UNIVERSIDAD DE GRANADA

PROGRAMA DE DOCTORADO EN BIOMEDICINA

Control of opioid analgesia and tolerance by sigma-1 receptors:

Studies on nociceptive and inflammatory joint pain

Tesis doctoral presentada por

ÁNGELES MONTILLA GARCÍA

Licenciada en Biología, para optar al grado de

DOCTOR CON MENCIÓN INTERNACIONAL

Editor: Universidad de Granada. Tesis Doctorales
Autor: Ángeles Montilla García
ISBN: 978-84-9163-960-2
URI: http://hdl.handle.net/10481/52761

UNIVERSIDAD DE GRANADA

Facultad de Medicina

Departamento de Farmacología e Instituto de Neurociencias

Directores**:**

ENRIQUE JOSÉ COBOS DEL MORAL

JOSÉ MANUEL BAEYENS CABRERA

COMPROMISO DE RESPETO DE LOS DERECHOS DEL AUTOR

La doctoranda Ángeles Montilla García y los directores de la tesis Enrique José Cobos del Moral y José Manuel Baeyens Cabrera:

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada 2 de Julio de 2018

Director/es de la Tesis	Doctoranda
Enrique José Cobos del Moral	Ángeles Montilla García
José Manuel Baeyens Cabrera	

D. ENRIQUE JOSÉ COBOS DEL MORAL, PROFESOR CONTRATADO DOCTOR DEL DEPARTAMENTO DE FARMACOLOGÍA E INVESTIGADOR DEL INSTITUTO DE NEUROCIENCIAS DE LA UNIVERSIDAD DE GRANADA

CERTIFICA:

Que el trabajo de investigación titulado, "CONTROL OF OPIOID ANALGESIA AND TOLERANCE BY SIGMA-1 RECEPTORS: STUDIES ON NOCICEPTIVE AND INFLAMMATORY JOINT PAIN" ha sido realizado por Dña. Ángeles Montilla García para optar al grado de Doctor por la Universidad de Granada, en el Instituto de Neurociencias y el Departamento de Farmacología de la Universidad de Granada, bajo mi dirección.

Y para que conste donde proceda se firma este certificado en Granada a 2 de Julio de 2018

Fdo. Enrique José Cobos del Moral

Fdo. Ángeles Montilla García

D. JOSÉ MANUEL BAEYENS CABRERA, CATEDRÁTICO DE FARMACOLOGÍA DE LA UNIVERSIDAD DE GRANADA E INVESTIGADOR DEL INSTITUTO DE NEUROCIENCIAS DE LA UNIVERSIDAD DE GRANADA

CERTIFICA:

Que el trabajo de investigación titulado, "CONTROL OF OPIOID ANALGESIA AND TOLERANCE BY SIGMA-1 RECEPTORS: STUDIES ON NOCICEPTIVE AND INFLAMMATORY JOINT PAIN" ha sido realizado por Dña. Ángeles Montilla García para optar al grado de Doctor por la Universidad de Granada, en el Instituto de Neurociencias y el Departamento de Farmacología de la Universidad de Granada, bajo mi dirección.

Y para que conste donde proceda se firma este certificado en Granada a 2 de Julio de 2018

Fdo. José Manuel Baeyens Cabrera

Fdo. Ángeles Montilla García

La realización de esta tesis ha sido posible gracias a la financiación de nuestro grupo de investigación por la Junta de Andalucía (grupo CTS-109), por el CEI BioTic Granada, por fondos FEDER, Ministerio de Ciencia e Innovación (proyectos SAF2013-47481P y SAF2016-80540-R), y Laboratorios Esteve.

A Rafa

"¡Y qué buena es la tierra de mi huerto!:
hace un olor a madre que enamora,
mientras la azada mía el aire dora
y el regazo le deja pechiabierto"

Miguel Hernández

Agradecimientos

El amor a la naturaleza es también amor al hombre que se integra en ella; pero no es el único sentimiento que comparten ambos mundos. La gratitud es otro. Amor en la labranza y gratitud por los resultados son precisos para que el huerto nos ofrezca sus mejores frutos. Y son ambas emociones las que siento al pensar en la multitud de personas que habéis contribuido de una u otra forma, a que concluya esta tesis con un grito de entusiasmo y satisfacción.

El primer reconocimiento es a la labor de mis directores, Kike y José Manuel. Sois los más prestigiosos jardineros que este huerto podría haber tenido. Habéis puesto las guías para que el tallo no se tuerza, abonado cuando era menester y eliminado las hojas secas que entorpecían el crecimiento. Quehaceres que habéis desempeñado con cariño, entrega y el conocimiento, lo que os agradezco profundamente.

A Kike, gracias por darme la oportunidad de poder realizar esta tesis. Todo empezó aquel día, paseando por el aparcamiento de la antigua facultad, rodeados de flores de azahar, sin apenas conocernos. Desde entonces, has contado conmigo para todo lo posible, siempre encauzando las aguas del arroyo para que no se pierda ni una gota. Por tus consejos, tu tiempo, palabras de apoyo, incluso cuando más perdida he andado. Has guiado este trabajo de una forma impecable, inteligente como el que más. Has sido y serás siempre el mejor jardinero que podría haber tenido. Me siento afortunada y más que agradecida.

A José Manuel, gracias por acogerme en el mejor grupo del mundo. Eres nuestro padre científico; constante como el que más, detalloso, sabio, cercano y amable. Qué decir que no se sepa. Gracias por cuidar con esmero cada paso, por confiar y ayudarme en tantas ocasiones, tanto profesional como personalmente. Por tu paciencia. Por demostrarnos cada día que la pasión y los pequeños detalles son los que hacen grandes diferencias. Por enseñarme tanto y ser un ejemplo a seguir para todos los que te rodeamos y admiramos.

A Inma y Mari Carmen, por ser tan maravillosas conmigo. Porque sois las preciosas mariposas del huerto; gracias por vuestras espléndidas alas, capaces de cubrirme en el día más lluvioso.

A Migue y Gloria, porque esta tesis sin vosotros no hubiera sido posible. Por el tiempo que me habéis dedicado. Por ayudarme tanto en el laboratorio como fuera de él y siempre con una sonrisa. Gracias por esos incontables momentos junto a mí.

Gracias a Paco por resolver mis dudas y tenderme tu mano en innumerables ocasiones.

A Paqui, por ser el árbol del amor del jardín. Por ser tan bondadosa. Gracias por escucharme, darme cobijo y tu cariño en infinidad de ocasiones.

Al Instituto de Neurociencias, en particular a Entrena, Bea, Cruzmi, Paco, Milagros, y especialmente a Juanmi, con quienes he compartido tanto. Os agradezco aquellos agradables ratos que me habéis hecho pasar.

A Antonio Tirado, Cristina, Lucía, Toñi y Luis, por compartir cantidad de inquietudes conmigo y junto a los que he convivido todos estos años en el laboratorio.

A Esperanza del Pozo, Jesús Torres, Rosa Montes, Ferrer, Ahmad y Agatángelo, por haber sido excelentes profesores de posgrado y compañeros.

A David Rincón, Raquel Durán, Pilar, Galindo, Sara, Laura Boyero, Geraldine, Lucía, Mahmoud, Ruth, Ehab y Cecilia, por vuestro cariño y por haber hecho más ameno el día a día en la facultad.

A Eduardo y Javier, por transmitirme conocimientos histológicos y enriquecer con vuestra experiencia nuestro trabajo.

A Paco Alba, Araceli y, en especial, a Antonio Torres, por enseñarme tanto.

A Antonio García, Iago, Sara, María, Fernando... y esa larga lista de investigadores que he conocido en congresos, por mostrarme la ciencia desde otra perspectiva.

A Esteve y su magnífico equipo científico, ya que sin vosotros este trabajo no podría haber cumplido todos los objetivos marcados.

A Patri, Carlos, Cris, Rodri, Miri, Sergio, Isa, Javi, Lupa, María, Tati y Tomás. Ese maravilloso grupo de "bostoñoles" que lograsteis cargar mi estancia predoctoral de momentos míticos.

A mi grupo "amiguitos", Isa, Antonio, Olivas, Jose, Pepico, Noe, Jose Antonio, Natalia, Álex, Migue, Jose León, Juan y Mari, porque sois para mí las flores preciosas que dan color al día más oscuro. Gracias por ayudarme a levantarme en cada tropiezo y por animarme en tantas ocasiones. Por arroparme y hacerme sentir genial a vuestro lado o lejos de él. Sé que seguiremos reuniéndonos siempre, porque la distancia no impide que en los huertos más amplios las flores sean lo más bonito y resplandeciente.

A mis amigos de la infancia, Manme, Nuria, Patri, Encarni, Ana, Samu y Eli. A mi amiga Ele, así como a Sergio, Tere, Kiki y los motrileños. Por supuesto a mi prima Maricella, en nombre de decenas de primos y titos fascinantes. Porque sois los pajaritos del jardín, me ayudáis a volar con vuestros tiernos y mágicos aleteos, con vuestros encantadores trinos. Gracias por quererme, acompañarme y escucharme, por compartir conmigo tanto.

A mis padres, Ángeles y José Luis, por inculcarme desde siempre la importancia del conocimiento y del trabajo bien hecho, la pasión por las ciencias y el valor de la cultura. Por enseñarme tanto, por darme todo lo que he necesitado y querido. Gracias por cuidarme como nadie, por caer como agua del cielo para darme tanta fuerza en esta última etapa. Por compartir mis inquietudes e intentar tranquilizarme una vez tras otra. Os debo todo lo que he conseguido, porque sin vosotros hubiera sido imposible.

A mis hermanos, Margarita y José Luis, sois mi alegría. Por vuestra confianza y todo aquello que compartimos. Por ser mis confidentes. Gracias por esta piña de la que formamos parte y de la que me siento tan orgullosa. Por ser ejemplo de esfuerzo y constancia. Debo confesaros que aunque sea la mayor siempre me he sentido diminuta a vuestro lado, ¡qué grandes sois!

A mis abuelos, por vosotros empecé a escribir estos agradecimientos. A mi abuela Petro, mi ejemplo a seguir como mujer. Valiente, optimista, trabajadora como la que más. Gracias por tus sabios consejos, refranes mil, tu apoyo incondicional, fundamental para

mí. Y a mi abuelo Antonio, que entre tanto, me inculcaste tu pasión por la música. Aún escucho la canción -he lives in you- y siento que a pesar de estar en otra vega (como tú dirías), nos has dejado una buena parte de ti. Gracias por mantenerme con los pies en el suelo, como buenas raíces que sois.

A Marisa, Tato, Manuel y Juan Antonio. Por ser el aire que respiro infinidad de veces. Si me he sentido ahogada, me habéis aportado oxígeno con vuestras ráfagas de amor y mimos. Si me he sentido flotando, os habéis ilusionado tanto como yo con cada pequeño logro. Gracias por vuestra ternura y ánimos diarios.

A mi otra familia, mi primo Manu, Antonio Manuel y Paqui. Por acogerme como un miembro más de vuestro maravilloso equipo. A mi abuela María, por darme tu cariño y quererme de esa manera tan especial para mí. A mi abuelo Manuel, porque a pesar de estar lejos te debo mucho, destapaste mi pasión por la naturaleza; eres el mejor capataz que un huerto podría tener. Y por supuesto, a Carmen, por adoptarme como si fuera una hija, mimarme y aconsejarme desde el corazón. Gracias familia.

A Rafa, mi todo, a quien dedico esta tesis. Porque pienso en ti y el corazón se me acelera, después de estos catorce años juntos. Porque todo esto es gracias a ti; y es que eres la luz solar de mi ecosistema. Desde el primer momento en que te vi me deslumbraste, me enamoré de tu brillo, y desde entonces, no he querido separarme de ti ni un solo segundo. En unos días volveremos a estar juntos y por siempre; porque una vida contigo se me queda corta y no hay tiempo que perder. Porque con tus rayos das energía al día más nublado; gracias por hacerme sentir que estoy a tu lado, a pesar de estar tan lejos, por hacerme reír como nadie, apoyarme y quererme tanto. Te amo mi vida y cada día más, y más, y más. ¡Hasta el infinito y más allá! ¡Hasta el techo!

Y por último, a todas aquellas ratoncillas que habéis hecho posible que este huerto florezca y dé sus mejores frutos. Gracias.

A veces la naturaleza se rebela, incluso en esta pequeña porción de naturaleza que es mi huerto (tesis). La ayuda y colaboración que me habéis prestado ha conseguido que esos percances no hayan pasado de contratiempos, sin consecuencias en la cosecha. A todos vosotros, amor y gratitud.

Acknowledgements

To Michael Costigan for the time that I spent in Boston. It was a pleasure to work in your group at Children´s Hospital Boston/Harvard Medical School. Thank you for your dedication during my stay, your patience, your constant and deep explanations and, of course, for being a great mentor. An unforgettable experience.

To Clifford Woolf for giving me the opportunity to work in your wonderful lab. It was an honour to do research in such an excellent environment.

To Nick Andrews for your kindness and patience with me. Among many other things, thank you for your attention and the help at the animal research facilities (and for the help with the paperwork).

To Eri, Evgeny, Lee, Ajay, Chelsea, Maud, Marco, Giulia and people from Woolf's lab, in particular to Priscilla and Alyssa. You made my stay very comfortable and were so friendly with me; thank you for everything.

INDEX

Index

RESUMEN

RESUMEN

Los receptores sigma-1 son una diana farmacológica prometedora para el tratamiento del dolor (Sánchez-Fernández et al., 2017). Entre los antagonistas selectivos sigma-1, el que ha sido caracterizado con mayor profundidad es el S1RA (Vela et al., 2015). Este fármaco ha sido evaluado en estudios de fase II para el dolor neuropático, con resultados prometedores (Bruna et al., 2018). Además, diversos estudios preclínicos muestran que la inhibición del receptor sigma-1 incrementa la analgesia opioide (Zamanillo et al., 2013; Sánchez-Fernández et al., 2017), y de hecho, otra indicación potencial para el uso clínico del S1RA en pacientes humanos es como adyuvante a la analgesia opioide (Vela et al., 2015). En esta Tesis Doctoral nos centramos en el estudio de la modulación de la analgesia opioide por los receptores sigma-1.

Inicialmente estudiamos esta modulación en modelos de dolor nociceptivo producido tanto por estímulos mecánicos como térmicos en el ratón. Encontramos que la administración local de los agonistas opioides μ morfina, oxicodona y buprenorfina producía efectos antinociceptivos locales frente a un estímulo térmico (placa caliente unilateral a 55 ºC) pero no mecánico (presión plantar con 450 g). La inhibición del receptor sigma-1, tanto en ratones desprovistos de este receptor como por su antagonismo farmacológico mediante el S1RA, indujo un gran incremento de la analgesia opioide periférica frente al estímulo mecánico pero no frente al estímulo térmico. Mediante ensayos inmunohistoquímicos, encontramos que el receptor sigma-1 se encuentra presente a niveles muy elevados en las neuronas C no peptidéricas ($IB4^+$) de los ganglios de las raíces dorsales espinales (donde se encuentran los somas de las neuronas aferentes primarias), mientras que las neuronas C peptidérgicas ($TRPV1^+$) presentaban unos niveles de receptores sigma-1 más reducidos. La eliminación de las neuronas C peptidérgicas mediante la administración *in vivo* de resiniferatoxina, un superagonista TRPV1, no alteró ni el dolor por el estímulo mecánico ni los efectos del antagonismo sigma-1 en la analgesia opioide periférica frente a este tipo de estimulación dolorosa. Sin embargo, el tratamiento con esta toxina inhibió las respuestas dolorosas frente al estímulo térmico, así como el efecto analgésico de la administración local de morfina frente a este tipo de estímulo. Por lo tanto, la analgesia opioide periférica frente al estímulo nociceptivo mecánico está inhibida de manera

tónica por la actividad de los receptores sigma-1, mientras que la analgesia opioide periférica frente al estímulo nociceptivo térmico (la cual es producida en neuronas C peptidérigicas) no es modulable por este receptor.

Además, estudiamos la modulación de la analgesia opioide por los receptores sigma-1 durante el dolor patológico, en concreto en un modelo murino de inflamación articular dolorosa. Exploramos no sólo los efectos de esta interacción en la alodinia mecánica (la medida estándar de dolor crónico en estudios preclínicos), sino que también estudiamos los efectos de la modulación de la analgesia opioide por el receptor sigma-1 en la alteración funcional inducida por el dolor articular. En concreto, focalizamos nuestros esfuerzos en la fuerza de agarre como indicador de la interferencia del dolor en la función física. Este parámetro se usa rutinariamente en las consultas de reumatología, y sus variaciones correlacionan no sólo con la progresión de la patología articular, sino también con el dolor percibido por el paciente (Fraser et al., 1999; Overend et al., 1999). Sin embargo, la vinculación entre el dolor y la fuerza de agarre está muy poco estudiada en el roedor, por lo que hicimos una caracterización profunda de este parámetro en animales con inflamación articular, previa al estudio del papel del receptor sigma-1 en el dolor inflamatorio.

Los animales con inflamación articular en los tobillos inducida por la administración periarticular de adyuvante completo de Freund (CFA, por sus siglas en inglés), mostraron un masivo infiltrado inmunitario a nivel periarticular y alteraciones en la membrana sinovial, junto con alodinia mecánica (medida mediante filamentos de von Frey) y una abrupta disminución en la fuerza de agarre. La alodinia mecánica fue mucho más duradera que las alteraciones en la fuerza de agarre, por lo que no es la responsable de la disminución funcional. De hecho, cuando las alteraciones sinoviales inducidas por la inflamación desaparecieron, los animales recuperaron valores de fuerza de agarre normales mientras que mantuvieron una marcada alodinia mecánica. Tanto la alodinia mecánica como la disminución en la fuerza de agarre fueron revertidas por analgésicos de grupos farmacológicos diferentes. Estos incluyen los fármacos opioides oxicodona y tramadol, los antiinflamatorios no esteroideos (AINEs) ibuprofeno y celecoxib, así como el paracetamol, indicando que tanto la alodinia mecánica como la disminución en la fuerza de agarre responden al tratamiento analgésico, y consecuentemente se deben a

las alteraciones sensoriales propias del proceso inflamatorio. Tanto la administración de rojo rutenio (un antagonista TRP), como de resiniferatoxina para eliminar las neuronas TRPV1+, inhibieron completamente la alodinia mecánica mientras que no alteraron la fuerza de agarre de los animales con la inflamación articular. Por consiguiente, la disminución en la fuerza de agarre por la inflamación articular se produce por mecanismos diferentes a la alodinia mecánica, y merece ser usada en estudios preclínicos como una medida adicional de dolor articular para conseguir una visión más global y a la vez más traslacional al humano de la eficacia de los fármacos analgésicos.

Por último, dentro del estudio del papel del receptor sigma-1 en el dolor inflamatorio articular, encontramos que el antagonismo del receptor sigma-1 por el S1RA no alteró la alodina mecánica inflamatoria, aunque incrementó levemente la fuerza de agarre de los animales con la inflamación articular. El antagonismo sigma-1 potenció en gran medida el efecto antialodínico de la morfina en los animales con inflamación, sin embargo no modificó el efecto de este opioide en la fuerza de agarre. Además, puesto que la tolerancia analgésica producida tras el tratamiento repetido con agonistas opioides es un problema de gran relevancia clínica (Morgan y Christie, 2011), estudiamos el papel del receptor sigma-1 en la tolerancia analgésica a la morfina. El S1RA, administrado a animales tolerantes a morfina, rescató la analgesia morfínica tanto en la alodinia mecánica como en la fuerza de agarre. Sin embargo, el tratamiento preventivo con S1RA durante la inducción de la tolerancia a la morfina impidió el desarrollo de la tolerancia morfínica únicamente en la fuerza de agarre. Estos resultados ponen de manifiesto tanto el papel del receptor sigma-1 como modulador de la analgesia opioide durante el dolor inflamatorio articular, como las diferencias entre la alodinia mecánica y la fuerza de agarre.

En conclusión, los receptores sigma-1 juegan un papel importante en la modulación de la analgesia y la tolerancia opioide (aunque sus efectos dependen del tipo de estímulo doloroso y del parámetro indicador de dolor utilizado). Estos hallazgos podrían tener interés terapéutico para el uso de los antagonistas sigma-1 como adyuvantes a los fármacos opioides.

Bibliografía

Bruna J., Videla S., Argyriou A.A., Velasco R., Villoria J., Santos C., Nadal C., Cavaletti G., Alberti P., Briani C., Kalofonos H.P., Cortinovis D., Sust M., Vaque A., Klein T., Plata-Salaman C., 2018. Efficacy of a novel sigma-1 receptor antagonist for oxaliplatin-induced neuropathy: a randomized, double-blind, placebo-controlled phase IIa clinical trial. Neurotherapeutics 15, 178-189.

Fraser A., Vallow J., Preston A., Cooper R.G., 1999. Predicting 'normal' grip strength for rheumatoid arthritis patients. Rheumatology 38, 521-528.

Morgan M.M., Christie M.J., 2011. Analysis of opioid efficacy, tolerance, addiction and dependence from cell culture to human. Br. J. Pharmacol. 164, 1322-1334.

Overend T.J., Wuori-Fearn J.L., Kramer J.F., MacDermid J.C., 1999. Reliability of a patient-rated forearm evaluation questionnaire for patients with lateral epicondylitis. J. Hand Ther. 12, 31-37.

Sánchez-Fernández C., Entrena J.M., Baeyens J.M., Cobos E.J., 2017. Sigma-1 receptor antagonists: a new class of neuromodulatory analgesics. Adv. Exp. Med. Biol. 964, 109-132.

Vela J.M., Merlos M., Almansa C., 2015. Investigational sigma-1 receptor antagonists for the treatment of pain. Expert. Opin. Investig. Drugs 24, 883-896.

Zamanillo D., Romero L., Merlos M., Vela J.M., 2013. Sigma 1 receptor: a new therapeutic target for pain. Eur. J. Pharmacol. 716, 78-93.

INTRODUCTION

Introduction

1. μ-OPIOID DRUGS

1.1. FROM THE EARLY USE OF OPIUM TILL THE DISCOVERY OF OPIOID RECEPTORS

Opium is obtained from the latex of the opium poppy capsule (*Papaver somniferum*). Archaeobotanical analyses place the first crops of the opium poppy even in the earliest Neolithic period (4000 B.C.) (e.g. excavations in Granada and Banyoles –Spain-, Milan – Italy- or Rhine river – Netherlands-), as they have been found in diverse settlements of that period (Nencini et al., 1997; Tetenyi, 1997; Antolín et al., 2014). The Sumerian clay tablet (dated in approximately 2100 B.C.) is considered to be the world's oldest recorded list of medical prescriptions, and it is believed by some researchers that the opium poppy is referred to on this tablet (Norn et al., 2005). However, the first reference to the milky juice of the poppy are attributed to Theophrastus in the ancient Greece at the beginning of the third century B.C., who refers its use as a strong analgesic (Macht, 1915). The antidiarrheal properties of opium are well documented in the Roman Empire where it was used in an epidemic of dysentery which occurred during the first century A.D. (Macht, 1915; Delgado-García et al., 2016). Much later, arabic physicians extended the use of opium from the Middle East to India and China. In fact, the extensive use of opium in China was relatively late (in the middle of the 1700s). The increased used of opium in China became a major political issue giving rise to the opium wars between China and England (Macht, 1915; Benyamin et al., 2008).

The active compounds of opium were later found to be alkaloids. The German pharmacist Serturner, in 1805, was the first to isolate one of these alkaloids from opium and named it Morphine, after Morpheus, the god of dreams from the Greek mythology (reviewed in Trang et al., 2015). Despite the abundant use of opioids during centuries, it was not until the early 1970s when the existence of an endogenous opioid system was demonstrated, with specific receptors and selective ligands of these receptors (Hughes, 1975; Pasternak et al., 1975; Terenius and Wahlstrom, 1975). Specifically, opioid research rose with the demonstration of opioid receptors in 1973 (Pert et al., 1973; Simon et al., 1973; Terenius, 1973). Shortly after the discovery of the opioid receptors, the first endogenous opioid peptide, enkephalin, was described (Hughes et al., 1975).

Endogenous opioids, which are now known to include several peptides such as endorphins, enkephalins, dynorphins and endomorphins, have opium-like activity and affinity for opioid receptors (Pasternak and Pan, 2013). The canonical opioid receptors include mu (μ), kappa (κ), and delta (δ) receptors. μ and κ opioid receptors are named after the representative drug which binds them: morphine and ketocyclazocine, respectively; δ opioid receptors were originally named after vas deferens, the tissue within which it was first isolated (Pathan and Williams, 2012). In addition to these classical opioid receptors, the opioid receptor like-1 (ORL-1) was much more recently identified (reviewed by Fioravanti and Vanderah, 2008). This ORL-1 shares an overall 65% structure homology to the other members of the opioid family, being nociceptin/orphanin FQ (N/OFQ) its endogenous ligand, although with an analgesic potential much more limited (if any) than the other opioid agonists (reviewed by Fioravanti and Vanderah, 2008). The μ-opioid receptors are the main target of the opioid drugs used in therapeutics (as it will be described in the next section). For this reason, in this chapter we will focus in μ-opioid drugs and their receptor.

1.2. CLASSIFICATIONS OF THE OPIOID DRUGS

Opioid drugs can be classified according to their origin in natural, semi-synthetic or synthetic compounds. Naturally occurring opioids are those found in the capsule of the opium poppy, such as morphine or codeine; semisynthetic opioid drugs are made by chemical modifications of those found in the opium poppy, such as buprenorphine or oxycodone, and fully synthetic compounds include drugs such as fentanyl or tapentadol (Pathan and Williams, 2012). These drugs can be also classified according to their chemical structure in: morphine analogs (e.g. heroin), benzomorphans (e.g. ketocyclazocine) or oripavines (e.g. buprenorphine) among others (Pasternak and Pan, 2013).

Based on the clinical use of analgesics, the World Health Organization elaborated the three-step ‘analgesic ladder’ to classify them based on their use according to the pain intensity, and this remains the most standard classification of analgesics in the clinical

practice. Step I drugs include non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen, and are used for pain of mild intensity. Codeine or tramadol, which are considered to be weak opioids, are used when pain intensity is too high to be appropriately managed with nonopioid analgesics (i.e. mild to moderate pain), and are named step II opioids. On the other hand, if pain is too intense (i.e. moderate to severe), strong opioids with higher analgesic efficacy are needed. These drugs are also called step III opioids and include drugs such as morphine, fentanyl, oxycodone or tapentadol (Pergolizzi et al., 2008; Ramaswamy et al., 2015; Mercadante, 2017). All these opioid drugs are μ-opioid agonists (Pathan and Williams, 2012), as previously pointed out.

There is another classification of opioids based on the intrinsic activity of the compound on opioid receptors (Flórez, 2008). This is important as it influences the effects of the drugs both in the preclinical and clinical assays. According to their intrinsic activity the opioid drugs can be classified as:

- Pure agonists: opioid drugs which display a maximum intrinsic efficacy and therefore the highest efficacy inducing signaling through its receptor. The known μ agonists morphine, oxycodone, fentanyl, heroin and methadone are included in this group (Flórez, 2008).
- Partial agonists: opioid drugs that act with a lower intrinsic efficacy than a pure agonist. The most relevant example of this group is the partial μ agonist buprenorphine (Khanna and Pillarisetti, 2015).
- Mixed agonist-antagonist: opioid drugs which act as agonists of a specific subtype of opioid receptor while acting as antagonists of a different subtype. Pentazocine is a known example, which is a κ agonist but also a μ antagonist (van Niel et al., 2016).
- Agonists with additional mechanisms: some drugs show opioid agonism and also additional mechanisms which contribute to their analgesic effects. Examples are tramadol and tapentadol, which in addition to μ agonism inhibit the reuptake of neurotransmitters. Specifically, tramadol inhibits the reuptake of both serotonin and noradrenalin (Raffa et al., 2012), and tapentadol inhibits the reuptake of noradrenalin (Vadivelu et al., 2013).
- Antagonists: drugs that have affinity for opioid receptors but lack of intrinsic

efficacy and therefore of analgesic efficacy. Naloxone is the most widely used opioid antagonist. This centrally-penetrant drug has affinity for all three opioid receptor subtypes (although with some preference for μ-opioid receptors) (Zádor et al., 2017). This drug is used in both preclinical research or in the clinical practice (in cases of opioid intoxication) to reverse the effect of opioid agonists (Sánchez-Fernández et al., 2014; Wermeling, 2015). In addition, there have been developed opioid antagonists with limited accessibility to the central nervous system, to reverse exclusively the peripheral effects induced by opioid agonists. This is the case of naloxone methiodide, which has been extensively used in preclinical research to dissect peripheral opioid effects (e.g. Tejada et al., 2017), or methylnaltrexone, which is used in the clinical practice to reverse peripherally-mediated opioid side effects (Mehta et al., 2016).

1.3. μ-OPIOID RECEPTOR SIGNALING

All μ, κ and δ opioid receptors, and also the ORL-1, are G-protein-coupled receptors with the typical seven transmembrane domains, an extracellular *N* terminus and an intracellular C terminus (Al-Hashani and Bruchas, 2011; Costantino et al., 2012; Pasternak and Pan, 2013). Interestingly, there has been reported several splice variants of the μ-opioid receptor, and it has been suggested that specific μ-agonists might signal preferentially through some of these specific splice variants of the receptor (reviewed in Pasternak, 2014). The G-proteins coupled to μ-opioid receptors are heterotrimeric proteins composed by a Gα subunit which is coupled to the receptor, and the Gβγ dimer which is anchored to the plasma membrane and binds to the Gα subunit (Raehal et al, 2011). After opioid receptors bind an agonist, the Gα and Gβγ subunits dissociate from the complex and subsequently act on various intracellular effector pathways. Guanosine triphosphate (GTP) is essential in this signal transduction, as it binds to the Gα subunit, triggering the dissociation of the receptor complex. The activated Gα subunits, which belong mainly to the Gi/o family (Sánchez-Blázquez et al., 2001), inhibit the activity of adenylate cyclase with the consequent decrease in cAMP production and thereby protein kinase A functioning (Pasternak et al., 2013). In addition to the inhibition of protein kinase A, the action of agonists on μ-opioid receptors can lead to the activation

of other Gα subtypes and to the modulation of other second messenger systems, including Gq-protein kinase C-phospholipase C pathway, and this is dependent on the specific μ-agonist used (Sánchez-Blázquez et al., 2001), indicating that the mechanisms to induce analgesia are not fully overlapping between opioid drugs, and this might be related with the previously mentioned splice variants of the μ-opioid receptor.

Upon agonist stimulation of the opioid receptor, not only the Gα subunit dissociates from the complex to induce biochemical effects, but also the Gβγ dimer is released from the complex to modulate the activity of several ion channels, including voltage-dependent Ca^{++} channels, which are inhibited by the Gβγ reducing the Ca^{++} current to the cytosol and therefore decreasing neurotransmitter release (Al-Hasani and Bruchas, 2011). Moreover, the Gβγ dimer are able to open G-protein-regulated inwardly rectifying K^{+} channels (GIRKs) to hyperpolarize neurons (Mark and Herlitze, 2000; Ocaña et al., 2004). Therefore, the activation of μ-opioid receptors decreases neuronal activity (Al-Hashani and Bruchas, 2011; Raehal et al., 2011). These actions of opioid agonism are schematically summarized below (Fig. 1).

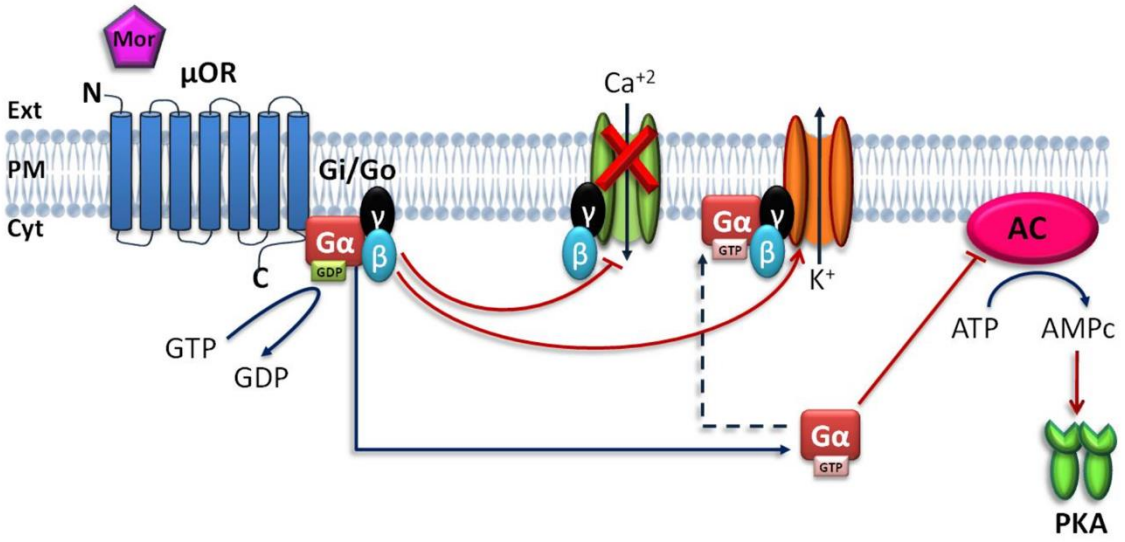

Fig. 1. Schematized mechanism of action of μ-opioid receptors. Activation of an opioid receptor by an agonist (e.g. morphine) inhibits the activity of adenylate cyclase resulting in a decrease in the production of cAMP, an increase in the efflux of K^{+} with the consequent cellular hyperpolarization, and a decrease in the influx of Ca^{++} which decreases neurotransmitter release. Taken from Sánchez-Fernández, 2014.

There are other effectors which also mediate opioid analgesia. For instance, ATP-sensitive K^+ channels are needed for the antinociceptive effect of morphine, buprenorphine or methadone. However, fentanyl or levorphanol do not need of the activation of these channels for inducing antinociception (Ocaña et al., 1995). This indicates again that not all μ-opioid agonists have identical mechanisms.

The μ-opioid receptors interact with other proteins, including other receptors, which impact in their signaling and in the intracellular events they trigger. The oligomerization includes the existence of homodimers (the association of two μ-opioid receptors) and heterodimers (between different classes of opioid receptors or even between opioid receptors and other GPCRs such as the cannabinoid receptor type 1) (Gomes et al., 2000; Ríos et al., 2006; Hojo et al., 2008). In addition to the dimerization of μ-opioid receptors with opioid and non-opioid GPCRs, they can also physically interact with ion channels, such as the ionotropic receptor *N*-methyl-*D*-aspartate (NMDA) receptor (Rodríguez-Muñoz et al., 2011a), and the transient receptor potential vanilloid 1 (TRPV1) (Scherer et al., 2017), as well as numerous cytoskeletal trafficking proteins, most of which participate in membrane protein endocytosis (Whistler et al., 2002; Milligan, 2005; Charlton et al., 2008). Moreover, μ-opioid receptors have been reported to directly interact with sigma-1 receptors (Kim et al., 2010; Rodríguez-Muñoz et al., 2015a and b). The interaction between sigma-1 receptors and the μ-opioid receptors involve a complex regulation between the NMDA receptor and the histidine triad nucleotide-binding protein 1 (HINT1) (Rodríguez-Muñoz, 2015a and b), resulting in a fine modulation of μ-opioid signalling, as will be described in the next Chapter "Sigma-1 receptors and pain" of this PhD Thesis (Section 2.5.1.4.).

1.4. μ-OPIOID ACTIONS AT THE PERIPHERAL AND CENTRAL NERVOUS SYSTEM

Opioid receptors are important modulators of neurotransmission, at both central and peripheral levels. Consequently, opioid drugs have effects both at central sites and in the peripheral nervous system. All opioid-induced effects, including their therapeutic and side events, derive from the ability of these drugs to decrease neuronal function

(Khansari et al., 2013). Although the main therapeutic use of opioid drugs is pain treatment, these drugs can induce a variety of non-analgesic effects, as they have antitussive and antidiarrheal properties, which can be used therapeutically (Khademi et al., 2016), but also several side effects including respiratory depression, constipation, sedation and confusion. Most of these effects have a mixed peripheral/central contribution, although for most of them the actions of the opioid predominates at only one of these locations (see Table 1).

