processos mal adaptativos nestas vias da dor, que perpetuam mecanismos de sinalização mesmo após a resolução da lesão iniciadora da dor, assim como modulam processamentos centrais (5).

Além dos tratamentos tradicionais utilizados em dor aguda como analgésicos, anti-inflamatórios não esteroides (AINES) clássicos e corticoides, nos quadros de dor crônica são utilizadas abordagens farmacológicas que envolvem fármacos adjuvantes como os antidepressivos, anticonvulsivantes, bloqueadores de canais de sódio e cálcio, β-bloqueadores e antagonistas de receptores N-metil-D-aspartato (NMDA). Entretanto, estes fármacos são relacionados a efeitos adversos que muitas vezes limitam o seu uso (6–8).

Desta forma, a busca por novas modalidades de tratamento que permitam uma melhor abordagem terapêutica dos quadros dor, com menos efeitos adversos e baixo custo torna-se de fundamental importância, sobretudo nas condições crônicas. Entre os sistemas endógenos moduladores do processo nociceptivo esta o sistema adenosinérgico. A adenosina atua no processamento da dor regulando a transmissão excitatória, a sinalização neuronal persistente e a ativação e proliferação glial (9). A adenosina apresenta funções como molécula sinalizadora por meio de quatro receptores distintos: A_1R, A_2AR, A_2BR e A_3R, que apresentam distintos perfis farmacológicos, distribuição tecidual e proteínas ligantes (10). Os receptores A_1 estão

envolvidos em antinocicepção enquanto que os A_2R podem apresentar atividade anti-inflamatória (11,12). Entretanto, o uso terapêutico de agonistas destes receptores é bastante restrito, devido ao risco de efeitos adversos cardiovasculares (13). Por outro lado, o uso de agonistas seletivos de receptor A_3 parece representar uma opção terapêutica viável para o tratamento da dor crônica de diversas etiologias, pois não tem sido relacionado a efeitos adversos cardíacos ou hemodinâmicos (14–16).

Recentes estudos têm sugerido que o efeito antinociceptivo do agonista adenosinérgico IB-MECA está relacionado à ativação de receptor GABA, de A_3R e de canais de potássio (K+) e de cloreto (Cl-) (17). Ren e colaboradores evidenciaram o papel da ativação do receptor A_3R após administração do agonista IB-MECA na modulação do processo pró-inflamatório com envolvimento da sinalização de NF-κB em modelo animal de colite (18,19). Adicionalmente, a ativação do receptor A_3R pelo IB-MECA promoveu alterações nas vias de NF-κB e proteína quinase ativada por mitógeno (ERK e P38) em modelo de dor neuropática induzida por quimioterápico, e redução em citocinas pró-inflamatórias (20).

Os mecanismos de sinalização do receptor A_3 frente ao seu papel analgésico e anti-inflamatório ainda estão pouco esclarecidos, sobretudo em condições dolorosas crônicas específicas, como a dor inflamatória crônica e a dor neuropática. Sabe-se que a administração de IB-MECA impede o desenvolvimento da dor neuropática em ratos submetidos a um modelo de constrição do nervo isquiático (14) e um recente trabalho publicado também mostrou que o tratamento crônico com IB-MECA alterou o curso de desenvolvimento do quadro doloroso neuropático, promovendo alterações na expressão de citocinas em células da glia (21). Ambos estudos abordam o tratamento da dor crônica neuropática durante o seu processo de estabelecimento e não após a consolidação do processo doloroso. Portanto, ainda existe uma lacuna de conhecimento relacionada à cascata de sinalização e aos mecanismos intrínsecos relacionados ao sistema adenosinérgico no tratamento de quadros de dor crônica, sobretudo nos modelos de dor crônica inflamatória e dor neuropática, principalmente na busca dos mecanismos de ação relacionados a este agonista.

II. REVISÃO DA LITERATURA

2. REVISÃO DA LITERATURA

2.1 DOR: CLASSIFICAÇÕES E MECANISMOS

A dor é definida pela *International Association for the Study of Pain* (IASP) como uma experiência sensorial e emocional desagradável, podendo ser associada a uma lesão real ou potencial dos tecidos, ou descrita em termos deste dano. Além disso, é notável que se trata de uma experiência subjetiva, em que cada indivíduo aplica o termo "dor" conforme suas experiências ao longo da vida, relacionadas ou não ao dano tecidual. Diversos critérios tem sido adotados para classificar a dor, como tempo de duração e etiologia, buscando organizar e tornar universais os termos utilizados para cada contexto (22).

Do ponto de vista da abordagem clínica, a dor pode ser classificada a partir de um critério temporal em aguda ou crônica. A dor aguda é reconhecida como um mecanismo fisiológico, tendo papel protetivo contra lesões mais graves, servindo como alerta para lesões teciduais e defesa do organismo; além disso, apresenta relação lesão-efeito geralmente bem determinada e tem duração breve (23). Em contraponto, a dor crônica apresenta-se como uma dor persistente acompanhada, na maioria dos casos, de inflamação tecidual e ou injúria do sistema nervoso, e frequentemente não há um estímulo extrínseco que a justifique. É geralmente acompanhada de hiperalgesia ou alodinia e não tem caráter de alerta ou defesa, sendo gradativamente incapacitante e relacionada a mecanismos mal adaptativos na sinalização do processamento nociceptivo em sistema nervoso central e periférico (22,24).

Considerando a etiologia e os mecanismos subjacentes, a dor pode ser dividida em três grandes grupos: aguda, pós-lesão inflamatória e pós-lesão nervosa, sendo as duas últimas, partes das síndromes dolorosas crônicas (25). A necessidade de uma taxonomia definida para a dor crônica levando em conta sua etiologia, já havia sido sugerida por Bonica em 1979, adotando, assim, termos universais para a classificação das síndromes dolorosas. Nesse contexto, em 2011, a IASP revisou e expandiu a descrição das síndromes dolorosas crônicas, já publicada em 1984, com ênfase particularmente em sua duração, local e padrão (26). Portanto, a dor crônica proveniente de uma lesão inflamatória é descrita com dor crônica inflamatória e a dor crônica proveniente de uma lesão nervosa é caracterizada como um dor neuropática.

Essas duas condições citadas acima são particularmente interessantes do ponto de vista do estudo dos mecanismos fisiopatológicos e de novas terapias farmacológicas e não-farmacológicas, em virtude do seu grande potencial incapacitante e prevalência relevante na população em geral. A primeira delas, a dor crônica inflamatória, é definida como uma

inflamação crônica, lenta e prologada, que persiste por meses ou anos. Em geral, a extensão dos efeitos variam de acordo com a causa da lesão e a habilidade do organismo em reparar e superar as repercussões do processo inflamatório (27). Condições dolorosas crônicas relacionadas à inflamação, como osteoartrite e atrite reumatoide apresentam prevalência de 16% e 6%, respectivamente, nos Estados Unidos conforme dados da IASP e as taxas de sucesso de tratamento giram em torno de 30% apenas (1).

Por outro lado, a dor neuropática é caracterizada pela IASP como uma dor causada por uma lesão ou doença no sistema somatossensorial (22) e representa, na sua forma mais típica, uma síndrome dolorosa crônica desencadeada após uma lesão traumática ou patológica em nervo e/ou em vias sensoriais na medula espinhal ou encéfalo. Estima-se que a prevalência de dor com características neuropáticas na população esteja entre 6,9 a 10%, variando de acordo com a disfunção relacionada (28,29). Quando uma lesão ocorre, pode ocorrer perda de sensibilidade na região de inervação correspondente aos nervos danificados ou às projeções periféricas destas estruturas no SNC e/ou alterar a representação sensorial da dor (30).

Fisiopatologicamente, a dor crônica envolve a modulação de diversos mecanismos relacionados ao processamento nociceptivo. A partir do local da lesão, o processamento agudo da nocicepção mantem conexões diretas pela via da dor seguindo uma "linha" de transmissão que restringe a sensação dolorosa ao local da injúria e prevê que, após a cicatrização, esta será abolida. Entretanto, a dor crônica que persiste após a resolução da lesão causadora, seja ela de etiologia inflamatória ou por lesão de nervo e que pode surgir sem nenhum causador patológico claro, tem sua fisiopatologia baseada no conceito de que os substratos neurais que medeiam a dor são plásticos, sofrendo modificações em diversos níveis moleculares e celulares a partir de múltiplos processos somatossensoriais (31–33).

A experiência dolorosa por si só é resultado da ativação do sistema nociceptivo e de influências genéticas e sensoriais intrincadas em uma rede complexa de sinalização. O gatilho desencadeador do processamento nociceptivo ocorre perifericamente por meio de fibras nervosas aferentes, denominadas nociceptores, encontradas em todo o organismo com exceção do SNC. Os nociceptores detectam e respondem a estímulos nocivos térmicos, mecânicos e químicos e transmitem essas informações ao SNC por meio de potenciais elétricos. Possuem um ramo axonal periférico que inerva o órgão alvo e um ramo axonal central que inerva a medula espinhal. Seus corpos celulares estão localizados no gânglio dorsal da medula espinhal para os nociceptores do corpo e no gânglio trigeminal para os nociceptores localizados na face (34).

Uma vez codificada a informação do estímulo nocivo e transformada em potencial de ação são desencadeadas as sinapses entre as projeções dos nociceptores da medula espinhal e os neurônios de segunda ordem na substância cinzenta do corno dorsal da medula espinhal. Os neurônios sensoriais nociceptivos que ativam os neurônios do corno dorsal da medula espinhal liberam dois tipos principais de neurotransmissores: o glutamato, o qual é o neurotransmissor primário liberado a partir dos terminais centrais dos nociceptores, tendo ação excitatória nos alvos pós-sinápticos, e os neuropeptídios, que são liberados como cotransmissores por vários nociceptores com axônios amielinizados (34,35). São os interneurônios excitatórios e inibitórios que atuam regulando o fluxo de informação nociceptiva, promovendo a interação entre o estímulo nociceptivo aferente e eferente e transferindo-o às estruturas supraespinhais (35).

A partir das projeções neuronais no corno dorsal da medula espinhal, os axônios dos neurônios de segunda ordem formam ramos aferentes que transmitem a informação nociceptiva para estruturas do tronco cerebral e do diencéfalo - o tálamo, substância periaquedutal, região parabraqueal, formação reticular da medula, complexo amigdaloide, núcleo septal e hipotálamo. Nesse contexto, as características sensoriais-discriminativas e afetivo-cognitivas referentes à dor são conjugadas ao impulso nociceptivo (36,37). Os demais axônios dos neurônios espinhais estão relacionados aos reflexos motores e autonômicos promovidos pelo estimulo nociceptivo. Finalmente, os tratos descendentes modulam a resposta dolorosa, reduzindo ou facilitando o processamento nociceptivo espinhal a partir dos núcleos do tronco cerebral, descendo pela porção dorsolateral da medula espinhal (35,38,39).

No contexto de estados dolosos crônicos, como a dor inflamatória crônica e a dor neuropática, o sistema nociceptivo e os processos decorrentes da sua ativação sofrem mudanças significativas tanto em nível periférico quanto em nível central (40). Com a persistência do processo inflamatório induzido pela lesão ao tecido, o limiar de excitação dos nociceptores poli modais sofre redução, o que leva à sua ativação mesmo considerados inócuos, caracterizando alodinia (22). Além disso, quando há a ativação destes nociceptores por estímulos nocivos, a resposta desencadeada será mais intensa do que a resposta em estados não sensibilizados (22,39), caracterizando hiperalgesia. Este fenômeno é consequente da ação das substâncias algogênicas, reduzindo o limiar de ativação dos nociceptores e o aumento da resposta a estímulos supraliminares (41,42). Nesse contexto, plasticidade induzida pela continuidade dos processos nociceptivos, traduzindo um estado crônico, ocorre em contexto mal-adaptativo e envolve dois principais mecanismos: sensibilização periférica e sensibilização central (40).

Embora os processos de perpetuação do processo doloroso tenham vias comuns para os casos de dor crônica, a dor inflamatória e a dor neuropática apresentam alguns mecanismos próprios de cada uma delas (43).

2.1.1 Dor crônica inflamatória

A inflamação é um mecanismo crítico de proteção em resposta à lesão, infecção ou irritação a um tecido e apresenta cinco sinais cardinais: calor, hiperemia, dor, edema e perda de função (44). Em condições fisiológicas, a inflamação permite a remoção ou reparação do tecido danificado após uma lesão. Nestas circunstâncias, o papel da dor inflamatória é protetor, limitando o uso da área afetada e evitando outros danos durante o processo de cicatrização. Entretanto, em pacientes com condições inflamatórias crônicas, a presença de mediadores algogênicos em longo prazo resulta em sensibilização dos nociceptores no tecido afetado promovendo a permanência da dor mesmo após a remoção do estímulo primário que originou a lesão tecidual (25,40).

No terminal periférico, a lesão leva a uma cascata imune inata, resultando na liberação de fatores ativos a partir do sangue, células inflamatórias locais e migratórias, e células lesadas (25). Os tipos de células imunes que contribuem para a dor inflamatória são específicos de acordo com cada condição, mas em geral vários tipos celulares podem ser recrutados e contribuir para a sensibilidade anormal ao estímulo doloroso em diferentes níveis. Os mastócitos e macrófagos são ativados e algumas células imunológicas, incluindo os neutrófilos, podem ser recrutadas. Vários mediadores inflamatórios são liberados, como fator de necrose tumoral alfa (TNF-α), interleucina1β (IL-1β), interleucina 6 (IL-6), óxido nítrico (NO), bradicinina e fator de crescimento nervoso (NGF), que exercem seus efeitos hiperalgésicos atuando diretamente nos nociceptores ou indiretamente por meio da liberação de outros mediadores, principalmente os prostanoides (45).

Os mediadores inflamatórios ligam-se aos receptores acoplados à proteína G (G-protein-coupled receptors – GPCR) para ativar as proteínas quinase A e C (PKA e PKC, respectivamente) que fosforilam receptores ou aumentam a sua expressão, o que leva a um aumento da sua sensibilidade, caracterizando a sensibilização periférica (46). Esse processo leva ao aumenta da excitabilidade e responsividade ao estímulo no corno dorsal da medula espinhal, caracterizando a facilitação da transmissão sináptica para a medula espinhal e centros

superiores. Os sistemas subjacentes a essa facilitação espinhal incluem i) a facilitação de transmissão pós-sináptica por meio dos receptores de glutamato, da ativação das quinases que fosforilam os canais da membrana e os receptores para viabilizar sua atividade e da ativação de quinases que ativam as enzimas intracelulares como a fosfolipase A2 aumentando a formação dos seus metabólitos; ii) a facilitação da transmissão sináptica nas projeções do corno dorsal da medula espinhal; iii) a perda da inibição local promovida pelos receptores GABAérgicos no corno dorsal da medula espinhal por meio da liberação de prostaglandinas; iv) ativação de astrócitos e microglia na medula espinhal levando à liberação de mediadores inflamatórios e v) migração de células inflamatórias não neuronais para o corno dorsal da medula espinhal (25).

Além disso, outros mediadores estão envolvidos com os mecanismos de sensibilização central. O aumento da expressão de IL-1β perifericamente e no gânglio dorsal da medula espinhal está associado a hiperalgesia mecânica e térmica em condições inflamatórias induzidas pela injeção de carragenina, lipopolissacarideo (LPS) ou adjuvante completo de Freund (CFA) (47,48). Em contrapartida, a IL-10 inibe citocinas pró-inflamatórias, principalmente TNF-α, IL-1β e IL-6, aumentando também a proliferação de mastócitos e impedindo a produção de outros mediadores inflamatórios pelas células natural killer (NK) (49). O fator neurotrófico derivado do encéfalo (brain derived neurotrophic fator – BDNF), uma vez liberado no corno dorsal da medula espinhal, induz um aumento rápido na fosforilação do seu receptor (TrκB) e os efeitos plásticos induzidos pelo BDNF estão relacionados ao tipo de célula afetada. Alguns estudos mostram um aumento agudo dos níveis de BDNF alguns dias após o estabelecimento da inflamação, entretanto o BDNF parece estar mais relacionado à plasticidade sináptica em medula espinhal, sendo pró- ou anti-nociceptivo de acordo com o contexto (50,51).

2.1.2 Dor crônica neuropática

Após lesão no nervo, ocorre uma sucessão de eventos envolvendo as aferências sensoriais primárias, sendo que especificamente após o dano axonal de um nervo periférico, ocorre a degeneração walleriana. Na degeneração walleriana, o axônio e a bainha de mielina são degradados e macrófagos e outras células imunes, como neutrófilos e células T, são liberados para o tecido (4,52). Somado a isso, há o envolvimento dos receptores da família TRP e de segundo mensageiros, como o AMP cíclico (adenosina 3,5-monofosfato cíclico - cAMP),

proteína quinases (como a mitogen-activated protein kinase – MAPK) e o óxido nítrico, que promovem mudanças na função e estrutura que levam à persistência do quadro de dor (53,54).

Além disso, os neurônios passam a apresentar atividade espontânea patológica por meio da geração de um potencial de ação ectópico nas vias nociceptivas na ausência de estímulo doloroso nos terminais periféricos (55). A atividade espontânea pode ter múltiplas origens, como o local da lesão com crescimento axonal interrompido - denominado neuroma, o corpo celular dos neurônios do gânglio da raiz dorsal e as aferências proximais intactas (56–58).

A sensibilização central é consequência de alterações dos impulsos periféricos, com aumento da excitabilidade de neurônios nociceptivos, descargas persistentes após estímulos repetidos e ampliação dos campos receptivos de neurônios do corno dorsal da medula espinhal. Essas alterações provocam mudanças no processamento sensorial cerebral, mau funcionamento do mecanismo antinociceptivo descendente, aumento da atividade da rota facilitatória da dor e somação temporal e espacial do estímulo doloroso (59).

No circuito neural ocorre modulação dos sinais nociceptivos gerados pelo dano nervoso por meio da inibição ou facilitação supra espinhal descendente, que converge nos neurônios do corno dorsal da medula espinhal (60,61). Além disso, a lesão neuronal periférica ativa os receptores excitatórios de glutamato aumentando a excitabilidade em medula espinhal, e diminuindo os transportadores de glutamato (62). O aumento da disponibilidade de glutamato regional, secundário à perda dos seus transportadores, resulta na ativação aumentada e persistente dos receptores ionotrópicos (NMDA e AMPA) e metabotrópicos (metabotropic glutamate receptors - mGluRs) de glutamato, levando à redução do limiar de ativação dos neurônios, e ao aumento da excitabilidade e da neurotoxicidade (63,64). Os receptores AMPA ativados nos neurônios do corno dorsal da medula espinhal desencadeiam a resposta ao estímulo doloroso agudo, uma vez que os receptores N-metil-D-aspartato (NMDA) estão fisiologicamente bloqueados pelo íon magnésio. Quando ocorre uma despolarização repetitiva, como nos casos de dor crônica, este bloqueio é removido, promovendo a amplificação e o prolongamento do processo nociceptivo no corno dorsal da medula espinhal (65). Além disso, a atividade glutamatérgica medular pode iniciar uma cascata de sinalização intracelular, incluindo a ativação da proteína quinase C, resultando em neuroplasticidade a longo prazo na medula espinhal (66–69).

A atividade contínua proveniente das aferências primárias pode ser uma das causa da degeneração dos interneurônios inibitórios do corno dorsal da medula espinhal que contém ácido gama-aminobutírico (GABA), contribuindo para a desinibição e aumento da sensitividade

(70). E importante salientar que a inibição medular desempenha função importante na transmissão do processo nociceptivo e o desequilíbrio entre a inibição descendente e a facilitação pode promover a perpetuação da dor neuropática, com participação dos centros supra espinhais, fundamentais nesse processo (30,71).

O sistema imune também apresenta um papel crítico no estabelecimento e manutenção da dor neuropática, assim como na dor associada à inflamação (72). Após a lesão nervosa periférica, as células gliais do SNC no corno dorsal de medula espinhal, os oligodendrócitos e os astrócitos em estado de "repouso" são ativados. A ativação da microglia é secundária à alteração na expressão de múltiplas moléculas, como receptores de membrana celular (incluindo receptores purinérgicos) e moléculas de sinalização intracelular (73). A microglia ativada pode desencadear diferentes respostas celulares, promovendo a liberação de neurotrofinas e de mediadores pró-inflamatórios, que modulam a transmissão nociceptiva liberando neurotransmissores pré-sinápticos ou alterando a excitabilidade pós-sináptica (74). As neurotrofinas, por sua vez, como o fator de crescimento neural (nerve growth factor- NGF), participam da gênese da dor neuropática aumentando a transmissão excitatória, reduzindo a transmissão inibitória e promovendo reforço da facilitação descendente no corno dorsal da medula espinhal (75,76). No SNC, o NGF é responsável por uma regulação positiva de vários genes, como o BDNF nos neurônios nociceptivos, modulando e sensibilizando uma população representativa de neurônios envolvidos no processo de dor neuropática. (77,78). Em modelos animais de dor neuropática, estudos mostram a modulação de várias citocinas e quimiocinas, como interleucina-10 (IL-10), IL-1β e TNF-α e prostaglandinas (79,80). As citocinas aumentam a ativação da microglia induzindo mecanismos autócrinos e podem agir diretamente nos neurônios do corno dorsal da medula espinhal, induzindo hipersensibilidade, assim como desencadeiam a cascata de respostas inflamatórias no SNC (74,81).

