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MODULAÇÃO DAS ALTERAÇÕES FUNCIONAIS E SINTOMÁTICAS RELACIONADAS À CISTITE HEMORRÁGICA INDUZIDA POR CICLOFOSFAMIDA EM CAMUNDONGOS ATRAVÉS DO BLOQUEIO MEDULAR DOS CANAIS DE CÁLCIO VOLTAGEM-DEPENDENTES DOS SUBTIPOS P/Q E N

Porto Alegre
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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Medicina e Ciências da Saúde. Área de concentração em Farmacologia Bioquímica e Molecular, da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Profa. Dra. Maria Martha Campos

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Dedico esta dissertação aos meus pais que, incansavelmente, estimularam e apoiaram para o meu crescimento profissional.

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"E quando o inesperado se manifesta, é preciso ser capaz de rever nossas teorias e ideias, em vez de deixar o fato novo entrar à força na teoria incapaz de recebê-lo." (MORIN, 2000, p. 30)

RESUMO

Os canais de cálcio voltagem-dependentes (CCVDs), ao nível medular, são um dos principais reguladores das alterações inflamatórias e dolorosas, representando um interessante alvo terapêutico. Com isso, avaliamos os efeitos da administração intratecal dos bloqueadores seletivos dos CCVD do subtipo P/Q e N, Tx3-3 e Phα1β, respectivamente, isolados do veneno da aranha *P. nigriventer*, nas mudanças sintomáticas, inflamatórias e funcionais, relacionadas com a cistite hemorrágica (CH), induzido pelo quimioterápico ciclofosfamida (CPA) em camundongos. Os efeitos dos peptídeos provenientes da aranha *P. nigriventer* foram comparados com os exibidos pelas toxinas MVIIC e MVIIA, obtidas do caramujo *C. magus*. A CH foi induzida por uma única administração intraperitoneal de CPA (300 mg/kg). O bloqueio medular dos CCVD do subtipo P/Q através da Tx3-3 e MVIIC ou, o bloqueio dos CCVD do subtipo N pela toxina Phα1β atenuou as respostas nociceptivas e inflamatórias associadas à CH, incluindo estresse oxidativo e produção de citocinas na bexiga. Além disso, a CPA produziu um aumento evidente na expressão do RNAm de TRPV1 e TRPA1 na bexiga, o qual foi virtualmente revertido por todas as toxinas. Notavelmente, a isoforma Phα1β reduziu a migração de neutrófilos para a bexiga, além de reverter as disfunções da bexiga relacionadas à CH. Finalmente, a co-administração medular do antagonista seletivo dos receptores NK1, CP-96345, com o peptídeo Phα1β, intensificou o efeito antinociceptivo do mesmo. Nossos resultados trazem novas evidências da função dos CCVD do subtipo P/Q e N ao nível medular na disfunção da bexiga, apontando a Phα1β como uma possível alternativa de tratamento para complicações associadas à CH, induzida por CPA.

Palavras-chave: Cistite Hemorrágica. Inflamação. Ciclofosfamida. *Phoneutria nigriventer*. *Conus magus*. Canais de cálcio voltagem-dependentes.

ABSTRACT

Spinal voltage-gated calcium channels (VGCC) are pivotal regulators of painful and inflammatory alterations, representing attractive therapeutic targets. We examined the effects of epidural administration of the selective P/Q- and N-type VGCC blockers Tx3-3 and Phα1β, respectively, isolated from the spider *P. nigriventer*, on symptomatic, inflammatory and functional changes allied to cyclophosphamide (CPA)-induced hemorrhagic cystitis (HC) in mice. The effects of *P. nigriventer*-derived toxins were compared to those displayed by MVIIC and MVIIA, extracted from the cone snail *C. magus*. HC was induced by a single intraperitoneal injection of CPA (300 mg/kg). The spinal blockage of P/Q-type VGCC by Tx3-3 and MVIIC, or N-type VGCC by Phα1β attenuated nociceptive and inflammatory events associated with HC, including bladder oxidative stress and cytokine production. Moreover, CPA produced an evident increase of bladder TRPV1 and TRPA1 mRNA expression, which was virtually reversed by all the tested toxins. Noteworthy, Phα1β strongly prevented bladder neutrophil migration, besides HC-related functional alterations. Finally, the spinal co-administration of the selective NK1 receptor antagonist CP-96345 heightened Phα1β antinociceptive effects. Our results shed new lights on the role of spinal P/Q and N-type VGCC in bladder dysfunctions, pointing out Phα1β as a promising alternative for treating complications associated to CPA-induced HC.

Keywords: Hemorrhagic Cystitis. Inflammation. Cyclophosphamide. *Phoneutria nigriventer*. *Conus magus*. Voltage-gated calcium channels.

LISTA DE ABREVIATURAS

ACR - Acroleína

CFA - Adjuvante Completo de Freund

Ca^{+2} - Cálcio

Canais de Cálcio Voltagem-Dependentes - CCVDs

COX - Cicloxigenase

CPA - Ciclofosfamida

CH - Cistite Hemorrágica

TNF-α - Fator de Necrose Tumoral Alpha

NFAT - Fator Nuclear de Células T Ativadas

NF-κB - Fator Nuclear Kappa B

FDA - Food and Drug Administration

DRG - Gânglio da Raiz Dorsal

IL - Interleucina

Mesna - 2-Mercaptoetanosulfonato de Sódio

NK - Neurocinina

PhTx - Phoneutriatoxina

K^{+} - Potássio

TRP - Receptor de Potencial Transitório

SNC - Sistema Nervoso Central

Na^{+} - Sódio

SP - Substância P

SUMÁRIO

1. INTRODUÇÃO

A cistite hemorrágica (CH) é um processo inflamatório que consiste em um sangramento agudo difuso ou insidioso na mucosa da bexiga. Apresenta como principais características, hematúria recorrente e sintomas de irritabilidade, incluindo disúria, urgência e frequência na micção, assim como dor supra-púbica (Traxer *et al.*, 2001; Cheuk *et al.*, 2007; Manikandan *et al.*, 2010). Embora a etiologia da CH seja variada, há duas causas predominantes: irritação química e radiação. Entre as substâncias químicas indutoras de CH está o grupo das oxazoforinas, que compreende a ciclofosfamida (CPA) e a ifosfamida (Giraud *et al.*, 2006; Manikandan *et al.*, 2010). Por outro lado, pacientes com carcinomas próximos à região pélvica e, sob tratamento radioterápico também podem desenvolver CH. Além desses fatores, infecções virais (poliomavírus e adenovírus), bacterianas, fúngicas ou parasitárias podem estar relacionadas a esta patologia (Traxer *et al.*, 2001; Arafa, 2009). Muitos casos de CH leve são resolvidos espontaneamente, sem complicações. No entanto, casos graves resultam em uma significativa morbidade, prolongamento nas internações hospitalares e, eventualmente, em mortalidade (Decker *et al.*, 2009).

Em 1959, a CPA foi aprovada pelo *Food and Drug Administration* (FDA) como um agente anti-câncer e, até os dias atuais, vêm sendo utilizada como medicamento de primeira linha para determinados tipos de câncer (Emadi *et al.*, 2009). Além de ser um potente quimioterápico, também possui atividade imunossupressora em doses baixas (Wong *et al.*, 2000). A CPA representa um agente alquilante, devido ao seu mecanismo de ação, pois interfere no funcionamento normal do DNA por alquilação, provocando ligações cruzadas dos filamentos que, por sua vez, impede a reprodução celular. Trata-se de um pró-fármaco, pois sua atividade só se manifesta após uma biotransformação enzimática, dando origem ao metabólito ativo, mostarda de fosforamida (Crocitto *et al.*, 1996; Wong *et al.*, 2000; Ozolins, 2010). A conversão da CPA para o metabólito ativo é dependente do citocromo P-450 (Fenselau *et al.*, 1977). Com isso, a metabolização ocorre no fígado, gerando a 4-hidroxiciclofosfamida e, seu tautômero acíclico, a aldofosfamida. Por conseguinte, a aldofosfamida e a 4-hidroxiciclofosfamida são transportadas pela circulação sanguínea até ao alvo tumoral. Chegando ao local, a aldofosfamida sofre clivagem produzindo a mostrada de fosforamida (agente ativo) e, a acroleína (ACR), um composto diretamente tóxico para epitélio da bexiga. Desta forma, importantes efeitos adversos da CPA como, por exemplo, a marcante urotoxicidade, acabam limitando, em alguns casos, seu uso na clínica (Fenselau *et al.*, 1977; West, 1997; Furlanut *et al.*, 2003; de Jonge *et al.*, 2005).

Na clínica, a CPA é amplamente utilizada no tratamento de tumores sólidos, neoplasias de células B, tais como linfoma, mieloma múltiplo, leucemia linfocítica crônica, macroglobulinemia de Waldenström e, em algumas doenças auto-imunes (lúpus e artrite reumatóide). Ademais, pode ser usada após transplante de medula óssea, devido a sua potente atividade imunossupressora, podendo ser administrada tanto por via oral, quanto endovenosa (Crocitto *et al.*, 1996; Serrano Frago *et al.*, 2005; Joensuu *et al.*, 2012). Em muitos casos, pode ser usada em combinação com outros fármacos, como por exemplo, associada ao metrotrexato e à 5-fluorouracila na terapia adjuvante, após cirurgia do carcinoma de mama (Traxer *et al.*, 2001; Davis *et al.*, 2011).

O metabólito urotóxico gerado pela CPA, a ACR, está presente na indústria, no meio-ambiente e, pode ser ocasionada por tratamentos farmacológicos, como mencionado acima. Industrialmente, a ACR é principalmente usada como um herbicida. Ambientalmente, ocorre naturalmente em alimentos e é formada durante a combustão de materiais orgânicos. Assim, a ACR é encontrada em todos os tipos de fumo, incluindo o do cigarro (Kehrer *et al.*, 2000). Além desses casos, a ACR foi identificada como um produto da CPA em 1971 (Alarcon *et al.*, 1971). É o aldeído α,β-insaturado mais reativo da classe, que rapidamente se liga e remove nucleófilos celulares, tais como a glutationa. Também pode reagir com alguns resíduos de proteínas e com locais nucleofílicos do DNA. O mecanismo pelo qual a ACR causa danos ao uroepitélio não está totalmente elucidado. Por outro lado, existem algumas propostas de mecanismos de ação, tais como: (i) a ACR atravessa rapidamente o uroepitélio, devido sua natureza química e, com isso, causa o aumento da produção de espécies reativas de oxigênio (ROS) no epitélio da bexiga e; (ii) induz a expressão de alguns fatores de transcrição intracelulares, tais como NF-κB e AP-1, ativando citocinas pró-inflamatórias como TNF-α e IL-1β (Korkmaz *et al.*, 2007). Em essência, a urotoxicidade é gerada devido ao contato direto do epitélio da bexiga com a ACR, o que leva à formação de edema, ulceração, neovascularização, hemorragia, necrose e expressão de enzimas inflamatórias (Macedo *et al.*, 2008b).

Atualmente, uma maneira de prevenir a CH é fazer hidratação do paciente, a fim de manter o fluxo urinário, além da irrigação da bexiga, prevenindo a obstrução do trato urinário (Cheuk *et al.*, 2007). Outra conduta essencial é associar CPA a agentes que neutralizam a ACR, tais como o 2-mercaptoetanosulfonato de sódio, mais conhecido como Mesna. O Mesna contém um composto sulfidrila, que se liga à ACR no interior do sistema urinário e, desta forma, recolhe e remove o metabólito tóxico através da urina. Com isso, o Mesna atua de maneira preventiva na CH (Brock *et al.*, 1983). Em contrapartida, a proteção da bexiga nem

sempre é alcançada, particularmente quando a lesão já está instalada. Em estudos recentes, foi observada a ocorrência de cistite, mesmo com a utilização do Mesna, tanto experimentalmente, quanto clinicamente (Vieira *et al.*, 2003; Lima *et al.*, 2007). Desta forma, na ausência de uma substância com propriedades uroprotetoras adequadas, a CH se torna dose-limitante, com uma incidência de 20-40%. Além disso, casos de hematúria, frequência na micção, disúria e alterações microscópicas e/ou macroscópicas, ou seja, características clássicas da CH, ainda têm sido observadas clinicamente (Korkmaz *et al.*, 2001; Macedo *et al.*, 2012). Esses fatos evidenciam, cada vez mais, a importância de estudos que investiguem os mecanismos envolvidos nas lesões da bexiga, resultantes do tratamento com agentes alquilantes.

A bexiga é um órgão altamente inervado e que expressa uma grande diversidade de receptores relacionados aos processos de nocicepção e inflamação, sendo que estes constituem importantes alvos farmacológicos (Black *et al.*, 2007). Entre os alvos, está o receptor de potencial transitório vanilóide-1 (TRPV1). O TRPV1 faz parte da grande família de receptores de potencial transitório (TRP) (Nilius *et al.*, 2005). O TRPV1 é caracterizado por ser um canal catiônico permeável e não seletivo a Ca^{2+} e, está sujeito à ativação ou à regulação positiva por uma série de estímulos, incluindo a despolarização da membrana, calor levemente nocivo, compostos vanilóides e endocanabinóides, prótons extracelulares, assim como, mediadores inflamatórios (Voets *et al.*, 2004). Portanto, não é surpreendente que o TRPV1 esteja ligado a um amplo espectro de doenças. Especialmente na bexiga, o TRPV1 é o receptor mais bem caracterizado entre os TRP (Nilius *et al.*, 2005), estando envolvido no desenvolvimento de hiperalgesia inflamatória da bexiga. Além disso, a ativação do TRPV1 pelo agonista endógeno, anandamida, evoca o aumento da atividade reflexiva da bexiga e da dor associada à CH. Esses efeitos estavam ausentes com a utilização de antagonistas de TRPV1, assim como pela utilização de animais *knockout* para estes receptores (Dinis *et al.*, 2004; Nilius *et al.*, 2005). Outro membro importante da família dos TRP nos processos nociceptivos e inflamatórios é o receptor de potencial transitório anquirina-1 (TRPA1). Assim como o TRPV1, o TRPA1 é caracterizado como um canal catiônico permeável e não seletivo a Ca^{2+} (Nilius *et al.*, 2005). Estudos recentes mostram que a utilização do antagonista de TRPA1 inibiu a alodínia mecânica mediada por CFA (Brain, 2011). Ademais, pesquisas recentes demonstram que a ativação dos TRPA1 induz a ativação de neurônios sensoriais primários, através da liberação de substância P (SP) via receptor de neurocinina-1 (NK1) (Nakamura *et al.*, 2012).

Outrossim, estudos realizados pelo nosso grupo demonstraram que após a administração intraperitoneal (i.p.) de CPA em camundongos, há um aumento significativo da expressão de c-Fos na medula espinhal e na região cortical do cérebro, em um processo dependente da ativação do receptor purinérgico, P2X7 (Martins *et al.*, 2012). Cabe ressaltar, que a c-Fos é uma importante proteína envolvida na modulação da transmissão central da dor. Além disso, estudos recentes mostram que a cicloxigenase-2 (COX-2), enzima envolvida no processo inflamatório, está expressa na bexiga, após a indução de CH pela aplicação de ifosfamida em ratos (Macedo *et al.*, 2008a).