Opioid-mediated analgesia has been classically attributed to the actions of these drugs in the central nervous system (Christie et al., 2000; Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008; Khalefa et al., 2012); however opioid receptors are located at different sites along the pain-processing pathway, including both the central and peripheral nervous system (Bigliardi-Qi et al., 2004; Khalefa et al., 2012), and it is currently well documented that opioid agonism is able to exert peripheral analgesic effects (Stein et al., 2003; Sehgal et al., 2011; Sánchez-Fernández et al., 2014). In fact, the use of peripherally-mediated opioid analgesia has been proposed as a way to minimize centrally-induced opioid side effects (Sehgal et al., 2011; Ringkamp and Raja, 2012). We will also focus this section exclusively in the analgesic actions of opioids at both peripheral and central levels, as well as the mechanisms for the tolerance to opioid analgesia after prolonged opioid therapy. We will briefly describe the main features of the pain pathways, at both the central and peripheral levels, to highlight the role of μ-opioid receptors on pain modulation at each level.

Table 1. Non-analgesic effects of opioid drugs, distributed by their primary location of induction (central or peripheral). The actions summarized in this table are observed for all clinically available opioid agonists. Although several of these effects are known to have both a central and a peripheral component, for clarity purposes only the predominant location of each side effect was considered for this table. Taken from Sánchez-Fernández, 2014, with modifications.

Location of action		Effects
Central Nervous System		↓ Cough reflex
		↑ Nausea and vomiting
		↑ Psychotomimetic alterations
		↑ Confusion
		↑ Sedation
		↓ Rate of respiration
		↑ Truncal rigidity
		↓ Pupil size (miosis)
		↑ Urinary retention
		↑ Pruritus
Peripheral Nervous System	Gastrointestinal system	↓ Gastric motility
		↑ Sphincter contraction
		↓ Intestinal secretion
		↓ Peristaltic waves in the colon
	Cardiovascular system	↓ Blood pressure and heart rate if cardiovascular system is stressed

1.5. GENERAL OVERVIEW OF THE PAIN PATHWAYS

In the XVII century, René Descartes published the theory of nerve reflexes in his book *Le traité de l'homme* (Fig. 2), which much later would be known as the "reflex arc". Descartes provided the first concept of sensory input-motor output which was mediated by the nervous system. His findings were undoubtedly meritorious at that time, taking into account that in depth anatomical descriptions of the structures involved could not be technically performed, and that neural activity was of course not yet understood (Brooks and Tracey, 2005). In recent times, our knowledge of the somatosensory system has been dramatically increased by in depth anatomical studies and by the analysis of the electrophysiological properties and molecular characteristics of sensory neurons, as it will be described in this section.

Fig. 2. Illustration of the theory of nerve reflexes by René Descartes. With great insight, he wrote: 'If for example fire *A* comes near the foot *B*, minute particles of this fire, which you know move at great velocity, have the power to set in motion the spot of skin on the foot which they touch, and by this means pulling on the delicate thread *c* which is attached to the spot of the skin, they open up at the same instant the pore *d* against which the delicate thread ends, just as by pulling on one end of a rope one makes to strike at the same instant a bell which hangs at the end.' Taken from Brooks and Tracey, 2005.

Ramón y Cajal described accurately the anatomical basis of many somatosensory structures of the **peripheral nervous system**. He represented some of the specialized

nerve endings typical of low threshold peripheral mechanoreceptors, as well as of bare nerve endings which nowadays we know to be typical of nociceptive fibers (as it will be described in the following paragraphs); also, he described the anatomy of the dorsal root ganglia (DRG), including the pseudounipolar sensory neurons which are located in this structure and which generate the peripheral nerve fibers and their central terminals (Willis, 2007).

Primary afferent fibers can be classified in three main types, named Aβ-, Aδ-, and C-fibers. Most **Aβ-fibers** are low-threshold mechanoreceptors (LTM) which detect pressure, stretch or hair movement, playing therefore an essential role for discriminative touch and proprioception (Priestley, 2009). Aβ-fibers have distinct specialized endings which detect specific types of non-noxious mechanical stimuli (such as light touch or joint rotation). These nerve endings include: Merkel´s disks, Meissner´s corpuscles, Ruffini´s endings and Pacini´s corpuscles (Hall, 2011a). The Aβ fibers are characterized by a large diameter, high myelination and the consequent rapid conduction velocity (Table 2) (Hall, 2011a). It is worth pointing out that in spite of the variety of the types of mechanical stimuli detectable by Aβ-fibers, they are insensitive to thermal stimuli.

Both Aδ- and C-fibers exhibit bare nerve endings able to detect noxious stimuli, and are hence termed nociceptive fibers. However, they are distinct fibers with unique properties. **Aδ-fibers** are characterized by their thinly myelinated axons, as opposed to the unmyelinated axons of the **C-fibers**. This confers faster conduction velocities for Aδ- than for C-fibers (Table 2) (Priestley, 2009). Therefore, whereas Aδ-fibers mediate acute, well-localized “first” or fast pain, C-fibers convey poorly localized, “second” or slow pain (Basbaum et al., 2009; Beissner et al., 2010).

Table 2. Main characteristics of peripheral sensory neurons (data from Hall, 2011a; Julius and Basbaum, 2001).

	Aβ-fibers	Aδ-fibers	C-fibers
Myelin	Highly myelinated	Lightly myelinated	Unmyelinated
Diameter	6-20 µm	1-5µm	0,2-0,5 µm
Somatic sensibility modality	Propioception and discriminative touch	Nociception	Nociception
Speed conduction	80-120 m/s	35-75 m/s	0,5-2 m/s

Both C- and Aδ-nociceptors can be categorized attending to the type of stimulus that activates them: mechanical (M) and heat (H) stimulus (Farquhar-Smith, 2008; Dubin and Patapoutian, 2010). However, some of them do not respond exclusively to a single type of sensory stimulation, so overlapping neuronal populations of both C- and Aδ-nociceptors are responsible for temperature sensing and the transmission of noxious stimuli. According to their sensitivity to mechanical stimuli, they are termed as mechanically sensitive and insensitive afferents (MSAs and MIAs, respectively). Both are subclassified attending to their sensitivity to heat stimuli. MSAs can also respond to both mechanical and heat stimuli (mechano-heat sensitive nociceptors, MH) or exclusively to mechanical stimulations (M) (Dubin and Patapoutian, 2010). MIAs, can be sensitive to heat (H), or may not respond to either mechanical or heat. In this latter case, they are defined as silent nociceptors (mechanically insensitive and heat insensitive afferents, MiHi) (Meyer et al., 2008; Dubin and Patapoutian, 2010), which are thought to develop sensitivity to noxious mechanical or heat stimuli after tissue injury or inflammation, when they are sensitized by inflammatory mediators (Farquhar-Smith, 2008; Dubin and Patapoutian, 2010). In addition to sensing heat and painful mechanical stimuli, these C-

and Aδ-nociceptors are also able to code for some types of non-noxious sensory information. For instance, C-fibers not only code for painful heat information but also for non-painful warm stimulus (Peier et al., 2002; Benham et al., 2003), and it is known that some specialized C-fibers, termed C-tactile afferents (CT), are able to respond to non-noxious stroking stimulus of the hairy skin (Olausson et al., 2008; McGlone and Reilly, 2010).

The type of stimulus which is coded by each subset of sensory neuron are determined by the differential expression of specific transducers. For instance, the expression of the transient receptor potential vanilloid 1 (TRPV1) give the ability to C-neurons to respond to noxious heat, as this cation channel is opened in response to temperatures in the painful range (equal or above to 45 ºC), and hence depolarizing the sensory neuron (Basbaum et al., 2009; Scherrer et al., 2009; Bardoni et al., 2014). These TRPV1-expressing C-neurons are therefore responsive to noxious heat, but interestingly, are not responsive to mechanical stimulation (Cavanaugh et al., 2009; Zhang et al., 2013). TRPV1-expressing C-neurons also express neuropeptides, such as substance P (SP) or calcitonin gene related peptide (CGRP), and are termed peptidergic C-nociceptors (Mishra and Hoon, 2010; Hsieh et al., 2012b; McCoy et al., 2012). As opposed to this subtype of sensory neurons, non-peptidergic C-neurons bind isolectin B4 (IB4) and express the Mas-related G-protein-coupled receptor D (Mrgprd) (Zylka et al. 2003, 2005; Basbaum et al. 2009; Rau et al., 2009). These non-peptidergic C-neurons do not code for noxious heat stimulus but sense noxious mechanical stimulation (Zylka et al., 2005; Cavanaugh et al. 2009; Zhang et al., 2013). Therefore, the sensory modalities which are coded by each type of sensory neuron are segregated in distinct labelled lines. However, this distinction is clear in the mouse (Zwick et al., 2002; Woodbury et al., 2004), but in the rat there is marked overlap between the $TRPV1^+$ and $IB4^+$ neurons (Woodbury et al., 2004; Chen et al., 2006). In addition, the sensory selectivity of these populations might be altered during pathological conditions, as it has been reported that TRPV1 neurons play an important role in mechanical pain hypersensitivity during inflammation (Borbély et al., 2015), although the exact inflammatory mediators responsible of this phenomena remain unknown. Therefore, although it is clear that the properties of each sensory neuron are determined by their molecular machinery, more studies are needed to

increase our knowledge on how each type of sensory stimulation is transduced.

Primary afferent fibers project to the dorsal horn of the **spinal cord**, which is organized into anatomically and electrophysiological distinct laminae, called Rexed laminae (Rexed, 1952; Todd, 2010). The low threshold, rapidly conducting Aβ afferents, which respond to light touch, project to deep laminae (III, IV, and V). Aδ nociceptors project to lamina I as well as to lamina V (deeper dorsal horn). By contrast, C nociceptors project more superficially to laminae I and II (Todd, 2010). Fig. 3 summarizes the projections of the different primary afferent fibers in the spinal cord.

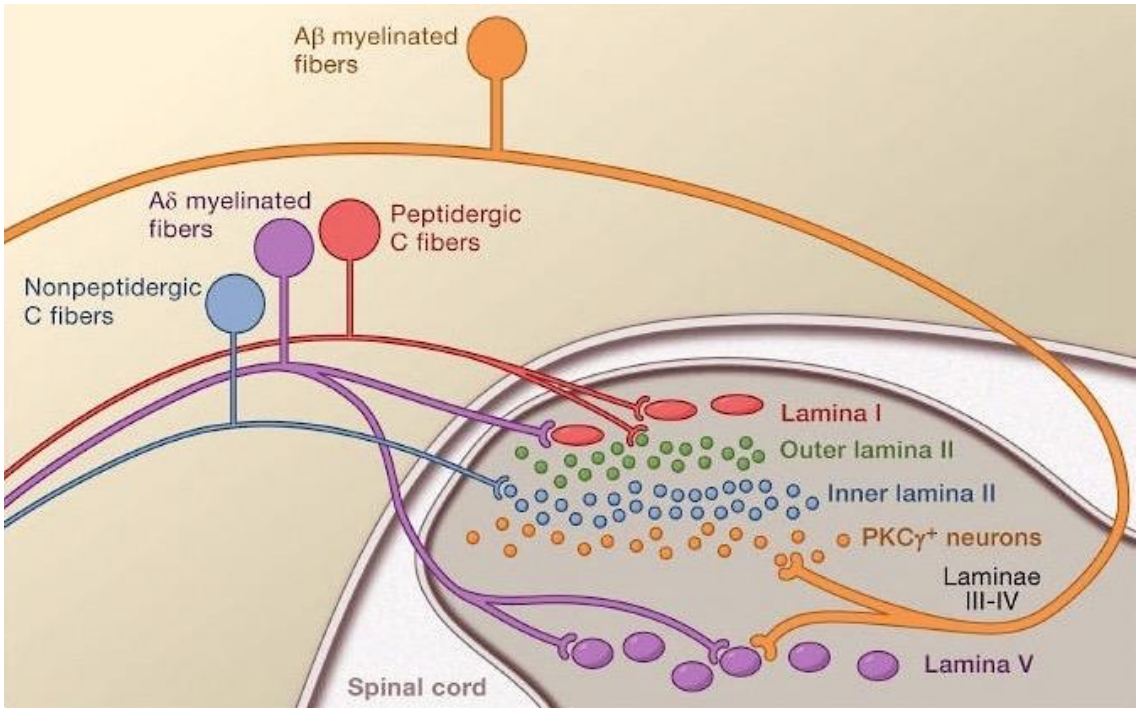

Fig. 3. Connections between primary afferent fibers and the spinal cord. There is a very precise laminar organization of the dorsal horn of the spinal cord; subsets of primary afferent fibers target spinal neurons within discrete laminae. Taken from Basbaum et al., 2009.

It should be noted the peptidergic C-fibers population target dorsal horn projection neurons in lamina I and interneurons in superficial lamina II (outer), whereas the non-peptidergic C-fibers population primarily target interneurons in inner lamina II (Fig. 3, red and blue fibers respectively) (Basbaum et al., 2009; Priestley et al., 2009). These anatomical differences are paralleled by modality-specific contributions of these

nociceptor populations to the processing of pain messages.

There are two **ascending pathways** that transmit the sensitive information received by primary afferents from spinal cord to thalamus: the dorsal columns and the anterolateral system. The dorsal columns of the spinal cord transmit information mainly from low threshold mechanoreceptor (Willis, 2007); therefore, it transmits mainly proprioceptive and fine tactile information (nonnoxious mechanical stimulation). Anatomically, the fibers in the dorsal columns are originated from collaterals of the primary afferents and decussate to the contralateral side in the medulla oblongata. On the other hand, the anterolateral system transmits the information of noxious (painful) stimuli to the brain; also, it indicates the location, intensity and type of stimulus (thermal or mechanical), and is involved in the affective-motivational component of the sensations, leading to the unpleasantness of the pain experience, and in the autonomous activation following a noxious stimulus (Martins and Tavares, 2017). Anatomically, ascending fibers in the anterolateral system come from second order neurons located primarily in the dorsal horn (postsynaptic to primary afferent fibers) and the fibers crosses in the spinal cord (Basbaum and Jessel, 2000). Ascending fibers of the anterolateral system can be classified depending on if they directly or indirectly project to the thalamus (Hall, 2011b). The tract which directly synapses with the thalamus is denominated neospinothalamic tract, and constitutes the classical contralateral spinothalamic tract (Martins and Tavares, 2017). The spinothalamic tract projecting indirectly to the thalamus is termed paleospinothalamic tract, where a higher proportion of fibers ascend bilaterally and before sending projections to the thalamus, synapse with other brain areas (Ness and Randich, 2010).

After synapsing in the thalamus, the pathway continues to the somatosensory cortex which is involved in the processing of somatic sensory information (Purves et al., 2006; Hall, 2011a). The somatosensory cortex comprises two different regions: the primary somatic sensory cortex that contributes to the discriminative analysis of painful stimuli (Martins and Tavares, 2017), and the secondary somatic sensory cortex, that plays a major role in pain-induced attention, learning and memory, as well as in the emotional aspects of pain experience (Chen et al., 2008). This is because the secondary somatic sensory cortex receives convergent projections from the primary somatic sensory cortex

and sends projections to limbic structures such as the amygdala and hippocampus (Martin and Jessel, 2000; Lorenz and Hauck, 2010), incorporating the emotional interpretation to the sensory information (García-Larrea, 2017).

The **descending pain pathways** are an endogenous analgesic system, consisting of a series of inhibitory circuits which can be activated by different stimuli (stress, pain, electrical stimulation or opioid agonism) (Reynolds, 1969; Calvino and Grilo, 2006; Sun et al., 2017).

The secondary somatosensory cortex projects to two important areas of limbic system: the amygdala and the hypothalamus which in turn send descending projections to the brainstem (Bonica et al., 1990). The main brainstem areas involved are: the periaqueductal gray matter (PAG), the locus coeruleus (LC) and the rostral ventromedial medulla (RVM). These areas are interconnected between them and project directly to the dorsal horn where they release neurotransmitters, which decrease painful ascending information at the dorsal horn (Benarroch, 2008; Ossipov et al., 2010; Llorca-Torralba et al., 2016). Specifically, the main neurotransmitters released are two: norepinephrine (NE) and serotonin (5-HT) (Commons et al., 2016; Llorca-Torralba et al., 2016) (Fig. 5).

Descending serotonergic and noradrenergic projections from the brainstem synapse with primary afferent terminals, ascending projection neurons and modulatory interneurons in the spinal cord, controlling therefore the “gate” of pain transmission (Ossipov et al., 2010). There are fibers that carries predominantly descending inhibitory signals (Ness and Randich, 2010); conversely, other fibers transmits predominantly facilitatory influences from supraspinal origin (Zhuo and Gebhart, 1992). Therefore, the balance between the inhibitory and facilitatory descending systems determines the overall level of excitability of the neurons in the dorsal horn (Kirkpatrick et al., 2015).

1.5.1. Opioid effects in the pain pathways

1.5.1.1. Peripheral opioid receptors and analgesia

Peripheral μ-opioid receptors are mainly present in cutaneous nociceptive peptidergic C-fibers which express TRPV1$^+$ (Lawson et al., 2008; Yamamoto et al., 2008); these afferents are heat sensitive and mechanically insensitive (Fig. 4). Moreover, μ-opioid receptors barely colocalizes with non-peptidergic C-fibers IB4$^+$ (Yamamoto el al., 2008). By contrast, δ-opioid receptors are expressed by non-peptidergic C-fibers IB4$^+$ and myelinated nociceptors that respond to mechanical stimulation (Fig. 4). Also, μ-opioid receptors are expressed by a relatively small population of myelinated NF200$^+$ neurons (where mostly are coexpressed with δ-opioid receptors) (Yamamoto et al., 2008; Scherrer et al., 2009; Bardoni et al., 2014; Chiu et al., 2014). All the results commented on in this section are referred to experiments in mouse, because in rat the population proportions differ a lot; since many peptidergic C-fibers which express TRPV1$^+$ are also IB4-binding neurons in rat (Guo et al., 1999; Michael and Priestley, 1999; Woodbury et al., 2004).

In the early 1990s evidence began to accumulate that peripheral antinociceptive effects of opioids were mediated by peripheral opioid receptors (Stein, 1995). Nowadays, for its advantages minimizing side effects, the activation of peripheral opioid receptors as a potential tool for the treatment of pain has aroused great scientific interest (Stein, 2003). However, it seems that opioid drugs induce much of their analgesic effects at the central level as has been traditionally asserted, in both humans or rodents (e.g. Christie et al., 2000; Thomas et al., 2008; Ringkamp and Raja, 2012). In addition, the contribution of the effect of peripheral opioid receptors to the analgesic effect of conventional systemic opioids with high central penetrability is controversial (Joshi et al., 2008; Khalefa et al., 2012).

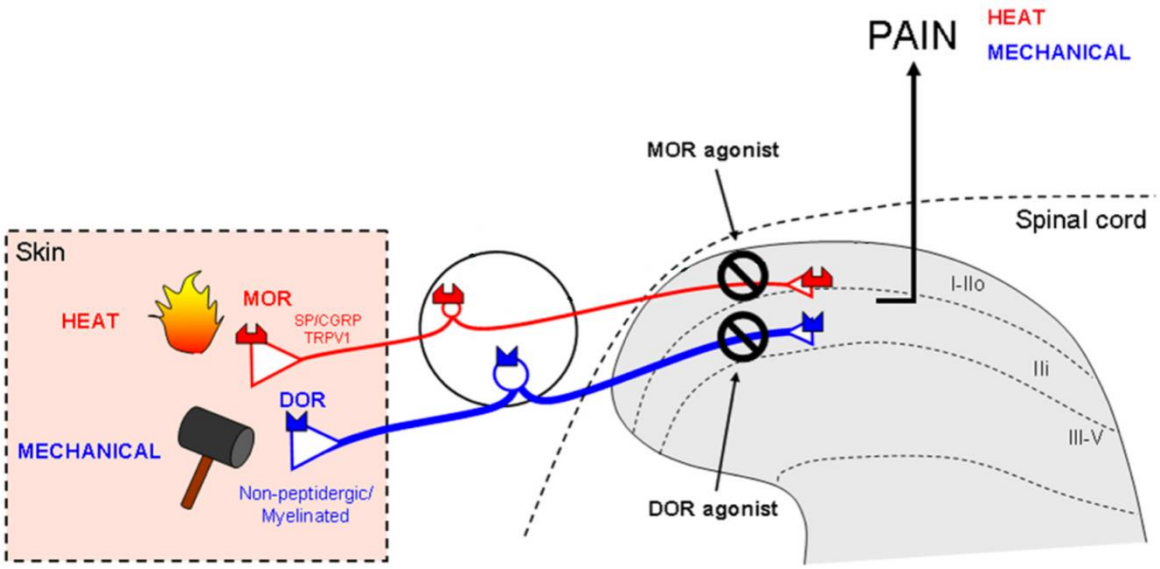

Fig. 4. Expression of μ-opioid receptors (MOR) in C-fibers. MOR are expressed mainly by heat-sensitive (TRPV1+), peptidergic (SP+ and CGRP+) unmyelinated nociceptors that innervate the skin (peripheral terminals in red; skin box). These sensory neurons project to the laminae I and outer II (I-IIo) of the spinal cord, where they activate neurons that transmit painful heat messages to the brain. Modified from Scherrer et al., 2009.

Classically, the majority of *in vivo* studies have focused on the analgesic effect of opioids preferably at the central level (e.g. Kuraishi et al., 1985; Tseng et al., 1995; Wegert et al., 1997; Sato et al., 1999). However, there are reports which show that local opioids are effective as analgesic (e.g. Kolesnikov et al., 2000; Menéndez et al., 2005; Curto-Reyes et al, 2008; Hervera et al., 2011). Moreover, i.t. administration of the μ-agonist DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) dose-dependently reduced heat pain responsiveness in mice; however, the same doses of DAMGO were without significant effect against mechanical pain (Scherrer et al., 2009). There results are explained by the segregated opioid receptor expression in nociceptors, but futher studies are needed where both types of sensory stimulation are compared more directly.

1.5.1.2. Descending pain pathways: opioid modulation.

PAG, LC and RVM are the main supraspinal structures that participate in opioid analgesia; the administration of opioids into any of these sites is enough to relieve pain

(e.g. Millan, 2002; Porreca et al., 2002; Mei and Pasternak, 2007). The PAG was the first brain region to have been explicitly demonstrated to activate an endogenous pain inhibitory system. Analgesia produced by PAG stimulation or microinjection of opioids is naloxone-reversible; therefore, it is a source of descending opioid-mediated inhibition of nociceptive inputs (Ossipov et al., 2014).

Descending projections from the brainstem synapse with primary afferent terminals and activate spinal interneurons that release endogenous opioid peptides (e.g. enkephalins) (Fig. 5). The opioids act presynaptically to decrease the release of pain transmitters from the central terminations of primary afferent neurons. They also act on postsynaptic receptors on spinothalamic tract neurons in the spinal cord to decrease the rostral transmission of the pain signal (Fig. 5). Moreover, opioid analgesics activate the descending inhibitory pathways, and they also directly activate opioid receptors in the spinal cord (reviewed by Ossipov et al., 2014).

The RVM exerts a bidirectional pain modulatory effect, both inhibiting and facilitating pain owing to the actions of the subpopulations of on- and off-cells (Fig. 6A). On-cells increase their activity immediately before a pain response occurs, and are thought to exert a facilitating role for the nociceptive transmission. However, off-cells show a tonic activity which is stopped immediately before the pain response, and are thought to be responsible for the descending inhibition of nociception (Fig. 6A). The net effect of RVM on pain modulation depends therefore on the balance between on- and off-cells activity (Fields, 1992; Porreca et al., 2002; Calvino and Grilo, 2006; Ossipov et al., 2010).

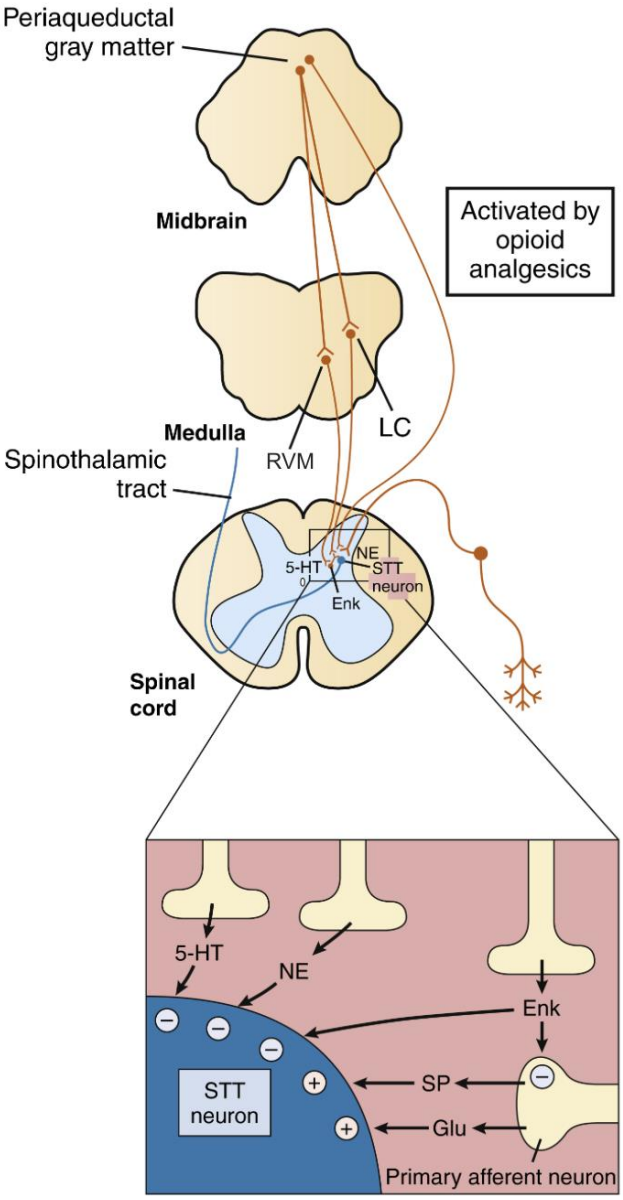

Fig. 5. Descending inhibitory pathways activated by opioids. Nerve fibers from brainstem areas (PAG, LC and RVM) release serotonin (5-HT) and norepinephrine (NE) in the spinal cord, where they inhibit dorsal spinal cord neurons that transmit pain impulses to supraspinal sites (i.e. inhibit the conduction of the painful information). Nerve fibers from brainstem areas also activate spinal interneurons that release endogenous opioid peptides (e.g. enkephalins, Enk). Opioids act to decrease the release of pain transmitters from the central terminations of primary afferent neurons. They also act on spinothalamic tract neurons (STT neurons) to decrease the rostral transmission of the pain signal. Modified from: https://basicmedicalkey.com/opioid-analgesics-and-antagonists-2/

In the presence of opioids (when morphine is administered systemically or into the PAG), the on-cells become silent and the off-cells fire continuously. Thus, opioids are able to decrease the activation of on-cells (and not that of off-cells), resulting in the disinhibition

of off-cells and therefore eliciting analgesia (reviewed by Fields, 2004; Ossipov et al., 2014) (Fig. 6B left). If naloxone is given following systemic administration of an analgesic dose of morphine, off-cell firing is shut down, on-cell firing increases and becomes continuous, and withdrawal reflexes are enhanced (Fig. 6B right) (Bederson et al., 1990; Kaplan and Fields, 1991).

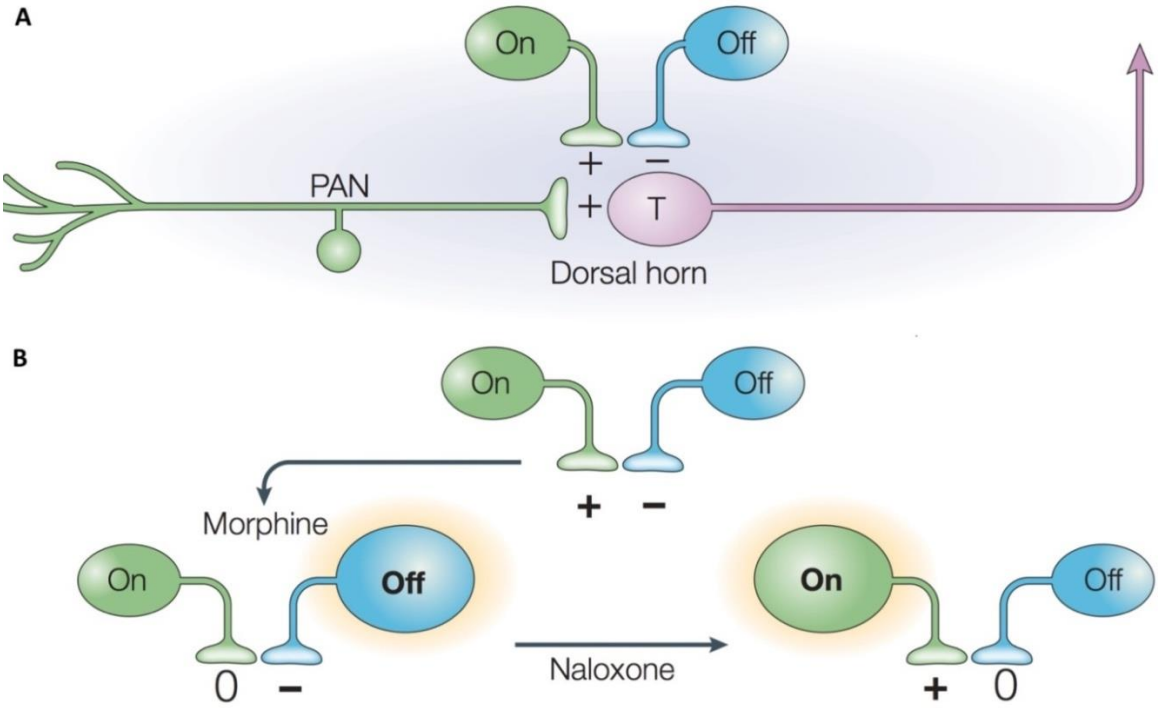

Fig. 6. Bidirectional control of pain transmission. (A) During the application of a nociceptive stimulus on-cells facilitate and off-cells inhibit nociceptive transmission at the level of the spinal cord dorsal horn. (B) Morphine facilitates off-cells firing to inhibit pain (lower left), whereas when naloxone is used to precipitate acute abstinence, on-cells are activated and produce a hyperalgesic state (lower right). PAN, primary afferent nociceptor; T, pain transmission neuron. Modified from Fields, 2004.

1.6. ANALGESIC TOLERANCE TO μ-OPIOID DRUGS

Tolerance is defined as a decrease in the pharmacological response following repeated or prolonged drug administration (Dumas and Pollack, 2008). This phenomenon occurs with opioid agonists and leads to the increase in the doses required to maintain the same level of analgesia (Raehal et al., 2011). The rate at which tolerance develops may

vary between individuals, and does not develop at the same speed and extent to all opioid effects (Pasternak and Pan, 2013). For instance, tolerance to the respiratory depression and constipation in response to opioid treatment is developed much more slowly than to analgesia (reviewed in Pasternak and Pan, 2013; and Hayhurst and Durieux, 2016). As a consequence, the previously mentioned increase in the dose of opioid agonist to maintain acceptable analgesia, leads to a marked increase in opioid-induced side effects, with the consequent decrease of the therapeutic index during chronic administration with opioids (reviewed in Morgan and Christie, 2011; Pasternak and Pan, 2013; Hayhurst and Durieux, 2016). Therefore, analgesic tolerance is a substantial drawback for the use of opioid analgesics. When opioid side effects due to opioid dose escalation are poorly tolerated, the so called "opioid rotation" is often used. Opioid rotation consists in the switch to a different μ-opioid, and the patient often regains some analgesic sensitivity because of incomplete cross-tolerance among the analgesic effects of μ-opioids (Stein, 2013; Williams et al., 2013). This might be influenced by the diversity of μ opioid receptors and the differences in the signalling and effectors between the different μ opioid drugs (see Section 1.3.), and also by intrinsic differences in the mechanisms for the development of analgesic tolerance by different μ-opioid agonists which will be commented in the next paragraphs.

Inherent inter-individual pharmacokinetic variability can influence analgesia within a population, but appears to play a minimal role in the development of opioid tolerance (Dumas and Pollack, 2008), as it seems that opioid analgesic tolerance is fundamentally a pharmacodynamic phenomenon (Al-Hasani and Bruchas, 2011). There are many biochemical events that contribute to tolerance, such as desensitization and internalization of the receptor, but also changes in second messenger systems. Some of these processes are summarized in Fig. 7, and detailed below.

Chronic opioid exposure leads to the desensitization of the receptor, with the consequent progressive reduction of signal transduction. The speed of receptor desensitization depends on the specific agonist used (Allouche et al., 2014). Receptor desensitization is triggered by the phosphorylation of the C-terminus of the μ-opioid receptor by G-protein receptor kinases (GRKs) in response to receptor activation by an agonist. This phosphorylation leads to the uncoupling of the receptor from the G

proteins, which attenuates the second messenger signal cascade, reducing agonist efficacy (Allouche et al., 2014). β-arrestins, and in particular β-arrestin 2, exhibit an increased affinity for the phosphorylated C-terminus of the receptor and facilitates the internalization of the receptor (Groer at al., 2011; Allouche et al., 2014). Once internalized, the receptor can follow different routes: recycling back to the cell surface as a resensitized (dephosphorylated) receptor, sequestration into endosomes, or degradation by lysosomes (if agonist exposure is persistent) (Dumas and Pollack, 2008; Allouche et al., 2014). The degradation leads to the downregulation of the number of opioid receptors available in the plasma membrane, and consequently decreases the effects of the opioid agonists (reviewed by Garzón et al., 2008; Pasternak and Pan, 2013). There are marked differences in the degree of internalization between agonists. For instance, morphine-receptor binding promotes minimal receptor internalization (Haberstock-Debic et al., 2005; Nowoczyn et al., 2013; Williams et al., 2013), whereas DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) or fentanyl induce a marked internalization (Whistler and Von Zastrow, 1998; Schulz et al., 2004; Garzón et al., 2008; Morgan and Christie, 2011).

Moreover, changes during tolerance not only involve phosphorylation and internalization of receptors, but also parallel changes in second messenger systems. Whereas activation of the μ-opioid receptor by an agonist such as morphine decreases adenylate cyclase activity, and hence cAMP production, chronic morphine induces the superactivation of adenylate cyclase, with the consequent increase in cAMP production. This triggers cAMP-responsive element binding protein (CREB) phosphorylation and overexpression, which in turn promotes gene expression changes (Dumas and Pollack, 2008). There are other processes that occur in conjunction with those described above, that are also opposed to the normal signaling of the receptor upon stimulation with an agonist, such as alterations in ion channel conductance, and an increase in neurotransmitter release (Dumas and Pollack, 2008). It is well described that there is an enhancement in glutamatergic neurotransmission, through the NMDA-nitric oxide cascade, during tolerance development, and that NMDA receptor blockade is able to rescue from opioid analgesic tolerance, indicating that during tolerance NMDA

receptors restrain opioid analgesia (reviewed by Waldhoer et al., 2004; Garzón et al., 2008; Pasternak and Pan, 2013).

Recent research has shown that phosphorylated arrestin-bound opioid receptor complex is not simply inactive, but that it can recruit alternate signal transduction cascades, including mitogen-activated protein kinases (MAPKs) (Lefkowitz and Shenoy, 2005). These MAPKs include the extracellular-related kinases 1/2 (ERK1/2), c-Jun *N*-terminal kinase 2 (JNK2), and p38 (reviewed by Al-Hasani and Bruchas, 2011). Interestingly, inhibition of MAPKs decreases morphine tolerance but not the tolerance induced by other opioid agonists such as fentanyl or oxycodone (Melief et al., 2011), indicating again that the mechanisms for the development of analgesic tolerance are not fully overlapping for all μ-agonists.