É importante ressaltar que os mediadores inflamatórios, assim como as neurotrofinas, podem exercer funções diversas, tanto adaptativas, quanto mal adaptativas no processamento da nocicepção, sobretudo na dor crônica. Portanto, o manejo e o tratamento farmacológico dessas condições requerem a exploração de novas terapias que não interfiram nas funções inatas desses mediadores (44). Além disto, é importante considerar que a resposta imune e glial na periferia e no SNC apresentam um padrão temporal na cascata de ativação das vias que as interconectam (4).

2.2 MODELOS ANIMAIS DE DOR

Para estudo dos mecanismos de transmissão e processamento doloroso, assim como novos alvos para o seu tratamento e potencial analgésico de novos fármacos, vários modelos experimentais tem sido desenvolvidos para mimetizar as condições patológicas humanas (82). Os modelos animais pré-clínicos permitem o estudo detalhado da dor crônica em nível celular e molecular, torna-se essencial a realização de estudos com modelos animais que envolvam sistemas relacionados aos mecanismos de dor e que explorem novas opções de tratamentos farmacológicos (83). O uso de modelos animais com roedores, pela complexidade comportamental, permite o estudo e a analise de componentes afetivos da dor. Nesse contexto, considerando a variedade de processos neurobiológicos envolvidos nos diferentes tipos de condições dolorosas crônicas, diversos modelos de dor pré-clínicos foram desenvolvidos, dentre eles os modelos de dor crônica inflamatória e dor neuropática (83,84).

2.2.1 Modelo de dor crônica inflamatória

Diversos modelos foram desenvolvidos para o estudo da dor crônica inflamatória, tanto para condições inflamatórias localizadas, quando para as generalizadas. As respostas inflamatórias localizadas são comumente desenvolvidas em modelos animais pela injeção de uma substância irritante, como a formalina, o adjuvante completo de Freund (Complete Freund Adjuvant - CFA) ou carregenina na pata (83). Cada uma dessas substâncias produz um curso de tempo diferentes para as respostas dolorosas, que varia de minutos quando utiliza-se a formalina até semanas se injetado o CFA, possibilitando estudos a curto e longo prazo envolvendo os efeitos da inflamação local (84,85).

O modelo de indução de inflamação pela injeção local de CFA tem sido utilizado pelo grupo de pesquisa Farmacologia da Dor e Neuromodulação: modelos pré-clínicos e demonstrou ser válido para a avaliação do efeito analgésico e anti-inflamatório da dexametasona (86) e melatonina (87,88). Para esse modelo experimental de dor crônica inflamatória, o edema é induzido por meio da injeção intradérmica (i.d.) no membro posterior esquerdo de 0,1 ml do adjuvante completo de Freund’s na concentração de 1 mg/ml em óleo mineral estéril (87,89). Na inflamação induzida pelo CFA há uma resposta inflamatória bifásica: inicial (inflamação

aguda) e tardia (inflamação crônica, considerado a partir do 14º dia após a injeção do CFA), permitindo o estudo da fisiopatologia na condição crônica dolorosa (90).

2.2.2 Modelo de dor crônica neuropática

Em roedores, a abordagem mais comum para indução de neuropatia periférica é lesão do nervo por meio de ligadura, transecção ou compressão do nervo ciático, ramos distais do nervo ciático, nervo infraorbital ou raízes nervosas do trigêmeo, que mimetizam as condições dolorosas neuropáticas advindas de traumas em nervos periféricos (71,82,91). Por outro lado,

os modelos pré-clínicos de dor neuropática central são escassos, com a maioria recorrendo a lesões mecânicas ou quimicamente induzidas na medula espinhal (92). Ainda, neuropatias induzidas por produtos químicos em roedores também têm sido usadas para tentar reproduzir em modelos animais dor neuropática periférica associada à diabetes, quimioterapia e terapia anti-retroviral (83).

O modelo de dor crônica neuropática por constrição do nervo isquiático procedimento de compressão crônica do nervo isquiático é realizado conforme técnica descrita por Bennett & Xie (93) e modificado por Cioato et al. (94), que consiste em três amarraduras leves no terço inicial do nervo, com 1 mm de distância entre os nós. Assim, o comprimento do nervo a ser afetado será aproximadamente de 5,0 mm (Figura 1). O grau de constrição desejado retarda, mas não interrompe totalmente a circulação sanguínea da superfície epineural. Os animais pertencentes ao grupo sham são submetidos a todo o processo, com exceção da compressão nervosa. Esse modelo permite mimetizar a dor crônica neuropática advinda de compressão de nervo isquiáticos nos humanos, condição patológica dolorosa prevalente e de difícil tratamento farmacológico, justificando a importância do seu estudo (28).

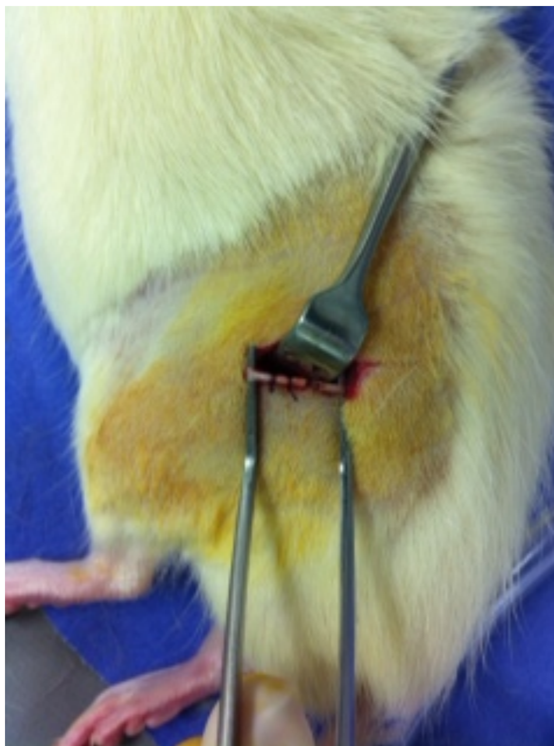

Figura 1. Modelo de dor neuropática por constrição do nervo isquiático. Arquivo pessoal.

2.3 SISTEMA ADENOSINÉRGICO

A adenosina é um nucleosídeo endógeno composto por uma adenina ligada a uma ribose. É molécula essencial para a sobrevivência, tendo caráter endógeno e dita onipresente, sendo distribuída na maioria dos tecidos e afetando quase todos os aspectos da fisiologia celular, incluindo a regulação do sono, atividade neuronal, a função vascular, a agregação plaquetária e a regulação das células sanguíneas, além de ter papel importante na homeostase de células do sistema nervoso (10,95–97). Considerando sua importância em vias metabólicas de funções essenciais, as concentrações intracelulares de adenosina não podem ser nulas, entretanto há a necessidade de uma quantidade finita de adenosina extracelular, na maioria das condições homeostáticas basais do organismo (98).

O principal mecanismo responsável pela geração extracelular de adenosina é a desfosforilação das suas entidades precursoras: adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP), que são liberadas em condições estressantes para o organismo (95). Essas moléculas são liberadas por vários tipos de células por meio das enzimas

hidrólise-específicas: ectonucleosídeo trifosfato difosfohidrolase (CD9) e ecto-5-nucleotidase (CD-73), sem as quais as concentrações de nucleotídeos seriam relativamente estáveis. Entretanto, em condições fisiológicas, a adenosina é originada no ambiente intracelular a partir da hidrólise do AMP e da S-adenosil-homocisteína (SAH). Uma vez gerada, a adenosina extracelular é capturada para o meio intracelular por meio da família de transportadores de nucleotídeos concentrados ligados ao cálcio SLC28 e da família de transportadores de nucleotídeos equilibrativos energia-dependente SLC29, que garantem a passagem livre de adenosina pela membrana celular. A direção da captação ou liberação de adenosina das células vai ser determinada pela diferença de concentração na membrana celular (95). Após a recaptação intracelular, a adenosina sofre rápida fosforilação gerando AMP pela adenosina quinase ou deaminação da inosina pela adenosina desaminase. Essas vias garantem a manutenção das concentrações intracelulares de adenosina por meio de um controle enzimático rigoroso. Portanto, existem três sistemas essenciais que são responsáveis pela inativação e ou remoção de adenosina nos tecidos: adenosina desaminase, adenosina quinase e o sistema de recaptação (97).

A adenosina atua como um modulador pré-sináptico da liberação de neurotrasmissores. Além disso, a atividade das vias locais de sinalização podem ser reguladas pelo equilíbrio entre os efeitos do ATP, adenosina e ectonucleotidases na transmissão sináptica, sendo que algumas subpopulações de neurônios e ou astrócitos podem liberar adenosina diretamente (99,100).

Os receptores de adenosina tem sido implicados em vários processos fisiológicos importantes, desde a regulação imunológica e da função vascular ao controle metabólico e neuromodulação (101). Análogos de adenosina atuam na resposta nociceptiva em vários contextos testados, incluindo a dor neuropática, em que processos de sinalização estão alterados e existe um potencial interesse no desenvolvimento de fármacos baseados na adenosina. Além disso, os subtipos de purinoceptores envolvidos na ativação nociceptiva foram recentemente identificados, clonados e demonstraram ter uma distribuição única nos neurônios sensoriais; isto pode permitir o desenvolvimento de alvos dos receptores de adenosina P1 para condições em que o ATP contribui para a etiologia da dor. É importante salientar que os efeitos das purinas no processo nociceptivo podem ser complexos, com efeitos dependendo de subtipos de receptores específicos ativados e da localização do receptor (102,103).

2.3.1. Receptores de Adenosina

Os receptores purinérgicos pertencem à família dos receptores acoplados à proteína G (*G-protein coupled receptors* – GPCR) e são classificados em dois subtipos: P1 e P2, de acordo com a suas propriedades farmacológicas e moleculares, sendo encontrados na maioria dos órgãos do corpo e apresentando um papel importante na contratilidade de vários vasos sanguíneos, entre outras funções (97,101,104). Os receptores P1 reconhecem a adenosina como seu principal ligante e seus subtipos são denominados A_1, A_2A, A_2B e A_3, sendo que cada um destes é caracterizado por clonagem molecular, perfil de ativação por agonista, perfil de ativação por antagonista, ligação à proteína G e sistemas efetores (98).

O subtipo de receptores A1 é expresso no SNC, principalmente no córtex, cerebelo, hipocampo, terminais de nervos autonômicos, medula espinhal e células gliais e, tendo em vista sua distribuição ampla, suas funções também abrangem diversos mecanismos fisiológicos. Dentre essas funções encontram-se a modulação da liberação de neurotransmissores, redução da excitabilidade neuronal, controle de sono e vigília, analgesia, sedação e ansiólise. Do ponto de vista cardiovascular, os receptores A1 de adenosina são encontrados em altos níveis no átrio, induzindo efeitos cronotrópicos e inotrópicos negativos, além de atuarem na regulação do fluxo sanguíneo renal e na liberação de renina. Nas vias aéreas, promove essencialmente efeitos pró-inflamatórios (10,105). Vários estudos tem demostrado a ação antinociceptiva desencadeada pela ativação de receptores A1; entretanto, o uso clínico é limitado principalmente pelos efeitos adversos cardiovasculares e respiratórios (106,107).

A adenosina tem alta afinidade pelos receptores A2A que apresentam distribuição limitada ao estriado e bulbo olfatório, em terminais pré e pós sinápticos de nervos (108). São altamente expressos em mastócitos, leucócitos e plaquetas e de forma intermediária no coração, pulmão e vasos sanguíneos. Na maioria das células, sua ativação promove inibição dos níveis de cálcio intracelular e parece estar relacionada a mecanismos anti-inflamatórios (97,101). Com relação aos receptores A2B, estes são altamente expressos no trato gastrointestinal, bexiga, pulmão e em mastócitos. Devido a menor afinidade da adenosina por este tipo de receptor, o receptor A2B permanece silenciado em condições fisiológicas (109). No SNC, o receptor A2B é expresso em baixos níveis nas células neuronais e gliais, como a microglia e os astrócitos (108) e sua ativação promove uma gama diversificada de efeitos, tanto pró- quanto anti-inflamatórios. No entanto, o papel destes receptores na dor é ambíguo (97,107,110).

Assim como em relação aos receptores A2B, a adenosina tem menor afinidade pelos receptores A_3, que estão distribuídos por todo o organismo (97). No SNC, são expressos no hipotálamo, hipocampo, córtex e terminais de nervos motores, assim como na microglia e astrócitos (101). Tem sido sugerido que sua ativação em microglia e astrócitos promove inibição da resposta neuroinflamatória que tem sido relacionada aos seus efeitos analgésicos (111). Também são encontrados em células inflamatórias, como mastócitos, células dendríticas, condrócitos e osteoblastos mediando efeitos anti-inflamatórios (95). Do ponto de vista cardiovascular, apresenta função cardioprotetora, entretanto, a sua localização precisa no coração não é conhecida.

2.3.2 Receptor de Adenosina A_3 e potencial terapêutico na dor crônica

Como é comum aos receptores pertencentes à superfamília GPCR, o receptor de adenosina A_3 é caracterizado por 7 domínios transmembrana e um uma região intracelular C-terminal, com resíduos Ser e Thr que representam sítios de fosforilação importantes para a dessensibilização rápida do receptor. Após a estimulação por agonista, os receptores A_3 são desfosforilados no C-terminal pelas GPCR quinases e internalizados, onde interagem com a β-arrestina 2. Além disso, o receptor preferencialmente se liga à proteína Gi para inibir o acúmulo de AMPc, mas também pode se ligar à proteína Gq para mediar a estimulação da fosfolipase C, aumentando as concentrações de cálcio (112).

Os receptores A_3 de adenosina tem um papel complexo na fisiopatologia da inflamação, modulando diversas funções das células imunes, como a produção de citocinas, degranulação, quimiotaxia e proliferação (113). O papel desses receptores na modulação do processo inflamatório foi confirmado por meio da deleção do receptor em modelos animais, uma vez que a adenosina apresenta papel na mediação na permeabilidade vascular, levando ao extravasamento de proteínas, mecanismo que tem papel importante na resposta inflamatória (114).

Os receptores de A_3 tem sido investigados nas condições dolorosas, sendo que alguns estudos já mostraram efeitos benéficos no uso de agonistas desses receptores em modelos de dor neuropática, inibindo a alodinia mecânica e promovendo aumento da potência antinociceptiva de analgésicos clássicos como a morfina e a gabapentina (115,116). Além disso, foi observada redução da resposta nociceptiva relacionada à redução de produção de citocinas

pró-inflamatórias e às vias de NF-κB e proteína quinase ativada por mitógeno (ERK e P38) em modelos de dor neuropática induzida por quimioterapia (20) (Figura 2).

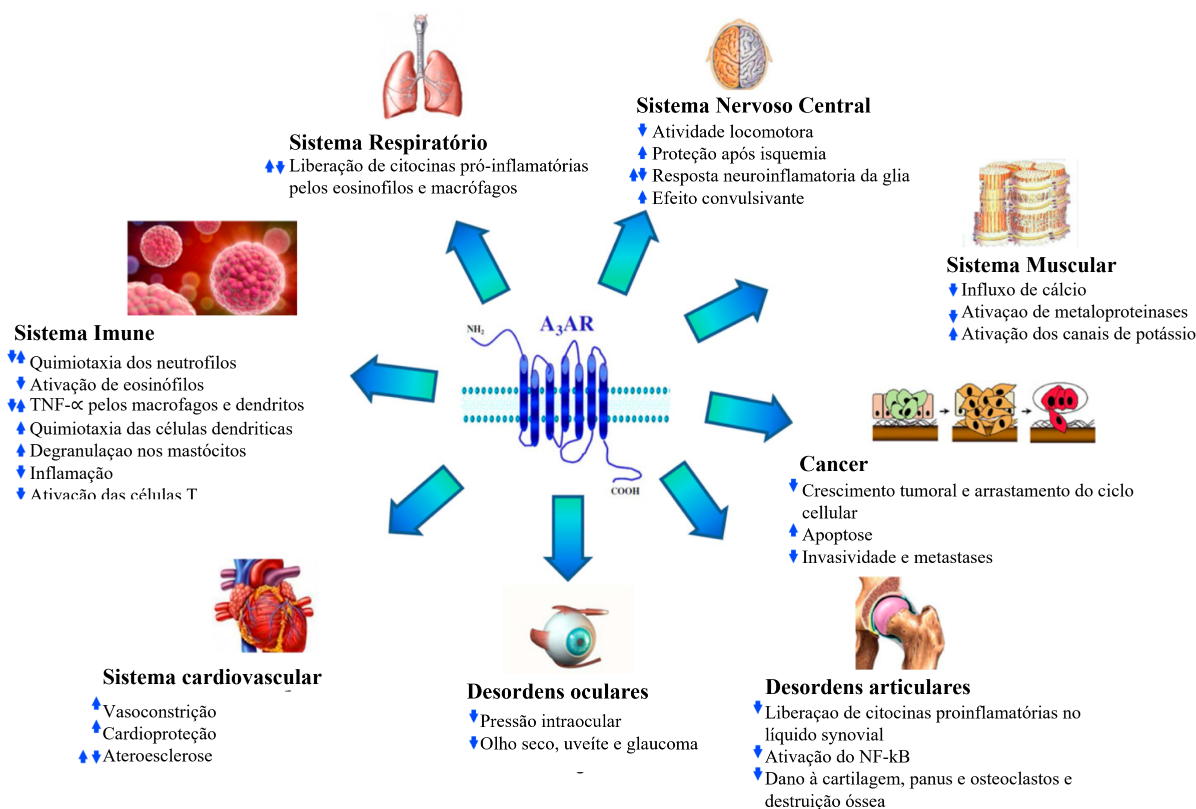

Figura 2. Diagrama esquemático que ilustra as ações dos receptores A3 de adenosina em diferentes sistemas. A ativação dos receptores A3 pela adenosina pode afetar vários órgãos, incluindo os sistemas respiratório, imune, cardiovascular, muscular e nervoso central, e modificar patologias como doenças oculares, articulares e câncer, modulando diversos desfechos biológicos. Adaptado de Borea et al. (95).

Estudo de Little (2012) demonstrou que a ativação de receptor de A3 em medula espinhal e em nível supraespinal reduz o comportamento de dor espontânea e sem produzir tolerância, como encontrado em modelos de dor neuropática. Ainda, reduz o processamento na medula espinhal pela redução da excitabilidade de neurônios espinhais de amplo espectro (wide dinamic range – WDR) e produz inibição supraespinhal dos mecanismos noceptivos espinhais por meio da ativação da circuitaria noradrenérgica supraespinhal (116).

O uso de agonista de receptor A3 também foi capaz de reduzir a resposta inflamatória no teste da formalina, sendo que a estimulação destes receptores em nível medular parece inibir a liberação de mediadores relacionados à sinalização da dor. Além disso, o uso de antagonista

reverteu o efeito antinociceptivo da adenosina neste mesmo estudo, sugerindo que o receptor A_3 tem contribuição na modulação da facilitação da transmissão do estímulo doloroso na medula espinhal (117). A ativação do receptor A_3 parece ter efeitos na inibição mediada pelo GABA por meio da atenuação da sinalização da sinalização do BDNF (118).

Vários dos estudos com os receptores de adenosina têm sido centrados na identificação de agonistas ou antagonistas competitivos capazes de interagir, com crescente seletividade, com os principais sítios de ligação dos receptores. Neste contexto, os estudos tem se concentrado principalmente na pesquisa com os subtipos de receptores que apresentam um papel crítico na fisiopatologia da inflamação, particularmente os receptores A_2A, A_2B e A_3 (119). No entanto, a ampla distribuição de receptores de adenosina por todo o corpo aumenta o risco de efeitos adversos após a administração de agonistas não-específicos. Por essa razão, nos últimos anos, o desenvolvimento de moduladores alostéricos de receptores de adenosina tem sido explorado, buscando a modulação da interação entre os receptores e o seu agonista endógeno.

Atualmente, a maioria dos compostos alostéricos disponíveis atuam como moduladores positivos dos receptores de adenosina, sendo capazes de aumentar a afinidade, a potência e ou a eficácia da adenosina endógena (120). Considerando os efeitos favoráveis da ativação dos receptores de adenosina A_3, a busca por agonistas seletivos levou ao desenvolvimento do fármaco N6-(3-Iodobenzil)adenosina-5′-N-metiluronamida, chamado IB-MECA, que é 50 vezes mais seletivo para o A_3 em relação dos receptores A1 e A2A (121).

Recentes estudos tem relatado que o efeito nociceptivo do agonista adenosinérgico IB-MECA está parcialmente relacionado com os receptores GABA e a sinalização envolvida com a ativação do A_3R e transportador neuronal de potássio (K+) e de cloreto (Cl-) (KCC2) (17). Além disso, sabe-se do envolvimento da glia no desenvolvimento e manutenção da dor crônica e que vários agentes que reduzem a estimulação glial podem prevenir ou atenuar os processos nociceptivos (122). Estudo de Janes e colaboradores (2015) demonstrou que o efeito analgésico da ativação dos receptores A_3 pelo IB-MECA pode ser resultante da modulação dos processos neuroinflamatórios na medula espinhal acarretando uma redução na hiperatividade astrocitária e na produção de citocinas pró-inflamatórias em modelo de dor neuropática (20,118).