Os venenos de aranhas, caramujos, caracóis, cobras e escorpiões contêm uma série de toxinas que bloqueiam receptores ou ativam canais iônicos, como meio de produção de choque, paralisia e morte da sua presa. Alternativamente, estes venenos têm levado a derivações bastante interessantes para o desenvolvimento de fármacos (Estrada *et al.*, 2007). Em especial, as aranhas são um grupo antigo e bem sucedido de animais invertebrados, amplamente distribuídos em todo o mundo. Possuem lugar marcante nos mitos populares e no folclore, devido a seus hábitos secretos, aspecto físico e comportamento predatório. Embora exista uma imensa diversidade (cerca de 40.000 espécies descritas e, provavelmente, mais de 100.000 não descritas), poucas espécies representam realmente um problema de saúde (Isbister *et al.*, 2011). Segundo a Organização Mundial da Saúde, os gêneros clinicamente importantes de aranhas são *Phoneutria*, *Latrodectus*, *Loxosceles*, (Araneomorphae) e *Atrax* (Mygalomorphoe). No Brasil, as aranhas perigosas pertencem aos gêneros *Phoneutria*, *Loxosceles* e *Latrodectus*, compreendendo cerca de 20 espécies. Dentro do gênero *Phoneutria*, as espécies de maior relevância clínica são: *Phoneutria fera*, *Phoneutria reidyi*, *Phoneutria keyserlingi* e *Phoneutria nigriventer* (Farsky *et al.*, 2005).

O gênero *Phoneutria* pertence à família da *Ctenidae*, estando presente na América do Sul e Costa Rica. No entanto, a maioria dos relatos de acidentes clinicamente relevantes são no Brasil (Bucaretchi *et al.*, 2000; Bucaretchi *et al.*, 2008). Em particular, a espécie *P. nigriventer* é conhecida popularmente como aranha armadeira, pela posição que toma ao se sentir ameaçada. Possui hábitos noturnos e permanece refugiada durante o dia. No período de acasalamento, esta espécie pode atingir o intradomicílio, quando os machos se tornam mais ativos à procura das fêmeas, acentuando-se os riscos de acidentes. A aranha *P. nigriventer* não constrói teia e seu sucesso como predadora pode ser explicado pela potência das diversas toxinas presentes em seu veneno (Gomez *et al.*, 2002). Os sintomas de envenenamento em animais e seres humanos pelo veneno da *P. nigriventer* incluem dor intensa e irradiada, salivação, perturbações visuais, sudorese, priapismo, arritmias cardíacas, taquicardia,

convulsões tônicas e paralisia espástica, podendo evoluir para a morte. Estes sintomas são causados por ações centrais e periféricas, como consequência da liberação maciça de neurotransmissores em terminações nervosas autonômicas e motora (Lucas, 1988; Farsky *et al.*, 2005).

Os peptídeos produzidos pela glândula da aranha *P. nigriventer* têm sido extensivamente investigados e, até o presente, são descritos cerca de 20 peptídeos ativos, atuando principalmente no funcionamento de canais de sódio (Na^+), cálcio (Ca^{2+}), potássio (K^+), assim como em receptores do sistema nervoso (Grishin, 1999; Gomez *et al.*, 2002). Além disso, os peptídeos têm peso molecular que varia de 3500 a 9000 Daltons (Gomez *et al.*, 2002).

Primeiramente, os pesquisadores observaram um potente efeito neurotóxico, uma das principais características do veneno da *P. nigriventer*. O efeito foi atribuído à ação sobre os canais de Na^+ voltagem-dependentes, através da indução de potenciais de ação repetidos em terminações nervosas e membranas de fibras musculares (Fontana *et al.*, 1985). Não obstante, foram encontradas outras atividades farmacológicas distintas relacionadas ao veneno e, consequentemente aos canais iônicos. Particularmente, isso foi possível devido ao fraciomento do veneno total (Diniz *et al.*, 1990). Com isso, alguns estudos conseguiram demonstrar que frações do veneno, quando administradas por via intracerebroventricular, produziram paralisia flácida. Portanto, um segundo efeito farmacológico do veneno foi estabelecido, ou seja, atividade bloqueadora dos canais de Ca^{2+} voltagem-dependentes (CCVD) (Gomez *et al.*, 1995; Prado *et al.*, 1996; Leao *et al.*, 2000).

Os CCVD são uma família de canais iônicos, classificados por propriedades farmacológicas e eletrofisiológicas. Têm sido geralmente divididos em canais de baixo limiar de ativação (subtipo T) e de alto limiar (subtipos L, N, P/Q e R) (Lai *et al.*, 2006). Além disso, a contribuição dos diferentes CCVD para processos nociceptivos e inflamatórios ganharam considerável interesse nos últimos anos (Bourinet *et al.*, 2005; Snutch, 2005). De fato, sua atividade modulatória, nas respostas nociceptivas, em áreas como a medula espinhal, gânglios da raiz dorsal (DRG) e do tronco cerebral, indicam o papel essencial destes CCVD no processamento de informações nociceptivas para o sistema nervoso central (Heinke *et al.*, 2004; Murakami *et al.*, 2004). Ademais, os CCVD parecem estar implicados na sensibilização da dor central, que ocorre em nervos lesionados e, durante os estados inflamatórios (Matthews *et al.*, 2001; Matthews *et al.*, 2007). Em modelos animais de lesão nervosa, foi visto que o bloqueio dos CCVD do subtipo N e P/Q reduziu os sinais comportamentais de nocicepção de origem neuropática (Matthews *et al.*, 2001). Os CCVD do subtipo N estão presentes no

terminal pré-sináptico de neurônios nociceptivos no corno dorsal da medula espinhal, regulando dessa forma, a liberação de neurotransmissores pró-nociceptivos como o glutamato e a SP. (Wen *et al.*, 2005). Além disso, o aumento da concentração de Ca^{2+} intracelular faz com que haja a translocação do fator nuclear de células T ativadas (NFAT) para o núcleo e, com isso, a inicialização da transcrição, resultando na secreção de citocinas e na proliferação de células T (Bradding *et al.*, 2009).

Logo após os primeiros achados do veneno da *P. nigriventer*, foram descritas as primeiras frações do veneno: Phoneutriatoxina-1 (PhTx1) e Phoneutriatoxina-2 (PhTx2), as quais causavam contração do íleo de cobaias (Rezende Junior *et al.*, 1991). Posteriormente, outra fração proteica foi isolada e estudada, a Phoneutriatoxina-3 (PhTx3). Essa fração, quando injetada em roedores, induziu paralisia flácida (Rezende Junior *et al.*, 1991), que ocorreu, provavelmente, devido à sua ação inibitória sobre a liberação de neurotransmissores (Gomez *et al.*, 1995; Prado *et al.*, 1996). A partir da fração PhTx3, seis diferentes isoformas foram purificadas (Tx3-1 a Tx3-6) (Cordeiro Mdo *et al.*, 1993) e, pelo menos três delas (Tx3-3, Tx3-4 e Tx3-6) bloqueiam o influxo de Ca^{2+} induzido por despolarização com altas concentrações de KCl, nas terminações nervosas (Prado *et al.*, 1996; Guatimosim *et al.*, 1997; Miranda *et al.*, 1998). Logo após, experimentos eletrofisiológicos demonstraram que a isoforma Tx3-3 é um potente bloqueador (IC_{50}= 0,7 nM) das correntes do subtipo P/Q e R localizadas no soma de células granulares do cerebelo, enquanto os subtipos N e L são parcialmente bloqueados em outros tipos celulares (Leao *et al.*, 2000). Além disso, estudos recentes mostam que o polipepetídeo Tx3-3 tem efeito antinociceptivo de longa duração em modelos de dor neuropática (Dalmolin *et al.*, 2011).

Por outro lado, recentemente, a isoforma Tx3-6, que foi patenteada e, então denominada Phα1β, foi capaz de bloquear seletivamente os CCVD do subtipo N (Vieira *et al.*, 2005). Ademais, pesquisas demonstraram que a Phα1β é tão potente quanto a ω-conotoxina MVIIA na resposta antinociceptiva, apresentando índice terapêutico maior do que ω-conotoxina MVIIA em experimentos pré-clínicos (Souza *et al.*, 2008; de Souza *et al.*, 2011). Cabe ressaltar que a versão sintética do peptídeo ω-conotoxina MVIIA é a ziconotida, um medicamento aprovado pelo FDA (Nome comercial: Prialt®; Azur Pharma International, Filadélfia, EUA) para o tratamento da dor em pacientes que necessitam de analgesia intratecal (i.t.) e são refratários à terapia opióide. Esta propriedade analgésica mostrou ser eficaz no tratamento de dor severa e crônica por bloquear os CCVD do subtipo N da medula espinhal (McGivern, 2007). Outrossim, a ω-conotoxina MVIIC, também extraída do caramujo *Conus magus*, é um potente bloqueador dos CCVD do subtipo P/Q (Hillman *et al.*, 1991).

Nesse contexto, o presente estudo teve como objetivo determinar os efeitos anti-inflamatórios e antinociceptivos desempenhados pelas isoformas Tx3-3 e Phα1β do veneno da aranha *P. nigriventer*, bem como, comparar esses efeitos com as toxinas MVIIC e MVIIA, extraídas do caramujo *C. magus*, no modelo de CH induzida pela aplicação de ciclofosfamida em camundongos. O desenvolvimento do estudo, dentro dos moldes propostos, permitiu definir a importância dessas toxinas na CH, assim como, identificar novas alternativas para o tratamento desta doença, contribuindo para o avanço científico na área de farmacologia, toxinologia e urologia.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar os efeitos anti-inflamatórios e antinociceptivos das frações purificadas Tx3-3 e Phα1β, obtidas do veneno da aranha *Phoneutria nigriventer*, bem como, a dos peptídeos do caramujo *Conus magus*, MVIIC e MVIIA, no modelo de cistite hemorrágica induzida por ciclofosfamida em camundongos.

2.2. Objetivos específicos

1) Comparar o efeito de diferentes doses dos peptídeos Tx3-3, Phα1β, MVIIC e MVIIA, nas alterações comportamentais nociceptivas, induzidas pela ciclofosfamida em camundongos;

2) Comparar o efeito de diferentes doses das frações Tx3-3, Phα1β, MVIIC e MVIIA, sobre as alterações inflamatórias vesicais (edema e hemorragia), após a aplicação de ciclofosfamida em camundongos;

3) Determinar os efeitos da administração intratecal das toxinas Tx3-3, Phα1β, MVIIC ou MVIIA, sobre a migração de neutrófilos para a bexiga, após aplicação de ciclofosfamida em camundongos;

4) Verificar os efeitos da injeção intratecal das toxinas Tx3-3, Phα1β, MVIIC ou MVIIA, sobre a produção local de citocinas (TNF-α, IL-1β, IL-4 e IL-10), após o tratamento com ciclofosfamida em camundongos;

5) Determinar os efeitos da aplicação intratecal da toxina Phα1β, sobre o estresse oxidativo na medula espinhal e na bexiga, após administração de ciclofosfamida em camundongos;

6) Analisar os efeitos do tratamento intratecal das toxinas Tx3-3, Phα1β, MVIIC e MVIIA, sobre expressão do RNAm de TRPV1, TRPA1 e NK1 na bexiga e na medula espinhal, após a administração de ciclofosfamida em camundongos;

7) Verificar o efeito do tratamento intratecal da toxina Phα1β, sobre a disfunção da bexiga através da técnica de cistometria, após a aplicação de ciclofosfamida em camundongos.

8) Avaliar o co-tratamento intratecal da toxina Phα1β com o antagonista dos receptores NK1, CP-96345, nas alterações comportamentais nociceptivas e inflamatórias, induzido pela administração de ciclofosfamida em camundongos.

3. MANUSCRITO DO TRABALHO EXPERIMENTAL

Os resultados do presente estudo foram submetidos à revista *The Journal of Experimental Medicine*, fator de impacto 13.214 (JCR: 2012).

P/Q- OR N-TYPE CALCIUM CHANNELS SPINAL BLOCKAGE MODULATES FUNCTIONAL AND SYMPTOMATIC CHANGES ALLIED TO MOUSE HEMORRHAGIC CYSTITIS

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Abbreviations used: CPA, cyclophosphamide; HC, hemorrhagic cystitis; TRP, transient receptor potential; VGCC, voltage-gated calcium channels.

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Abstract

Spinal voltage-gated calcium channels (VGCC) are pivotal regulators of painful and inflammatory alterations, representing attractive therapeutic targets. We examined the effects of epidural administration of the selective P/Q- and N-type VGCC blockers Tx3-3 and Phα1β, respectively, isolated from the spider *P. nigriventer*, on symptomatic, inflammatory and functional changes allied to cyclophosphamide (CPA)-induced hemorrhagic cystitis (HC) in mice. The effects of *P. nigriventer*-derived toxins were compared to those displayed by MVIIC and MVIIA, extracted from the cone snail *C. magus*. HC was induced by a single intraperitoneal injection of CPA (300 mg/kg). The spinal blockage of P/Q-type VGCC by Tx3-3 and MVIIC, or N-type VGCC by Phα1β attenuated nociceptive and inflammatory events associated with HC, including bladder oxidative stress and cytokine production. Moreover, CPA produced an evident increase of bladder TRPV1 and TRPA1 mRNA expression, which was virtually reversed by all the tested toxins. Noteworthy, Phα1β strongly prevented bladder neutrophil migration, besides HC-related functional alterations. Finally, the spinal co-administration of the selective NK1 receptor antagonist CP-96345 heightened Phα1β antinociceptive effects. Our results shed new lights on the role of spinal P/Q and N-type VGCC in bladder dysfunctions, pointing out Phα1β as a promising alternative for treating complications associated to CPA-induced HC.

Keywords: Hemorrhagic Cystitis. Inflammation. Cyclophosphamide. *Phoneutria nigriventer*. *Conus magus*. Voltage-Gated Calcium Channels.

Introduction

Voltage-gated calcium channels (VGCCs) are a family of integral membrane calcium-selective proteins found in all excitable and in many nonexcitable cells (Stock et al., 2013). These channels are classified as low voltage- (T-type), and high voltage-activated (L-, N-, P/Q-, and R-types) channels, according to their electrophysiological and pharmacological proprieties (Lai et al.,, 2006; Turner et al., 2011). In the past 10 years, VGCCs have been recognized as potential targets for inflammatory and neuropathic pain, manly by modulating the calcium influx, and the consequent release of neurotransmitters, such as glutamate, calcitonin gene-related peptide (CGRP) and substance P (SP) from primary afferent neurons (Rahman et al., 2013; Vink et al., 2012). Supporting this, the selective N-type VGCC blocker MVIIA peptide (named Ziconotide or Prialt®), isolated from the marine cone snail *Conus magus*, was approved by the Food and Drug Administration (FDA) as an intrathecal analgesic for managing chronic intractable pain, particularly in patients refractory to opioids (Adams *et* al., 2012). Nevertheless, the clinical use of MVIIA has been related nervous system side effects, such as memory impairment, dizziness or speech disorders. In addition to MVIIA, the ω-conotoxin MVIIC has been demonstrated to display marked spinal antinociceptive effects in diverse pre-clinical models, an effect likely related to the inhibition of P/Q-type calcium currents (Nimmrich et al., 2012).

