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Ação da Phα1β, peptídeo purificado do veneno da aranha *Phoneutria nigriventer*, sobre os efeitos analgésicos e adversos causados pela morfina em camundongos

TESE DE DOUTORADO

Raquel Tonello

Santa Maria, RS, Brasil,

2015

Ação da Phα1β, peptídeo purificado do veneno da aranha *Phoneutria nigriventer*, sobre os efeitos analgésicos e adversos causados pela morfina em camundongos

Por

Raquel Tonello

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Orientador: Prof. Dr. Juliano Ferreira

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“Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
A presença distante das estrelas!”

Mario Quintana em “Espelho Mágico”.

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

Ação da Phα1β, peptídeo purificado do veneno da aranha *Phoneutria nigriventer*, sobre os efeitos analgésicos e adversos causados pela morfina em camundongos

AUTOR: Raquel Tonello
ORIENTADOR: Juliano Ferreira
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Os opióides são os medicamentos mais comuns prescritos em todo o mundo para aliviar as dores moderadas a intensas. No entanto, a utilização de opióides está associada com o desenvolvimento de tolerância ao efeito analgésico e de efeitos adversos, tais como hiperalgesia paradoxal, síndrome de abstinência e constipação. Um alvo importante para a analgesia induzida pela morfina é o bloqueio dos canais de cálcio regulados por voltagem (CCRV). Porém pouco se sabe sobre o papel desses canais na tolerância e nos efeitos adversos produzidos pela morfina. Assim, o presente estudo foi realizado com o intuito de avaliar as possíveis ações da Phα1β, um inibidor peptídico dos CCRVs purificado do veneno da aranha *Phoneutria nigriventer*, sobre os efeitos antinociceptivos e adversos produzidos pela administração única ou repetida de morfina. Foi avaliado o efeito da administração intratecal da Phα1β (0.01-30 pmol/site) sobre a hiperalgesia térmica e mecânica, tolerância, síndrome de abstinência e constipação induzidos pelo tratamento único (10 mg/kg) ou repetido (doses crescentes, 3 vezes ao dia, durante três dias) de morfina por via subcutânea em camundongos C57BL/6. Observamos que uma única administração de morfina foi capaz de reduzir a nocicepção térmica mas não a mecânica em camundongos, bem como reduzir o trânsito gastrointestinal. A antinocicepção, mas não a constipação, causada por uma única dose de morfina foi levemente aumentada pela administração intratecal da Phα1β. O tratamento repetido com morfina não causou somente tolerância analgésica como também induziu hiperalgesia, síndrome de abstinência e constipação. A Phα1β foi capaz de reverter a tolerância, a síndrome de abstinência, a hiperalgesia mecânica e térmica e a constipação induzidas pelo tratamento repetido de morfina. Finalmente, os efeitos produzidos pela forma nativa Phα1β foram totalmente mimetizados por uma versão recombinante do presente peptídeo. Em conclusão, nossos resultados sugerem que a Phα1β é efetiva em potencializar a analgesia, bem como, reduzir a tolerância e os efeitos adversos induzidos pela morfina. Desta maneira, a Phα1β apresenta um uso potencial como uma droga adjuvante na terapia opióide.

Palavras-chave: canais de cálcio, constipação, hiperalgesia, opióide, síndrome de abstinência, tolerância.

ABSTRACT

PhD Thesis

Graduate Course in Biological Sciences: Toxicological Biochemistry

Federal University of Santa Maria, RS, Brazil

Action of Phα1β, a peptide from the venom of the *Phoneutria nigriventer* spider, on analgesic and adverse effects caused by morphine in mice

AUTHOR: Raquel Tonello

ADVISOR: Juliano Ferreira

PLACE AND DATE OF THE DEFENSE: Santa Maria, June, 19th, 2015.

Opioids are the most common drugs prescribed worldwide for alleviating moderate to severe pain. However, the use of opioids is associated with the development of tolerance to the analgesic effect and potential adverse effects, such as paradoxical hyperalgesia, withdrawal syndrome and constipation. An important target for morphine-induced analgesia is the blockade of voltage-gated calcium channels (VGCCs). However, the participation of VGCCs in the tolerance and adverse effects caused by morphine is poorly understood. Thus, the present study was conducted in order to evaluate the possible actions of Phα1β, a peptide inhibitor of VGCCs purified from the venom of the *Phoneutria nigriventer* spider on the antinociceptive and adverse effects produced by single or repeated administration of morphine. It was evaluated the effect of intrathecal injection Phα1β (0.01-30 pmol/site) on mechanical and heat hyperalgesia, tolerance, withdrawal syndrome and constipation induced throught single (10 mg/kg) or repeated (increasing doses, 3 times a day, for 3 consecutive days) subcutaneous treatment of morphine in C57BL/6 mice. We observed that a single administration of morphine was able to reduce heat but not mechanical nociception as well as decrease gastrointestinal transit. The antinociception, but not the constipation, caused by a single injection of morphine was slightly increased by an intrathecal injection of Phα1β. Repeated treatment with morphine caused not only tolerance to its antinociceptive effect but also induced paradoxical heat and mechanical hyperalgesia, withdrawal syndrome and constipation. Phα1β was able to reverse the tolerance, withdrawal syndrome, mechanical and heat hyperalgesia and constipation induced by repeated morphine treatment. Finally, the effects produced by the native form of Phα1β were fully mimicked by a recombinant version of this peptide in naïve mice. Our results suggest that Phα1β is effective in potentiating the analgesia as well as in reducing tolerance and the adverse effects induced by morphine, indicating its potential use as an adjuvant drug in combination with opioids.

Keywords: calcium channel blocker, constipation, hyperalgesia, opioid, tolerance, withdrawal syndrome.

LISTA DE FIGURAS E TABELAS

INTRODUÇÃO

ARTIGO

CONCLUSÃO

APÊNDICE

LISTA DE ABREVIATURAS

ACP	Área cinzenta periaquedutal
IASP	Associação Internacional para o Estudo da Dor
CAMKII	Proteína quinase dependente de cálcio calmodulina II
CCRV	Canal de cálcio regulado por voltagem
GIRK	Canal de potássio
GRD	Gânglio da raiz dorsal
HIO	Hiperalgesia induzida por opióide
LC	Locus coeruleus
Go/Gi	Proteína G inibitória
NMDA	N-metil-D-aspartato
DOR	Receptor delta opióide
KOR	Receptor kappa opióide
MOR	Receptor mu opióide
SNC	Sistema nervoso central
SP	Substância P

LISTA DE APÊNDICES

SUMÁRIO

1. APRESENTAÇÃO

No item **INTRODUÇÃO** consta uma revisão sucinta da literatura sobre os temas abordados nesta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigo científico que foi publicado, o qual se encontra no item **ARTIGO**. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio artigo e representam a íntegra deste estudo.

Os itens **DISCUSSÃO**, **CONCLUSÕES** e **REFERÊNCIAS BIBLIOGRÁFICAS** encontram-se no final desta dissertação.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente as citações que aparecem nos itens Introdução e Discussão desta tese.

No item **APÊNDICE** consta um manuscrito submetido, que é requisito necessário para obtenção do título de doutor.

2.1. Dor e nocicepção

Os processos dolorosos afetam milhões de pessoas por todo o mundo, sendo uma razão muito comum para a procura por cuidados médicos e uso de fármacos. Porém, o tratamento adequado da dor ainda é considerado um desafio terapêutico, tanto em países desenvolvidos como em desenvolvimento. É estimado que 80% da população mundial tem acesso limitado a medicamentos para tratamento da dor moderada a intensa, o que reduz consideravelmente a qualidade de vida destes pacientes (Brennan et al., 2007; Lohman et al., 2010; Cousins e Lynch, 2011; King e Fraser, 2013).

De acordo com a Associação Internacional para o Estudo da Dor (IASP), a dor é definida como "uma experiência sensorial e emocional desagradável associada a uma lesão tecidual real, ou potencial; ou descrita em termos de tal dano" (Loeser e Treede, 2008). A dor pode ser classificada por função, etiologia, duração, intensidade e expressão (Esquema 1). Etiologicamente a dor pode ser classificada como nociceptiva, inflamatória, neuropática, relacionada ao câncer e disfuncional.

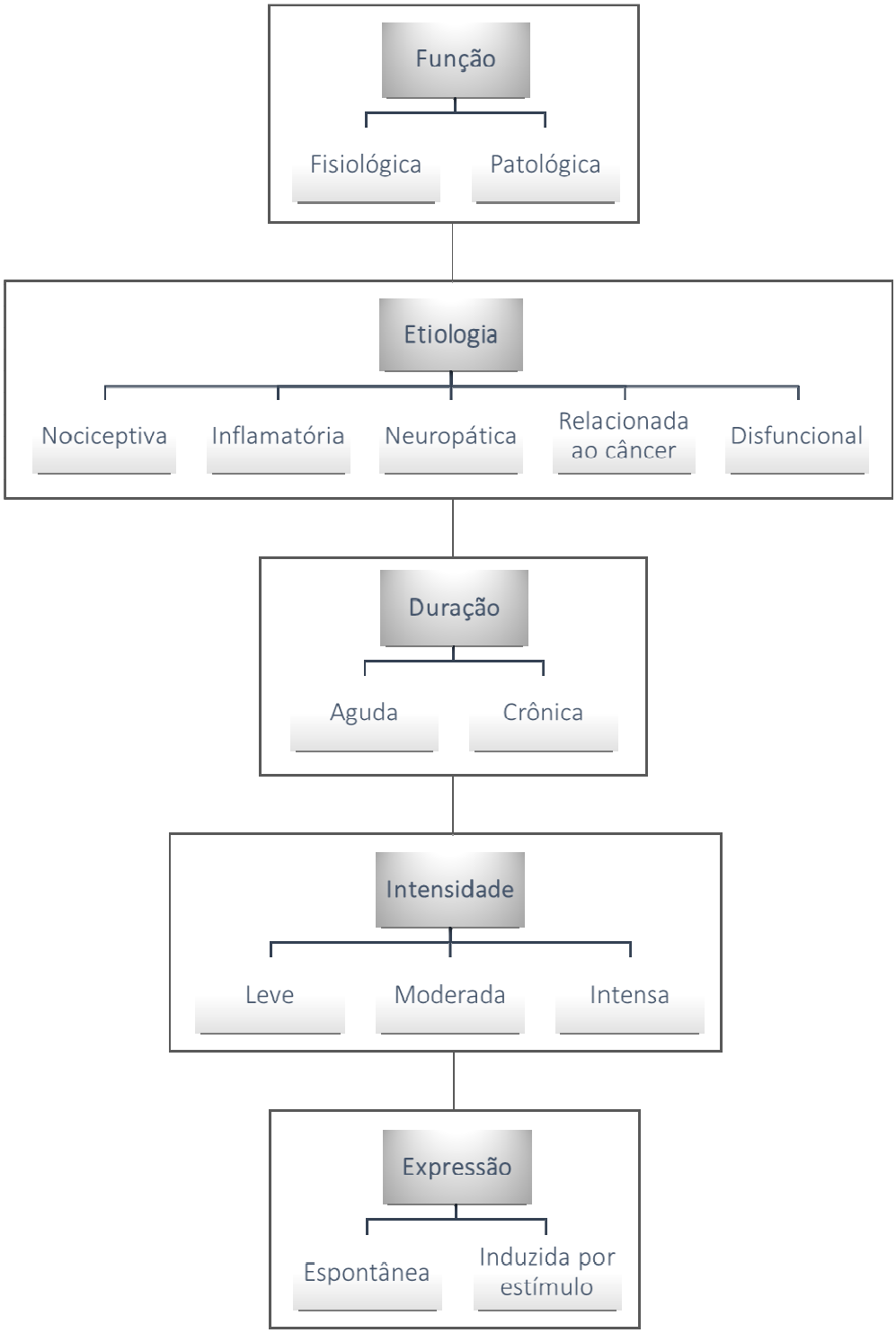
Inicialmente, a dor desempenha um papel protetor do organismo, como um sistema de alerta ativado em resposta a impedir lesão, e é definida como dor nociceptiva e tem função fisiológica. Neste caso a dor funciona como um sinal de aviso, que é essencial para se detectar e minimizar o contato com estímulos nocivos, como no caso de superfícies muito quentes ou frias, ou ainda cortantes (Woolf, 2010). A dor nociceptiva é gerada pela ativação dos nociceptores que são receptores sensoriais capazes de serem ativados por estímulos nocivos e de transmitir estas informações até estruturas supra-espinhais envolvidas na percepção da dor, resultando em uma resposta dolorosa no organismo.

Existem duas principais classes de nociceptores, uma composta por subtipos de neurônios não mielinizados, as fibras C nociceptivas, e outra formada por subtipos de neurônios finamente mielinizados, as fibras Aδ nociceptivas. As fibras Aδ diferem consideravelmente das fibras mielinizadas de grande diâmetro, Aβ, que respondem a estímulos mecânicos inócuos, como um leve toque, por exemplo. Os corpos celulares dos neurônios dos nociceptores encontram-se no gânglio da raiz dorsal (GRD - para neurônios

que inervam o corpo), trigeminal (para neurônios que inervam a cabeça) e nos gânglios nodoso e vagal (para as vísceras). Sendo neurônios pseudo-unipolares os neurônios nociceptivos possuem terminações centrais (medula espinhal e tronco cerebral) e também periféricas (tecido onde possui inervações), e então são capazes de detectar a informação na periferia e transmitir a estruturas centrais (Loeser e Treede, 2008; Basbaum et al., 2009; Ossipov et al., 2010; Woolf, 2010).

Estímulos nocivos mecânicos, térmicos ou químicos, ativam estes nociceptores na periferia e geram então um potencial de ação que é conduzido até o corno dorsal da medula espinhal (sobretudo as lâminas I, II e IV), onde então ocorre a liberação de neurotransmissores excitatórios, principalmente substância P (SP) e glutamato, que estimulam neurônios de segunda ordem. Esses neurônios de segunda ordem formam vias que irão distribuir informações para circuitos cerebrais. Os axônios ascendem através da via espinotalâmica, responsável pela atividade discriminatória do estímulo, e terminam dentro do tálamo ventroposterior e ventrobasal, e posteriormente são projetados para o córtex. A segunda via percorrida pelos axônios provenientes do corno dorsal da medula espinhal é a via espinoparabraquial amidalóide que termina no núcleo parabraquial e têm projeções para hipotálamo e amígdala, e é relevante para modulação das dimensões afetivas da experiência dolorosa (Loeser e Treede, 2008; Basbaum et al., 2009; Ossipov et al., 2010; Woolf, 2010).

Diferente da dor nociceptiva, uma segunda forma de dor, denominada dor inflamatória, tem função primordial de proteger o organismo (função fisiológica), mas pode perder esta função quando não existe resolução da inflamação (função patológica). Neste caso a sensibilidade sensorial é aumentada após o dano aos tecidos de maneira a auxiliar a recuperação do local lesado. Durante este processo, pode ser observado nos pacientes o aparecimento de dor induzida por estímulos antes inócuos (alodínia), como um leve toque, ou ainda, ocorre a percepção exacerbada da dor a estímulos dolorosos (hiperalgesia) e também aparecimento da dor espontânea, descrita como dor em queimação ou choque. E esta dor inflamatória, tanto fisiológica como patológica, deve ser reduzida, como no caso de pacientes com dor pós-cirúrgica, e em casos de lesão extrema ou grave (Woolf, 2010).



Esquema 1. Classificações da dor

Alodínia, hiperalgesia e dor espontânea também são observados na dor neuropática, relacionada ao câncer e disfuncional. A dor neuropática é causada por uma lesão ou dano das fibras nervosas. Já a dor relacionada ao câncer

pode surgir a partir de diferentes processos, tanto por envolvimento direto do tumor, como resultado de procedimentos diagnósticos ou terapêuticos (tais como biópsias), ou ainda como efeito colateral de toxicidade relacionada a terapias utilizadas para o tratamento do câncer (por exemplo, quimioterapia, ou radioterapia). E ainda a dor disfuncional, em condições em que não há dano ou inflamação, como na fibromialgia e síndrome do intestino irritável (Mantyh, 2006; Woolf, 2010).

De acordo com sua duração e intensidade (que pode ser leve, moderada ou intensa), a dor é comumente descrita como aguda ou crônica. A dor aguda é caracterizada por ter início recente e duração limitada e geralmente tem uma relação temporal e causal com a lesão ou doença. Entre as dores agudas, incluem a dor pós-cirúrgica, pós-traumática e dor de cabeça, sendo considerado o tipo de dor mais tratado na clínica (Carr e Goudas, 1999).

Ao contrário do propósito claramente protetor da dor aguda, a dor pode se tornar crônica quando o organismo não é capaz de produzir resolução de uma lesão ou quando a doença mantiver a dor após a resolução da lesão. A dor é considerada crônica quando persiste durante pelo menos 3 meses, sendo uma dor que não responde ou responde parcialmente ao tratamento. A dor crônica é frequentemente observada em pacientes com câncer, fibromialgia, dor das costas, artrite reumatoide e dor neuropática. A dor crônica difere substancialmente da dor aguda não somente em relação ao seu caráter persistente, mas está principalmente associada com alterações adaptativas, tais como à neuroplasticidade em vários níveis do sistema nervoso, sendo de difícil tratamento (Mantyh et al., 2002; Loeser e Treede, 2008; Basbaum et al., 2009; Ossipov et al., 2010; Woolf, 2010).

Uma vez que a dor associada a diferentes condições patológicas representa o sintoma que mais causa sofrimento aos pacientes, diferentes abordagens terapêuticas têm sido utilizadas com o objetivo de atenuá-la e promover a qualidade de vida dos pacientes (Brennan et al., 2007; Tracey e Mantyh, 2007). Dentre os principais medicamentos utilizados na clínica para produzir analgesia estão os opióides, amplamente utilizados como tratamento de escolha para dores moderadas e intensas, como a dor relacionada ao câncer e a dor pós-cirúrgica (Angst e Clark, 2006; Rosenblum et al., 2008; Trescot et al., 2008; Mugabure Bujedo, 2012).

2.2. Sistema opióide

2.2.1. Receptores opióides

Os receptores opióides têm sido alvo para o tratamento da dor e doenças relacionadas por milhares de anos e continuam sendo os analgésicos mais utilizados na clínica atualmente. Registros egípcios já relatavam o uso do ópio (extraído das sementes da papoula – *Papaver somniferum* – cujo principal ingrediente ativo é a morfina) para o alívio da dor. Em 1973, o estudante Candace Pert (e o seu orientador Solomon H. Snyder) identificou o sítio de ligação para opióides através do uso de naloxona tritiada radioativa em preparação de membrana de cérebro de ratos. Dois anos mais tarde, foram descobertos os primeiros ligantes endógenos por John Hughes e Hans Kosterlitz (1975), chamados encefalinas. E a partir destes, outros ligantes endógenos foram identificados, como dinorfinas e endorfinas (Trescot et al., 2008; Al-Hasani e Bruchas, 2011; Bian et al., 2012).

Os receptores opióides são amplamente expressos no sistema nervoso central (SNC) e periférico, e regulam diversas funções fisiológicas, como a respiração, o trânsito gastrointestinal, euforia, dependência e respostas imunológicas e endócrinas. Sobretudo, esses receptores desempenham um papel central na dor. Eles estão expressos na via descendente da modulação da dor, que inclui o córtex cerebral, núcleo talâmico, área cinzenta periaquedutal (ACP), corpo estriado e corno dorsal da medula espinhal nas lâminas I e II. A ativação dos receptores opióides nestes locais inibe diretamente os neurônios, que por sua vez inibe a transmissão da dor na medula espinhal, resultando na analgesia (Trescot et al., 2008; Al-Hasani e Bruchas, 2011; Bian et al., 2012).

O sistema opióide consiste de três receptores: MOR, DOR E KOR (também referidos como mu - μ, delta - δ e kappa - κ). São da família dos receptores acoplados a proteína G, caracterizados por uma estrutura de sete domínios transmembrana. A ativação desses receptores, que são acoplados à proteína G inibitória (Go/Gi), causa a inibição dos canais de cálcio regulados por voltagem (CCRV), a ativação de canais de potássio retificadores de influxo de corrente e bloqueia a liberação de neurotransmissores. A redução da liberação de neurotransmissores como glutamato, SP e peptídeo relacionado ao gene da calcitonina das fibras nociceptivas causa uma redução da

excitabilidade neuronal, culminando com o alívio da dor (Trescot et al., 2008; Al-Hasani e Bruchas, 2011; Bian et al., 2012).

Logo, não é por acaso que poucos analgésicos são tão potentes e eficazes quanto os opióides. Dentre os opióides mais utilizados está a morfina, considerada o tratamento "padrão ouro" em dores moderadas a intensas, como as dores agudas e relacionada ao câncer bem como em dores crônicas não malignas (ex. dor nas costas, fibromialgia, dor musculoesquelética e inflamatória) (Angst e Clark, 2006; Stein, 2013). Entretanto, uma preocupação comum em relação ao uso de opióides é o desenvolvimento de tolerância analgésica e de efeitos adversos, como constipação e síndrome de abstinência. Além disso, outro efeito adverso relacionado ao consumo de opióides é a hiperalgesia induzida por opióides (HIO), que recentemente tem despertado interesse e apresenta um número crescente de estudos sobre seu mecanismo e significado clínico (Angst e Clark, 2006; Gallantine e Meert, 2008; Rubovitch, Pick e Sarne, 2009; Greenwood-Van e Standifer, 2008; Low, Clarke e Huh, 2012; Lee et al., 2011).

2.2.2. Efeitos adversos relacionados ao uso de opióides

2.2.2.1 Hiperalgesia e tolerância induzida por opióides

A HIO é definida como um estado de sensibilização nociceptiva causada pela exposição a opióides. Ela pode se manifestar como hiperalgesia ou alodínia, e pode também ser acompanhada por outros sinais causados por opióide, como delírio, mioclonias e convulsões. É uma condição caracterizada por uma resposta paradoxal, onde um paciente que recebe opióide para o tratamento da dor, na verdade, pode tornar-se mais sensível aos estímulos dolorosos. A HIO é descrita tanto na exposição aguda como crônica, em altas e baixas doses e com diferentes tipos de opióides e vias de administração (Angst e Clark, 2006; Lee et al., 2011; Low, Clarke e Huh, 2012).

Pesquisas clínicas sugerem que alguns pacientes apresentam um aumento dos sintomas dolorosos como consequência da terapia com opióides. Este quadro clínico foi observado não somente em estudos com voluntários submetidos a uma curta exposição a potentes opióides, como também em pacientes submetidos à cirurgia, onde potencializou a dor pós-cirúrgica, e em pacientes com câncer ou dores crônicas (Davis, Shaiova e Angst, 2007; Lee et

al., 2011). Similar às evidências clínicas, a HIO também é descrita em vários modelos animais. Estudos pré-clínicos induzem alterações na alodínia mecânica e na hiperalgesia térmica dos roedores após exposição aguda ou crônica a opióides, por via intratecal ou sistêmica (Mao, 2002; Angst e Clark, 2006; Lee et al., 2011).

Ao contrário da HIO, onde a exposição a opióides induz um aumento da sensibilidade dolorosa, na tolerância, doses crescentes de opióide são necessárias para promover o mesmo nível de alívio da dor (Pasero e McCaffery, 2012). Tolerância analgésica pode ser definida então como uma redução da resposta farmacológica após a exposição a opióide, uma situação que contribui notavelmente para os problemas sociais que envolvem abuso recreativo de opióides (Dumas e Pollack, 2008; Garzón, Rodríguez-Muñoz e Sánchez-Blázquez, 2008).