Considerando os estudos já conduzidos e tendo em vista que sua ativação parece ser capaz de modificar o curso dos processos dolorosos patológicos e não os processos protetores, o receptor de A_3 de adenosina parece uma possibilidade terapêutica promissora, uma vez que não apresenta as complicações clássicas dos opioides relacionadas à tolerância e ao potencial

de abuso. Além disso, seu papel nos processos dolorosos merece ser estudado, a fim de determinar seu envolvimento nas vias patológicas de perpetuação da dor crônica e buscar opções terapêuticas que possam lançar mão da ativação dos receptores de adenosina A_3.

III. JUSTIFICATIVA

3. JUSTIFICATIVA

O manejo dos quadros dolorosos crônicos permanece ainda sendo um desafio, uma vez que muitos pacientes não apresentam alívio adequado da dor, como descrito tanto por dados clínicos, quanto experimentais (123).O uso de moduladores alostéricos parece ser um caminho promissor, pois esses compostos, ao potencializar o efeito da adenosina endógena, principalmente em contextos inflamatórios, onde há liberação intensa de mediadores, podem levar a respostas específicas locais com diminuição de efeitos adversos sistêmicos (113).

Estudos experimentais utilizando modelos animais permitem a investigação a fisiopatologia da dor crônica, assim como de novas terapias farmacológicas, avaliando o seu impacto na resposta nociceptiva, seus efeitos neuroquímicos e moleculares. Portanto, considerando a escassez de estudos que explorem os mecanismos de sinalização relacionados à ativação de receptores A_3 de adenosina e seus efeitos antinociceptivos em modelos animais de dor crônica, torna-se essencial a realização de pesquisas que envolvam a utilização dessa terapêutica, avaliando o seu impacto na resposta nociceptiva e quantificando marcadores centrais de inflamação e plasticidade.

IV. OBJETIVOS

4. OBJETIVOS

4.1 OBJETIVO GERAL

Investigar o efeito antinociceptivo de tratamento com agonista de receptor A_3 de adenosina em modelos animais de dor crônica inflamatória e de dor neuropática, avaliando a resposta nociceptiva e quantificando marcadores centrais de inflamação e plasticidade em ratos.

4.2 OBJETIVOS ESPECÍFICOS

A) Avaliar o efeito da administração de agonista de receptor A_3 adenosina em ratos submetidos a modelos de dor crônica inflamatória e neuropática, por meio da:

1) avaliação da resposta nociceptiva utilizando:

a) uma curva de tempo da hiperalgesia térmica no teste de placa quente;

b) a hiperalgesia mecânica no teste de von Frey;

c) a hiperalgesia mecânica no teste de Randall Selitto;

2) avaliação da resposta inflamatória por meio da quantificação dos níveis de interleucinas IL-1β e IL-10 em córtex cerebral, tronco encefálico, hipocampo, medula espinhal.

3) avaliação da neuroplasticidade por meio da quantificação dos níveis BDNF e NGF em córtex cerebral, tronco encefálico, hipocampo, medula espinhal.

B) Utilizar ensaio de complementação por Nano-Bit para caracterizar interação de receptores acoplados à proteína G com β-arrestina 2: receptores de adenosina A_3 e receptores de peptídeo liberador de gastrina

V. REFERÊNCIAS DA PARTE I

5. REFERÊNCIAS DA PARTE I

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PARTE II

Artigos Científicos, Discussão, Conclusão e Perspectivas

VI. ARTIGOS CIENTÍFICOS

MANUSCRITO 1

IB-MECA acute treatment relieves pain in CFA chronic inflammatory model in rats

Periódico: Cytokine Journal

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IB-MECA acute treatment relieves pain in CFA chronic inflammatory model in rats

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ABSTRACT

It is known that IB-MECA compound, an agonist of adenosine A_3 receptor (A_3AR), is involved with pain relief and modulation in the inflammatory process; however, its action mechanisms are not completely elucidated. The aim of this study was to evaluate the antinociceptive effect of IB-MECA) in a chronic inflammatory pain model, and the involvement of neurotrophins and cytokines central levels in this effect. Chronic inflammatory pain was induced using Complete Freund's Adjuvant (CFA) in the hind paw of male adult Wistar rats. Thermal and mechanical hyperalgesia/allodynia was measured by Hot plate, Von Frey and Randal Selitto tests. Neurochemical measured were brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), interleukin 1β (IL-1β) and IL-10. The establishment of pain model, decrease of latency withdrawal, was observed 10 and 14 days after CFA injection. And, IB-MECA was effective to revert mechanical and thermal hyperalgesia, in a totally or partially way. We observed CFA pain model effects in IL-1β and IL-10 spinal cord and brainstem levels. Also, we showed that IB-MECA administration in controls increased the interleukin levels. And, we did not find any involvement neurotrophins in this effect, at least those we measured in spinal cord and brainstem of rats. Studies have shown that CFA increases the IL-1β in the hind paw of injection when evaluated acutely after the induction of model. But, there is a lack of knowledge about the neuroinflammatory effects induced by CFA pain model. Also, the adenosine A_3 receptor seems to have complexes effects in the central nervous system, with pro-inflammatory and anti-inflammatory roles, specially in healthy conditions.

Keywords: CFA model; rats; IB-MECA; nociceptive tests.

1 INTRODUCTION

Different neurotransmitters systems are involved with pain transmission and relief. The adenosinergic signaling has reached large interest in the last decades. The adenosine receptors were subdivided in A_1, A_2A, A_2B and A_3 [1]. Each receptor presented a specific distribution in the body, as well as, functionality. Specifically, A_3 adenosine receptor (A_3AR) is distributed for both peripheral and central nervous systems, including glial cells; and it has been linked to antinociceptive and anti-inflammatory roles [2]. A selective agonist for A_3AR has been developed, the N6- (3-iodobenzyl) adenosine-5'-methyluronamide (IB-MECA) compound; and there is an increase interest in studying this compound in different pain conditions. In acute model of pain, after intrathecal administration of IB-MECA, the authors conclude that A_3A receptor may be involved in the regulation of the late phase response of the formalin test in mice [3]. Chen and colleagues have reported that IB-MECA is effective in neuropathic pain models indexed by different chemotherapeutic agents [4].

Previous study showed an overexpression of A_3AR in the synovia, in the peripheral blood mononuclear cells, and in the drain lymph node in the Complete Freund's Adjuvant (CFA) inflammatory model in female rats; and these receptors were down-regulated after oral chronic treatment with IB-MECA [5]. Diverse mechanisms have been investigated from the A_3 activation, with the characteristic of cell-dependent effect [6].

Considering all exposed before, the objective of the current study was to increase knowledge about the action mechanisms of IB-MECA in a pain model in rats, thus we evaluated the antinociceptive effects of a single dose of an adenosine A_3 receptor agonist (IB-MECA) in a CFA chronic inflammatory pain model, assessing the nociceptive responses and the neurochemical levels in central nervous system structures.

2 MATERIALS AND METHODS

2.1 Animals

A total of 64 adult male Wistar rats (55–65 days old; weight 200–250 g) were used. The number of animals was calculated as 8 rats per group for behavioral experiments [7] and 6 rats per group for biochemical analysis, to determine a difference between the variables of 1.5 of standard deviation and $\alpha = 0.05$ [8–10]. The animals housed in groups of three per polypropylene cage (49 cm x 34 cm x 16 cm) with sawdust-covered flooring. All animals were maintained in a controlled environment (22±2°C) under a standard light-dark cycle (lights-on at 7 a.m. and lights-off at 7 p.m.), with water and chow (Nuvital, Porto Alegre, Brazil) *ad libitum.* All experiments and procedures were approved by the institutional Animal Care and Use Committee (GPPG-HCPA protocol no.150530) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th ed. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines [11]. The experiment used the number of animals necessary to produce reliable scientific data.

2.2 Experimental design

The rats were acclimated to the maintenance room for two weeks before the experiment began. Initially, rats were divided into two groups: control (CT) and pain (P). Rats were randomized by weight and by paw withdrawal latency measured through Hot Plate test to ensure that all rats have similar nociceptive behavior (Figure 1). After the establishment of chronic inflammation assessed by nociceptive tests, fourteen days after chronic constriction, rats were subdivided into eight groups (eight animals per group) according the treatment: control (CT); control + vehicle (CTV); control + morphine (CTM); control + IB-MECA treatment (CTT);

pain (P); pain + vehicle pain + morphine (PM) and pain + IB-MECA treatment (PT). Randall Selitto, von Frey and Hot plate tests were performed at baseline, 10 days and 14 days after the inflammation induction, and 30 minutes after treatment with one dose of vehicle, morphine or IB-MECA. Hot plate test was repeated 60, 90 and 120 minutes after treatment. The rats were killed by 6 hours after the treatment; spinal cord and brainstem were collected for after analyses of Brain Derived Neurotrophic Factor (BDNF), Neuronal Growth Factor (NGF), Interleukin 1β (IL-1β) and Interleukin 10 (IL-10). For all procedures (nociceptive tests and neurochemical assays), the experimenter was blinded to group and treatment of rats being tested.

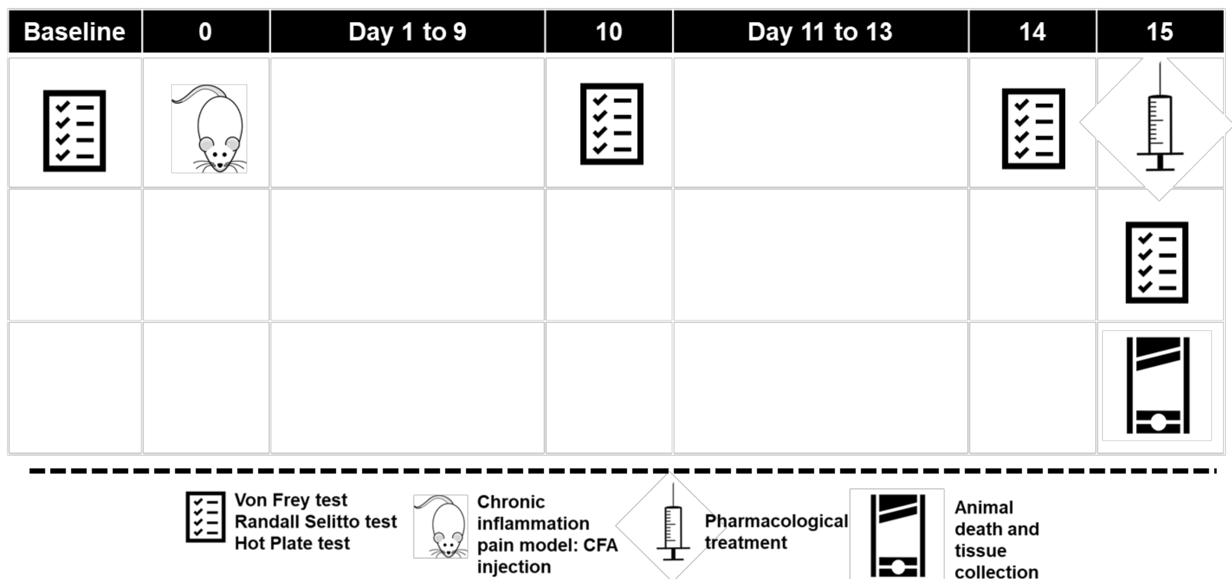

Figure 1. Timeline of experimental design.

2.3 Chronic inflammation pain model

The chronic inflammation was induced using model previously described by Laste et al. [13]. Rats were anesthetized with isoflurane (5% for induction, 2.5% for maintenance) and placed in the dorsal position. In the pain group, the inflammation was induced with a single intradermal injection in the left footpad of 100 µl of Complete Freund's Adjuvant (CFA) diluted in saline solution 1 mg/ml [12]. This group received tramadol 5 mg/kg intraperitoneally for

analgesia immediately and every 12 hours for 2 days after the pain induction [14]. The control group did not undergo any intervention.

2.4 Pharmacological treatment

The rats of treatment groups received a selective agonist of the adenosine A_3 receptor N6- (3-iodobenzyl) adenosine-5'-methyluronamide (IB-MECA) [15] dissolved in 3% Dimethyl sulfoxide (DMSO) and applied by intraperitoneal (i.p.) injection. The acute treatment protocol consisted in one dose of 0.5 µmol/kg i.p. [4].The animals of vehicle groups received one dose of DMSO 3% in saline solution under i.p. injection, and rats from positive control group received one dose of morphine 5 mg/kg i.p. [16] as gold standard of analgesia.

2.5 Hot Plate test

The hotplate (HP) test was carried out to confirm the effectiveness of inflammatory pain model, and to assess the effects of IB-MECA treatment on the thermal nociceptive threshold [17] . This test was conducted at baseline, 10 and 14 days after CFA injection as well as 30 minutes after the IB-MECA treatment. We used the hot-plate test to determine changes in latency of behaviors such as jumping and hind paw-licking as indicator of modifications of the supraspinal pain process [18], considered results of supraspinal sensory integration [19,20]. All rats were exposed to the HP for 5 minutes, 24 hours prior to testing in order to avoid the analgesia induced by the novelty of the apparatus [21]. The surface of the HP was pre-heated and kept at a constant temperature of 55±0.1°C. AS described previously by Cioato (2015) [22], rats were placed inside glass funnels on the heated surface and the time in seconds between placement of the rat and the first response (foot-licking, jumping, or rapidly removing paws) was recorded as the latency of nociceptive response. The cut-off time was 20 seconds to prevent tissue damage.

2.6 Randall Selitto test

This test was assessed at baseline, 10 and 14 days after CFA injection and 30 minutes after the IB-MECA treatment. The rats were subjected to mechanical stimuli in paw withdrawal test described by Randall and Selitto (1947) [23]. Analgesymeter, progressively increasing pressure stimulus (type 7200, apparatus Ugo-Basile Biological Research, Comerio-Varese, Italy) was used. For mechanical stimulation, which gradually increases the pressure was applied to the dorsal surface of the rat paw. The nociceptive threshold was defined as the force in grams that produces the withdraw of hind paw. A cut-off value was used for 100g.

2.6 Von Frey Test

Mechanical allodynia was assessed at baseline, 10 and 14 days after the CFA injection, and 30 minutes after the IB-MECA treatment. We used similar methodology as described before by Cioato et al. (2015)[22]. The von Frey aesthesiometer (Insight, São Paulo, Brazil) is an adaptation of the classical von Frey filaments test in which pressure intensity is recorded automatically after paw removal [24]. This device automatically records the pressure intensity after paw withdrawal. Test was performed in polypropylene cages (12cm × 20cm × 17cm) with wire grid flooring. Rats were habituated to cages for 10 minutes 24 hours prior to test and 5 minutes daily before test to prevent analgesia induced by apparatus novelty. For testing, a polypropylene tip was inserted perpendicularly from underneath the floor grid and applied to plantar side of right hind paw at gradually increasing pressure. A tilted mirror below the grid provided a clear view of the animal's hind paw. The intensity of the stimulus supported up to paw withdrawal, in grams (s), was automatically recorded. Three successive readings were measured between interval periods of 5 s and averaged. The averages were used as the final measurements and the paw withdrawal threshold was expressed in grams (g) [24].

2.7 Tissue collection

Rats were killed by decapitation six hours after IB-MECA treatment; the spinal cord and the brainstem were collected and frozen at -80°C until the assays were performed.

2.8 Neurochemical assays

Spinal cord and brainstem IL-1β, IL-10, BDNF and NGF levels were determined by sandwich ELISA using monoclonal antibodies specific for IL-1β, IL-10, BDNF and NGF (R&D Systems, Minneapolis, United States). Procedures were performed in accordance to the manufacturer's protocol. Optical density was measured using an ELISA reader at wavelength of 450 nm. The data were expressed in pg/mg of protein. Total protein was measured by the Bradford method using bovine serum albumin as the standard [25].

2.9 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (S.E.M.). *P*-values of less than 0.05 were considered significant. The Statistical Package for the Social Sciences (SPSS) 20.0 for Windows was used for the statistical analysis. Generalized Estimated Equations followed by the Bonferroni test was used to compare nociceptive tests data across groups to confirm the establishment of pain model before the treatment. One-way ANOVA test followed by Student-Newman-Keuls (SNK) was used to compare the nociceptive tests and neurochemical data across groups after treatment.

3 RESULTS

3.1 Establishment of CFA inflammatory chronic pain model

According to Figure 2, it is possible to observe the establishment of the CFA inflammatory pain model after 10 days of CFA administration, and lasting until 14 days, and the reduction of pain latency were observed at all three behavioral tests assessed (Hot Plate, Randal Selitto, electronic Von Frey). In the Hot Plate test, the Randal Selitto, and the electronic Von Frey, we found interaction between time and group (GEE/Bonferroni, Wald $\chi2=107.363$, Wald $\chi2=102.198$, Wald $\chi2=67.495$, respectively, and $P<0.001$ for all). At baseline time, rats presented similar pain threshold between control and pain groups; however, at 10 and 14 days after CFA injection, pain group presented lower pain threshold than control group, as showed at Figure 2 Panel A, B and C.

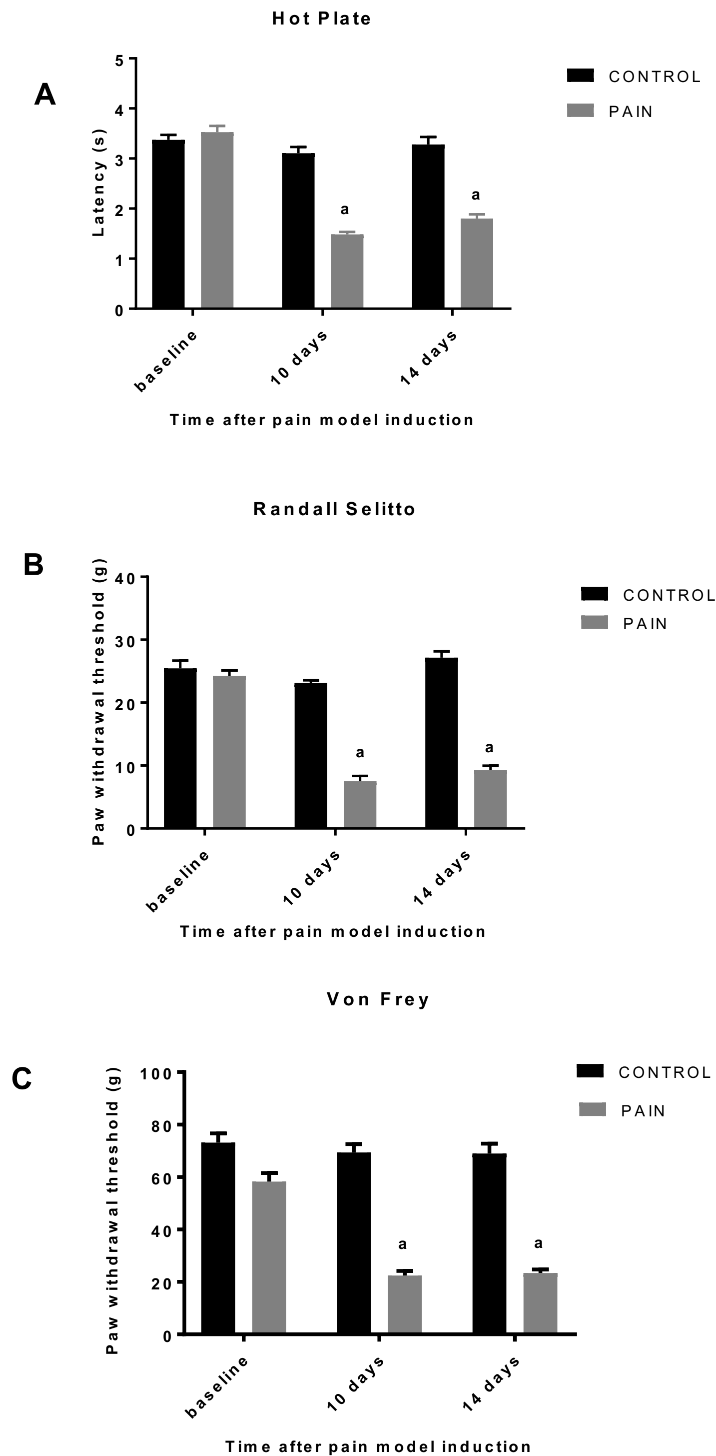

Figure 2. Establishment of chronic inflammatory model in male Wistar rats after CFA injection in the right hind paw.

Panel A. Latency of jumping and hind paw-licking in seconds assessed by Hot Plate test. Panel B. Paw withdrawal threshold in grams assessed by Randal Selitto. Panel C. Paw withdrawal threshold in grams assessed by Von Frey test. A= significant interaction between group *vs* time (GEE/ Bonferroni, P=0.001 for all).