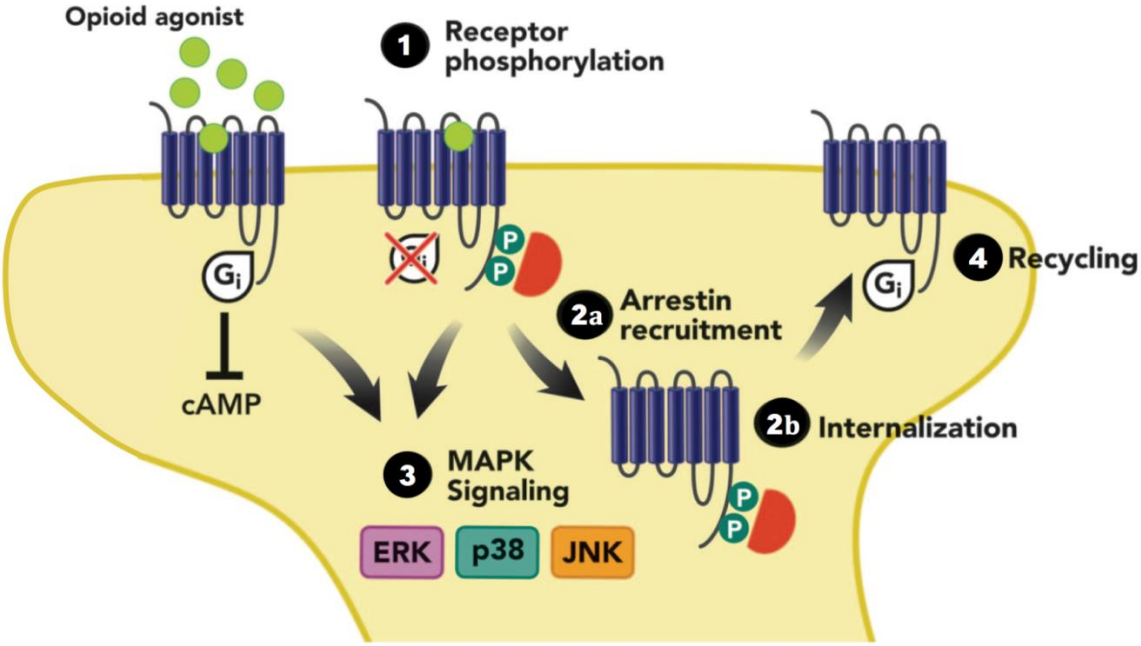

Fig. 7. Mechanisms of analgesic tolerance induced by chronic opioid administration. These mechanisms include receptor phosphorylation and uncoupling from G proteins, arrestin recruitment, internalization and recycling or degradation of the receptor, and the signaling through MAPKs. βγ = G protein β-γ subunit; cAMP = cyclic adenosine monophosphate; ERK = extracellular signal-regulated kinase; JNK = c-jun *N*-terminal kinase; MAPKs = mitogen-activated protein kinases; P = phosphorylation. Modified from Al-Hasani and Bruchas, 2011.

Although opioid analgesic tolerance it is much more deeply studied at central levels, and the mechanisms described above correspond to studies performed at central sites, it is known that tolerance to the analgesic effect of opioid drugs can also be produced at peripheral levels (Kolesnikov and Pasternak, 1999; Inoue and Ueda 2000; Meuser et al., 2003; Williams et al., 2013). Interestingly, and similar to the analgesic tolerance at central levels, peripheral morphine tolerance was effectively blocked by NMDA antagonism, suggesting that the central and peripheral mechanisms of opioid analgesic tolerance might share some characteristics (Kolesnikov and Pasternak, 1999). In addition, it is known that peripheral opioid tolerance is also contributed by the release of excitatory neurotransmitters including glutamate, CGRP, and Substance P from nociceptive primary afferent fibers within the spinal cord (reviewed by Waldhoer et al., 2004; King et al., 2005). All these findings indicate that analgesic tolerance has also a prominent peripheral component.

2. SIGMA-1 RECEPTORS AND PAIN

2.1. HISTORICAL OVERVIEW OF SIGMA-1 RECEPTOR

Sigma receptors were initially described as a subtype of opioid receptors to account for the psychotomimetic actions of (±)-SKF-10,047 (*N*-allylnormetazocine) and other racemic benzomorphans (Martin et al., 1976). The confusion on the classification of sigma-1 receptors as an opioid receptor subtype was due to the mixed pharmacological profile of the racemic mixture of (±)-SKF-10,047, as the (-)-isomer was later known to be a κ agonist, whereas the (+)-isomer was devoid of opioid effects but preserved a high affinity for a binding site which retained the name of sigma receptors (reviewed by Cobos et al., 2008).

Sigma receptors were then pharmacologically characterized in two subtypes, termed sigma-1 and sigma-2 (reviewed by Matsumoto et al., 2003; Cobos et al., 2008). Sigma-1 receptor was first cloned from guinea pig liver in 1996 (Hanner et al., 1996) and later on several other species, including humans (reviewed in Cobos et al., 2008). On the other hand, sigma-2 receptors have only very recently been identified as TMEM97 (Alon et al., 2017). With the cloning of the sigma-1 receptor, it was found to be a unique protein with more than 90% amino acid identity across various species (including several rodents and humans) (Romero et al., 2016). The gene that encodes for the human sigma-1 receptor is located on chromosome 9 band p13 and contains four exons and three introns (Chu et al., 2016). Interestingly, the sequence of sigma-1 protein does not show homology with opioid receptors or any other known mammalian protein; it is therefore currently considered a unique entity (Romero et al., 2016). The cloning of the sigma-1 receptor lead to the development of sigma-1 knockout mice (Langa et al., 2003) which are undoubtedly powerful tools to improve our knowledge of the biochemical and functional characteristics of the sigma-1 receptor.

Sigma-1 receptors play an important role in multiple therapeutic fields such as drug addiction, depression, anxiety, epilepsy, amnesia, schizophrenia and pain (Cobos et al., 2008; Davis, 2015). In this chapter of the Introduction section, we will focus on the role

of sigma-1 receptors on pain modulation, but we will firstly briefly describe the pharmacology, structure and subcellular and anatomical distribution of these receptors.

2.2. SIGMA-1 RECEPTOR PHARMACOLOGY

The sigma-1 receptor is able to bind numerous endogenous and exogenous chemical substances. Neurosteroids such as pregnenolone, dehydroepiandrosterone (DHEA), their sulphate esters, progesterone and allopregnenolone, and the natural hallucinogen *N*,*N*-dimethyltryptamine (DMT) are currently considered the most probable putative endogenous sigma-1 ligands (Cobos et al., 2008; Fontanilla et al., 2009; Maurice et al., 2009). Neurosteroids can be sigma-1 agonists (e.g. estradiol, pregnenolone sulfate or DHEA sulfate) or antagonists (e.g. allopregnenolone or progesterone), and DMT is considered an agonist (Davis, 2015). Whether neurosteroids are true endogenous ligands of the sigma-1 receptor remains controversial, because the affinity of most of them for sigma-1 receptors does not appear to be high enough for an endogenous ligand (Schwarz et al., 1989; Cobos et al., 2008; Zamanillo et al., 2013). The same can be applied to DMT. More recent studies suggest the possibility that sphingosine and monoglycosylated ceramide might possess a high affinity for the sigma-1 receptor (Hayashi and Fujimoto, 2010; Ruoho et al., 2012), pointing to that these lipids might be the endogenous sigma-1 ligands.

Unlike sigma-2 receptors, sigma-1 receptor displays stereospecificity towards dextrorotatory isomers of benzomorphans (such as SKF-10,047 or pentazocine) (Hellewell and Bowen, 1990; Quirion et al., 1992). An interesting aspect of sigma-1 receptor pharmacology is that these receptors can bind, with high to moderate affinity, a wide spectrum of known compounds of very different structural classes and with different therapeutic and pharmacological applications, such as neuroleptics (e.g. haloperidol), antidepressants (e.g. fluvoxamine), antitussives (carbetapentane, dimemorfan), drugs for the treatment of neurodegenerative disorders such as Parkinson's disease (amantadine) or Alzheimer's disease (memantine, donepezil), and

drugs of abuse (cocaine, methamphetamine) (see Cobos et al., 2008 and Su et al., 2010 for references).

Some selective and high-affinity sigma-1 drugs have been developed and are considered prototypical sigma-1 ligands. Among these ligands, are worth noting the sigma-1 agonists PRE-084 and (+)-pentazocine, and the sigma-1 antagonists BD-1047, BD-1063 and NE-100 (Cobos et al., 2008). The number of sigma ligands is increasing rapidly with the development of new compounds (Díaz et al., 2015; Franchini et al., 2016; Sun et al., 2016; Zampieri et al., 2016; Prezzavento et al., 2017; Amata et al., 2018). The sigma-1 antagonist S1RA deserves special mention, as it has demonstrated an exquisite selectivity for sigma-1 receptors, lacking affinity for 170 additional targets (Romero et al., 2012).

2.3. STRUCTURE OF SIGMA-1 RECEPTOR

Although the sigma-1 receptor was first cloned 20 years ago, its structure is still controversial. It is now known that in contrast to the seven transmembrane domains of opioid receptors, which are heavy proteins of 75-80 kDa, the sigma-1 receptor is a 29-kDa single polypeptide composed by only 223 amino acids. Most models of the sigma-1 receptor show only two transmembrane domains (TM1 and TM2, Fig. 8 (reviewed in Cobos et al., 2008; Su et al., 2010). Significant additional structural features of TM2 include a canonical membrane-imbedded GxxxG dimerization sequence, at positions 87–91, which is thought to be involved in oligomerization of the sigma-1 receptor (Chu et al., 2016; Merlos et al., 2017).

Some studies have reported the existence of two additional hydrophobic segments (one of them partially overlapping the second transmembrane domain) corresponding to steroid binding domain-like sites (SBDLI and SBDLII shown in red, Fig. 8) and purportedly responsible for ligand binding (Brune et al., 2013). It has been shown that the *N*-terminus sequence before entrance into the membrane bilayer involves approximately 9 amino acids, including an endoplasmic reticulum (ER) retention sequence (Chu et al., 2016). Finally, the sigma-1 receptor has been shown to bind cholesterol through the

cholesterol recognition motifs in the *C*-terminus (CRM I and II shown in green, Fig. 8) (Palmer et al, 2007), which might aid to the location of this receptor in the lipid rafts, as it will be described in the section below. Both the *N* and *C* termini of this protein are located on the same side of the membrane. However, in mammalian endoplasmic reticulum (ER) membranes the *N* and *C* termini are both located in the ER lumen (Hayashi and Su, 2007), while in the plasma membrane are located outside the cell.

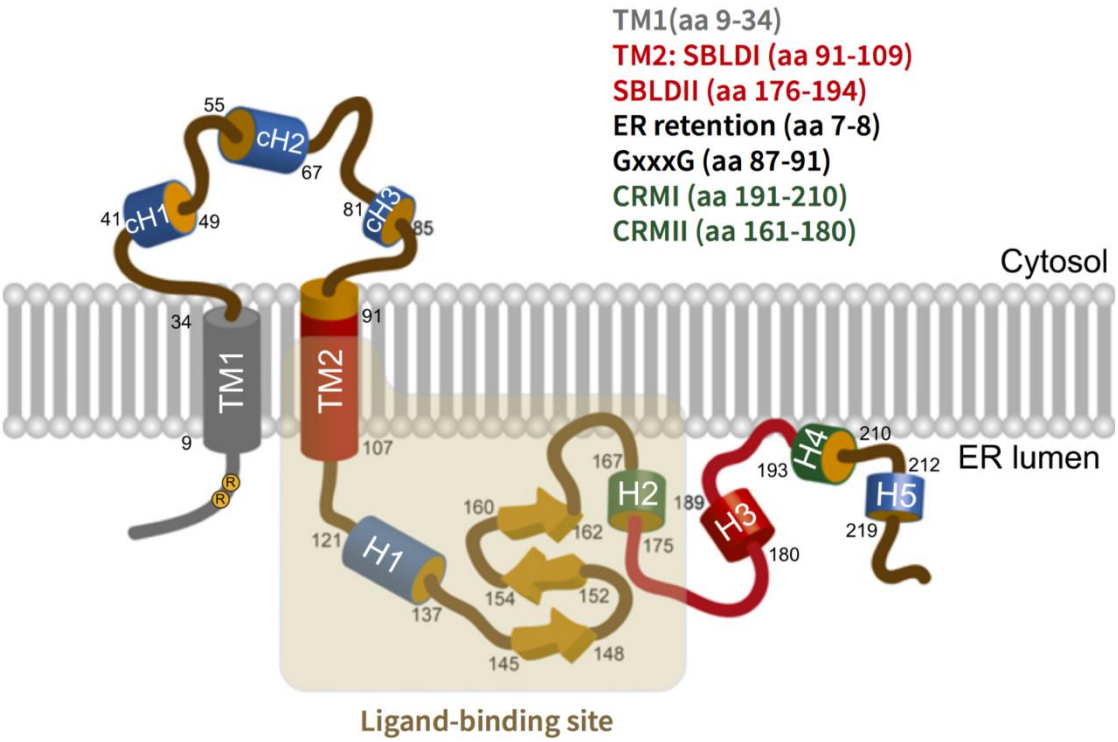

Fig. 8. Two transmembrane domains model of the sigma-1 receptor. TM1 and TM2: Transmembrane domain 1 and 2; SBDL I and II: Steroid binding domain-like 1 and 2; ER: endoplasmic reticulum; GxxxG: canonical membrane-imbedded dimerization sequence; CRM I and II: cholesterol recognition motif I and II; H: helical region; ER: endoplasmic reticulum Modified from Chu et al., 2016.

The precise structure of the human sigma-1 receptor is still an active area of research. This human receptor has been crystallized recently, showing that it has a trimeric architecture with a single transmembrane domain in each protomer (Schmidt et al., 2016), contrary to the prevailing models of the two transmembrane domains which are described above. As the two transmembrane domains model and this new one with a

trimeric architecture are difficult to reconcile, it could be concluded that the molecular architecture of the sigma-1 receptor remains still enigmatic and further studies are needed to be fully elucidated.

Moreover, 10 years ago the existence of a chaperone domain within the sigma-1 receptor was shown (Hayashi and Su, 2007). The chaperone domain is located at the *C*-terminus (residues 112–223) and contains a predicted membrane associated region with the two cholesterol recognition motifs already referenced (CRMI and CRMII) (Chu et al., 2016; Crottes et al, 2013; Su et al., 2010). This chaperone domain confers to this receptor the ability to bind to and modify the function of a variety of target proteins, which underlies the neuromodulatory actions of sigma-1 receptors. It has recently been identified a truncated splice variant of the sigma-1 receptor (short form sigma-1) (Shioda et al., 2012). Furthermore, there is evidence of sigma-1 receptor polymorphisms that might have functional consequences in diseases such as schizophrenia, Alzheimer's disease, alcoholism or myotrophic lateral sclerosis (Miyatake et al., 2004; Takizawa et al., 2009; Huang et al., 2011; Ruscher and Wieloch, 2015).

2.4. SUBCELLULAR AND ANATOMICAL DISTRIBUTION OF SIGMA-1 RECEPTORS

At the subcellular level, the sigma-1 receptor is located in cell membranes, including microsomal, mitochondrial, nuclear and plasma membranes (Alonso et al., 2000; Hayashi and Su, 2004). These proteins are immersed in lipid rafts and especially in lipid rafts of the endoplasmic reticulum (ER) where they interact with mitochondria at the mitochondria-associated ER membrane domain (MAM) (Palmer et al., 2007; Ruscher et al., 2015). The association between sigma-1 receptors located at MAM and inositol 1,4,5- trisphosphate (IP_3) receptors leads to enhancement of Ca^{2+} signalling from the ER to the mitochondria (Fig. 9) (Sánchez-Fernández et al., 2017). In stress conditions, the sigma-1 receptor is translocated to other areas of the cell, such as the extended ER reticular network or plasma membrane (Su et al., 2010).

Anatomically, sigma-1 receptors are extensively distributed in different areas of the central nervous system, where they have been thoroughly studied. They are widely distributed in the brain, including important areas for pain control such as the periaqueductal gray matter (PAG), locus coeruleus (LC) and rostroventral medulla (RVM) (Roh et al., 2011; Sánchez-Fernández et al., 2013; Zamanillo et al., 2013). Sigma-1 receptors are also present in the spinal cord, mainly in the superficial layers of the dorsal horn (Alonso et al., 2000), and in motoneurons (Mavlyutov et al., 2010). In these locations the sigma-1 receptor is found in several types of cells such as neurons, astrocytes, oligodendrocytes and microglia (Ruscher et al., 2015). In the peripheral nervous system, it was recently found that sigma-1 receptors are also present in neuronal bodies of the DRG (Bangaru et al., 2013; Mavlyutov et al., 2016), and at a much higher density than in pain-related central nervous system areas (Sánchez-Fernández et al., 2014). The presence of these receptors in the peripheral nervous system is not exclusively neuronal, as they have also been shown in Schwann cells (Palacios et al., 2004).

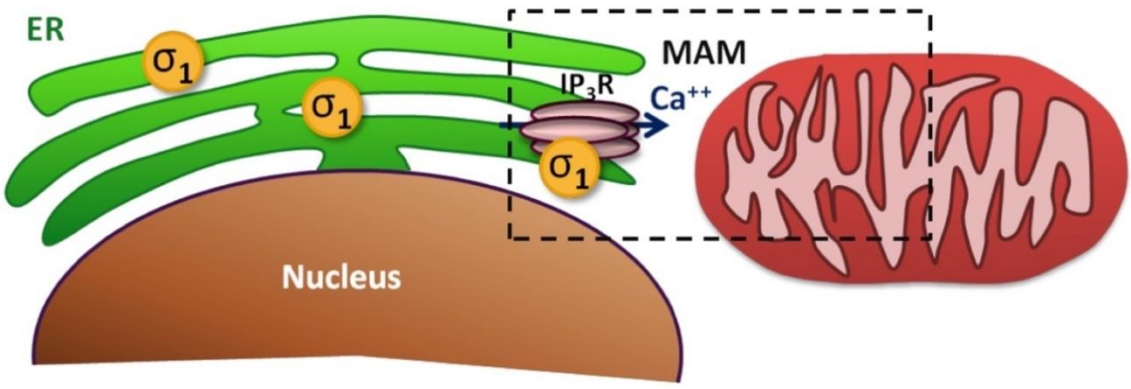

Fig. 9. Subcellular location of sigma-1 receptors. Sigma-1 receptors are located at the mitochondrion-associated ER membrane (MAM) and they lead to the potentiation of Ca^{++} influx from the endoplasmic reticulum (ER) to mitochondria. Taken from Sánchez-Fernández, 2014.

Sigma-1 receptors have also been found in nonnervous organs, such as the gastrointestinal tract, liver, kidney, spleen, heart and bladder where they might be of major physiological importance (e.g. Bowen, 2000; Stone et al., 2006; Bhuiyan and Fukunaga, 2011), although their precise functional role remains largely unknown.

2.5. SIGMA-1 RECEPTORS AND PAIN

There is accumulated evidence strongly supporting that sigma-1 receptors play a pivotal role on pain neurotransmission. The involvement of sigma-1 receptors has been studied under two different perspectives: the modulation of the effects induced by opioid drugs, and pain modulation in the absence of opioid drugs. Accordingly, the sigma-1 antagonist S1RA has been developed with an intended indication for pain treatment, specifically as an adjuvant therapy for opioid analgesia and as a stand-alone analgesic treatment for certain painful pathologies, specifically for neuropathic pain treatment (reviewed in Sánchez-Fernández et al., 2017). S1RA completed Phase I studies with excellent kinetics and tolerability results (Abadías et al., 2013), and has been evaluated recently in several Phase II clinical trials for neuropathic pain treatment with positive results, specifically for chemotherapy-induced neuropathic pain (Bruna et al., 2018). The most important preclinical findings on the role of sigma-1 receptors in pain are summarized below.

2.5.1. Sigma-1 receptor modulation of the effects induced by opioid drugs

The neurochemical mechanisms triggered by opioids on mechanical and thermal antinociception are different (Kuraishi et al., 1985, Tseng et al., 1995; Wegert et al., 1997; Sato et al., 1999), justifying the study of the modulation by sigma-1 receptors of both heat and mechanical antinociception induced by opioid drugs, which might not be equal. Most studies focused on the modulation of opioid antinociception to heat stimulus, but more recently it has been reported that sigma-1 receptors also modulate mechanical antinociception induced by opioid drugs. Furthermore, the possible modulation of non-analgesic opioid effects by sigma-1 receptors has also been explored. These experiments are detailed in this section.

2.5.1.1. Modulation by sigma-1 receptors of opioid-induced thermal antinociception.

In the early 1990s, Pasternak and colleagues were the first to report the modulation of opioid antinociception by sigma-1 receptor acting-drugs. They studied the effects on morphine antinociception of haloperidol, a compound that at the time was considered a prototypical sigma antagonist because no other more selective drug was available. They demonstrated that systemic administration of haloperidol (a non-selective sigma-1/dopaminergic antagonist), while lacking analgesic activity alone, increased morphine antinociception to acute noxious heat stimulation (tail-flick test) (Chien and Pasternak, 1993, 1994 and 1995). More, recent experiments systemically administering other more selective sigma-1 antagonists (including S1RA) replicated the results on the potentiation of morphine-induced thermal antinociception by haloperidol (Piergentili et al., 2010; Vidal-Torres et al., 2013). Furthermore, pharmacological sigma-1 antagonism with S1RA also improve the antinociceptive effect to acute thermal stimuli induced by the administration of other clinically relevant μ-opioids including fentanyl, oxycodone, codeine, buprenorphine and tramadol (Vidal-Torres et al., 2013). On the opposite side, the sigma-1 agonist (+)-pentazocine was able to decrease morphine antinociception to heat stimulus (Chien and Pasternak, 1994).

Sigma-1 KO mice did not show any deficit in response to acute thermal stimuli (Vidal-Torres et al., 2013), which is consistent with the lack of effect of sigma-1 receptor blockade on thermal nociception in wild-type mice in the absence of opioids (De la Puente et al., 2009; Vidal-Torres et al., 2013; Tejada et al., 2014). However, in contrast to the improvement of opioid-induced thermal antinociception produced by sigma-1 pharmacological inhibition in WT mice, sigma-1 KO mice do not exhibit any enhancement in the effect induced by systemic administration of morphine, sufentanil, fentanyl or buprenorphine (Vidal-Torres et al., 2013). Although these results are apparently contradictory, they could be explained by the development of compensatory mechanisms in mutant mice (Vidal-Torres et al., 2013). Previous studies of pain mechanisms that have used pharmacological and genetic inhibition of other receptors have also reported conflicting outcomes (eg. Petrus et al., 2007; Bonin et al., 2011), which have been attributed to the development of compensatory mechanisms in pain

pathways in mutant animals. Development of these purported compensatory mechanisms has also been appreciated in other fields of study (e.g., Guscott et al. 2005; Voss et al., 2010). It is worth pointing out that sigma-1 antagonism by S1RA did not enhance opioid antinociception in sigma-1 KO mice, indicating the selectivity of the effects induced by S1RA in wild-type mice, and that sigma-1 KO mice can be used to test for the selectivity of sigma-1 drugs (Vidal-Torres et al., 2013).

A number of studies have been designed to determine the anatomical location of the modulation by sigma-1 receptors of μ-opioid-induced heat antinociception, mainly at supraspinal levels. It was shown that the antinociceptive effect of supraspinal morphine was enhanced by the intracerebroventricular (i.c.v.) administration of specific antisense oligodeoxynucleotides to inhibit the expression of sigma-1 receptors (Pan et al., 1998; Mei and Pasternak, 2002). In this context, it was also shown that the antinociceptive effect induced by the i.c.v. administration of the μ-opioid agonist DAMGO was enhanced by the systemic antagonism of sigma-1 receptors in acute thermal nociception (Marrazzo et al., 2006). On the other hand, the i.c.v. administration of the sigma-1 agonist (+)-pentazocine decreased morphine antinociception (Mei and Pasternak, 2002), indicating that supraspinal sigma-1 activation or inhibition modulate morphine antinociception in opposed directions. Other studies were aimed to determine the specific central sites at which this interaction occurs. They were focused on three key areas of the descending pain modulatory pathways: PAG, LC and RVM. It was found that although all these three areas were able to be modulated by sigma-1 drugs, the RVM was probably the most prominent site for the sigma-1 modulation of morphine analgesia (Mei and Pasternak, 2007).

Although it is clear that central sigma-1 receptors play a prominent role on the modulation of μ-opioid antinociception to heat stimulus, sigma-1 receptors are found at a much higher density in the peripheral nervous system (DRG) than in central pain-related areas (Sánchez-Fernández et al., 2014), but up to now, there are no studies exploring the possible modulation of opioid-induced heat antinociception by peripheral sigma-1 receptors, which has been one of the objectives of this PhD Thesis.

The modulation of μ-opioid antinociception by sigma-1 receptors is not limited to this opioid receptor subtype, as it has been shown that sigma-1 antagonism also enhances the effects induced by κ- and δ-opioid agonists, including the κ-opioid agonists U50,488H, naloxone benzoylhydrazone (NalBzoH), and (-)-pentazocine, as well as the δ-agonist DPDPE ([D-Pen^2, D-Pen^5]enkephalin) (Chien and Pasternak 1995b; Pan et al., 1998; Mei and Pasternak, 2002;Marrazzo et al., 2006).

2.5.1.2. Modulation by sigma-1 receptors of opioid-induced mechanical antinociception

The role of sigma-1 receptor in mechanical opioid antinociception has been much less studied than in heat opioid antinociception. Systemic administration of sigma-1 antagonists (including S1RA, BD-1063, BD-1047 and NE-100, and derivatives of MR200) enhanced the antinociception induced by μ-agonists (Sánchez-Fernández et al., 2013 and 2014; Amata et al., 2018). The μ-agonists whose antinociceptive effects to mechanical stimulus are known to be enhanced by sigma-1 antagonism include morphine, fentanyl, oxycodone, buprenorphine and tramadol. (Sánchez-Fernández et al., 2013 and 2014; Amata et al., 2018). Furthermore, it was recently reported that both enantiomers of phenazocine showed affinity for μ-opioid receptors and sigma-1 receptors, and induced higher antinociceptive effects due to a simultaneous μ-opioid agonism and sigma-1 antagonism (Prezzavento et al., 2017). These results suggest that this type of dual compounds might offer high analgesic effects.

It was reported that morphine, fentanyl, oxycodone, buprenorphine and tramadol produced greater antinociception in sigma-1-KO mice than in WT mice (Sánchez-Fernández et al., 2013 and 2014). These data do not agree with those observed in thermal antinociception, where the effect of opioids in sigma-1 KO mice were not modulated by this receptor, due to possible compensatory mechanisms already commented (Vidal-Torres et al., 2013), and suggest that the role of sigma-1 receptors on opioid-induced mechanical and heat antinociception might differ.

Interestingly, sigma-1 inhibition was sufficient to unmask strong antinociceptive effects by loperamide (Sánchez-Fernández et al., 2014), a peripherally restricted μ-opioid agonist used in the clinical practice as an antidiarrheal drug (Nee et al., 2015; Lääveri et al., 2016). In agreement with these findings, local (intraplantar) treatment of wild-type mice with the selective sigma-1 antagonists BD-1063 or S1RA potentiated mechanical antinociception induced by the above mentioned μ-opioid agonists (including loperamide) (Sánchez-Fernández et al., 2013 and 2014). The potentiation of μ-opioid antinociception by inhibition of sigma-1 receptor in sigma-1-KO mice or after pharmacological antagonism was sensitive to the peripheral opioid antagonist naloxone methiodide (Sánchez-Fernández et al., 2013 and 2014; Prezzavento et al., 2017). Finally, the potentiation of μ-opioid antinociception induced by sigma-1 antagonists, either systemically or i.pl. administered, was reversed by PRE-084 treatment, supporting the involvement of sigma-1 receptors in these effects (Sánchez-Fernández et al., 2013 and 2014; Prezzavento et al., 2017). Therefore, these results indicated the important role of peripheral opioid receptors in the enhanced mechanical antinociception, as well as the involvement of peripheral sigma-1 receptors.

2.5.1.3. Modulation by sigma-1 receptors of non-analgesic effects of opioids

Opioid-induced non-analgesic effects can be produced either centrally or peripherally (or with the participation of both central and peripheral sites), and together to the development of analgesic tolerance are important drawbacks to opioid use in the clinical practice. Interestingly, some opioids induced non-analgesic effects are different in mice and humans: although opioids produce myosis and sedation in humans (Al-Hasani and Bruchas, 2011), they induce mydriasis (Vidal-Torres et al., 2013) and hyperlocomotion in mice (Sánchez-Fernandez et al., 2013).

Sigma-1 receptor inhibition did not increase opioid-induced hyperlocomotion, mydriasis or physical dependence (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013), and morphine-induced lethality (Chien and Pasternak, 1994). In addition, opioid-induced constipation (one of the most worrisome peripheral side effect of μ-opioids) induced by

either the opioid analgesics morphine or fentanyl, or by the antidiarrheic drug loperamide was shown not to be modulated by either sigma-1 receptor antagonists or in sigma-1 KO mice (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). Finally, it has been shown that sigma-1 antagonism enhances morphine antinociception to heat stimulus once analgesic tolerance is completely established, although it was unable to prevent its development (Vidal-Torres et al., 2013). Therefore, the modulation of opioid effects by sigma-1 receptors appear to be limited to their analgesic effects. Hence, the use of sigma-1 receptor antagonists as opioid adjuvants could represent a promising pharmacological strategy to enhance opioid analgesia without modifying its side effects and, therefore, to increase the therapeutic index of opioids.

However, the potentiation of the antinociception induced by opioid drugs, as well as those findings on the rescue of opioid antinociception in tolerant animals by sigma-1 antagonism, have been demonstrated in acute nociception to heat stimulus, but no studies have been performed in models of pathological pain. Since it is known that plastic changes occur in both the nociceptive system and opioid functioning during pathological pain, for instance during inflammation (Latremoliere and Woolf, 2009; Grau et al., 2014; Kuner and Flor, 2016) it is interesting to test whether the ameliorative effects of sigma-1 inhibition on opioid-induced analgesic tolerance seen in a model of acute nociception can be confirmed in a more clinically relevant pain model, which has been another objective of this PhD Thesis.

2.5.1.4. Molecular mechanisms involved in the modulation of opioid antinociception by sigma-1 receptors

At the plasma membrane, μ-opioid receptors and NMDARs establish physical interactions forming a macromolecular complex where the μ-opioid receptor carries the histidine triad nucleotide-binding protein 1 (HINT1) (Rodríguez-Muñoz et al., 2015a). When an agonist binds to μ-opioid receptor, protein kinase C γ (PKCγ) is activated and phosphorylates NMDARs. Phosphorylation releases NMDARs from the μ-opioid receptor-HINT1 complex, enhancing NMDAR activity, which increases the permeation

of Ca^{2+} ions into the cytosol. This increase in intracellular Ca^{2+} activates Ca^{2+}–calmodulin (CaM) complex to enhance the activity of calmodulin-dependent kinase II (CaMKII), which in turn acts on the μ-opioid receptor decreasing its activity (reviewed in Rodríguez-Muñoz 2015a and b). Moreover, increased Ca^{2+} levels enhanced binding of the Ca^{2+}–CaM complex to NMDARs to decrease their activity to avoid excessive Ca^{2+} entry into the cytosol (Wang et al., 2008). The sigma-1 receptor is located in the complex formed by the μ-opioid receptor, HINT1 and NMDAR (Fig. 10A). When NMDARs are active, increased Ca^{2+} influx induces the binding of sigma-1 receptors to NMDARs, which protects the latter from the inhibitory effect of Ca^{2+}–CaM, thereby improving the activity of NMDARs, and consequently decreasing the activity of the μ-opioid receptor (Rodríguez-Muñoz 2015a and b) (Fig. 10B). When a sigma-1 antagonist binds to its receptor, the sigma-1 receptor stays close to the μ-opioid receptor and facilitates the transfer of HINT1 to NMDARs. When NMDARs lack the protection provided by sigma-1 receptors against the binding of Ca^{2+}–CaM, they decrease their activity (Rodríguez-Muñoz 2015a and b) (Fig. 10C). Therefore, the enhanced opioid signaling by sigma-1 antagonists (Kim et al., 2010) is a consequence of decreased NMDAR activity, and this process might explain both the enhancement of opioid analgesia and the rescue from opioid tolerance by sigma-1 antagonists (Rodríguez-Muñoz 2015a and b).

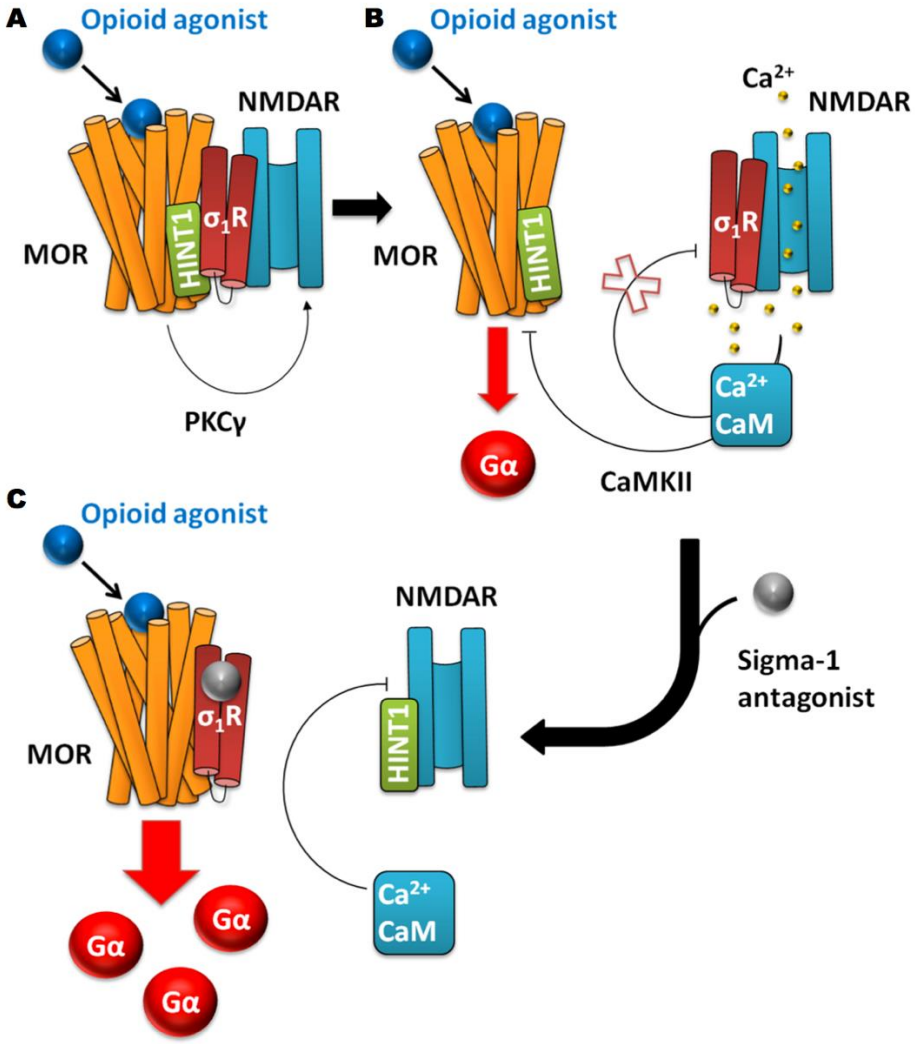

Fig. 10. Schematic representation of the interaction between sigma-1 receptor (σ_1R), μ-opioid receptor (MOR), NMDA receptor (NMDAR) and histidine triad nucleotide-binding protein 1 (HINT1). (A) MOR activation by an agonist leads to the activation of NMDARs through protein kinase C γ (PKCγ). (B) NMDAR activation promotes Ca^{++} influx and facilitates the interaction of σ_1R with NMDARs, which impedes the inhibitory action of Ca^{++}-calmodulin (CaM) on NMDARs. Ca^{++}-CaM impacts negatively on MOR signaling by the activation of calmodulin-dependent kinase II (CaMKII). (C) Sigma-1 antagonists stabilize the association of σ_1R to MOR, and promote the transfer of HINT1 to NMDARs. When NMDARs do not bind σ_1Rs, they increase their susceptibility to the inhibitory effect of Ca^{++}-CaM. As result their inhibitory influence on MOR signaling is reduced. Taken from Sánchez-Fernández et al., 2017.