Embora existam muitos estudos, o preciso mecanismo celular e molecular da HIO e da tolerância ainda não foi elucidado. Geralmente estes fenômenos resultam de alterações neuroplásticas no sistema nervoso periférico e central que levam à sensibilização das vias pró-nociceptivas. Acredita-se que para a HIO primeiramente haja um aumento da estimulação neuronal. Posteriormente ocorre o envolvimento do sistema glutamatérgico central; o aumento dos níveis de dinorfinas espinhais que causam a liberação de neuropeptídios excitatórios centrais; a ativação da via descendente da dor; alterações genéticas e a diminuição da recaptação de neurotransmissores envolvidos na resposta nociceptiva (Mao, 2002; Lee et al., 2011; Low, Clarke e Huh, 2012). Já para a tolerância sugere-se uma resposta adaptativa no receptor MOR e na sinalização após interação com o receptor que progressivamente neutraliza o efeito do opióide, envolvendo dessensibilização, internalização, “downregulation” e fosforilação dos receptores opióides ou heterodimerização com outros receptores (Dumas e Pollack, 2008; Garzón, Rodríguez-Muñoz e Sánchez-Blázquez, 2008).

Embora sejam fenômenos clínicos distintos, a tolerância e a hiperalgesia induzidos por opióide, e possivelmente a síndrome de abstinência, podem ter mecanismos relacionados. De fato, o sistema glutamatérgico central é considerado o mecanismo celular comum entre estes processos (Mao, 2002). Além disso, tanto a hiperalgesia como a alodínia são sintomas clássicos da

síndrome de abstinência e da HIO, que podem ser ambas consequências de uma mesma alteração nociceptiva precipitada pela exposição a opióides. Desta forma, podemos dizer que a HIO também é definida como uma sensibilização nociceptiva causada pela parada abrupta da administração prolongada de opióide; neste caso, conhecida como hiperalgesia induzida pela síndrome de abstinência (Li e Clark, 2002; Low, Clarke e Huh, 2012).

Potenciais estratégias utilizadas como tratamento de tais efeitos adversos induzidos por opióides incluem rotação de opióides e esforços para a redução da dose de opióides pela administração concomitante de analgésicos não opióides e adjuvantes. Os analgésicos não opióides que são utilizados incluem fármacos anti-inflamatórios, agonistas do receptor α_2 adrenérgico e antagonistas do receptor NMDA glutamatérgico. No entanto, na maioria das vezes, este tratamento pode ser longo e por vezes, impraticável. Consequentemente, muitos pacientes simplesmente desistem da terapia adjuvante e procuram retomar à terapêutica opióide, sendo um tratamento não satisfatório, pois este não proporciona o alívio completo da dor (Garzón, Rodríguez-Muñoz e Sánchez-Blázquez, 2008; Lee et al., 2011).

2.2.2.2 Síndrome de abstinência

A dependência a opióides é caracterizada por um conjunto de fenômenos fisiológicos, comportamentais e cognitivos de intensidade variável, que resultam de uma compulsão pelo consumo de opióides. Uma manifestação debilitante da dependência do opióides é a síndrome de abstinência, que possui uma baixa resposta às terapias clínicas disponíveis. A síndrome de abstinência é um traço característico da dependência a opióides, observada após a interrupção ao uso crônico de opióides ou precipitada pela administração de antagonistas opióides, como a naloxona. Ela é acompanhada por sintomas aversivos físicos e emocionais. Em humanos, os sinais e sintomas da abstinência incluem dores de estômago, diarreia, rinorreia, sudorese, elevação da frequência cardíaca e aumento da pressão arterial, irritabilidade, disforia, hiperalgesia e insônia. Estes sintomas se manifestam dentro de 24 horas e geralmente persistem por um período de uma semana a 10 dias (Ouyang et al., 2012; Scavone, Sterling e Van Bockstaele, 2013).

Os sinais e sintomas da síndrome de abstinência encontrados nos humanos também podem ser reproduzidos em modelos animais. Estes sinais são prontamente observados e quantificados após a administração de antagonistas, tais como a naloxona (denominado retirada precipitada pela naloxona) ou após a interrupção abrupta do tratamento com opióide (denominado retirada espontânea). Estão associados ao sistema simpático e parassimpático, e incluem tremor do corpo tipo "wet-dog shake", pulos, hiperatividade, postura anormal, ranger dos dentes, piloereção, lacrimejamento, rinorreia, diarreia, perda de peso abrupta e ejaculação (Koob, Maldonado e Stinus, 1992; Ouyang et al., 2012).

Diferentes regiões cerebrais são responsáveis pelos sinais/sintomas físicos e emocionais da síndrome de abstinência. Da mesma forma, alguns sinais físicos podem ser mediados por receptores opióides periféricos. Regiões cerebrais como a ACP, amígdala, área tegmental ventral, núcleo acumbens e hipotálamo estão diretamente envolvidas; porém o locus coeruleus (LC) é considerado o principal sítio cerebral que provoca o aparecimento da síndrome de abstinência induzida por opióide. Neurônios do LC, que são de natureza noradrenérgica, possuem uma elevada densidade de receptores opióides, que quando ativados, reduzem a liberação de noradrenalina. Contudo, uma exposição contínua a opióides causa uma tolerância deste efeito inibitório do opióide, aumentando a atividade noradrenérgica e resultando nos sinais de abstinência. (Koob, Maldonado e Stinus, 1992; Ouyang et al., 2012; Scavone, Sterling e Van Bockstaele, 2013; Rehni, Jaggi e Singh, 2013).

Agentes terapêuticos que diminuam a transmissão da via simpática no SNC, demostraram eficácia clínica em atenuar a hiperatividade noradrenérgica que é observada durante a retirada de opióides. No entanto, em alguns indivíduos, a utilização de agonistas do receptor α_2 adrenérgico é limitada pela presença de efeitos colaterais, tais como hipotensão, sedação, e comprometimento cognitivo, ou por supressão incompleta dos sintomas de abstinência. Isso torna importante a pesquisa de outros sistemas que interajam com o sistema opióide em regiões do cérebro e possam servir como potenciais alvos terapêuticos (Ouyang et al., 2012; Rehni, Jaggi e Singh, 2013).

Além do uso de medicamentos não opióides α_2 adrenérgicos, o tratamento farmacológico usualmente utilizado para aliviar os sintomas de

abstinência associada a dependência de opióides inclui a desintoxicação utilizando antagonistas opióides (naltrenoxa e naloxona), cessação gradual do agonista opióide (metadona) e a utilização a curto prazo de um agonista opióide parcial (buprenorfina). Infelizmente, os dados de eficácia existentes sobre tais tratamentos não são encorajadores, visto que o índice de reincidência após a descontinuação do tratamento é de 80% (Rehni, Jaggi e Singh, 2013).

2.2.2.3. Constipação

Outro efeito indesejável causado pelo uso de opióides é a constipação, que reduz consideravelmente a qualidade de vida dos pacientes. Estima-se que entre 40 – 95% dos pacientes tratados com opióides desenvolvem constipação, sendo assim considerado o efeito adverso mais frequente causado por opióides. Este efeito pode ocorrer não somente após uso prolongado de opióide, como também, com uma única dose (Khansari, Sohrabi e Zamani, 2013; Kumar, Barker e Emmanuel, 2014).

Agonistas opióides são capazes de mediar alguns efeitos gastrointestinais: inibem o esvaziamento gástrico, aumentam o tônus do esfíncter, causam mudanças nos padrões de motilidade e bloqueiam o peristaltismo, que resultam na constipação. Estes efeitos gastrointestinais surgem pela ação dos opióides no SNC e trato gastrointestinal. No entanto, a elevada densidade de receptores μ opióides no sistema entérico é que parece mediar a maior parte dos efeitos gastrointestinais dos agonistas opióides, reduzindo o tônus e a contractilidade intestinal, prolongando o tempo de trânsito (Khansari, Sohrabi e Zamani, 2013; Kumar, Barker e Emmanuel, 2014).

Ao contrário da analgesia, o opióide não produz tolerância na constipação, devendo esta ser monitorada e tratada. No entanto, as opções de tratamento para constipação induzida por opióides ainda são limitadas. Atualmente a abordagem farmacológica utilizada para constipação engloba os agentes laxativos e antagonistas opióides. Porém estes não apresentam um alívio adequado dos sintomas e são acompanhados de outros efeitos adversos gastrointestinais, não causando uma melhora na qualidade de vida dos pacientes (Khansari, Sohrabi e Zamani, 2013; Kumar, Barker e Emmanuel, 2014).

Novos alvos farmacológicos vêm sendo estudados na tentativa de reversão dos feitos adversos causados pelos opióides. Evidências indicam que canais de cálcio regulados por voltagem (CCRV) estão envolvidos no desenvolvimento da hiperalgesia e tolerância induzidos por opióides, bem como na síndrome de abstinência (Yokoyama, 2004; Dogrul et al., 2005; Meng et al., 2008). Além do mais, um importante alvo da analgesia induzida pelos opióides são os CCRV (Ruskin e Moises, 1995; Law, Wong e Loh, 2000).

2.3. Canais de cálcio regulados por voltagem - CCRV

Já é bem estabelecido que CCRVs são proteínas mediadoras importantes na sinalização nociceptiva em neurônios aferentes primários. Juntamente com uma série de canais iônicos e receptores, os CCRVs modulam a propagação e o processamento de sinais dolorosos. Esses canais de cálcio são altamente expressos em terminais nervosos pré-sinápticos; são ativados em resposta a potenciais de ação e medeiam o influxo de cálcio responsável pela liberação de neurotransmissores pró-nociceptivos que consequentemente ativam receptores neuronais pós-sinápticos que irão distribuir a informação nociva até centros supra espinhais (Zamponi et al., 2009; Park e Luo, 2010; Bourinet et al., 2014).

Os CCRV são classificados de acordo com suas propriedades biofísicas e farmacológicas em canais tipo L, N, P, Q, R (ativados por alta voltagem: ~ -30 mV) e T (ativados por baixa voltagem: ~ -60 mV). Os CCRVs são ainda agrupados em 3 famílias, de acordo com o gene que codifica sua subunidade α1, que forma o poro do canal. Os genes da família Cav1 codificam canais do tipo L (Cav1.2, Cav1.3 e Cav1.4); os da família Cav3 codificam os canais do tipo T (Cav3.1, Cav3.2 e Cav3.3) e os da família Cav2 codificam os canais do tipo N (Cav2.2), R (Cav2.3) e P/Q, sendo que os canais do tipo P e Q são variantes de "splicing" do gene Cav2.1 (Catterall, 2000; Evans e Zamponi, 2006; Simms e Zamponi, 2014).

Os CCRVs são complexos proteicos heteromultiméricos, são compostos de uma subunidade α_1 (formadora do poro) e subunidades auxiliares como β, α2δ e γ que regulam a expressão e as propriedades biofísicas desses canais. A subunidade formadora do poro permeável a íons cálcio é estruturalmente organizada em 4 domínios homólogos (I-IV), sendo que cada um contém 6

regiões transmembrana (1-6). O segmento transmembrana 4, rico em resíduos de arginina, é o sensor de voltagem do canal, cujo poro é formado pela união dos segmentos transmembrana 5 e 6 dos 4 domínios. Tanto a porção N- quanto a C-terminal são citoplasmáticas (Evans e Zamponi, 2006; Simms e Zamponi, 2014) (Figura 1).

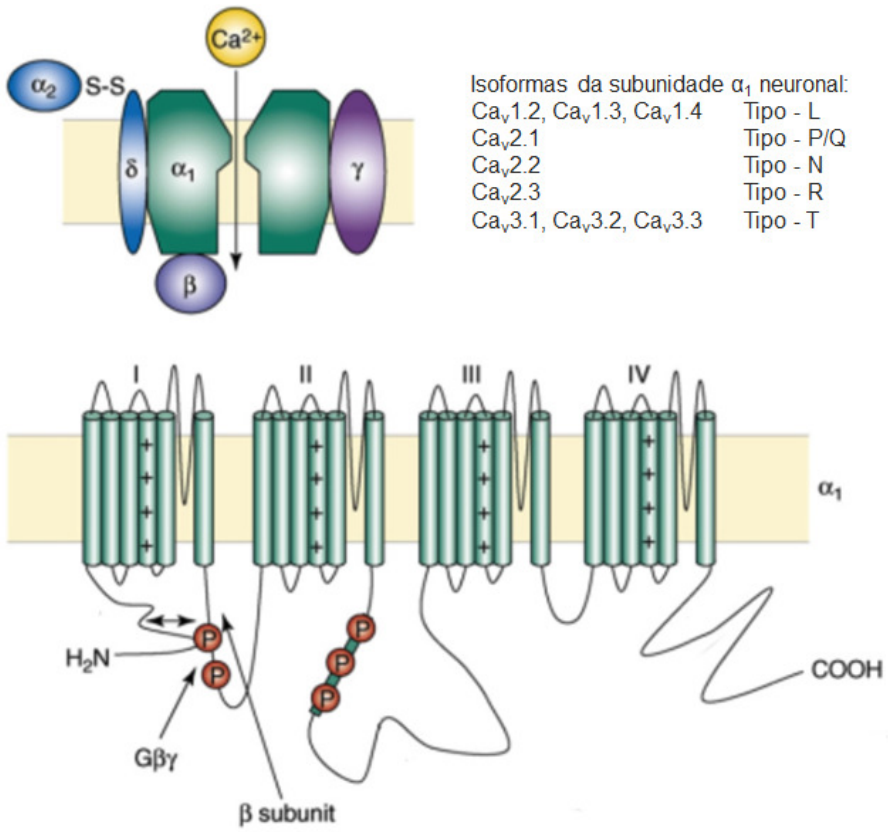


Figura 1 – Subtipos, estrutura e subunidades que compõem os canais de Ca^{2+} regulados por voltagem (adaptado de Evans e Zamponi, 2006).

Dados pré-clínicos demostram o envolvimento dos subtipos dos CCRV no processamento da dor. De fato, bloqueadores de CCRV do tipo N e do tipo R apresentaram atividade antinociceptiva em modelos de dor neuropática (Miljanich, 2004; Matthews et al., 2007). Além disso, foi observado que a ablação de qualquer um dos genes da família Cav2 - Cav2.1, Cav2.2 ou Cav2.3 - que codificam a subunidade α_1 dos canais do tipo P/Q, N ou R, respectivamente, reduz a hiperalgesia presente em modelo animal de neuropatia (Saegusa, Matsuda e Tanabe, 2002; Luvisetto et al. 2006). Ainda, o CCRV do tipo T está envolvido no desenvolvimento na síndrome da dor

musculoesquelética crônica, bem como na neuropatia diabética e na lesão do nervo ciático (Latham et al., 2009; Chen et al., 2010; Takahashi et al., 2010). Também foi evidenciado que os CCRV do tipo L apresentam uma regulação positiva em neurônios da medula espinhal em condições de dor crônica (Favereaux et al., 2011).

Além de estarem envolvidos em diferentes tipos de dor, os CCRV também estão relacionados com o desenvolvimento dos efeitos adversos causados pelos opióides. De fato, foi demostrado que após administração crônica de morfina, a expressão dos CCRV do tipo L e do tipo N estava significativamente aumentada na lâmina superficial da medula espinhal (Verma et al., 2009). Do mesmo modo, evidências indicam que bloqueadores dos CCRV do tipo L, N e R são capazes de inibir o desenvolvimento de hiperalgesia, tolerância e síndrome de abstinência induzidos por opióides (Yokoyama, 2004; Dogrul et al., 2005; Meng et al., 2008).

Além disso, um inibidor dos CCRV do tipo N, a ziconotida (versão sintética da ω-conotoxina MVIIA, um peptídeo isolado do veneno do caracol marinho *Conus magus*) foi aprovada para o uso intratecal em pacientes com dores intensas e refratárias ao tratamento com morfina e a outros tratamentos (Staats et al., 2004). A ziconotida é também frequentemente utilizada na clínica em associação com opióides (Wallace et al., 2010). Entretanto, ela ainda não é um analgésico ideal, já que apresenta uma janela terapêutica pequena e produz vários efeitos adversos em doses analgésicas, tais como: hipotensão postural, sedação, agitação e diarreia (Penn e Paice, 2000 e Staats et al., 2004).

Com isso, na tentativa de se desenvolver um bloqueador de CCRV tão eficaz quanto a ziconotida, porém mais seguro, há um crescente interesse por toxinas peptídicas presentes no veneno de animais peçonhentos como possíveis tratamentos para a dor.

2.4. Phα1β – peptídeo isolado do veneno da aranha *Phoneutria nigriventer*

O veneno da aranha *Phoneutria nigriventer*, conhecida popularmente como aranha armadeira, apresenta uma vasta gama de toxinas peptídicas com ações sobre canais iônicos (Gomez et al., 2002).

A aranha armadeira é assim conhecida pois assume uma posição bem característica; quando se sente ameaçada costuma “arma-se para o bote” (Figura 2).

Figura 2. Aranha *Phoneutria nigriventer*

A *Phoneutria nigriventer* é comumente encontrada no sul e sudeste do Brasil. É um ser solitário, irascível – não tolerando nem mesmo a presença de companheiras da mesma espécie, errante – não constrói teia, seu sucesso como predadora se dá pela potência do seu veneno. Apresenta hábitos noturnos ou crepusculares, esconde-se durante o dia em locais escuros, entre folhagem de arbustos, dentro das bainhas das bananeiras, não raro nos sapatos, daí a ocorrência de um grande número de acidentes com essa aranha (Gomez et al., 2002).

A picada de uma armadeira é muito dolorosa. A dor é intensa e imediata no local a princípio, irradiando-se por todo o membro após alguns minutos e persistindo durante horas. Os sintomas tóxicos são caracterizados por febre, sudorese, câimbras, tremores, paralisia espástica, priapismo, arritmias, distúrbios visuais e, em casos raros, morte, sendo mais graves em crianças se não for devidamente tratada (Gomez et al., 2002).

O poder tóxico do seu veneno se dá pelas diversas toxinas, capazes de interagir com canais iônicos, que o compõe. Seu principal efeito neurotóxico parece estar relacionado a sua ação sobre canais de Na^+ regulados por voltagem (Araújo et al., 1993; Romano-Silva et al., 1993). Entretanto, outras

atividades farmacológicas, relacionadas à ação em canais iônicos, podem ser encontradas em todo o veneno. A porção tóxica do veneno é dividida em 4 frações: PhTx1, PhTx2, PhTx3 e PhTx4 com peso molecular entre 3500 a 9000 Da (Rezende et al., 1991; Cordeiro et al., 1993; Gomez et al., 2002). As primeiras descrições de ações farmacológicas foram a partir das frações PhTx1 e PhTx2, as quais causaram contração do íleo de porcos da índia (Rezende et al., 1991).

Outra fração tóxica que foi isolada e estudada foi o PhTx3. A administração intracerebroventricular dessa fração produziu paralisia flácida em camundongos (Rezende et al., 1991), o que mais tarde foi atribuído à sua ação inibitória na liberação de neurotransmissores (Gomez et al.,1995; Prado et al., 1996). Seis diferentes toxinas foram purificadas a partir da fração PhTx3 (Tx3-1 até Tx3-6) e pelo menos três delas (Tx3-3, Tx3-4, Tx3-6) atuam quase que exclusivamente em canais de cálcio (Cordeiro et al., 1993). A toxina Tx3-3 foi uma das primeiras a ser investigada no veneno da *Phoneutria nigriventer* e bloqueia os CCRV de maneira não seletiva, inibindo preferencialmente correntes do tipo P/Q e R (Leão et al., 2000; Gomez et al., 2002). Além disso, mostrou-se um potente inibidor da liberação de glutamato e acetilcolina (Gomez et al., 1995; Prado et al., 1996). Mais recentemente foi demonstrado que Tx3-3 apresentou efeitos antinociceptivos em modelos de dor neuropática sem causar efeitos adversos em doses eficazes (Dalmolin et al., 2011).

Outra toxina presente na fração PhTx3 do veneno da aranha *Phoneutria nigriventer*, a Tx3-6, hoje conhecida como Phα1β é alvo do presente estudo. A Phα1β é um polipeptídeo básico, constituído de 55 aminoácidos (sequência de aminoácidos: ACIPRGEICT DDCECCGCDN QCYCPPGSSL GIFKCSCAHA NKYFCNRKKE KCKKA) e possui um peso molecular de 6044 Da (Cordeiro et al., 1993). Esta toxina mostrou-se eficaz na inibição da liberação de glutamato e no influxo de cálcio intrassinaptossomal (Vieira et al., 2003). Além disso foi observado que a Phα1β inibe correntes de cálcio do tipo Cav2.2 (que permeiam correntes de cálcio do tipo N), mas não era seletivo bloqueando também Cav2.3 (que permeiam correntes de cálcio do tipo R), Cav 2.1 (que permeiam correntes de cálcio do tipo P/Q) e por último Cav1.2 (que permeiam correntes de cálcio do tipo L), nesta ordem de potência (Vieira et al., 2005).

Assim como para a Tx3.3, trabalhos do grupo demostraram que a Phα1β apresenta potencial analgésico em diferentes modelos de dor (Tabela 1). De uma maneira geral, a Phα1β apresentou eficácia similar, mas índice terapêutico superior à ω-conotoxina MVIIA nestes modelos pré-clínicos de dor, como a pós-cirúrgica, a neuropática e a relacionada ao câncer (Souza et al., 2008; Rigo et al., 2013a). Além disso, a Phα1β produziu um efeito analgésico sobre a dor do câncer mesmo em animais tolerantes à morfina (Rigo et al., 2013b). No entanto, a utilização da Phα1β como uma droga adjuvante na terapia com opióides ainda não foi avaliada.

Tabela 1. Resumo do efeito analgésico das toxinas peptídicas em diferentes modelos de dor (retirado de Gomez et al., 2014)

MODELOS DE DOR	**TOXINAS PEPTÍDICAS**		
	Phα1β (Tx3.6)	Tx3.3	ω-conotoxin MVIIA
Nociceptiva			
Calor[a]	+	+	+
Mecânica[b]	-	-	-
Inflamatória			
Desencadeado por um agente irritante[c]	+	N.T.*	+
Artrítica[d]	+	-	+
Pós-operatória	+	N.T.	+
Neuropática			
Traumática[e]	+	+	+
Induzida por quimioterápicos[f]	+	N.T.	+
Relacionada ao Diabetes	N.T.	+	+
Câncer	+	-	+
Relacionada ao Tumor[g]			
Induzida por opióides	-	+	N.T.
Disfuncional			
Fibromialgia[h]	+	N.T.	N.T.

[a]Placa quente ou teste de imersão da cauda; [b]Filamentos de von Frey; [c]Teste da formalina ou capsaicina; [d]Inflamação induzida por adjuvante completo de Freund; [e]Lesão parcial do nervo ciático ou lesão por constrição crônica; [f]Síndrome dolorosa induzida por paclitaxel; [g]Inoculação de melanoma; [h]Causada pelo tratamento repetido com reserpina; *Não-testado

Portanto, a toxina Phα1β apresenta características que a tornam um peptídeo em potencial para o desenvolvimento de novos fármacos analgésicos. Assim, o presente estudo foi realizado com o intuito de avaliar as possíveis ações da Phα1β sobre os efeitos antinociceptivos e adversos produzidos pela administração única ou repetida de morfina em camundongos.