3.2 Effect of single administration of IB-MECA in the hyperalgesia thermal and mechanical induced by CFA inflammatory chronic pain model in rats

All behavioral tests were assessed 30 min after IB-MECA administration at 14 days post CFA injection; in the hot plate test, the control-morphine group presented an increase in the pain threshold in relation to pain, pain plus DMSO, and pain plus IB-MECA groups (one way ANOVA, $F_{(7,53)}$=4.923, P<0.001). The control plus IB-MECA group showed an increase in the pain threshold in relation to pain and pain plus DMSO groups (one way ANOVA, P<0.001), and it was possible to observe that morphine applied in the pain group reverted the reduction in the pain threshold. In the Randal Selitto test, we observed that pain groups (pain, pain plus DMSO and pain plus morphine) presented lower pain threshold in relation to other groups. However, IB-MECA administration in the pain group was able partially to reversed the decrease in the pain threshold (one way ANOVA, $F_{(7,53)}$ =20.284, P=0.001). In the von Frey test, pain and pain plus DMSO groups presented a decrease in the pain threshold in relation to other groups (one way ANOVA, $F_{(7,52)}$=5.868, P=0.001); thus we found that morphine or IB-MECA when applied in pain group revert the reduction in the pain threshold (Figure 3).

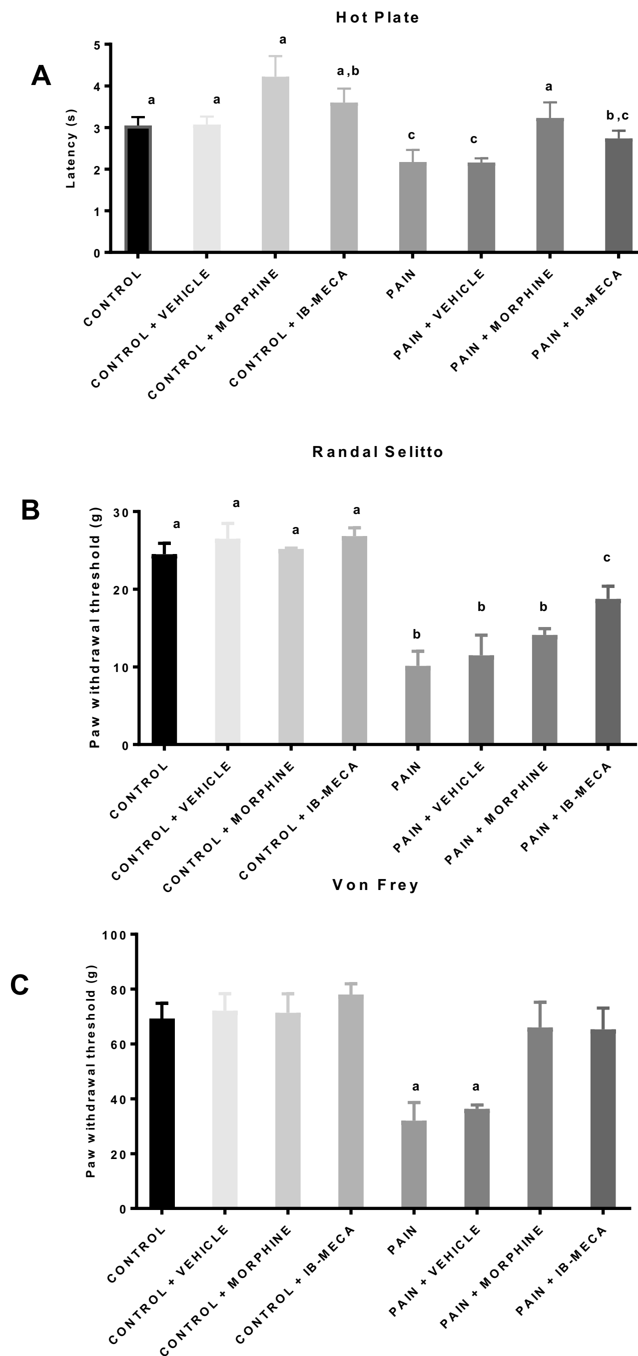

Figure 3. Effect of IB-MECA acute treatment upon behavioral response in rats subjected to CFA inflammatory model.
Panel A. Latency of jumping and hind paw-licking in seconds assessed by Hot Plate Panel B. Paw withdrawal threshold in grams assessed by Randal Selitto, Panel C. Paw withdrawal threshold in grams assessed by von Frey test
In Panel A, B, C equal letters showed no significant difference between groups,(one way ANOVA/ SNK, $P>0.05$) and different letter showed a significant difference between groups (one way ANOVA/ SNK, $P=0.001$).

3.3 Effect of single administration of IB-MECA on biomarkers levels in spinal cord and brainstem of rats subjected to CFA inflammatory chronic pain model

3.3.1. IL-1β levels

The statistic analysis of brainstem IL-1β levels (Figure 4) showed an increase in the levels of this interleukin in the group control plus IB-MECA when compared to all other groups (one way ANOVA, $F_{(7,44)}$=4.348, P=0.001). However, in the spinal cord, it was observed a decrease in the levels of IL-1 β levels in pain, pain plus vehicle, pain plus morphine and pain plus IB-MECA groups when compared with all control groups (one way ANOVA, $F_{(7,45)}$=19.770, P=0.

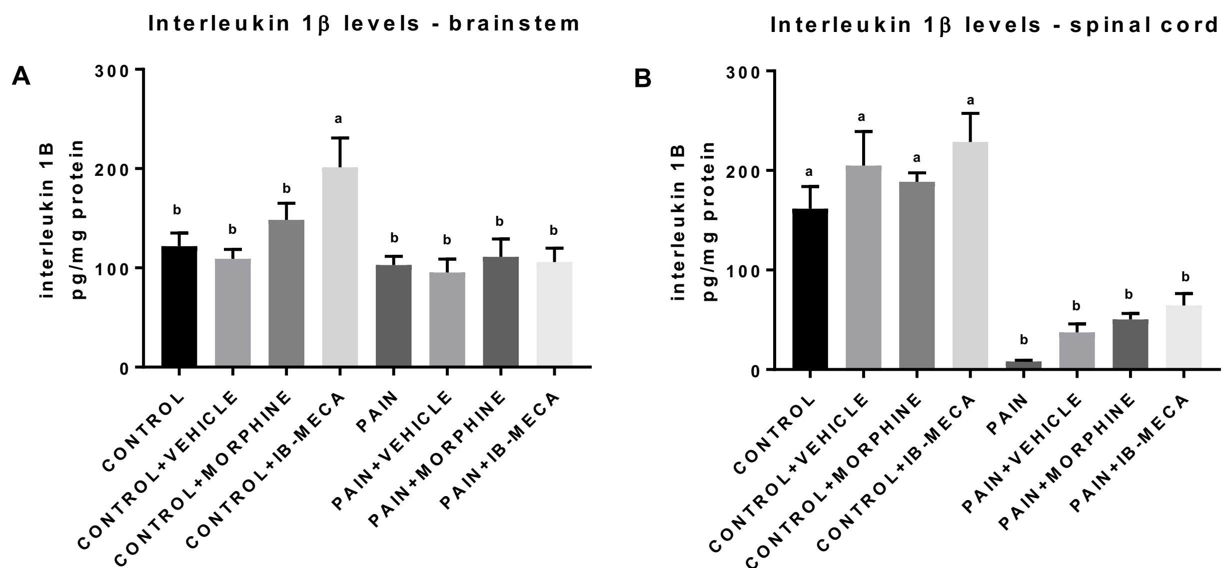

Figure 4. Effect of single administration of IB-MECA on IL-1β levels in brainstem and spinal cord of rats subjected to CFA inflammatory pain model. Panel A. IL-1β levels in brainstem. Panel B. IL-1β levels in spinal cord.
In both panels equal letters showed no significant difference between groups,(one way ANOVA/ SNK, P>0.05) and different letter showed a significant difference between groups, (one way ANOVA/ SNK, P=0.001).

3.3.2. IL-10 levels

According to the figure 5, it was observed a decrease in the brainstem IL-10 levels in pain group when compared with all other groups (one way ANOVA, $F_{(7,47)}$=6.583, P=0.001); and the administration of vehicle, morphine or IB-MECA in the pain presence induced to an increase in the IL-10 levels in brainstem to same levels of control, control plus vehicle and control plus morphine groups (one way ANOVA, P=0.001).

Interestingly, in spinal cord was observed the same pattern of results in the IL-10 levels. The pain group presented lower levels of IL-10 when compared with all other groups (one way ANOVA, $F_{(7,39)}$=15.531, P<0.001); as well as the pain plus vehicle, pain plus morphine and pain plus IB-MECA groups showed an increase in the IL-10 levels in this structure to same levels of control group, control plus vehicle and control plus morphine (one way ANOVA, P=0.001).

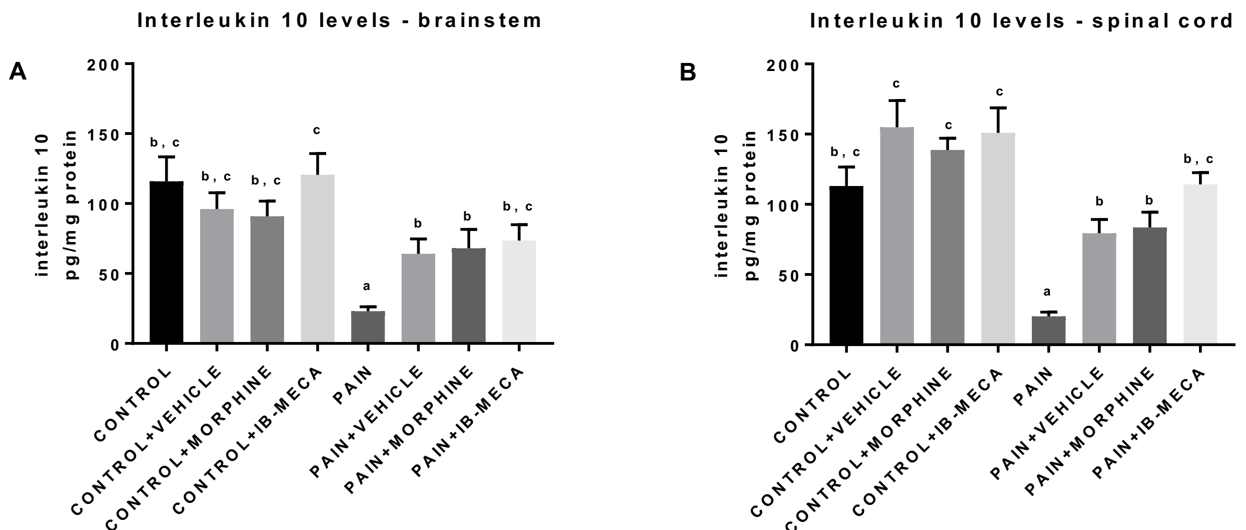

Figure 5. Effect of single administration of IB-MECA on IL-10 levels in brainstem and spinal cord of rats subjected to CFA inflammatory pain model.
Panel A. IL-10 levels in brainstem. Panel B. IL-10 levels in spinal cord. In both panels equal letters showed no significant difference between groups,(one way ANOVA/ SNK, P>0.05) and different letter showed a significant difference between groups, (one way ANOVA/ SNK, P=0.001).

3.3.3 BDNF levels

The statistic analysis of brainstem and spinal cord BDNF levels (Figure 6) showed no difference between groups analyzed both, brainstem (one way ANOVA, $F_{(7,43)}=0.949$, $P>0.05$) and spinal cord (one way ANOVA, $F_{(7,45)}=1.561$ $P>0.05$).

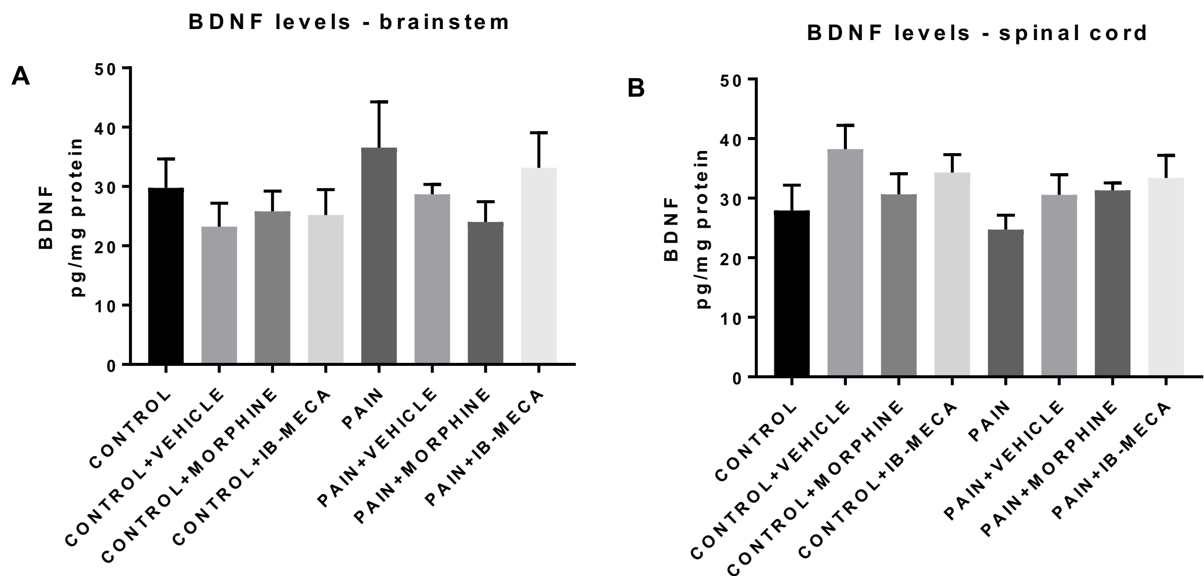

Figure 6. Effect of single administration of IB-MECA on BNDF levels in brainstem and spinal cord of rats subjected to CFA inflammatory pain model.
Panel A. BDNF levels in brainstem. No significant difference between groups (one way ANOVA, P>0.05).
Panel B. BDNF levels in spinal cord, No significant difference between groups (one way ANOVA, P>0.05).

3.3.4 NGF levels

The statistic analysis of brainstem and spinal cord NGF levels (Figure 7) showed no difference between groups (one way ANOVA, $F_{(7,44)}=0.702$ for brainstem and $F_{(7,45)}=1.561$ for spinal cord, $P>0.05$).

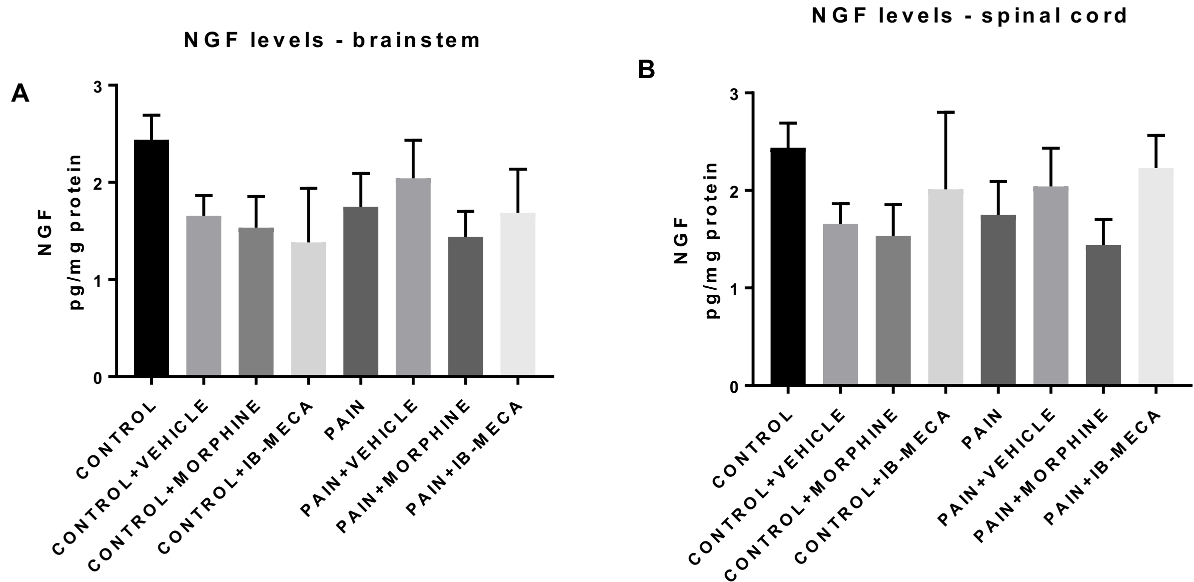

Figure 7. Effect of single administration of IB-MECA on NGF levels in brainstem and spinal cord of rats subjected to CFA inflammatory pain model.
Panel A. NGF levels in brainstem. No significant difference between groups (one way ANOVA, P>0.05).
Panel B. NGFlevels in spinal cord. No significant difference between groups (one way ANOVA, P>0.05).

DISCUSSION

In the present study, we showed that CFA, a chronic inflammatory model in rats is well established in the literature, after 10 days of CFA injection there was a reduced latency, and paw withdrawal threshold in all tests evaluated; and this behavior lasts until 14 days after CFA; when we have tested the IB-MECA effect, the IB-MECA was effective in the von Frey and Randal Selitto tests increasing the latency of paw withdrawal. And, the positive group, morphine administration, it was observed that the pain group increased the pain threshold, at all nociceptive tests evaluated. However, the neurochemical measures (BDNF, NGF, IL-1β and IL-10) in brainstem and spinal cord structures were not related with the IB-MECA role indexed to analgesic effect.

The action mechanism of IB-MECA has been studied, and different pathways have been described, however it is a tissue specific action-response. IB-MECA promoted down-regulation of A_3AR expression after its overexpression induced by incomplete Freund's adjuvant injection, with decreased expression level of Phosphoinositide 3-kinase (PI3K), protein kinase B (PKB ou Akt), Nuclear factor κB (NF-κB), inhibitor of NF-κB (IKK), and tumor necrosis factor α (TNF-α) in synovia and drain lymph node (DLN) protein extracts, whereas the caspase-3 level was up regulated [5]. IB-MECA showed efficacy in the relief of neuropathic pain induced by chronic constriction injury (CCI) model or chemotherapeutic agents in rats (Chen et al., 2012). The mechanisms of IB-MECA upon neuropathic pain induced by paclitaxel has been involved with the inhibition of spinal nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase. This prevents the activation of NF-κB and mitogen activated protein kinases as extracellular-signal-regulated kinase (ERK) and p38, associated to decrease of the production of TNF-α and IL-1β, and increase of the formation of IL-10. In addition, its mechanism has been linked to glutamate transporter 1 (GLT-1) and glutamine synthetase [27].

The A_3AR is a G protein-coupled receptor mainly associated to $G_{i/o}$ protein [28]. Previous study suggests low level of A_3AR expression in CNS [29]. The A_3AR suffers rapid desensitization after activation by agonists, it is associated with phosphorylation of GRKs family [1]. The A_3AR recruits a pathway that involves βγ release from Gi/o, PI3K, Ras, and MEK to induce ERK1/2 phosphorylation and activation, whereas signaling is independent of calcium^{2+}, PKC, and c-Src in CHO cells expressing human A_3 receptor [30]. Previous report show that the A_3AR mechanism did not alter nociceptive thresholds in non-neuropathy animals;

however produced selective relief of persistent neuropathic pain states [31], corroborating the current study.

Our group has investigated the effect of different pain model and treatments upon neuroimmunomodulatory biomarkers. It is well known that, in a chronic pain states, with persistent noxious input, occur a central sensitization with activation of astrocytes and microglia. This phenomenon induces the release of pro-inflammatory factor, as IL-1β and Interleukin 6 (IL-6) [32], as well as IL-10, which plays a classic anti-inflammatory role [33].

However, the relationship between pain, interleukins and neurotrophins were ambiguous. It is well know that nervous and immune systems influence which other in the control of homeostasis [34]. Also, pain and hyperalgesia crosstalk with inflammation, once mast cells and microglia have a role as interlocutors for pain neurons at periphery, spinal and supraspinal levels. When the pain is persistent, alterations in these cells promotes a longstanding inflammation process with alterations in neuronal functionality [35].

In the current study, we demonstrated lower levels of IL-1β in the spinal cord and in the IL-10 levels in both structures analyzed (brainstem and spinal cord) in the CFA pain groups when compared to controls. Studies have shown that CFA increases the IL-1β in the hind paw of injection, when evaluated acutely after the induction of model [36,37], as well as a study of our research group [38]. The study published by Raghavedra and colleagues [39] is the only one that described a glial activation and pro-inflammatory cytokines activation induced by CFA inflammatory pain model in central nervous system structures . In this way, there is a lack of knowledge about the neuroinflammatory effects induced by CFA pain model. In relation to neurotrophins levels, we showed that there is no influence of CFA pain model and/or IB-MECA in the BDNF and NGF levels in both structures evaluated.

This study also showed that IB-MECA is able to increase the IL-1β in the control group when compared to all other groups. It has been described the role of adenosine in the regulation of homeostasis and immune system through the activation of A_{2A}, A_{2B}, and A_3 receptor subtypes [40]. Also, there are evidences demonstrating the influence of A_3 receptor in rheumatoid arthritis, Crohn’s disease and psoriasis, with direct role in the inflammation [6]. However, the adenosine A_3 receptor seems to have complexes effects in the central nervous system, with pro-inflammatory and anti-inflammatory roles [41].