2.5.2. Pain modulation by sigma-1 receptors in the absence of opioid drugs

In addition to the participatation of sigma-1 receptors in the modulation of opioid analgesia, as described above, they can modulate nociception under specific conditions

in the absence of opioid drugs. This is the case of pain induced by chemical irritants, and in rodent models of clinically relevant painful pathologies such as neuropathic and inflammatory pain. Below we will summarize the main findings from the studies carried out on all these types of pain.

2.5.2.1. Sigma-1 receptors and pain induced by chemical irritants

Thirteen years ago, our group showed the first evidence on the role of sigma-1 receptors in pain control in the absence of the association to opioid drugs, specifically in formalin-induced pain. It was shown that sigma-1 KO mice exhibited less pain-like responses after intraplantar administration of formalin (Cendán et al., 2005b). Later studies showed that sigma-1 antagonists produced dose-dependent antinociceptive effects in this pain model using either non-selective drugs such as haloperidol or selective sigma-1 antagonists such as S1RA or BD-1047 (Cendán et al., 2005a; Kim et al., 2006; Romero et al., 2012; Díaz et al., 2013; Gómez Soler et al., 2014; Lan et al., 2014; Vidal-Torres et al., 2014; Tejada et al., 2017). Since altered pain processing at central levels is involved in the second phase of formalin-induced pain (a process called central sensitization), further studies have focused on the role of sigma-1 receptors at the spinal level in this pain model. It was found that the i.t. administration of sigma-1 antagonists reduced formalin-induced pain (Kim et al., 2006; Vidal-Torres et al., 2014), and this was accompanied by a reduction in the phosphorylation (activation) of NMDA receptors (Kim et al. 2006), which plays a key role in central sensitization (Haley and Dickenson, 2016). Moreover, peripheral sigma-1 receptors also participate in the decrease of this type of pain, since when S1RA was administered i.pl., it attenuated pain-like behaviors in the formalin model (Vidal-Torres et al., 2014). These results indicate that sigma-1 receptors facilitate formalin-induced pain at central sites but also peripherally.

Sigma-1 receptors have also being tested in the secondary mechanical allodynia developed after the subcutaneous injection of capsaicin and in the cutaneous-refereed mechanical hyperalgesia after the visceral administration of this algogenic chemical. These types of mechanical hypersensitivity are widely considered as behavioral features

of central sensitization (Baron, 2000; Cervero and Laird, 2009), and might have a higher translatability than the formalin test, since mechanical hypersensitivity after capsaicin administration, either intradermally or intracolonically, is performed in human volunteers to test for novel analgesics (Park et al., 1995; Drewes et al., 2003; Schmidt et al., 2004; Arendt-Nielsen et al., 2008). The pharmacological blockade of the sigma-1 receptor, by the non-selective drug haloperidol or by the selective sigma-1 antagonists BD-1063 and S1RA, as well as the lack of this receptor in sigma-1 KO mice reduced sensitization to mechanical stimuli in response to intradermal capsaicin (Entrena et al., 2009a and b; Romero et al., 2012; Díaz et al., 2013). Conversely, sigma-1 agonism was able to enhance the sensitizing effect of capsaicin (Entrena et al., 2016). When capsaicin was administered intracolonically, the antagonism of sigma-1 receptor attenuated both acute pain-related behaviours and cutaneous-referred mechanical hyperalgesia (González-Cano et al., 2013). Therefore, it seems clear that sigma-1 receptors markedly modulate these behavioral models of central sensitization.

It is noteworthy that the effects of sigma-1 inhibition in either the secondary mechanical hypersensitivity induced by subcutaneous capsaicin or in formalin-induced pain, were not reversed by the opioid antagonist naloxone (Cendán et al., 2005b; Entrena et al., 2009a and b; Tejada et al., 2017). This definitively indicates that these effects are independent of modulation of the opioidergic system and that sigma-1 receptors can act by means of non-opioid mechanisms to decrease pain transmission.

2.5.2.2. Sigma-1 receptors and neuropathic pain

Since central sensitization is prominent during neuropathic pain after peripheral nerve injury (Meacham et al., 2017), the role of sigma-1 receptors in this type of pain has also been tested. Sigma-1 KO mice did not develop signs of either cold or mechanical allodynia after traumatic nerve injury (De la Puente et al., 2009). Similarly, the administration of sigma-1 antagonists (including S1RA) has also been shown to ameliorate neuropathic pain after mechanical injury of the sciatic nerve (Díaz et al., 2012; Romero et al., 2012; Bura et al., 2013; Espinosa-Juárez et al., 2017a and b) or the

infraorbital nerve (Gris et al., 2016). Interestingly the repeated administration of this drug did not induce analgesic tolerance (Romero et al., 2012), which is in contrast to the known development of analgesic tolerance that occurs during repeated opioid administration (e.g. Lavand'homme and Steyaert, 2017). Several studies of neuropathic pain and sigma-1 receptors have focused on the role of these receptors at the spinal level, showing that the i.t. administration of the sigma-1 antagonist BD-1047 in the early days after nerve injury prevented the full development of neuropathic mechanical allodynia (Roh, 2008; Choi et al., 2013). Importantly, neuropathic rodents were shown to freely self-administer S1RA once neuropathy was established, to ameliorate not only sensory hypersensitivity but also neuropathic anhedonia (measured as decreased preference for a sweetened liquid) as an indicator of the negative emotional state induced by pain (Bura et al., 2103). These behavioral results were supported by the decreased phosphorylation of NMDA receptors at the spinal level (Roh et al., 2008), a decrease in the production of reactive oxygen species in the spinal cord (Choi et al., 2013), as well as a decrease in the activation of spinal astrocytes (Moon et al., 2015), since all of these processes are known to participate on neuropathic pain development and maintenance (Inoue and Tsuda, 2018). In addition to the plastic changes in the nociceptive system at the spinal level, it is known that sigma-1 antagonists induce the dissociation of sigma-1 receptors and NMDA receptors in the PAG of animals with traumatic nerve injury, and this process may have an inhibitory effect on NMDAR activity (Rodriguez-Muñoz et al., 2015a). Therefore, sigma-1 receptors might modulate at several anatomical sites neuropathic pain development after mechanical nerve injury, not exclusively at the spinal level.

In addition, the role of sigma-1 receptors on central neuropathic pain after mechanical spinal cord injury has also been very recently tested. It was found that sigma-1 inhibition decreased mechanical allodynia and heat hyperalgesia (Choi et al., 2016; Castany et al., 2018), as well as several markers of sensitization in the spinal cord, including astrocytic activation (Choi et al., 2016), TNF-α, IL-1β and the phosphorylation of NMDA receptors and ERK1/2 (Castany et al., 2018). Therefore, central sigma-1 receptors appear to be a promising target for the treatment of pain induced by trauma of either peripheral nerve or spinal cord.

Similar results on the role of sigma-1 receptors on neuropathic pain were found after chemically-induced nerve injury. Sigma-1 KO mice did not develop sensory hypersensitivity (mechanical- and cold-allodynia) after the administration of the antineoplastic drug paclitaxel (Nieto et al., 2012 and 2014). The pharmacological antagonism of sigma-1 receptor also abolished mechanical and cold allodynia once paclitaxel-induced neuropathic pain was fully developed (Nieto et al., 2012). Interestingly, when the sigma-1 antagonists were administered during the treatment with the antineoplastics paclitaxel or oxaliplatin, they were able to prevent the development of neuropathic pain (Nieto et al., 2012 and 2014; Gris et al., 2016). At least during paclitaxel-induced neuropathic pain, sigma-1 inhibition was shown to decrease the central sensitization marker pERK1/2 at the spinal cord (Nieto et al., 2012), but also decreased mitochondrial abnormalities in the peripheral myelinated A-fibers (Nieto et al., 2014), indicating that both central and peripheral sigma-1 receptors participated in the modulation of antineoplastic-induced neuropathy. Importantly, these results have a translation in humans, since a recent study in patients treated with the antineoplastic oxaliplatin showed an increase in their cold pain threshold when treated with S1RA, indicating the amelioration of cold allodynia (Bruna et al., 2018).

Finally, it was recently shown that sigma-1 antagonism was also able to ameliorate behavioral signs (mechanical and thermal hypersensitivity) of diabetic neuropathic pain in rodents, which were accompanied by a decrease in the hyperexcitability of the peripheral nerve (Gris et al., 2016; Paniagua et al., 2017), supporting again modulatory effects of peripheral sigma-1 receptors in neuropathic pain induced by peripheral nerve toxicity.

In summary, sigma-1 antagonism is a novel promising approach for the treatment of neuropathic pain.

2.5.2.3. Sigma-1 receptors and inflammatory pain

The role of sigma-1 receptors in inflammatory pain has also been very recently described, and with promising results. In contrast to neuropathic pain, in which as described above the altered central processing of the nociceptive information is thought to be more prominent than the peripheral component, inflammatory pain is characterized by a more pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the inflammatory mediators released at the inflammation site (Scholz and Woolf, 2002; Patapoutian et al., 2009).

The first works of the modulation of inflammatory pain by sigma-1 receptors were published only 4 years ago. Up to now, several sigma-1 antagonists have been tested in inflammatory pain models, including S1RA, BD-1063, BD-1047 and MR200 (and its derivatives) (Gris et al., 2014; Parenti et al., 2014a and b; Tejada et al., 2014; Jeong et al., 2015). Systemic sigma-1 antagonism reversed mechanical and heat hypersensitivity in mice with paw inflammation induced by carrageenan, complete Freund´s adjuvant (CFA) or zymosan (Gris et al., 2014; Jeong et al., 2015; Parenti et al., 2014 a and b; Tejada et al., 2014). It should be noted that sigma-1 KO mice did not develop mechanical hyperalgesia (Tejada et al., 2014). However, the lack of receptor was not able to alter mechanical allodynia or thermal hyperalgesia in inflamed animals (Gris et al., 2014; Tejada et al., 2014). These results with sigma-1 KO mice which are in contrast with those found with sigma-1 antagonists, could be explained by the development of compensatory mechanisms in mutant mice (Sánchez-Fernández et al., 2017), as described in previous sections.

In addition to these effects induced by systemic sigma-1 inhibition, the role of sigma-1 receptors on inflammatory pain at specific locations (peripheral or central) has also been studied. The i.t. administration of sigma-1 antagonist was able to reverse zymosan-induced inflammatory hyperalgesia (Jeong et al., 2015), suggesting that spinal sigma-1 receptors might play a role on inflammatory pain hypersensitivity. Interestingly, it has been shown that the administration of sigma-1 antagonists at the inflamed site was also able to reverse inflammatory hyperalgesia (Tejada et al., 2014 and 2017), indicating that peripheral sigma-1 receptors participate on these effects. A detailed mechanism of the

modulation of inflammatory pain by sigma-1 receptors has been described. Immune cells infiltrated into the inflamed site are able to produce endogenous opioid peptides, whose analgesic actions can be enhanced by peripheral sigma-1 receptor antagonism (Tejada et al., 2017). Although the sigma-1 modulatory effects on the antinociceptive effects of opioid drugs is extensively reported (as shown in the Section 2.5.1.1. and 2.5.1.2.), this was the first report on the modulation of the endogenous opioid system by sigma-1 receptors (Fig. 11). It is unclear whether the modulation by sigma-1 receptors of the immune-mediated opioid analgesia accounts for the effects of sigma-1 antagonists on other pathological pain models, for instance in neuropathic pain, in which the activity of the immune system is prominent (Tejada et al., 2018).

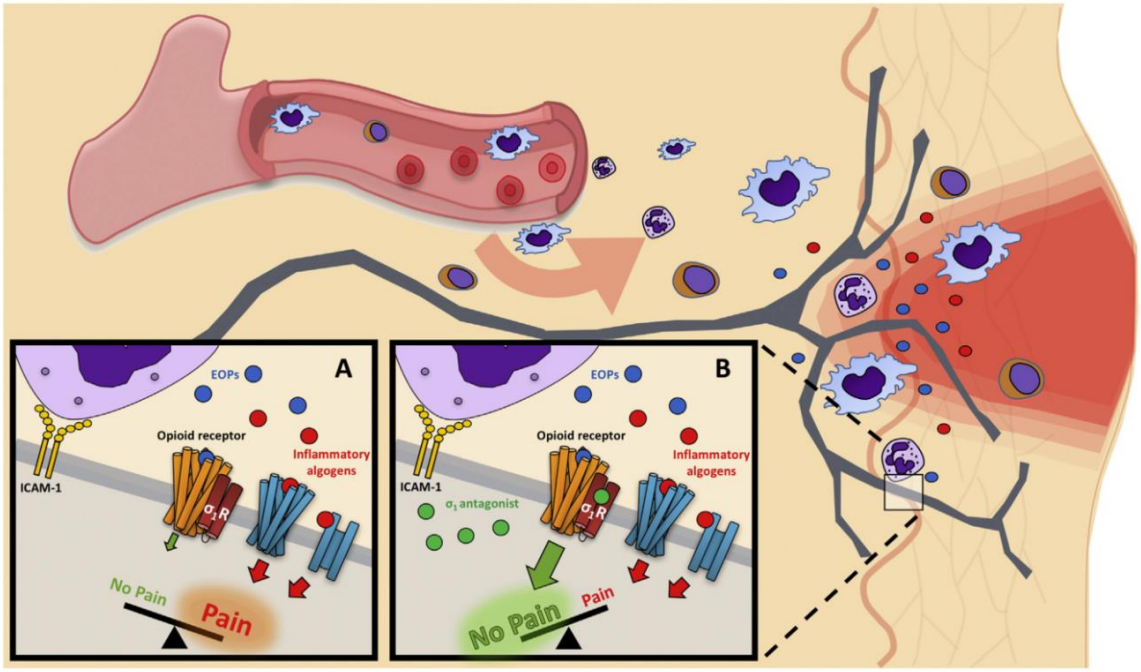

Fig. 11. Effect of sigma-1 antagonism on immune-driven opioid analgesia. Immune cells migrate to the inflamed tissue, where they interact with peripheral nerve terminals which express ICAM-1 (intercellular adhesion molecule-1). These immune cells release both inflammatory algogens which promote pain, and endogenous opioid peptides (EOPs). (A) Under normal conditions the overall result of this immune–neuron interaction is pain, because sigma-1 receptors (σ_1-R) tonically inhibit opioid receptors, and hence decrease the analgesic effect of EOPs. (B) In the presence of a sigma-1 antagonist, the effects of opioid receptors are enhanced, potentiating the effects of EOPs of immune origin, and resulting in immune-driven opioid analgesia (B). Taken from Tejada et al., 2017.

3. PAIN BEHAVIOR IN PRECLINICAL STUDIES: THE SEARCH FOR TRANSLATIONAL PAIN OUTCOMES TO REFINE ANALGESIC DEVELOPMENT

Pain is undoubtedly an important global health problem, with millions of people with chronic pain (Goldberg and McGee, 2011; Pina et al., 2017). Current analgesics (including opioids, nonsteroidal anti-inflammatory drugs and gabapentinoids) show limited efficacy in many pain conditions or a number of side effects which limit their use (Yaksh et al., 2015; Phillips et al., 2017). Therefore, there is a strong need for novel analgesics. However, despite major advances in our understanding of the somatosensory system in recent decades, there has been limited translation of new analgesics from bench to bedside (Hill, 2000; Mao, 2009; Kissin, 2010; Huggins et al., 2012; Barrett, 2015; Fallon et al, 2017), and most "new" pain medications consist of refined delivery methods for known analgesic drugs, or combination therapies between well known analgesics (Kissin, 2010; Mao et al., 2011; Gilron et al., 2013; Dale and Stacey, 2016; Wolkerstorfer et al., 2016).

For the purposes of translation, animal testing should mimic as closely as possible routine clinical practice and clinical trials. This has been the reasoning for the constant refining of the animal models of pain (defining them as the process aimed to mimic a pathological condition seen in humans) which has been intensely pursued since decades ago, and there are currently several models of inflammatory, neuropathic and cancer pain which are thought to resemble these clinical pain conditions (Gregory et al., 2013; Burma et al., 2017). However, the previously commented lack of recent real breakthrough analgesic drugs rise questions on the predictive validity of these pain models (Mogil and Crager, 2004; Negus et al., 2006; Cobos and Portillo-Salido, 2013; Negus, 2013; Percie and Rice, 2014). It might be thought that for an effective translation from the bench to bedside in addition to successfully mimic the pathological condition, it should also be necessary to use translational pain outcomes. This is challenging, since the absence of verbal communication in animals is undoubtedly an obstacle to the evaluation of pain in preclinical studies.

We do not intend to make an extensive review of all literature but to provide the reader a sense on how the field evolved to approach rodent experimental pain models to the

human pain phenotype, and in particular regarding the evolution of pain outcomes in rodents.

3.1. ACUTE NOCICEPTIVE PAIN, TONIC PAIN AND SENSORY HYPERSENSITIVITY

To get a historical perspective of the evolution of pain behavior assessment, we tracked in Medline original pain research articles, written in English and published since the 70's till current times. We selected a period of 5 years per decade to obtain a representative value of each period (1973-1977, 1983-1987, 1993-1997, 2003-2007, 2013-2017).

In 1940 Hardy and coworkers published their results using a new device to reliably measure heat pain threshold in human subjects, by using a light beam controlled by a rheostat (Hardy et al., 1940). This served as inspiration for preclinical pain researchers who soon developed a similar device to study pain and analgesia on rodents (D'Amour and Smith, 1941). In this test, the animal is restrained and a light beam is directed against the tail of the rodent until provokes an abrupt movement of the tail; that made this test to be named as the "tail flick" test. The reaction time can be monitored as an index of pain or analgesia. Variants of this test were developed using hot water instead of a light beam as a source of heat stimulus, but the behavioral outcome evaluated remained identical (e.g. Ben-Bassat et al., 1959). Soon after the first description of the tail flick test, the hot plate was developed (Woolfe and MacDonald, 1944). The hot plate test has the advantage over the tail flick in that the animal is not restrained, but it is able to move freely on a plate heated to a constant temperature surrounded by an open-ended evaluation cage. However, and in contradistinction to the tail flick test, heat stimulation in the hot plate is not restricted to a specific body area, as the four limbs, the tail and even the genitals of the rodent can be simultaneously stimulated. This stimulation of multiple body areas might trigger diffuse inhibitory controls that are likely to disturb the responses observed (Le Bars et al., 2001). The behavioral outcome most often used in the hot plate test is licking or flinching of the paws, and the jumping response if the exposure to the nociceptive stimulus is prolonged. Hot plate test has been improved in recent years in the "so-called" unilateral hot plate test, in which the animal is gently hold

by the skin of the interscapular region, and only one paw is placed on the hot surface, avoiding the previously commented triggering of diffuse inhibitory pain controls (Menéndez et al., 2002). These tests for acute heat nociception clearly dominated preclinical pain research till 1990´s (See Fig. 12).

Years later, the first models of tonic pain were developed. Possibly, the first of these tests was the writhing test (also named abdominal constriction test) which was described in 1957 (Siegmund et al., 1957). The intraperitoneal injection of chemical irritants induced a stereotyped behavior in the rodents characterized by abdominal stretching together with full extensions of the hindlimbs. Although it was originally described using 2-phenyl-1,4-benzoquinone (Siegmund et al., 1957), several other irritants have been used with the same purpose, including acetylcholine, dilute hydrochloric or acetic acid, bradykinin, adenosine triphosphate, potassium chloride, tryptamine and oxytocin, as all these compounds induce this stereotyped behavior when administered intraperitoneally (reviewed by Le Bars et al., 2001). Twenty years after the first description of the writhing test, formalin-induced pain was reported (Dubuisson and Dennis 1977). The intraplantar injection of formalin produces a biphasic behavioral reaction, with an initial phase lasting 5 minutes (or less) and a quiescent period followed by a second tonic pain phase which ranges typically from 10 to 40 min (reviewed in Sawynok and Liu, 2004). Pain-like responses induced by formalin include licking/biting or flinching of the injected paw (reviewed by Le Bars et al., 2001). The sensitivity to analgesics of acute and tonic pain models was strikingly different. Opioid drugs showed more potent analgesic effects in both the writhing test and formalin-induced pain than in tail flick and hot plate tests (e.g. Abbott et al., 1982; Schmauss et al., 1985). In addition, mild analgesics such as nonsteroidal antiinflammatory drugs (NSAIDs), which hardly induced analgesic effects on nociceptive heat pain, were found to induce robust analgesic-like effects in these tonic pain models (e.g. Miranda et al., 2001; Nieto et al., 2001). Therefore, tonic pain by these chemical irritants approached more to clinical pain than the tests of acute heat nociception (Le Bars et al., 2001; Cobos and Portillo-Salido, 2013). This probably influenced the continuous shift on the use of tonic pain models in detriment of the use of the classic models of heat nociception. In 1993-1997 formalin-induced pain/writhing test were used in a much lower number than

the studies which employed the tail flick/hot plate tests. This analysis agrees with a previous one covering each year of the 1990´s decade (Le Bars et al., 2001). However, in 2003-2007 these models of tonic pain were most often used than the tests for acute heat nociception, and this difference was more prominent in the last period examined (2013-2017) (see Fig. 12).

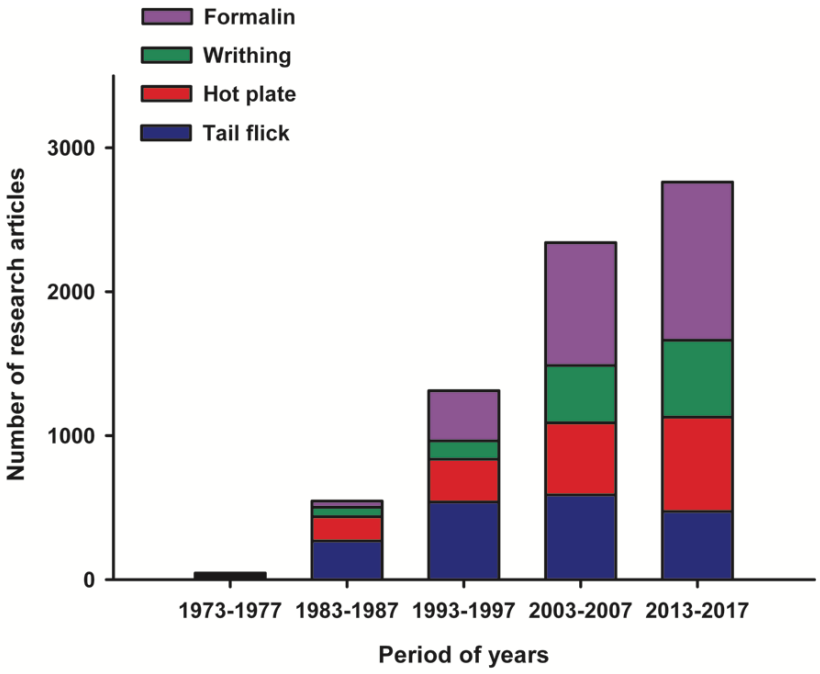

Fig. 12. Temporal evolution of the number of original articles written in English language which used heat pain nociceptive tests (hot plate and tail flick tests) and tonic pain models (writhing and formalin tests).

To decrease the distance between clinical pain and preclinical pain research, animal models of pathological pain conditions were also developed. Inflammatory pain has been modeled using the administration of several types of inflammatory compounds, such brewer's yeast, which was used as early as in 1957 (Randall and Selitto, 1957), and was followed by several other agents such as carrageenan, complete Freund's adjuvant (CFA), bacterial lipopolysaccharide and croton oil, among many others (reviewed in Le Bars et al., 2001; Cobos and Portillo-Salido, 2013). These chemical substances can be injected to induce local inflammation into the soft tissue or into the joint (to model

inflammatory arthritis) (Fischer et al., 2017). More recently, collagen-induced arthritis has been added to the repertoire of inflammatory pain models, and it is considered to be a highly translational model of inflammatory arthritis (e.g. Nieto et al., 2016).

Much later than inflammatory pain models, neuropathic pain models were developed. In most neuropathic pain models, neuropathy is induced by mechanical injury of the sciatic nerve, and this include the chronic constriction injury (CCI), which was the first to be developed in 1988 (Bennett and Xie, 1988), and was followed by the spinal nerve ligation in 1992 (Kim and Chung, 1992) and the spared nerve injury in 2000 (Decosterd and Woolf, 2000), among many other neuropathic pain models, including variants by mechanical injury of the infraorbital nerve to induce facial neuropathic pain (e.g. Vos et al., 1994). In addition, neuropathic pain induced by chemical injury of the peripheral nerves by antineoplastics, including paclitaxel, vincristin, cisplatin or oxaliplatin, has also been used in preclinical research (reviewed in Sisignano et al., 2014). Furthermore, central neuropathic pain models have also been developed, and they are induced by mechanical contusion or electrolytic lesions of the spinal cord (Kumar et al., 2018).

Further efforts were made to mimic other pathological pain conditions. Osteoarthritis (non-inflammatory arthritis) can be induced experimentally by several methods, including the injection of monoiodoacetate (a chondrocyte glycolytic inhibitor) or by surgical destabilization of the medial meniscus (O'Brien et al., 2017). Cancer pain is modeled by injecting tumor cells into the bone marrow (femur, calcaneus or tibia), and it is accompanied by bone destruction (reviewed in Wang et al., 2003), To model postoperative pain, the first proposed model was plantar incision (Brennan et al., 1996), although more recently more realistic and complex surgical procedures have been used, such as laparotomy (e.g. Martin et al., 2005; Sotocinal et al., 2011); or thoracotomy (Buvanendran et al., 2004). Other types of pain have also been explored, such as burn injury-induced pain which can be induced by constant application of the paw to a hot plate (under anesthesia) (e.g. Morgan et al., 2008), or ischemic pain which can be induced by acute occlusion of the femoral artery (Sher and Mitchell, 1990).

The search for adequate models of painful pathological conditions was accompanied by the search of adequate pain outcomes. As sensory hypersensitivity (including allodynia

and hyperalgesia) is an important feature of clinical pain, several behavioral tests attempted to quantify this enhanced sensitivity to sensory stimuli in models of pathological pain. The first measure of sensory hypersensitivity was made in 1957 by Randall and Selitto. They used an inflammatory pain model (induced by brewer's yeast) to demonstrate mechanical hyperalgesia applying a blunt mechanical stimulus, and quantifying the reduced latency to struggle (Randall and Selitto, 1957). This test was termed the Randall-Selitto test after the authors who developed it, and it is also known as the paw pressure test. Much later, in 1988, Hargreaves et al. complemented the Randall-Selitto test by measuring heat hyperalgesia during inflammation using a light beam directed to the inflamed site (the paw) in freely moving rats placed on a glass floor. Heat hyperalgesia is shown as a decreased latency to paw withdrawal during inflammation (Hargreaves et al., 1988). This test was named after the first author who developed it (Hargreaves test), and also called the plantar test. This measure of heat hyperalgesia has been extensively used in pain research since the 90's, and to a much higher extent than the paw pressure test (Fig. 13).

In early 90's, and inspired by the sensory testing made by neurologists in human patients, the von Frey filaments were used in neuropathic rodents to asses for their mechanical threshold (Shirand Seltzer, 1990; Kupers and Gybels, 1993; Chaplan et al., 1994). This test is based on the sequential application of calibrated filaments till the animal makes a pain-like response (fast withdrawal, licking or flinching of the stimulated paw), to determine the threshold to mechanical stimulation and hence the presence of mechanical allodynia. The use of von Frey filaments gained popularity not only for evaluating the mechanical threshold of neuropathic rodents but also for virtually all types of pathological pain models (reviewed in Mogil, 2009; Cobos and Portillo-Salido, 2013), and measures of tactile allodynia clearly currently dominate pain behavior research over all other measures of hypersensitivity (Fig. 13), but also over all other types of sensory testing (compare Fig. 12 and 13).

Finally, as cold allodynia is also a feature of neuropathic patients, cold stimuli has also been used to study changes in cold sensitivity during pathological pain. The most widely used on this modality is the acetone drop test, which is based on the sensation of cold produced by the evaporation of acetone, which might elicit pain-like responses under

pathological conditions. The outcome measures vary from the number or frequency of brisk foot withdrawals to the time spent reacting by licking or shaking the stimulated paw (reviewed in Sandkuhler, 2009; Deuis et al., 2017). Since this test was developed in mid 90's (Choi et al., 1994), other liquids such as ethyl chloride have been used for the same purpose, and also more sophisticated methods to measure cold hypersensitivity have been developed. The latter include the application of dry ice through a glass platform, the cold plate test and the temperature preference test (Deuis et al., 2017). Although the responses for cold stimulus in rodents have been used since more than 20 years, cold measures have not gained the popularity of the Hargreaves test or the evaluation of the von Frey threshold in preclinical pain research, as shown in Fig. 13.

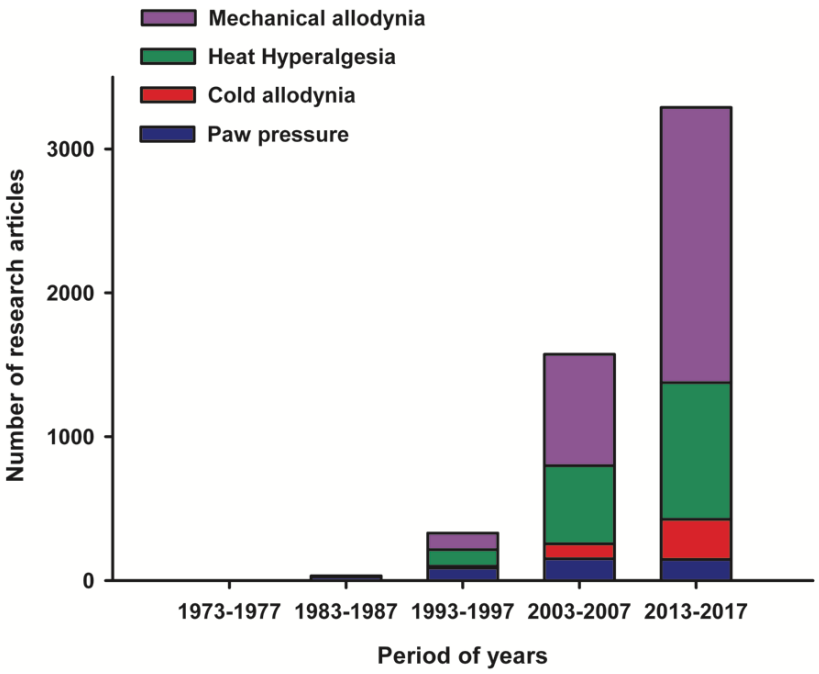

Fig. 13. Temporal evolution of the number of original articles written in English language which used measures of sensory hypersensitivity including mechanical allodynia, heat hyperalgesia, cold allodynia and the paw pressure (Randall-Selitto) test.

3.2. NEW BEHAVIORAL OUTCOMES IN PAIN RESEARCH: IN THE SEARCH FOR MEASURES CLOSER TO THE HUMAN PAIN PHENOTYPE

Although von Frey testing, the most widely behavioral test currently used in preclinical pain research, is reverenced by neurologists and is undoubtedly useful to detect sensory alterations in neuropathic patients (e.g. Bennett, 2001; Bouhassira et al., 2005; Moharic et al., 2012), the use of von Frey filaments in other human pain conditions such as during rheumatic diseases or cancer pain is virtually absent. As an example, we found only three published studies which used von Frey filaments in patients with rheumatoid arthritis (Morris et al., 1997; Hendiani et al., 2003; van Laarhoven et al., 2013), one of the most worrisome painful conditions that occurs with joint inflammation (Scott et al., 2000; Lee, 2013). Therefore, although von Frey testing has been established as the standard of preclinical pain testing for practical reasons, it should be taken into account that it is not an extended pain measure in patients with chronic painful diseases.

Furthermore, it is worth mentioning that all pain responses typically evaluated in rodents by using the standard methods described in the section above, including licking, flinching, biting or abdominal stretching, can be decreased by non-analgesic (sedative) drugs giving false-positive results (reviewed by Le Bars et al., 2001; Cobos and Portillo-Salido et al., 2013; Gregory et al., 2013; Deuis et al., 2017). In addition, all these pain outcomes are reflex measures which persist even in decerebrated animals (reviewed in Mogil, 2009). Therefore, although sensory hypersensitivity can be evaluated in rodents by tools such as the von Frey filaments, and it is a feature of clinical pain, the human pain phenotype is far more complex than a simple reflex response. In fact, consensus-based recommendations for the main outcomes that should be measured in clinical trials of treatments for pain include not only pain itself but also alterations in physical and emotional functioning, as well as diverse parameters to quantify the impact of pain in the daily life of chronic pain patients (Dworkin et al., 2008). All these limitations of the standard pain outcomes have recently led to pain researchers around the globe to search for additional outcomes to approach preclinical pain research to the human pain phenotype.

Several of these new pain outcomes aim to provide a wider view of the rodent pain

phenotype by measuring alterations in physical functioning induced by pain. The impact of pain on the physical functioning can be assessed in rodents by measuring postural changes (by either weight bearing asymmetry or gait alterations) and grip strength deficits. The weight bearing distribution in humans or rodents is approximately equal in each lower extremity in normal conditions. However, after unilateral hindlimb injury, body weight support is shifted to the noninjured side. Weight bearing asymmetry has long been targeted in preclinical pain research, in particular since 1994 when Schott and coworkers developed a device consisting on two adjacent force plates able to measure the weight bearing of the hindlimbs of the rat (Schott et al., 1994). Recently, new versions of this test have been developed in which rodents can move freely in a chamber with the floor covered by pressure sensors allowing to simultaneously measure the weight bearing by the four limbs (Tetreault, et al., 2011). Changes in weight bearing distribution can be observed during hindlimb inflammation induced by inflammatory agents such as carrageenan or CFA (e.g. Schott et al., 1994; Munro et al., 2008; Huntjens et al., 2009; Cobos et al., 2012), in rodent models of osteoarthritis (e.g. Ivanavicius, et al., 2007; Okun et al., 2012; Pajak et al., 2017; Philpott et al., 2017), cancer pain (Tetreault, et al., 2011), after paw incision (Whiteside et al., 2004; Luk et al., 2018). or during peripheral neuropathy (e.g. Manzhulo et al., 2015; Nakazato-Imasato, et al., 2009). In addition, it has been shown that mice with visceral pain increased their weight distribution toward their front paws, indicating that this outcome can be useful in other pain models apart from those based on the unilateral injury of a hindlimb (Laux-Biehlmann et al., 2016).

The study of the gait patterns has been used in the past to study the functional motor consequences of neurological deficits in rodents (reviewed by Cobos and Portillo-Salido, 2013). More recently, these methodologies have been adapted for measuring adaptive dynamic postural changes during a pain condition. There have been reported alterations in gait parameters during unilateral inflammation (Angeby-Moller et al., 2008; Piesla et al., 2009) or peripheral nerve injury (Vrinten et al., 2003; Piesla et al., 2009; Mogil et al., 2010).

It is worth pointing out that although both weight bearing changes and gait alterations can be recovered by analgesic treatment in inflammatory pain models in rodents, as well

as during osteoarthritis pain, these outcomes are resistant to change by analgesic administration in models of traumatic peripheral neuropathic pain (reviewed by Cobos and Portillo-Salido et al., 2013). The differential sensitivity to analgesic drugs of both weight bearing asymmetry and gait alterations in inflammatory/osteoarthritis pain and peripheral nerve injury suggest that pain is the primary driver of postural changes in models of inflammatory and osteoarthritis pain, but not in peripheral nerve injury (which might reflect motor deficits due to surgical damage to the motor axons, rather than pain). Assessing postural changes as pain indicators is therefore not free from motor confounders. In fact, sedative drugs can also have an impact on weight bearing asymmetry and might result in false-positive analgesic-like effects (Munro et al., 2008), similar to what occurs with the standard reflexive outcomes, as previously mentioned. In addition, several drugs are known to alter gait patterns (Authier et al., 2016), which might influence the results on gait analysis when used as a surrogate pain measure.