A series of peptides derived from the Brazilian armed spider *Phoneutria nigriventer* have been investigated, particularly concerning their ability to modulate VGCCs in nociceptive and inflammatory processing (Gomez et al., 2002). One example is the purified fraction Tx3-3 that inhibits P/Q-type VGCC in cerebellar granule neurons (Leao et al., 2000). Indeed, it has been recently shown that intrathecal injection of Tx3-3 attenuates nociceptive effects related to neuropathic pain in rodents, without causing adverse motor effects (Dalmolin et al., 2011). Of high interest, the patented toxin Tx3-6 isolated from the venom of

P. nigriventer (named Phα1β) was characterized by means of electrophysiological assays, as a selective inhibitor of N-type calcium currents (Vieira et al., 2005). Recent studies conducted by de Souza *et al*. demonstrated long-lasting analgesic effects for Phα1β toxin in several animal models of nociception and inflammation, in comparison to ω-conotoxin MVIIA (de Souza et al., 2013; de Souza et al., 2011).

Cyclophosphamide (CPA) is an antineoplastic alkylating agent broadly indicated for the treatment of lymphomas and solid tumors, such as breast cancer. However, CPA has many serious side effects, limiting its use (Joensuu et al., 2012). Among these, hemorrhagic cystitis (HC) is the main adverse effect related to CPA chemotherapy, due to the urinary metabolite acrolein, which causes bladder fibrosis, edema, severe hemorrhage with obstructive renal failure, as well as pain. Moreover, HC can develop within weeks to months in 20-25% of patients who receive high doses of CPA (Emadi et al., 2009). Currently, Mesna is the reference drug used to prevent acute HC, by limiting uroepithelial exposure to acrolein. However, recent studies showed the occurrence of cystitis, both experimentally and clinically, even under the use of Mesna (Lima et al., 2007; Vieira et al., 2003).

It is well established that the central nervous system (CNS) has an important integrative role with the urinary bladder, by mediating distinct functions, such as mechanosensation, bladder filling, micturition reflex, and pain (Birder et al., 2013). There is increasing evidence that afferent Aδ and C fibers can be modulated by urothelial cells, which are able to respond to various types of stimuli such as physiological, psychological and disease-related factors (Birder, 2013). Furthermore, several receptors and ion channels are known to be expressed throughout bladder tissues (urothelium, nerve endings, detrusor muscle and lamina propria), including VGCCs, transient receptor potential (TRP) channels, besides purinergic, cholinergic and neurokinin (NK) receptors (Birder et al., 2013; Jiang et al., 2013a; Moran et al., 2011; Pailleux et al., 2012). Changes in the uroepithelial sensory system

can be associated with detrusor muscle overactivity, neurogenic injury and urinary incontinence (Birder et al., 2013; Everaerts et al., 2010). Noteworthy, it has been recently demonstrated that selective inhibition of L-type VGCC is able to prevent the contractile responses in mice with diabetic bladder dysfunction (Jiang et al., 2013a). In addition, Su et al. (2008) previously showed that spinal blockage of P/Q-type VGCC by ω-conotoxin MVIIC reduced acute bladder nociception induced by mechanical distention in rats. However, there is no previous study investigating whether the inhibition of VGCC could interfere with the symptoms allied to CPA-induced HC. In this regard, the present study investigated, for the first time, the effects of spinal administration of the P/Q- and N-type VGCC blockers Tx3-3 and Phα1β, respectively, isolated from the spider *P. nigriventer*, on painful, inflammatory and functional alterations related to HC induced by CPA. Special attempts have been made in order to compare the effects of *P. nigriventer*-derived toxins, to those displayed by MVIIC and MVIIA extracted from the marine cone snail *C. magus*.

Results

Antinociceptive effects of P/Q and N-type VGCC inhibitors in HC

CPA-induced HC is frequently associated to the occurrence of severe pain in clinical setting. Accordingly, a single i.p. administration of CPA (300 mg/kg) led to marked nociceptive behavior in mice (as evaluated through 4 h), which was inhibited by treating animals with the reference drug Mesna (given i.p., 60 mg/kg, 30 min before CPA) (Figure 1). Of note, the i.t. administration of P/Q-type VGCC inhibitors MVIIC from *C. magus*, or Tx3-3 from *P. nigriventer* (given 2 h post-CPA, at 10, 30 and 50 pmol/site) produced a significant and dose-related inhibition of CPA-elicited visceral nociception in mice. The observed percentages of inhibition were 30 ± 4%, 55 ± 8% and 69 ± 5% for MVIIC, and 25 ± 9%, 38 ± 3% and 68 ± 6% for Tx3-3 toxin, respectively (Figure 1A, 1B and 1C).

Next, to examine whether the selective blockage of N-type VGCC might interfere in pain transmission in this model of HC, two selective N-type VGCC inhibitors were tested: the drug used in clinics for cancer pain MVIIA from *C. magus*, and the toxin from the spider *P. nigriventer* Phα1β. The i.t. administration of MVIIA (10 pmol/site, 2 h post-CPA) promoted a significant decrease of CPA-elicited nociception, with 26 ± 5% of inhibition (Figure 1D). When the animals received Phα1β by i.t. route (50, 100 or 200 pmol/site, 2 h post-CPA), the nociceptive behavior responses were reduced by 29 ± 6%, 18 ± 4% and 39 ± 8%, respectively, without clear dose-dependent effects (Figure 1D and 1E).

Additional assessment of time-related effects of P/Q- and N-type VGCC inhibitors revealed that both MVIIC and Tx3-3 (50 pmol/site) lacked significant effects when administered at 1 or 3 h post-CPA (Figure 1F). Conversely, the N-type blockers MVIIA (10 pmol/site) and Phαβ (50 pmol/site) also displayed significant inhibitory effects on HC-related nociception, when dosed 1 h or 3 h after CPA (Figure 1G).

Modulation of macroscopic inflammation

As it can be observed from Figure 2, CPA-induced HC was associated to high scores of macroscopic edema and hemorrhage, as well as to increased bladder wet weight, in comparison to PBS control groups, according to evaluation at 6 h after HC induction. These inflammatory alterations were sensitive to the administration of Mesna (60 mg/kg, i.p.), given 30 min before and 4 h after CPA. The P/Q-type inhibitors MVIIC or Tx3-3 failed to significantly alter any evaluated indicative of macroscopic inflammation, when administered i.t., at 10 pmol/site, 2 h after CPA (Figure 2A, 2D and 2G). A lack of effect was also observed for MVIIC, given at 30 pmol/site, although the same dose of Tx3-3 significantly inhibited both macroscopic edema (60 ± 10%) and bladder wet weight increase (38 ± 5%) (Figure 2B, 2E and 2H). Either MVIIC or Tx3-3 (50 pmol/site) produced significant reductions of edema scores (by 49 ± 9%, for both toxins) (Figure 2F), whilst only Tx3-3 was able to significantly decrease the hemorrhage index at this dose (63 ± 12%; Figure 2C). Nonetheless, 50 pmol/site of both P/Q-type VGCC inhibitors visibly brought the bladder wet weight near to PBS control values, although this effect was not statistically significant (Figure 2I).

We also tested the potential anti-inflammatory effects of three doses of *P. nigriventer*-derived toxin Phα1β (50, 100 or 200 pmol/site, 2 h post CPA), using MVIIA (10 pmol/site, 2 h post-CPA) as a positive control for selective N-type VGCC inhibition. Interestingly, mice treated i.t. with Phα1β, at 50 pmol/site, displayed a significant reduction of macroscopic edema and bladder wet weight (37 ± 8% and 25 ± 6%, Figure 3D and 3F, respectively), although hemorrhage remained unaffected (Figure 3B). However, this toxin was not effective in reducing any evaluated macroscopic inflammatory marker, when administered i.t., at the doses of 100 and 200 pmol/site. Likewise, a lack of effect was observed for *C. magus* toxin MVIIA (10 pmol/site) against CPA-induced macroscopic inflammation (Figure 3A, 3C and 3E). Further time-course evaluation showed that either tested toxins failed to display

significant effects on CPA-elicited macroscopic inflammation, when administered 1 h and 3 h after CPA (Figure 4A to 4F).

Assessment of neutrophil migration

In order to support data described above on macroscopic inflammation, we assessed the neutrophil migration by determining the MPO activity in bladder tissues, 6 h after CPA-induced HC. CPA administration was associated with a striking increase of MPO activity, compared to the PBS control group. The pretreatment with Mesna (60 mg/kg, i.p., 30 min before and 4 h after CPA) produced a significant reduction of MPO activity. Of high interest, the treatment with N-type VGCC blocker Phα1β (50 pmol/site, 2 h post-CPA), caused a remarkable and significant inhibition of MPO activity (60 ± 9%). Regardless, the other tested toxins, namely MVIIC and Tx3-3 (50 pmol/site) or MVIIA (10 pmol/site) did not alter CPA-related MPO activity in a significant manner (Figure 5A).

Effects of P/Q and N-type blockers on bladder cytokines

As another approach, we evaluated the production of TNF-α, IL-1β, IL-4 and IL-10 in the bladder tissues, 6 h after HC induction by CPA. It is possible to observe that i.p administration of CPA (300 mg/kg) resulted in a prominent increase in the levels of both pro-inflammatory cytokines TNF-α (Figure 5B) and IL-1β (Figure 5C), whereas the levels of anti-inflammatory cytokines IL-4 (Figure 5D) and IL-10 (Figure 5E) remained unchanged. Interestingly, the treatment with Mesna (60 mg/kg), MVIIC (50 pmol/site), Tx3-3 (50 pmol/site) or Phα1β (50 pmol/site) reversed TNF-α to the basal levels (Figure 5B). Furthermore, CPA-elicited IL-1β production was significantly diminished by all tested inhibitors, except MVIIA. The percentages of inhibition were 38 ± 10, 36 ± 12 and 58 ± 6%, for MVIIC, Tx3-3 and Phα1β, respectively (Figure 5C). Neither tested VGCC inhibitors were

able to modify IL-4 levels (Figure 4C), although IL-10 production in bladder tissues was markedly increased in the Phα1β-treated group (50 pmol/site, i.t.). This effect occurred in a significant manner, with a raise percentage of 62 ± 4% (Figure 5E).

Analysis of spinal and peripheral oxidative stress

Considering the remarkable effects of Phα1β from *P. nigriventer* on MPO activity and cytokine formation, we decided to investigate the actions of this toxin on CPA-caused oxidative stress. To this aim, we assessed MDA levels in bladder and spinal cord tissues. HC induced by CPA was accompanied by a moderate, but significant elevation of MDA production in bladder tissues, according to evaluation 6 h post-CPA administration. Of high interest, the treatment with Phα1β (50 pmol/site, 2 h-post CPA), by i.t. route, was able to reduce the MDA levels in bladder tissues by 30 ± 6%, similarly to the reference compound Mesna (29 ± 4%) (Figure 5F). On the other hand, no significant changes of MDA levels were detected in spinal cord tissues, either at 2 ½ or 6 h following CPA administration (results not shown).

Expression of genes related to neurogenic inflammation

We have also examined whether the effects of toxins might be related to the modulation of TRPV1, TRPA1 or NK1 mRNA receptor expression in either bladders or spinal cords of CPA-injected mice. Data depicted in Figure 6 (A and B) demonstrates that HC caused by CPA was associated to a slight increase of TRPV1 and TRPA1 mRNA receptor expression, in the urinary bladder, according to the evaluation at 6 h. The increased expression of TRPV1 mRNA in bladders was virtually prevented by pre-treating animals with Tx3-3 (50 pmol/site), MVIIC (50 pmol/site), Phα1β (50 pmol/site) or MVIIA (10 pmol/site), 2 h after HC induction (Figure 6A). The tested toxins, except MVIIC, also produced a visible

reduction of TRPA1 mRNA expression in the bladder tissues (Figure 6B) in CPA-treated mice, although significant differences were not observed. Nevertheless, bladder NK1 receptor expression was not modified by CPA administration or by any of the tested compounds (Figure 6C). Furthermore, no marked changes of TRPV1, TRPA1 and NK-1 receptor expression were observed in the spinal cords in either tested experimental condition, in relation to the basal expression levels (Figure 6 D to 6F).

Assessment of bladder functional parameters

Subsequently, we investigated to what extent the remarkable effects played by Phα1β on several inflammatory parameters might be correlated to functional cystometry alterations in CPA-induced HC. The i.p. administration of CPA resulted in a marked and significant decrease of the following urodynamic parameters: mean amplitude (Figure 7B), intercontraction interval (Figure 7C), voided volume (Figure 7D), bladder capacity (Figure 7F), and voiding efficiency (Figure 7G). In addition, CPA caused a significant increase of basal pressure (Figure 7A) and number of NVCs (Figure 7E). Of high interest, the i.p. treatment with the reference drug Mesna (60 mg/kg, 30 min before and 4 h post-CPA), or the i.t. administration of the toxin Phα1β (50 pmol/site, 2 h post-CPA), was able to strongly reverse all the evaluated urodynamic parameters (Figure 7A to 7G), except the intercontraction interval in Phα1β-treated animals (Figure 7C). Representative traces for this set of experiments are provided in Figure 7H to 7K.

Interplay between N-type VGCC inhibition and NK1 receptor antagonism

The spinal activation of NK1 receptors by SP represents a pivotal mechanism implicated in bladder overactivity (Seki et al., 2005). In an attempt to examine the effects of Phα1β on NK1 receptor activation, the selective antagonist of this receptor, namely CP-96345

(50 µg/site) was co-administrated with Phα1β toxin (50 pmol/site), via a single i.t. injection, 2 h after CPA-evoked HC. As it can be observed from Figure 8, the i.p. administration of CPA (300 mg/kg) produced a marked increase of nociception scores, allied to decrease of mouse activity (as evaluated through 4 h) and high scores of macroscopic inflammation (6 h post-induction). The isolated i.t. treatment with either CP-96345 (50 µg/site) or Phα1β toxin (50 pmol/site), given 2 h post-CPA, reversed all the evaluated parameters (Figure 8A to 8E). Interestingly, the animals that received the association of CP-96345 plus Phα1β displayed a remarkable reduction of nociception scores, which was significantly different from the inhibition obtained with Phα1β given alone. However, this combined strategy did not produce additive effects on locomotion (measured by activity) (Figure 8B), hemorrhage (Figure 8C), edema (Figure 8D) or bladder wet weight (Figure 8E), in comparison to the isolated treatment with Phα1β toxin.

Discussion

HC is the main toxic effect caused by the widely used chemotherapeutic agent CPA, via generation of the metabolite acrolein, representing a major clinical challenge for the urologists. Besides the occurrence of severe hemorrhage, HC is accompanied by edema, bladder pain, voiding dysfunction, as well as changes at the cellular level, including the increased production of TNF-α, IL-1β and C-reactive protein (Decker et al., 2009; Jiang et al., 2013b). Ion channels, especially VGCCs, are implicated in the modulation of nociceptive and inflammatory alterations, manly by regulating calcium influx and release of neurotransmitters and inflammatory neuropeptides (Park et al., 2010). Relevantly, VGCCs are constitutively expressed in urothelium, interstitial cells and afferent bladder nerves (Birder et al., 2013). A previous study from our group demonstrated that either genic or pharmacological inhibition of purinergic ionotropic P2X7 receptors was able to prevent inflammatory and nociceptive changes allied to CPA-induced HC (Martins et al., 2012). However, there is no previous study investigating whether the spinal inhibition of VGCC could interfere with the symptoms related to CPA-induced acute HC.