It is interesting to note that our work presented some limitations, we used unique session of IB-MECA for treating chronic condition; the morphine did not present a good positive

control in naïve rats; our sample is composed only for male Wistar rats, discarding hormonal influence; the vehicle DMSO showed effect in pain group when IL-10 biomarker was analyzed.

CONCLUSION

In summary, we conclude according to our results that IB-MECA was able to revert the reduced latency in pain group assessed by mechanical tests, however no effect in the nociceptive response assessed by thermal test. In addition, this effect was not linked directly interleukins and neurotrophins measured in the current research. However, further studies should be developed to a better understanding of the mechanisms related to the effect of IB-MECA upon behavior response.

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MANUSCRITO 2

IB-MECA acute treatment relieves pain in neuropathic chronic model in rats

Periódico: Neuroimmunomodulation Journal

Status: *a ser submetido*

IB-MECA acute treatment relieves pain in neuropathic chronic model in rats

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ABSTRACT

The main hypothesis of the present study is that a single dose of A3 adenosinergic agonist (IB-MECA) is able to alleviate nociceptive behavior in rats undergoing a neuropathic pain model. Also, the treatment with IB-MECA is able to modify inflammatory and neurotrophic parameters. The model for the induction of neuropathic pain used was the chronic constriction injury (CCI) of sciatic nerve. Thermal and mechanical hyperalgesia was measured by Hot plate, Von Frey and Randal Selitto tests. The acute treatment protocol consisted in one dose of 0.5 μmol/kg i.p. of a selective agonist of the adenosine A_3 receptor N6- (3-iodobenzyl) adenosine-5'-methyluronamide (IB-MECA) dissolved in 3% Dimethyl sulfoxide (DMSO) by intraperitoneal (i.p.) injection. The positive control group received as gold standard of analgesia one dose of morphine 5mg/kg i.p. Neurochemical measured were brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and interleukin 1β (IL-1β). The establishment of pain model by decrease of latency withdrawal, was observed 14 days after CCI model. The behavior results obtained in this study have shown that IB-MECA adenosinergic A_3 agonist receptor presented analgesic effect upon the hypernociceptive behavior induced by CCI surgery; however this effect was not linked to central levels of neurotrophins or cytokines, at least, those were measured (BDNF, NGF and IL-1beta). IB-MECA represents a potential therapeutic target in neuropathic pain condition; once it was able to relief the hypernociceptive behavior induced by CCI model in rats. However, the exact action mechanism of IB-MECA needs to be investigated, thus we extremely encouraged further findings using IB-MECA agonist.

Keywords: neuropathic pain; rats; IB-MECA; nociceptive tests.

1 INTRODUCTION

Pain is an essential mechanism to survival, acting as a warming sign of actual or potential tissue damage [1]. However, an injury to neural tissue can induce neuropathic pain that lasts for months or years after the injury, even if the primary lesions have already been treated [2]. The neuropathic pain presents a high prevalence rate and an absence of effective treatments. This mainly occurs for the inability of the pharmacological therapy to target the precise mechanism of this painful syndrome [1].

In this context, the neuropathic pain is characterized by pain in the absence of a stimulus, and many alterations in the pain pathways are responsible by the maintenance of painful symptoms [3]. Excessive and sustained inflammatory response in the peripheral and central nervous systems has been associated with the initiation and maintenance of persistent pain [4]. Also, the role of glial and immune cells activation, cytokines, as interleukin 1β (IL-1β) and interleukin 10 (IL-10) have been investigated in the establishment of neuropathic pain [5]. Also, the neurotrophins seems to contribute to pathogenesis of neuropathic pain, through the Brain Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF), which present key roles in the peripheral and central sensitization [6].

In the same line, it has been shown that adenosine receptors are expressed in inflammatory cells and could modulate the inflammatory and immune responses at different stages [7]. Recent studies showed that adenosine A_3 receptors (A_3R) agonists can produce antinociceptive effects in neuropathic pain, but the mechanisms triggered by this activation are not clear [8–10].

The main hypothesis of the present study is that a single dose of A_3 adenosinergic agonist (IB-MECA) is able to alleviate nociceptive behavior in rats undergoing a neuropathic pain model. Also, the treatment with IB-MECA is able to modify inflammatory and

neurotrophic parameters. Thus, our aim was to investigate the antinociceptive and anti-inflammatory effects of IB-MECA, an A_3 adenosine receptor agonist, in a neuropathic pain model in rats.

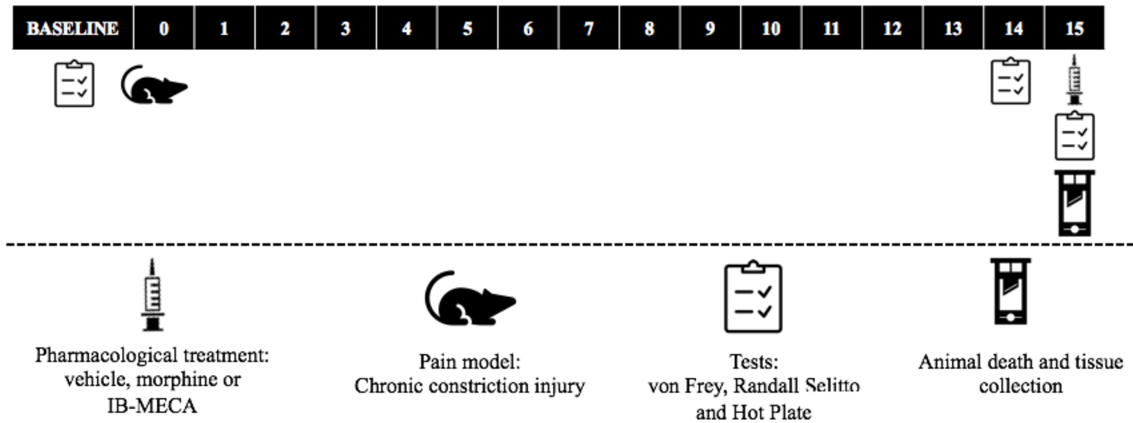
2 MATERIALS AND METHODS

2.1 Animals

A total of 72 adult male Wistar rats (55–65 days old; weight 200–250 g) were used. The number of animals was calculated as 8 rats per group for behavioral and biochemical analysis, to determine a difference between the variables of 1.5 of standard deviation and α= 0.05 [11–13]. The animals were housed in groups of three per polypropylene cage (49 cm x 34 cm x 16 cm) with sawdust-covered flooring in a controlled environment (22±2°C) under a standard light-dark cycle (lights-on at 7 a.m. and lights-off at 7 p.m.), with water and chow (Nuvital, Porto Alegre, Brazil) *ad libitum.* All experiments and procedures were approved by the institutional Animal Care and Use Committee (GPPG-HCPA protocol no.2018-0377) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th ed. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines [14].

2.2 Experimental design

The rats were acclimated to the maintenance room for two weeks before the experiment began. Rats were divided into nine groups: control (CT); sham (S); sham + vehicle (SV); sham + morphine (SM); sham + IB-MECA treatment (ST); pain (P); pain + vehicle pain (PV); pain + morphine (PM) and pain + IB-MECA treatment (PT). Rats were randomized by weight and by paw withdrawal latency measured through Hot Plate test to ensure that all rats have similar nociceptive behavior. Randall Selitto, von Frey and Hot plate tests were performed at baseline, 14 days after the chronic constriction injury, and 30 minutes after treatment with one dose of vehicle, morphine or IB-MECA. Hot plate test was repeated 60 and 90 minutes after treatment. The rats were killed by 6 hours after the treatment; spinal cord, cerebral cortex, hippocampus and brainstem were collected for after analyses of Brain Derived Neurotrophic Factor (BDNF), Neuronal Growth Factor (NGF) and Interleukin1β (IL-1β). The experimenter was blinded to group and treatment of rats being tested for all procedures including nociceptive tests and



neurochemical assays (Figure 1).

Figure 1. Experimental design

2.3 Neuropathic pain model

The model for the induction of neuropathic pain used was the chronic constriction injury (CCI) of sciatic nerve described by Bennett & Xie (1988) [15] and adapted by Cioato et al. (2015) [16]. Rats were anesthetized with isoflurane (5% for induction, 2.5% maintenance) and placed in dorsal position for the trichotomy of left thigh and skin antisepsis with 2 % iodine alcohol. After skin incision of the left hind limb, in the middle third of the thigh to expose the femoral biceps muscle, the common sciatic nerve was exposed. Three ligatures were tied (Vycril 4.0) separated by an interval of 1mm. The length of nerve affected was approximately 5.0 mm and the ligatures reduced the diameter of the nerve, but did not interrupt the epineural circulation. The same investigator performed the ligatures in all rats to ensure equal level of constriction. The skin was sutured using Mononylon 4.0 thread. The sham groups were submitted to anesthesia and the sciatic nerve was exposed similarly to CCI model, but it was not ligated. After surgery and anesthetic recovery, the animals were allowed in their home cages where they will remain until the day of death. The control group did not undergo anesthesia and surgical procedure.

2.4 Pharmacological treatment

The acute treatment protocol consisted in one dose of 0.5 µmol/kg i.p. [9] of a selective agonist of the adenosine A_3 receptor N6- (3-iodobenzyl) adenosine-5'-methyluronamide (IB-MECA)[17] dissolved in 3% Dimethyl sulfoxide (DMSO) and applied by intraperitoneal (i.p.) injection. The animals of vehicle groups received one dose of DMSO 3% in saline solution intraperitoneally. The positive control group received as gold standard of analgesia one dose of morphine 5mg/kg i.p. [18].

2.5 Hot Plate test

The hot plate (HP) test was used to confirm the effectiveness neuropathic pain model and to assess the effects of IB-MECA treatment on the thermal nociceptive threshold [19]. This test was carried out at baseline, 10 and 14 days after chronic constriction injury as well as at 30, 60 and 90 minutes after the IB-MECA administration. The hot plate test is used to determine changes in latency of behaviors, such as jumping and hind paw-licking, as indicator of modulation of the supraspinal pain process [20], considered results of supraspinal sensory integration [21,22]. All rats were adapted to the HP apparatus 24 hours prior to testing for 5 minutes, in order to avoid the analgesia induced by the novelty [23]. As described previously by Cioato (2015) [16] rats were placed inside glass funnel on the heated surface at a constant temperature of 55±0.1°C and the time in seconds between placement of the rat and the first response (foot-licking, jumping, or rapidly removing paws) was recorded as the latency of nociceptive response. The cut-off time was 20 seconds to prevent tissue damage.

2.6 Randall Selitto test

The rats were subjected to mechanical stimuli in paw withdrawal test described by Randall and Selitto (1947) [24]. This test was assessed at baseline, 14 days after chronic constriction injury, and 30 minutes after the IB-MECA treatment. Analgesymeter, which gradually increases the pressure, was applied to the dorsal surface of the rat paw (type 7200, apparatus Ugo-Basile Biological Research, Comerio-Varese, Italy). All rats were adapted to the HP apparatus 24 hours prior to testing for 5 minutes, in order to avoid the analgesia induced by the novelty [23]. The force in grams that produces the withdraw of hind paw was defined as the nociceptive threshold. A cut-off value was used for 100g to avoid damage to the paw.

2.6 Von Frey Test

The von Frey aesthesiometer (Insight, São Paulo, Brazil) is an adaptation of the classical von Frey filaments test and we used similar methodology as described before by Cioato et al. (2015)[16]. This test was assessed at baseline, 14 days after the chronic constriction injury, and 30 minutes after the IB-MECA treatment. Test was performed in polypropylene cages (12cm × 20cm × 17cm) with wire grid flooring and rats were habituated to cages for 10 minutes 24 hours prior to test and 5 minutes before test to prevent analgesia induced by apparatus novelty. For testing, a polypropylene tip was inserted perpendicularly from underneath the floor grid and applied to plantar side of right hind paw at gradually increasing pressure. The intensity of the stimulus supported up to paw withdrawal, in grams (s), was automatically recorded. Three successive readings were measured between interval periods of 5 s and averaged. The averages were used as the final measurements and the paw withdrawal threshold was expressed in grams (g) [25].

2.7 Tissue collection

Rats were killed by decapitation six hours after IB-MECA administration. The spinal cord, cerebral cortex, hippocampus and brainstem were collected and frozen at -80°C until the assays were performed.

2.8 Neurochemical assays

The spinal cord, cerebral cortex, hippocampus and brainstem IL-1β, BDNF and NGF levels were determined by sandwich ELISA using monoclonal antibodies specific for each cytokine and neurotrophin (R&D Systems, Minneapolis, United States) using manufacturer's protocol. Optical density was measured using an ELISA reader at wavelength of 450 nm. Total

protein was measured by the Bradford method using bovine serum albumin as the standard [26]. The data were expressed in pg/mg of protein.

2.9 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) 20.0 for Windows was used for the statistical analysis. Data were expressed as the mean ± standard error of the mean (S.E.M.). *P*-values of less than 0.05 were considered significant. Generalized Estimated Equations followed by the Bonferroni test was used to compare nociceptive tests data across groups to confirm the establishment of pain model before the treatment and to confirm the effects of treatment. One-way ANOVA test followed by Bonferroni was used to compare the neurochemical data across groups after treatment.

3 RESULTS

3.1 Effect of single administration of IB-MECA in the hyperalgesia thermal and mechanical induced by neuropathic chronic pain model in rats

To confirm the establishment of neuropathic pain model, the nociceptive response was evaluated using GEE analysis. There was not difference between groups at the baseline time from thermal and mechanical latency withdrawal in the Hot Plate, Randall Selitto and von Frey tests; however at the fourth day after the CCI model these animals presented decreased latency withdrawal in all three tests performed (Figures 2, 3 and 4).

In relation to thermal hyperalgesia, our results shown that IB-MECA was able to reverse partially the decrease in latency to nociceptive response induced neuropathic pain conditions 30 minutes after injection; and this effect remains until 60 and 90 minutes after the administration. We also shown that morphine was able to induce analgesia in sham and pain groups and its effects were observed 30 and 60 minutes after administration (Wald $\chi 2= 730,64$) (Figure 2).

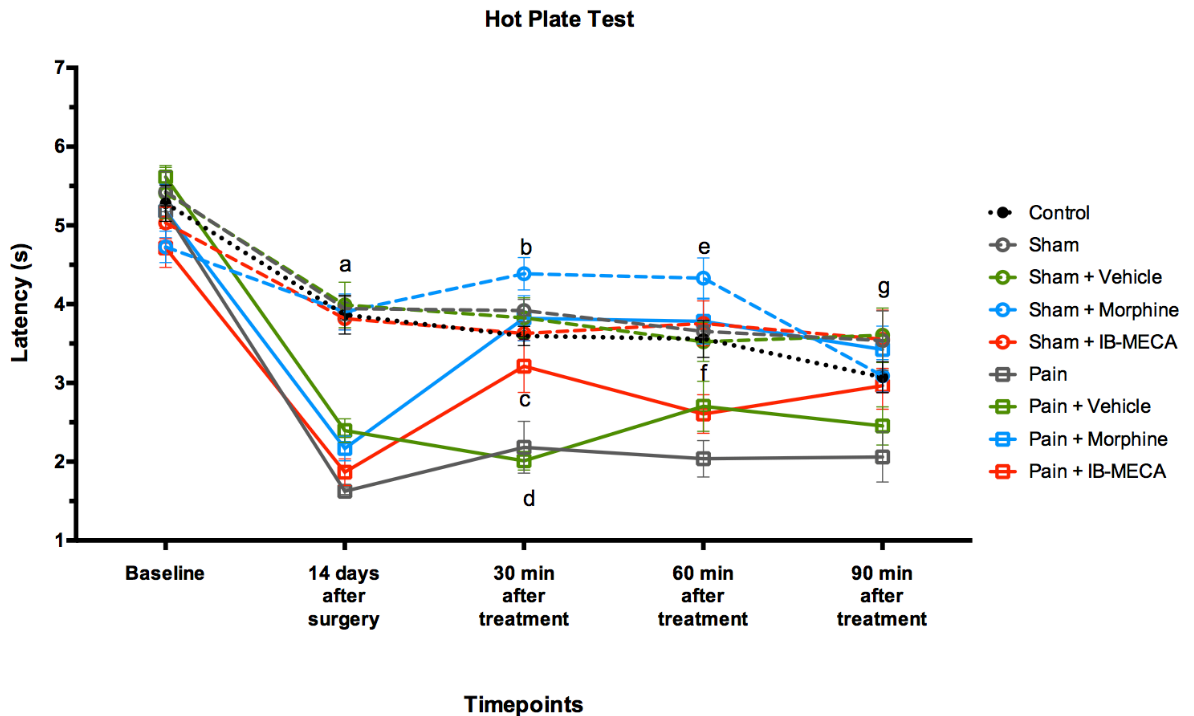


Figure 2. Thermal hyperalgesia by Hot Plate test: basal measure, 14 days after CCI model and 30, 60 and 90 minutes after pharmacological treatment (n= 7-8 per group). Data presented as mean±S.E.M of latency in seconds (s).
[a]significant difference between: Control, Sham, Sham + Vehicle, Sham + Morphine groups from Sham + IB-MECA; Pain, Pain + Vehicle, Pain + Morphine groups and Pain + IB-MECA group (GEE,$P<0.05$).
[b]significant difference between: Sham group + Morphine and Control group; Sham, Sham+ Vehicle, Sham + IB-MECA, Pain, Pain + Vehicle groups from Pain + IB-MECA group (GEE, $P<0.05$).
[c]significant difference of Pain + IB-MECA group from other groups (GEE, $P<0.05$).
d. significant difference of Pain and Pain + Vehicle from other groups (GEE, $P<0.05$).
[e]significant difference between: Sham + Morphine and Sham groups from Sham + Vehicle, Sham + IB-MECA, Pain, Pain + Vehicle and Pain + IB-MECA groups (GEE, $P<0.05$).
[f]significant difference between Pain, Pain + Vehicle and Pain + IB-MECA groups from other groups (GEE, $P<0.05$).
[g]significant difference between Pain and Pain + Vehicle groups from other groups (GEE, $P<0.05$).

When it was analyzed the mechanical hyperalgesia in Randall Selitto test was observed that IB-MECA was able to reverse completely the nociceptive behavior induced by chronic constriction of sciatic nerve 30 minutes after the its administration. Also, as expected, the sham group that received morphine showed an analgesic response in Randall Selitto test in comparison with the other groups, including the pain group treated with morphine (Wald $\chi 2$= 193,656) (Figure 3). A similar result was found when we analyzed the von Frey test. However,

in this text, the IB-MECA partially reversed the hyperalgesia induced by CCI when compared with pain groups untreated or treated with vehicle (Wald $\chi2= 356,832$) (Figure 4)

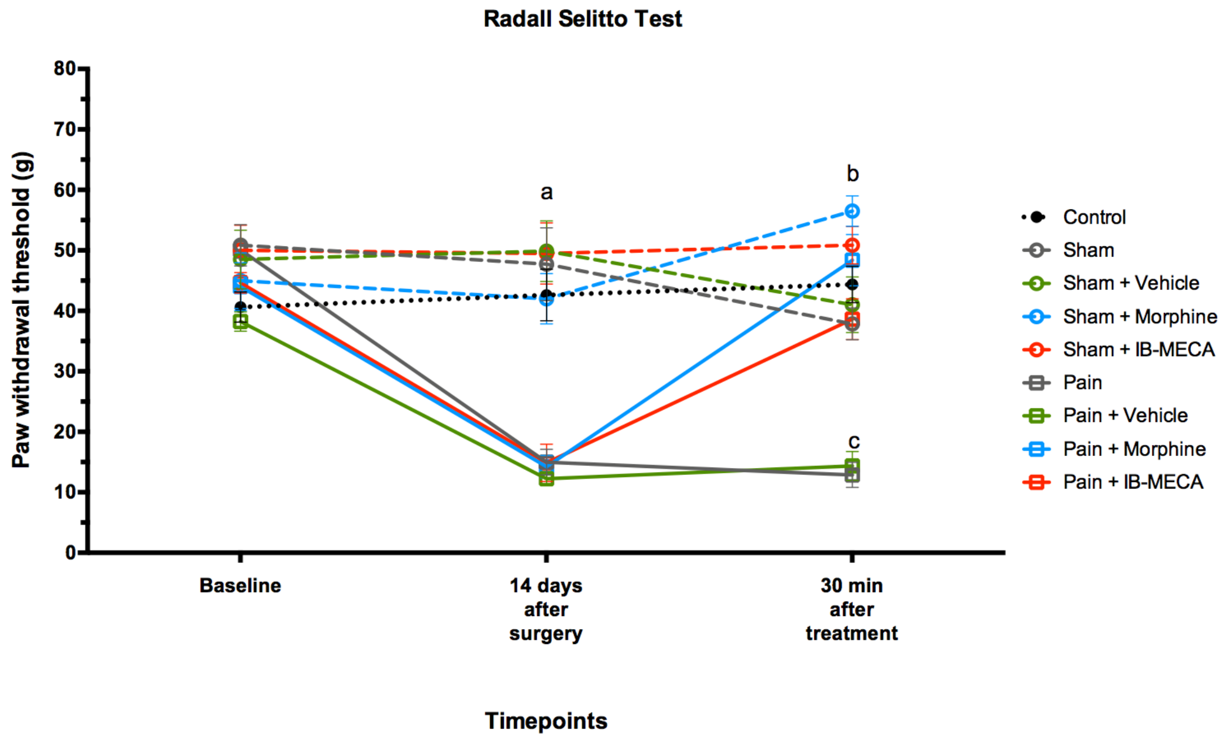


Figure 3. Mechanical hyperalgesia by Randall Selitto test: basal measure, 14 days after CCI model and 30 minutes after pharmacological treatment (n= 7-8 per group). Data presented as mean±S.E.M of paw withdrawal threshold in grams (g).