3. OBJETIVOS

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar as possíveis ações da toxina Phα1β isolada do veneno da aranha *Phoneutria nigriventer* sobre os efeitos antinociceptivos e adversos produzidos pela morfina em camundongos.

3.2 Objetivos Específicos

1. Verificar o desenvolvimento de antinocicepção e de efeitos adversos induzidos pelo tratamento único ou repetido com morfina em camundongos;
2. Avaliar o efeito da injeção intratecal da Phα1β sobre os efeitos antinociceptivos e adversos produzidos pela morfina;
3. Averiguar se a forma recombinante da Phα1β (CTK 01512-2) apresenta efeitos semelhantes ao peptídeo nativo.

4. ARTIGO

Os resultados inseridos nesta tese apresentam-se sob a forma de artigo científico, o qual se encontra aqui estruturado. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no artigo. O artigo está disposto conforme aceito para publicação na revista The Journal of Pain.

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Action of Phα1β, a Peptide From the Venom of the Spider *Phoneutria nigriventer*, on the Analgesic and Adverse Effects Caused by Morphine in Mice

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Abstract: **Opioids are standard therapy for the treatment of pain; however, adverse effects limit their use. Voltage-gated calcium channel blockers may be used to increase opioid analgesia, but their effect on opioid-induced side effects is little known. Thus, the goal of this study was to evaluate the action of the peptide Phα1β, a voltage-gated calcium channel blocker, on the antinociceptive and adverse effects produced by morphine in mice. A single administration of morphine (3–10 mg/kg) was able to reduce heat nociception as well as decrease gastrointestinal transit. The antinociception caused by a single injection of morphine was slightly increased by an intrathecal injection of Phα1β (30 pmol/site). Repeated treatment with morphine caused tolerance, hyperalgesia, withdrawal syndrome, and constipation, and the Phα1β (.1–30 pmol/site, intrathecal) was able to reverse these effects. Finally, the effects produced by the native form of Phα1β were fully mimicked by a recombinant version of this peptide. Taken together, these data show that Phα1β was effective in potentiating the analgesia caused by a single dose of morphine as well as in reducing tolerance and the adverse effects induced by repeated administration of morphine, indicating its potential use as an adjuvant drug in combination with opioids.**

Perspective: ***This article presents preclinical evidence for a useful adjuvant drug in opioid treatment. Phα1β, a peptide calcium channel blocker, could be used not only to potentiate morphine analgesia but also to reduce the adverse effects caused by repeated administration of morphine.***

Key words: *Calcium channel blocker, constipation, hyperalgesia, opioid, tolerance, withdrawal syndrome.*

Opioids are the most common drugs prescribed worldwide for alleviating moderate to severe pain, such as cancer-related pain and chronic pain.[2] However, the use of opioids is associated with the development of tolerance and potential adverse effects, such as constipation and withdrawal syndrome.[13,15,16] Accumulating evidence indicates that withdrawal syndrome may be related to another problem, often referred to as opioid-induced hyperalgesia.[2,24,36] Opioid-induced hyperalgesia is defined as a state of nociceptive sensitization as a result of systemic or intrathecal (i.t.) exposure to opioids or after the chronic use of these drugs when administration is abruptly stopped (in the latter case, it is called withdrawal-induced hyperalgesia).[23,24] Thus, adjuvant therapies could be useful not only to increase analgesia but also to reverse tolerance and the adverse effects caused by opioid drugs.[22]

An important target for opioid-induced analgesia is the blockade of voltage-gated calcium channels (VGCCs).[21,37]

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VGCCs are a family of ion channels classified according to their electrophysiological and pharmacological properties as L-, N-, P-, Q-, or R-type channels (high-voltage-activated: approximately −30 mV) or T-type channels (low-voltage-activated: approximately −60 mV).[4,9] As an indication of the usefulness as opioid adjuvant drugs, spinal blockade or gene deletion of certain VGCCs, such as N, P/Q, R, and L, increases the antinociceptive effect as well as reduces the tolerance induced by morphine.[8,12,47,48] However, the participation of VGCCs in the adverse effects caused by acute or repeated opioid administration is poorly understood. Repeated treatment with morphine resulted in an increase in the expression of both L- and N-type VGCCs in the superficial laminae of the spinal cord, indicating a possible role of VGCCs in this process.[43]

The very selective N-type VGCC blocker ziconotide (the synthetic form of the ω-conotoxin MVIIA) is clinically used for the treatment of pain in patients who require i.t. medication and are refractory to opioids.[26] Preclinical studies have demonstrated that there is no cross-tolerance between ziconotide and morphine. The former was unfortunately not capable of reversing opioid-induced tolerance.[46] Moreover, the clinical use of ziconotide is limited by the manifestation of serious adverse effects at analgesic doses (small therapeutic window).[41,45] Thus, although VGCCs seem to be interesting targets for the development of analgesic and opioid adjuvant drugs, novel safe and efficacious drugs are needed.

Phα1β is a peptide purified from the venom of the Brazilian armed spider *Phoneutria nigriventer* and blocks high-voltage calcium currents in type N, R, P/Q, and L VGCCs (in this order of potency).[15,44] As an indication of its therapeutic potential, i.t. Phα1β has similar efficacy but a higher therapeutic index than ziconotide in preclinical models of chronic neuropathic and cancer pain.[33,40] However, the potential use of Phα1β as an adjuvant drug in opioid therapy has not been investigated. Thus, the goal of this study was to evaluate the possible actions of Phα1β on antinociception, tolerance, hyperalgesia, withdrawal syndrome, and constipation produced by single or repeated administration of morphine in mice.

Methods

Animals

Male C57BL/6 mice (20–30 g) bred in-house were used in all experiments. The experimental protocols were authorized by the local ethics committee of the Universidade Federal de Santa Maria (process number: 23081.005024/2010-88) and were in accord with the current ethical guidelines of the International Association for the Study of Pain for the investigation of experimental pain in conscious animals.[49] In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Drugs

Native Phα1β was purified as previously described and had the following amino acid sequence: ACIPRGEICT DDCECCGCDN QCYCPPGSSL GIFKCSCAHA NKYFCNRKKE KCKKA.[5] The recombinant form of Phα1β (CTK 01512-2) was purchased from Giotto Biotech S.r.l. (Florence, Italy). The stock solutions of the drugs were prepared in phosphate-buffered saline (PBS, pH 7.4) in siliconized plastic tubes, maintained at −20°C, and diluted to the desired concentration just before use. Morphine sulfate and naloxone hydrochloride were purchased from Cristália (São Paulo, Brazil).

Drug Treatments

Phα1β (.1–100 pmol/site) or CTK 01512-2 (30 pmol/site) were administered by the i.t. route at a volume of 5 μL/site according to the technique described by Hylden and Wilcox.[17] Morphine (1–25 mg/kg) was administered subcutaneously (s.c.). Naloxone (2 mg/kg) was administered by the intraperitoneal route. Phα1β and CTK 01512-2 were dissolved in PBS, and morphine and naloxone were dissolved in saline (NaCl .9%). Behavioral testing was performed by an experienced observer (R.T.) blinded with respect to drug administration.

Experimental Design

Protocols Using a Single Injection of Morphine

First, a single injection of morphine (1, 3, or 10 mg/kg, s.c.) or Phα1β (30 or 100 pmol/site, i.t.) was evaluated on thermal and mechanical models of nociceptive pain in mice at different times (.25–4 hours) after treatment. Then, 2 doses of morphine (10 + 10 mg/kg, s.c.) were administered at 4-hour intervals to evaluate the acute tolerance on thermal test. As controls, different groups of animals were injected with the appropriate vehicle (PBS, 5 μL/site, i.t., or saline, 10 mg/mL, s.c.). The dose of drugs and evaluation times were based on those of previous studies.[13,33]

Next, we evaluated the effects of the combined treatment of Phα1β with a single dose of morphine on thermal and mechanical nociception tests in mice. Mice were injected with morphine (3 mg/kg, s.c.) immediately after being injected with Phα1β (30 pmol/site, i.t.) and were tested from .25 to 4 hours after treatment. Separate groups of animals were injected with PBS (5 μL/site, i.t.) and saline (10 mg/mL, s.c.), Phα1β (30 pmol/site, i.t.) and saline (s.c.), or PBS (i.t.) and morphine (3 mg/kg, s.c), as controls. A similar experimental design was used to assess the effect of Phα1β on a single dose of morphine-induced constipation (measured by gastrointestinal transit) or withdrawal syndrome (measured by naloxone-precipitated withdrawal), with the exceptions that morphine was used at 10 mg/kg and tested 1 (gastrointestinal transit) or 2 (naloxone-precipitated withdrawal) hours after treatment. For these tests, animals were treated with morphine (10 mg/kg, s.c.) immediately after being injected with Phα1β (30 pmol/site, i.t.). Separate groups of animals were treated with PBS (5 μL/site, i.t.) and saline (10 mg/mL, s.c.), PBS and morphine (10 mg/kg, s.c.), or Phα1β (30 pmol/site, i.t.) and saline, as controls. The dose of drugs and time evaluated were based on those of previous studies.[13]

Protocols Using Repeated Injections of Morphine

To evaluate the antinociceptive and adverse effects produced by repeated administration of morphine in mice, an experimental design adapted from Marshall and Weinstock[25] was used. The protocol consisted of administration of increasing doses of morphine 3 times each day for 3 days (10 + 10 + 15, 15 + 15 + 20, and 20 + 20 + 25 mg/kg, s.c.). Briefly, mice received 3 s.c. injections of morphine per day, with an interval of 4 to 5 hours between each injection (first injection 9:00 AM). On day 1, 10, 10, and 15 mg/kg of morphine were administered, followed by 15, 15, and 20 mg/kg on day 2 and 20, 20, and 25 mg/kg on day 3. The 5-mg/kg increase in the last injection each day of treatment was aimed to minimize the induction of withdrawal syndrome overnight. On day 4, the animals received a challenge dose of morphine (10 mg/kg, s.c.). As a control, a different group of animals was injected with the vehicle (saline, 10 mg/mL, s.c.).

Before each morning injection of morphine, thermal and mechanical nociception tests were carried out in animals to check the development of hyperalgesia induced by the opioid. On days 1 and 4, mice were subjected to the thermal nociception test 30 minutes after the morning injection of morphine (10 mg/kg) to check the development of analgesic tolerance.

On the fourth day, 2 hours after the morphine challenge (a time when it has lost its effect), animals were treated with Phα1β (.1–30 pmol/site, i.t.) or vehicle (PBS). Thirty minutes to 6 hours after its injection, animals were evaluated with the thermal and mechanical tests to assess the effect of Phα1β on morphine-induced hyperalgesia.

In addition, we tested whether Phα1β could restore the analgesic effect of morphine in tolerant mice. To do this, a dose of Phα1β that was ineffective against hyperalgesia (.1 pmol/site, i.t.) was administered on the fourth day of treatment, immediately before the morphine challenge (10 mg/kg, s.c.). As controls, separate groups of animals were injected with PBS (5 μL/site, i.t.) and saline (10 mg/mL, s.c.), PBS and morphine, or Phα1β and saline. The thermal and mechanical nociception tests were then evaluated from .25 to 4 hours after treatment.

Additionally, we assessed the effect of Phα1β on morphine-induced withdrawal syndrome and constipation (measured by the naloxone-precipitated withdrawal and gastrointestinal transit, respectively) caused by repeated doses of morphine. To do this, increasing doses of morphine were administered in mice for 3 days, as previously described, and on day 4, naloxone-precipitated withdrawal or gastrointestinal transit was evaluated. The animals were treated with morphine (10 mg/kg, s.c.) immediately after being injected with Phα1β (30 pmol/site, i.t.) and tested 1 (gastrointestinal transit) or 2 (naloxone-precipitated withdrawal) hours after treatment. Separate groups of animals were treated with PBS (5 μL/site, i.t.) and saline (10 mg/mL, s.c.), PBS and morphine (10 mg/kg, s.c.), or Phα1β (30 pmol/site, i.t.) and saline, as controls.

Assessment of Analgesic and Hyperalgesic Effects

Tail-Flick Test

Measurement of thermal nociceptive pain was carried out as previously described.[6] Briefly, after an environmental habituation period, the mice were gently handled and had two-thirds of their tail dipped into a bath containing water kept at 48 $\pm$ 1 °C. This low-intensity stimulus yields baseline latencies (8–10 seconds) that are long enough to observe hyperalgesia or analgesia. If no response occurred within 24 seconds, the test was interrupted to avoid tissue damage. The results were expressed in latency to tail flick or as a percentage of the maximum possible effect (MPE), which was calculated as %MPE = [(test latency $-$ control latency)/(24 $-$ control latency)] $\times$ 100. Percent MPE was calculated for at least 6 mice per group.

Von Frey Test

Mechanical nociceptive pain was assessed by measurement of the paw withdrawal threshold (PWT) using the "Up-and-Down" paradigm, as previously described.[6] Briefly, mice were first acclimatized (1–2 hours) in individual clear acrylic glass boxes on an elevated wire mesh platform to allow access to the plantar surface of the hind paws. Von Frey filaments of increasing stiffness (.02–10 g) were applied to the hind paw plantar surface of the animals with a pressure high enough to bend the filament. The 50% mechanical PWT response was then calculated from the resulting scores as described previously by Dixon.[7] The PWT was expressed in grams (g) and was evaluated before and several times after s.c. injection of morphine or i.t. injection of Phα1β. A significant decrease in PWT compared to baseline values was considered mechanical hyperalgesia.

Assessment of Side Effects

Naloxone-Precipitated Withdrawal

This test was conducted to assess the possible effect of Phα1β on withdrawal syndrome induced by acute and chronic injections of morphine. Mice were acclimatized to a clear acrylic glass testing chamber 1 hour before naloxone administration. Two hours after treatment, mice received naloxone (2 mg/kg) to precipitate withdrawal. Signs of withdrawal were the same as those previously described, with some modifications.[10] Briefly, jumping, headshaking, wet-dog shaking, abdominal writhing, and grooming behavior were evaluated at 10-minute intervals for a total testing period of 30 minutes, and a standardized score of 0 to 3 was assigned (0 = absent; 1 = 1–3 bouts; 2 = 4–6 bouts; 3 = 7 bouts and greater). Paw tremors, piloerection, salivation, and ejaculation were also evaluated, with 1 point being given for the presence of each sign during each 10-minute interval. The number of periods showing the latter signs were then counted (maximum score of 3 per behavioral sign), and the scores were

added together to yield a final cumulative withdrawal score.

Gastrointestinal Transit

This test was conducted to determine the possible effect of Phα1β on constipation induced by acute and chronic injections of morphine. In this study, the mice were fasted for 18 to 24 hours (with water ad libitum) prior to analysis of gastrointestinal transit, as previously described by Andrade and coworkers.[1] Thirty minutes after treatment, a mixture of activated charcoal standard (5% activated carbon, 20% gum Arabic, .3 mL) was given to mice by gavage. Twenty minutes after the administration of the mixture of activated charcoal, the animals were euthanized, and their stomachs and small intestines were removed to measure the length of the intestine (from the pyloric sphincter to the ileum-cecal junctions, considered the total gut length) and the distance traveled by the charcoal meal. The propulsive activity of the gut was determined by the percentage of the gastrointestinal tract that the charcoal traveled, calculated as % traveled = 100 × (distance charcoal traveled/total gut length).

Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM), with the exception of the ED_{50} value (the dose of compound that produces 50% of the effect relative to the control value), which was reported as the geometric mean accompanied by respective 95% confidence limits. The percentages of maximum effect (E_{max}) and maximum inhibition (I_{max}) were reported as the means ± SEM for each individual experiment in relation to the control values. The data were analyzed using Student's t-test and 1- or 2-way analysis of variance followed by the Student-Newman-Keuls or Bonferroni's post hoc tests, when appropriate. The ED_{50} value was determined by nonlinear regression analysis using a sigmoid dose-response equation using GraphPad Software, version 5.0 (Graph Pad, La Jolla, CA). The level of significance was set to $P < .05$.

Results

Effect of Phα1β on the Antinociceptive Effect Produced by a Single Injection of Morphine

To determine the possible effect of i.t. administration of Phα1β or s.c. administration of morphine on the thermal and mechanical nociception test in mice, we used the tail-flick and von Frey tests, respectively. Phα1β (30 and 100 pmol/site, i.t.) treatment did not alter the results of the thermal and mechanical tests of naïve mice (Supplementary Fig 1). For the next experiments, the maximum tested dose of Phα1β was 30 pmol/site because Rigo and coworkers[33] showed that Phα1β 100 pmol/site produced adverse sensory effects.

In contrast, a single injection of morphine (10 mg/kg, s.c.), but not saline, was able to induce antinociception in the tail-flick test, an effect that started at .25 hours, peaked at .5 hours and lasted up to 1 hour after treatment (Fig 1A). Morphine (1–10 mg/kg, s.c.) caused an increase in thermal latency with a mean ED_{50} value (and its 95% confidence limits) of 3.1 (2.9–3.4) mg/kg and an E_{max} of 100% at a dose of 10 mg/kg (Fig 1B). However, we did not observe any effect of morphine (1–10 mg/kg, s.c.) on the mechanical threshold (Figs 1C and 1D).

The administration of Phα1β (30 pmol/site, i.t.) with a single submaximal dose of morphine (3 mg/kg, s.c.) slightly increased (20 ± 7% at .5 hours) the antinociceptive effect of morphine in the thermal test (Fig 2A). We did not observe any effect of Phα1β (30 pmol/site, i.t.) and morphine (3 mg/kg, s.c.) on the mechanical threshold (Fig 2B).

Effect of Phα1β on Constipation Produced by a Single Injection of Morphine

A single dose of morphine (10 mg/kg, s.c.) but not saline (s.c.) or Phα1β (30 pmol/site, i.t.) alone caused a 70 ± 7% reduction in gastrointestinal transit 1 hour after treatment (Fig 3C). I.t. administration of Phα1β (30 pmol/site) did not alter the constipation induced by a single dose of morphine (Supplementary Fig 2). However, the single injection of neither morphine (up to 10 mg/kg, s.c.) nor Phα1β (up to 100 pmol/site) caused hyperalgesia until 4 hours after injection or naloxone-precipitated withdrawal syndrome 2 hours after treatment. The same way, morphine (10 mg/kg, s.c.) did not produce tolerance to its antinociceptive effect when the second dose administered 4 hours after the first (data not shown).

Effect of Phα1β on the Hyperalgesia and Tolerance Produced by Repeated Injections of Morphine

Repeated administration of increasing doses of morphine caused a progressive decrease in the thermal nociceptive response (thermal hyperalgesia) from 3 to 4 days after morphine treatment was started (reduced from 8.5 ± .4 seconds on day 1 to 5.7 ± .6 seconds 4 days after morphine treatment started; $P < .001$, Student's t-test) and a decrease in the mechanical threshold (mechanical hyperalgesia) 4 days after morphine treatment was started (reduced from 1.75 ± .24 g on day 1 to .67 ± .16 g on day 4; $P < .001$, Student's t-test), whereas saline injection had no effect (Figs 3A and 3C). Morphine challenge on day 4 not only fully reverted thermal hyperalgesia but also slightly increased the latencies above those observed with vehicle treatment (ie, had an antinociceptive effect) (Fig 3B). In contrast, morphine challenge did not alter the mechanical hyperalgesia of morphine-tolerant animals (Fig 3D).

Moreover, repeated treatment with morphine greatly reduced its antinociceptive efficacy, indicating the development of tolerance. The MPE values 30 minutes after a morphine challenge (10 mg/kg, s.c.) were 97 ± 2% on day 1 and 23 ± 18% on day 4 ($P < .01$, Student's t-test) (Fig 3E).

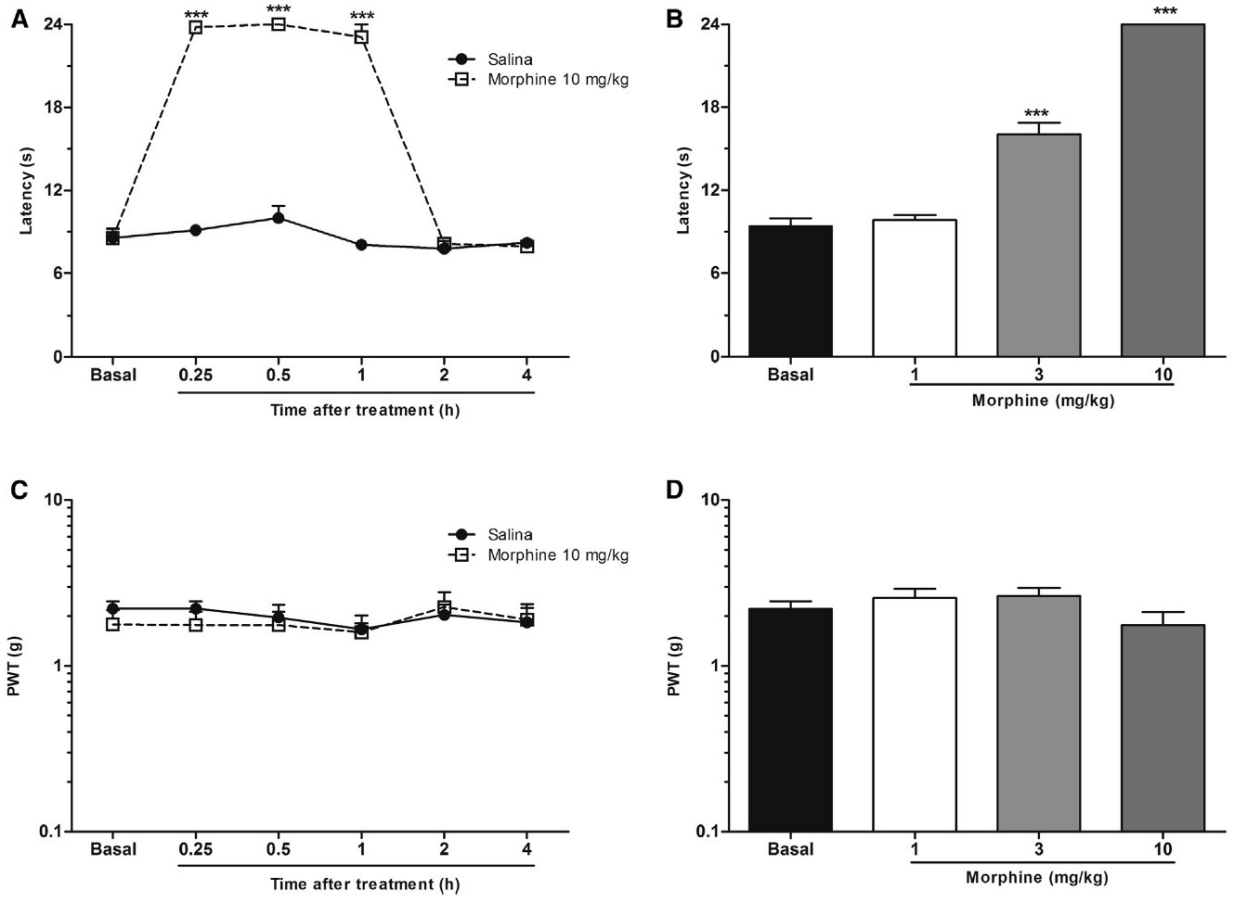

Figure 1. Effect of s.c. injection of morphine on thermal and mechanical nociception in mice. **(A)** Time course and **(B)** dose-response curve of the effect of morphine (1–30 mg/kg, s.c.) on thermal nociception. **(C)** Time course and **(D)** dose-response curve of the effect of morphine (1–30 mg/kg, s.c.) on mechanical nociception. Data are expressed as **(A** and **B)** latency(ies) or **(C** and **D)** PWT (g). Each point and column represents the mean of 5 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance followed by Bonferroni's post hoc test **(A** and **C)** or 1-way analysis of variance followed by the Student-Newman-Keuls post hoc test **(B** and **D)**. ***$P < .01$ when compared to baseline values.