Grip strength assessment in experimental animals (Fig. 14A) was developed to assess the effects of muscle relaxants and to test drug-induced toxicity (Tilson et al., 1990; Nevins et al., 1993; Savilampi et al., 2014). This technique has been adapted recently for the study of pain resulting from muscle inflammation (Kehl et al., 2000 and 2003), muscle pain induced by repeated exercise (Fujiwara et al., 2017), musculoskeletal hyperalgesia induced by stress (Goudie-DeAngelis et al., 2016), bone cancer (Kehl et al., 2003; Wacnik et al., 2003), osteoarthritis (Chandran et al., 2009; Honore et al., 2009; Lee et al., 2011), and in a model of sickle cell disease (Calhoun et al., 2015). Decreased grip strength under painful conditions is known to be reversed by analgesics (reviewed by Cobos and Portillo-Salido, 2013), although a comparison between drug effects in the grip strength and other more conventional pain measure has not been performed, and, therefore, it has been another objective of this PhD Thesis. One potential advantage of grip strength as an indicator of the analgesic activity of drugs is that, in contradistinction to the other behavioral tests described above, substances producing significant sedation or muscle weakness might decrease this behavior, and therefore would not produce false positive results due to these confounders when evaluating molecules as potential analgesics.

Other studies evaluate the decrease in the performance of certain behaviors during

painful conditions, the termed “pain-depressed behaviors”. These behaviors include those which are normally performed by the rodents and change in frequency or intensity during a pain condition (Negus et al., 2006), such as exploratory activity, home cage activity, burrowing behavior, wheel running, nesting behavior and intracranial self-stimulation (reviewed by Cobos and Portillo-Salido, 2013; Negus, 2018).

Exploratory behavior in a novel environment has consistently been reported to be affected by pain in rodents, in fact, there are even studies from mid-80´s showing pain-induced decrease of exploratory locomotion (e.g. Larsen et al., 1985). In more recent years the number of studies using this parameter as a pain surrogate appears to be increased in rodents with pain conditions such as inflammatory, osteoarthritis or peripheral and central neuropathic pain, among others (e.g. Vos et al., 1994; Mills et al., 2001; Bon et al., 2003; Martin et al., 2005; Stevenson et al., 2006; Matson et al., 2007; Gregoire et al., 2012; Zhu et al., 2012). Decrease in home cage activity has also been proposed as pain index in rodents with chronic pain. This parameter has been explored in animals with chronic inflammation, osteoarthritis or neuropathic pain, but whereas some studies show alterations in home cage activity, others did not (Larsen et al., 1985; Guingamp et al., 1997; Philippe et al., 1997; Urban et al., 2011; Pitzer et al., 2016). These discrepancies might indicate that exploratory locomotion might be more sensitive than home cage activity to detect pain-induced behavioral changes, which might be due to the fact that a novel environment boosts the locomotion on rodents, giving a wider window to detect pain-induced depression of activity.

Similar to exploratory locomotion, rodents spontaneously perform other ethologically relevant behaviors, such as burrowing, wheel running or nesting (Fig. 14B, 14C and 14D, respectively). All these measures have been used to detect pain interference in rodent behavior. Depression of burrowing has been used as a surrogate measure of pain in rodents with painful inflammation, osteoarthritis, neuropathic pain (in either the hindlimb, the orofacial region or in a model of discogenic low back pain, or chemically-induced neuropathy by stavudine treatment), postsurgical pain, visceral pain or radiation-induced oral mucositis (Jirkof et al., 2010; Andrews et al., 2012; Huang et al., 2013; Jirkof et al., 2013; Lau et al., 2013; Rutten et al., 2014; Bryden et al., 2015; Muralidharan et al., 2016; Wodarski et al., 2016; Nolan et al., 2017; Shi et al., 2017;

Deseure and Hans, 2018; Rutten et al., 2018). Depression of wheel running activity has been proposed as an index of pain from diverse etiology, including inflammatory, neuropathic, visceral, headache pain, and in a model of osteogenesis imperfecta (Miller et al., 2011; Cobos et al., 2012; Grace et al., 2014; Abdelaziz et al., 2015; Pitzer et al., 2016; Kandasamy et al., 2017a and b; Whitehead et al., 2017). Nesting behavior has been successfully tested as a pain measure during visceral pain, cancer pain, and after surgical procedures (Jirkof et al., 2013; Negus et al., 2015; Forte et al., 2016; Lewter et al., 2017; Oliver et al., 2018). Therefore, although the use of all these measures as pain indicators is recent, they all have been extensively validated in several pain conditions.

Intracranial self-stimulation (ICSS) is a type of operant conditioning. ICSS has also been shown to be markedly affected by the intraperitoneal injection of lactic acid (Kwilasz and Negus, 2012; Altarifi et al., 2015; Altarifi and Negus, 2015; Miller et al., 2015; Hillhouse and Negus, 2016; Lazenka et al., 2017 and 2018) or by intraplantar formalin (Leitl et al., 2014). However, when longer-lasting pain states were tested such as CFA-induced inflammation, neuropathic pain by spinal nerve ligation or paclitaxel-induced neuropathy, minimal or no change in ICSS was detected (Ewan and Martin, 2014; Leitl et al., 2014; Legakis et al., 2018). Therefore, it appears that this outcome measure is more adequate for the evaluation of tonic rather than chronic pain.

All these pain-depressed behaviors, similar to the assessment of grip strength, have the potential to discriminate substances producing significant sedation from true analgesic effects, since sedative drugs are expected to decrease even more the target behavior and not to restore it (which is used as an index of drug-induced analgesia).

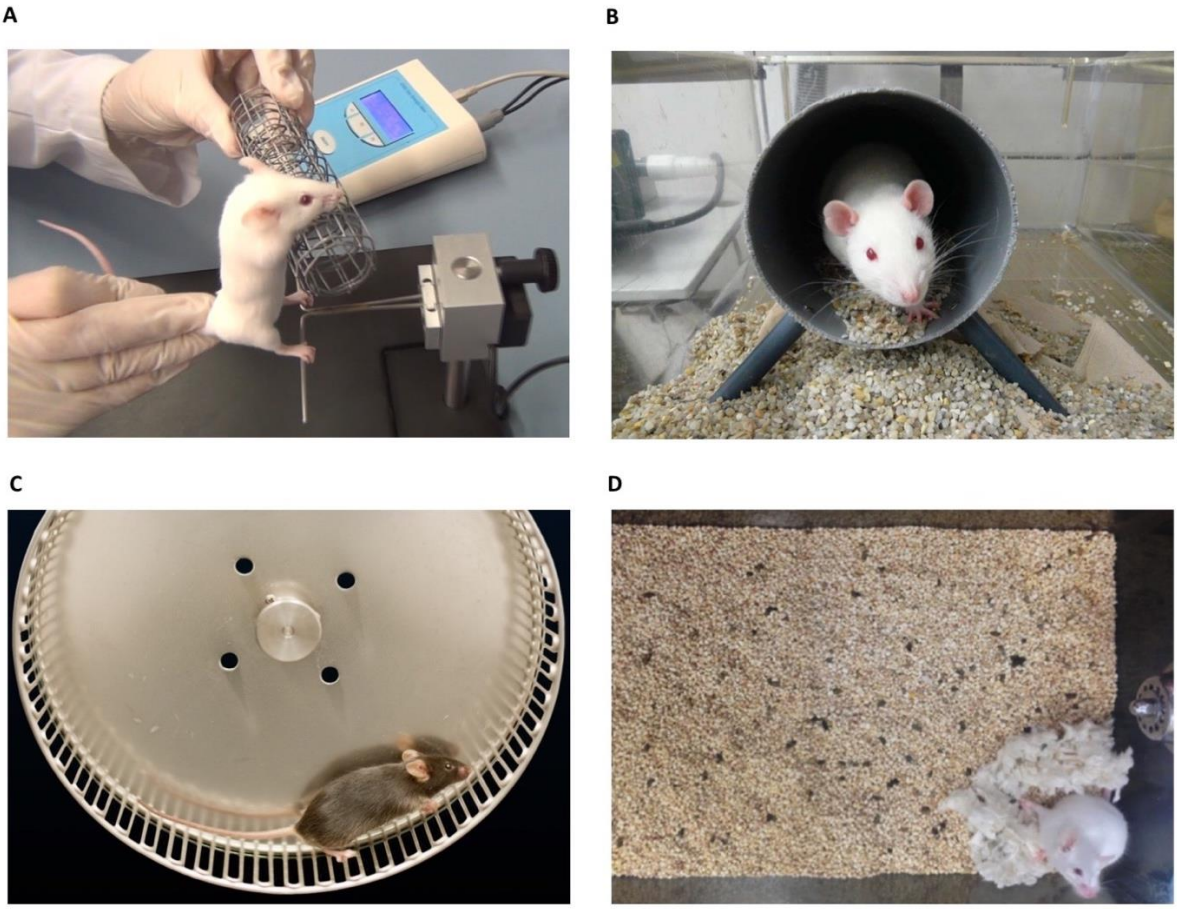

Fig. 14. Some examples of behavioral tests that can be used as pain indicators in rodents. (A) Grip strength, (B) burrowing, (C) wheel running and (D) nesting. The picture of the grip strength meter (A) was taken in our lab, using the device provided by Ugo Basile. The other images are taken from Wodarski et al., 2016 (B); Bioseb activity wheels used in Cobos et al., 2012 (C) and Negus, 2018 (D).

Interestingly, some of these pain-depressed behaviors have been proved not to be equivalent to the most standard reflex-based outcomes. For instance, it is known that the time-course of mechanical allodynia/hyperalgesia do not match weight bearing changes or wheel running depression during inflammatory conditions (Huntjens et al., 2009; Cobos et al., 2012). In addition, drug sensitivity between reflex-based outcomes and pain-depressed behaviors are markedly different, being the latter more sensitive than the former (reviewed by Cobos and Portillo, 2013), and it has been recently found that rodents developed at a faster pace analgesic morphine tolerance to abdominal stretching than to ICSS (Altarifi and Negus, 2015). Altogether, these differences might

reflect that the neurobiological mechanisms involved differ between the different outcomes explored. However, mechanistic studies should be formally performed to shed light on this issue.

In addition to these behavioral outcomes, the impact of pain on anxiety or depressive behaviors in rodents have also been assessed, as well as the interference of pain on sleep architecture (reviewed by Cobos and Portillo-Salido, 2013). The aversive quality of pain and the rewarding properties of analgesia during a painful condition have also been used as a surrogate measure of pain in several studies (reviewed by Cobos and Portillo-Salido, 2013). Even grimaces have also been scored in rodents under painful conditions as a direct measure of pain (Langford et al., 2010; Sotocinal et al., 2011; Akintola et al., 2017; Tuttle et al., 2018). Although these latter measures have not been extensively described in this chapter, as they fall out of the scope of this PhD Thesis, altogether they provide a compelling set of behavioral outcomes which can be readily used by preclinical research laboratories to complement the standard pain outcomes.

3.3. WHAT WE HAVE LEARNT ON THE EVOLUTION OF PAIN MODELS AND OUTCOMES

In conclusion, pain models have been evolving since the beginning of preclinical pain research, always attempting to approach to the evaluation of pain in humans and to the human pain phenotype. Although some non-reflex pain measures have been tested decades ago (such as exploratory locomotion or weight bearing changes), the use of reflex outcomes gained in popularity to a point that most pain studies use the von Frey as the standard pain outcome in preclinical research. In recent years a new wave of pain measures evaluating the impact of pain on normal rodent behaviors, attempting to approach the behavioral changes observed in human patients, is increasing in popularity among pain researchers. To explicitly demonstrate distinct mechanisms between the standard and non-reflexive pain outcomes is important, since an excessive focus on a single reflex-based outcome, such as the von Frey threshold, might lead to serious misinterpretations of the relevance of novel pain targets. Not exploring whether the

novel putative analgesic drugs are able to improve relevant aspects of the human pain phenotype, such as physical or emotional functioning is certainly risky, since we might be selecting drugs with antiallodynic activity but no effects on several important pain components. We believe that the exploration of these new pain measures has the potential to profoundly benefit pain research and analgesic development by providing non-overlapping information on new pain targets.

RATIONALE AND GOALS

RATIONALE AND GOALS

Sigma-1 receptors are a promising novel pharmacological target for pain treatment (Sánchez-Fernández et al., 2017). Among the selective sigma-1 antagonists available, the best characterized is S1RA (Vela et al., 2015). This compound has been already evaluated in several Phase II clinical trials for pain treatment with positive results, specifically for neuropathic pain (Bruna et al., 2017). In addition, it is known from preclinical studies that sigma-1 inhibition enhances the antinociception induced by opioid drugs (reviewed in Zamanillo et al., 2013; Sánchez-Fernández et al., 2017), and in fact a further potential indication for S1RA in human patients might be the enhancement of opioid analgesia (Vela et al., 2015).

For this PhD Thesis project, we focused on the study of the modulation of opioid antinociception by sigma-1 receptors. We initially studied this modulation in nociceptive pain induced by mechanical and heat stimuli in mice. Then, we aimed to study the modulation of opioid analgesia by sigma-1 receptors during pathological pain, specifically in a mouse model of painful joint inflammation. We aimed to explore not only the standard measure of cutaneous hypersensitivity (tactile allodynia evaluated by von Frey filaments), but also the decrease in physical function (measured as grip strength deficits) characteristic of patients with joint inflammation. Since pain-induced grip strength deficit is poorly characterized in rodents, we made a full characterization of this pain outcome before exploring the role of sigma-1 receptors on the modulation of opioid effects during inflammatory pain.

The detailed rationale and specific goals of each of these parts of this PhD Thesis are described below.

Modulation by sigma-1 receptors of μ-opioid effects on heat and mechanical nociceptive pain

Opioid agonists, particularly those of the μ-receptor subtype such as morphine, oxycodone and buprenorphine, are widely used to treat moderate to severe pain (Al-Hasani and Bruchas, 2011; Pasternak and Pan, 2011). Opioid receptors are located at

different sites along the pain-processing pathway, including both the central (spinal cord and different supraspinal nuclei) and peripheral nervous system (dorsal root ganglion [DRG] and peripheral nerve terminals) (Bigliardi-Qi et al., 2004; Khalefa et al., 2012).

Although opioid analgesia is thought to be stronger at central levels (Porreca et al., 2002), peripherally administered opioid agonists can undoubtedly exert analgesic effects under some circumstances. In fact, morphine induces robust topical antinociceptive effects to heat stimulation in rodents (Kolesnikov and Pasternak, 1999), whereas this drug was unable to induce antinociception to a mechanical stimulus when administered locally (Sánchez-Fernández et al., 2013). These preclinical results are consistent with the preferential peripheral actions of morphine on heat rather than mechanical nociception in human volunteers (Koppert et al., 1999). However, it remains unknown whether this modality-specific peripheral effect of morphine is a general characteristic of μ-opioid drugs and, therefore, also occurs with other clinically relevant μ-agonists. Taking into account these antecedents, our **first goal** was to compare peripheral antinociceptive actions in response to heat and mechanical stimuli induced by clinically relevant μ-agonists of different intrinsic efficacy.

Sigma-1 receptors are located in several areas of the central nervous system of great relevance for pain control (Alonso et al., 2000; Kitaichi et al., 2000; Roh et al., 2008; Sánchez-Fernández et al., 2014; Rodríguez-Muñoz et al., 2015a); nonetheless, the greatest concentration of sigma-1 receptors is found in the DRG (Sánchez-Fernández et al., 2014), pointing to a prominent role for these peripheral receptors in pain modulation. We recently reported that peripheral sigma-1 antagonism was able to greatly potentiate the peripheral opioid antinociception induced by a variety of μ-agonists in response to mechanical stimulation (Sánchez-Fernández et al., 2013 and 2014), findings which suggests that peripheral sigma-1 receptors constantly inhibit opioid functioning and thus constitute a tonically active biological brake to opioid mechanical analgesia (Sánchez-Fernández et al., 2017). However, distinct subsets of high-threshold peripheral sensory neurons are activated by mechanical or thermal stimuli (Zylka et al., 2005; Cavanaugh et al., 2009; Scherrer et al., 2009; Bardoni et al., 2014) and it is unknown whether peripheral sigma-1 receptors similarly modulate μ-opioid antinociception to mechanical or thermal stimulation. Therefore, the **second goal**

of this PhD Thesis was to compare the effect of pharmacological and genetic inactivation of sigma-1 receptors on peripherally-mediated μ-opioid-induced heat and mechanical antinociception.

Heat-sensitive nociceptors correspond mainly to peptidergic C-type neurons that express transient receptor potential vanilloid 1 (TRPV1), whereas the C non-peptidergic subtype (which can be labelled with isolectin B4 [IB4]) is sensitive to mechanical stimuli (Zylka et al., 2005; Cavanaugh et al., 2009; Scherrer et al., 2009; Bardoni et al., 2014). Peripheral μ-opioid receptors are densely expressed in TRPV1 C-type neurons, but they are also present in other peripheral sensory neurons (Scherrer et al., 2009; Bardoni et al., 2014; Chiu et al., 2014). Since in the studies performed to fulfill the aims described in the paragraph above, we found that peripheral sigma-1 receptors modulate peripheral opioid antinociception to mechanical but not to heat stimuli, we hypothesized that the modality-specific peripheral effects of μ-opioid agonists might be related to a differential modulation by peripheral sigma-1 receptors of the opioid effects in different subsets of peripheral neurons. Therefore, the **third goal** of this PhD Thesis was to compare the effect of peripheral sigma-1 receptor blockade on peripheral μ-opioid antinociception in animals after the ablation of TRPV1-expressing peptidergic C-type peripheral sensory neurons.

Grip strength in mice with joint inflammation: a rheumatology function test sensitive to pain and analgesia

Despite major advances in our understanding of pain mechanisms in recent decades, there has been little translation of new analgesics from bench to bedside (Mao, 2009; Kissin, 2010; Barret, 2015). The predictive validity of animal models of pain has been intensely debated, and one possible reason for the limited translation of pain research is the differences in outcome measures used to evaluate pain and analgesia in experimental animals and human patients (Mogil and Crager, 2004; Negus et al., 2006; Cobos and Portillo-Salido, 2013; Negus, 2013; Percie and Rice, 2014). Ideally, for the purposes of translation, animal testing should mimic as closely as possible routine clinical practice and clinical trials. Standard outcome measures used in preclinical chronic pain research have been adapted from quantitative sensory testing (QST)

designed for the evaluation of patients with chronic pain, and von Frey filaments are one of the most widely used QST instruments to determine the mechanical pain threshold in preclinical research. In human patients, QST procedures are used to detect sensory alterations during neuropathic pain (e.g. Bennet, 2001; Bouhassira et al., 2005; Moharic et al., 2012). However, the use of QST in patients with rheumatic diseases is rare. To our knowledge only three published studies used von Frey filaments in human patients with rheumatoid arthritis (Morris et al., 1997; Hendiani et al., 2003; van Laarhoven et al., 2013), one of the most worrisome painful conditions that occurs with joint inflammation (Scott et al., 2000; Lee, 2013). This low number of clinical reports with von Frey testing is in marked contrast to the hundreds of preclinical studies that have used this technique in animal models of joint pain.

Pain is a complex phenomenon. Part of the core of the human pain phenotype includes alterations in physical functioning, which negatively impacts several aspects of daily life in patients with painful diseases (Turner et al., 2005; Romera et al., 2011). Because of the important relationship between pain and physical functioning, one set of consensus-based recommendations advocates measuring physical function as one of the main outcomes in clinical trials of treatments for pain (Dworkin et al., 2008). In this connection, grip strength has been widely and routinely evaluated for decades in rheumatology as a functional measure in patients with joint inflammation (e.g. Bijlsma et al., 1987; Pincus and Callahan, 1992; Lee, 2013), and remarkably, it is known to correlate to pain (Callahan et al., 1987; Fraser et al., 1999; Overend et al., 1999). Despite the widespread use of grip strength in rheumatology, this outcome is poorly characterized as a pain measure in experimental animals. However, as noted above, preclinical studies of tactile allodynia are abundant. It is known that transient receptor potential (TRP) channels or TRP-expressing nociceptors participate in inflammatory cutaneous hypersensitivity (Szallasi et al., 2007), but much less is known about the neurobiological mechanisms leading to pain-induced functional disability.

In light of these antecedents, our **fourth goal** was to compare the sensitivity of grip strength in mice with joint inflammation *vs.* inflammatory tactile allodynia to the effects of several analgesic drugs of different pharmacological classes, and to test whether the

appearance of grip strength deficits and tactile allodynia arose from the same mechanisms.

Modulation of morphine-induced antinociceptive effects and tolerance by sigma-1 receptors: studies on nociceptive heat pain and on tactile allodynia and grip strength deficits during joint inflammation

It is known from preclinical studies that sigma-1 inhibition not only enhances the antinociception induced by opioid drugs, including morphine (reviewed in Zamanillo et al., 2013; Sánchez-Fernández et al., 2017), but it is also able to rescue morphine antinociception in mice rendered tolerant to this opioid (Vidal-Torres et al., 2013; Rodríguez-Muñoz et al., 2015b). Therefore, sigma-1 antagonists are promising tools as opioid adjuvants (Vela et al., 2015; Sánchez-Fernández et al., 2017). However, all these preclinical findings on the enhancement of opioid antinociception by sigma-1 inhibition have been exclusively demonstrated on models of nociceptive pain. It is known that during inflammation there are changes in opioid receptor functioning (reviewed in Stein et al., 2009); therefore, the involvement of sigma-1 receptor on the modulation of opioid antinociception during inflammation might not be the same than in a non-injured condition.

Taking into account these antecedents, the **fifth goal** of this PhD Thesis was to test whether the sigma-1 receptor antagonist S1RA could enhance morphine antinociception or modulate morphine analgesic tolerance during inflammatory pain. We measured both the antiallodynic effect of morphine and the recovery on grip strength deficits induced by this opioid, as a measure of cutaneous sensory hypersensitivity and the impact of pain on physical function, respectively. As a control for the known effects of sigma-1 antagonism on nociceptive pain, we also performed experiments on heat antinociception in animals without inflammation. This would allow us to compare the effects of morphine, S1RA, and their association, in nociceptive heat pain, inflammatory tactile allodynia and functional deficits (grip strength) induced by inflammatory pain.

MATERIAL AND METHODS, RESULTS AND DISCUSSION

1. MODULATION BY SIGMA-1 RECEPTORS OF μ-OPIOID EFFECTS ON HEAT AND MECHANICAL NOCICEPTIVE PAIN

1.1. MATERIAL AND METHODS

1.1.1. Experimental animals

Female CD1 wild-type (WT) (Charles River, Barcelona, Spain) and homozygous sigma-1 receptor knockout mice (sigma-1-KO) (Laboratorios Esteve, Barcelona, Spain) were used in all experiments. Knockout mice were generated on a CD-1 background as previously described (Entrena et al., 2009b). Animals weighing 25–30 g were tested randomly throughout the estrous cycle. They were housed in colony cages with free access to food and water prior to the experiments, and were maintained in temperature- and light-controlled rooms (22 ± 2 °C, lights on at 08:00 h and off at 20:00 h). The experiments were performed during the light phase (from 9:00 h to 15:00 h). The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments. For behavioral experiments, after habituation the mice were randomized to treatment groups. Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, Granada, Spain), regional (Junta de Andalucía, Spain) and international standards (European Communities Council directive 2010/63).

1.1.2. Drugs and drug administration

We used the μ-opioid agonists morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), oxycodone hydrochloride and buprenorphine hydrochloride (Sigma-Aldrich Química SA, Madrid, Spain). We also tested the effects of the opioid antagonists naloxone hydrochloride and naloxone methiodide (Sigma-Aldrich Química SA). Naloxone is a centrally-penetrant opioid antagonist, whereas its quaternary derivative naloxone methiodide is unable to cross the blood–brain barrier and is therefore a useful tool to determine peripheral opioid

effects (e.g. Menéndez et al., 2005; Sánchez-Fernández et al., 2014). S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)1H-pyraol-3-yl]oxy]ethyl]morpholine hydrochloride) was used as a selective sigma-1 antagonist (Romero et al., 2012) (DC Chemicals, Shanghai, China). PRE-084 ([2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate) hydrochloride]) was used as a selective sigma-1 agonist (Hayashi and Su, 2004; Cobos et al., 2008) (Tocris Cookson Ltd., Bristol, United Kingdom). All drugs were dissolved in sterile physiologic saline (0.9% NaCl); the solution of PRE-084 was alkalinized as appropriate with NaOH. To evaluate the effects of systemic treatments, drugs or their solvents were injected s.c. into the interscapular area in a volume of 5 mL/kg. When the effect of the association of two drugs administered s.c. was tested, each drug was injected into different parts of the interscapular area. To test for local effects of treatments, drugs or their solvents were administered i.pl. in a volume of 20 µL with a 1710 TLL Hamilton micro-syringe (Teknokroma, Barcelona, Spain) and a 30G × 1/2-inch gauge needle. When opioids and S1RA were associated i.pl., they were dissolved in the same solution and injected (in a volume of 20 µL) together to avoid paw lesions due to multiple injections in the same paw, except in experiments in which opioids were injected in one hindpaw and S1RA in the other.

1.1.3. Evaluation of mechanical nociception (paw pressure)

Mechanical stimulation was applied to the hindpaw of animals with a pressure analgesimeter (Model 37215, Ugo-Basile, Varese, Italy) as previously described (Menéndez et al., 2005; Sánchez-Fernández et al., 2013 and 2014). Briefly, mice were gently pincer grasped between the thumb and index fingers by the skin above the interscapular area. Then a blunt cone-shaped paw-presser exerting a pressure of 450 g was applied to the dorsal surface of the hindpaw until the animal showed a struggle response (see Supplementary Video 1, which demonstrates the procedure used to measure mechanical nociception). The latency in seconds from paw stimulation to the struggle response was measured with a digital chronometer.

1.1.4. Evaluation of heat nociception (unilateral hot plate)

Heat nociception was assessed as previously described (Menendez et al., 2002). To obtain comparable results between mechanical and thermal nociception, the mice were held gently in the same manner as described above. Then the plantar side of the stimulated hindpaw was placed on the surface of a thermal analgesiometer (Model PE34, Series 8, IITC Life Science Inc., Los Angeles, USA) previously set at 55 ± 1 °C until the animal showed a paw withdrawal response. The latency in seconds from paw stimulation to the behavioral response was measured with a digital chronometer. Only a clear unilateral withdrawal of the paw was recorded as the nociceptive response. We avoided simultaneous heat stimulations in both hindpaws by placing the plantar side of the tested hindpaw on the hot plate while the other hindpaw was placed on filter paper (off the hot plate) during observations (see Supplementary Video 2, which demonstrates the procedure used to measure heat nociception).

1.1.5. General procedures for behavioral assays and drug treatments

All handling of the rodents before and during the evaluation, as well as the test environment, were identical for evaluations of mechanical and heat nociception with the exception of the sensory stimulus used (see Supplementary Video 1 and Video 2). The animals were placed in the experimental room for a 1-h acclimation period before starting the experiments. The behavioral responses of untreated mice tested with 55 °C and 450 g pressure force were very similar (non-significantly different) (See Fig. 1, dose 0). The similarities between the evaluation procedures and baseline responses of the mice to both types of sensory stimulation facilitated comparisons of drug effects on mechanical and heat nociception. To study the effects of systemic opioids, morphine and oxycodone were administered s.c. 30 min before paw stimulation; buprenorphine was administered s.c. 1 h before the evaluation, because its antinociceptive effect is much slower than the other opioids (Yassen et al., 2005). To explore the contribution of peripheral opioid receptors to μ-opioid antinociception, the effects of naloxone and naloxone methiodide (or saline) were compared. These opioid antagonists were

administered s.c. 5 min before the μ-agonist. To test the effect of local treatments (S1RA, μ-agonists or the association of both), the animals were evaluated 5 min after i.pl. injection to minimize possible systemic effects induced by drug treatments. To study the contribution of sigma-1 receptors to the effects induced by the local administration of opioids alone or in association with S1RA, PRE-084 was administered s.c. 30 min before i.pl. administration. This sigma-1 agonist was administered at a dose of 32 mg/kg, since it was shown in previous studies to reverse the effect of the local administration of sigma-1 antagonists (Sánchez-Fernández et al., 2014). Control groups received the same volume of drug vehicle.

All mice were used in only one experimental procedure (mechanical or thermal nociception). Evaluations were done twice alternately in each hindpaw at intervals of 1 min between each stimulation. A 50-s cut-off was used for each measurement to prevent tissue damage. The mean value of the two averaged measurements for each hindpaw was used to analyze the effects of systemic (s.c.) treatment, because animals showed similar response latencies during stimulation in each hindpaw. However, in local (i.pl.) drug treatment experiments, the average of the two values was considered independently for each paw (ipsi- and contralateral). The experimenters who evaluated the behavioral responses were blinded to the treatment group and genotype of each experimental animal.

1.1.6. In vivo ablation of TRPV1-expressing nociceptors

To investigate the effects of the ablation of TRPV1-expressing neurons on mechanical and thermal nociception, we administered resiniferatoxin (RTX) (Tocris Cookson Ltd.) dissolved in vehicle (10% Tween 80 and 10% ethanol in normal saline). Each animal received a single dose of RTX (50 μg/kg) via i.p. injection (Hsieh et al., 2008; Montilla-García et al., 2017). The control group received an equal volume of vehicle. To minimise suffering, RTX or vehicle was injected under isoflurane anaesthesia (IsoVet®, B. Braun, Barcelona, Spain). After the i.p. injection, the mice were returned to their home cages for 5 days before behavioral testing and sample collection for immunostaining assays.

1.1.7. Immunohistochemistry

Mice were anesthetized with isoflurane and perfused transcardially with formaldehyde solution 4.0% wt/wt (Scharlab, Sentmenat, Spain). The L4 DRGs were dissected and post-fixed in formaldehyde solution for 1 h at room temperature. Samples were dehydrated and embedded in paraffin with standard procedures. Tissue sections 5 µm thick were cut with a sliding microtome, deparaffinized in xylol (Panreac Quimica, Castellar del Vallès, Spain) and rehydrated before antigen retrieval (steam heating for 22 min with 1% citrate buffer, pH 8). Sections were incubated for 1 h in blocking solution (5% normal donkey serum or goat serum, depending on the experiment, 0.3% Triton X-100, 0.1% Tween 20 in Tris buffer solution). After blocking, the slides were incubated for 1 h at room temperature with the primary antibodies in blocking solution. The primary antibodies used were: TRPV1 goat anti-mouse (sc-12498, 1:100, Santa Cruz Biotechnology, Heidelberg, Germany), sigma-1 mouse anti-mouse (sc-137075, 1:200, Santa Cruz Biotechnology) or NeuN rabbit anti-mouse (ABN78, 1:500, Merck Millipore, Madrid, Spain). NeuN was used as a pan-neuronal marker which labels neuronal nuclei, perikarya, and to a lesser extent the cytoplasm (Mullen et al., 1992; Wolf et al., 1996). After incubation with the primary antibodies, tissue sections were washed three times for 10 min and incubated for 1 h with the secondary antibodies Alexa Fluor-488 donkey anti-goat (A11055, 1:500), Alexa Fluor-488 goat anti-mouse (A11029, 1:500) or Alexa Fluor-594 goat anti-rabbit (A11012, 1:500) (all from Life Technologies, Alcobendas, Spain). We also stained tissue sections with Bandeiraea simplicifolia lectin I, isolectin B4 (IB4) conjugated with Dylight-594 (DL-1207, 1:200, Vector Laboratories Ltd., Peterborough, United Kingdom). The slides were then washed three times for 10 min and mounted with ProLong® Gold Antifade Mountant (Life Technologies). In some experiments, slides were incubated for 5 min with Hoechst 33342 for nucleic acid staining (Life Technologies, 1:1000), and washed three times before the mounting procedure. Staining patterns were examined with a confocal laser-scanning microscope (Model A1, Nikon Instruments Europe BV, Amsterdam, Netherlands). Fluorescence intensity of sigma-1 receptor staining in IB4+ neurons and other small DRG neurons (soma size similar to or smaller than IB4+ neurons) was quantified with ImageJ 1.50g image analysis software (National Institutes of Health, Bethesda, MD, USA).

Quantification was performed in DRG slices from 5 different animals. Fluorescence intensity was measured in 30 random small neurons in each slice by an experimenter blinded to the IB4 labelling.

1.1.8. Data and statistical analysis

When several means were compared, statistical analysis was done with one- or two-way ANOVA depending on the experiment; a Bonferroni post-hoc test was done in both cases. To compare fluorescence intensity of sigma-1 receptor staining in IB4+ neurons and other small DRG neurons, we used the unpaired Student's t test. Data were analyzed with the SigmaPlot 12.0 application (Systat Software Inc., San Jose, CA, USA). In all tests, the differences between means were considered significant when the P value was below 0.01.

1.2. RESULTS

1.2.1. Effects of systemic μ-opioid agonists on mechanical and heat nociception in wild-type mice: differential contribution of peripheral opioid receptors

The response latency in control mice exposed to mechanical or thermal stimuli was similarly short, and less than 5 s in both cases (Fig. 1, dose 0). The s.c. administration of buprenorphine (0.0625–0.5 mg/kg), oxycodone (1–4 mg/kg) or morphine (4–16 mg/kg) induced dose-dependent antinociceptive effects in WT mice to both mechanical or thermal stimuli (Fig. 1, closed and open symbols, respectively). The antinociceptive effects induced by the full μ-agonists morphine and oxycodone were particularly robust, and at the highest doses tested the response latencies were close to the cut-off time of 50 s for heat antinociception and about 20 s for the mechanical stimulus (Fig. 1).

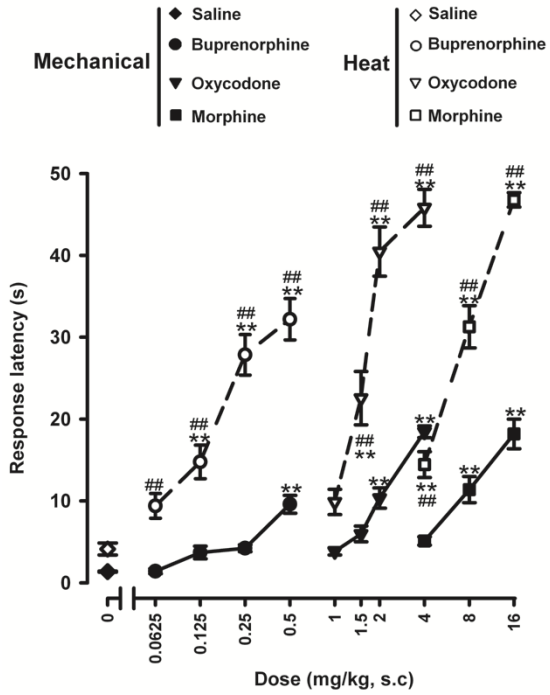

Fig. 1. Comparison of the antinociceptive effects induced by the systemic administration of different μ-opioid agonists on nociceptive pain induced by mechanical and heat stimulation in wild-type mice. The results represent the response latency during stimulation of the hindpaws with 450 g pressure or 55 °C. Each animal was evaluated in only one pa in test and received only one s.c. dose of μ-opioid agonist (or saline). Each point and vertical line represents the mean ± SEM of values obtained in both hindpaws in 8 animals. Statistically significant differences between the values obtained in saline- and opioid-treated groups: **P < 0.01; and between the values obtained for mechanical and heat stimulation at the same dose of opioid: ##P < 0.01 (two-way ANOVA followed by Bonferroni test).

The partial μ-agonist buprenorphine showed more limited antinociceptive effects, but as observed for the other opioid drugs tested, its effects were more prominent for heat than for mechanical antinociception (Fig. 1).