[a] significant difference between Control, Sham, Sham + Vehicle, Sham + Morphine and Sham + IB-MECA from Pain, Pain + Vehicle, Pain + Morphine and Pain + IB-MECA groups (GEE, $P<0.05$).

[b]significant difference between Sham + Morphine and Control, Sham, Sham+Vehicle, Pain + IB-MECA groups from Pain, Pain + Vehicle groups (GEE, $P<0.05$).

[c]significant difference between Pain and Pain + Vehicle from other groups (GEE, $P<0.05$).

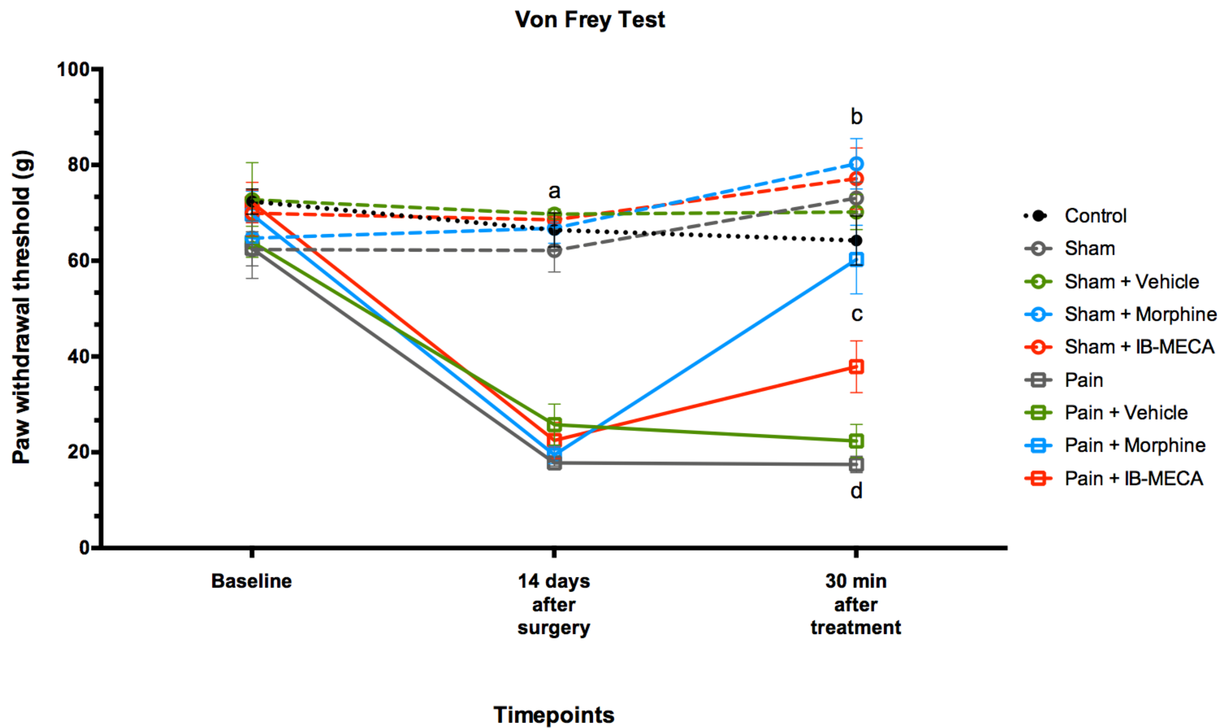


Figure 4. Mechanical hyperalgesia by von Frey test: basal measure, 14 days after CCI model and 30 minutes after pharmacological treatment (n= 7-8 per group). Data presented as mean±S.E.M of paw withdrawal threshold in grams (g).
[a]significant difference between Control, Sham, Sham + Vehicle, Sham + Morphine and Sham + IB-MECA groups from Pain, Pain + Vehicle, Pain + Morphine and Pain + IB-MECA groups (GEE, $P<0.05$).
[b]significant difference between Sham + Morphine, Pain, Pain + Vehicle, Pain + Morphine groups from Pain + IB-MECA group (GEE, $P<0.05$).
[c]significant difference between Pain + IB-MECA from other groups (GEE, $P<0.05$).
[d]significant difference between Pain and Pain + Vehicle from other groups (GEE, $P<0.05$).

3.3 Effect of single administration of IB-MECA on biomarkers levels in cerebral cortex, brainstem, hippocampus and spinal cord of rats subjected to neuropathic chronic pain model

There was no difference in the IL-1β brainstem and spinal cord levels in (one-way ANOVA, $F_{(8,62)}= 1.245$ and $F_{(8,62)}= 1.215$ respectively, $P>0.05$) however we observed an effect of vehicle administration in the cerebral cortex and hippocampus in the its levels (one-way ANOVA, $F_{(8,62)}= 2.692$ and $F_{(8,62)}= 3.182$ respectively, $P<0.05$). Is interesting to note that the effect in these structures is inverse: there is a decrease of IL-1β cerebral cortex levels, while there is an increase in the IL-1β hippocampus levels (Figure 5).

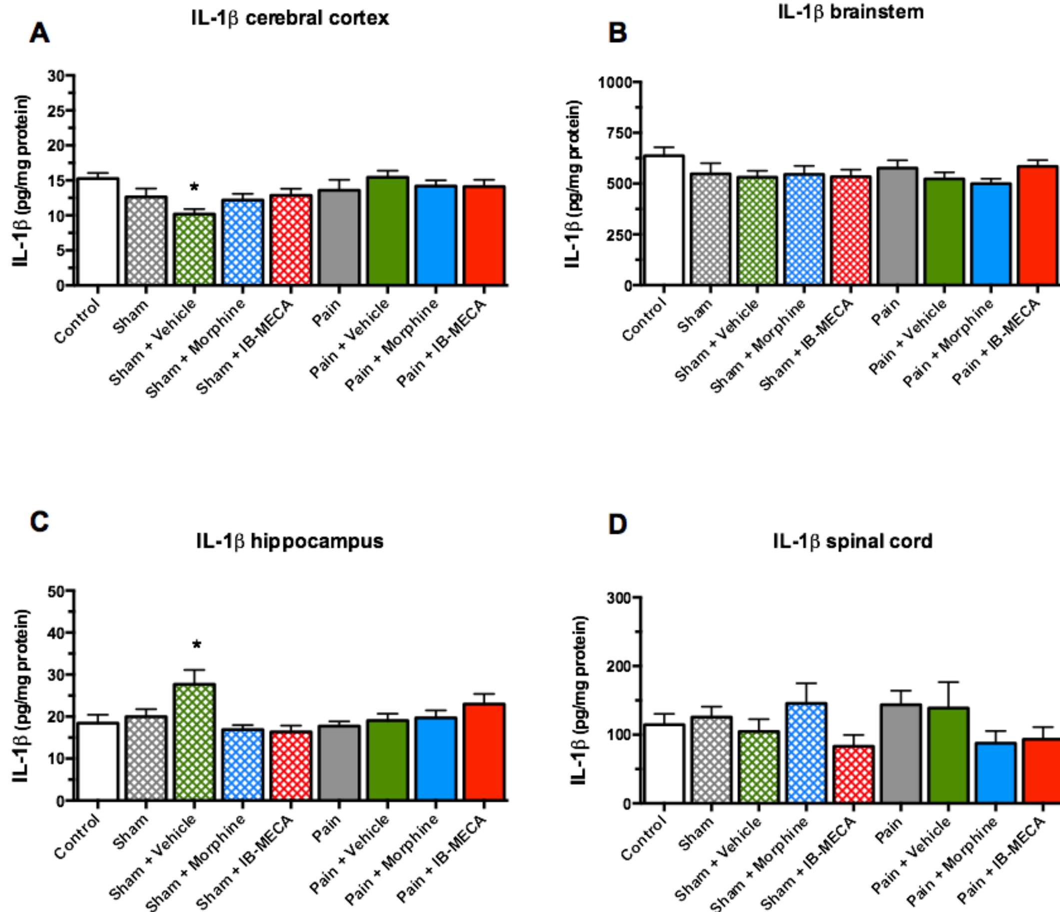


Figure 5. Effect of single administration of IB-MECA on IL-1β levels in cerebral cortex, brainstem, hippocampus and spinal cord of rats subjected to neuropathic pain model.
Panel A. IL-1β levels in cerebral cortex. * significant difference between Sham + Vehicle from Control and Pain + Vehicle groups (one way ANOVA/ Bonferroni, $P<0.05$).
Panel B. IL-1β levels in brainstem. There was not significant difference between groups (one way ANOVA/Bonferroni, $P>0.05$).
Panel C. IL-1β levels in hippocampus. * significant difference between Sham + Vehicle from all other groups (one way ANOVA/Bonferroni, $P<0.05$).
Panel D. IL-1β levels in spinal cord. There was not significant difference between groups (one way ANOVA/Bonferroni, $P>0.05$).

In relation to the BDNF levels, there was no difference in cerebral cortex and spinal cord (one-way ANOVA, $F_{(8,62)}$= 1.744 and $F_{(8,62)}$= 0.647 respectively, $P>0.05$). In the brainstem, it was observed an increased level of this neurotrophin in the sham group treated

with IB-MECA and pain group and pain groups treated with vehicle and morphine when compared with sham group (one-way ANOVA, $F_{(8,62)}$= 2.060, P<0.05). The sham group treated with vehicle presented an increase in the BDNF hippocampus levels compared to control group (one-way ANOVA, $F_{(8,62)}$= 2.098, P<0.05) (Figure 6).

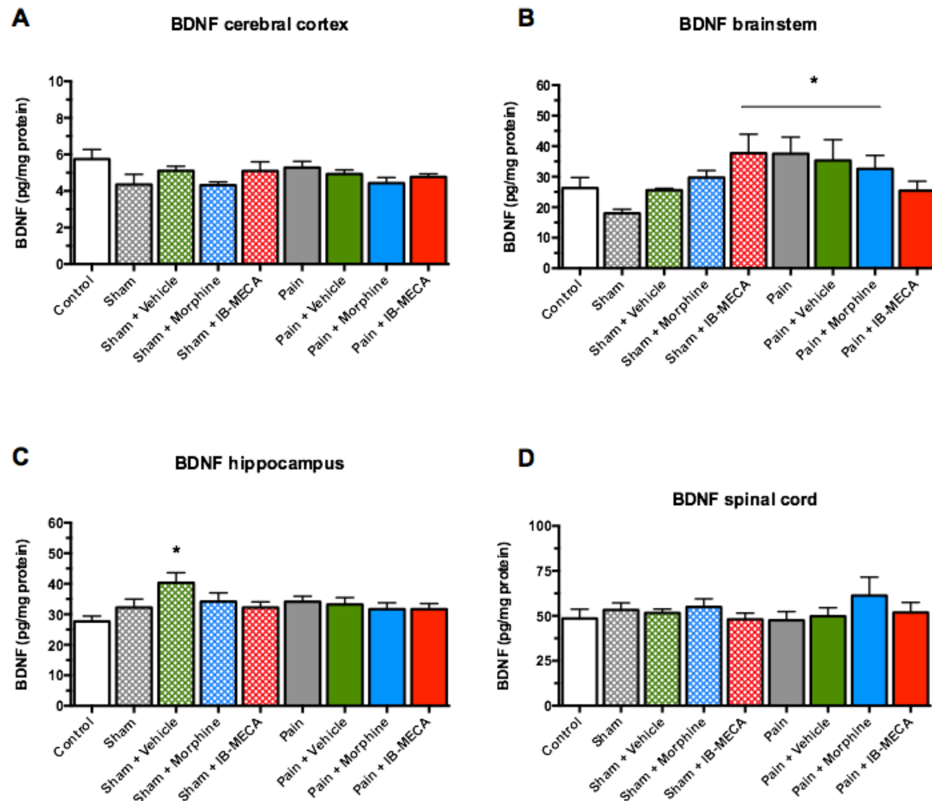


Figure 6. Effect of single administration of IB-MECA on BDNF levels in cerebral cortex, brainstem, hippocampus and spinal cord of rats subjected to neuropathic pain model.
Panel A. BDNF levels in cerebral cortex. There was not significant difference between groups (one way ANOVA/Bonferroni, P>0.05).
Panel B. BDNF levels in brainstem. * significant difference between Sham + IB-MECA, Pain, Pain + Vehicle and Pain + Morphine from Sham (one way ANOVA/Bonferroni, P<0.05).
Panel C. BDNF levels in hippocampus. * significant difference between Sham + Vehicle from Control group (one way ANOVA/Bonferroni, P<0.05).
Panel D. BDNF levels in spinal cord. There was not significant difference between groups (one way ANOVA/Bonferroni, P>0.05).

We did not observe any statistical difference in NGF brainstem or spinal cord levels (one-way ANOVA, $F_{(8,62)}$= 1.458 and $F_{(8,62)}$= 1.045 respectively, P>0.05). Our results showed an effect of IB-MECA in the sham group when compared to the control group in cerebral cortex

levels (one-way ANOVA, $F_{(8,62)}= 2.100$, $P<0.05$). And in the hippocampus NGF levels, it was observed that the sham group treated with vehicle presented increased levels of this neurotrophin when compared to the sham group without any pharmacological treatment (one-way ANOVA, $F_{(8,62)}= 2.392$, $P<0.05$) (Figure 7).

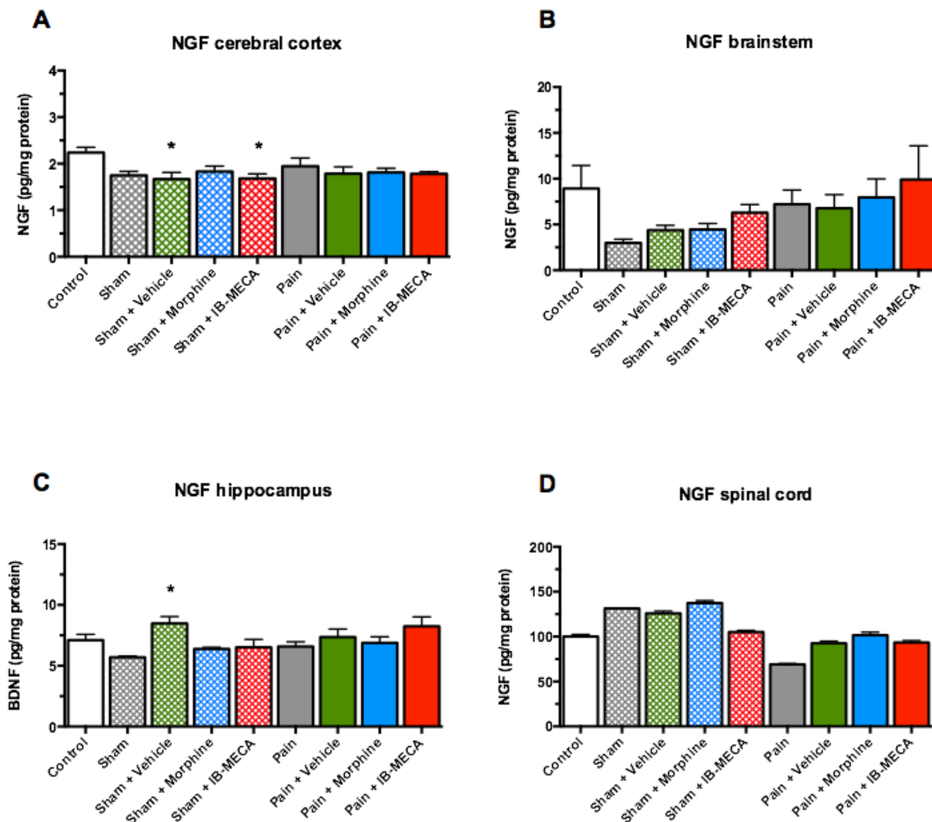


Figure 7. Effect of single administration of IB-MECA on NGF levels in cerebral cortex, brainstem, hippocampus and spinal cord of rats subjected to neuropathic pain model.
Panel A. NGF levels in cerebral cortex (one way ANOVA/Bonferroni, P>0.05). * significant difference between Sham + Vehicle and Sham + IB-MECA from Control
Panel B. NGF levels in brainstem. There was not significant difference between groups (one way ANOVA/Bonferroni, *P*>0.05).
Panel C. NGF levels in hippocampus (one way ANOVA/Bonferroni, P<0.05). * significant difference between Sham + Vehicle from Sham group (one way ANOVA/ Bonferroni, P<0.05).
Panel D. NGF levels in spinal cord. There was not significant difference between groups (one way ANOVA/Bonferroni, *P*>0.05).

DISCUSSION

The behavior results obtained in this study have shown that IB-MECA adenosinergic A_3 agonist receptor presented analgesic effect upon the hypernociceptive behavior induced by CCI surgery; however this effect was not linked to central levels of neurotrophins or cytokines, at least, those were measured (BDNF, NGF and IL-1beta). It is interesting to note that the intensity of analgesic effect observed by A_3 agonist receptor is dependent of the test applied, i.e., IB-MECA totally reversed the mechanical hypernociceptive behavior in the Randal Selitto test at 30 min after its administration; and partially reversed in the Von Frey test. In the same way, IB-MECA was effective in partially relieving the thermal hypernociceptive behavior measured in the Hot Plate test at 30 min after its administration.

It is known that the neuropathic pain is usually characterized by allodynia and hyperalgesia [27]; and CCI surgery represents a reproducible pain model to study neuropathic pain, once the rats presented thermal and mechanical hyperalgesia at fourteenth day after surgery in the current study and in our previous studies [28,29]. Some authors describe that the mechanisms of CCI neuropathic pain involves gene expression of a family of pro-inflammatory cytokines, like IL-1beta, IL-6, and TNF; and a delayed expression of IL-10 seems to be involved with resolution of hyperalgesia [30]. Our previous studies found a relationship between central levels of IL-1beta and IL-10 [28], BDNF [29] and neuropathic pain after CCI surgery. Is suggested the role of P2X4R in neuropathic pain development, with up-regulation of these receptors, and its interaction with neuroimmune biomarkers [31]. Thus, considering the complexity frame linked to neuropathic pain, it is important to investigate new therapies for this condition.

In this context, adenosine receptors comprise four distinct extracellular G-protein coupled receptors, known A_1R, A_2AR, A_2BR, A_3R [32] The A1R was clearly involved with analgesic response obtained using agonists' compounds [33] otherwise some studies also have

reported an antinociceptive profile for A2ARs [34]. A2BR antagonists' drugs are being investigated as potential therapeutics for treating immune and inflammatory disorders [35,36]. And previous studies described the involvement of A_3R in a pronociceptive response [37]. Thus, efforts were made to comprehend the involvement of A_3R in the nociceptive transmission or modulation. In this way, IB-MECA has been studied as a potential pharmacological treatment for neuropathic pain [38–43]. IB-MECA has presented to be a more a selective agonist for A_3 adenosine receptor rather than for A1 and A2 adenosine receptors [44]. Different mechanisms have been proposed for IB-MECA; for example, it attenuates neuropathic pain by suppressing microglial activation in tibial nerve injury [43]. IB-MECA alleviated mechanical hyperalgesia and thermal hypoalgesia in mice with diabetes induced by streptozocin injection by inhibition of the activation of nuclear factor-κB, decreasing the generation of tumor necrosis factor-α [45]. In addition, IB-MECA was able to prevent the establishment of neuropathic pain induced by CCI in rodents [41]. In our current study, IB-MECA relieves hypernociceptive behavior in rats with neuropathic pain, however the exact mechanism is still not clear. The antinociceptive effect linked to A_3R presented supraspinal and spinal components [34].

The analgesic effect of IB-MECA may be linked to A_3 receptors expressions, but its effects in inflammation are not clear. A_3 receptor agonists effects have been studied through its modulation of spinal neuroinflammatory processes, as IB-MECA treatment showed by reduction of hyperactive astrocytes and decrease in pro-inflammatory/neuroexcitatory cytokines levels in models of neuopathic pain induced by chemotherapy [39]. Previous studies have suggested that the A_3R is expressed in astrocytes and microglia [10], and it is known that these cells are closed involved with characteristics of initiation or perpetuation of neuropathic pain [43]. In this context, we have suggested that the analgesic effect of IB-MECA found in the hypernociceptive behavior of rats may be has the role of interleukins and neurotrophins.