Next, we evaluated the effect of i.t. injection of Phα1β on morphine-induced hyperalgesia in mice. The thermal and mechanical hyperalgesia induced by morphine were reverted by injection of Phα1β (30 pmol/site, i.t.) from 1 up to 2 hours after treatment (Figs 4A and 4C). A lower dose of Phα1β (i.e., 3 pmol/site, i.t.) presented similar efficacy and time course (data not shown). The injection of Phα1β (1–30 pmol/site, i.t.) was capable of reversing the thermal hyperalgesia induced by morphine with a mean ED_{50} value of .6 (.1–3.3) pmol/site and I_{max} of 100% at a dose of 30 pmol/site (Fig 4B). In addition, i.t. injection of Phα1β (3–30 pmol/site) was capable of reversing the mechanical hyperalgesia induced by morphine with a mean ED_{50} value (and its 95% confidence limits) of 2.5 (1.7–3.8) pmol/site and an I_{max} of 98 ± 8% at a dose of 30 pmol/site (Fig 4D).

In addition, Phα1β (.1 pmol/site, i.t.), at a dose that was not effective in altering either the thermal or mechanical hyperalgesia induced by morphine, was largely able to reverse morphine-induced tolerance (MPE increased by 84 ± 9% at 30 minutes) when concomitantly administered with the morphine challenge (10 mg/kg, s.c.). Moreover, Phα1β (.1 pmol/site, i.t.) induced an increase in the duration of the morphine effect, from .25 up to 2 hours after treatment, in the thermal test (Fig 4E). In addition, the morphine challenge did not have an effect on mechanical hyperalgesia in mice by itself. However, when administered with Phα1β (.1 pmol/site, i.t.), morphine demonstrated an effect from .25 up to .5 hours (Fig 4F).

Effect of Phα1β on Withdrawal Syndrome Produced by Repeated Injection of Morphine

Compared to the saline challenge, a challenge with morphine (10 mg/kg, s.c.) increased the naloxone-precipitated withdrawal syndrome in animals that had received repeated treatment with morphine by 86 ± 18% (Fig 5A). I.t. injection of Phα1β (30 pmol/site), but not PBS, was capable of fully reversing the naloxone-precipitated withdrawal syndrome induced by the morphine challenge (100% inhibition) (Fig 5A). In contrast, Phα1β (30 pmol/site) did not alter the induction of naloxone-precipitated withdrawal syndrome in animals challenged with saline (Fig 5A).

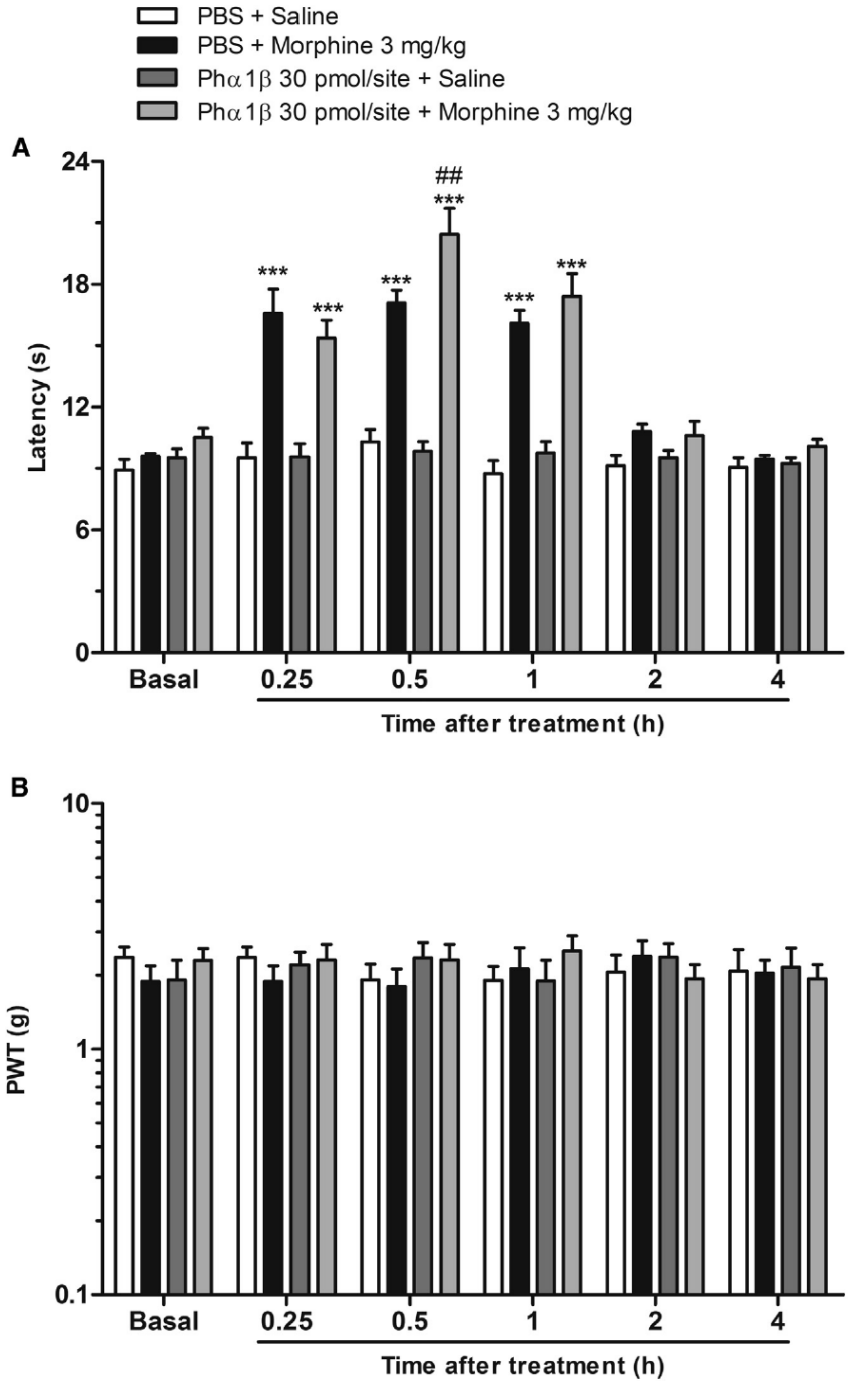

Figure 2. Effect of i.t. injection of Phα1β on the antinociceptive effect produced by a single dose of morphine in mice. **(A)** Time course of the effect of Phα1β (30 pmol/site, i.t.) or PBS (5 μL/site, i.t.) and morphine (3 mg/kg, s.c.) or saline (10 mg/mL, s.c.) on thermal nociception in mice. **(B)** Time course of the effect of Phα1β (30 pmol/site, i.t.) or PBS (5 μL/site, i.t.) and morphine (3 mg/kg) or saline (10 mg/mL, s.c.) on mechanical nociception in mice. Data are expressed as **(A)** latency(ies) or **(B)** PWT (g). Each column represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance followed by Bonferroni's post hoc test. ***$P < .001$ when compared to the PBS and saline group; ##$P < .05$ when compared to the PBS and morphine group.

Effect of Phα1β on Constipation Produced by Repeated Morphine Injections in Mice

A morphine challenge (10 mg/kg, s.c.) largely reduced (76 ± 2% inhibition) gastrointestinal transit in animals that had previously received repeated treatment with morphine (Fig 5B). A morphine challenge (10 mg/kg, s.c.) also decreased gastrointestinal transit in animals that previously received repeated treatment with saline (results not shown). I.t. injection of Phα1β (30 pmol/site) but not PBS was able to partially reverse the constipation induced by the morphine challenge (23 ± 4% inhibition)

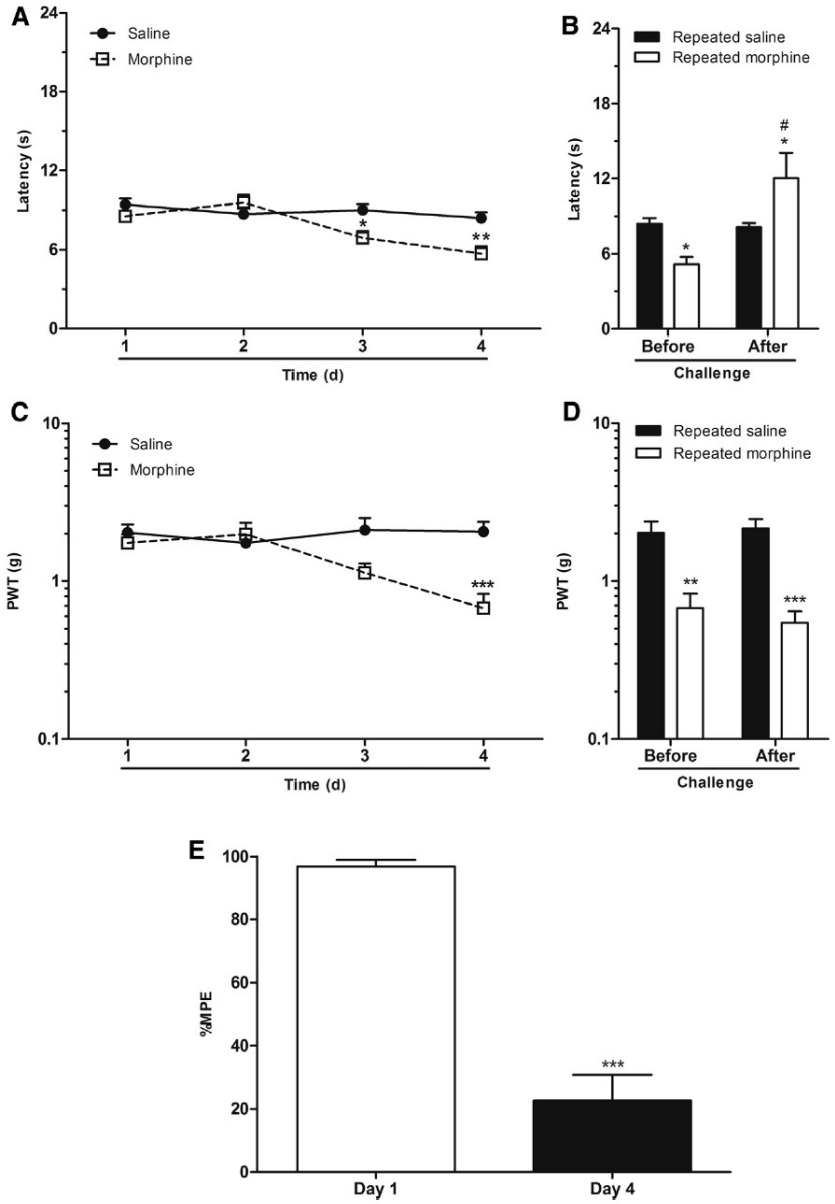

Figure 3. Effect of repeated injection of morphine on thermal and mechanical nociception in mice. Time course of thermal **(A)** and mechanical **(C)** nociception just before the first of the daily morphine or saline repeated injections. Thermal **(B)** and mechanical **(D)** nociception 4 days after repeated morphine or saline treatment, before and 30 minutes after the morphine (10 mg/kg) or saline (10 mL/kg) challenge. **(E)** Maximum possible antinociceptive effect (MPE) of the morphine challenge (10 mg/kg, 30 minutes after injection) on day 1 or 4 of repeated morphine treatment. Data are expressed as **(A** and **B)** latency(ies), **(C** and **D)** PWT (g), or **(E)** MPE (%). Each point or column represents the mean of 10 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way analysis of variance followed by Bonferroni's post hoc test **(A–D)** or Student's t-test **(E)**. $*P < .05$, $**P < .01$, or $***P < .001$ when compared to the saline group **(A–D)** or to day 1 **(E)**; $\#P < .05$, when compared to values before morphine treatment.

(Fig 5B). In contrast, Phα1β (30 pmol/site) did not alter gastrointestinal transit in animals challenged with saline (Fig 5B).

The Recombinant Form of Phα1β (CTK 01512-2) Mimicked the Effects of the Native Peptide on the Antinociceptive and Adverse Effects Produced by Single or Repeated Injection of Morphine

CTK 01512-2 (30 pmol/site, i.t.) treatment did not alter the thermal and mechanical nociception tests of naïve mice (Supplementary Figs 3A and 3B). Moreover, i.t. administration of CTK 01512-2 (30 pmol/site) did not alter the constipation induced by a single dose of morphine (Supplementary Fig 3C).

We also evaluated the effect of i.t. injection of CTK 01512-2 on morphine-induced hyperalgesia in mice. The thermal and mechanical hyperalgesia induced by morphine were reverted by injection of CTK 01512-2 (30 pmol/site, i.t.) at 1 and 2 hours, with an inhibition of 100% and 85 $\pm$ 18%, observed at 1 hour for thermal and mechanical stimulus, respectively (Figs 6A and 6B).

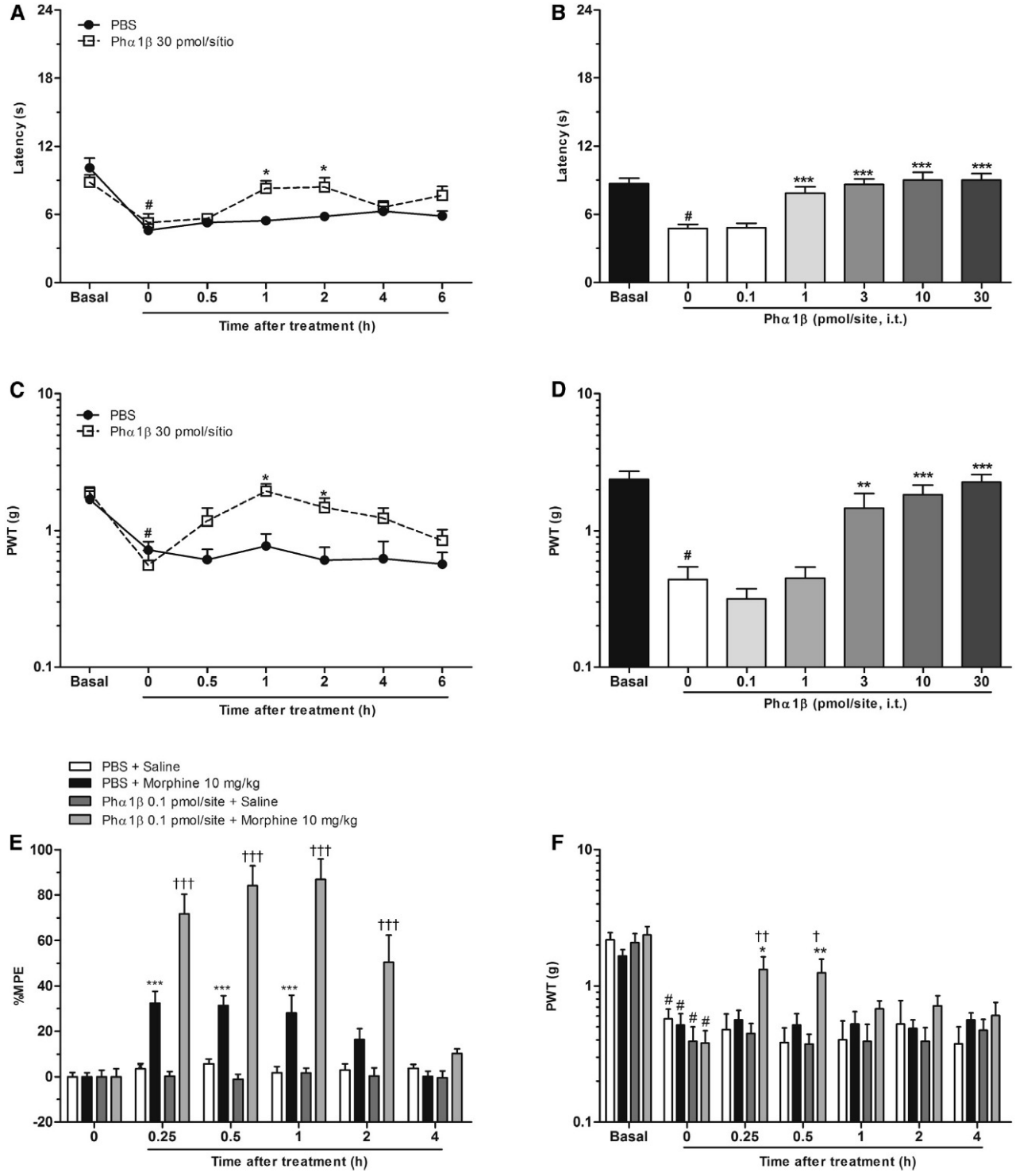

Figure 4. Effect of i.t. injection of Phα1β on hyperalgesia and tolerance produced by repeated injection of morphine in mice. **(A** and **C)** Time course and **(B** and **D)** dose-response curve of the effect of Phα1β (.1–30 pmol/site, i.t.) on thermal **(A** and **B)** or mechanical **(C** and **D)** hyperalgesia induced by repeated morphine treatment. **(E)** Time course of the maximum possible antinociceptive effect (MPE) of Phα1β (.1 pmol/site, i.t.) or PBS (5 μL/site, i.t.) and morphine (10 mg/kg, s.c.) or saline (10 mg/mL, s.c.) challenge on tolerance to the antinociceptive effect caused by morphine in thermal nociception. **(F)** Time course of the effect of Phα1β (.1 pmol/site, i.t.) or PBS (5 μL/site, i.t.) and morphine (10 mg/kg, s.c.) or saline (10 mg/mL, s.c.) challenge on mechanical nociception in mice. Data are expressed as **(A** and **B)** latency(ies), **(C**, **D**, and **F)** PWT (g), or **(E)** MPE (%). Each point and column represents the mean of 6 to 8 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance followed by Bonferroni's post hoc test **(A**, **C**, **E**, and **F)** or 1-way analysis of variance followed by Student-Newman-Keuls post hoc test **(B** and **D)**. *$P < .05$, **$P < .01$, or ***$P < .001$ when compared to dose 0 values **(B** and **D)**, to the PBS group **(A** and **C)**, or to the PBS and saline group **(E** and **F)**; #$P < .05$, when compared to baseline (basal) values **(B**, **D**, and **F)**; †$P < .05$, ††$P < .01$, or †††$P < .001$ when compared to the PBS and morphine group **(E** and **F)**.

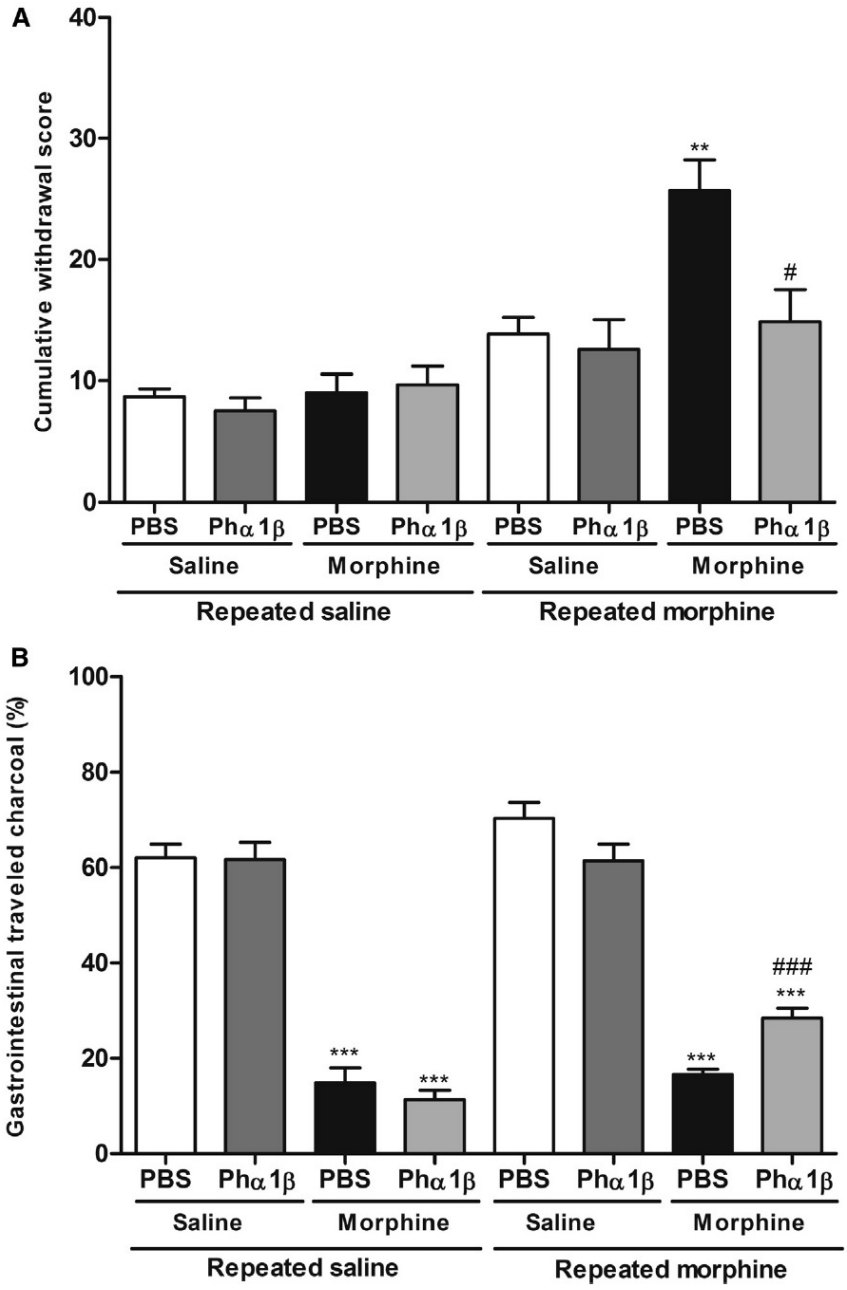

Figure 5. Effect of i.t. injection of Phα1β on withdrawal syndrome and constipation produced by repeated injection of morphine in mice. **(A)** Effect of Phα1β (30 pmol/site, i.t.) on withdrawal syndrome induced by repeated morphine or saline injection in mice. **(B)** Effect of Phα1β (30 pmol/site, i.t.) on gastrointestinal transit inhibition induced by repeated morphine or saline injection in mice. Data are expressed as **(A)** cumulative withdrawal score or **(B)** percentage of the gastrointestinal tract that charcoal traveled. Each column represents the mean of 5 to 9 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way analysis of variance followed by Bonferroni's post hoc test. $^{**}P < .01$ when compared to the PBS and saline group in repeated morphine treatment **(A)**; $^{***}P < .001$ when compared to the PBS and saline group **(B)**; and $\#P < .05$ or $\#\#\#P < .001$ when compared to the PBS and morphine group in repeated morphine treatment **(A** and **B)**.