We compared the sensitivity of the effects induced by systemic opioids to the peripherally restricted opioid antagonist naloxone methiodide. Animals were treated s.c. with morphine (16 mg/kg), oxycodone (3 mg/kg), buprenorphine (0.5 mg/kg) or their solvent in association with naloxone methiodide (2–6 mg/kg, s.c.) or its solvent. Naloxone methiodide at any dose tested did not alter the increase in struggle response latency induced by morphine or buprenorphine to mechanical stimulation (Fig. 2A). However, the antinociceptive effect induced by oxycodone was slightly reversed by the highest dose of this peripheral opioid antagonist (Fig. 2A).

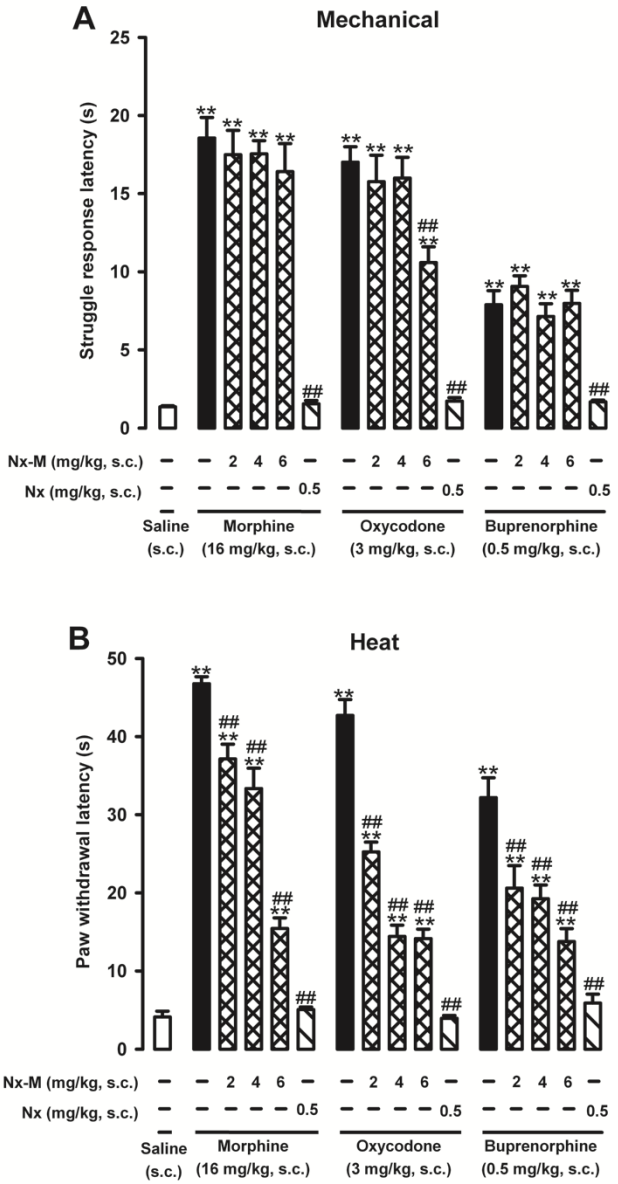

Fig. 2. Contribution of peripheral opioid receptors to the antinociception induced by the systemic administration of different μ-opioid agonists on pain induced by mechanical and heat stimulation in wild-type mice. The results represent the response latency during stimulation of the hindpaws with (A) 450 g pressure or (B) 55 °C. Animals were treated s.c. with an opioid agonist (morphine 16 mg/kg, oxycodone 3 mg/kg, or buprenorphine 0.5 mg/kg) or its solvent (saline), associated with the s.c. injection of naloxone (Nx, 0.5 mg/kg), naloxone methiodide (Nx-M, 2–6 mg/kg) or their solvent (saline). Each animal was evaluated in only one pain test and received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in both hindpaws in 8 animals. Statistically significant differences between the values obtained in animals treated with saline and the μ-opioid agonists: $^{**}P < 0.01$; and between the groups treated with the different μ-opioid agonists alone and associated with Nx or Nx-M: $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

In contrast, the antinociceptive effect on thermal stimulation in animals treated with all three μ-opioid agonists was markedly and dose-dependently reversed by naloxone methiodide (Fig. 2B).

The centrally-penetrant opioid antagonist naloxone (0.5 mg/kg, s.c.) completely reversed the antinociceptive effect induced by all μ-agonists in mice exposed to the mechanical or thermal stimulus (Fig. 2A and 2B, respectively).

Therefore, the contribution of peripheral opioid receptors to the antinociceptive effects induced by systemic μ-opioid analgesics differed depending on the type of stimulus, and was more prominent for heat than for mechanical antinociception.

1.2.2. Effects of local (intraplantar) μ-opioid agonists on mechanical and thermal nociception in wild-type and sigma-1-knockout mice

In contrast to the antinociceptive effects induced by systemic opioids to mechanical stimulation in WT mice described above, the i.pl. administration of buprenorphine (3.125–25 μg), oxycodone (12.5–50 μg) or morphine (25–200 μg) did not induce significant mechanical antinociceptive effects (Fig. 3A, closed symbols). However, the same doses of i.pl. opioids induced marked and dose-dependent antinociceptive effects to heat stimulation (Fig. 3B closed symbols). These results highlight the modality-specific effects of peripheral opioid antinociception.

We then compared the antinociceptive effects induced by the i.pl. administration of buprenorphine, oxycodone or morphine in WT and sigma-1-KO mice. The response latency to mechanical and heat stimuli was similar in WT and sigma-1-KO mice treated with the solvent of the opioid analgesics (Fig. 3A and B, dose 0). Despite the absence of local antinociceptive effects of i.pl. opioids in response to mechanical stimulation in WT mice, sigma-1-KO animals showed robust dose-dependent antinociceptive effects with each of the three μ-opioid agonists tested. The antinociceptive effects after mechanical stimulation in sigma-1-KO mice were particularly prominent for oxycodone and morphine, but weaker for buprenorphine (Fig. 3A). In marked contrast, the antinociceptive effects of i.pl. opioid agonists after heat stimulation were similar in WT and sigma-1-KO mice (Fig. 3B). None of the treatments tested here modified the response latency when the stimulus was applied to the hindpaw contralateral to the injection, with the exception of the highest dose of oxycodone, which induced a

significant antinociceptive effect after mechanical stimulation in sigma-1-KO mice, and an antinociceptive effect after heat stimulation in mice of both genotypes (Fig. 4A and 4B, respectively).

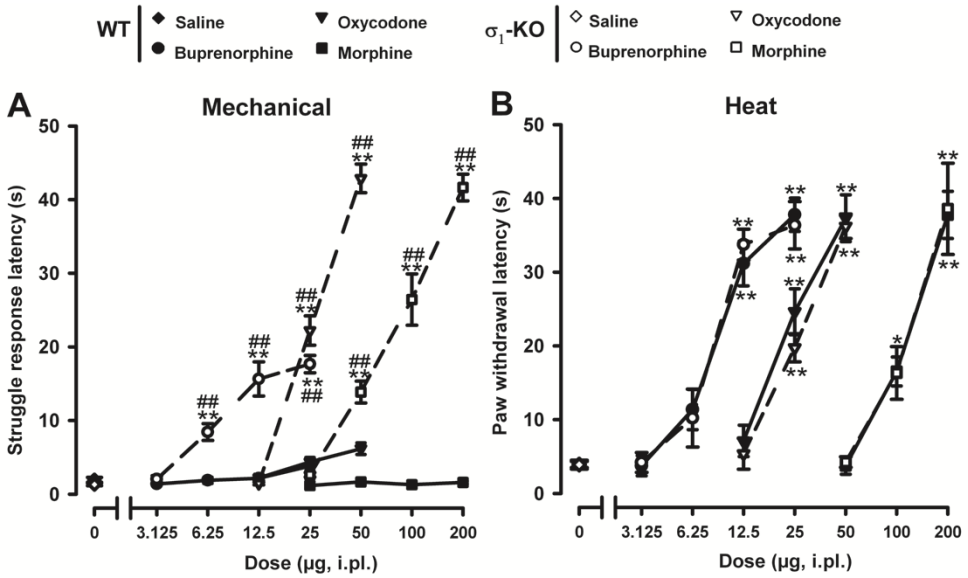

Fig. 3. Mechanical and heat antinociception induced by the local administration of different μ-opioid receptor agonists in wild-type (WT) and sigma-1 knockout (σ_1-KO) mice. The results represent the response latency during stimulation with (A) 450 g pressure or (B) 55 °C of the injected hindpaw in mice treated intraplantarly (i.pl.) with the μ-opioid agonists. Each animal was evaluated in only one pain test and received only one dose of drug or saline. Each point and vertical line represents the mean ± SEM of values obtained in the injected hindpaw in 8 animals. Statistically significant differences between the values obtained in saline- and opioid-treated groups: $^{**}P < 0.01$; and between the values obtained in WT and σ_1-KO mice at the same dose of a given opioid: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test).

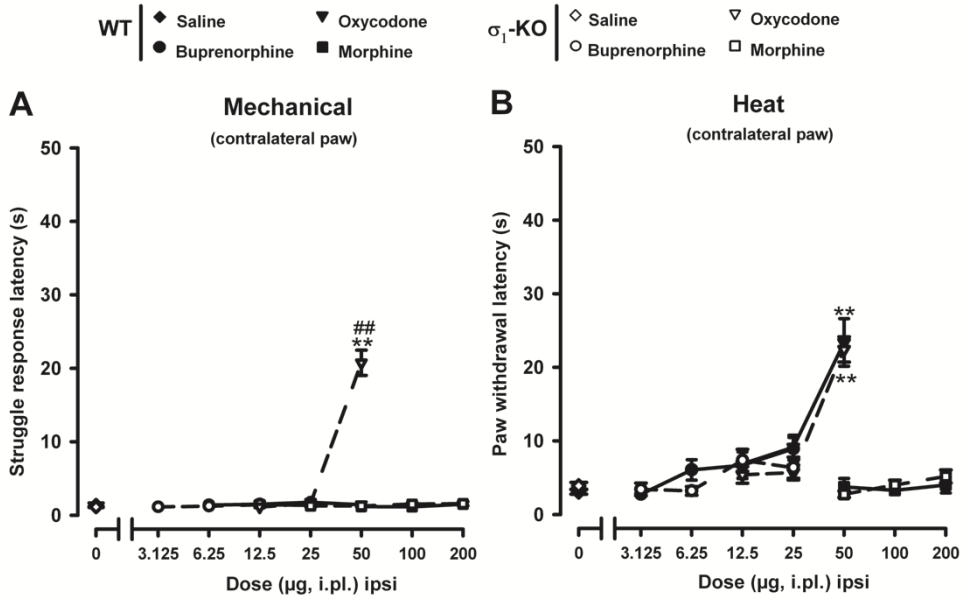

Fig. 4. Mechanical and heat nociception in the paw contralateral to the site of intraplantar administration of different μ-opioid agonists in wild-type (WT) and sigma-1 knockout (σ_1-KO) mice. The results represent the response latency during stimulation with (A) 450 g pressure or (B) 55 °C on the hindpaw contralateral to the site of drug injection in mice treated intraplantarly (i.pl.) in the ipsilateral (ipsi) paw with the μ-opioid agonists. Each animal was evaluated in only one pain test and received only one dose of drug or saline. Each point and vertical line represents the mean ± SEM of values obtained in the non-injected hindpaw in 8 animals. Statistically significant differences between the values obtained in saline- and opioid-treated groups: **P < 0.01; and between the values obtained in WT and σ_1-KO mice at the same dose of a given opioid: ##P < 0.01 (two-way ANOVA followed by the Bonferroni test).

1.2.3. Effects of sigma-1 drugs on mechanical antinociception induced by local (intraplantar) administration of μ-opioid agonists

To test whether the local pharmacological blockade of sigma-1 receptors in WT mice replicated the phenotype seen in sigma-1-KO mice exposed to mechanical stimulation, we studied the effects of the co-administration of S1RA with the opioids (or their solvents) to the hindpaw. The response latency to mechanical stimulation was similarly short in WT mice stimulated in the paw treated with morphine 200 μg alone or in the contralateral (untreated) hindpaw, indicating the absence of antinociceptive effects of morphine to mechanical stimulation (Fig. 5A, dose 0 of S1RA).

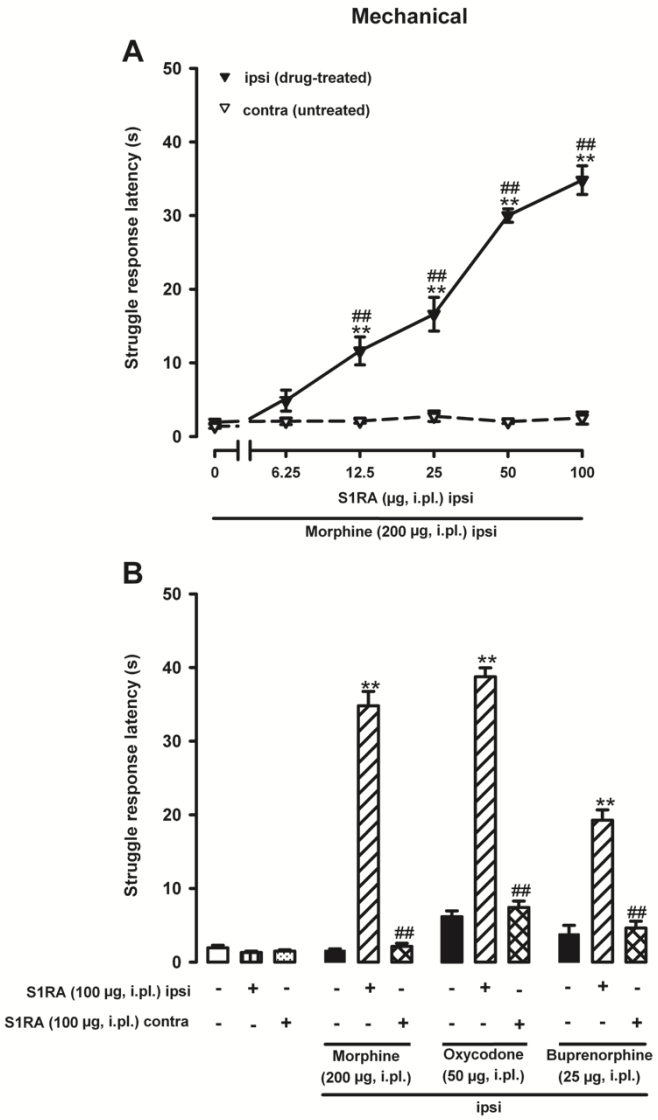

Fig. 5. Effects of the local administration of the sigma-1 receptor antagonist S1RA on mechanical antinociception induced by the local administration of different µ-opioid agonists in wild-type mice. The results represent the struggle response latency during mechanical stimulation in: (A) mice treated intraplantarly (i.pl.) with morphine (200 µg) co-administered with S1RA (6.25–100 µg) or its solvent (saline, dose 0), and stimulated in the injected (ipsilateral, ipsi) and non-injected (contralateral, contra) hindpaw; (B) the hindpaw ipsilateral to the site of µ-opioid agonist injection (morphine 200 µg, oxycodone 50 µg or buprenorphine 25 µg) alone or associated with S1RA (100 µg) in the same or in the contralateral paw. (A and B) Each animal received only one treatment. Each point or bar and vertical line represents the mean ± SEM of values obtained in 8 animals. (A) Statistically significant differences between the values obtained in the groups treated with S1RA and its solvent: **P < 0.01, and between the values obtained in the ipsilateral and contralateral hindpaw at the same dose of S1RA (or saline): $^{\#\#}$P < 0.01 (two-way ANOVA followed by Bonferroni test). (B) Statistically significant differences between the values obtained in the groups treated with S1RA or its solvent in the paw ipsilateral to the opioid injection: **P < 0.01; and between the groups treated with S1RA in the paw ipsilateral or contralateral to the opioid injection: $^{\#\#}$P < 0.01 (one-way ANOVA followed by Bonferroni test).

The co-administration of S1RA (6.25–100 μg) with this dose of morphine induced a dose-dependent increase in struggle response latency after mechanical stimulation in the injected hindpaw (Fig. 5A, closed symbols), without altering the behavioral response in mice stimulated in the hindpaw contralateral to the site of drug administration (Fig. 5A, open symbols), suggesting that these effects were not produced systemically. Treatment with S1RA (100 μg) alone was devoid of antinociceptive effects (1.9 ± 0.3 s in controls vs 1.3 ± 0.2 s in S1RA-treated mice).

We also compared the effects of the i.pl. administration of oxycodone (50 μg) and buprenorphine (25 μg) alone or associated with S1RA (100 μg). As observed for the results with morphine, the local administration of oxycodone or buprenorphine alone was devoid of antinociceptive effects in response to mechanical stimulation, but the co-administration of S1RA (at a dose which had no effect per se) with these opioids induced a robust antinociceptive effect (Fig. 5B). This enhanced antinociceptive effect was weaker for buprenorphine compared to morphine or oxycodone (Fig. 5B). None of the treatments produced any effect in the non-injected paw (Fig. 6A), and importantly, when S1RA was administered i.pl. in the hindpaw contralateral to the morphine, oxycodone or buprenorphine injection, there was no increase in the struggle response latency in the paw in which the opioids were injected (Fig. 6A).

These results rule out any possible systemic effect of the i.pl. administration of S1RA and demonstrate that this sigma-1 antagonist is able to locally potentiate opioid-induced mechanical antinociception at peripheral sites.

We also evaluated the effects of the sigma-1 agonist PRE-084 (32 mg/kg, s.c.) on the mechanical antinociception induced by the local co-administration of S1RA with the opioid agonists, and found that treatment with the sigma-1 agonist abolished the S1RA-induced potentiation of μ-opioid antinociception to mechanical stimulation (Fig. 7). These results support the selectivity of the effects induced by S1RA.

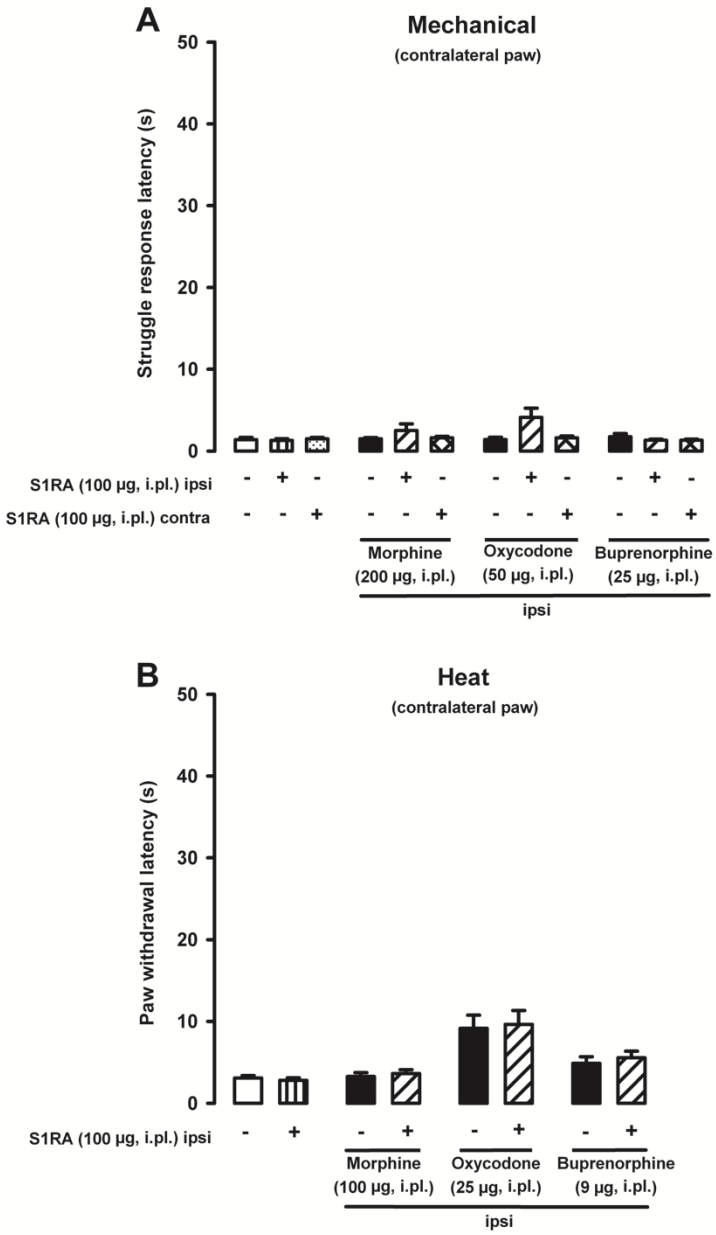

Fig. 6. Mechanical and heat nociception in the paw contralateral to the site of intraplantar co-administration of S1RA with different µ-opioid agonists in wild-type mice. The results represent the response latency during (A) mechanical stimulation of the hindpaw contralateral to the injection of the µ-opioid agonist when the opioid agonist was injected in the ipsilateral (ipsi) paw alone or associated to S1RA, and when the opioid agonist was injected in the ipsilateral paw and S1RA in the contralateral (contra) paw, and (B) heat stimulation of the hindpaw contralateral to the site of injection of the µ-opioid agonist alone or associated to S1RA, when the drugs were injected in the ipsilateral (ipsi) paw. (A and B) Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in 8 animals. None of the treatments produced any statistically significant effect in the paw contralateral to the opioid injection (one-way ANOVA followed by Bonferroni test).

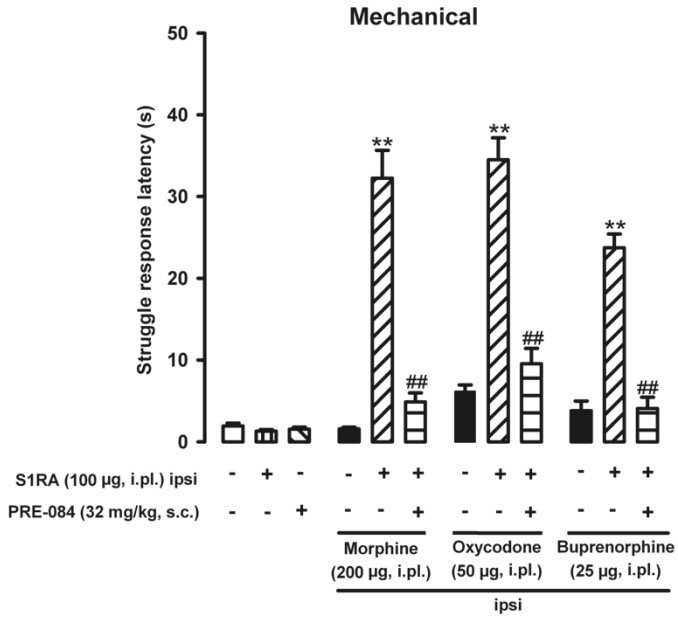

Fig. 7. Effects of the systemic administration of the sigma-1-receptor agonist PRE-084 on mechanical antinociception induced by local co-administration of different µ-opioid agonists and S1RA in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure in the hindpaw of mice given an intraplantar (i.pl.) injection of a µ-opioid agonist (morphine 200 µg, oxycodone 50 µg or buprenorphine 25 µg) alone or with S1RA (100 µg, i.pl.) and associated with the s.c. administration of PRE-084 (32 mg/kg) or its solvent. Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in 8 animals. Statistically significant differences between the values obtained in the groups treated with the opioids + S1RA and animals treated with the opioids alone or their solvent: **P < 0.01; and between the values obtained in the groups treated with opioids + S1RA alone or associated with PRE-084: ##P < 0.01 (one-way ANOVA followed by Bonferroni test).

1.2.4. Effects of sigma-1 drugs on heat antinociception induced by local (intraplantar) administration of µ-opioid agonists

We tested the effects on heat nociception of the co-administration of S1RA (25–100 µg) with morphine, oxycodone or buprenorphine (or their solvent) to the hindpaw. The i.pl. administration of morphine (200 µg) induced an increase in the response latency to heat stimulation applied to the injected paw, whereas the latency in the hindpaw contralateral to the site of drug administration remained short (Fig. 8A, dose 0 of S1RA). In contrast to the marked potentiation by the co-administration of S1RA of the local mechanical antinociceptive effect induced by morphine 200 µg as reported above in the previous section, S1RA did not increase the antinociceptive effect to heat stimulation induced by the same dose of morphine (Fig. 8A).

Because this i.pl. dose of morphine exerted a marked antinociceptive effect to heat stimulation, we also tested the effects of S1RA co-administration with a lower dose of morphine (100 μg) to avoid a possible ceiling effect which would make it difficult to detect an increase in morphine antinociception induced by S1RA. Although the antinociceptive effect of morphine 100 μg was lower than morphine 200 μg, co-administration with S1RA was still unable to modify the effect of this opioid (Fig. 8A). The co-administration of S1RA with morphine did not modify the response latency in mice stimulated in the hindpaw contralateral to the drug-treated paw (Fig. 8A, open symbols).

As seen regarding the absence of effects of S1RA on heat antinociception induced by local morphine, this sigma-1 antagonist did not increase the local antinociceptive effect to heat stimulation induced by submaximal doses of oxycodone (25 μg) or buprenorphine (9 μg) (Fig. 6B). None of the treatments had any effect in the non-injected paw (Fig. 6B). These results clearly showed that peripheral sigma-1 antagonism was not able to modulate heat antinociception induced by peripheral μ-opioid agonism.

We also tested whether sigma-1 agonism was able to decrease peripheral μ-opioid antinociception in response to heat stimulation. The administration of PRE-084 (32 mg/kg, s.c.) did not result in alterations in the antinociceptive effects induced by i.pl. morphine (200 μg), oxycodone (50 μg) or buprenorphine (12.5 μg) (Fig. 9). Therefore, neither sigma-1 antagonism nor agonism led to modifications in peripheral μ-opioid antinociception to heat stimulation.

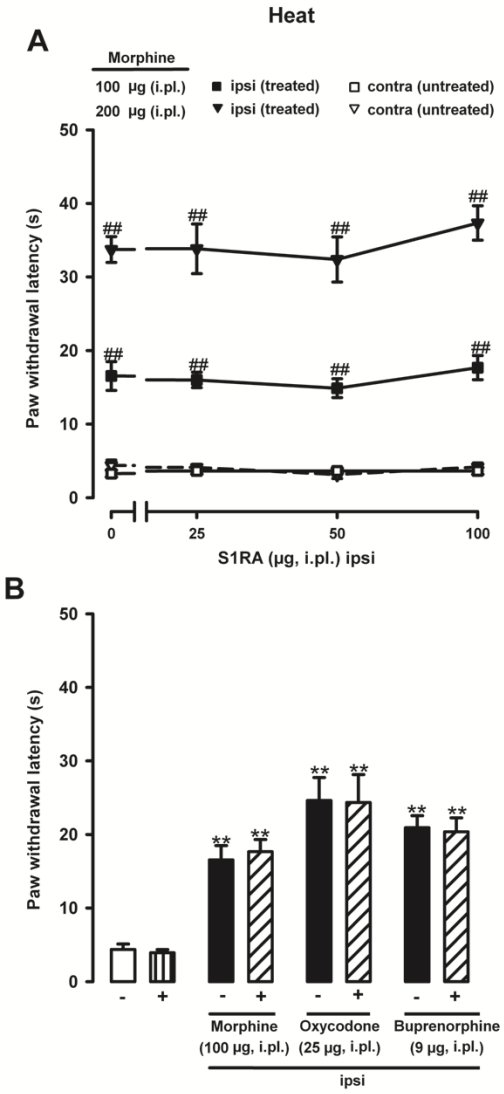

Fig. 8. Effects of the local administration of the sigma-1 receptor antagonist S1RA on heat antinociception induced by the local administration of different µ-opioid agonists in wild-type mice. The results represent the paw withdrawal latency during heat stimulation in: (A) mice treated intraplantarly (i.pl.) with morphine (100 or 200 µg) associated with S1RA (6.25–100 µg) or its solvent (saline, dose 0), and stimulated in the injected (ipsilateral, ipsi) and non-injected (contralateral, contra) hindpaw; (B) the hindpaw ipsilateral to the site of µ-opioid agonist injection (morphine 100 µg, oxycodone 25 µg or buprenorphine 9 µg) alone or co-administered with S1RA (100 µg). (A and B) Each animal received only one treatment. Each point or bar and vertical line represents the mean ± SEM of values obtained in 8 animals. (A) Statistically significant differences between the values obtained in the hindpaw ipsilateral and contralateral to the same dose of morphine: $^{\#\#}P < 0.01$. There were no statistically significant differences between the values obtained in the groups treated with S1RA or its solvent (two-way ANOVA followed by Bonferroni test). (B) Statistically significant differences between the values obtained in the groups treated with the opioids or their solvent: $^{**}P < 0.01$. There were no statistically significant differences between the values obtained in the groups treated with the opioid agonists injected alone or with S1RA (one-way ANOVA followed by Bonferroni test).

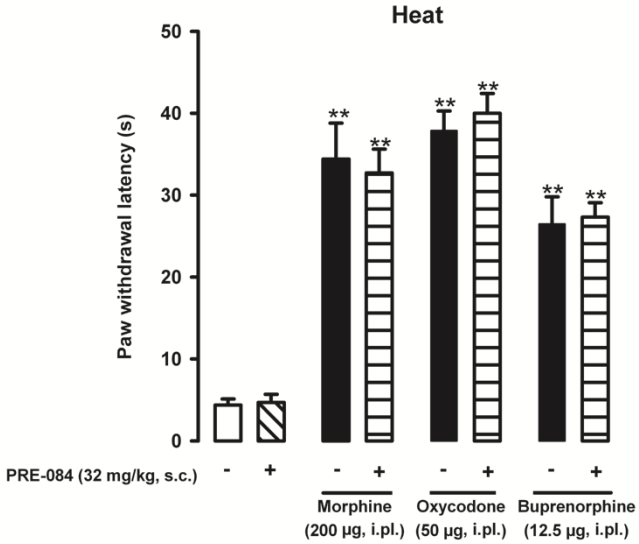

Fig. 9. Effects of the systemic administration of the sigma-1-receptor agonist PRE-084 on heat antinociception induced by the local administration of opioid agonists in wild-type mice. The results represent the paw withdrawal latency during heat stimulation of the ipsilateral hindpaw of mice treated intraplantarly (i.pl.) with an opioid agonist (morphine 200 µg, oxycodone 50 µg or buprenorphine 12.5 µg) alone or associated with the s.c. administration of PRE-084 (32 mg/kg) or their solvent. Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in the injected (ipsilateral) hindpaw in 8 animals. Statistically significant differences between the values obtained in the groups treated with the opioids or their solvent: **P < 0.01. There were no statistically significant differences between the values obtained in the groups treated with PRE-084 or its solvent (one-way ANOVA followed by Bonferroni test).

1.2.5. Contribution of TRPV1-expressing neurons to mechanical and heat nociception, and to the modulatory effects of sigma-1 receptors on peripheral µ-opioid antinociception

We then tested whether the behavioral effects seen were mediated by TRPV1-expressing neurons. Staining for IB4 and TRPV1 showed minimal overlap among DRG neurons from intact mice (Fig. 10A, top panels). Treatment with RTX completely abolished TRPV1 labelling although IB4 staining remained (Fig. 10A, bottom panels), confirming the specificity of the ablation procedure. In addition, RTX treatment did not alter response latency to nociceptive mechanical stimulation in either of the two hindpaws (Fig. 10B).

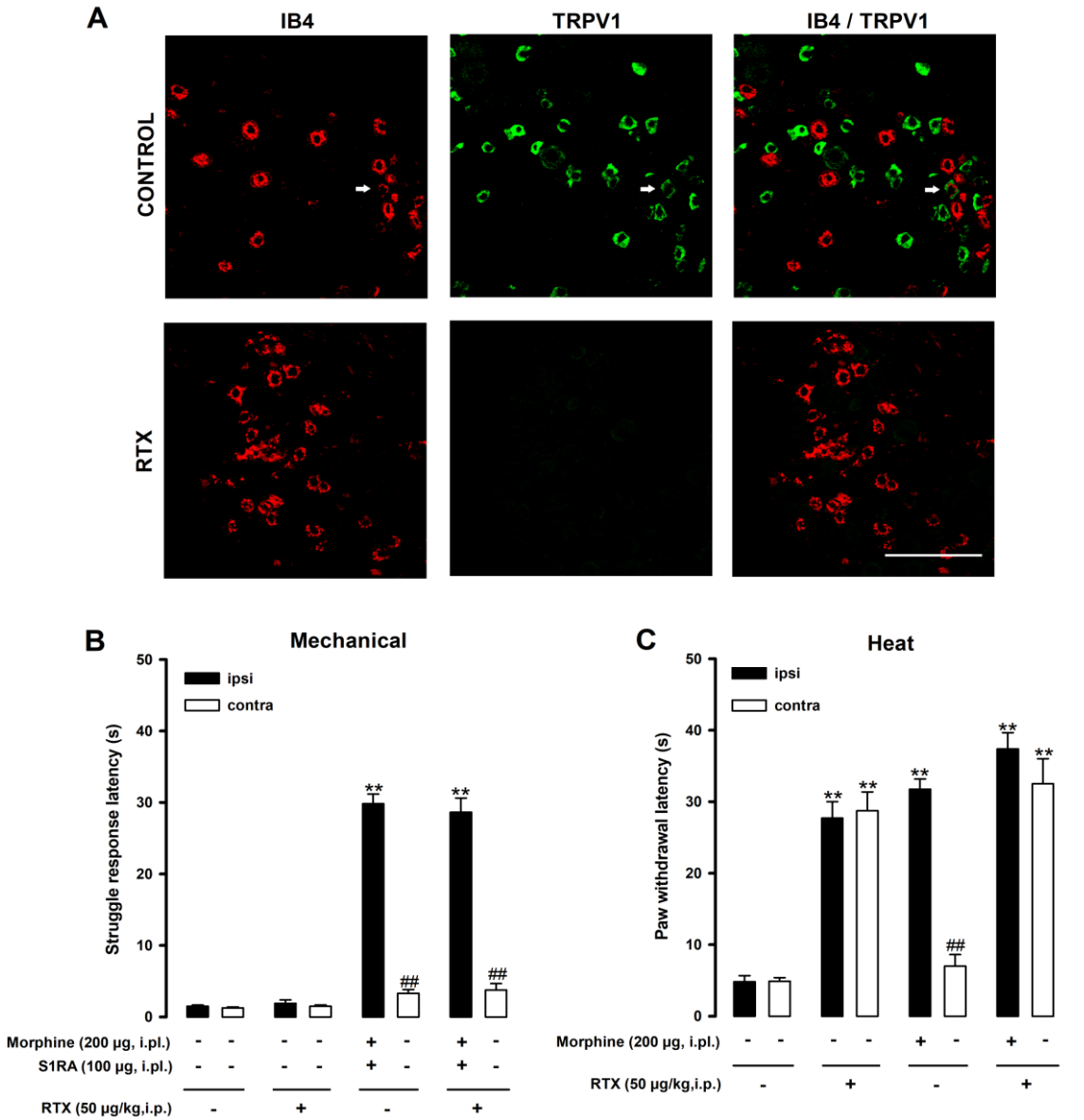

Fig. 10. Effect of the ablation of TRPV1-expressing neurons on mechanical and heat nociception, and on the modulatory effects of sigma-1 receptors on peripheral μ-opioid antinociception. Animals were treated i.p. with resiniferatoxin (RTX, 50 μg/kg) or its solvent five days before obtaining samples or performing the behavioral experiments. (A) Double labelling of isolectin B4 (IB4, red) and TRPV1 (green) in the L4 dorsal root ganglion. Top panels: samples from control (solvent-treated) wild-type (WT) mice. White arrow indicates co-localization of both markers. Bottom panels: samples from WT-mice treated with RTX. Scale bar, 100 μm. (B and C) The behavioral results represent: (B) the struggle response latency during stimulation with 450 g pressure in the hindpaw of WT-mice pretreated with RTX or its solvent, and treated with an intraplantar (i.pl.) injection of morphine (200 μg) co-administered with S1RA (100 μg) or their solvent; (C) the paw withdrawal latency during stimulation with 55 °C in the hindpaw of WT-mice pretreated with RTX or its solvent, and i.pl. injected with morphine (200 μg) or its solvent. (B and C) Mice were stimulated in the paw ipsilateral (ipsi) or contralateral (contra) to the opioid injection. Each animal was evaluated in only one pain test and received only one treatment. Each bar and vertical line represents the mean ± SEM of 8 animals. Statistically significant differences compared to mice treated with solvent only: **P < 0.01 (one-way ANOVA followed by Bonferroni test). Statistically significant differences between the values obtained in the hindpaw ipsilateral and contralateral to the opioid injection for each group: ##P < 0.01 (one-way ANOVA followed by Bonferroni test).