Herein, we provide novel evidence about the relevance of spinal P/Q and N-type VGCC in CPA-evoked HC in mice, by evaluating the effects of the purified peptides from the Brazilian spider *P. nigriventer*, Tx3-3 and Phα1β, respectively, or MVIIC and MVIIA toxins, obtained from the marine cone snail *C. magus*, when given intrathecally. The following major findings were achieved: (i) the spinal blockage of P/Q-type VGCC by Tx3-3 and MVIIC, and the selective inhibition of N-type calcium currents by Phα1β peptide attenuated the nociceptive and inflammatory events associated with HC in mice, including bladder oxidative stress and cytokine production; (ii) a single i.p injection of CPA produced an evident increase of bladder TRPV1 and TRPA1 mRNA expression, an effect that was virtually reversed by all the tested toxins; (iii) the inhibition of N-type VGCC by Phα1β strongly prevented the

functional alterations of mouse bladder; and (iv) the spinal co-administration of the selective NK1 receptor antagonist CP-96345 heightened the antinociceptive effects of Phα1β in the mouse model of acute HC.

Severe pain is one of the most relevant symptoms observed in CPA-elicited HC. It is well known that VGCCs play an important role in peripheral pain transmission to CNS, by facilitating the propagation of action potentials along the primary afferent nerves into sensory neurons in the dorsal root ganglia (DRG), via calcium influx (Park et al., 2010). VGCC-blocking toxins, including Tx3-3, Phα1β, MVIIC and MVIIA, have been widely investigated as pharmacological targets for development of new analgesic and anti-inflammatory drugs (Gomez et al., 2002; Lewis et al., 2012). Corroborating this series of studies, our first set of data revealed that i.t. treatment of mice, 2 h post-CPA injection, with the selective P/Q-type VGCC blockers Tx3-3 or MVIIC clearly prevented the nociceptive behavior related to CPA-evoked HC, with a dose-related profile of inhibition for Tx3-3 toxin, and an efficacy similar to Mesna, the most used drug to treat HC in clinics. Earlier evidence has demonstrated that both toxins Tx3-3 and MVIIC displayed antinociceptive effects in inflammatory and neuropathic pain models, albeit Tx3-3 peptide exhibited long-lasting analgesic effects in mice (Dalmolin et al., 2011). Furthermore, the nociceptive visceral score induced by CPA was also reduced by the spinal administration of the N-type VGCC blockers Phα1β and MVIIA, both given 2 h after CPA application. Notably, it has been shown that i.t. administration of Phα1β and MVIIA reduced the acute and chronic pain evoked by paclitaxel in rats, with Phα1β toxin showing less adverse effects, when compared to the FDA-approved drug MVIIA (ziconotide) (Rigo et al., 2013).

As a next step, we examined the time-related effects of *P. nigriventer*- or *C. magus*-derived toxins in the same experimental parameters of nociception, when these toxins were administered 1 and 3 h after CPA injection. As noted, the treatment with the selective P/Q-

type VGCC blockers Tx3-3 and MVIIC did not alter the nociception parameters, when dosed 1 or 3 h after CPA application. Otherwise, the spinal pharmacological inhibition of N-type VGCC by Phα1β or MVIIA greatly decreased CPA-elicited nociception, when given 1 h or 3 h after CPA. Thus, it is feasible to suggest that blockage of N-type calcium channels by Phα1β from *P. nigriventer* or MVIIA from *C. magus* showed a superior antinociceptive activity, with long-lasting effects. Nonetheless, all the tested toxins were found effective to alleviate HC-related bladder nociception, probably by modulating P/Q and N-type calcium currents and peripheral pain transmission to CNS. Beyond CPA-elicited HC, there is a series of other clinical conditions associated to severe bladder pain, such as bladder overactivity (OAB) or interstitial cystitis (Kim et al., 2013). In this context, it is tempting to suggest that spinal inhibition of P/Q, and mainly N-type VGCCs, might be an attractive therapeutic option, especially when unresponsiveness to the currently available analgesic drugs is observed.

The activation of TRP-related channels is responsible for regulating bladder sensory perception, as well as bladder function (Yu et al., 2011). It has been demonstrated that TRPA1 receptor exerts an important role in OAB after spinal cord injury (SCI) (Andrade et al., 2011). Furthermore, Dornelles *et al.* (2013) showed that TRPV1 mRNA is upregulated in the rat bladder after CPA administration, and the systemic pharmacological inhibition of TRPV1 was capable of diminishing pain behavior and bladder dysfunction related to CPA. Allied to these findings, the results presented herein demonstrate that HC caused by CPA was associated to an increase of TRPV1 and TRPA1 mRNA receptor expression in the urinary bladder of mice. Of relevance, the increased expression of TRPV1 mRNA in the bladders was virtually prevented by treating animals with Tx3-3, MVIIC, Phα1β or MVIIA after HC induction. These toxins, except MVIIC, also produced a clear reduction of TRPA1 mRNA expression in the bladder tissues in CPA-treated mice, although significant differences were not observed. TRP channels have a complex and heterogenic distribution throughout the mouse urinary

bladder (considering the urothelium, basement membrane and lamina propria) or in the sensory neurons that innervate the lower urinary tract, what might well explain the absence of significant effects in our study (Everaerts et al., 2010; La et al., 2011; Vandewauw et al., 2013; Yu et al., 2011). However, it is worth mentioning that i.p CPA administration or i.t. treatment with all the tested toxins did not cause any alteration in the expression of TRPV1 and TRPA1 mRNA in the spinal cord. Even though, our data indicates that antinociceptive action follow-on the spinal inhibition of either P/Q- or N-type VGCC is related to the peripheral modulation of TRPV1 and TRPA1 receptors.

It is well known that SP acting via NK1 receptor activation facilitates bladder nociception and inflammatory responses (Chien et al., 2003; Maggi, 1995). Nonetheless, in this study, our data revealed that NK1 receptor expression was not modified by CPA administration or by any of the tested compounds, as assessed in bladders or spinal cords. This allows us to suggest that CPA-induced HC and spinal inhibition of VGCC might lead to changes of NK1 receptor function, rather than expression. In fact, this hypothesis was tested in the final part of our study.

Calcium channels have been shown to be consistently involved in the pathophysiology of inflammation (Dianzani et al., 2001; Rahman et al., 2013; Yamamoto et al., 2010). For instance, it was demonstrated that selective inhibition of N-type VGCC by N-triazole oxindole (TROX-1) was able to prevent inflammatory hyperalgesia in rats, with an efficacy similar to that seen for a series of non-steroidal anti-inflammatory drugs (Abbadie et al., 2010). Extending this notion, the results of the present study revealed, for the first time, the involvement of both P/Q- and N-type VGCC in bladder inflammation induced by CPA. Accordingly, the hemorrhage or edema induced by CPA was greatly diminished in mice that had been treated with the selective P/Q-type VGCC blockers Tx3-3 and MVIIC, while the N-type VGCC inhibitor Phα1β produced a significant reduction of edema and bladder wet

weight, when these toxins were dosed 2 h after CPA. Of note, the drug clinically used for refractory pain MVIIA failed to alter macroscopic inflammation, what allow to us to propose additional beneficial effects for Tx3-3, MVIIC and Phα1β. Therefore, these latter toxins were found effective in preventing both painful and inflammatory alterations associated to CPA-induced HC, supporting their possible therapeutic application for treating cystitis, especially in clinical conditions resistant to the reference drug Mesna.

Data on macroscopic inflammation prompted us to examine whether the i.t. administration of Tx3-3 and Phα1β from *P. nigriventer*, or MVIIC and MVIIA from *C. magus* might affect neutrophil migration and cytokine production elicited by CPA. Our results are in full accordance with previous publication by our group, demonstrating that CPA administration to mice resulted in a marked increase of MPO activity, as well as TNF-α and IL-1β levels in bladder samples (Martins et al., 2012). Strikingly, the i.t. treatment with the selective N-type VGCC blocker Phα1β caused a marked and significant inhibition of MPO activity, while the other tested toxins, MVIIC, Tx3-3 or MVIIA did not alter this parameter in a significant manner. Indeed, the relevance of N-type VGCC have been extensively reported under inflammatory pain, due to their potential distribution throughout the central and peripheral terminals, contributing to inflammatory signaling, principally in organs innervated by spinal cord, such as the urinary bladder (Brittain et al., 2011; Matthews et al., 2001; Pradhan et al., 2013; Waterman, 1996). In addition, our data also revealed that i.t. treatment with MVIIC, Tx3-3 or Phα1β brought TNF-α to the basal levels. Furthermore, CPA-elicited IL-1β production was significantly diminished by all the tested inhibitors, except by MVIIA, supporting our previous results on edema and hemorrhage.

We also attempted to determine to what extent the spinal blocking of P/Q- or N-type VGCC could modulate the expression of anti-inflammatory cytokines, such as IL-4 and IL-10, in the bladder of CPA-injected mice. Previous literature data indicate that IL-4 was able to

widely reduce the severity of HC induced by ifosfamide in mice, probably via inhibition of TNF-α and IL-β production (Macedo et al., 2012; Malley et al., 2002). In addition, recent evidence showed that herpes simplex virus vector-mediated interleukin-4 expression in the bladder and bladder afferent pathways widely prevented inflammation, bladder overactivity and nociceptive behavior caused by resiniferatoxin in rats (Oguchi et al., 2013). Nevertheless, we failed to demonstrate any significant change of IL-4 levels in our study, even under treatment with either evaluated VGCC-blocking toxin. Noticeably, IL-10 levels in bladder tissues were found markedly increased in the Phα1β-treated group. It was demonstrated prior that epidural administration of the neurotoxin HWTX-I, derived from the Chinese bird spider *O. huwena*, produced a marked inhibition of joint inflammatory pain and TNF-α production, allied to an increase of IL-4 and IL-10 serum levels, in a rat model of rheumatoid arthritis (Wen Tao et al., 2011). An overall analysis of this data permits us to suggest that anti-inflammatory and analgesic effects displayed by the peptide toxin Phα1β in the CPA model of HC, are mediated, at least partially, by the ability to increase the IL-10 production in mouse bladder tissues. It is tempting to propose that *P. nigriventer*-derived toxin Phα1β might well represent a promising alternative to control the symptoms allied to HC, or other inflammation-related diseases affecting the urinary bladder.

During tissue damage and inflammation, reactive oxygen species (ROS), such as hydrogen peroxide, are generated endogenously by infiltrating macrophages and neutrophils (Rhee, 2006; Winterbourn, 2002). Exposure of cellular membranes to inflammatory ROS or exogenous oxidants (including the metabolite acrolein derived from CPA) causes membrane lipid peroxidation (Sayre et al., 2006). Hence, we sought to investigate the MDA levels, a final product of lipid peroxidation, in bladder and spinal cord tissues as indicative of oxidative stress in mice with HC caused by CPA. Considering the remarkable effects of Phα1β from *P. nigriventer* on MPO activity and cytokine formation, we exclusively investigated this toxin in

MDA production. HC induced by CPA was accompanied by a moderate, but significant elevation of MDA production in bladder tissues. Of high interest, the treatment with Phα1β, by i.t. route, greatly reduced the MDA levels in bladder tissues, similarly to the reference compound Mesna. On the other hand, no significant changes of MDA levels were detected in spinal cord tissues, either at 2 ½ or 6 h following CPA administration. Thus, we surmise that blockage of N-type VGCC by Phα1β reduces neutrophil migration, and consequently attenuates ROS production. This hypothesis is supported by previous evidence showing that ROS generation is abolished in MPO knockout mice with spinal cord injury (Kubota et al., 2012).

The next aim of the present study was to investigate the potential actions of the N-type VGCC blocker Phα1β on urodynamic alterations caused by CPA in mice, on the basis of its remarkable effects on several inflammatory parameters. Distinctly, the i.t. administration of Phα1β was able to robustly reverse all the evaluated urodynamic parameters, including basal pressure, voided volume, bladder capacity, voiding efficiency and number of NVCs. Curiously, this inhibitory effect was similar to that seen for the reference prophylactic agent Mesna. Of interest, it has recently been shown that bladder dysfunction can develop in clinics, as a result of several neurological conditions, such as diabetic neuropathy, human immunodeficiency virus (HIV)-associated neuropathy, amyloid neuropathy and SCI (Burakgazi et al., 2012; Chen et al., 2011; Nardulli et al., 2012), what might point out Phα1β as a promising therapeutic alternative for treating these disorders. Moreover, a similar result was obtained from recent experiments showing that N-type VGCC blockage improves the number of NVCs, bladder capacity and voided volume in rats after bladder outlet obstruction (Igawa et al., 2013). Allied to literature data, our results clearly indicate that spinal blockage of N-type VGCC, through Phα1β toxin derived from the spider *P. nigriventer*, reverses

bladder dysfunction induced by CPA in mice. This evidence may open avenues for drug development, especially for conditions related to bladder dysfunction, such as cystitis.

As a final goal of this study, we sought to investigate the possible role of SP and NK1 receptor activation, in modulating either nociceptive or inflammatory responses evoked by CPA in mice, by the use of the selective antagonist CP-96345, directly injected into spinal cord, in combination with Phα1β toxin. As expected, the isolated i.t. treatment with CP-96345 improved all the evaluated parameters. However, the animals that received the association of CP-96345 plus Phα1β displayed a remarkable reduction of nociception scores, which was significantly higher than the inhibition obtained for Phα1β given alone. This combined strategy also displayed a tendency for additive effects on locomotion and inflammatory parameters, in comparison to the isolated treatment with Phα1β toxin, but this effect was not significant. We could therefore conclude that analgesic effects of Phα1β are mediated, at least partially, by modulation of NK1 receptors activity. Supporting this hypothesis, a recent study conducted by Malykhina et al. (2013) showed that SP contributes to nerve damage in bladder outlet obstruction and triggers secondary changes in the contraction/relaxation mechanisms in the lower urinary tract.

In conclusion, as a major novelty of this study, we have demonstrated, for the first time, that spinal P/Q-type VGCC (Tx3-3, MVIIC) or N-type VGCC (Phα1β, MVIIA) blockers are effective in controlling nociceptive and inflammatory processing in the mouse model of CPA-induced HC, probably by interfering with TRPV1 and TRPA1 receptor expression, and by inhibiting the production of TNF-α and IL-1β. In the case of the preferential N-type inhibitor Phα1β from *P. nigriventer*, the analgesic, the anti-inflammatory and recovering-functional actions appear to rely on the reduction of neutrophil migration, which in turn might diminishes lipid peroxidation. Moreover, the beneficial effects of Phα1β seem to be likely dependent on the bladder increase of the anti-inflammatory cytokine IL-10,

as well as to the modulation of NK1 receptor activity. Taken together our data suggests that epidural administration of P/Q- or N-type VGCC inhibitors would represent a new attractive therapeutic option for treating patients with severe symptoms allied to HC. Concerning particularly the N-type VGCC blocker Phα1β, we put forward a promising application for the treatment of distinct diseases related to bladder dysfunction, such as SCI, diabetic neuropathy, as well as cystitis.