The i.t. injection of CTK 01512-2 (30 pmol/site) but not PBS was able to partially reverse the constipation induced by the morphine challenge (24 ± 7% inhibition) (Fig 6C) and to fully reverse naloxone-precipitated withdrawal syndrome (100% inhibition) (Fig 6D). CTK 01512-2 (30 pmol/site) did not alter gastrointestinal transit or the naloxone-precipitated withdrawal syndrome in animals challenged with saline (Figs 6C and 6D).

Discussion

Opioids are the standard therapy for the management of several types of pain; however, their use is limited by adverse effects.[24] In the present study, we observed that the i.t. administration of Phα1β was effective in potentiating the analgesic effect caused by a single dose of morphine and reducing the tolerance,

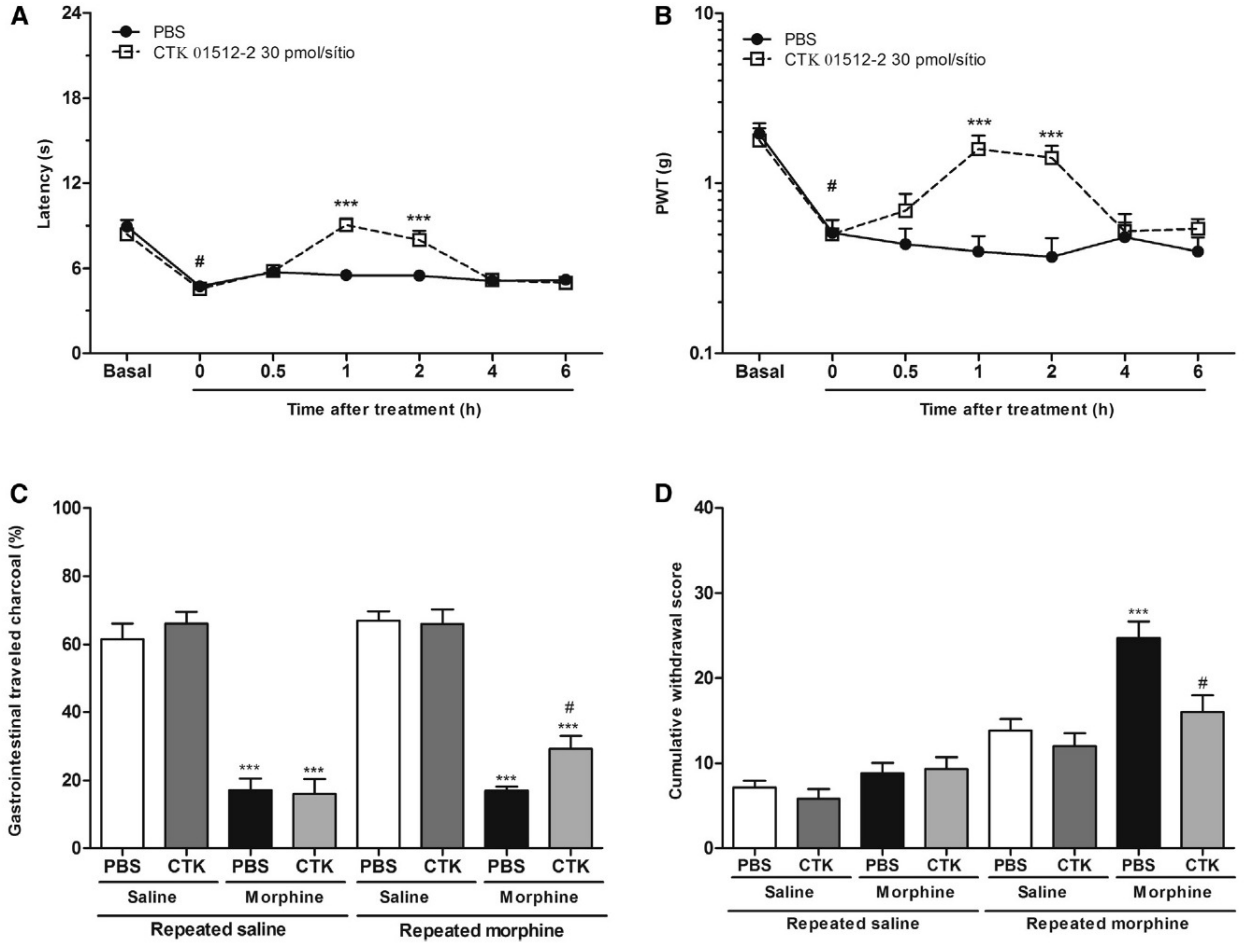

Figure 6. Effect of i.t. injection of the recombinant form of Phα1β (CTK 01512-2) on hyperalgesia, constipation, and withdrawal syndrome produced by repeated injection of morphine in mice. **(A** and **B)** Time course of the effect of CTK 01512-2 (.1–30 pmol/site, i.t.) on thermal **(A)** and mechanical **(B)** hyperalgesia induced by repeated morphine treatment. **(C** and **D)** Effect of CTK 01512-2 (30 pmol/site, i.t.) on gastrointestinal transit **(C)** and withdrawal syndrome **(D)** induced by repeated morphine or saline injection in mice. Data are expressed as **(A)** latency(ies), **(B)** PWT (g), **(C)** percentage of the gastrointestinal tract that charcoal traveled, or **(D)** cumulative withdrawal score. Each point and column represent the mean of **(A** and **B)** 10 mice and of **(C** and **D)** 6 to 7 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance followed by Bonferroni's post hoc test. ***$P < .001$ when compared to the PBS group **(A** and **B)** or to the PBS and saline group in repeated saline and morphine treatment **(C** and **D)**; #$P < .05$, when compared to baseline (basal) values **(A** and **B)** or to the PBS and morphine group in repeated morphine treatment **(C** and **D)**.

constipation, hyperalgesia, and withdrawal syndrome induced by repeated administration of morphine in mice.

First, we observed that a single administration of morphine showed an antinociceptive effect on heat but not on mechanical nociception in control animals. This effect was expected and can be explained by the segregated expression pattern of the μ-opioid receptor in heat-sensitive pain pathways but not in mechanical-sensitive pathways.[38] Morphine antinociceptive action is caused by the activation of μ-opioid receptors, which may lead to both activation of potassium channels (G-protein-activated inwardly rectifying K [GIRK]) and inhibition of VGCCs, resulting in reduced neuronal excitability in several central nervous system (CNS) structures.[21] In our study, the blockade of VGCCs in the spinal cord by Phα1β treatment alone was not sufficient to alter mechanical or heat nociception. In fact, the spinal participation of VGCCs in the perception of thermal and mechanical nociception is controversial.[12,27,31] Thus, our results indicate that G-protein-activated inwardly rectifying K activation and/or the involvement of VGCCs at supraspinal sites are more relevant than those in the spinal cord in mediating the antinociceptive effect produced by a single systemic administration of morphine on thermal nociception tests, as has been described previously.[18,19] In contrast to the effect of either drug administered alone, the concomitant administration of Phα1β with a single dose of morphine slightly increased the analgesia produced by morphine in the thermal nociception test. It has been described that i.t. injection of selective N-, P/Q-, and L-type VGCC blockers potentiate morphine analgesia in rodent thermal nociception tests.[12,31] Therefore, the blockade of VGCCs by Phα1β in the spinal cord was not sufficient to produce antinociception itself, but it was able to increase the analgesia induced by a single systemic administration

of morphine as expected for a blocker of several high-voltage VGCCs.

Opioids are the standard therapy for alleviating several types of moderate to severe pain.[2] However, the repeated use of opioids is hampered by tolerance, withdrawal syndrome, and hyperalgesia.[2,24] In the present study, we showed that repeated treatment with morphine not only caused tolerance to its antinociceptive effect but also paradoxically produced both heat and mechanical hyperalgesia and caused withdrawal syndrome. Although they are distinct clinical phenomena, opioid-induced tolerance and hyperalgesia may be intricately related mechanistically.[24] One may be concerned that the repeated opioid-induced hyperalgesia is related to repeated daily exposure of mice to the tail-flick test, because a latency decrease occurred on thermal latency after 5 consecutive days of testing in rats.[28] However, it seems not to be the case here, once we were unable to observe thermal latency reduction in repeated saline-treated mice.

Moreover, opioid-induced hyperalgesia may be related to withdrawal syndrome.[24,36] In our study, repeated treatment with morphine for 3 days induced thermal and mechanical hyperalgesia observed at day 4 (approximately 12 hours after the last dose of morphine). Curiously, a morphine challenge on day 4 did not alter mechanical hyperalgesia but fully reversed thermal hyperalgesia, indicating that the latter phenomenon but not the former is related to opioid withdrawal. Accordingly, we also observed that in addition to hyperalgesia, repeated morphine treatment caused other signals of withdrawal syndrome. Naloxone treatment precipitated the occurrence of jumping, wet-dog shaking, and paw tremors—behavioral expressions that are dependent on both supraspinal and spinal stimulus.[20,23,34,36]

Strategies used for opioid treatment include the concomitant administration of nonopioid adjuvant analgesics, which may increase opioid-induced analgesia and reduce opioid-related side effects.[22] In our study, Phα1β was able to reverse both the mechanical and heat hyperalgesia induced by repeated morphine administration and completely reversed the signs of opioid withdrawal syndrome. Phα1β reversed morphine-induced thermal and mechanical hyperalgesia, although it exhibited more potent inhibition of the former, again suggesting that there are differences in the mechanisms involved in thermal and mechanical opioid-induced hyperalgesia. Furthermore, a low dose of Phα1β that was not able to alter opioid-induced hyperalgesia per se also largely reversed the antinociceptive tolerance produced by repeated morphine treatment. There is evidence indicating that the development of hyperalgesia, tolerance, and withdrawal syndrome due to opioid treatment is associated with the activation of L-, N- and R-type VGCCs.[8,27,47,48] In contrast to the effects of Phα1β treatment, i.t. injection of the selective N-type VGCC ziconotide does not reverse the antinociceptive tolerance produced by repeated morphine treatment.[46] This difference may be explained by the fact that Phα1β inhibits not only N-type but also R- and L-type VGCCs.[44] Thus, it is possible that Phα1β, by blocking several VGCCs, decreases the activation of VGCCs during repeated morphine treatment, thus reversing hyperalgesia, analgesic tolerance, and withdrawal syndrome.

A limitation of the use of toxin to reverse the tolerance and the adverse effects induced by repeated administration of morphine is that its effect was obtained just after i.t. injection, which has some disadvantages in comparison with other routes of administration. Parenterally administered toxin peptides, including ziconotide (.005%), have been demonstrated in the literature to have lower CNS penetration and high peripheral side effects because of their susceptibility to degradation, reduced blood-brain barrier penetration, and a high molecular weight.[30] Because Phα1β has a higher molecular weight (6,044 Da) than ziconotide (2,639 Da), it will be improbable that the toxin Phα1β could reach the CNS, causing low peripheral adverse effects after parenteral injection, even with its high potency to reverse tolerance, hyperalgesia, and withdrawal induced by morphine. Currently, we are developing ways to improve the stability and CNS penetration of toxin Phα1β.

Another side effect caused by opioid treatment is constipation.[13,16,32] We also observed that single or repeated administration of morphine produced a reduction in gastrointestinal transit in mice. Moreover, although morphine induced tolerance to its analgesic effect, we observed that repeated morphine did not produce tolerance to constipation, as previously reported.[42] In contrast to ziconotide, which potentiates morphine-induced inhibition of gastrointestinal transit,[46] Phα1β did not reduce gastrointestinal transit when administered alone or alter the effect of a single dose of morphine. However, in contrast to its effect after administration of a single dose of morphine, i.t. Phα1β was able to partially reverse the constipation produced by repeated administration of morphine. Of note, opioid-induced constipation may be induced by the activation of the μ-opioid receptor in both peripheral and spinal cord sites.[29,39] Sympathetic innervation from the thoracic region of the spinal cord alters gastrointestinal transit, and repeated administration of morphine leads to altered noradrenergic neurotransmission.[11,35] Because spinal VGCC blockade is sympatholytic,[3,14] spinally administered Phα1β could inhibit the sympathetic effect on gastrointestinal transit, partially reversing the repeated morphine-induced constipation. However, additional studies are required to further investigate the involvement of spinal VGCCs in opioid-induced constipation.

Although native Phα1β treatment produces interesting results on opioid effects, its clinical use could be hampered by its low yield from spider venom.[15] As an alternative to this problem, we found that the recombinant form of Phα1β (CTK 01512-2) presented effects similar to the native peptide, that is, showed the same time course and efficacy for reversing thermal and

mechanical hyperalgesia as well as reduced withdrawal syndrome and gastrointestinal transit. Collectively, these results showed that the recombinant form of Phα1β could be used as an analgesic drug instead of native Phα1β.

In summary, we observed that the i.t. injection of Phα1β was effective in potentiating the analgesic effect caused by a single dose of morphine as well as reducing the hyperalgesia, tolerance, constipation, and withdrawal syndrome induced by repeated administration of morphine in mice. Thus, Phα1β, possibly by blocking VDCCs, could be a useful adjuvant drug in opioid treatment.

Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpain.2014.02.007.

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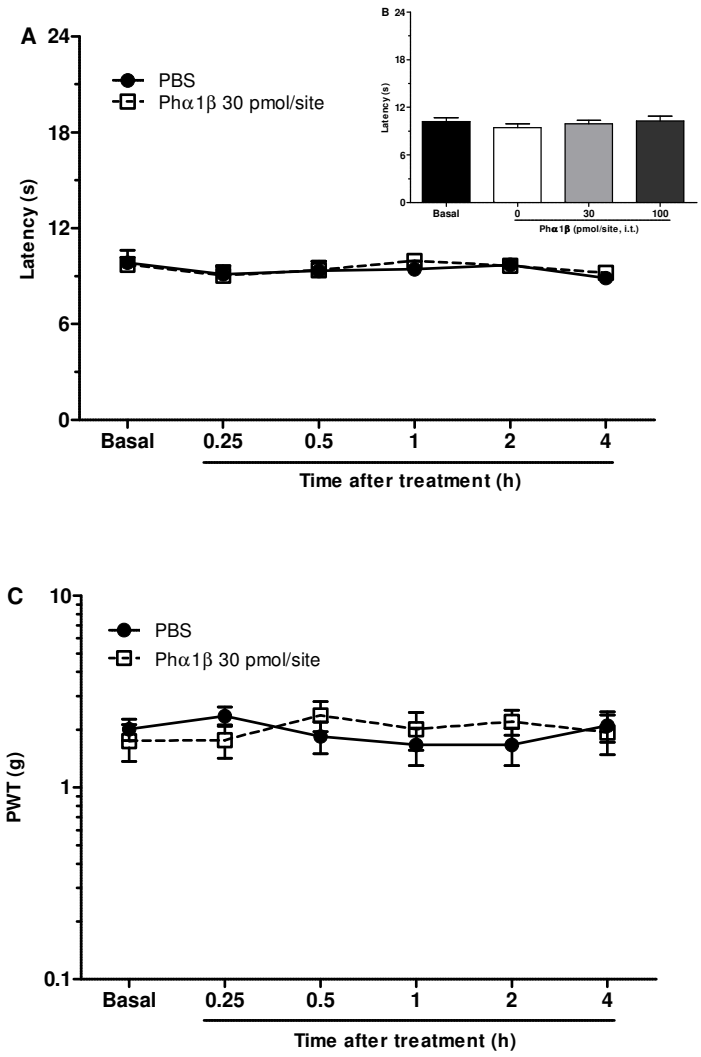
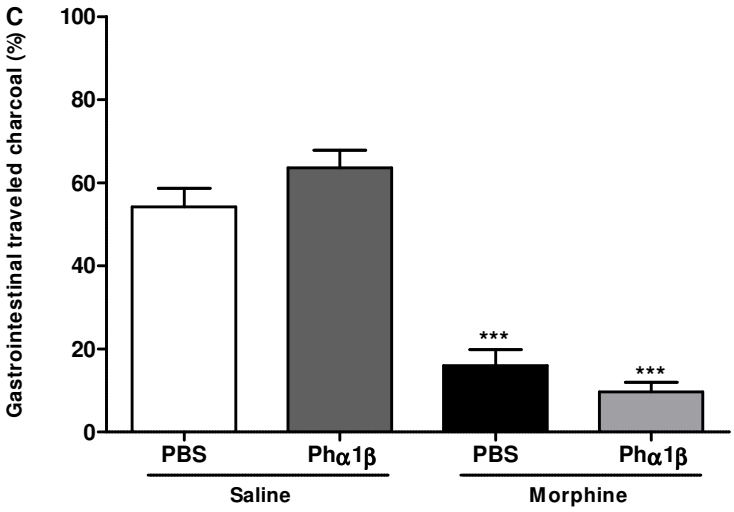
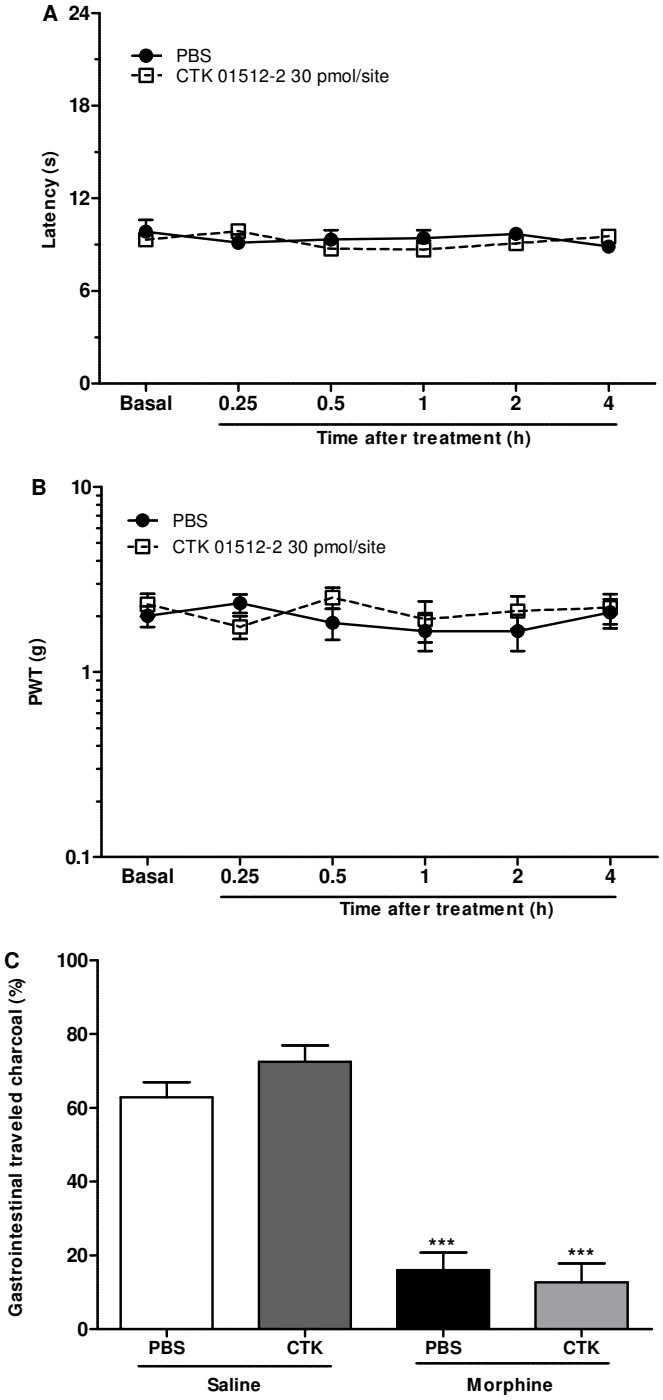
Supplementary Data



Supplemental Fig. 1. Effect of intrathecal (i.t.) injection of Phα1β on thermal and mechanical nociception in mice. (A) Time course (B) and dose-response curve of the effect of Phα1β (30 and 100 pmol/site, i.t.) on thermal nociception. (C) Time course of the effect of Phα1β (30 pmol/site) on mechanical nociception. Data are expressed as (A and B) latency (s) or (C) PWT (g). Each point represents the mean of 5 mice, and vertical lines show the SEM. Statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-hoc test (B) or 2-way repeated measures ANOVA followed by Bonferroni's post-hoc test (A and C).



Supplemental Fig. 2. Effect of intrathecal (i.t.) injection of Phα1β on the constipation produced by a single dose of morphine in mice. Effect of Phα1β (30 pmol/site, i.t.) on gastrointestinal transit after a single injection of saline (vehicle, 10 ml/kg) or morphine (10 mg/kg). Data are expressed as the percent of gastrointestinal tract that charcoal traveled. Each column represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. ***$p<0.001$ when compared to the PBS and saline group.



Supplemental Fig. 3. Effect of intrathecal (i.t.) injection of the recombinant form of Phα1β (CTK 01512-2) on thermal and mechanical nociception and constipation produced by a single injection of morphine in mice. Time course of the effect of CTK 01512-2 (30 pmol/site, i.t.) on thermal (A) and on mechanical nociception (B). (C) Effect of CTK 01512-2 (30 pmol/site, i.t.) on gastrointestinal transit after a single injection of saline (vehicle, 10 ml/kg, s.c.) or morphine (10

mg/kg, s.c.). Data are expressed as (A) latency (s), (B) PWT (g) or (C) percent of the gastrointestinal tract that charcoal traveled. Each point and column represents the mean of 5 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. ***$p<0.001$ when compared to the PBS and saline group.

5. DISCUSSÃO

Os opióides são considerados a terapia padrão para o tratamento de diversos tipos de dor, como a dor relacionada ao câncer e as dores crônicas (Angst e Clark, 2006). Porém o uso de opióides está associado ao desenvolvimento de tolerância e potenciais efeitos adversos, como constipação e síndrome de abstinência (Greenwood-Van e Stadifer, 2008; Low, Clarke e Huh, 2012). Ainda, evidências indicam que a síndrome de abstinência está relacionada a outro problema, conhecido como HIO (Angst e Clark, 2006; Rubovitch, Pick e Sarne, 2009; Low, Clarke e Huh, 2012). A HIO é definida como um estado de sensibilização nociceptiva, causada pela exposição a opióides, podendo se manifestar como hiperalgesia ou alodínia (Low, Clarke e Huh, 2012). Assim, poderia ser útil o uso concomitante de adjuvantes terapêuticos ao tratamento opióide (Lee et al., 2011). Bloqueadores dos CCRVs podem ser utilizados para aumentar a analgesia dos opióides, mas suas ações sobre os efeitos adversos causados pela morfina são pouco conhecidas (Law et al., 2000; Ruskin e Moises, 1995).

Inicialmente avaliamos o efeito da morfina em testes de nocicepção térmica e mecânica. Observamos que uma única administração de morfina mostrou um efeito antinociceptivo sobre a nocicepção térmica, mas não sobre a mecânica em animais controle. Este efeito era esperado e pode ser explicado pelo padrão de expressão segregado do receptor MOR em fibras termosensíveis, mas não em fibras mecanosensíveis (Scherrer et al., 2009). A morfina é um agonista dos receptores MOR e a ativação destes receptores resulta na redução da excitabilidade neuronal em várias estruturas do SNC, culminando com o alívio da dor (Law, Wong e Loh, 2000; Trescot et al., 2008).