It is well reported that BDNF acts in pain by modulation at spinal and supraspinal levels through the fast excitatory and inhibitory signals mediated by glutamatergic and GABAergic systems, respectively [46]. And, study of Coull and colleague [47] showed the crucial involvement of BDNF as signaling molecule between microglia and neurons and the blockade of this pathway can be a new strategy to treatment of neuropathic pain. Our results showed that the CCI model increase the BDNF levels in the hippocampus and the IB-MECA treatment was able to reduce these levels. In relation to IB-MECA effects in this signaling pathway, studies have shown that the enhanced of convergent inputs was correlated to inhibitory effects on nerve injury-induced microglial activation and behavioral responses. Also, the activation of A_3 receptor could attenuate the microglial activation which involves a decreasing in BDNF release and contribute to restoration of GABA signaling in the spinal dorsal horn [43]. This theory can explain the decreased levels of BDNF induced by IB-MECA found in this work, however it is necessary to access the GABA levels and other markers involved in this signaling cascade to confirm this hypothesis.

Also, our biochemical data showed the vehicle used to IB-MECA can alter NGF and IL-1β levels in the cerebral cortex and hippocampus with the same pattern in both structures: increased levels in the first one and decreased in the second in the sham groups. It is interesting to note that these alterations were state dependent, once that was observed just in the sham groups, not in the animals subjected to CCI model. These results can be explained by the potential effects of DMSO in biochemical pathways involved in the inflammation, including in naïve animals, despite of its effects depends of the administration route i.e. orally, in the site of injury, intraperitoneally [48]. And, considering that these alterations were observed just in the sham groups, we can suggest that the concentration used in this study (3% to dilute the IB-MECA) can not promote any effect in the inflammation process induced by CCI model, discarding bias induced by vehicle in pain groups.

Unfortunately, our current study presents some limitations. First, we have tested the analgesic effect of acute administration of IB-MECA in neuropathic pain, however we have considered this as first step before repeated administrations. Second, we have discarded hormonal interference, because our results are from only male adult rats. Third, it is possible to observe that rats presented an indicative of habituation in the Hot Plates test, once rats from control group presented a decreased latency along the time assessment [49].

In summary, IB-MECA represents a potential therapeutic target in neuropathic pain condition; once it was able to relief the hypernociceptive behavior induced by CCI model in rats. Here, we failed to show that the analgesic effect of IB-MECA acts through neurotrophins, at least, like BDNF and NGF, or IL-1beta, in central nervous structures as cerebral cortex, brainstem, hippocampus and spinal cord. However, the exact action mechanism of IB-MECA needs to be investigated, thus we extremely encouraged further findings using IB-MECA agonist.

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MANUSCRITO 3

Characterization of G-coupled receptor - β-arrestin2 recruitment using NanoBiT luciferase complementation

Periódico: Current Pharmacology Protocols

Status: *a ser submetido*

Characterization of G-coupled receptor - β-arrestin2 recruitment using NanoBiT luciferase complementation

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Conflict of Interest: All authors declared no financial or commercial interests in the outcome of the study.

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Abstract

Background: New technologies came to increase knowledge in the pharmacology field, mainly, linked to signalling and biased agonism. It is known that G protein coupled receptors (GPCRs) are the main target from the pharmacological treatments available nowadays. And, these receptors trigger different intracellular effectors, for example G proteins and β-arrestins, corroborating the biased agonism response. Thus, the main objective this study was to test the new technology using luciferase complementation (NanoBiT) assay to measure recruitment of β-arrestin2 to the A_3 adenosine receptor (A_3AR) and the gastrin-releasing peptide receptor (GRPR) by agonist and antagonist compounds.

Methods: HEK293T cells were transiently transfected (lipofectamine) with SNAP-tagged A_3AR or SNAP-tagged GRPR with C terminals fused to the large luciferase fragment (LgBiT) and β-arrestin2 with a N-terminal fused to small fragment (Sm114 or Sm86). The assays were performed 24h later. Briefly, for agonist assay, 5min furimazine substrate pre-incubation in HBSS/0.1% BSA at 37°C, followed by agonist stimulation; for antagonist assay, 10min antagonist preincubation in HBSS/0.1% BSA at 37°C was used before furimazine substrate addition followed by agonist stimulation. Luminescence was measured 30 min after agonist stimulation using Pherastar2 at 37°C. Concentration response curves were fitted in GraphPad Prism for apparent pEC50 values, as mean±s.e.m (n=8).

Results: HEMADO, IB-MECA and NECA showed luminescence response linked to interaction between A_3AR and the β-arrrestin2 in the NanoBiT assay with peak at 3 min, and a sustained recruitment component at 30min.

Discussion: The NanoBiT complementation technology allows us to measure real time interaction between agonist and its receptor, and the recruitment of β-arrestin2.

Keywords: GPCR, β-arrestin2, HEK cells, luminescence

Introduction

The G coupled-protein receptors (GPCRs), the major targets of pharmacological drugs, are involved with many physiological processes (Sriram & Insel, 2018). They are characterized by seven transmembrane domains (7TMDs)(Luo et al., 1994); with N-terminal and C-terminal localized extracellular and intracellular for A_3 receptor, respectively (Gaspar et al., 2011). It is a large family of receptors linked to G protein which is classified into four families, Gs, Gi/o, Gq/11, and G12/13 (Wettschureck et al., 2004), and it activates different second messengers, for example, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), inositol trisphosphate (IP3), diacylglycerol (DAG).

In the drug discovery field the role of the β-arrestins, important for intracellular signaling, has been investigated. These proteins are involved with the desensitization of GPCRs, the control of GPCR intracellular trafficking, and the activation of GPCRs to multiple signalling pathways (Lefkowitz & Shenoy, 2005; Gurevich & Gurevich, 2006; Moore et al., 2007; Tian et al., 2014). They are characterized by two isoforms, β-arrestin1 and β-arrestin2 (also denoted as arrestin-2 and arrestin-3, respectively)(Attramadal et al., 1992). The discovery of β-arrestin recruitment highlights the selectivity of some agonists to activate or block different pathway signaling. This phenomenon is known as biased-agonism, which is the ability of certain agonists to signal through different pathways of a GPCR with different efficacies (Rajagopal et al., 2010).

In this way, many technologies are being developed to understand the interaction between agonist-receptor-intracellular signaling and conformational changes, which activates different intracellular cascades. Some studies have described that when the agonist binds the receptor there is a conformational change and this interaction is able to stimulate or inhibit an intracellular signal (Ghanouni et al., 2001; Han et al., 2005). One interesting technology is molecular docking method that means the computational reconstruction of actual ligand-

receptor or protein-protein interaction (Bartuzi et al., 2017). In addition, different approaches regarding probes for GPCRs signaling and protein-protein interactions have been developed. Among them, we highlight designer receptors exclusively activated by designer drug (DREADDs)(Wess et al., 2013), Förster Resonance Energy Transfer (FRET) (Shrestha et al. 2015), bioluminescence resonance energy transfer (BRET) (Marullo & Bouvier, 2007), Fluo4, a new fluorescent dye for quantifying cellular Ca^{2+} concentration (Gee et al., 2000).

Taken all together, the main objective was to test the new technology using luciferase complementation (NanoBiT) assay to measure recruitment of β-arrestin2 to the A_3 adenosine receptor (A_3AR) by their interactions with agonist drugs.

Methodology

a. Technology: the technology tested is based on protein-protein interaction using a luminescence signal, and the main focus is to test interaction between two proteins, the first one (A) is fused to LargeBit (LgBit), and the second one (B) is fused to SmallBit (SmBit), and both expressed in cells. Interaction between protein A and B generates a luminescent signal detected by Pherastar FS (Figure 1).

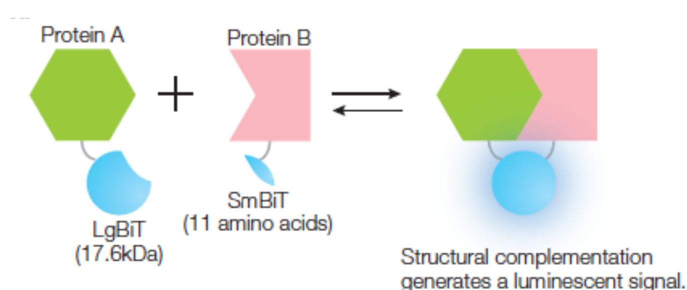

Figure 1. Structural design for protein-protein interaction using NanoBit system (source: www.promega.com/protocols/).

b. Materials: The expression vector pcDNA3.1 and restriction enzymes (EcoRI and XhoI) were from Thermo Fisher Scientific. The custom primers, Lipofectamine was from Invitrogen (Invitrogen). QuickStrange site-directed mutagenesis kit was purchased from Stratgene. Human

embryonic cells 293T (HEK) were from laboratory. HEMADO [2-(1-hexynyl)-N-methyladenosine], NECA [5'-N-Ethylcarboxamidoadenosine], IB-MECA [N6-(3-iodobenzyl)-adenosine-5¢-N-methylcarboxamide] were purchased from Tocris Bioscence (UK). All drugs were stored in experiment aliquots at -20°C. Other consumables were obtained from Sigma-Aldrich (UK).

c. Molecular Biology: SNAP-tagged A_3AR human receptor cDNA were inserted between restrictions sites EcoRI (3') and XhoI (5') in pcDNA^{TM}3.1 vector (Thermo Fisher Scientific). The A_3R cDNA was fused at the C terminus to the LgBiT Nanoluc fragment, with an intervening 5 GlySer linker. The 11 amino acid SmBiT114 peptide (MVSGYRLFEEIL) was used as the N terminal tag for human ß-arrestin2. Both cDNAs, in pcDNA3.1, were transiently co-transfected in HEK293T cells.

d. Cells culture: HEK293 cells were maintained in a humidified environment at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) added with 10% fetal serum bovine (FBS) and when confluent were trypsinized. SNAP-tagged human A_3 receptor cells were seed one day before the experiments in 96-well white plate coated with poly-D-lysine.

e. Transfection: Lipofectamine (Invitrogen) was used in accordance with manufactures' instructions.

f. Protocol assays:

Protocol 1:

Protocol steps of Luminescence Curve-Response Assay for agonist.

Cells: HEK A_3AR-LgBIT and Sm114-β-arrestin2 (A2) cells.

Materials: HEPES-buffered saline solution (HBSS), 0.1% bovine serum albumin (BSA, Sigma-Aldrich), NanoBit commercial kit (Promega), pipettes, microtubes, dry bath, vortex mixer, Pherastar

Reagents: concentration curve from 10^{-4}M until 10^{-11}M for HEMADO, IB-MECA and NECA.

For both assays, buffer sample was prepared using HBSS and 0.1% BSA warmed at 37°C. The concentration curve of agonist and antagonist were prepared using sample buffer, and all reagents were maintained at 37°C, except the Live Cell Substrate, prepared just before added.

Before starting the experiment:

1) Check the cells, it needs to be 70% confluent.
2) Make up enough HBSS (with glucose added) + 0.1% BSA for your experiment. A guide is 20 ml per plate.
3) Prepare fresh ligand dilutions according as suggested in Table 1.

Table 1. Example of dilution for agonists.

Concentration in compound plate	*Log [con] in assay*	*Made by*
Vehicle		*HBS/BSA*
100 µM	-4	333 µl of HBS + 20 µl 10^{-2}M
10 µM	-5	450 µl of HBS + 50 µl 10^{-4}M
1 µM	-6	450 µl of HBS + 50 µl 10^{-5}M
100 nM	-7	450 µl of HBS/BSA + 50 µl 10^{-6}M
10 nM	-8	450 µl of HBS + 50 µl 10^{-7}M
1 nM	-9	450 µl of HBS/BSA + 50 µl 10^{-8}M
0.1 nM	-10	450 µl of HBS/BSA + 50 µl 10^{-9}M
0.01 nM	-11	450 µl of HBS/BSA + 50 µl 10^{-10}M

4) Prepare the compound plate: 1 column with vehicle and 1 column with which agonist (120 µl per well).

5) Switch on the Pherastar and choose the right protocol and set up the temperature at 37°C.

Start the experiment:

1) Remove the medium from all wells.
2) Wash with 50 µl of HBS/BSA 0.1% and remove the buffer.
3) Add 40 µl of HBS/BSA 0.1% per well.

4) Prepare the substrate just before using: 10µL of Live Cell Substrate + 690µL HBS/BSA 0.1%. And add 10µL per well and read 3 times as baseline.

5) Take plate out from the Pherastar and immediately add 10 µl of vehicle, HEMADO, IB-MECA and NECA per well (multichannel pipette) according to plate design as suggested Figure 2.

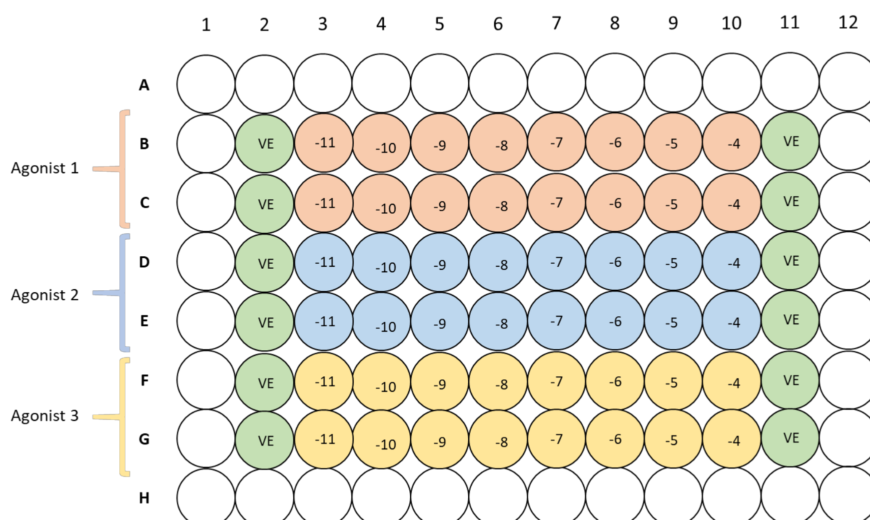

Figure 2. Example of plate design for luminesce assay, avoiding the borders.

6) Read at 30 minutes.

g. Statistical analysis: concentration response curves were fitted in GraphPad Prism for apparent pEC50 values, as mean±s.e.m (n=8). Data were normalized by 100nM HEMADO. Results were analyzed using one-way ANOVA followed by Student Newman Keuls (SNK) or Student t test, and P less than 0.05 was considered significant difference.

Results and Discussion

According to our results, the transient transfection of SNAP-tagged A_3AR and Sm114-β-arrestin2 worked well, once the time course curve presented in the Figure 3, it showed the peak of luminescence signal at 3 min (Figure 2), with a sustained recruitment component at 30 min (Figure 3) for all three agonists evaluated (HEMADO, IB-MECA and NECA). Important

to highlight that luminescence response is closely related to the interaction between A_3AR and β-arrestin2, in the current study the LgBIT was fused in the C-terminal of A_3AR, and the SmBIT 114 was fused in the N terminal tag for human ß-arrestin2. HEK293T cells were selected due rapid growth characteristics and high transient transfection efficiency. Additionally, this technology was chosen because the interaction between the human A_3AR and β-arrestin2 can be monitored in a kinetic way, and it can be used for different GPCRs and intracellular proteins signaling. It is based on splitted Nanoluc parts, called Large Bit (LgBit;18 kDa) and Small Bit (SmBit; 1 kDa), can be fused to two proteins of interest, making a reversible interaction possible. The Nanoluc luciferase is a small (19.1 kDa) enzyme, producing high intensity, glow-type luminescence by conversion of the coelenterazine-derived cell permeable substrate furimazine (Dixon et al., 2016).

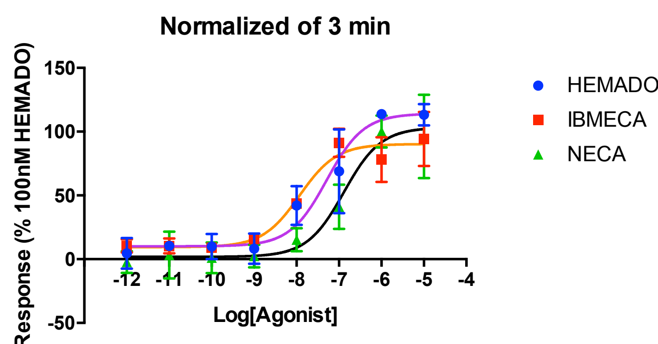

Figure 3. Graphic representative of normalized data measured at 3 min after agonist administration.

Recent studies have been exploring the A_3 adenosine receptor agonism and the biased allosteric modulation involved with the ligands and the profile of each one in terms of cAMP accumulation and β-arrestin recruitment, as well as concerning to their coupling to G-protein coupling dependent or independent pathways (Jacobson et. Al, 2006; Stott et al., 2016). Despite that, in the Figure 4 we have shown the pEC50 for all drugs, and no differences were found among them at 3min and at 29min, i.e., all three drugs presented similar potency in both times assessed, and the potency was not increased along time in comparison each drug at 3min and 29min (Figure 5 and Figure 6). Our results showed that all three agonists were able to link to

β-arrestin2 and this ligation is maintained during the 29 minutes that comprises the kinetic evaluation of A_3 receptor link to drugs.

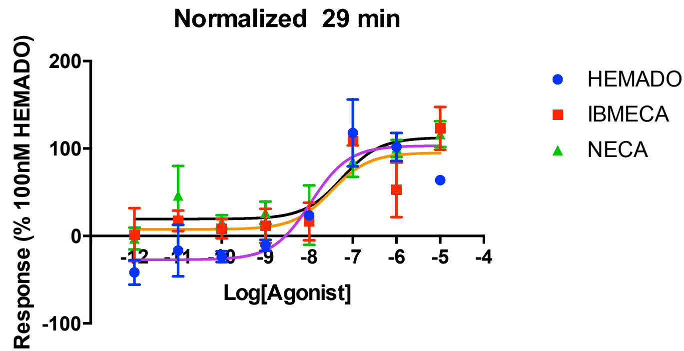

Figure 4. Graphic representative of normalized data measured at 29 min after agonist administration.

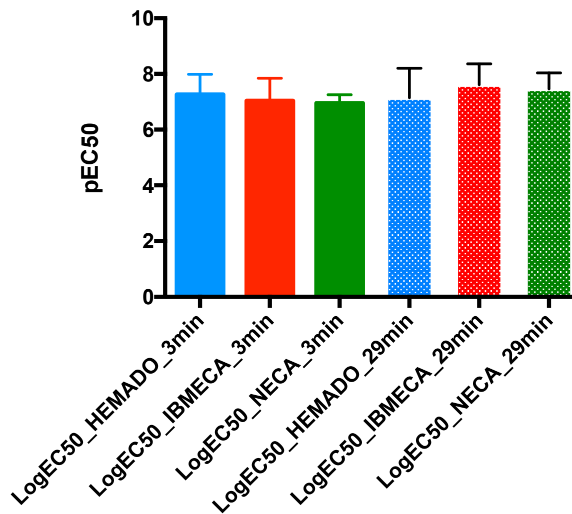

Figure 5. pEC50 from different adenosinergic agonists in HEK cells expressing A_3-LgBIT and Sm114-β-arrestin2 at 3 min and 29 min after agonist administration (no differences were found, one-way ANOVA, $P>0.05$; $n=8$).