Materials and Methods

Drugs and reagents

The following drugs were used: cyclophosphamide (Genuxal - Baxter Oncology GmbH; Halle/Westfalen, Germany) and 2-mercaptoethanol sodium sulfonate (Mesna – Eurofarma; São Paulo, Brazil) were purchased from Medilar (Porto Alegre, Brazil); ω-conotoxins MVIIA and MVIIC were purchased from Latoxan (Valence, France); Phα1β and Tx3-3 were purified by a combination of gel filtration, reverse phase FPLC/FPLC and ion exchange high-performance liquid chromatography (HPLC), as previously described (Cordeiro et al., 1993). All the samples used in this study presented a purity superior to 95%. Phα1β and Tx3-3 have molecular weights of 6044.39 and 6300.00 Da, respectively, and their amino acid sequences are ACIPRGEICTDDCECCGCDNQCYCPPGSSLGIFKCSCAHANKYFCNRKKEKCKKA and GCANAYKSCNGPHTCCWGYNGYKKACICSGXNWK, respectively (Gomez et al., 2002); (2S,3S)-*N*-(2-Methoxyphenyl)methyl-2-diphenylmethyl-1-azabicyclo[2.2.]octan-3-amine (CP-96345) was purchased from Tocris Bioscience (Bristol, United Kingdom). Cyclophosphamide and Mesna were diluted in distilled water. All toxins (MVIIA, MVIIC, Phα1β and Tx3-3) were prepared in phosphate-buffered saline (PBS) in siliconized plastic tubes and maintained at -18 °C. CP-96345 was solubilized in 1% DMSO with gentle warming.

Experimental animals

Male Swiss mice (25-30 g) were used throughout this study. Swiss mice were obtained from Federal University of Pelotas (UFPEL; Pelotas, Brazil). The animals were housed in groups of five per cage and maintained in controlled temperature (22 ± 1 ºC) and humidity (60-70%), under a 12 h light-dark cycle (lights on 08:00 AM), with food and water *ad libitum*.

Animals were acclimatized to the experimental room for at least 1 h before the experiments. All the tests were performed between 8:00 AM and 8:00 PM. The experimental procedures reported in this manuscript followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 85-23, revised 1996), and Ethical Guidelines for the Investigation of Experimental Pain in Conscious Animals (Zimmermann, 1983). The protocols were approved by the Local Animal Ethics Committee (protocol number: 12/00292). Euthanasia was carried out under deep anesthesia by isoflurane inhalation. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. For all tests, the animals were distributed randomically throughout the experimental groups.

Pharmacological treatments

In this study, HC was induced by a single intraperitoneal (i.p.) administration of CPA (300 mg/kg) (Martins et al., 2012). The animals were treated intrathecally (i.t.) with toxins obtained from the spider *P. nigriventer* Phα1β (50, 100 or 200 pmol/site; 5 µl) or Tx3-3 (10, 30 or 50 pmol/site; 5 µl), or with toxins from the marine cone snail *C. magus* MVIIA (10 pmol/site; 5 µl) or MVIIC (10, 30 or 50 pmol/site; 5 µl). In some cases, the animals received the selective NK1 receptor antagonist CP-96345 (50 µg/site; 5 µl). The reference drug Mesna (60 mg/kg, i.p.) was given in two doses, 30 min before and 4 h after injection of CPA. The toxins Phα1β, Tx3-3, MVIIA and MVIIC were administered as a single i.t. dose, at 1, 2 or 3 h after CPA administration. CP-96345 was co-administrated with Phα1β via a single i.t. injection, 2 h after CPA-evoked HC. The control groups received the respective vehicle used to dissolve the drugs. The schemes of administration for all drugs were selected on the basis of literature data (Chien et al., 2003; Dalmolin et al., 2011; de Souza et al., 2011; Martins et al., 2012). The experimenters were unaware of the pharmacological treatment.

Behavioral assessment of nociception

The experimental protocols were carried out according to the method described by Martins et al., (2012), with minor modifications. These experiments were performed between 8:30 and 12:30 AM to minimize the potential circadian variations in the behavioral responses. One day before, the mouse dorsum was shaved for allowing intrathecal injection. On the day of experiments, the animals were adapted in individual new cages, without sawdust bedding, for a period of 30 min. Subsequently, each animal received an i.p. injection of CPA and it was returned to the respective cage. The nociception spontaneous behavior was measured for 2 min, every 30 min, over a total period of 4 h. During the 2-min period, each animal was evaluated for: (i) activity (walking, rearing, climbing and grooming); (ii) immobility; and (iii) behaviors indicative of visceral nociception (‘crises’). The nociception behavior was scored according to (Olivar and Laird, 1999): 0 = normal; 1 = piloerection; 2 = strong piloerection; 3 = labored breathing; 4 = licking of the abdomen; or 5 = stretching and contractions of the abdomen. If the animal presented more than one behavioral alteration, the scores were summed. For nociception assessment, 6 to 8 Swiss mice per group were used. The same animals were employed for evaluating macroscopic inflammation and measurement of mieloperoxidase (MPO) activity.

Evaluation of bladder macroscopic inflammation

For this analysis, the animals were euthanized 6 h after CPA administration. The macroscopic examination was based on criteria previously established by Gray et al., (1986). All bladders were dissected free from connecting tissues, and transected at the bladder neck. Each bladder was macroscopically assessed, by two examiners with experience in this technique, blinded to the experimental groups. The edema formation was categorized: 0 = absent, 1 = mild edema in the internal mucosa, 2 = edema confined to the all internal mucosa,

or 3 = fluid externally in the walls of the bladder, as well as internally. Moreover, the bladders were also surveyed for hemorrhage, according to the respective score: 3 = presence of intravesical clots, 2 = mucosal hematomas, 1 = dilatation of the bladder vessels, or 0 = normal aspect. As an additional measure of edema, the wet weight of each bladder was registered and expressed as milligram per 100 g of animal body weight (Gray et al., 1986).

Measurement of myeloperoxidase (MPO) activity

Neutrophil recruitment to the mouse bladder was quantified indirectly by tissue MPO activity, according to the method described by Martins et al., (2012), with minor modifications. Bladders were removed at 6 h after CPA injection and were immediately frozen at -80 °C. After, the tissues were homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 5000 rpm for 20 min, at 4 °C. The pellets were resuspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were re-centrifuged (5000 rpm, 20 min, 4 °C). Twenty-five µl of the supernatant were used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$ and 0.3 mM hydrogen peroxide. The absorbance was measured at 595 nm, and the results are expressed in optical density per milligram of tissue.

Cytokine production in bladder tissue

The procedure used was similar to the method described by Fernandes et al., (2005). The animals were treated with CPA (300 mg/kg, i.p.), and the bladders were collected at 6 h. The tissues were homogenized in PBS containing: 0.05% Tween-20, 10 mM EDTA, 0.4 M NaCl, 0.5% BSA, 0.1 mM benzethonium chloride and 0.1 M PMSF. Afterward, the samples were centrifuged at 6900 rpm for 10 min, at 4 ºC, and the supernatant (100 µl) were used for the assay. TNF-α, IL-1β, IL-4 and IL-10 levels in bladder tissues were analyzed by sandwich

enzyme-linked immunosorbent assays (ELISA) using dual-set ELISA kits according to the manufacturer's instructions (R&D Systems; Minneapolis, USA). The results are expressed in picograms per milligram of tissue. For these experiments, 5 to 6 animals per group were used.

Malondialdehyde (MDA) assay

The levels of MDA were measured as indicative of oxidative stress, according to the method described by Boeira et al., (2011), with minor modifications. The bladders of 5 mice per group were collected 6 h after CPA-injection. Tissue homogenates 1:19 (w/v) were prepared in 0.9% saline solution. Immediately after, the samples were centrifuged at 3000 rpm for 10 min, at 4 ºC, and the supernatant (200 µl) was homogenized with 1M NaOH (100 µl). Alkaline hydrolysis of protein-bound MDA was achieved by incubating this mixture in a 60-°C water bath for 30 min, followed by addition of milli-Q water (200 µl), 39.9 mM thiobarbituric acid (250 µl), 440 mM phosphoric acid (750 µl), and incubation in a 95 °C water bath for 60 min. After, the samples were chilled on ice. Before the injections into HPLC, 500 µl of sample and 9:1 (v/v) methanol/NaOH 1M were mixed, and the resulting products were centrifuged at 12000 rpm for 30 s, 4 ºC. Finally, 20 µl of the supernatant were injected into an HPLC equipped with ultraviolet detector (Agilent Technologies Inc.; Santa Clara, USA). The protein content in the supernatant was determined with a commercial kit (Labtest; Lagoa Santa, Brazil). MDA levels were calculated from the standard curve using the 1,1,3,3- tetraethoxy propane (97%) and expressed in nanomoles per milligram of protein.

Expression of TRPV1, TRPA1 and NK1 by RT-qPCR analysis

Bladders and spinal cords were dissected 6 h after CPA-induced HC, and were stored in 300 µl of TRIzol Reagent® (Sigma; St Louis, USA). Immediately after, the tissues were frozen at -80 ºC for assays. The total RNA was isolated with TRIzol Reagent® in accordance

with the manufacturer's instructions. Total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System from 1 µg of total RNA, in accordance with manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen; Carlsbad, USA) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µl using 12.5 µl of diluted cDNA (1:50), containing a final concentration of 0.2× SYBR Green I, 100 µM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum Taq DNA Polymerase (Invitrogen; Carlsbad, USA) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting curve analysis was included and fluorescence was measured from 60 °C to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems; Carlsbad, USA), and the $2^{-\Delta\Delta ct}$ method was used for data analysis. The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the reference genes Tbp and Hprt (M-value) (Pernot et al., 2010) and the optimal number of reference genes according to the pair wise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). For the expression assays, the experimental N was 8 per group.

In vivo cystometric parameters

The urodynamic functional analysis was performed 6 h after CPA-evoked HC, following the method described in rats by Andrade et al., (2011), and adapted for mice (N = 4-6/group). The animals were anesthetized with urethane (1.2 g/kg, i.p.). After 30 min, a polyethylene catheter-10 (Clay Adams; Parsippany, USA) was inserted via a midline abdominal incision into the bladder through the bladder dome. The intravesical catheter was

connected via a three-way stopcock to a pressure transducer (ADInstruments; Castle Hill, Australia) and to a micro-infusion pump (Insight Equipamentos Científicos; São Paulo, Brazil) to record intravesical pressure and to infuse saline into the bladder, respectively. Intravesical pressure was recorded continuously using data acquisition software (PowerLab 8/30 ADInstruments; Castle Hill, Australia). After catheter implantation, mice were left untouched for 30 min for bladder stabilization. After this period, the animals received a continuous infusion of 0.9% NaCl at a rate of 20 µl/min, during 30 min.

We assessed the basal pressure (BP; the lowest bladder pressure between micturitions), micturition pressure (MP; maximum bladder pressure during micturition) and the intercontraction interval (ICI). The mean amplitude (maximum bladder pressure, less, threshold pressure) and the number of non-voiding contractions (NVCs) were also measured. NVCs were defined as rhythmic intravesical pressure increases greater than 5 mmHg from baseline pressure without release of fluid from the urethra. Saline voided from urethral meatus was collected and the voided volume (VV) was measured. In order to determine the residual volume (RV), saline infusion was stopped at the beginning of the voiding contraction, and the RV was measured by withdrawing saline through the intravesical catheter and then manually expressing the remaining intravesical contents by exerting pressure on the bladder abdominal wall. The bladder capacity (BC) was calculated as mean VV plus RV. The voiding efficiency (VE) was estimated as a percentage using the following equation: VE = [(VV/BC) x 100].

Statistical analysis

The results are presented as the mean ± standard error mean of 4 to 8 animals per group, depending on the experimental protocol. The percentages of inhibition were calculated as the mean of inhibitions obtained for each individual experiment. Statistical comparison of the data was performed by one-way analysis of variance (ANOVA) followed by Newman-

Keuls post hoc test. *P*-values less than 0.05 ($P < 0.05$) were considered significant. All tests and the production of graphs were performed using the GraphPad 5 Software (San Diego, USA).

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Conflict of interest

The authors have no competing financial interests.

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Figure 1

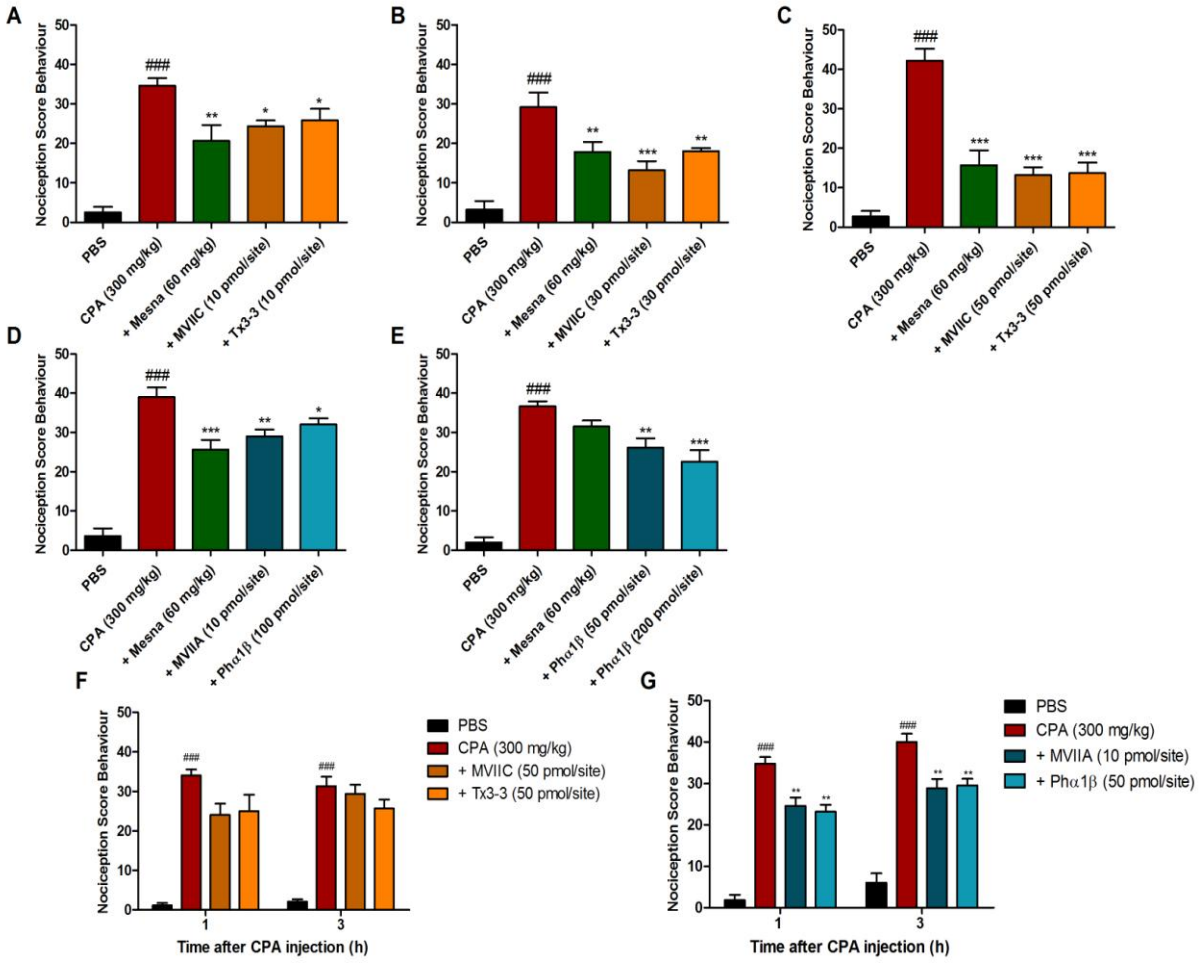

Figure 1. Epidural P/Q and N-type VGCC blockage modulates visceral nociception following HC induction. Nociception score behavior was measured for 2 min, at 30 min intervals, over a total period of 4 h after intraperitoneal (i.p.) injection of cyclophosphamide (CPA). (A to C) Effects of treatment with the reference drug Mesna (60 mg/kg, i.p., given 30 min before CPA), P/Q-type voltage-gated calcium channel (VGCC) blockers MVIIC from *Conus magus*, or Tx3-3 from *Phoneutria nigriventer* (10, 30 or 50 pmol/site, respectively, 2 h post-CPA), given by intrathecal (i.t.) route, on the nociceptive responses in the model of CPA-evoked hemorrhagic cystitis (HC) in Swiss mice. (D, E) Effects of treatment with the reference compound Mesna (60 mg/kg, i.p., given 30 min before CPA), (D) N-type VGCC blockers MVIIA from *Conus magus* (10 pmol/site, i.t., 2 h post-CPA), or (D, E) Phα1β from

Phoneutria nigriventer (50, 100 or 200 pmol/site, i.t., 2 h post-CPA), on the nociceptive responses in CPA-induced HC. (F) Time-related effects of MVIIC or Tx3-3 (50 pmol/site, i.t.), dosed 1 h or 3 h after CPA injection, on the nociceptive responses in CPA-induced HC in Swiss mice. (G) Time-related effects of MVIIA (10 pmol/site, i.t.) or Phα1β (50 pmol/site, i.t.), dosed 1 h or 3 h after CPA injection, on the nociceptive responses in CPA-induced HC in Swiss mice. Differences in the nociceptive score behavior was determined by one-way analysis of variance, followed by Newman-Keuls post-hoc test. Each column represents the mean of 6–8 animals, and the vertical lines show the standard error mean. $^{###}P < 0.001$ significantly different from PBS values. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ significantly different from CPA values. CPA = cyclophosphamide; PBS = phosphate buffered saline.