Previamente, o nosso grupo de pesquisa demostrou que a administração intratecal de Phα1β apresenta efeito antinociceptivo em diferentes modelos de dor (Rigo et al., 2013; de Souza et al., 2013). De fato, o papel dos CCRVs é bem estabelecido na transmissão dos impulsos dolorosos na medula espinhal, sendo altamente expressos em terminais pré-sinápticos (CCRV do tipo L e do tipo N) de neurônios nociceptivos, permitindo o influxo de cálcio e a liberação de neurotransmissores algogênicos (Cao, 2006). Também, a Phα1β apresentou eficácia similar, mas índice terapêutico superior à ω-conotoxina MVIIA, um peptídeo bloqueador seletivo dos CCRV do tipo N, em testes pré-clínicos de dor (Vieira et al., 2005; Souza et al., 2008). Contudo, evidenciamos

que o bloqueio dos CCRVs pela administração intratecal da Phα1β não foi suficiente para alterar a nocicepção em animais controle, não apresentando efeito *per se*. Assim, o bloqueio dos CCRVs em neurônios nociceptivos pela Phα1β poderia ser interessante no controle da dor, o que a torna um peptídeo em potencial para o desenvolvimento de novos fármacos analgésicos.

Sabendo do potencial efeito analgésico da Phα1β, buscamos analisar a ação deste peptídeo sobre o efeito antinociceptivo produzido por uma única dose de morfina. A analgesia mediada por opióides, tanto endógenos quanto exógenos está, em grande parte, relacionada com a inibição da liberação de neurotransmissores, através da ação inibitória em CCRV (Law, Wong e Loh, 2000; Trescot et al., 2008). No presente estudo observamos que a administração concomitante de Phα1β com uma sub dose de morfina foi capaz de produzir um leve aumento da analgesia sobre a nocicepção térmica da morfina nos animais. Estes resultados corroboram com estudos anteriores que descrevem que a injeção intratecal de bloqueadores seletivos dos CCRVs do tipo N, P/Q, e do tipo L potencializam a analgesia da morfina em roedores (Omote et al., 1996; Fukuizumi, Ohkubo e Kitamura, 2003). Desta forma, esta potencialização do efeito analgésico da morfina pela Phα1β nos leva a crer a que a Phα1β poderia ser utilizada como um adjuvante para a analgesia da morfina.

Os opióides são amplamente utilizados para o alívio da dor, sendo o tratamento habitual tanto da dor aguda como crônica. No entanto, o uso prolongado de opióides é limitado pelo desenvolvimento de tolerância, síndrome de abstinência e hiperalgesia, que prejudicam a qualidade de vida dos pacientes (Mao, 2002; Angst e Clark, 2006; Alam et al., 2012; Low et al., 2012). No presente estudo, nós mostramos que o tratamento repetido com morfina não causou somente tolerância analgésica como também induziu hiperalgesia e síndrome de abstinência em camundongos. De fato, a tolerância analgésica é uma limitação do uso prolongado de mofina (Lee, Wanigasekera e Tracey, 2013). Além disso, dados recentes sugerem que a exposição sistêmica ou intratecal ao opióide ou a retirada abrupta de opióides pode causar um estado hiperalgésico, onde os opióides podem, paradoxalmente, piorar o quadro da dor (Li e Clark, 2002; Low, Clarke e Huh, 2012; Bannister e Dickenson, 2010).

Embora existam vários mecanismos propostos para explicar o desenvolvimento da HIO e tolerância, o preciso mecanismo molecular envolvido nestes fenômenos ainda não é bem estabelecido. Uma série de conceitos e teorias sobre tais fenômenos tem sido abordada nos diversos estudos da área. Dentre estes, há alguns estudos que demostraram o envolvimento de proteínas quinase, incluindo a proteína quinase dependente de cálcio calmodulina II (CaMKII), na hiperalgesia e tolerância induzida por opióides (Tang et al., 2006; Chen et al., 2010). De fato, uma alternativa para explicar a HIO e tolerância a opióides seria a dessensibilização dos receptores MOR via fosforilação (Liu e Anand, 2001). O receptor MOR exibe sítios de consenso à fosforilação pela CaMKII. E a ativação dessas quinases está relacionada à dessensibilização destes receptores (Mestek et al., 1995; Koch et al., 1997). A ação da CaMKII se dá em resíduos de serina (S_{261} e S_{266}) localizados na terceira alça intracelular do receptor MOR, num sítio muito próximo àquele relacionado com o acoplamento da proteína G (Koch et al., 1997; Wang et al., 2001), o que implica num possível desacoplamento do receptor à proteína G via fosforilação. Além disso, a CaMKII está co-localizada com receptores MOR em diversas áreas relacionadas ao processamento da dor, como as lâminas superficiais do corno dorsal da medula espinhal e GRD (Bruggemann et al., 2000). Esta co-localização da quinase com o receptor foi interpretada como um possível facilitador da fosforilação do receptor MOR. Desta forma, é possível acreditar que, em situações particulares como na HIO e tolerância, o receptor MOR sofra ação dessas quinases, que o tornam inativo. No presente estudo, o bloqueio de CCRV desencadeado pela administração i.t. de Phα1β foi capaz de reverter em grande parte a hiperalgesia e a tolerância induzida pela morfina e completamente a síndrome de abstinência em camundongos. Logo, é possível sugerir que o bloqueio dos CCRVs reduza a ação dessas quinases - cuja ativação é dependente de cálcio - sobre o receptor MOR.

O envolvimento dos CCRVs no desenvolvimento de tolerância e efeitos adversos induzidos pela morfina já foi demostrado em diversos estudos. Em um destes estudos a administração crônica de morfina aumentou significativamente a expressão dos CCRVs do tipo L e N (Verma et al., 2009). Além disso, bloqueadores de CCRVs dos tipos L, N e R em roedores inibiram o

desenvolvimento de hiperalgesia, tolerância e síndrome de abstinência induzidas por opióides (Yokoyama, 2004; Dogrul et al., 2005; Meng et al., 2008). Assim, a capacidade da Phα1β de bloquear vários CCRV poderia explicar a sua boa eficácia em reverter a hiperalgesia, a tolerância analgésica e a síndrome de abstinência induzida pelo tratamento repetido de morfina.

Embora sejam fenômenos clínicos distintos, a tolerância e a hiperalgesia induzidas por opióide, e possivelmente a síndrome de abstinência, podem ter mecanismos relacionados (Low, Clarke e Huh, 2012). De fato, o sistema glutamatérgico central é considerado o mecanismo celular comum entre estes processos (Mao, 2002). Além disso, a parada abrupta da administração crônica de opióides está relacionada com a hiperalgesia (neste caso é chamada de hiperalgesia induzida por retirada) (Rubovitch, Pick e Sarne, 2009). Ainda, observou-se que no dia 4 (cerca de 12 horas após a última dose de morfina), além de hiperalgesia térmica e mecânica, o tratamento repetido com morfina causou outros sinais da síndrome de abstinência, como saltar, tremor do corpo "wet-dog shaking" e tremores da pata. Todos esses comportamentos são expressos na síndrome de abstinência precipitada pela naloxona em camundongos (Koob, Maldonado e Stinus, 1992). Deste modo, a relação entre estes efeitos adversos causados pelo uso de opióides, pode explicar o efeito relevante apresentado pela Phα1β tanto sobre a hiperalgesia, como na tolerância e síndrome de abstinência induzidos pela morfina.

Outro efeito indesejável causado pelo uso de opióides é a constipação, que reduz consideravelmente a qualidade de vida dos pacientes (Khansari, Sohrabi e Zamani, 2013; Kumar, Barker e Emmanuel, 2014). Nós também observamos que a administração única ou repetida de morfina produziu uma redução no trânsito gastrointestinal em camundongos. Além disso, embora a morfina induza tolerância analgésica, observamos que a administração repetida de morfina não produz tolerância à constipação, como relatado anteriormente (Khansari, Sohrabi e Zamani, 2013). Em contraste com a ziconotida, que potencializa a inibição induzida por morfina sobre o trânsito gastrointestinal (Wang e Scott Bowersox, 2000), a Phα1β não reduziu o trânsito gastrointestinal quando administrada sozinha ou alterou o efeito de uma dose única de morfina. Porém, a Phα1β intratecal foi capaz de reverter parcialmente a constipação produzida pela administração repetida de morfina. De fato, a constipação

induzida pelos opióides pode ser resultado da ativação do receptor MOR tanto a nível periférico como central (Shook et al., 1987; Mori et al., 2013). Ainda, sabe-se que a inervação simpática da região torácica da medula espinal altera o trânsito gastrointestinal, e a administração repetida de morfina leva a uma alteração na neurotransmissão noradrenérgica (Fuentealba et al., 2000; Roman e Gonella, 1981). Então, como o bloqueio espinhal dos CCRVs é simpaticolítico (Bowersox et al., 1992; Gaur et al., 1994), a administração intratecal de Phα1β poderia inibir o efeito simpático sobre o trânsito gastrointestinal, revertendo parcialmente a constipação induzida pela administração repetida de morfina. No entanto, o envolvimento dos CCRVs espinhais sobre a constipação induzida por opióides não foi elucidado, sendo necessários estudos adicionais.

Embora o tratamento com a Phα1β nativa produza resultados interessantes sobre os efeitos dos opióides, o seu uso clínico pode ser prejudicado devido ao seu baixo rendimento a partir do veneno de aranha (Gomez et al., 2002). Como uma alternativa para este problema, verificou-se que a forma recombinante da Phα1β (CTK 01512-2) apresenta efeitos semelhantes ao peptídeo nativo, isto é, apresentou o mesmo decurso temporal e eficácia na reversão da hiperalgesia térmica e mecânica, bem como reduziu a síndrome de abstinência e o aumentou o trânsito gastrointestinal em camundongos. Juntamente, estes resultados indicam que a forma recombinante do Phα1β poderia ser utilizada como um adjuvante analgésico ao invés da Phα1β nativa.

Em conclusão, nossos resultados sugerem que a Phα1β é efetiva em potencializar a analgesia, bem como, reduzir a tolerância e os efeitos adversos induzidos pela morfina. Desta maneira, a Phα1β apresenta um uso potencial como uma droga adjuvante na terapia opióide.

6. CONCLUSÕES

Tendo em vista os resultados obtidos no presente estudo, pode-se concluir que:

1. A administração subcutânea de uma única dose de morfina apresentou efeito antinociceptivo e reduziu o trânsito gastrointestinal e o tratamento repetido com morfina causou tolerância analgésica, hiperalgesia paradoxal, síndrome de abstinência e constipação em camundongos.
2. A administração de Phα1β foi capaz de potencializar o efeito analgésico da morfina e também, de reverter em grande parte a hiperalgesia e a tolerância, completamente a síndrome de abstinência e parcialmente a constipação induzida por doses repetidas de morfina em camundongos.
3. A forma recombinante da Phα1β (CTK 01512-2) apresenta efeitos semelhantes ao peptídeo nativo, isto é, apresentou o mesmo decurso temporal e eficácia na reversão da hiperalgesia, bem como reduziu a síndrome de abstinência e a constipação em camundongos.

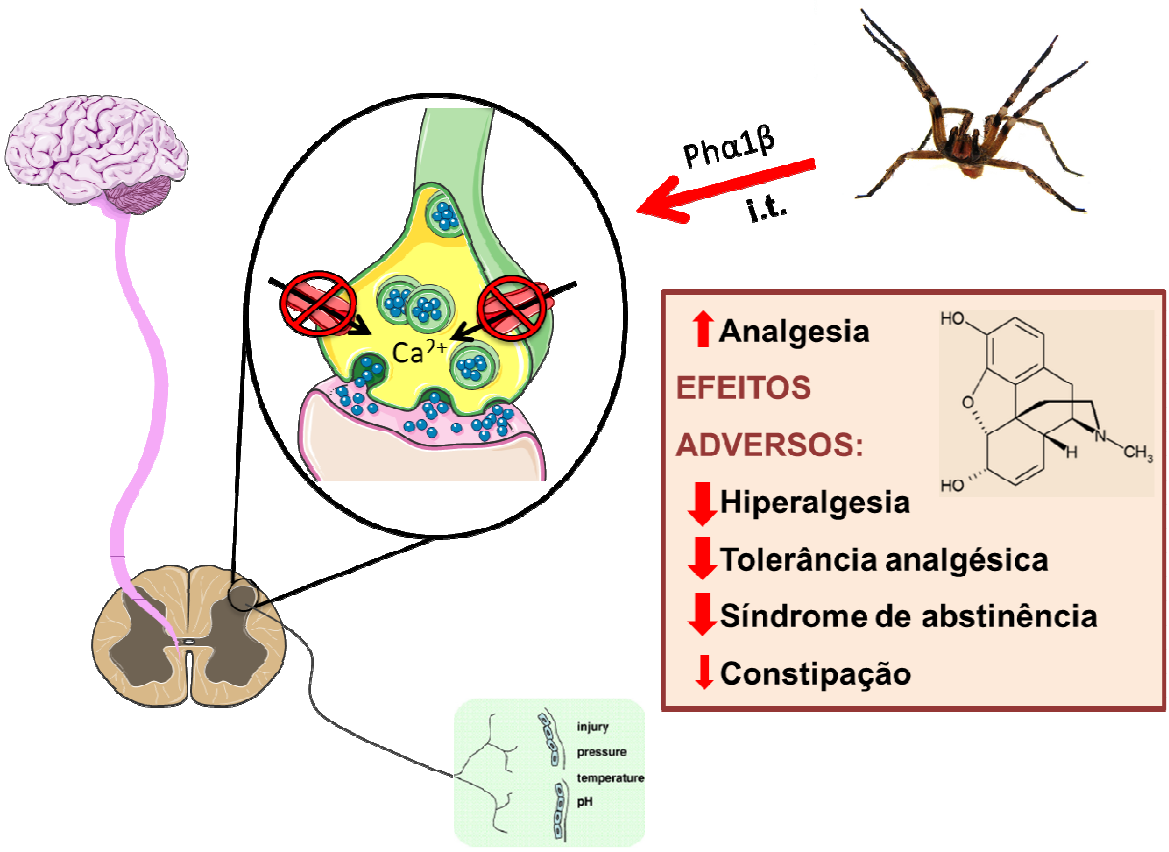


Figura 3. Efeito da Phα1β sobre a analgesia e desenvolvimento dos efeitos adversos da morfina: hiperalgesia, tolerância, sinais de abstinência e constipação. O bloqueio espinhal dos canais de

cálcio pela Phα1β foi capaz não só de potencializar o efeito analgésico da morfina, mas também reverteu amplamente a hiperalgesia, tolerância analgésica e a síndrome de abstinência e parcialmente a constipação induzida por doses repetidas de morfina.

A Phα1β por potencializar o efeito analgésico além de reverter a tolerância e os efeitos adversos induzidos pela morfina, possui potencial como droga adjuvante na terapia opióide.

7. PERSPECTIVAS

Perspectivas:

- Verificar o efeito do peptídeo Phα1β sobre os efeitos antinociceptivos e adversos produzidos pela morfina em modelos de dor, como em modelo de dor pós-cirúrgica (Em anexo o manuscrito)
- Investigar os mecanismos de ação pelos quais a Phα1β exerce tais ações sobre os efeitos antinociceptivos e adversos produzidos pela morfina

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9. APÊNDICE

Apêndice – Manuscrito submetido à revista Anesthesiology: “Effects of Phα1β on the analgesic and adverse effects induced by repeated morphine treatment in a mouse model of postoperative pain.”

Effects of Phα1β on the analgesic and adverse effects of repeated morphine treatment in a mouse model of postoperative pain

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Number of words: Abstract (238) Introduction (384) Discussion (1175)

Abbreviated Title: Phα1β as an adjuvant drug in postoperative opioid therapy

What We Already Know about This Topic

- Opioids are the “gold standard” treatment for postoperative pain, but these drugs also have limiting adverse effects.
- The intrathecal administration of Phα1β, the voltage-gated calcium channel (VGCC) blocker, presented efficacious and safe analgesic effects in different models of pain.

What This Article Tells Us That Is New

- Phα1β presented an analgesic effect and potentiated the analgesia of morphine in a mouse model of postoperative pain.
- Phα1β reversed hyperalgesia, tolerance and withdrawal syndrome induced through the repeated postoperative administration of morphine to mice.

Abstract

Background: Opioids are the “gold standard” treatment for postoperative pain, but these drugs also have limiting adverse effects. Thus, adjuvant drugs might be useful in opioid therapy for postoperative pain. The aim of the present study was to evaluate the effects of Phα1β, a voltage-gated calcium channel (VGCC) blocker, on the antinociceptive and adverse effects of morphine in a mouse model of postoperative pain.

Methods: In this randomized and blinded study, we evaluated the effect of intrathecal (i.t.) injection of Phα1β on the antinociceptive (reduction of mechanical hyperalgesia and guarding behavior) and adverse effects (tolerance, constipation, hyperalgesia and withdrawal syndrome) induced through single or repeated (increasing doses, 3 times a day for 3 consecutive days) subcutaneous (s.c.) injections of morphine in C57/BL6 mice subjected to right hind plantar incision.

Results: Single injections of Phα1β (100-300 pmol/site, i.t.) or morphine (3-10 mg/kg, s.c.) reversed postoperative nociception in mice. Moreover, in doses that did not produce antinociception when administered separately, Phα1β (30 pmol/site, i.t.) and morphine (1 mg/kg, s.c.) produced an expressive analgesic effect when concomitantly administered. Repeated treatment with morphine in operated mice caused constipation, tolerance, hyperalgesia and withdrawal syndrome, with exception of constipation, Phα1β (30 pmol/site, i.t.) reversed these adverse effects.

Conclusions: Taken together, these findings demonstrated that Phα1β not only presented analgesic effects but also potentiated the analgesia and reversed

some adverse effects of morphine on operated mice, indicating the potential use of this agent as an adjuvant drug in opioid therapy for postoperative pain.

Keywords: constipation, hyperalgesia, opioids, voltage-gated calcium channels, tolerance, withdrawal syndrome.

1. INTRODUCTION

Postoperative pain is a common form of acute pain in clinical practice. In recent years, the treatment of acute pain in surgical patients has been widely recognized as an important issue in health care. However, approximately 50-70% of patients still experience moderate to severe pain after surgery.[1,2,3] Moreover, inadequate treatment of acute pain might also contribute to the development of persistent postsurgical pain and could be associated with a negative impact on the quality of life of the patient.[4]

Opioids are the “gold standard” treatment for postoperative pain, and morphine is one of the most commonly used opioids in surgical patients.[5,6] However, some patients misuse or persistently use this opioid for longer periods, which impairs the postsurgical quality of life.[4,7,8] In addition, the prolonged use of opioids has been associated with tolerance to the analgesic effect of these drugs and various adverse effects, including the development of dependence, opioid-induced hyperalgesia (OIH) and constipation.[9,10] Adjuvant therapeutics are used to minimize the effects of opioids, but are not effective.[5] Thus, the development of new strategies is important to prevent and reverse analgesic tolerance and opioid-related adverse effects.

An important target for opioid-induced analgesia is the blockade of voltage-gated calcium channels (VGCCs).[11,12] Phα1β, a peptide purified from the venom of the Brazilian armed spider *Phoneutria nigriventer,* blocks high-voltage calcium currents in type N, R, P/Q and L VGCCs (in this order of potency).[13,14] The intrathecal (i.t.) administration of Phα1β presented antinociceptive effects in different models of pain.[15,16,17] Differently from people without painful diseases, patients with cancer or nonmalignant-related pain

might develop differential opioid-induced adverse effects.[18,19] We recently reported that Phα1β was effective in potentiating analgesia and reducing tolerance, and the adverse effects of morphine in naïve mice[20], but the potential use of Phα1β as an adjuvant drug in opioid therapy on postoperative pain in mice has not been investigated.

Thus, the present study was designed based on a rodent model of postoperative pain induced through plantar incision in mice. This model facilitated the examination of the mechanisms of increased sensitivity following surgical incision and the investigation of novel treatments for postoperative pain.[2,21] The aim of the present study was to evaluate the possible actions of Phα1β on antinociception, tolerance, hyperalgesia, withdrawal syndrome and constipation resulting from the use of morphine in a model of postoperative pain in mice.

2. MATERIAL AND METHODS

2.1. Ethical statement

All animal handling and experimental procedures were performed in accordance with the current ethical guidelines of the International Association for the Study of Pain (IASP) for the investigation of experimental pain in conscious animals.[22] The experimental protocols were authorized through the Committee on the Ethical Use of Animals of the Federal University of Santa Maria (CEUA process number: 23081.005024/2010-88) and the CEUA of the Federal University of Santa Catarina (Process No. 117/CEUA/PROPESQ/2013. Procedure PP00872). All animals subjected to plantar incision were first deeply anesthetized (2% isoflurane, 100% O_2 1 L/min). In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

2.2. Study design

The present study was conducted in accordance with the ARRIVE guidelines[23] (Suppl. Figure 1). Allocation concealment was performed using a randomization procedure (http://www.randomizer.org/). Experimenters were blinded to the drug treatment when performing tests. No animals were excluded from the analysis. Each experiment was repeated 2 to 3 times (using 3 or 2 animals in each repetition) between 8:00 a.m. and 5:00 p.m.

Sample size

For the experiments, the primary outcome was postoperative mechanical hyperalgesia and the secondary outcomes were postoperative guarding pain,

gastrointestinal transit and withdrawal scores. The group size for each experiment was determined through sample size estimation[24] based on the primary outcome. The expected standard deviation values were based on previous results.[25] The minimum effect size was considered as a reversion of at least 30% of the hyperalgesia, for a significance level of 5%, with a test power of 90% and a two-tailed hypothesis test. Thus, we estimated a group size of six mice for all tests, except for experiments using Phα1β in combination with morphine, where the sample size was increased to nine to obtain statistical significance on the guarding behavior using a non-parametric test.

Experimental Animals

The experiments were performed using male C57BL/6 mice (20-25 g, bred in house). The animals were housed in a controlled-temperature environment in individually ventilated cages (10 animals per cage), with wood shaving bedding and nesting material, maintained at 22±1 ℃. The animals were housed with a 12-hour light/dark cycle (lights on at 7:00 am) and fed with rodent chow (Puro Lab 22 PB pelleted form, PuroTrato, Rio Grande do Sul, Brazil) and tap water *ad libitum*. The animals were acclimatized to the housing environment for at least 7 days prior to experimentation and the experimental room for 1 hour prior to experiments.

Drugs

Native Phα1β was purified as previously described and had the following amino acid sequence: ACIPRGEICT DDCECCGCDN QCYCPPGSSL GIFKCSCAHA NKYFCNRKKE KCKKA.[26] The stock solutions of the drugs were

prepared in phosphate-buffered saline (PBS, pH 7.4) in siliconized plastic tubes, maintained at –20°C and diluted to the desired concentration immediately prior to use. Morphine sulfate and naloxone hydrochloride were purchased from Cristália (São Paulo, Brazil).

Drug treatments

Phα1β (30-300 pmol/site) was administered through an intrathecal (i.t.) route at a volume of 5 µL/site according to the technique of Hylden and Wilcox.[27] Morphine (1-25 mg/kg) was administered through a subcutaneous (s.c.) route. Naloxone (2 mg/kg) was administered through an intraperitoneal route (i.p.). Phα1β was dissolved in PBS, and morphine and naloxone were dissolved in saline (NaCl 0.9%).