The i.pl. co-administration of S1RA and morphine induced a marked increase in the response latency to mechanical stimulation in the injected paw compared to solvent-treated mice, and this increase was similar in RTX-treated and RTX-untreated mice (Fig. 10B, black bars). Latency values in the contralateral non-injected paw remained unchanged and indistinguishable from control values in all experimental groups (Fig. 10B, white bars). Therefore, TRPV1-expressing neurons did not participate in the response to nociceptive mechanical stimuli or in the modulatory effect induced by sigma-1 receptors on peripheral μ-opioid antinociception to mechanical stimulation.

In contrast, TRPV1 neuron ablation induced a bilateral increase in the response latency to the nociceptive heat stimulus (Fig. 10C). The i.pl. administration of morphine induced a significant increase in the time to response to heat stimulation in the injected paw (but not in the contralateral hindpaw) in mice treated with the RTX solvent. When we tested the effects of i.pl. morphine in RTX-treated mice, we found that the response latency was equally high in the injected and the non-injected hindpaw and non-significantly different from that obtained in animals treated with RTX and the solvent of morphine (Fig. 10C), which indicates that local peripheral morphine administration was not able to produce antinociception in RTX-treated mice.

The lack of effect of i.pl. morphine was not due to the high latency values observed in animals treated with RTX, since the systemic administration of morphine (16 mg/kg, s.c.) induced a marked increase in the response latency after heat stimulation despite ablation of the TRPV1 neurons (Fig. 11). This indicated that successful analgesic treatment was able to increase the response latency in TRPV1-ablated mice. Increased response latency was thus achieved by systemic morphine, which is able to act both centrally and peripherally, but not by the i.pl. administration of this opioid. These results suggested that morphine acted on TRPV1 neurons to induce a local peripheral antinociceptive effect on the response to heat stimulation.

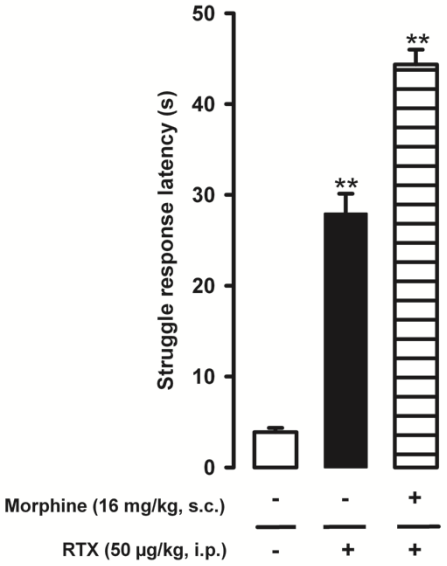

Fig. 11. Effect of systemic morphine on thermal nociception after ablation of TRPV1-expressing neurons. Animals were treated i.p. with resiniferatoxin (RTX, 50 µg/kg) or its solvent five days before the behavioral experiments. The results represent the paw withdrawal latency during stimulation with 55 °C in the hindpaw of wild-type mice pretreated with RTX or its solvent, and treated with a subcutaneous (s.c.) injection of morphine (16 mg/kg) or its solvent. Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of 8 animals. Statistically significant difference compared to mice treated with solvent only: **P < 0.01; and between the values obtained in RTX-treated mice treated with morphine or its solvent: $^{\#\#}$P < 0.01 (one-way ANOVA followed by Bonferroni test).

1.2.6. Expression of sigma-1 receptors in the dorsal root ganglion of wild-type and sigma-1-knockout mice

To study the expression of sigma-1 receptors in the DRG, we used immunohistochemical double labelling for the neuronal marker NeuN and sigma-1 receptors (Fig. 12). The pan-neuronal marker NeuN was located in the somas of all neurons from WT mice. Sigma-1 receptor immunoreactivity was also found in DRG cells. Merging the two images showed that cells that expressed NeuN and sigma-1 receptor formed completely overlapping cell populations (Fig. 12, top panels). These results indicated that all sensory neurons expressed sigma-1 receptors. In sigma-1-KO mice, NeuN staining was preserved but sigma-1 receptor labelling was completely absent (Fig. 12, bottom panels), supporting the specificity of the sigma-1 receptor antibody used.

Higher magnification photomicrographs showed that NeuN staining was more intense within a rounded area in DRG neurons, which overlapped with Hoechst 33342 staining, indicating the location of neuronal nuclei. This area was devoid of sigma-1 immunostaining, clearly indicating that sigma-1 receptors were not detected inside neuronal nuclei (see Fig. 13).

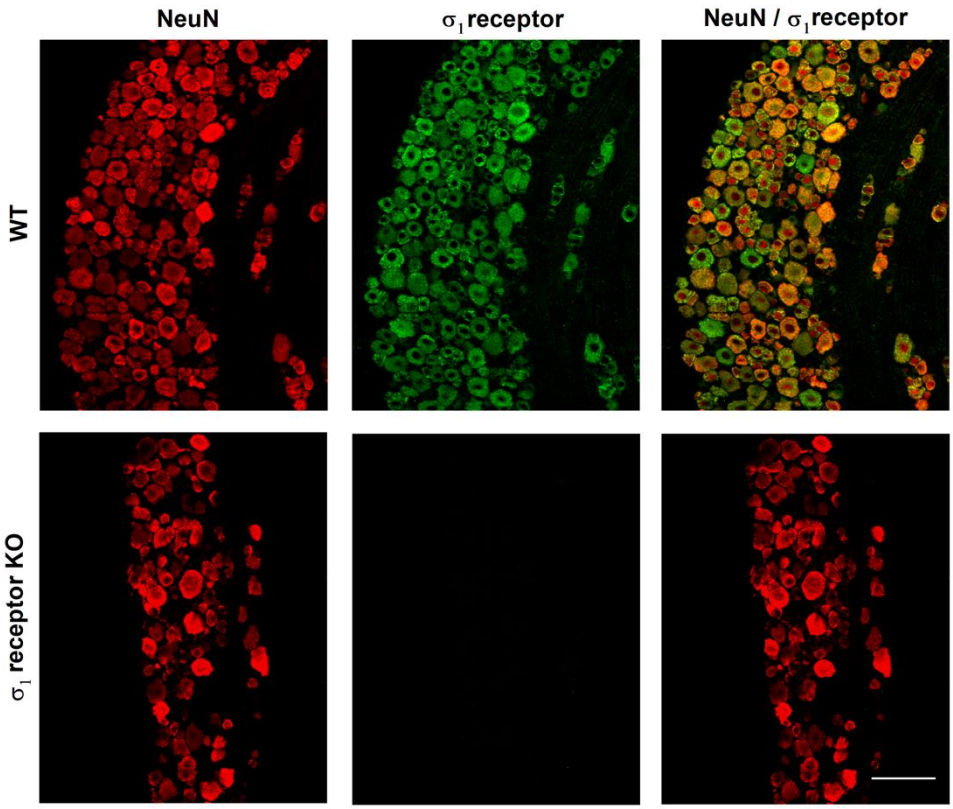

Fig. 12. Sigma-1 receptors are expressed in all dorsal root ganglion neurons. Double labelling of NeuN (red) and sigma-1 (σ_1) receptors (green) in the L4 dorsal root ganglion. Top panels: samples from wild-type (WT) mice. Bottom panels: samples from σ_1-knockout (σ_1-KO) mice. Scale bar, 100 µm.

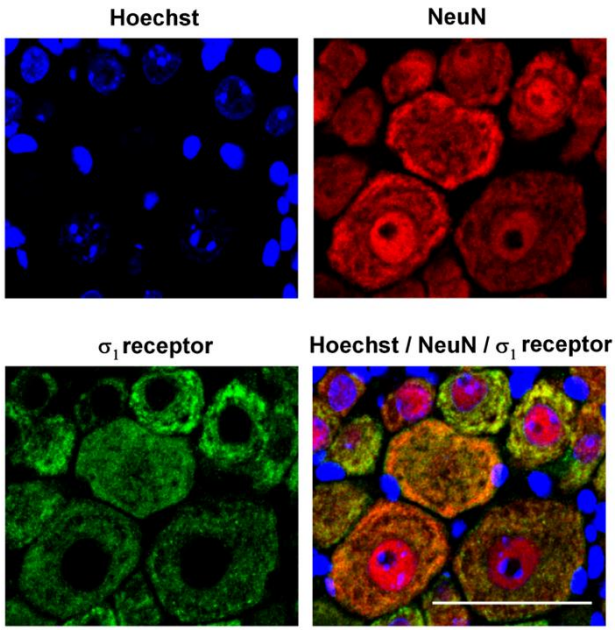

Fig. 13. Sigma-1 receptors are expressed selectively in dorsal root ganglion neurons, with wide distribution in the cytoplasm and plasma membrane, but not inside the neuronal nuclei. Hoechst 33342 (Hoechst) staining (blue), and NeuN (red) and sigma-1 (σ_1) receptor (green) labelling in the L4 dorsal root ganglion. Scale bar, 50 µm.

Interestingly, other Hoechst-stained cell nuclei were seen in non-neuronal cells (NeuN-negative cells) in close proximity to NeuN-labelled neurons, and these cells were devoid of sigma-1 receptor staining, as this was completely restricted to NeuN-labelled cells (see Fig. 13).

Although we found that sigma-1 receptors were expressed in all DRG neurons, the intensity of sigma-1 receptor labelling appeared to be much higher in IB4+ neurons than in all other small nociceptive neurons in the DRG (Fig. 14A). In fact, when we quantified signal intensity, we found that it was 2.3-fold higher in IB4+ neurons than in other small DRG neurons (Fig. 14B). These distinct expression levels of sigma-1 receptors in subsets of nociceptive neurons may account for the differential modulation by sigma-1 receptor inhibition of μ-opioid agonist-induced mechanical and heat antinociception.

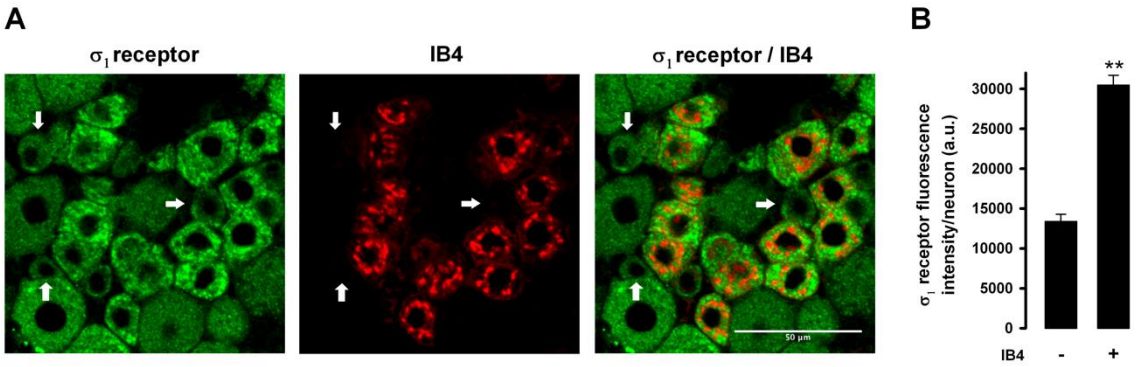

Fig. 14. Sigma-1 receptors are expressed at higher density in IB4+ neurons than in other small dorsal root ganglion (DRG) neurons. (A) Double labelling of sigma-1 (σ_1) receptors (green) and IB4 (red) in the L4 DRG. White arrows indicate the location of IB4− small neurons. (B) Fluorescence intensity of sigma-1 receptor staining in IB4+ and IB4− small DRG neurons, expressed in arbitrary units (a.u.). Statistically significant differences between sigma-1 receptor expression in the two groups of neurons: **P < 0.01 (unpaired Student's t test).

1.3. DISCUSSION

In this study we show that the clinically relevant μ-opioid analgesics morphine, buprenorphine and oxycodone induced prominent peripheral antinociceptive effects to a heat stimulus but little (oxycodone) or no peripheral antinociceptive effects to a mechanical stimulus under control conditions (in the absence of sigma-1 receptor

inhibition). However, sigma-1 inhibition markedly boosted peripherally-mediated mechanical antinociception induced by μ-opioid agonists without affecting peripheral μ-opioid effects on heat nociception.

We found that peripheral opioid receptor antagonism (with naloxone methiodide) did not alter mechanical antinociception induced by systemic buprenorphine or morphine and only slightly inhibited oxycodone effect. These results are consistent with our previous findings (Sánchez-Fernández et al., 2014), and are compatible with the presumed central analgesic effects of opioids (Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008). In marked contrast, the antinociceptive effects of buprenorphine, morphine and oxycodone on the response to a heat stimulus were highly sensitive to naloxone methiodide, indicating that peripheral opioid receptors play a more prominent role in heat- than in mechanical-antinociception induced by μ-opioid agonists. This notion is further supported by our finding that the local peripheral administration of the opioid agonists tested induced a robust antinociceptive effect at the site of administration in response to heat- but not to mechanical-stimulus. Our results are congruent with previous studies showing that peripheral morphine exerts analgesia to heat but not to mechanical nociceptive pain in both humans (Koppert et al., 1999) and rodents (Kolesnikov and Pasternak, 1999; Sánchez-Fernández et al., 2013), and extend these earlier findings by showing that the striking modality-specific peripheral effects seen with morphine are also a characteristic of other clinically relevant μ-agonists.

Although the local peripheral administration of buprenorphine, morphine or oxycodone was unable to induce mechanical antinociception in WT mice, all these opioids induced a robust antinociceptive effect to mechanical stimulation in sigma-1-KO mice. These results were replicated in assays based on the pharmacological inhibition of peripheral sigma-1 receptors: the co-administration of S1RA with the μ-opioids resulted in a strongly synergistic mechanical antinociceptive effect (greater than the sum of the individual effects of μ-opioids or S1RA alone) at the site of administration of the combined drug solution, but not in the contralateral paw. This finding suggests that the interaction was produced locally. The effects induced by S1RA were reversed by the known sigma-1 agonist PRE-084, and this finding argues in favor of a sigma-1-mediated

action on the effects induced by S1RA. All these results indicate that peripheral sigma-1 receptors constitute a biologic brake to peripheral μ-opioid antinociception in the response to a mechanical stimulus, and extend our previous finding of the synergistic effects of sigma-1 antagonism and peripheral opioid antinociception (Sánchez-Fernández et al., 2013 and 2014).

In marked contrast to the results for mechanical nociceptive pain, the local peripheral administration of buprenorphine, morphine or oxycodone exhibited a similar level of thermal antinociception in WT and sigma-1-KO mice, and the heat antinociception induced by the local peripheral administration of these μ-opioid agonists was not modified by their co-administration with S1RA. These results differ from those obtained when sigma-1 antagonists were administered systemically: both the non-selective sigma-1 drug haloperidol and the selective sigma-1 antagonist S1RA clearly enhanced opioid antinociception to heat stimulation (Chien and Pasternak, 1994 and 1995; Marrazzo et al., 2006; Vidal-Torres et al., 2013). In addition, central sigma-1 inhibition is known to markedly increase opioid antinociception to a heat stimulus (Pan et al., 1998; Mei and Pasternak, 2002 and 2007). Together, these results show that although sigma-1 receptors tonically inhibit opioid antinociception to heat stimulation, this must occur at the central but not at the peripheral level.

It was reported that sigma-1 agonism is able to decrease heat antinociception induced by systemic or central opioids (Chien and Pasternak, 1994). We therefore hypothesized that although the peripheral tonic inhibitory activity of the sigma-1 system might be too weak to allow sigma-1 antagonism to enhance opioid antinociception to heat, treatment with the sigma-1 agonist PRE-084 might be able to decrease heat antinociception induced by peripherally administered opioids. However, we show here that sigma-1 agonism did not modify peripheral μ-opioid-induced heat antinociception. Our findings thus show for the first time that peripherally-mediated μ-opioid antinociception to heat stimuli is clearly dissociated from the actions of sigma-1 receptors.

In the mouse TRPV1+ and IB4+ cells constitute separate neuronal populations, as shown here and in previous studies (Zwick et al., 2002; Woodbury et al., 2004), and correspond to peptidergic and non-peptidergic C-nociceptors, respectively (Priestley et al., 2009).

Heat-sensitive nociceptors correspond mainly to TRPV1-expressing C-nociceptors (Scherrer et al., 2009; Bardoni et al., 2014), whereas non-peptidergic C-nociceptors are mechanosensitive (Cavanaugh et al., 2009; Scherrer et al., 2009). The ablation of TRPV1-expressing neurons by RTX in our mice resulted in marked impairment of the nociceptive responses to heat stimulation, as also reported in other studies (Menéndez et al., 2006; Hsieh et al., 2012a; Zhang et al., 2013). Moreover, we show that although the local peripheral administration of morphine induced marked peripheral antinociception in response to heat stimulation in intact mice, it was unable to induce further antinociceptive effects in TRPV1-ablated mice, indicating that this opioid acts on TRPV1 neurons to induce a local antinociceptive effect on this type of stimulus. It is known that there is heavy crosstalk between TRPV1 and μ-opioid receptors (reviewed in Bao et al., 2015), so the prominent effects induced by the local administration of morphine in the heat nociception assays reported here may be due to modulation of the action of TRPV1 receptors in peptidergic C-nociceptors.

In marked contrast to the results discussed above, the ablation of TRPV1-expressing neurons by RTX in our mice left IB4+ cells unaltered and did not affect the responses to mechanical stimulation, indicating that the TRPV1-expressing neuronal population is dispensable for mechanical nociception, as shown here and in previous studies (Mishra and Hoon, 2010; Zhang et al., 2013). In addition, we show that RTX treatment did not modify the effect of the local administration of morphine injected alone or with S1RA on mechanical stimulation, indicating that sigma-1 receptors tonically inhibit peripheral μ-opioid antinociception in sensory neurons other than TRPV1-expressing afferents. Therefore, we hypothesized that differential expression of the sigma-1 receptor in subsets of peripheral nociceptive neurons might underpin the differential modulation by sigma-1 receptor antagonism of μ-opioid peripherally mediated heat- and mechanical-antinociception.

We found that sigma-1 receptors were located in the somas of all DRG neurons, with a neuron-specific distribution. These results are in agreement with a recent study of the location of sigma-1 receptors in the DRG (Mavlyutov et al., 2016). Although the antibody used in our study (sc-137075) was recently reported to intensely stain tissue from sigma-1-KO mice (Mavlyutov et al., 2016), we show that sigma-1 receptor immunostaining was

completely absent in our mutant mice, supporting the specificity of the antibody. The marked differences between our findings and previous studies may be due to the use of frozen sections embedded in optimal cutting temperature (OCT) compound by Mavlyutov and colleagues vs paraffin-embedded samples in the present study, and to a possible effect of our antigen retrieval procedure, which might improve antibody selectivity. It is worth pointing out that the knockout mice used in the two studies were generated with different procedures, which may also account for the differences. However, staining with the sc-137075 antibody was also seen in our sigma-1 knockout mice when the procedure was performed with frozen sections embedded in OCT (data not shown). These results suggest that the differences in antibody selectivity may be attributable to the experimental procedures rather than to the type of sigma-1 knockout mouse used.

Although sigma-1 receptors were located in all DRG neurons, the distribution of these receptors was not homogeneous: we show that the expression levels of sigma-1 receptors were much higher in IB4+ neurons than in all other small nociceptive neurons in the DRG. As previously noted, IB4+ neurons are well known to code for mechanical nociception. Therefore, the enrichment of sigma-1 receptor expression in this type of sensory neuron may account for our finding that sigma-1 antagonism acted preferentially on opioid-induced peripheral antinociception in response to mechanical stimuli.

Our results on nociceptive pain may seem to contradict a recent study showing that sigma-1 antagonism fully ameliorated both mechanical and thermal hyperalgesia in mice with carrageenan-induced inflammation, by enhancing the peripheral actions of endogenous opioid peptides (Tejada et al., 2017). The interaction between sigma-1 receptors and their protein targets is known to be Ca^{2+}-dependent (Rodríguez-Muñoz et al., 2015b), and inflammatory mediators can induce a marked increase in intracellular Ca^{2+} concentration, leading to nociceptor sensitization (Linhart et al., 2003; Richardson and Vasko, 2002). Therefore, inflammation might trigger plastic changes in heat-sensing peripheral neurons that enable opioid modulation by sigma-1 receptors, although further studies are needed to clarify this issue. It is worth noting that the potency of sigma-1 antagonists in ameliorating inflammatory mechanical hyperalgesia is much

higher than for heat hyperalgesia (Tejada et al., 2107). This suggests that even under inflammatory conditions, sigma-1 receptors play a more prominent role in the modulation of peripheral opioid effects in response to mechanical than to heat stimulation, which is consistent with our results.

2. GRIP STRENGTH IN MICE WITH JOINT INFLAMMATION: A RHEUMATOLOGY FUNCTION TEST SENSITIVE TO PAIN AND ANALGESIA

2.1. MATERIAL AND METHODS

2.1.1. Experimental animals

Experiments were done in female CD1 mice (Charles River, Barcelona, Spain) weighing 28–30 g at the beginning of the study. We chose female animals because it has been reported that women may be at greater risk for pain-related disability than men (e.g. Unruh, 1996; Stubbs et al., 2010), but no previous studies have evaluated grip strength as a measure of pain-induced functional disability in female animals. Animals were tested randomly throughout the estrous cycle. They were housed in colony cages with free access to food and water prior to the experiments, and were kept in temperature- and light-controlled rooms (22 ± 2°C, and light–dark cycle of 12 h). The experiments were done during the light phase (from 9:00 AM to 3:00 PM). All experimental protocols were carried out in accordance with international standards (European Communities Council directive 2010/63), and were approved by the Research Ethics Committee of the University of Granada. To decrease the number of animals in this study, we used the same mice for behavioral studies, histological analysis and immunostaining, when possible.

2.1.2. CFA-induced periarticular inflammation

Mice were injected periarticularly with complete Freund's adjuvant (CFA) (Sigma-Aldrich, Madrid, Spain) or sterile physiological saline (0.9% NaCl) as a control around the tibiotarsal joint. CFA (or saline) was administered subcutaneously in two separate injections to the inner and outer side of the joint in a volume of 10 or 15 µL/injection (20 or 30 µL/paw), to obtain homogeneous inflammation (Chen et al., 2009; Lolignier et al., 2011). We used a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a 30½-gauge needle under isoflurane anesthesia (IsoVet®, B. Braun, Barcelona, Spain). CFA-treated mice had prominent inflammation that appeared to be restricted to

the administration site and nearby areas (heel), whereas the paw pad did not appear to be affected. This allowed us to test the mechanical threshold in these two distinct areas. See “Results” for details. Because weight loss or delayed weight gain are considered signs of ongoing distress (Blackburn-Munro, 2004), body weight was monitored daily to ensure that our protocol did not induce excessive harm to the animals. Inflammatory edema was monitored by measuring ankle thickness with an electronic caliper (e.g. Croci and Zarini, 2007).

2.1.3. Drugs and drug administration

We used the following prototypic analgesics: the nonsteroidal antiinflammatory drug (NSAID) ibuprofen sodium salt (10–80 mg/kg), the cyclooxygenase-2 (COX-2) inhibitor celecoxib (40–160 mg/kg), and acetaminophen (40–320 mg/kg) (all from Sigma-Aldrich), and the opioids tramadol (10–80 mg/kg) and oxycodone hydrochloride (1–8 mg/kg) (supplied by Laboratorios Esteve, Barcelona, Spain). We also tested the effects of the antispastic baclofen (5–20 mg/kg) (Sigma-Aldrich). All drugs were dissolved in 0.5% hydroxypropyl methylcellulose (HPMC) with the exception of celecoxib and acetaminophen, which were suspended in HPMC supplemented with 1% Tween 80 (both from Sigma-Aldrich). These drugs or their solvents were administered orally (p.o.) in a volume of 10 mL/kg.

In addition, we also tested the effects of ruthenium red (1–2 mg/kg) (Sigma-Aldrich), a nonselective TRP antagonist (St Pierre et al., 2009). Ruthenium red was dissolved in saline and administered subcutaneously (s.c.) into the interscapular zone in a volume of 5 mL/kg. The control group received an equal volume of saline.

In all cases, behavioral evaluations after drug administration were recorded by an observer blinded to the treatment.

2.1.4. *In vivo ablation of TRP vanilloid 1 (TRPV1)-expressing nociceptive neurons*

We used resiniferatoxin (RTX) to selectively ablate TRPV1-expressing neurons. The drug (Tocris Cookson Ltd, Bristol, UK) was dissolved in 10% Tween 80 and 10% ethanol in normal saline. Animals received a single dose of RTX (50 µg/kg) via intraperitoneal injection, which has been previously reported to ablate all peripheral TRPV1+ neurons (Hsieh et al., 2012a). The control group received an equal volume of vehicle. All procedures were done under isoflurane anesthesia to minimize distress, 5 days before behavioral testing or sample collection.

2.1.5. *Measurement of grip strength*

Grip strength was measured with a computerized grip strength meter (Model 47200, Ugo-Basile, Varese, Italy). The apparatus consisted of a T-shaped metal bar connected to a force transducer. To measure grip strength in the hindpaws of the mice, the experimenter held the mouse gently by the base of the tail, allowing the animal to grasp the metal bar with its hindpaws. To prevent mice from gripping the metal bar with their forepaws during the recording, the animals were first allowed to grasp a wire mesh cylinder with their forepaws. As soon as the mice grasped the transducer metal bar with their hindpaws, the experimenter pulled the animals backwards by the tail until grip was lost (see Supplemental Video 3, which demonstrates the procedure used to measure hindlimb grip strength). The peak force of each measurement was automatically recorded in grams (g) by the device. Hindlimb grip strength in each mouse was measured in triplicate. Basal grip strength values were recorded for each animal as the average of two determinations on different days before the administration of CFA or saline. This value was considered as 100% of grip strength and used as a reference for subsequent determinations.

2.1.6. Measurement of von Frey threshold

Mechanical allodynia to a punctate stimulus was determined with a slight modification of a previously described method (Chaplan et al., 1994). Briefly, animals were acclimated for 2 h in methacrylate test compartments (7.5 cm wide x 7.5 cm long x 15 cm high) placed on an elevated mesh-bottomed platform, to provide access to the plantar surface of the hindpaws. Plantar stimulation is the standard site of application of von Frey filaments in animals with joint inflammation (e.g. Nieto et al., 2015; Mangione et al., 2016). A logarithmic series of calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA), with bending forces that ranged from 0.02 to 1.4 g, were applied using the up–down paradigm, starting with the 0.6 g filament. Filaments were applied two times for 2–3 s, with inter-application intervals of at least 30 s to avoid sensitization to the mechanical stimuli. The response to the filament was considered positive if immediate licking/biting, flinching or rapid withdrawal of the stimulated paw was observed. As for grip strength determinations, basal values were recorded for each animal as the average of two determinations on different days, before the administration of CFA or saline. This value was considered 100% of the von Frey threshold and used as a reference for subsequent determinations.

2.1.7. Locomotor activity measurements

Ambulatory locomotion was measured with an infrared detector (Med associated Inc., St. Albans, VT, USA) equipped with 48 infrared photocell emitters and detectors, according to a previously described method (Sánchez-Fernández et al., 2013) with slight modifications. Briefly, animals were placed individually in transparent evaluation chambers (27.5 cm wide x 27.5 cm long x 20 cm high) and the distance traveled (horizontal activity) and the number of rears (vertical activity) were recorded during 30 min. Animals were tested only once to avoid habituation to the evaluation chambers, which markedly decreases their locomotor activity. All experiments were done in a sound-proofed room with dim light (7–10 lux; Luxometer SM700, Milkwaukee 4

Electronics Kft., Szeged, Hungary). No experimenters were present in the testing room during the evaluation period.

2.1.8. Histology

Mice were anesthetized with isoflurane (IsoVet®, B. Braun, Barcelona, Spain) and perfused intracardially with 20 mL saline followed by 4% formaldehyde solution. After perfusion, the paws were dissected and fixed with 10% buffered formalin for 48 h at room temperature. Joints were then decalcified in Anne Morse solution (50 mL formic acid and 50 mL 20% sodium citrate) at room temperature for 14 days. Next, they were sectioned longitudinally, dehydrated with alcohol, and embedded in paraffin. We used a similar protocol to stain paw pad samples, but obtained the tissue with a 3-mm punch, and omitted the decalcification step. Tissue sections (7–10 µm) were stained with hematoxylin and eosin. Images were acquired with a Nikon Eclipse 50i microscope equipped with a DS-Ri1 camera.

2.1.9. Immunohistochemistry

Mice were transcardially perfused as above, and the L4 dorsal root ganglion and the spinal cord lumbar enlargement were dissected, postfixed, dehydrated and embedded in paraffin using standard procedures. Tissue sections (5 µm) were deparaffinized in xylol (Panreac Quimica, Castellar del Vallès, Spain) and rehydrated. Antigens were retrieved by steam heating with 1 % citrate buffer. Sections were incubated for 1 h in blocking solution (5% normal donkey serum, 0.3% Triton X-100, 0.1% Tween 20 in Tris buffer solution). Then the slices were incubated for 1 h at room temperature with a goat anti-TRPV1 antibody (sc-12498, 1:100, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in blocking solution. After incubation, the sections were washed three times for 10 min and incubated for 1 h with the secondary donkey anti-goat Alexa Fluor-488 antibody (A11055, 1:500, Life Technologies, Alcobendas, Spain) and a conjugated mouse anti-NeuN antibody (MAB377A5, 1:500, Merck Millipore, Madrid, Spain). The slices were

then washed three times for 10 min and mounted with ProLong® Gold Antifade Mountant (Life Technologies, Alcobendas, Spain). Images were acquired with a confocal laser-scanning microscope (Model A1, Nikon Instruments Europe BV, Amsterdam, Netherlands).

2.1.10. Data analysis

The data were analyzed with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA). Two-way repeated-measures analysis of variance (ANOVA) or one-way ANOVA were used depending on the experiment. The Student–Newman–Keuls post-test was used in all cases. The differences between means were considered significant when the P value was below 0.05.

2.2. RESULTS

2.2.1. Behavioral phenotyping of mice with CFA-induced joint inflammation: grip strength, mechanical allodynia, body weight and locomotor activity

The administration of 20 or 30 µL/ankle of CFA induced volume-dependent decreases in hindlimb grip strength (about 35% for 20 µL and 55% for 30 µL) during the first 3 days after the induction of inflammation. In both dose groups, grip strength values returned to normal levels, although the recovery period was longer in animals given the higher volume of CFA (Fig. 1A). Untreated (naïve) animals and mice treated periarticularly with saline (30 µL/ankle) did not show significant changes in grip strength at any time-point tested for up to 21 days (Fig. 1A).

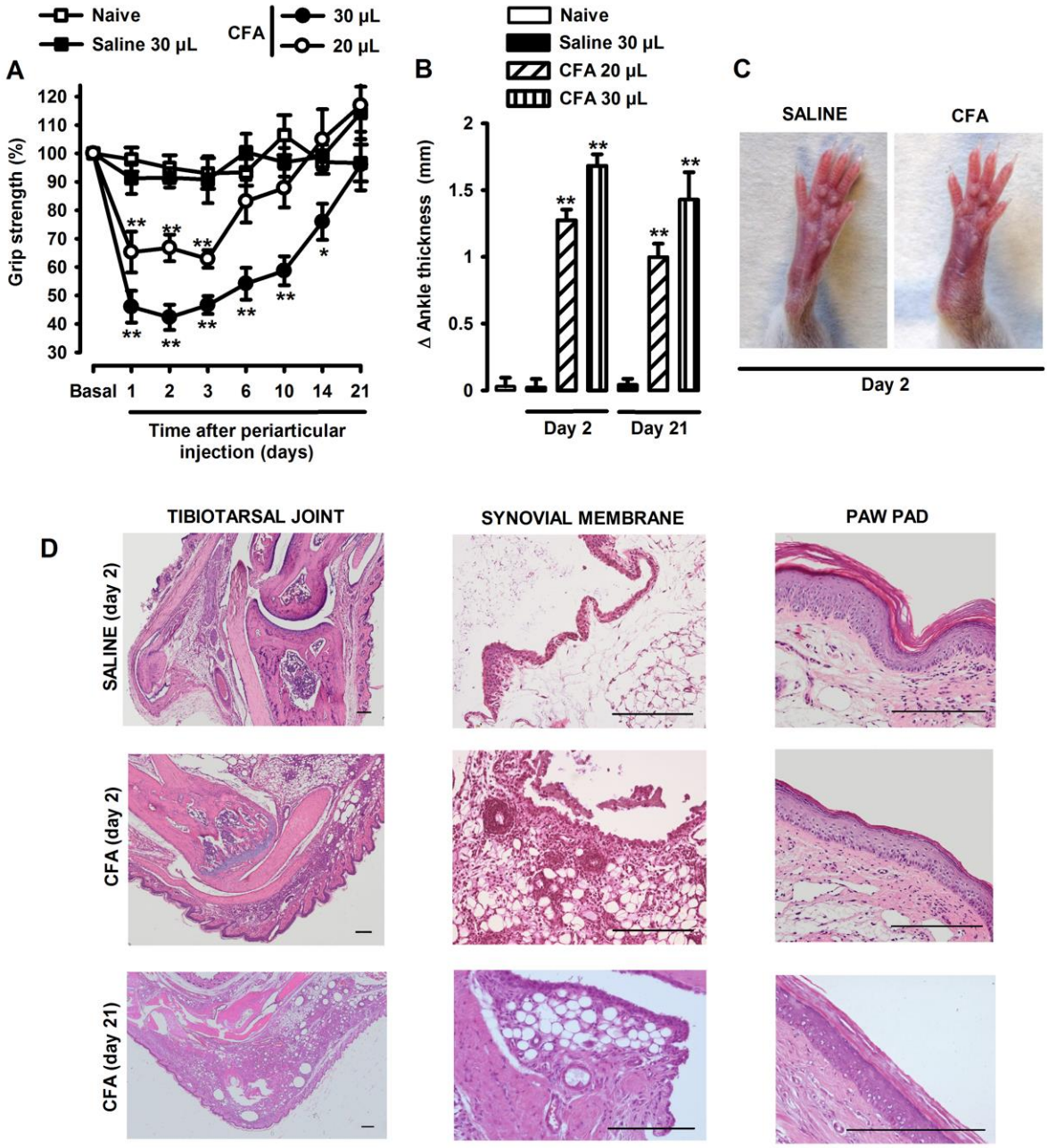

Fig 1. Grip strength deficits in mice given periarticular injections of CFA were accompanied by edema and localized immune infiltrate. (A) Time-course of hindlimb grip strength in animals treated with CFA (20 or 30 µL/ankle), compared to naïve mice and animals treated with saline (30 µL/ankle). (B) Increase in ankle thickness after the administration of CFA or saline (20 or 30 µL/ankle). (C) Representative pictures of mice treated periarticularly with 30 µL/ankle of saline or CFA (left and right panels, respectively). (D) Representative photomicrographs of hematoxylin and eosin-stained tibiotarsal joint, synovial membrane and paw pad in mice after the administration of saline or CFA (30 µL/ankle); the scale bars represent 200 µm in all panels. Values are the mean ± SEM (10–12 animals per group). Statistically significant differences in (A) and (B): **P < 0.01 versus the values from naïve animals (two-way repeated measures ANOVA in (A) and one-way ANOVA in (B) both followed by Student–Newman–Keuls test). There were no statistically significant differences between values from naïve animals and saline-treated mice. Data for ankle swelling and histological analyses were obtained from the same mice.

CFA induced persistent ankle swelling that lasted for at least 21 days, which was also dependent on the volume administered. Animals treated with 30 µL/ankle of CFA had thicker ankles than those given 20 µL, whereas those treated with saline (30 µL/ankle) showed no significant increase in ankle thickness (Fig. 1B). We used 30 µL CFA (or saline)/ankle in the rest of the experiments.