Figure 2

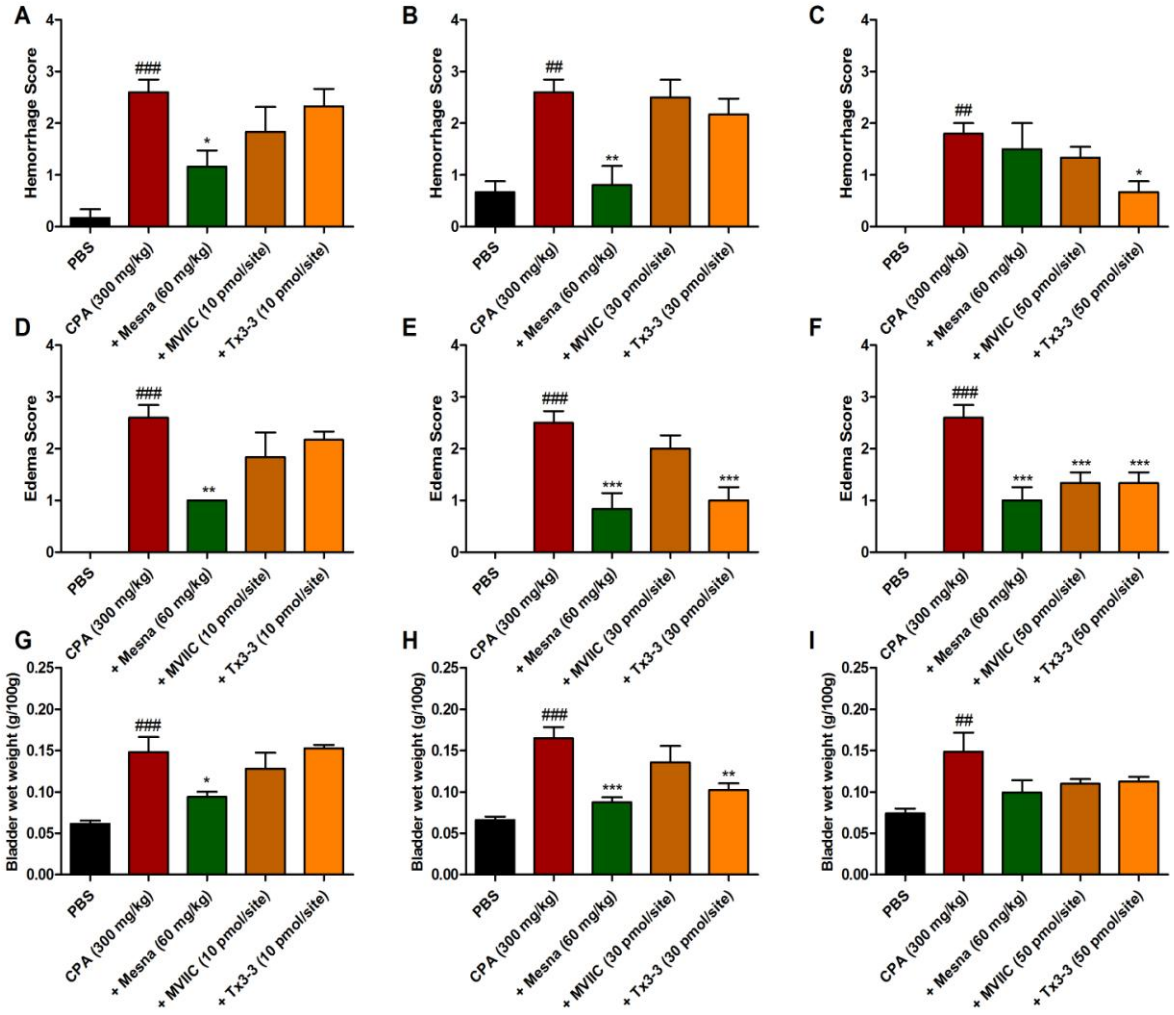

Figure 2. P/Q-type VGCC is required for bladder inflammation associated to CPA. Macroscopic inflammation was assessed 6 h after intraperitoneal (i.p.) administration of cyclophosphamide (CPA). Effects of treatment with the reference drug Mesna (60 mg/kg, i.p., given 30 min before and 4 h post-CPA), P/Q-type voltage-gated calcium channel blockers MVIIC from *Conus magus*, or Tx3-3 from *Phoneutria nigriventer* (10, 30 or 50 pmol/site, 2 h post-CPA), by intrathecal (i.t.) route, on macroscopic hemorrhage (A to C), edema (D to F) and on bladder wet weight (G to I), respectively, in the model of CPA-evoked hemorrhagic cystitis in Swiss mice. Differences in the macroscopic inflammation scores were determined by one-way analysis of variance followed by Newman-Keuls post-hoc test. Each column

represents the mean of 5–6 animals, and the vertical lines show the standard error of the mean value. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ significantly different from PBS values. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ significantly different from CPA values. CPA = cyclophosphamide; PBS = phosphate buffered saline.

Figure 3

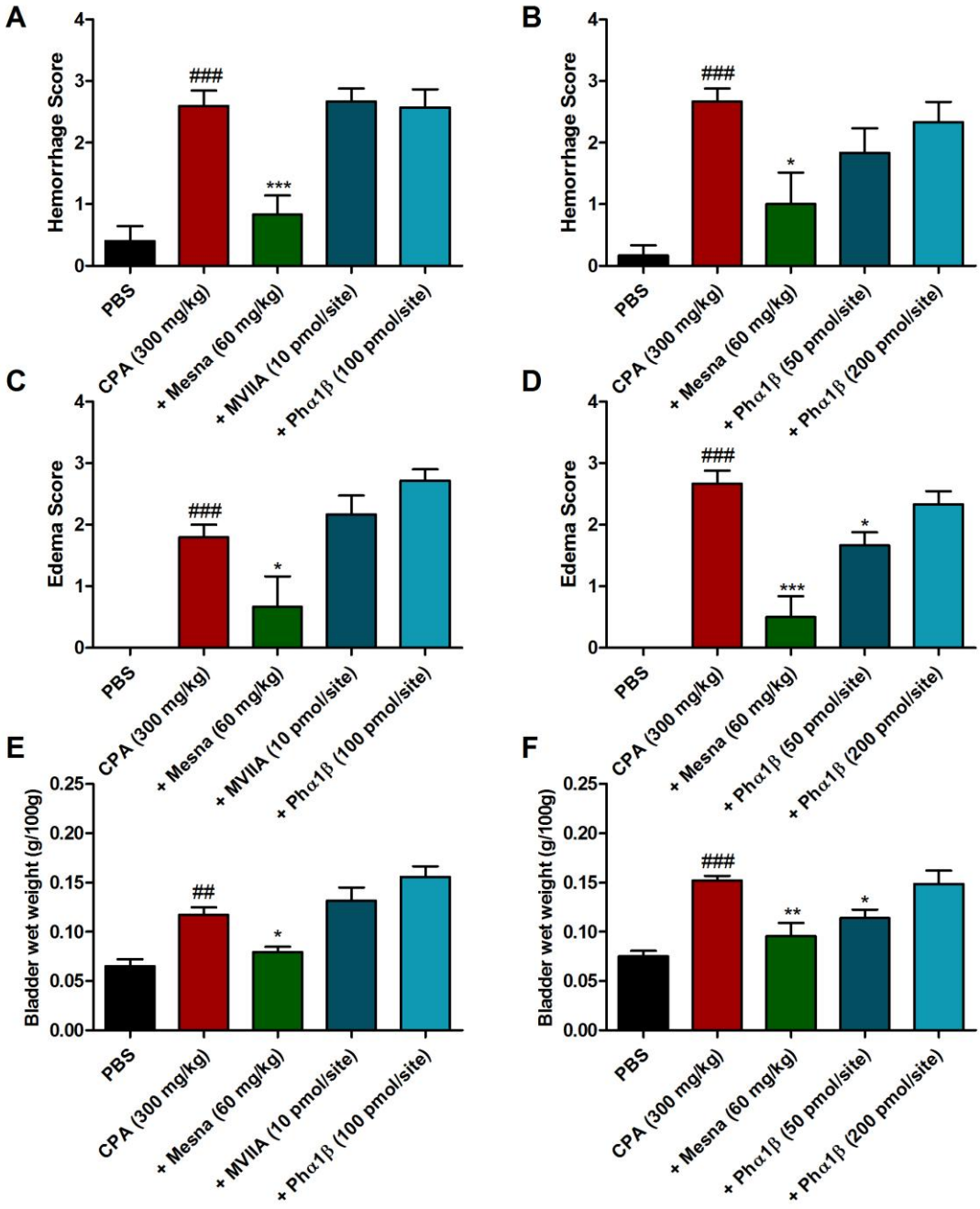

Figure 3. Preferential N-type VGCC inhibitor promotes bladder edema repair after CPA injection. Macroscopic inflammation was evaluated 6 h after intraperitoneal (i.p.) administration of cyclophosphamide (CPA). Effects of treatment with the reference drug Mesna (60 mg/kg, i.p., given 30 min before and 4 h post-CPA), N-type voltage-gated calcium channel blockers MVIIA from *Conus magus* (10 pmol/site, 2 h post-CPA), or *Phoneutria*

nigriventer-derived Phα1β toxin (50, 100 or 200 pmol/site, 2 h post-CPA), by intrathecal (i.t.) route, on macroscopic hemorrhage (A, B), edema (C, D) and on bladder wet weight (E to F), respectively, in the model of CPA-evoked hemorrhagic cystitis in Swiss mice. Differences in the macroscopic inflammation scores were determined by one-way analysis of variance followed by Newman-Keuls post-hoc test. Each column represents the mean of 5–7 animals, and the vertical lines show the standard error of the mean value. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ significantly different from phosphate buffered saline (PBS) values. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from CPA values. CPA = cyclophosphamide; PBS = phosphate buffered saline.

Figure 4

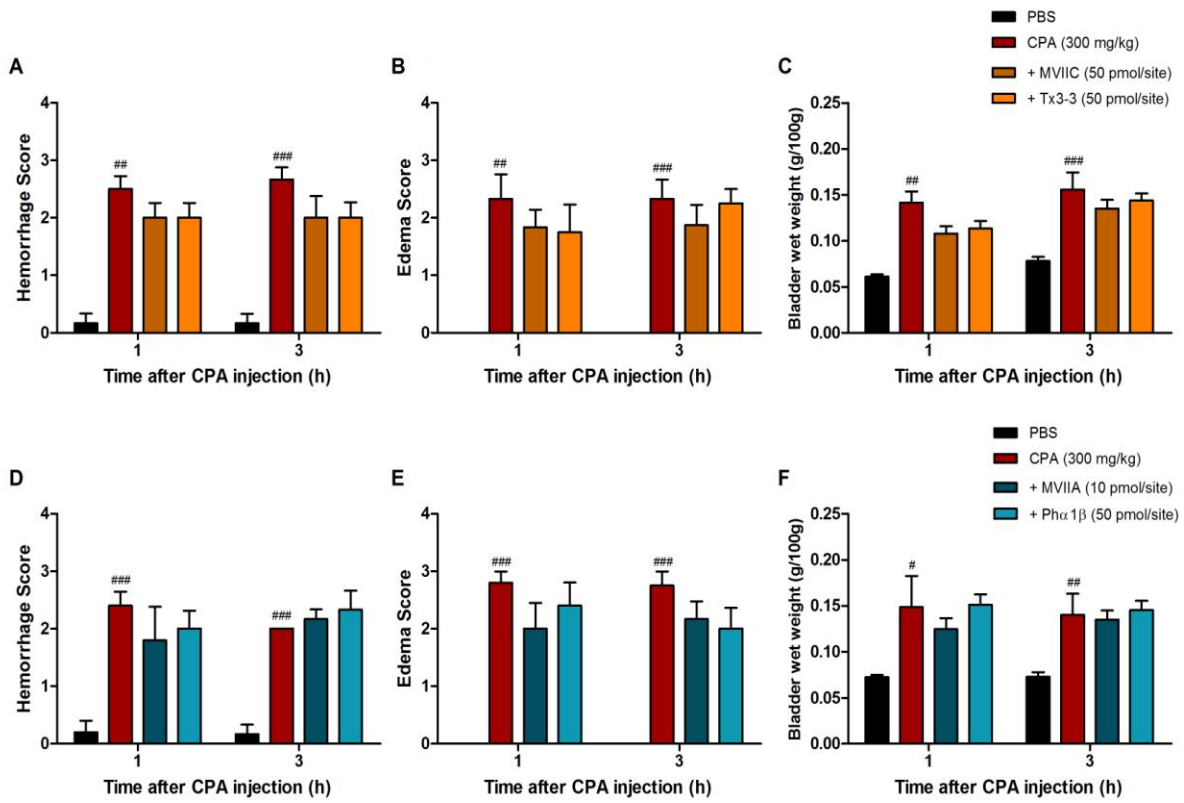

Figure 4. **Evaluation of VGCC blockers on bladder inflammation, given 1 or 3 h after CPA administration.** Time-course profile of voltage-gated calcium channel blockers on macroscopic inflammation induced by intraperitoneal (i.p.) injection of cyclophosphamide (CPA). Effects of treatment with P/Q-type voltage-gated calcium channel (VGCC) blockers MVIIC from *Conus magus*, or *Phoneutria nigriventer*-derived Tx3-3 toxin (50 pmol/site), given by intrathecal (i.t.) route 1 or 3 h after CPA, on macroscopic hemorrhage (A), edema (B) and on bladder wet weight (C), in the model of CPA-evoked hemorrhagic cystitis (HC) in Swiss mice. Effects of treatment with N-type VGCC blockers MVIIA from *Conus magus* (10 pmol/site, i.t.), or *Phoneutria nigriventer*-derived Phα1β toxin (50 pmol/site, i.t.), dosed 1 or 3 h after CPA, on macroscopic hemorrhage (D), edema (E) and on bladder wet weight (F), in the model of CPA-evoked HC in Swiss mice. Differences in the macroscopic inflammation scores were determined by one-way analysis of variance followed by Newman-Keuls post-

hoc test. Each column represents the mean of 6–8 animals, and the vertical lines show the standard error of the mean value. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ significantly different from phosphate buffered saline (PBS) values. CPA = cyclophosphamide; PBS = phosphate buffered saline.