2.3. Experimental design

Protocols using a single injection of morphine

After measuring the baseline threshold (Basal), the mice were subjected to plantar incision, and 30 minutes later, the mechanical nociception test was performed to evaluate the postoperative mechanical hyperalgesia (Figure 1A). One hour after surgery, a single injection of morphine (1, 3 or 10 mg/kg, s.c.) or Phα1β (30, 100 or 300 pmol/site, i.t.) was performed and the mechanical hyperalgesia at different times (0.5-4 hours) after treatment, and the spontaneous pain 1 hour after treatment were evaluated (Figure 1A). Two doses of morphine (10 + 10 mg/kg, s.c) were administered at 4-hour intervals to evaluate acute tolerance in the mechanical test. As a control, different groups of animals were injected with the appropriate vehicle (PBS, 5 µL/site, i.t. or saline,

10 mg/mL, s.c.). The drug dosages and evaluation times were based on previous studies.[16,28]

Next, we evaluated the effects of the combined treatment of Phα1β with a single dose of morphine using mechanical and spontaneous nociception tests, performed 1 hour after the plantar incision in mice. The mice were injected with morphine (1 mg/kg, s.c.) immediately after injection with Phα1β (30 pmol/site, i.t.) and subsequently tested from 0.5 to 4 hours after treatment using a mechanical nociception test and at 1 hour after treatment using a spontaneous test. Separate groups of animals were injected with PBS (5 µL/site, i.t.) and saline (10 mg/mL, s.c.), Phα1β (30 pmol/site, i.t.) and saline (s.c.) or PBS (i.t.) and morphine (1 mg/kg, s.c), as controls. A similar experimental design was used to assess the effects of Phα1β on a single dose of morphine-induced constipation (measured through gastrointestinal transit) or withdrawal syndrome (measured through naloxone-precipitated withdrawal) at 1 hour (gastrointestinal transit) or 2 hours (naloxone-precipitated withdrawal) after treatment. For these tests, the animals were treated with morphine (1 or 10 mg/kg, s.c.) immediately after injection with Phα1β (30 pmol/site, i.t.). Separate groups of animals were treated with PBS (5 µL/site, i.t.) and saline (10 mg/mL, s.c.), PBS and morphine (1 mg/kg, s.c.) or Phα1β (30 pmol/site, i.t.) and saline, as controls.

Protocols using repeated injections of morphine

To evaluate the antinociceptive and adverse effects produced by the repeated administration of morphine in a mouse model of postoperative pain, we used an experimental design adapted from Marshall and Weinstock[29]. The protocol involved the administration of increasing doses of morphine at 3 times

each day for 3 days (10+10+15, 15+15+20, 20+20+25 mg/kg, s.c.).[20] Briefly, 1 hour after plantar incision, the mice received 3 subcutaneous injections of morphine per day, with an interval of 3-4 hours between each injection (first injection 9:00 a.m.). On day one, 10, 10 and 15 mg/kg of morphine were administered, followed by 15, 15 and 20 mg/kg on day two, and 20, 20 and 25 mg/kg on day three. The 5 mg/kg increase in the last injection on each day of treatment minimized the induction of withdrawal syndrome overnight. On day 4, the animals received a challenge dose of morphine (10 mg/kg, s.c.) at 9 a.m. As a control, a different group of animals was injected with vehicle (saline, 10 mg/mL, s.c.).

Before each morning injection of morphine, the mechanical nociception test was performed to assess the development of opioid-induced hyperalgesia. On days 1 and 4, the mice were subjected to the mechanical nociception test at 30 minutes after the morning injection of morphine (10 mg/kg, s.c.) to assess the development of analgesic tolerance. On the fourth day, the animals were treated with Phα1β (30 pmol/site, i.t.) or vehicle (PBS), and 0.5 to 4 hours after injection, the animals were evaluated to mechanical threshold to assess the effect of Phα1β on morphine-induced hyperalgesia. We did not use the guarding behavior test in this protocol because the mice did not present spontaneous pain on the second day after the surgical procedure (data not shown).

In addition, we examined whether Phα1β could restore the analgesic effect of morphine in tolerant mice. To this end, a dose of Phα1β (30 pmol/site, i.t.) was administered on the fourth day of treatment, immediately prior to the morphine challenge (10 mg/kg, s.c.). As controls, separate groups of animals

were injected with PBS (5 µL/site, i.t.) and saline (10 mg/mL, s.c.), PBS and morphine or Phα1β and saline. The mechanical nociception test was subsequently evaluated at 0.5 to 4 hours after treatment (Figure 5A).

Additionally, we assessed the effect of Phα1β on morphine-induced withdrawal syndrome and constipation (measured through the naloxone-precipitated withdrawal and gastrointestinal transit, respectively) in response to repeated doses of morphine. To this end, increasing doses of morphine were administered in mice for 3 days, as previously described, and on day 4, naloxone-precipitated withdrawal or gastrointestinal transit was evaluated. The animals were treated with morphine (10 mg/kg, s.c.) immediately after injection with Phα1β (30 pmol/site, i.t.) and tested at 1 hour (gastrointestinal transit) or 2 hours (naloxone-precipitated withdrawal) after injection on day 4. Separate groups of animals were treated with PBS (5 µL/site, i.t.) and saline (10 mg/mL, s.c.), PBS and morphine (10 mg/kg, s.c.) or Phα1β (30 pmol/site, i.t.) and saline, as controls.

Postoperative pain model

The postoperative pain model was used according to the procedure described for mice[30,31], with some modifications.[21] The mice were anesthetized with 2% isoflurane, 100% O_2 1 L/min via a nose cone. After anti-septic preparation of the right hind paw with 10% povidone-iodine solution (PVPI), a 5 mm longitudinal incision was made through the skin, underlying fascia and plantar flexor digitorumbrevis muscle using a number 11 blade. The incision was initiated 2 mm from the proximal edge of the heel and extended toward the toes. Blunt curved forceps were subsequently inserted through the incision into

the muscle to further divide and retract the muscle. The muscle origin and insertion remained intact. The skin was opposed with a single mattress suture of 6.0 nylon. Control mice underwent a sham procedure involving anesthesia and antiseptic preparation without an incision.

2.4. Nociceptive parameters

Von Frey test

Mechanical hyperalgesia was assessed through the measurement of the paw withdrawal threshold using the "Up-and-Down" paradigm, as previously described.[20] Briefly, the mice were first acclimatized (1-2 hours) in individual clear Plexiglass boxes on an elevated wire mesh platform to facilitate access to the plantar surface of the hind paws. Von Frey filaments of increasing stiffness (0.02-10 g) were applied to the hind paw plantar surface of the animals with a pressure high enough to bend the filament. The 50% mechanical paw withdraw threshold (PWT) response was calculated from the resulting scores as previously described.[32] The PWT, expressed in grams (g), was evaluated before and 30 minutes after the plantar incision and several times after the s.c. injection of morphine or i.t. injection of Phα1β. A significant decrease in PWT compared with baseline values was considered mechanical hyperalgesia.

Guarding behavior test

Spontaneous pain was assessed through the observation of the guarding behavior induced after plantar incision, as previously described, with some modifications.[33] The mice were individually placed in clear Plexiglass boxes on an elevated wire mesh platform to facilitate access to the plantar surface of the

hind paws. The incised hind paws were closely observed during a 1 minute period repeated every 5 minutes for 30 minutes. According to the hind paw position during the majority of the 1-minute scoring period, a score of 0, 1, or 2 was given. Zero was scored when the incised area was touching the mesh, and the area was blanched or distorted by the mesh; 1 was scored when the incised area touched the mesh without blanching or distortion; and 2 was scored when the incised area was completely off of the mesh. For each hind paw, a sum score was obtained after adding the 6 scores during the 30-minute testing period and was evaluated at 1 hour after the s.c. injection of morphine or i.t. injection of Phα1β.

2.5. Side effects

Naloxone-precipitated withdrawal

This test was conducted to assess the possible effects of Phα1β on withdrawal syndrome induced through acute and chronic injections of morphine. The mice were acclimatized to a clear Plexiglass testing chamber for 1 hour prior to naloxone administration. Two hours after treatment, the mice received naloxone (2 mg/kg, i.p.) to precipitate withdrawal. The signs of withdrawal were the same as those previously described.[34] Briefly, jumping, headshaking, wet-dog shaking, abdominal writhing and grooming behavior were evaluated at 10-minute intervals for a total testing period of 30 minutes and a standardized score of 0 to 3 was assigned (0 = absent; 1 = 1–3 bouts; 2 = 4–6 bouts; 3 = 7 bouts and greater). Paw tremors, piloerection, salivation and ejaculation were also evaluated, with one point scored for the presence of each sign during each 10-minute interval. The number of periods showing the latter signs was counted

(maximum score of 3 per behavioral sign), and the scores were added together to yield a final cumulative withdrawal score.

Gastrointestinal transit

This test was conducted to determine the possible effects of Phα1β on constipation induced through acute and chronic injections of morphine. In this study, the mice were fasted for 18-24 hours (with water ad libitum) prior to the analysis of the gastrointestinal transit, as previously described.[35] Thirty minutes after treatment, a mixture of activated charcoal standard (5% activated charcoal, 20% gum Arabic, 0.3 mL) was administered to the mice using gavage. Twenty minutes after the administration of the activated charcoal mixture, the animals were euthanized, and the stomachs and small intestines were removed to measure the length of the intestine (from the pyloric sphincter to the ileum-cecal junctions, considered the total gut length) and the distance the charcoal meal traveled. The propulsive activity of the gut was determined as the percentage of the gastrointestinal tract that the charcoal traveled, calculated as %traveled = 100 × (distance charcoal traveled/total gut length).

2.6. Statistical methods

The effects of different doses of Phα1β or morphine on mechanical hyperalgesia at one time point were analyzed using one-way analysis of variance (ANOVA), followed by Newman-Keuls post-test. We evaluated the effects of drugs on mechanical hyperalgesia at different times, the development of hyperalgesia and tolerance-induced through repeated morphine administration, the effect of Phα1β on day 4 after paw incision or on

hyperalgesia and tolerance-induced through repeated morphine administration, and the interaction between Phα1β and morphine on withdrawal syndrome and constipation using two-way ANOVA, followed by Bonferroni's post-test. The difference between tolerance on day 1 and 4 was analyzed using Student's t test. Non-parametric measures (guarding behavior) were analyzed using the Kruskal-Wallis test, followed by Dunn's post hoc test. The maximum possible effect (MPE) was used to demonstrate the percentage reduction of the morphine effect (tolerance) and the percentage of reversion of tolerance through Phα1β.

Statistical analysis was performed using GraphPad Software 5.0 (GraphPad Software, San Diego, CA, USA). The results are expressed as median or means ± S.E.M. when appropriate, with the exception of the ED_{50} value (the dose of compound that produces 50% of the effect relative to the control value), which was reported as the geometric mean accompanied by respective 95% confidence limits. The percentage maximum effect (E_{max}) of the drugs was reported as the means ± S.E.M. for each individual experiment with respect to the control values. *P* values less than 0.05 were considered significant. To meet the ANOVA assumptions, the mechanical hyperalgesia data were log transformed prior to statistical analysis.

3. Results

Effect of Phα1β and morphine single administration on postoperative pain

From 1-5 to five hours after plantar incision, the mice showed a significant reduction of the mechanical paw withdrawal threshold (PWT), indicating the development of mechanical hyperalgesia. Phα1β (300 pmol/site, i.t.) but not PBS (i.t.) reversed postoperative mechanical hyperalgesia in mice from 0.5 to 2 hours after treatment (Figure 1B). Phα1β (100-300 pmol/site, but not 30 pmol/site, i.t.) caused an anti-hyperalgesic effect with a mean ED_{50} value (and its 95% confidence limits) of 106 (84-134) pmol/site and an E_{max} of 77±4% at the dose of 300 pmol/site (Fig. 1D). Similarly, a single injection of morphine (10 mg/kg, s.c.) but not saline (s.c.) was able to reverse incision-induced hyperalgesia from 0.5 to 2 hours after treatment (Fig. 1C). Morphine (3-10 mg/kg but not 1 mg/kg, s.c.) caused anti-hyperalgesia with a mean ED_{50} value (and its 95% confidence limits) of 3 (2-4) mg/kg and an E_{max} of 100% at a dose of 10 mg/kg (Fig. 1E).

In addition to mechanical hyperalgesia, operated mice also presented an ongoing guarding nociception at 2 hours after plantar incision (Fig. 1F), similar to pain at rest in postoperative patients. Compared with the PBS group, Phα1β (100 and 300 pmol/site, i.t.) reduced (46±8% of inhibition at 300 pmol/site) spontaneous pain in the plantar incision at 1 hour after treatment (Fig. 1F). Moreover, morphine (3 and 10 mg/kg, s.c.) nearly abolished (100% of inhibition at both doses) the guarding behavior in response to incision at 1 hour after treatment (Fig. 1G).

Effect of Phα1β in combination with a single injection of morphine on postoperative pain

As described above, the administration of low doses of either Phα1β (30 pmol/site, i.t.) or morphine (1 mg/kg, s.c.) alone did not present *per se* effects on postoperative pain in mice. However, the combination of Phα1β (30 pmol/site, i.t.) with a single dose of morphine (1 mg/kg, s.c.) reduced both the mechanical hyperalgesia (67±5% at 0.5 hour, Fig. 2A) and guarding behavior (56±9%, Fig. 2B) in response to plantar incision in mice.

Effect of Phα1β on adverse effects produced through a single injection of morphine in operated mice

A single dose of morphine alone (1 mg/kg, s.c.) but not Phα1β (30 pmol/site, i.t.) caused a 26±6% reduction in gastrointestinal transit at 1 hour after treatment (Fig. 3). The combination of Phα1β administration (30 pmol/site, i.t.) did not alter the constipation induced after a single dose of morphine (Fig. 3).

Regarding the other adverse effects assessed, neither the single injection of morphine (to 10 mg/kg, s.c.) nor Phα1β (to 100 pmol/site) caused hyperalgesia until 4 hours after injection or naloxone-precipitated withdrawal syndrome at 2 hours after treatment. Similarly, morphine (10 mg/kg, s.c.) did not produce tolerance to the antinociceptive effect after a second dose was administered at 4 hours after the first dose (data not shown).

Effect of Phα1β on the hyperalgesia and tolerance produced in response to repeated injections of morphine in the plantar incision model in mice

To assess the potential development of opioid-induced hyperalgesia in sham or operated animals, we verified PWT just prior to the first of the daily repeated injections with saline and morphine (i.e., more than 12 hours after the last daily injection). In sham control mice, the administration of increasing doses of morphine caused mechanical hyperalgesia at 4 days after morphine treatment was initiated (PWT reduced from 2.28±0.38 g at day 1 to 0.44±0.15 g at 4 days after morphine treatment was initiated; $p<0.001$, Student's t-test)(Fig. 4A). The repeated administration of saline did not significantly alter the PWT values in sham-operated mice from 1 to 4 days after treatment was initiated (Fig. 4A). Compared with sham-operated animals that received saline, operated animals repeatedly treated with saline, presented mechanical hyperalgesia, which peaked after 1 day and gradually decreased with time, but remained significant after 4 days (Fig. 4A). The repeated administration of increasing doses of morphine did not alter postoperative hyperalgesia at 1 to 3 days after treatment was initiated, but rather exacerbated hyperalgesia compared with saline repeated treatment at 4 days after treatment was initiated (reduced from 0.26±0.08 g in saline-repeated treatment to 0.11±0.03 g in morphine-repeated treatment; $p<0.001$, Student's t-test) (Fig. 4A).

We also assessed the development of tolerance after the repeated injections of drug, verifying the antinociceptive effect of morphine 0.5 hour after a challenge dose (10 mg/kg, s.c.) on days 1 and 4. However, the antinociceptive efficacy of morphine was greatly reduced after repeated treatment in operated mice, indicating the development of tolerance. Indeed, after repeated administration of increasing doses of morphine but not saline, morphine challenge (10 mg/kg, s.c.) on day 4 presented a decrease in

antinociceptive efficacy (reduced from 2.36±0.24 g on day 1 to 0.45±0.11 g 4 days after morphine treatment started; $p<0.001$, Student's t-test), whereas saline challenge injection had no effect (Fig. 4B). The MPE values obtained at 0.5 hours after a morphine challenge (10 mg/kg, s.c.) were 100% on day 1 and 17±5% on day 4 ($p<0.01$, Student's t test) (Fig. 4B inset).

Next, we evaluated the effect of the intrathecal (i.t.) injection of Phα1β on postoperative pain on day 4 after plantar incision in mice. Phα1β (30 pmol/site, i.t.) did not affect postoperative pain on day 4 (Fig. 5B). Thus, we evaluated the effect of intrathecal (i.t.) injection of Phα1β on repeated morphine-induced exacerbated hyperalgesia in operated mice. The mechanical hyperalgesia induced through morphine was largely reverted (83±6%, at 1 hour) after the injection of Phα1β (30 pmol/site, i.t.), an effect that was initiated after 0.5 hours, peaked after 1 hour and lasted for 2 hours after treatment (Fig. 5C).

Furthermore, we evaluated the effect of the intrathecal (i.t.) injection of Phα1β on repeated morphine-induced tolerance in operated mice. Tolerance was largely reversed when Phα1β (30 pmol/site, i.t.) was concomitantly administered with the morphine challenge (10 mg/kg, s.c.) from 0.5 to 1 hour after treatment (MPE increased by 66±13% at 1 hour – Fig. 5D inset). When administered separately, only a slight reversion of morphine-induce tolerance (MPE increased by 17±6% and 22±5% at 1 hour, respectively) was produced with morphine (10 mg/kg, s.c.) or Phα1β (30 pmol/site, i.t.).

Effect of Phα1β on withdrawal syndrome produced through repeated morphine injections in plantar incision mice

In animals repeatedly treated with saline, a challenge dose of morphine (10 mg/kg, s.c.) did not induce naloxone-precipitated withdrawal syndrome. Repeated treatment with morphine induced naloxone-precipitated withdrawal syndrome, which remained present after saline challenge, but was increased (60±27%) after morphine challenge (Fig. 6A). Intrathecal injection of Phα1β (30 pmol/site) but not PBS completely reversed the naloxone-precipitated withdrawal syndrome induced through morphine challenge in repeated morphine treated animals (Fig. 6A). In contrast, Phα1β (30 pmol/site) did not alter naloxone-precipitated withdrawal syndrome in animals challenged with saline and repeatedly treated with morphine (Fig. 6A).

Effect of Phα1β on constipation produced through repeated morphine injections in plantar incision mice

A morphine challenge (10 mg/kg, s.c.) largely reduced gastrointestinal transit in animals that had previously received repeated treatment with either saline or morphine (Fig. 6B). Neither the intrathecal injection of Phα1β (30 pmol/site) nor PBS reversed the constipation induced through morphine challenge (Fig. 6B). Additionally, Phα1β (30 pmol/site) did not alter gastrointestinal transit in animals challenged with saline (Fig. 6B).

4. Discussion

Opioids are the “gold standard” treatment of postoperative pain; however, the use of these drugs is limited by adverse effects.[5,6,36] The prolonged use and misuse of opioids might also contribute to the development of persistent postsurgical pain, which impairs patients quality of life[4,7,8] In the present study, we observed that the i.t. administration of Phα1β produced a antinociceptive effect on postoperative pain and reduced tolerance, hyperalgesia and withdrawal syndrome induced through the repeated administration of morphine in operated mice.

Previously, we showed that the i.t. administration of Phα1β produced an antinociceptive effect in different models of pain.[16,17] Here, we observed that Phα1β presented an adequate analgesic effect on postoperative pain, reducing not only mechanical hyperalgesia but also spontaneous pain. Indeed, the role of VGCCs has been well established in the transmission of painful impulses at the spinal cord, where these proteins are highly expressed in presynaptic terminals (N and L-type VGCCs) of nociceptive neurons, facilitating calcium influx and releasing algogenic neurotransmitters.[37] Moreover, studies have demonstrated that ziconotide, a selective blocker peptide of the N-type VGCC, showed mechanical anti-hyperalgesic effects in plantar incisional rats and reduced morphine consumption during the postoperative period in humans.[38,39] However, ziconotide is not an ideal analgesic as this drug presents a short therapeutic index and several adverse effects.[40] Phα1β presented similar efficacy but with a higher therapeutic index than ziconotide in preclinical models of neuropathic and cancer pain.[16,41] Therefore, blocking VGCCs in nociceptive neurons through

Phα1β could be interesting in controlling pain associated with surgical procedures.

As expected, morphine also presented an antinociceptive effect on postoperative pain as this drug completely abolished the mechanical nociception and guarding behavior induced after plantar incision. Indeed, clinical studies have demonstrated that morphine is one of the most commonly used opioids in surgical patients.[5,6] Morphine antinociceptive action reflects the activation of MOR receptors, potentially leading to both the activation of potassium channels (GIRK) and the inhibition of VGCCs, resulting in reduced neuronal excitability in several central nervous system (CNS) structures.[12] Thus, the blocking of VGCCs could potentiate the antinociceptive effects of morphine. Here, we observed that the concomitant administration of Phα1β with a single non-effective dose of morphine produced analgesia, including both mechanical nociception and guarding behavior, in plantar incision mice. It has been suggested that the i.t. injection of selective N-, P/Q-, and L-type VGCCs blockers potentiates morphine analgesia in rodents.[42,43] Indeed opioid-related adverse drug events are common in postoperative patients[8,44], thus Phα1β could be used as an adjunct to morphine analgesia, facilitating the use of a lower dose of morphine with greater efficacy and less adverse effects.

Although opioids are the usual treatment for postoperative pain, the misuse of these drugs after surgery can result in adverse effects that can lead to long-term use of opioid therapy.[7,8,44] In the present study, we showed that repeated treatment with an escalating dosing of morphine in plantar incision mice not only caused tolerance to the antinociceptive effect of this drug but also exacerbated hyperalgesia and caused withdrawal syndrome. Indeed, analgesic

tolerance is a limitation of chronic opioid therapy.[45] Additionally, recent studies have suggested that chronic opioid administration or withdrawal causes a hyperalgesic state, where opioids can paradoxically worsen pain.[46,47,48] Here, we observed that the incisional pain and the OIH exacerbated hyperalgesia, resulting in an additional effect between them. Accordingly, the misuse of repeated and escalating doses of morphine can worsen, instead of improve, post-surgical hyperalgesia in animals, suggesting that this procedure must be carefully managed in patients.

Different methods to prevent the adverse effects of opioids have been tested in patients after surgery, but the clinical benefit in the immediate postoperative period is either absent or limited to a moderate opioid-sparing effect and not to a reversal of these effects.[49] In the present study, the i.t. administration of Phα1β largely reversed the hyperalgesia and tolerance induced through morphine and completely reversed the withdrawal syndrome induced after morphine challenge on day 4. Accordingly, ziconotide also possesses analgesic efficacy after repeated administration of morphine, but was unable to restore morphine tolerance.[39] Although these drugs have a paradoxical effect, opioid-induced tolerance and hyperalgesia might be intricately mechanistically related.[10] Additionally, the abrupt termination of the chronic administration of opioids is associated with hyperalgesia (called withdrawal-induced hyperalgesia).[50] Accordingly, we observed that on day 4 (approximately 12 hours after the last dose of morphine), in addition to hyperalgesia, the plantar incision mice presented withdrawal syndrome, with a higher score after morphine challenge. Furthermore, other signals of withdrawal syndrome were observed, including jumping, wet-dog shaking and paw tremors.