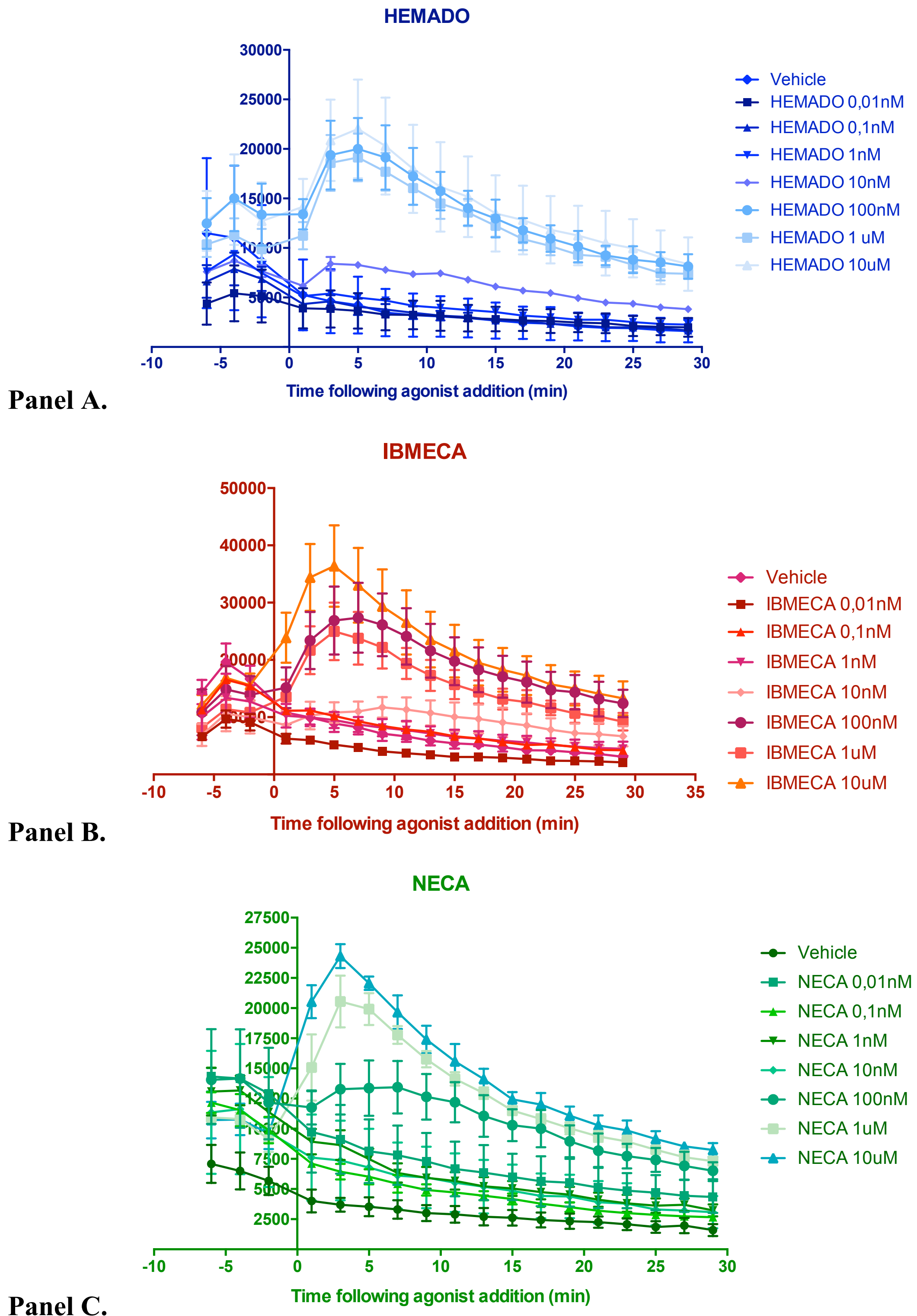

Figure 6. Time course curve measured during 30 min after agonist administration for HEMADO (Panel A), IB-MECA (Panel B) and NECA (Panel C).

Also, an important action of arrestin is the ability to start a downstream G protein-independent signaling pathway, with β-arrestin2 acting as a regulator of GPCR activation.

However, the mechanism linking agonist-induced GPCR conformation to the coupling and activation of β-arrestin2 still unknown. This way, is assumed that β-arrestin2 recruitment is induced by phosphorylation of serine/threonine residues thought G-protein kinases or second messengers after the agonist link to the receptor (Storme et al 2018a, Storme et al, 2018b).

In conclusion, we have tested NanoBit technology using A_3AR receptor and β-arrestin2 and this technique seems to be useful to evaluate the molecular behaviour of GPCR, especially of the adenosine receptor. However, more assays were necessary to elucidate the precise intracellular pathway unleashed by the activation of A_3 receptor induced by IB-MECA, i.e. internalization and calcium mobilization assay. Another suggestion is the modification of the A_3 receptor using molecular biology to enlarge the knowledge about the ligand mechanisms and improve the development of new agonists, considering the therapeutic potential of this receptor.

Acknowledgements

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VII. DISCUSSÃO

7. DISCUSSÃO

Ao longo dos dois últimos séculos, a ciência tem se dedicado ao estudo da dor e da compreensão dos seus mecanismos periféricos e centrais. Especialmente nos últimos anos, surgiram evidências de que muitos receptores e vias estão envolvidos nesse processamento e que a busca por novas terapêuticas abrange diversos sistemas e moléculas sinalizadoras (1). Entretanto, apesar de toda a expansão no conhecimento na fisiopatologia da dor, como dito anteriormente, a dor crônica ainda dispõe de poucas opções terapêuticas e, considerando disponíveis, ainda existem muitos efeitos adversos relacionados à elas, motivando a busca por novas opções farmacológicas (2).

Esta tese, utilizando dois modelos diferentes de dor crônica bem estabelecidos na literatura e no nosso grupo de pesquisa – dor crônica inflamatória e dor crônica neuropática – demonstrou que uma única administração de agonista de receptor de adenosina A_3, o IB-MECA, é capaz de alterar o comportamento hiperalgésico térmico e mecânico induzidos por esses modelos de dor. Do ponto de vista bioquímico e molecular, pode-se elucidar alguns mecanismos neuroimunomodulatorios envolvidos na cascata de sinalização do receptor A_3 nestes modelos de dor. Além disso, por meio de ensaio de *in vitro* foi possível observar a farmacologia do agonista IB-MECA e sua interação com a β-arrestina2.

No modelo de dor inflamatória uma dose de IB-MECA reverteu a hiperalgesia mecânica avaliada nos testes de Randall Selitto e de von Frey, no entanto não reverteu a hiperalgesia térmica avaliada no teste de Placa Quente. Por outro lado, no modelo de dor neuropática, a intensidade do efeito analgésico observado pela ativação do receptor A_3 é dependente do teste aplicado: IB-MECA reverteu totalmente o comportamento hipernociceptivo mecânico no teste de Randal Selitto e parcialmente no teste de von Frey. Da mesma forma, o IB-MECA reverteu parcialmente o comportamento hipernociceptivo térmico no teste da Placa Quente. Provavelmente as diferentes respostas ao tratamento nas respostas nociceptivas em cada um dos modelos estejam relacionadas aos diferentes mecanismos desencadeados pela ativação do receptor A_3 em cada um dos modelos de dor crônica estudados. É importante ressaltar também que as respostas e os mecanismos relacionados aos três testes nociceptivos são diferentes, uma vez que a resposta mediada pela placa quente depende de respostas supraespinhais (3), o que pode explicar as diferenças encontradas nas respostas nociceptivas os testes utilizados.

No modelo de dor crônica inflamatória induzida pela injeção de CFA, o agonista IB-MECA parece induzir *down-regularion* dos receptores de adenosina A_3, reduzindo os níveis de

proteína kinase B, fator nuclear-κB (NF- κB) e fator de necrose tumoral α (TNF-α) na sinovial e nos linfonodos (4). Além disso, em prévio estudo foi observado que o uso agonista de receptor A_3 reduziu resposta inflamatória no teste da formalina, que tem sido relacionados em nível espinhal a inibição da liberação de mediadores algogénicos; e a administração de antagonista reverteu o efeito antinociceptivo da adenosina neste mesmo estudo, sugerindo que o receptor A_3 contribui para a inibição da transmissão do estímulo doloroso em medula espinhal (5). Nesse contexto, fármacos com perfil anti-inflamatório parecem ter menos efetividade no teste de placa quente, corroborando nossos resultados. Importante salientar que a morfina, um fármaco opioide, reverte o comportamento nociceptivo induzido pela injeção de CFA nos três testes utilizados nesta tese. Desta forma é possível sugerir o mecanismo de ação do IB-MECA para seu efeito analgésico esteja mais relacionado a sua ação anti-inflamatória no processo nociceptivo (6).

Apesar dos testes nociceptivos demonstrarem a efetividade do agonista IB-MECA na hiperalgesia mecânica, não há correlação entre os dados comportamentais e os parâmetros neuroquímicos investigados, uma vez que apenas a injeção de CFA foi capaz de alterar os níveis de IL-1β e IL-10 nas estruturas do SNC. Nesse contexto, apenas o estudo conduzido por Raghavedra e colaboradores (7) descreve a ativação glial e a ativação de citocinas inflamatórias decorrente do modelo de dor induzido por CFA. sendo assim, nosso estudo contribui para a descrição dos efeitos centrais desencadeados por esse modelo de dor inflamatória, apesar de ainda serem necessários estudos mais abrangentes com relação aos mecanismos envolvidos na modulação das neurotrofinas e interleucinas em SNC.

O modelo de dor neuropática, uma condição caracterizada por hiperalgesia térmica e mecânica, vem sendo estudada no nosso grupo de pesquisa na busca de mecanismos e potenciais tratamentos, farmacológicos ou não-farmacológicos (8,9). Nesta tese nos demonstramos que uma dose de IB-MECA reduziu a resposta hiperalgesica induzida pela CCI, entretanto o mecanismo exato ainda não está elucidado, mas sabe-se que existem componentes supra-medulares e medulares relacionados (10). Estudos demonstram que o uso de IB-MECA é capaz de reduzir a resposta hiperalgésica induzida pela dor neuropática em modelo de CCI, como o conduzido por Chen e colaboradores (11), em que o tratamento com o IB-MECA inicia-se logo após a constrição do nervo. É importante ressaltar que neste período até o décimo quarto dia após a cirurgia deve-se considerar que a resposta dolorosa é composta pela dor nociceptiva aguda induzida pelo procedimento cirúrgico e a dor neuropática ainda em estabelecimento (8).

Estudos sugerem que os efeitos dos agonistas do receptor A_3 exercem modulação dos processos neuroinflamatórios na medula espinhal, uma vez que o tratamento com IB-MECA reduziu os astrócitos hiperativos e diminuição dos níveis de citocinas pró-inflamatórias em modelos de dor neuropática induzida por quimioterapia(12). Além disso, existem evidências de que o receptor A_3 de adenosina é expresso em astrócitos e micróglia, células que estão relacionadas com a iniciação e perpetuação da dor neuropática (13,14). Neste contexto, sugerimos que o efeito analgésico da IB-MECA encontrado no comportamento hipernociceptivo de roedores submetidos à dor neuropática pode ser devido à modulação das interleucinas e neurotrofinas.

Nossos resultados mostraram que o modelo de CCI aumenta os níveis de BDNF no hipocampo e o tratamento com IB-MECA foi capaz de reduzir esses níveis. O BDNF atua na dor por modulação nos níveis espinhais e supraespinhais apor meio de sinais excitatórios e inibitórios mediados pelos sistemas glutamatérgico e GABAérgico, respectivamente (15). Coull e colaboradores (16) mostraram o envolvimento do BDNF como sinalizador entre a microglia e os neurônios, sendo que o bloqueio dessa via pode ser uma nova estratégia para o tratamento da dor neuropática. Estudos tem mostrado o envolvimento de receptores A_3 nesta via de sinalização, uma vez que o IB-MECA aumentou os níveis de BDNF o que foi relacionado aos efeitos inibitórios na ativação microglial induzida pela lesão nervosa. Além disso, a ativação do receptor A_3 poderia atenuar a ativação microglial, reduzindo a liberação de BDNF e contribuindo para a restauração da sinalização de GABA no corno dorsal da medula espinhal (17). Esta hipótese pode explicar a diminuição dos níveis de BDNF induzida pela administração de IB-MECA encontrados no nosso estudo. No entanto, é necessário explorar os mecanismos relacionados ao GABA e outros marcadores envolvidos nesta cascata de sinalização.

Em resumo, IB-MECA representa um potencial alvo terapêutico na dor inflamatória crônica e na dor neuropática; uma vez foi capaz de aliviar o comportamento hipernociceptivo induzido por ambos os modelos utilizados em roedores. Entretanto, nossos estudos sugerem que não há uma correlação direta do efeito analgésico deste agonista às interleucinas ou neurotrofinas, pelo menos nas que foram exploradas nesse estudo (IL-1β, IL-10, BDNF e NGF) em estruturas nervosas centrais como córtex cerebral, tronco cerebral, hipocampo e medula espinhal.

VIII. CONCLUSÃO

8. CONCLUSÃO

A partir dos estudos desenvolvidos nesta tese podemos concluir que o IB-MECA foi capaz de reverter o comportamento nociceptivo induzido pelos dois modelos de dor crônica utilizados, inflamatório e neuropático, demonstrando o benefício de sua ulização como uma abordagem farmacológica nestas condições dolorosas. Entretanto, não podemos correlacionar diretamente os efeitos comportamentais às alterações neuroquímicas encontradas nos experimentos conduzidos, uma vez que, de forma geral, o IB-MECA não foi capaz de modificar de forma expressiva a sinalização de citocinas e neutrofinas em SNC. Ainda, foi possível observar por meio do ensaio de complementação que o IB-MECA envolve a β-arrestina 2 e que sua potência é similar aos outros agonistas não seletivos de A_3 analisados.

É importante ressaltar que esses efeitos foram obtidos apenas com uma dose aguda do fármaco, o que reforça o potencial analgésico deste fármaco e sugere que o tratamento crônico possa apresentar efeitos satisfatórios. Portanto, o desenvolvimento de estudos que utilizem modelo de dor crônica de etiologias distintas, como a dor inflamatória e a dor neuropática, e que façam uso do agonista de receptor A_3 IB-MECA comparando sua analgesia com os fármacos padrão ouro pode contribuir para o melhor entendimento do papel deste receptor nesses processos dolorosos. Além disso, análises bioquímicas que abordem as citocinas pró e anti-inflamatórias e as neutrofinas, assim como as moléculas envolvidas na sinalização intracelular desencadeada pela ativação do receptor A_3 pelo IB-MECA, nessas condições de dor, são necessárias para a elucidação dos mecanismos intrínsecos relacionando os processos nociceptivos e o papel dos receptores A_3 nestes contextos.

IX. PERSPECTIVAS

9. PERSPECTIVAS

A partir deste trabalho, novas pesquisas serão desenvolvidas buscando esclarecer os efeitos antinociceptivos e anti-inflamatórios da administração repetida de agonista adenosinérgico A_3 em ratos submetidos a modelos de dor crônica, bem como os parâmetros inflamatório e de neuroplasticidade envolvidos nos efeitos deste fármaco. Além disso, entende-se serem necessários estudos *in vitro* que explorem aspectos moleculares da ativação do receptor de A_3 pelo IB-MECA buscando elucidar as vias ativadas relacionadas aos processos antinociceptivos observados in vivo.

A) Avaliar o efeito da administração repetida com um agonista de receptor A_3 de adenosina em ratos submetidos a modelos de dor crônica inflamatória e dor crônica neuropática, avaliando:
1) a resposta nociceptiva por meio da avaliação da hiperalgesia térmica por meio do teste de placa quente; hiperalgesia mecânica por meio do teste de von Frey; avaliação motora por meio do teste de rota rod.
2) a resposta inflamatória por meio da avaliação dos níveis de TNF-α e interleucinas (IL-1β, IL-10, IL-4) em soro, córtex cerebral, tronco cerebral, hipocampo e medula espinal;
3) a neuroplasticidade por meio da avaliação dos níveis BDNF e NGF em soro, córtex cerebral, tronco cerebral, hipocampo e medula espinal;
4) expressão de receptores A_3 em estruturas do SNC (córtex cerebral, medula espinhal, tronco encefálico e hipocampo) utilizando a técnica de RT-PCR;
5) quantificação de receptor A_3 e marcadores da cascata de sinalização como ERK1 e ERK-2, NFkB por meio da técnica de Western Blot;
6) níveis de mediadores como TNF-alfa, IFN-gama, NFkB, AKt, p38, ERK-1/2 por meio da técnica de citometria de fluxo.

B) Avaliar vias de sinalização intracelular do receptor A_3 após administração de IB-MECA em cultura de células:
1) níveis de marcadores como interleucinas (TNF-alfa, IFN-gama, IL-4, Il-6, Il-10), NFkB, AKt, p38, ERK-1/2 por meio da técnica de citometria de fluxo.
2) ensaio de internalização de receptor A_3 por meio de microscópio confocal.

X. REFERÊNCIAS DA PARTE II

10. REFERÊNCIAS

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PARTE III

Divulgações e Anexos

XI. DIVULGAÇÕES

11. DIVULGAÇÕES

SALVI, A. A.; CIOATO, S. G.; LOPES, B. C.; MEDEIROS L.F.; TORRES, I.L.S. Tratamento agudo com agonista de receptor A_3 de Adenosina altera resposta hiperalgésica térmica induzida por modelo animal de dor crônica inflamatória In: 37a Semana Científica do HCPA, 2017, Porto Alegre- RS.

LOPES, B. C.; CIOATO, S. G.; SALVI, A. A.; MEDEIROS L.F.; TORRES, I.L.S. Efeito do tratamento agudo com agonista de receptor A_3 de Adenosina na hiperalgesia mecânica induzida por modelo animal de dor crônica In: 37a Semana Científica do HCPA, 2017, Porto Alegre- RS.

LOPES, B. C.; CIOATO, S. G.; SALVI, A. A.; MEDEIROS L.F.; TORRES, I.L.S. Tratamento agudo com agonista de receptor A_3 de adenosina altera resposta hiperalgésica térmica induzida por modelo animal de dor crônica inflamatória. In: XIII Congresso Brasileiro de Dor, 2017, Fortaleza- CE.

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CIOATO SG, MEDEIROS LF, LOPES B, SALVI AA, DE SOUZA A, ROESLER R, CAUMO W, TORRES ILS. IB-MECA Acute Treatment Relieves Pain in CFA Chronic Inflammatory Model in Rats. . In: 50th Brazilian Congress of Pharmacology and Experimental Therapeutic, 2018, Ribeirão Preto- SP.

XII. ANEXOS

A) APROVAÇÕES DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais (CEUA/HCPA) analisou o projeto:

Projeto: 150530
Data da Versão do Projeto: 15/01/2016

Pesquisadores:
IRACI LUCENA DA SILVA TORRES
STEFANIA GIOTTI CIOATO

Título: INVESTIGAÇÃO DO POTENCIAL EFEITO ANALGÉSICO E MECANISMO DE AÇÃO DE UM AGONISTA DE RECEPTOR A3 DE ADENOSINA EM MODELOS ANIMAIS DE DOR CRÔNICA INFLAMATÓRIA: TRATAMENTO AGUDO

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

- Os membros da CEUA/HCPA não participaram do processo de avaliação de projetos onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Porto Alegre, 24 de fevereiro de 2016.

Biol. Michael Everton Andrades
Coordenador CEUA/HCPA

HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

Grupo de Pesquisa e Pós Graduação

Carta de Aprovação

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 2018/0377

Título: INVESTIGAÇÃO DO POTENCIAL EFEITO ANALGÉSICO E MECANISMO DE AÇÃO DE UM AGONISTA DE RECEPTOR A3 DE ADENOSINA EM MODELO ANIMAL DE DOR CRÔNICA NEUROPÁTICA: TRATAMENTO AGUDO

Pesquisador Responsável: **IRACI LUCENA DA SILVA TORRES**

Equipe de Pesquisa:

LICIANE FERNANDES MEDEIROS | BETTEGA COSTA LOPES | RAFAELA PEDROSO DE AGUIAR

STEFANIA GIOTTI CIOATO

Data de Aprovação:

Data de Término: 01/04/2019

Espécie/Linhagem	Sexo/Idade	Quantidade
RATO HETEROGÊNICO	M/60 Dia(s)	86

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Assinado digitalmente por:
PATRICIA ASHTON PROLLA
Grupo de Pesquisa e Pós-graduação
06/11/2018 13:38:17
https://aghuse-externo.hcpa.edu.br/pesquisa/publico/cadastroapoio/conferenciaArquivo.xhtml?codarq=428602

B) CARTAS DE SUBMISSÃO DE ARTIGOS

Elsevier Editorial System(tm) for Cytokine
Manuscript Draft

Manuscript Number: CYTO-18-941

Title: IB-MECA acute treatment relieves pain in CFA chronic inflammatory model in rats

Article Type: Full length article

Keywords: CFA model; rats; IB-MECA; nociceptive tests.

Corresponding Author: Dr. Iraci Lucena da Silva Torres, Ph.D.

Corresponding Author's Institution: Universidade Federal do Rio Grande do Sul

First Author: Stefania G Cioato

Order of Authors: Stefania G Cioato; Liciane F Medeiros; Bettega C Lopes; Artur A Salvi; Andressa Souza; Rafael Roesler; Wolnei Caumo; Iraci Lucena da Silva Torres, Ph.D.

Abstract: It is known that IB-MECA compound, an agonist of adenosine A3 receptor (A3AR), is involved with pain relief and modulation in the inflammatory process; however, its action mechanisms are not completely elucidated. The aim of this study was to evaluate the antinociceptive effect of IB-MECA) in a chronic inflammatory pain model, and the involvement of neurotrophins and cytokines central levels in this effect. Chronic inflammatory pain was induced using Complete Freund's Adjuvant (CFA) in the hind paw of male adult Wistar rats. Thermal and mechanical hyperalgesia/allodynia was measured by Hot plate, Von Frey and Randal Selitto tests. Neurochemical measured were brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), interleukin 1β (IL-1β) and IL-10. The establishment of pain model, decrease of latency withdrawal, was observed 10 and 14 days after CFA injection. And, IB-MECA was effective to revert mechanical and thermal hyperalgesia, in a totally or partially way. We observed CFA pain model effects in IL-1β and IL-10 spinal cord and brainstem levels. Also, we showed that IB-MECA administration in controls increased the interleukin levels. And, we did not find any involvement neurotrophins in this effect, at least those we measured in spinal cord and brainstem of rats. Studies have shown that CFA increases the IL-1β in the hind paw of injection when evaluated acutely after the induction of model. But, there is a lack of knowledge about the neuroinflammatory effects induced by CFA pain model. Also, the adenosine A3 receptor seems to have complexes effects in the central nervous system, with pro-inflammatory and anti-inflammatory roles, specially in healthy conditions.

C) CO-AUTORIAS DE ARTIGOS CIENTÍFICOS NO PEÍODO DE DOUTORADO

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