Figure 5

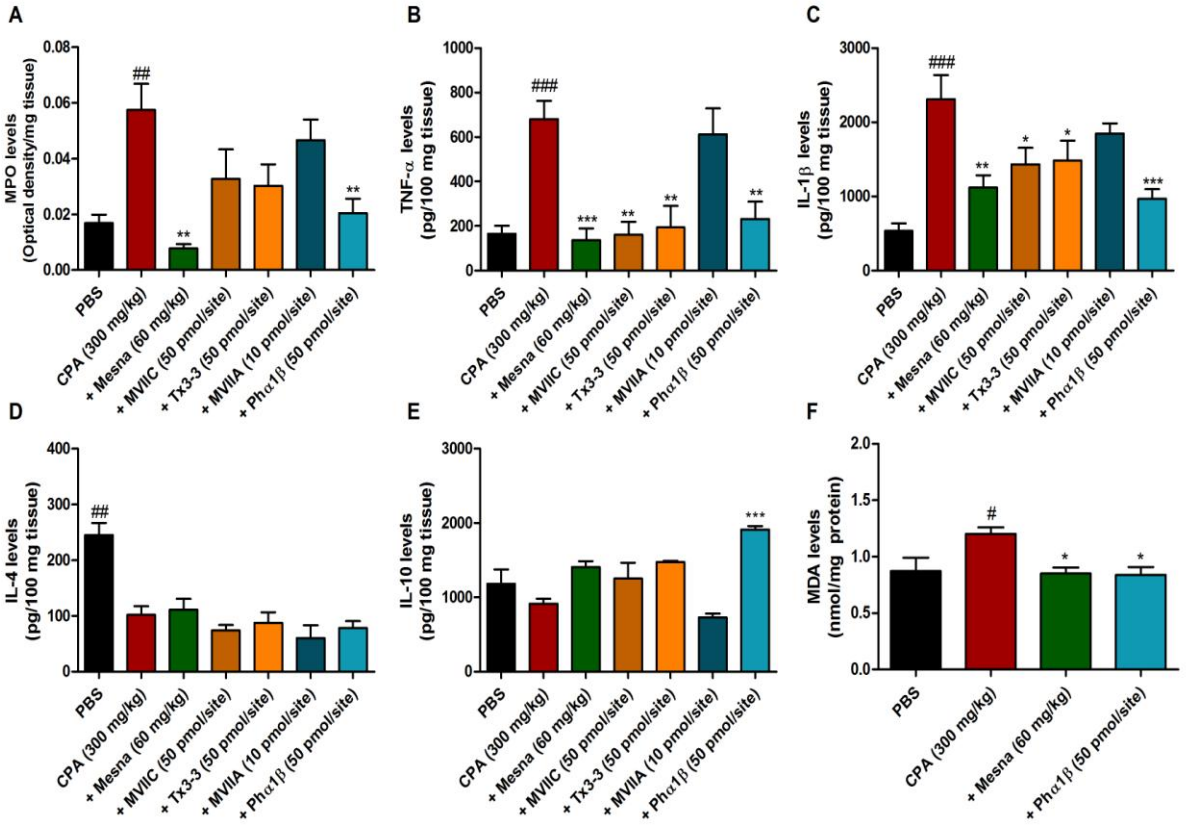

Figure 5. **Neutrophil migration, cytokine production and oxidative stress in the bladder tissue are modulated by epidural P/Q or N-type calcium channel blockage.** Neutrophil migration, cytokine formation and oxidative stress were measured in bladder tissues at 6 h after intraperitoneal (i.p.) administration of cyclophosphamide (CPA). Effects of treatment with the reference drug Mesna (60 mg/kg, i.p., given 30 min before and 4 h post-CPA), P/Q-type voltage-gated calcium channel (VGCC) blockers MVIIC from *Conus magus*, given by intrathecal (i.t.) route, *Phoneutria nigriventer*-derived Tx3-3 toxin (50 pmol/site, i.t.), N-type VGCC blockers MVIIA from *Conus magus* (10 pmol/site, i.t.) or *Phoneutria nigriventer*-derived Phα1β toxin (50 pmol/site, i.t.), on myeloperoxidase (MPO) activity (A), generation of TNF-α (B), IL-1β (C), IL-4 (D) and IL-10 (E), or production of malondialdehyde (MDA) levels (F), in the model of CPA-caused hemorrhagic cystitis in Swiss mice. Differences in the MPO levels, cytokine production and MDA formation were determined by one-way analysis

of variance followed by Newman-Keuls post-hoc test. Each column represents the mean of 5–6 animals, and the vertical lines show the standard error of the mean value. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $\#\#\#P < 0.001$ significantly different from phosphate buffered saline (PBS) values. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ significantly different from CPA values. CPA = cyclophosphamide; MPO = myeloperoxidase; MDA = malondialdehyde; PBS = phosphate buffered saline.

Figure 6

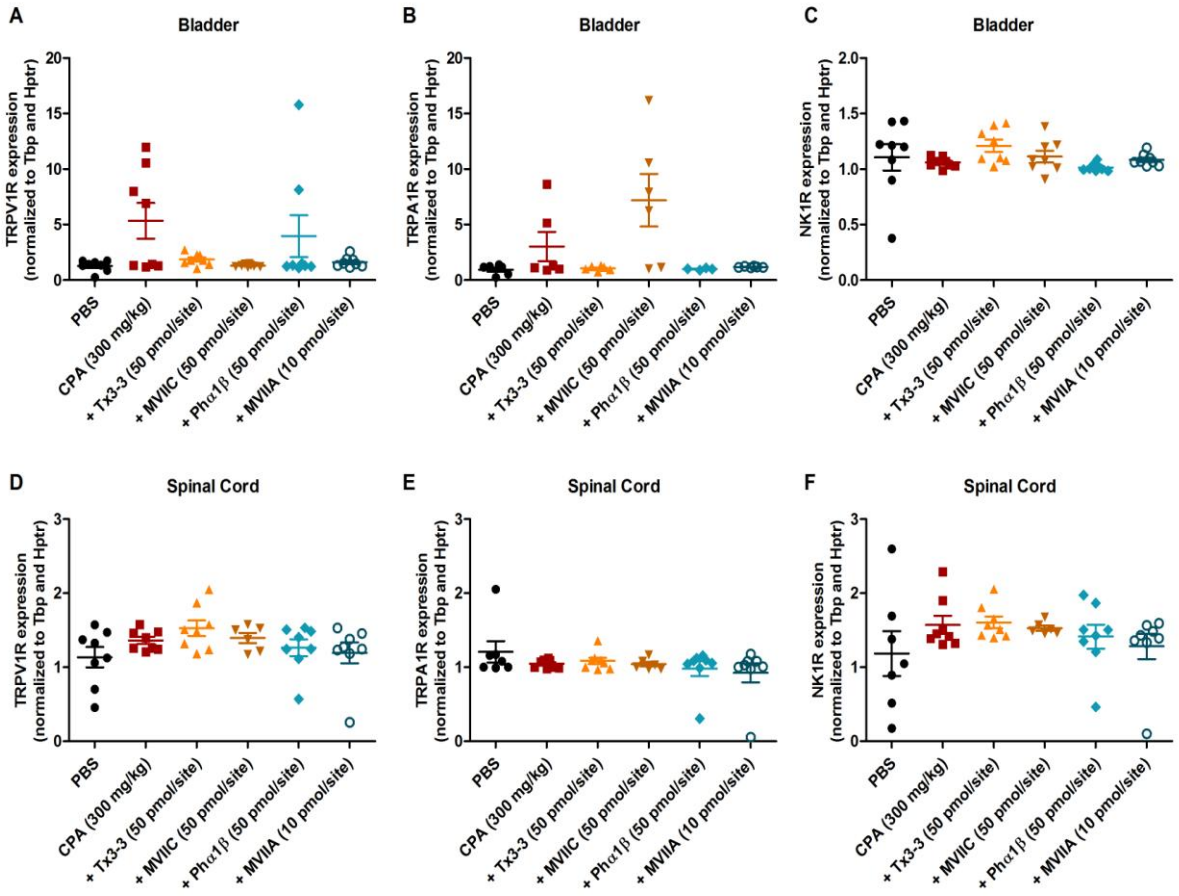

Figure 6. **TRPV1, TRPA1 or NK1 mRNA receptor expression in bladder or spinal cord of CPA-injected mice.** Expression of transient receptor potential vanilloid 1 (TRPV1), transient receptor potential ankyrin 1 (TRPA1) or neurokinin 1 (NK1) receptors was measured by quantitative polymerase chain reaction, in bladder and spinal cord tissues, at 6 h after intraperitoneal (i.p.) administration of cyclophosphamide (CPA). Effects of treatment with P/Q-type voltage-gated calcium channel (VGCC) blockers MVIIC from *Conus magus*, given by intrathecal (i.t.) route, *Phoneutria nigriventer*-derived Tx3-3 toxin (50 pmol/site, i.t.), N-type VGCC blockers MVIIA from *Conus magus* (10 pmol/site, i.t.) or *Phoneutria nigriventer*-derived Phα1β toxin (50 pmol/site, i.t.), on TRPV1 (A, D), TRPA1 (B, E) or NK1 (C, F) receptor mRNA levels in bladder and spinal cord tissues, respectively. Data has been normalized to the levels of Tbp and Hptr expression using the same sample. Each column

represents the mean of 8 samples, and the vertical lines show the standard error of the mean value. TRPV1R = Transient Receptor Potential Vanilloid 1; TRPA1R = Transient Receptor Potential Ankyrin 1; NK1R = Neurokinin 1 receptor; CPA = cyclophosphamide; PBS = phosphate buffered saline.

Figure 7

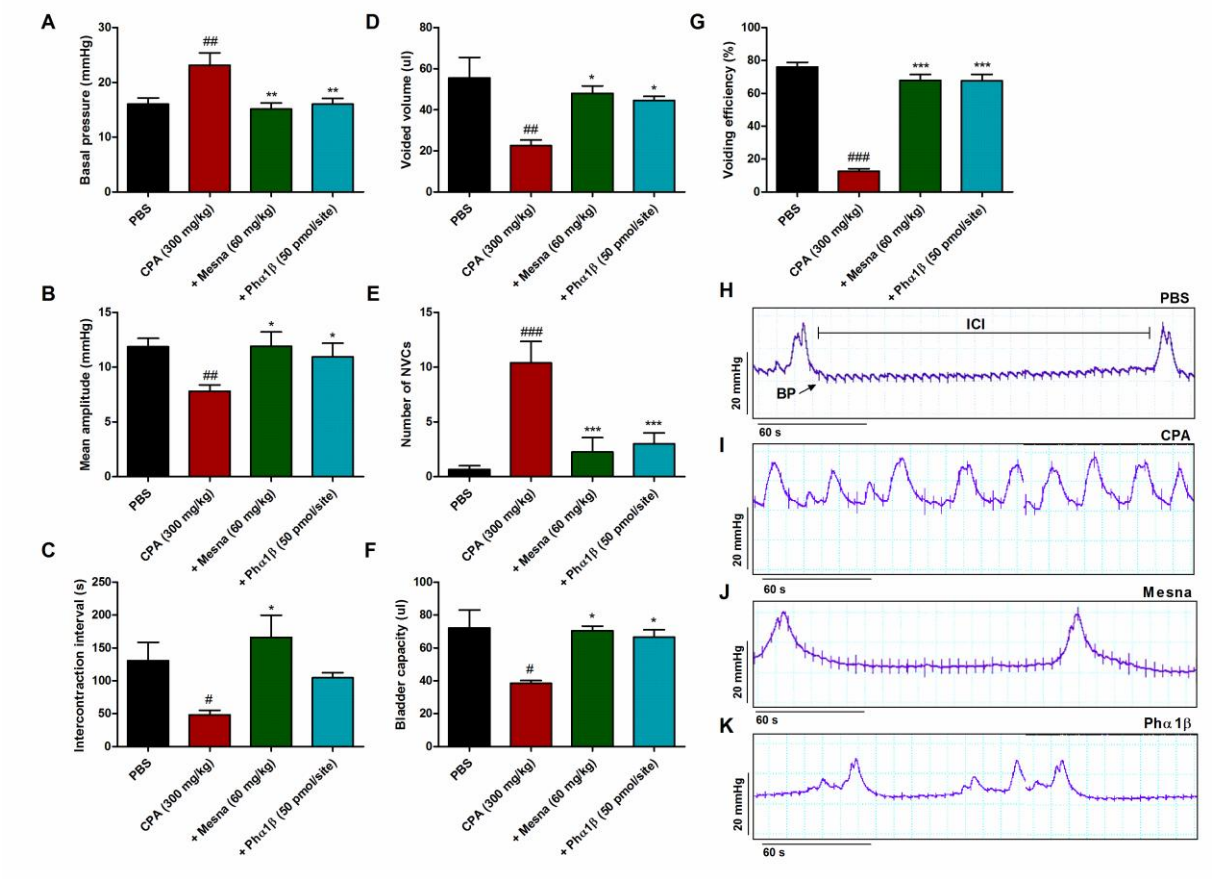

Figure 7. N-type VGCC blocking by Phα1β attenuates cystometric parameters in CPA-evoked HC. Changes in urodynamic parameters allied to cyclophosphamide (CPA) administration in hemorrhagic cystitis (HC) model were assessed by functional cystometry assay, during 30 min, 6 h after intraperitoneal (i.p.) administration of CPA. Effects of treatment with the reference drug Mesna (60 mg/kg, i.p., given 30 min before and 4 h post-CPA) or N-type voltage-gated calcium channel blockers Phα1β from *Phoneutria nigriventer* (50 pmol/site, i.t.), given 2 h post-CPA, on basal pressure (A), mean amplitude (B), intercontraction interval (C), voided volume (D), number of non-voiding contractions (NVCs) (E), bladder capacity (F) and on voiding efficiency (G) in the model of CPA-induced hemorrhagic cystitis in Swiss mice. Representative traces of cystometry of phosphate buffered saline (PBS) (H), CPA (I), Mesna (J) or Phα1β-administration (K) in HC model. Differences in the urodynamic parameters were determined by one-way analysis of variance followed by

Newman-Keuls post-hoc test. Each column represents the mean of 4–6 animals, and the vertical lines show the standard error of the mean value. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ significantly different from PBS values. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ significantly different from CPA values. BP = basal pressure; CPA = cyclophosphamide; ICI = intercontraction interval; NVCs = non-voiding contractions; PBS = phosphate buffered saline.