These behaviors are also expressed after naloxone precipitate treatment in mice.[51] Clinical studies with nonmalignant pain patients demonstrated that chronic opioid analgesic therapy leads to dependence or addiction in a small percentage of patients, and only a few patients presented withdrawal signals.[52,53] However, in contrast to patients receiving opioid for cancer pain or severe states, nonmalignant pains have been associated with opioid misuse and consequent dependence.[18]

A previous study demonstrated that after chronic morphine administration, the expression of L- and N-type VGCCs was significantly increased.[54] In addition, accumulating evidence indicates that L-, N- and R-type VGCCs blockers in healthy rodents modulate the development of hyperalgesia, tolerance and withdrawal syndrome induced through opioids.[55,56,57] Thus, the ability of Phα1β to block several VGCCs might reflect the efficacy of this molecule in reversing hyperalgesia, analgesic tolerance and withdrawal syndrome induced through repeated morphine treatment. Although, there are no studies showing the involvement of VGCCs in the adverse effects induced through opioids in a model of incisional pain, the effects of the peptide Phα1β on hyperalgesia and tolerance caused by morphine have been demonstrated in a mouse model of cancer pain.[16] Thus, Phα1β not only produces analgesia but is also useful to reverse the hyperalgesia, tolerance and withdrawal syndrome induced through morphine in postoperative pain.

Another uncomfortable post-surgery effect of opioid treatment is constipation.[58] Indeed, we also observed that the single or repeated administration of morphine in incisional mice reduced gastrointestinal transit. Unlike analgesia, we observed that repeated morphine did not produce

tolerance to constipation, as previously reported.[59] The i.t. administration of Phα1β alone did not reduce gastrointestinal transit and did not alter the effect of a single or repeated dose of morphine. In a recent study, we demonstrated that this peptide produced a slight reversion of repeated morphine-induced constipation in healthy mice.[20] Nevertheless, despite the gastrointestinal effects from opioid-mediated activity on both peripheral and central sites, the high density of μ receptors in the enteric system mediates most of the gastrointestinal effects of opioid agonists [60], consistent with the results of the present study as the administration of the Phα1β was intrathecally.

In summary, the findings of the present study showed that the i.t. injection of Phα1β not only presented analgesic effects and potentiated the analgesic effects of a single dose of morphine on postoperative pain but also reduced the hyperalgesia, tolerance and withdrawal syndrome induced through the repeated administration of morphine in operated mice. Thus, the blockage of VGCCs by Phα1β, could represent an useful analgesic and adjuvant drug in opioid therapy for postoperative pain.

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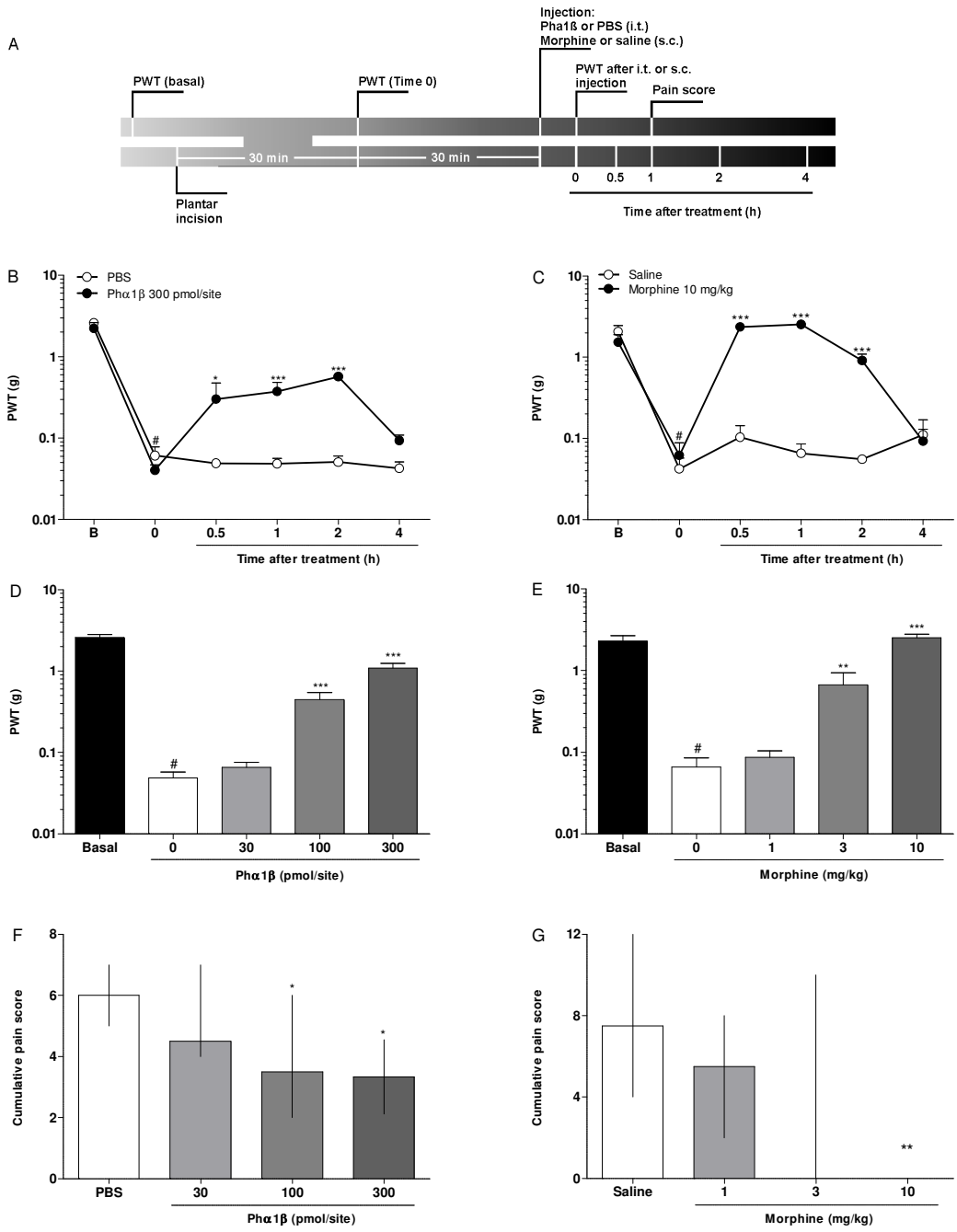

Figure 1. Effect of Phα1β and morphine injection on postoperative pain in mice. (A) Representative scheme of the experimental protocol. (B and C) Time course of the effect of (B) Phα1β (300 pmol/site, i.t.) or (C) morphine (10 mg/kg, s.c.) on mechanical nociception. (D and E) Dose-response curve of the effect of (D) Phα1β (30-300 pmol/site, i.t.) or (E) morphine (1-10 mg/kg, s.c.) on mechanical nociception. (F and G) Dose-response curve of the effect of (F) Phα1β (30-300

pmol/site, i.t.) or (G) morphine (1-30 mg/kg, s.c.) on guarding nociception. The treatment was performed at 1 hour after the paw incision. The data are expressed as (B, C, D and E) paw withdrawal threshold (PWT, g) or (F and G) cumulative pain score. Each column represents the mean of 6 mice, and vertical lines show the S.E.M. or median. Statistical analysis was performed using 2-way repeated measures analysis of variance (ANOVA), followed by Bonferroni's post hoc test (B and C), 1-way ANOVA, followed by the Student-Newman-Keuls post hoc test (D and E) or Kruskal-Wallis, followed by Dunn's post hoc test (F and G). *p < 0.05, **p<0.01, or ***p<0.001 compared with vehicle (B and C), to dose 0 values (D and E), or to control group (Saline or PBS) (F and G), #p<0.05, compared with baseline (B) values (B, C, D and E).

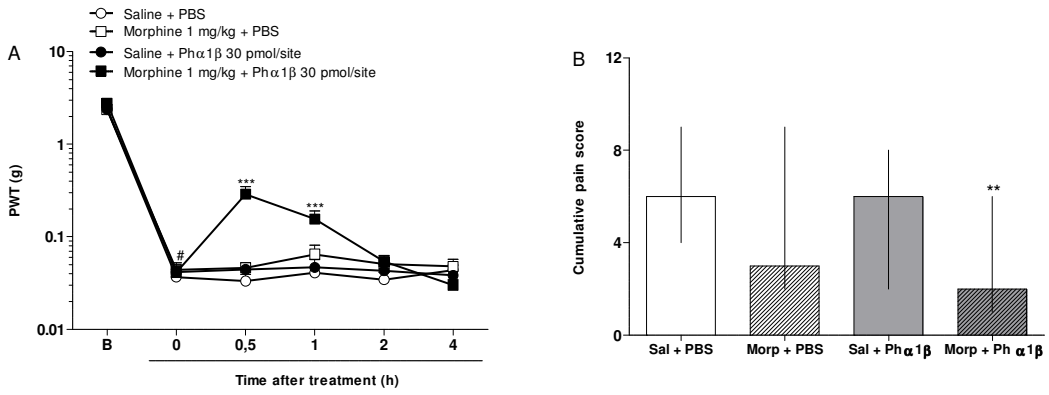

Figure 2. Effect of intrathecal (i.t.) injection of Phα1β with a single not effective dose of morphine on postoperative pain in mice. (A) Time course of the effect of Phα1β (30 pmol/site, i.t.) or PBS (5 µL/site, i.t.) and morphine (1 mg/kg, s.c.) or saline (10 mg/mL, s.c.) on mechanical nociception. (B) Effect of Phα1β (30 pmol/site, i.t.) or PBS (5 µL/site, i.t.) and morphine (1 mg/kg) or saline (10 mg/mL, s.c.) on guarding behavior. The data are expressed as (A) paw withdrawal threshold (PWT, g) or (B) cumulative pain score. Each column

represents the mean of 6 or 9 mice, and vertical lines show the SEM or median. Statistical analysis was performed using 2-way repeated measures analysis of variance, followed by Bonferroni's post hoc test (A) or Kruskal-Wallis, followed by Dunn's post hoc test (B). **p<0.01 or ***p<0.001 compared with the PBS and saline group (A and B); #p<0.05 compared with baseline (B) values (A).

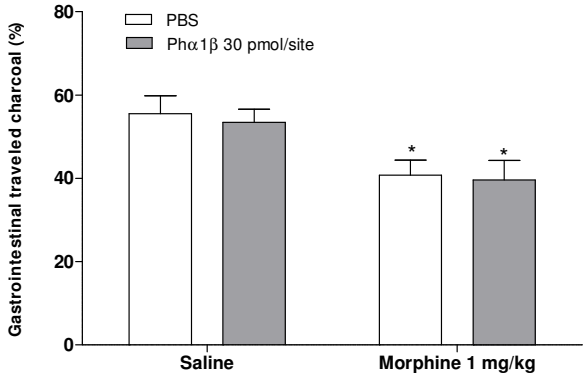

Figure 3. Effect of intrathecal (i.t.) injection of Phα1β on the constipation produced after a single dose of morphine in mice. Effect of Phα1β (30 pmol/site, i.t.) on gastrointestinal transit after a single injection of saline (vehicle, 10 mL/kg) or morphine (1 mg/kg). The data are expressed as the percent of gastrointestinal tract that the charcoal meal traveled. Each column represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. *p<0.05 compared with the PBS and saline group.

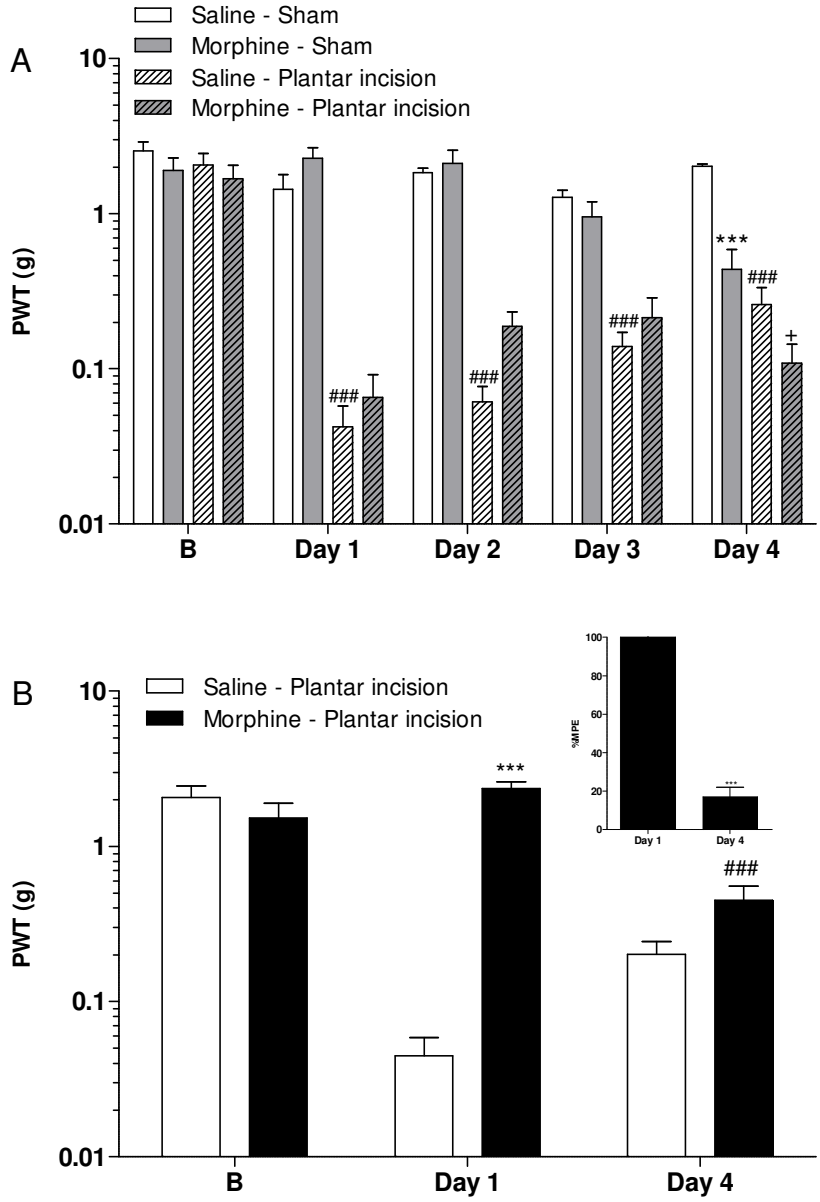

Figure 4. Effect of repeated injection of morphine after plantar incision in mice. (A) Morphine-induced hyperalgesia. Time course of mechanical nociception prior to the first of repeated daily morphine or saline injections in sham or plantar incision mice. (B) Morphine-induced tolerance. Mechanical nociception and maximum possible antinociceptive effect (MPE) of the morphine challenge (10 mg/kg, 30 minutes after injection) on day 1 or 4 of repeated morphine treatment. The data are expressed as (A and B) paw withdrawal threshold

(PWT, g), or (B inset) MPE (%). Each column represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way analysis of variance, followed by Bonferroni's post hoc test (A and B) or Student's t-test (B inset). ***$p<0.001$ compared with the saline-sham group (A), the saline-plantar incision group (B) or to day 1 (B inset); $^{+}p < 0.05$, compared with the saline-plantar incision group (A); $^{###}p<0.001$, compared with the morphine-plantar incision group (Day 1) (B).

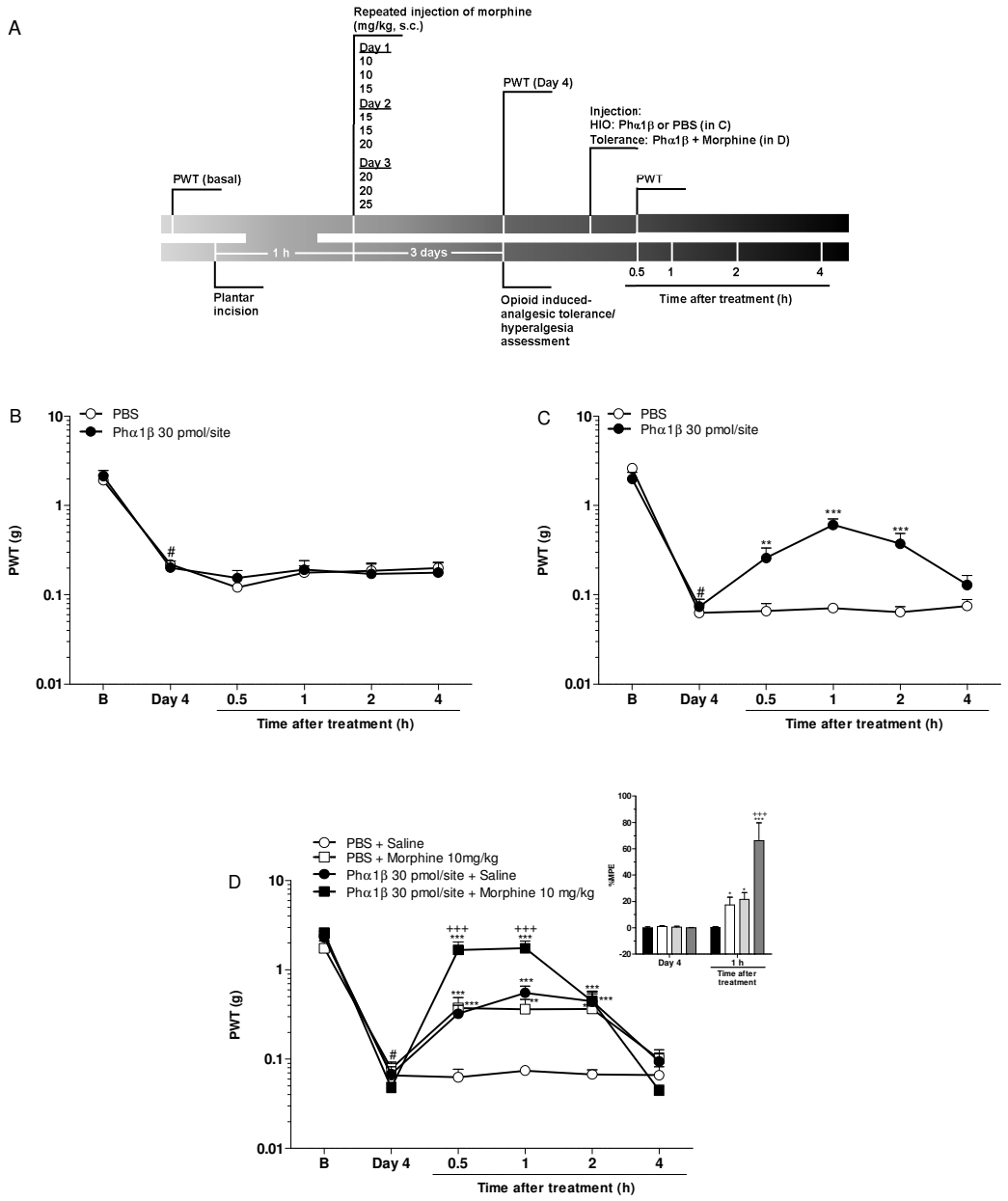

Figure 5. Effect of intrathecal (i.t.) injection of Phα1β on hyperalgesia and tolerance produced through repeated injection of morphine in plantar incision mice. (A) Representative scheme of the experimental protocol. (B) Phα1β effect on day 4 after plantar incision. Time course of the effect of Phα1β (30 pmol/site, i.t.) on mechanical nociception at 4 days after plantar incision. (C) Phα1β effect on hyperalgesia induced through morphine treatment in plantar incision mice. Time course of the effect of Phα1β (30 pmol/site, i.t.) on mechanical

hyperalgesia induced through repeated morphine treatment. (D and D inset) Phα1β effect on tolerance induced through morphine in plantar incision mice. Time course of the effect and maximum possible antinociceptive effect (MPE) of Phα1β (30 pmol/site, i.t.) or PBS (5 µL/site, i.t.) and morphine (10 mg/kg, s.c.) or saline (10 mg/mL, s.c.) challenge on tolerance induced through morphine on day 4. The data are expressed as (B, C and D) paw withdrawal threshold (PWT, g), or (D inset) MPE (%). Each column represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way repeated measures analysis of variance, followed by Bonferroni's post hoc test. *$p<0.05$, **$p<0.01$, or ***$p<0.001$ compared with the PBS group (B and C), or to the PBS and saline group (D); $^{\#}p<0.05$, compared with baseline (B) values (B, C and D); $^{+++}p<0.001$ compared with the PBS and morphine group (D).

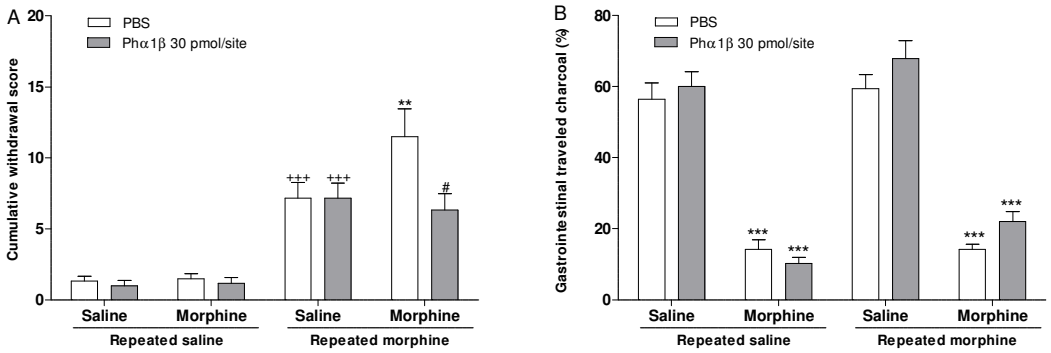

Figure 6. Effect of intrathecal (i.t.) injection of Phα1β on withdrawal syndrome and constipation produced through repeated injection of morphine in plantar incision mice. (A) Effect of Phα1β (30 pmol/site, i.t.) on withdrawal syndrome induced through repeated morphine or saline injection in plantar incision mice. (B) Effect of Phα1β (30 pmol/site, i.t.) on gastrointestinal transit inhibition induced through repeated morphine or saline injection in plantar incision mice. The data are expressed as (A) cumulative withdrawal score or (B) percentage of the gastrointestinal tract that the charcoal meal traveled. Each column represents the mean of 6 mice, and vertical lines show the SEM. Statistical analysis was performed using 2-way analysis of variance, followed by Bonferroni's post hoc test.**$p<0.01$ or ***$p<0.001$ compared with the PBS and saline group in repeated morphine treatment (A) or to the PBS and saline group (B); and $^{\#}p<0.05$ compared with the PBS and morphine group in repeated morphine treatment (A); $^{+++}p<0.001$ compared with the PBS and saline group in repeated saline treatment (A).

ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

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	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	1/1
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	a) Introductio n/ paragraph 1, 2 and 3 b) Introductio n/ paragraph 4
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	introductio n/ paragraph 4
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Methods Ethical statement/ paragraph 1
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	a) Methods Study design/sam ple size and Figure legends b and c) Methods study design/ paragraph 1 and study design/ experiment al animals
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day).	b) Methods: all subtitle include information s about the relative experiment

		c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	methods
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Methods Study design/experimetal animals

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	b) Methods: Study design/experimental animals
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	a) Methods: Study design/sample size and Figure legends b) Methods: Study design/sample size
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	a) Methods: Study design/paragraph 1 b) Methods: Experimental design (protocols)
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Methods: Study design/sample size
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Methods / Statistical analysis and Figure legends
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Results
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Figure legends
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Results and Figure legends
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	No reported
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the	Discussion

		replacement, refinement or reduction (the 3Rs) of the use of animals in research.	
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Discussion: several paragraphs
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Acknowled gment

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.