Figure 8

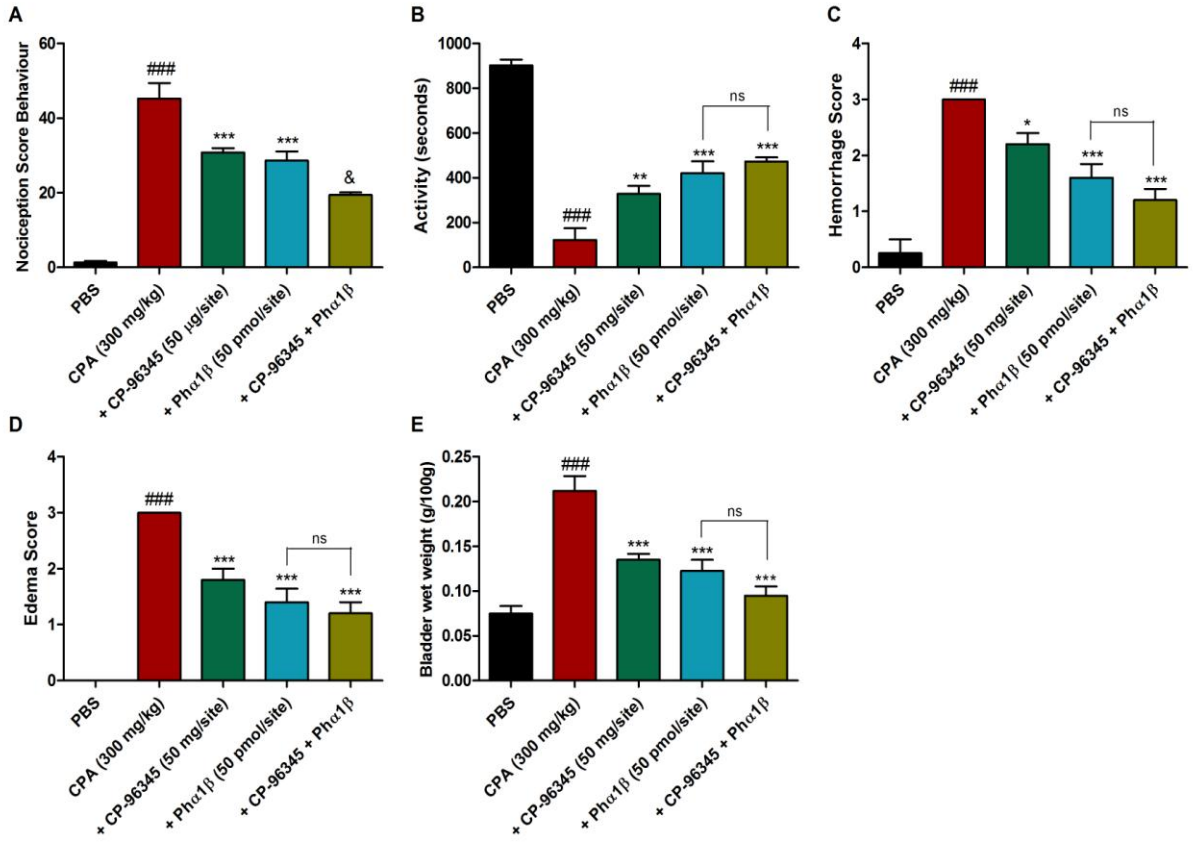

Figure 8. Antinociceptive effects of Phα1β are related to spinal NK1 receptor activation. Nociception score behavior and activity were measured for 2 min, at 30 min intervals, over a total period of 4 h after intraperitoneal (i.p.) injection of cyclophosphamide (CPA), whereas macroscopic inflammation was evaluated 6 h post-CPA. Effects of treatment with the selective NK1 receptor antagonist CP-96345 (50 µg/site, 2 h post-CPA), N-type voltage-gated calcium channel blocker Phα1β from *Phoneutria nigriventer* (50 pmol/site) or CP-96345 (50 µg/kg) plus Phα1β (50 pmol/site), given by intrathecal (i.t.) route, on the nociceptive responses (A), activity (B), hemorrhage (C), edema (D), and on bladder wet weight (E) in the model of CPA-caused hemorrhagic cystitis (HC). Differences in the nociception score, activity and macroscopic inflammation were determined by one-way analysis of variance followed by Newman-Keuls post-hoc test. Each column represents the mean of 4–5 animals, and the vertical lines show the standard error of the mean value. ###$P < 0.001$ significantly

different from phosphate buffered saline (PBS) values. *P < 0.05, **P < 0.01 and ***P < 0.001 significantly different from CPA values. $^{\&}P$ < 0.05 significantly different from Phα1β values. CPA = cyclophosphamide; PBS = phosphate buffered saline; ns = no significant.

Table 1. Quantitative PCR primers design.

Primers	Sequences (5´–3´)	PCR product (bp)	GenBank Accession Number
Tbp[a]	F CCGTGAATCTTGGCTGTAAACTTG	118	NM_013684
	R GTTGTCCGTGGCTCTCTTATTCTC		
Hprt[a]	F CTCATGGACTGATTATGGACAGGAC	123	NM_013556
	R GCAGGTCAGCAAAGAACTTATAGCC		
TRPV1[b]	F GGCAAGGATGACTTCCGGTGGTG	125	NM_001001445
	R AAGCTCAGGGTGCGCTTGACG		
TRPA1[b]	F GTATCATCTTCGTGTTGCCCTTGTTC	196	NM_177781
	R AGGAAGATAAACACTCCGGTCGATC		
NK1[b]	F AATGACAGGTTCCGTCTGGGCTTC	133	NM_009313
	R GGCTGACCTTGTACACGCTGCTCTG		

[a]According to Pernot, 2010; [b]Designed by authors, using Oligos.

4. CONSIDERAÇÕES FINAIS

Nos últimos anos, diversos estudos têm sido realizados para identificar peptídeos ativos naturais de venenos de diferentes espécies, que possam ser usados em uma variedade de aplicações médicas, em especial, nas doenças relacionadas à dor e inflamação. Essa busca se deve à seletividade por uma variedade de subtipos de canais iônicos. Além de possíveis alvos terapêuticos, esses peptídeos são usados como ferramentas farmacológicas, podendo ser usados para modular ou autorregular os canais iônicos (Rajendra *et al.*, 2004).

Outrossim, o interesse na caracterização bioquímica e farmacológica de toxinas, como por exemplo, da aranha *P. nigriventer*, cresce de forma vertiginosa. A relevância destas toxinas está relacionada à sua capacidade de bloquear os CCVDs. Com isso, podem produzir diversas respostas em níveis moleculares e celulares (Gomez *et al.*, 2002). Ademais, investiga-se o veneno da *P. nigriventer* por sua habilidade em afetar um grande número de sistemas fisiológicos, em particular, os relacionados aos processos dolorosos e inflamatórios (Costa *et al.*, 2002). De fato, o aumento da concentração intracelular de Ca^{2+} faz com que haja liberação de glutamato e substância P nos terminais nervosos, além da secreção de citocinas nas células (McGivern, 2007; Bradding *et al.*, 2009).

A CH é o principal efeito adverso causado pelo quimioterápico CPA, devido ao contato do metabólito ACR com o epitélio da bexiga e, assim, representa um grande desafio clínico para os urologistas. Além da grave hemorragia, a CH é acompanhada de edema, dor na bexiga, disfunção na micção, assim como, mudanças ao nível celular e molecular, incluindo o aumento da produção de TNF-α, IL-1β e proteína C reativa (Decker *et al.*, 2009; Jiang *et al.*, 2013). Os canais iônicos, especialmente, os CCVDs, estão implicados na modulação das respostas nociceptivas e inflamatórias, principalmente, pela regulação do influxo de cálcio e, por conseguinte, a liberação de neurotransmissores e neuropeptídeos pró-inflamatórios (Park *et al.*, 2010). Relevantemente, os CCVDs são constitutivamente expressos no urotélio, células intersticiais e nos nervos aferentes da bexiga (Birder *et al.*, 2013). Estudos prévios do nosso grupo demonstraram que a inibição farmacológica ou gênica dos receptores purinérgicos P2X7, preveniu alterações inflamatórias e nociceptivas associadas à CH, induzida por CPA (Martins *et al.*, 2012). No entanto, até o presente momento, não havia estudos investigando se o bloqueio medular dos CCVD pode interferir nos sintomas relacionados à CH aguda**.**

O presente estudo traz novas evidências sobre a relevância dos CCVDs dos subtipos P/Q e N ao nível medular, no modelo animal de CH induzida por CPA, através da avaliação dos peptídeos purificados da aranha brasileira *P. nigriventer*, Tx3-3 e Phα1β, respectivamente

ou, por meio das toxinas MVIIC e MVIIA, obtidas do caramujo *C. magus*, pela administração i.t. Os principais achados do presente estudo foram os seguintes: (i) o bloqueio medular dos CCVD do subtipo P/Q pela toxina Tx3-3 e MVIIC, e a inibição seletiva dos CCVD do subtipo N pelo peptídeo Phα1β, atenuaram os eventos nociceptivos e inflamatórios relacionados à CH em camundongos, incluindo o estresse oxidativo e produção de citocinas na bexiga; (ii) a injeção i.p. de CPA produziu um evidente aumento na expressão do RNAm de TRPV1 e TRPA1 na bexiga, um efeito no qual foi virtualmente revertido por todas as toxinas testadas; (iii) a inibição do CCVD do tipo N através da Phα1β, claramente, preveniu as alterações funcionais da bexiga em camundongos; e (iv) a co-administração i.t. do antagonista seletivo dos receptores NK1, CP-96345, potencializou o efeitos antinociceptivo da Phα1β no modelo agudo de CH.

Desta forma, considerando a gravidade dos quadros de CH associados ao tratamento com o quimioterápico, CPA e, ainda, o número limitado de opções terapêuticas para controlar as alterações inflamatórias, dolorosas e funcionais relacionadas à esta patologia. O nossos resultados trazem, pela primeira vez, novas evidências dos efeitos antinociceptivos e anti-inflamatórios produzidos pelas toxinas Tx3-3 e Phα1β da aranha *P. nigriventer* na CH induzida por CPA em camundongos, bem como, a recuperação na função da bexiga, especialmente pela toxina Phα1β, sendo estes efeitos similar ao produzido pelo medicamento utilizado na clínica, Mesna.

Em essência, o conjunto de dados, sugere que administração epidural dos inibidores dos CCVD dos subtipos P/Q e N pode representar uma nova e atrativa opção terapêutica para pacientes com sintomas graves relacionados à CH. Particularmente, o bloqueador dos CCVD do subtipo N, Phα1β, apresenta-se como uma promessa para o tratamento de patologias associados com a disfunção da bexiga, como LM, neuropatia diabética, assim como a cistite.

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ANEXO A – Aprovação da CEUA

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

Ofício 0121/12 – CEUA

Porto Alegre, 11 de outubro de 2012.

Senhora Pesquisadora:

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 12/00292, **"Efeitos anti-inflamatórios e antinociceptivos de toxinas da aranha Phoneutria nigriventer em modelo de cistite hemorrágica induzida por ciclofosfamida em camundongos".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora da CEUA/PUCRS

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Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

AVALIAÇÃO DE PROJETOS DE PESQUISA

TITULO DO PROJETO
Efeitos anti-inflamatórios e antinociceptivos de toxinas da aranha Phoneutria nigriventer em modelo de cistite hemorrágica induzida por ciclofosfamida em camundongos
Projeto nº 12/00292
Pesquisador: Maria Martha Campos
AVALIAÇÃO GERAL DO PROJETO
(X) Aprovado ( ) Aprovado com recomendação ( ) Pendente ( ) Não aprovado
Questões levantadas pelo CEUA – PUCRS
Todas as questões levantadas pelo CEUA foram atendidas

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ANEXO B – Submissão do manuscrito

JEM

MANUSCRIPT HOME | AUTHOR INSTRUCTIONS | REVIEWER INSTRUCTIONS | HELP | TIPS | LOGOUT | JOURNAL HOME

Detailed Status Information

Manuscript #	20132624
Current Revision #	0
Submission Date	2013-12-18 07:44:13
Current Stage	Initial QC Started
Title	P/Q- OR N-TYPE CALCIUM CHANNELS SPINAL BLOCKAGE MODULATES FUNCTIONAL AND SYMPTOMATIC CHANGES ALLIED TO MOUSE HEMORRHAGIC CYSTITIS
Running Title	Calcium channel inhibition prevents bladder damage
Manuscript Type	Research Article
Corresponding Author	Dr. Maria Martha Campos (PUCRS)
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Abstract	Spinal voltage-gated calcium channels (VGCC) are pivotal regulators of painful and inflammatory alterations, representing attractive therapeutic targets. We examined the effects of epidural administration of the selective P/Q- and N-type VGCC blockers Tx3-3 and Phα1β, respectively, isolated from the spider P. nigriventer, on symptomatic, inflammatory and functional changes allied to cyclophosphamide (CPA)-induced hemorrhagic cystitis (HC) in mice. The effects of P. nigriventer-derived toxins were compared to those displayed by MVIIC and MVIIA, extracted from the cone snail C. magus. HC was induced by a single intraperitoneal injection of CPA (300 mg/kg). The spinal blockage of P/Q-type VGCC by Tx3-3 and MVIIC, or N-type VGCC by Phα1β attenuated nociceptive and inflammatory events associated with HC, including bladder oxidative stress and cytokine production. Moreover, CPA produced an evident increase of bladder TRPV1 and TRPA1 mRNA expression, which was virtually reversed by all the tested toxins. Noteworthy, Phα1β strongly prevented bladder neutrophil migration, besides HC-related functional alterations. Finally, the spinal co-administration of the selective NK1 receptor antagonist CP-96345 heightened Phα1β antinociceptive effects. Our results shed new lights on the role of spinal P/Q and N-type VGCC in bladder dysfunctions, pointing out Phα1β as a promising alternative for treating complications associated to CPA-induced HC.
Subject Areas	Immunology, Metabolism, Neurobiology
Conflict of Interest	**No**, there is no conflict of interest that I should disclose, having read the above statement.
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ANEXO C – Ata de apresentação de dissertação nº 354

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE MEDICINA
PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE

ATA DE APRESENTAÇÃO DE DISSERTAÇÃO Nº 354

Aos vinte e quatro dias do mês de janeiro do ano de dois mil e quatorze, no Curso de Mestrado em Medicina e Ciências da Saúde, área de concentração em Farmacologia Bioquímica e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul foi concluído o processo de avaliação da dissertação intitulada **"MODULAÇÃO DAS ALTERAÇÕES FUNCIONAIS E SINTOMÁTICAS RELACIONADAS À CISTITE HEMORRÁGICA INDUZIDA POR CICLOFOSFAMIDA EM CAMUNDONGOS ATRAVÉS DO BLOQUEIO MEDULAR DOS CANAIS DE CÁLCIO VOLTAGEM-DEPENDENTES DOS SUBTIPOS P/Q E N"** de autoria do pós-graduando **Rodrigo Braccini Madeira da Silva** sob orientação da Professora Doutora **Maria Martha Campos**. A comissão examinadora foi constituída pelos professores: Dr. Giles Alexander Rae (UFSC), Dr. Domingos Otavio Lorenzoni d' Avila (PUCRS), Ivan Carlos Ferreira Antonello (PUCRS) e Dr. Jarbas Rodrigues de Oliveira, suplente (PUCRS). O aluno foi **APROVADO**. Para constar, lavrou-se esta ata que deverá ser anexada à documentação exigida para posterior expedição do diploma. A presente ata foi assinada pelo Coordenador em Exercício do Programa de Pós-Graduação em Medicina e Ciências da Saúde. Porto Alegre, aos vinte e quatro dias do mês de janeiro do ano de dois mil e quatorze.

Prof. Dr. Alexandre Vontobel Padoin