Macroscopically, sa line-treated animals showed no signs of paw inflammation (Fig. 1C, left panel). However, CFA-treated mice had prominent inflammation that appeared to be restricted to the administration site (ankle joint) and nearby areas, whereas the paw pad did not appear to be affected (Fig. 1C right panel).

Histological examination of the tibiotarsal joint, synovial membrane or paw pad from mice 2 days after the administration of saline did not disclose any histological anomalies (Fig. 1D upper panels); however, 2 days after CFA administr ation we found massive periarticular immune infiltrate which extended to the heel, as well as an inflammatory process in the synovial membrane characterized by prominent immune infiltrate accompanied by intraarticular exudate, but no histological alterations in the paw pad (Fig. 1D, middle panels). Twenty-one days after CFA administration, the animals still showed marked immune infiltration in the heel and periarticular structures, but the synovial membrane showed no immune infiltrate, and we did not observe appreciable intraarticular exudate, and the paw pad remained unaffected (Fig. 1D, lower panels).

We then measured the mechanical threshold in an inflamed (heel) and in a noninflamed area (pad) of the paw. Mice given CFA showed a marked decrease in the von Frey threshold in the heel, denoting the presence of tactile allodynia, and the decrease was maintained throughout the time-course of study (21 days) (Fig. 2A). Joint inflammation did not induce alterations in the mechanical threshold in the paw pad (Fig. 2A), indicating that the sensory alterations appeared to be restricted to the inflamed area. Control mice treated periarticularly with saline showed no alterations in their mechanical thresholds in either the heel or the paw pad (Fig. 2A).

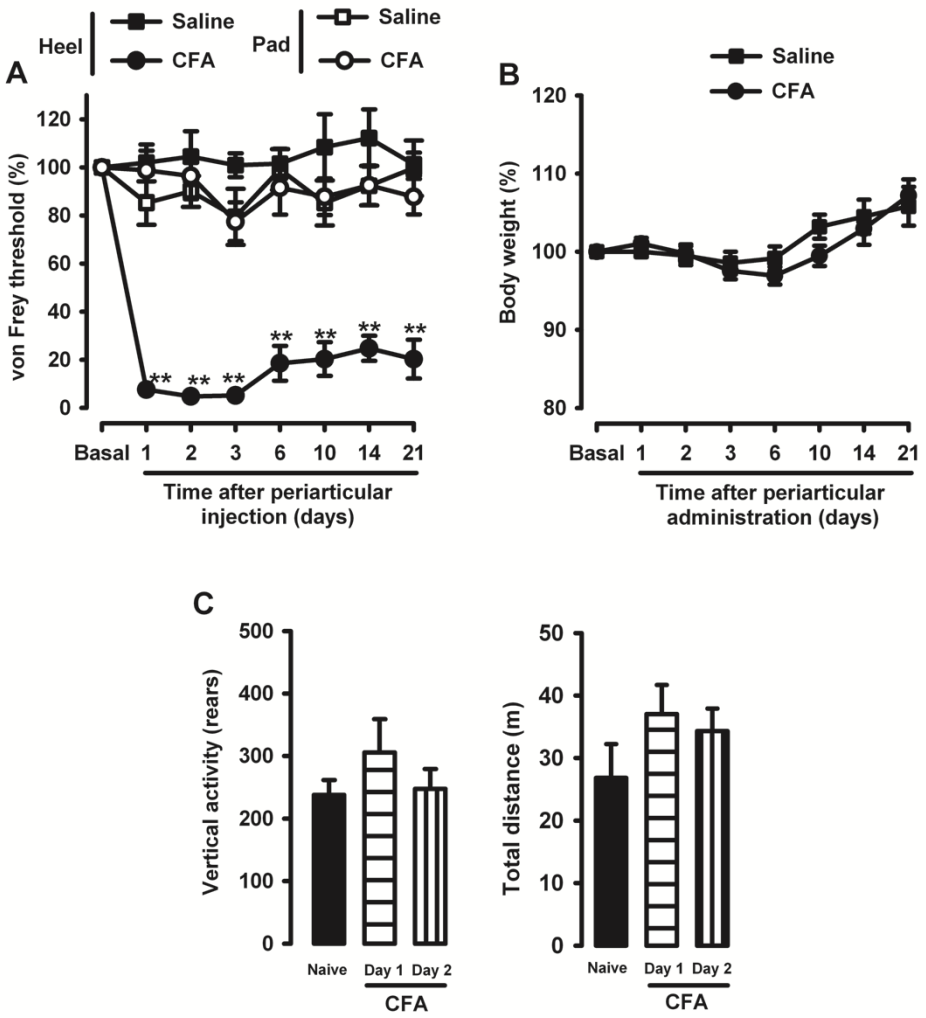

Fig. 2. Mechanical allodynia in mice given periarticular injections of CFA did not parallel alterations in body weight or locomotor activity. (A) von Frey threshold in the heel and the paw pad in mice treated periarticularly with CFA or saline. (B) Time-course of body weight after the administration of CFA or saline. (C) Vertical (rears) and horizontal (dis t ance traveled) locomotor activity of naïve mice and CFA-treated animals, 1 or 2 days after the induction of periarticular inflammation. A volume of 30 µL/ankle was used in all experiments. Values are the mean ± SEM (10–12 animals per group). Statistically significant differences in: (A) **P < 0.01 between the values from mice with and without inflammation stimulated in the heel at each time-point (two-way repeated measures ANOVA followed by Student–Newman–Keuls test); (B) no statistically significant differences between values from saline- or CFA-treated animals at any time-point tested (two-way repeated measures ANOVA); (C) no statistically significant differences between values from naïve- or CFA-treated animals (one-way ANOVA).

We also determined whether joint inflammation under our experimental conditions induced other alterations such as changes in body weight or locomotion. Animals with induced inflammation showed no changes in body weight in comparison to saline-

treated mice (Fig. 2B). In addition, CFA administration did not in duce alterations in either vertical or horizontal locomotor activity in comparison to control mice (Fig. 2C).

Therefore, this indicates that although CFA administration induced an apparent decrease in grip strength and mechanical threshold, it did not significantly affect the animals' general state or general mobility.

2.2.2. Effects of conventional oral analgesics on inflammation-induced grip strength deficits and tactile allodynia

To test whether grip strength deficits were related to pain, we evaluated the effects of conventional analgesics. Oral administration of the NSAIDs ibuprofen and celecoxib, as well as acetaminophen, produced a dose-dependent increase in grip strength in animals with joint inflammation (Fig. 3A, B and C, respectively). The maximum effect of all three drugs peaked at 90 min after administration, but even at this time-point grip strength in mice with induced inflammation failed to fully recover basal values. Subsequently, the increase in grip strength in animals with joint inflammation induced by the NSAIDs or acetaminophen gradually reverted (Fig. 3A, B and C).

We also tested the effects of oral administration of the opioids tramadol and oxycodone (Fig. 4A and B, respectively). These drugs induced a rapid recovery of physical function peaking at 45 min after administration, which decreased gradually with time. In contrast to the effects of NSAIDs or acetaminophen, both tramadol and oxycodone induced full recovery from grip strength deficits in mice with joint inflammation (Fig. 4A and B, respectively). None of these analgesics was able to modify grip strength values in animals without inflammation, even when administered at doses that had maximal effects in mice with induced inflammation (Fig. 3A, B and C and Fig. 4A and B). These results suggest that the analgesics tested improved grip strength deficits by pain-specific effects rather than by altering normal motor function.

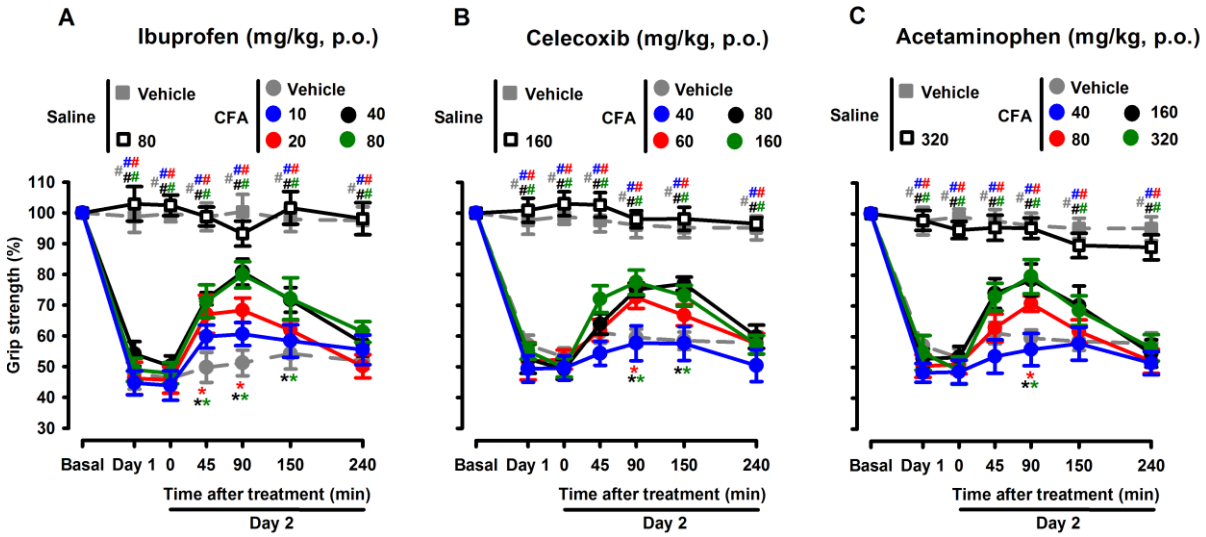

Fig. 3. Effects of oral NSAIDs and acetaminophen on grip strength deficits induced by the periarticular administration of CFA. Ibuprofen (A), celecoxib (B), acetaminophen (C) or their solvents (HPMC for ibuprofen, and 1% Tween 80 in HPMC for celecoxib and acetaminophen) were given orally (p.o.) 2 days after the administration of CFA or saline (30 µL/ankle). Values are the mean ± SEM (10–12 animals per group). Statistically significant differences in (A), (B) and (C): *P <0.05 between the values from mice with inflammation treated with the drug or solvent at each time-point; #P < 0.05 between the values from mice without inflammation treated with the drug solvents and mice with inflammation at each time-point (two-way repeated measures ANOVA followed by Student–Newman–Keuls test). For clarity, significances of P < 0.01 are not labeled with double symbols. There were no significant differences between mice without inflammation treated with the drugs or their solvents at any time-point tested.

We then constructed the dose-response curves of drug effects at their time of maximum effect (90 min for the NSAIDs and acetaminophen, and 45 min for the opioid drugs), to facilitate comparisons of the effects of different drugs on the recovery of grip strength in mice with joint inflammation.

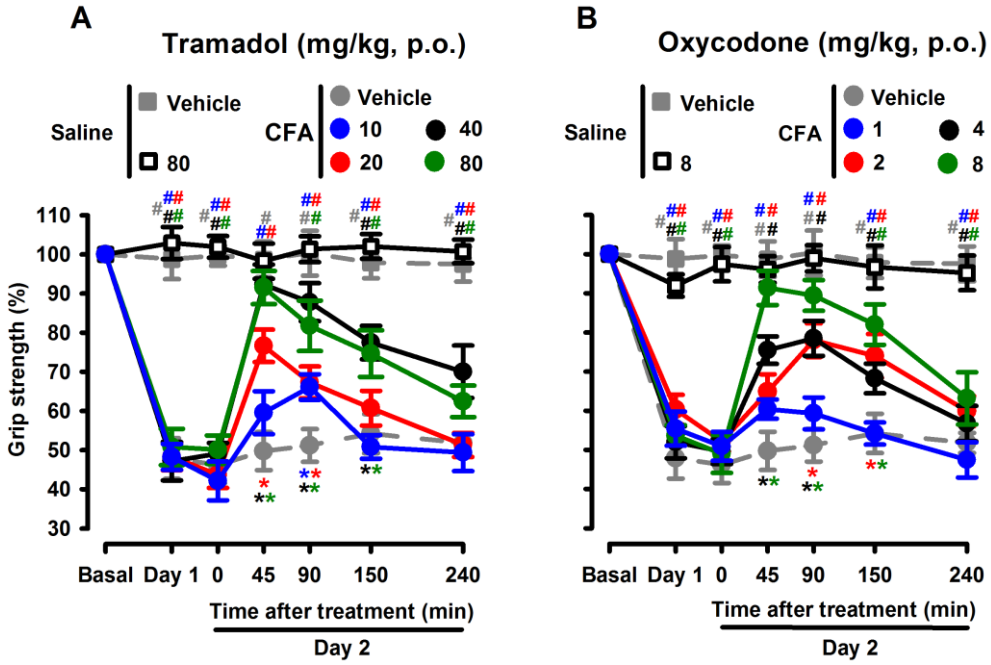

Fig. 4. Effects of oral opioids on grip strength deficits induced by the periarticular administration of CFA. Tramadol (A), oxycodone (B) or their solvent (HPMC) were administered orally (p.o.) 2 days after the administration of CFA or saline (30 µL/ankle). Val ues are the mean ± SEM (10–12 animals per group). Statistically significant differences in (A) and (B): *P < 0.05 between the values from mice with inflammation treated with the drug or solvent at each tim e-point; #P < 0.05 between the values from mice without inflammation treated with the drug solvents and mice with inflammation at each time-point (two-way repeated measures ANOVA followed by Student–Newman–Keuls test). For clarity, significances of P < 0.01 are not labeled with double symbols. There were no significant differences between mice without inflammation treated with the drugs or their solvents at any time-point tested.

Maximal effects of ibuprofen, celecoxib and acetaminophen led to a recovery of grip strength of about 60% of pre-inflammation values, whereas in animals treated with the opioids oxycodone or tramadol, grip strength recovered to approximately 90% of control values (Fig. 5A).

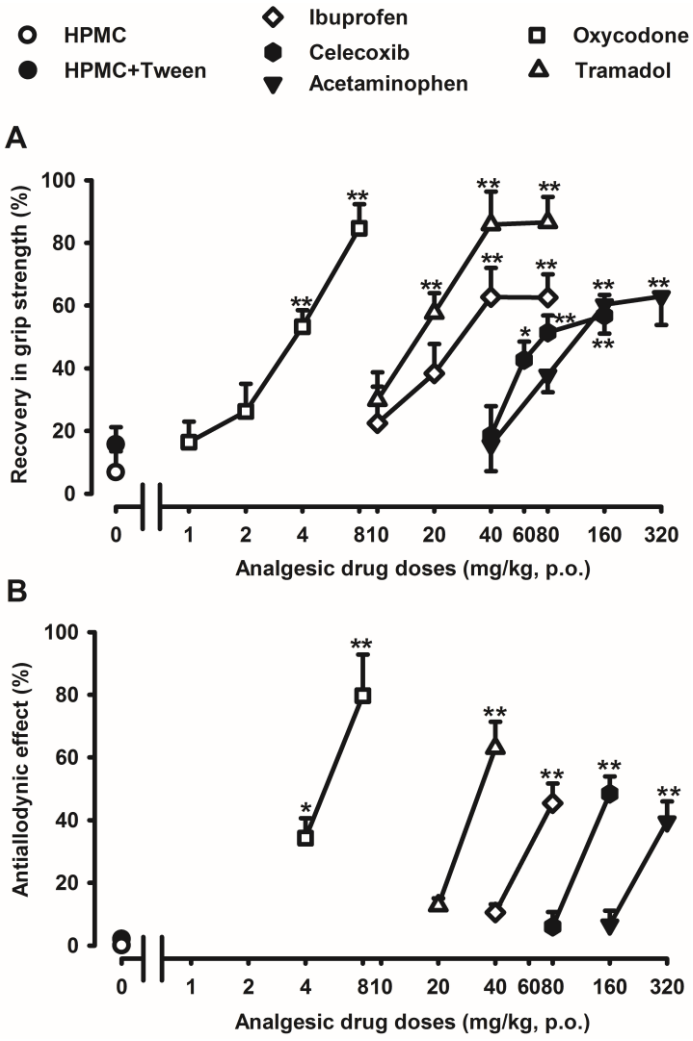

Fig. 5. Comparison of the effects induced by the oral administration of analgesic drugs on grip strength deficits and tactile allodynia in mice treated periarticularly with CFA. (A) Effects of analgesic drugs on grip strength deficits and (B) on the von Frey threshold in the heel of animals with inflammation. In all cases, drugs or their solvents were administered orally (p.o.) 2 days after CFA administration (30 μL/ankle). HPMC was used as the solvent for ibuprofen, tramadol and oxycodone (white symbols), and 1% Tween 80 in HPMC for celecoxib and acetaminophen (black symbols). Values are the mean ± SEM (10–12 animals per group). Data are expressed as the percent recovery of grip strength or percent antiallodynic effect, considering as 0% effect the values obtained immediately before drug administration, and a 100% effect the values obtained before CFA injection in each individual mouse. Values were obtained 45 min after tramadol or oxycodone administration, or 90 min after ibuprofen, celecoxib, or acetaminophen administration. Statistically significant differences in (A) and (B): $*P < 0.05$, $**P < 0.01$ between the values from mice with inflammation treated with the drugs or their solvents (one-way ANOVA followed by Student–Newman–Keuls test).

We then tested the effects of all drugs on tactile allodynia in mice with induced inflammation. Doses of ibuprofen, celecoxib, acetaminophen or tramadol that induced ≈60% recovery of grip strength (40 mg/kg, 80 mg/kg, 160 mg/kg and 20 mg/kg, respectively) were devoid of effect on tactile allodynia at any time-point tested between

45 and 240 min (Fig. 6A for NSAIDs and acetaminophen, and Fig. 6B for tramadol). On the other hand, oxycodone 4 mg/kg induced a modest but significant amelioration of mechanical allodynia at 45 mi n (Fig. 6B), which coincided with its peak effect on grip strength deficits (Fig. 4B).

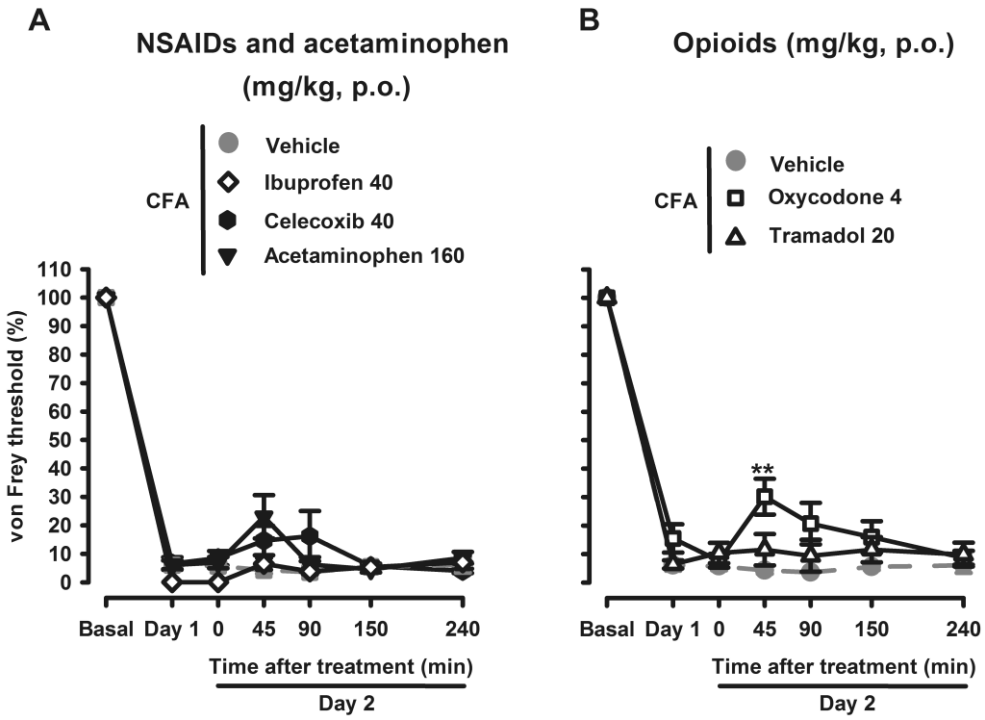

Fig. 6. Time-course of oral analgesic drugs on tactile allodynia in mice treated periarticularly with CFA. NSAIDs and acetaminophen (A), opioids (B), or their solvents were given orally (p.o.) 2 days after the administration of CFA or saline (30 µL/ankle). HPMC was used as the solvent for ibuprofen, tramadol and oxycodone, and 1% Tween 80 in HPMC for celecoxib and acetaminophen. Values are the mean ± SEM (10–12 animals per group). Statistically significant differences in (A) and (B): $^{**}P < 0.01$ between the values from mice with inflammation treated with the drug or solvent at each time point (two-way repeated measures ANOVA followed by Student–Newman–Keuls test). There were no statistically significant differences between values from HPMC- and 1% Tween 80 in HPMC-treated mice. For clarity, the latter group is not shown in the figure.

This effect rapidly disappeared, which is in contrast to the longer duration of its effects on grip strength deficits, which lasted for 90 min (Fig. 6B and Fig. 4B). To facilitate comparisons of the effects of these drugs on the recovery of grip strength and mechanical allodynia only the time-point of 90 min for the NSAIDs and acetaminophen, and 45 min for the opioid drugs, are shown in Fig. 5B. When we doubled the doses of these drugs all of them were then able to ameliorate tactile allodynia (Fig. 5B). Therefore, although all analgesics tested were able to improve both physical function

and tactile allodynia, grip strength was a more sensitive indicator of drug-induced analgesia than tactile allodynia.

2.2.3. *Effects of the muscle relaxant baclofen on inflammation-induced grip strength deficits and mechanical allodynia*

To determine the impact of motor impairment on grip strength and tactile allodynia, we tested the effects of the muscle relaxant baclofen. This drug induced opposite effects on grip strength and tactile allodynia: it dose-dependently increased the von Frey threshold in mice with inflammation, inducing a marked antiallodynic-like effect (to an extent similar to oxycodone), and did not ameliorate grip strength deficits, but induced a parallel decrease in this functional measure (Fig. 7).

In addition, the active doses of baclofen also decreased grip strength in noninjured animals (data not shown).

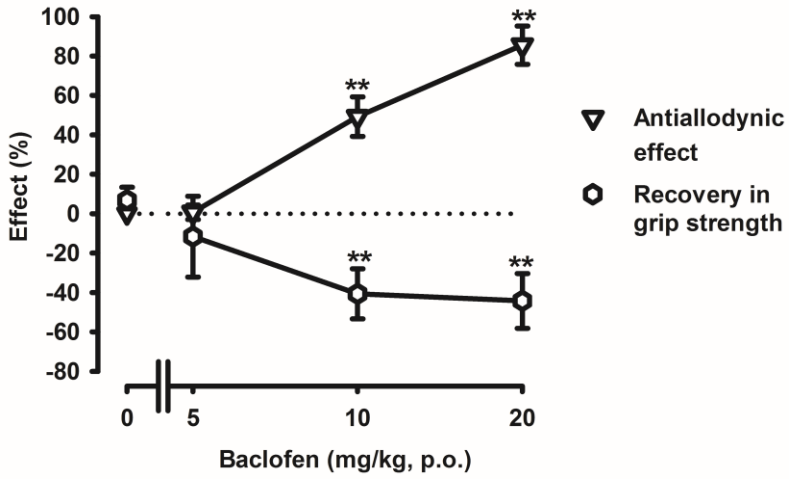

Fig. 7. Comparison of the effects induced by the oral administration of baclofen on grip strength deficits and tactile allodynia in mice treated periarticularly with CFA. Baclofen or its solvent (HPMC) was administered orally (p.o.) 2 days after CFA administration (30 µL/ankle). Values are the mean ± SEM (10–12 animals per group). The data are expressed as the percent recovery of grip strength or percent antiallodynic effect, considering the values obtained immediately before drug administration as 0%, and the values obtained before CFA injection in each individual mouse as 100%. All values were obtained 45 min after the administration of baclofen or its solvent. Statistically significant differences: *P < 0.05, **P < 0.01 between the values from mice with inflammation treated with the drugs or their solvents (one-way ANOVA followed by Student–Newman–Keuls test).

2.2.4. *Effects of the TRP antagonist ruthenium red on inflammation-induced grip strength deficits and mechanical allodynia*

We also evaluated whether grip strength deficits and mechanical allodynia during inflammation were sensitive to the TRP antagonist ruthenium red. The systemic administration of this compound (1–2 mg-kg) did not affect grip strength in injured or noninjured mice at any time-point tested between 30 and 180 min (Fig. 8). For clarity, only the data for 2 mg/kg 30 min post-administration are shown in Fig. 9A.

However, ruthenium red (administered 30 min before the behavioral evaluation) was able to abolish, in a dose-dependent manner, mechanical allodynia in mice with inflammation but without affecting the mechanical threshold in noninjured mice (Fig. 9B). We were unable to test a higher dose of ruthenium red (4 mg/kg) because it induced prominent side effects. The differential effects of ruthenium red on grip strength deficits and tactile allodynia suggest that their mechanisms differ.

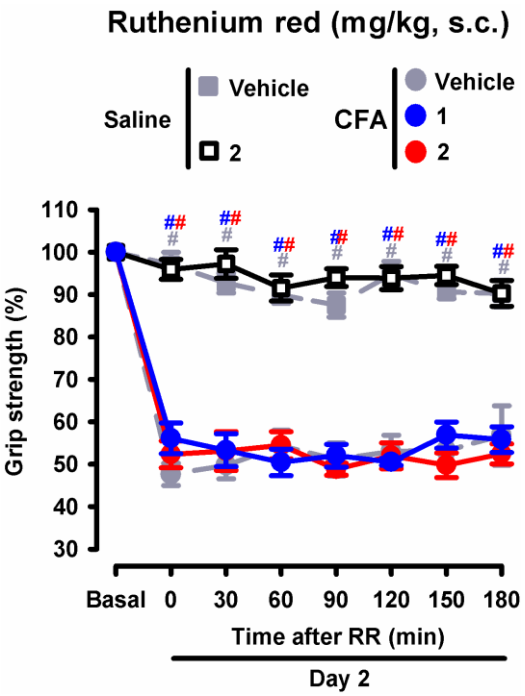

Fig. 8. Absence of effect of the subcutaneous administration of ruthenium red on grip strength deficits in mice treated periarticularly with CFA. Ruthenium red (RR) or its solvent (saline) was administered subcutaneously (s.c.) 2 days after CFA administration (30 µL/ankle). Values are the mean ± SEM (10–12 animals per group). There were no significant differences between mice without or with inflammation treated with RR or its solvent at any time point tested (two-way repeated measures ANOVA).

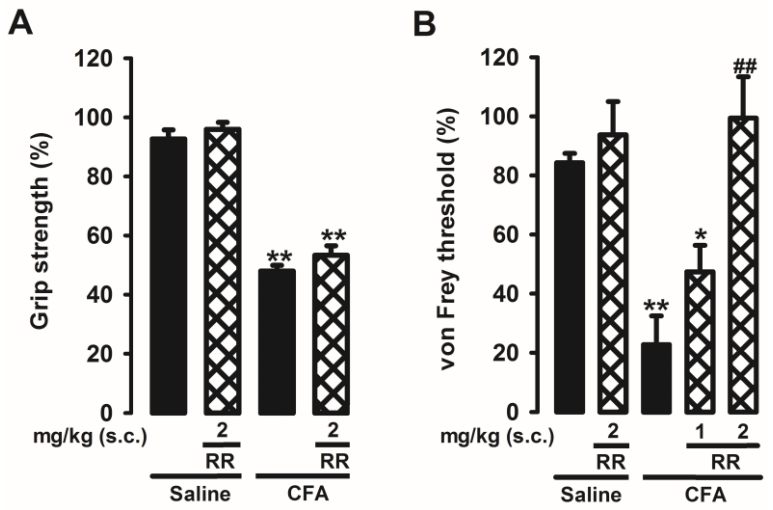

Fig. 9. Effects of the subcutaneous administration of ruthenium red on grip strength deficits and von Frey threshold in mice treated periarticularly with CFA. (A) Absence of effect of ruthenium red (RR) on grip strength deficits induced by CFA. (B) RR, administered 30 min before the behavioral evaluation, attenuated the decrease in von Frey threshold induced by CFA. RR or its solvent (saline) was administered subcutaneously (s.c.) 2 days after CFA administration (30 µL/ankle). Values are the mean ± SEM (10–12 animals per group). Statistically significant differences in (A) and (B): $^{**}P < 0.01$ between the values from mice with and without inflammation treated with ruthenium red or its solvent; $^{\#\#}P < 0.01$ between the values from mice with inflammation treated with ruthenium red or its solvent (one-way ANOVA followed by Student–Newman–Keuls test).

2.2.5. Contribution of TRPV1-expressing neurons to inflammation-induced grip strength deficits and tactile allodynia

To investigate whether grip strength deficits and mechanical allodynia during inflammation depend on the same type of nociceptive neurons, we compared the effects on grip strength and mechanical threshold of the in vivo ablation of TRPV1-expressing neurons. TRPV1 staining is present in the somas of small DRG neurons and in the superficial layers of the spinal cord dorsal horn (see controls in Fig. 10A and B, respectively). After RTX treatment we were unable to detect TRPV1 staining in either the DRG (Fig. 10A) or the spinal cord dorsal horn (Fig. 10B), reflecting the ablation of TRPV1-expressing neurons including their central terminals. The ablation of this nociceptive population did not affect either grip strength or mechanical threshold in noninjured mice (Fig. 10C and D, respectively).

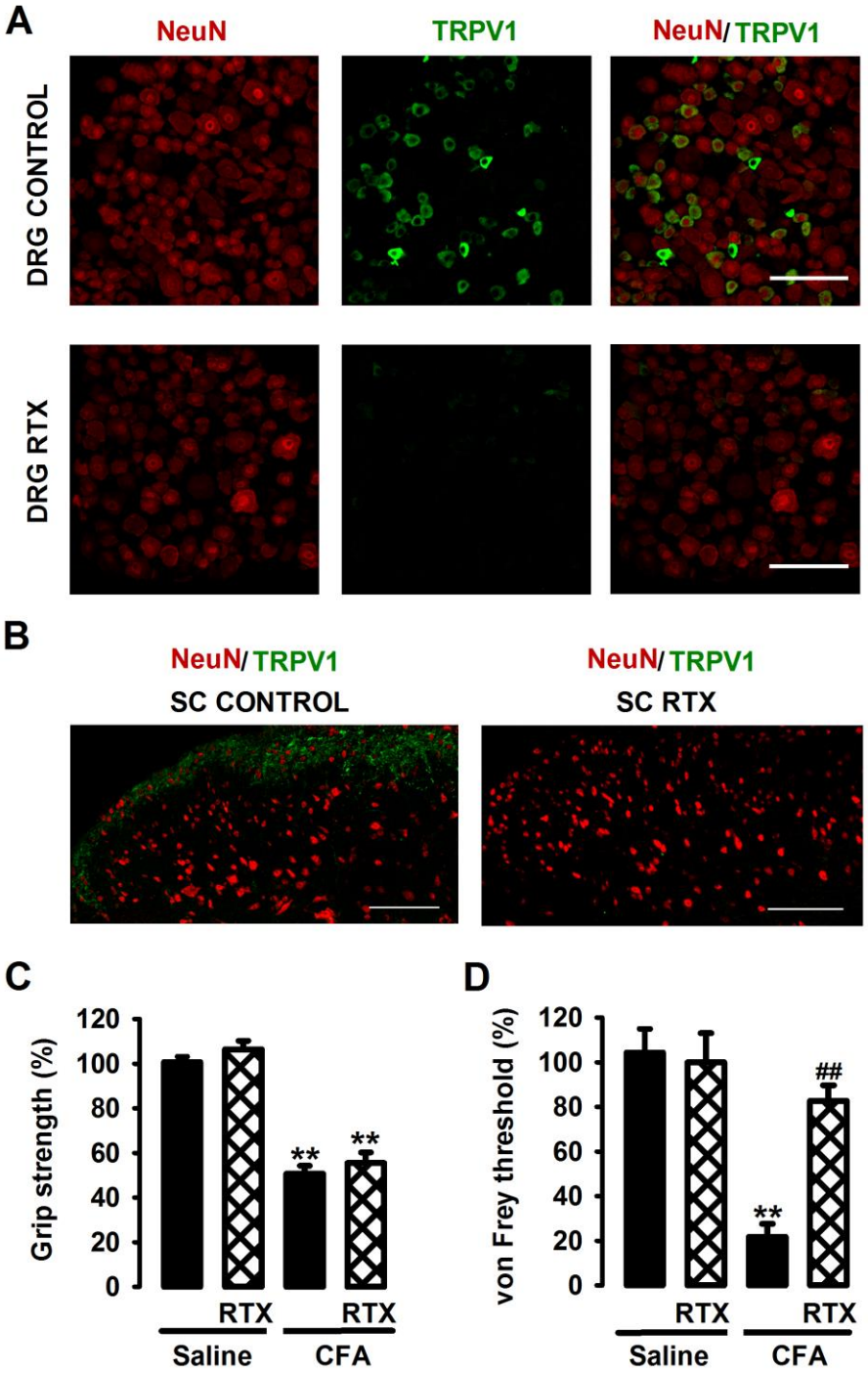

Fig. 10. Differential effects of the ablation of TRPV1-expressing neurons on grip strength deficits and von Frey threshold in mice treated periarticularly with CFA. (A) and (B) Double labeling of NeuN (red) and TRPV1 (green) in the L4 dorsal ro ot ganglion (DRG) and in the spinal cord (SC) lumbar enlargement from CFA-treated mice. Top panels: samples from saline-treated mice (control). Bottom panels: samples from mice treated with resiniferatoxin (RTX). The scale bars represent 100 µm. (C) Absence of effect of RTX on grip strength deficits induced by CFA. (D) RTX attenuated the decrease in von Frey threshold induced by CFA. Sample collection and behavioral testing were done 2 days after the periarticular administration of CFA or saline (30 µL/ankle), in mice treated with RTX (50 µg/kg) or its solvent. Values are the mean ± SEM (10–12 animals per group). Statistically significant differences in (C) and (D): **P < 0.01 between the values from mice with and without inflammation treated with RTX or its solvent; ##P < 0.01 between the values from mice with inflammation treated with RTX or its solvent (one-way ANOVA followed by Student–Newman–Keuls test). The same mice were used for the behavioral and immunofluorescence assays.

However, it was able to prevent the development of mechanical allodynia in mice with inflammation (Fig. 10D), although it had no effect on their grip strength deficits (Fig. 10C). Therefore, grip strength deficits and the decrease in the von Frey threshold during joint inflammation involve the participation of different populations of primary afferents.

2.3. DISCUSSION

In this study we show that in mice with experimentally-induced joint inflammation, grip strength decreased markedly and for a prolonged period. Grip strength deficit and mechanical allodynia (measured with von Frey filaments) in the inflamed area differed in both their time-courses of evolution and in their sensitivity to conventional analgesics. In addition, we show that although tactile allodynia was abolished by ruthenium red or by the ablation of TRPV1-expressing neurons, deficits in grip strength in mice with joint inflammation were not.

The time-courses of recovery from grip strength deficits and mechanical allodynia differed, as the latter persisted longer than the functional deficit. The different time-courses of evolution in these two outcomes indicate that pronounced tactile hypersensitivity in the inflamed area does not necessarily imply a significant alteration in physical function. We show that the periarticular administration of CFA in the ankle joint induced prominent, long-lasting ankle swelling. However, this sustained ankle swelling was accompanied by histological alterations which differed in duration. Both the inflammatory process in the synovial membrane (i.e. immune infiltrate and intraarticular exudates) and grip strength deficits were prominent 2 days after inflammation was induced, whereas they both became attenuated 21 days after CFA injection. These results suggest that synovial membrane alterations may be related to the functional deficits observed, and in this connection, synovitis has been strongly linked to both joint dysfunction and pain in human studies (reviewed in Rice et al., 2015; Scanzello and Goldring, 2012; Scott et al., 2000). Under our experimental conditions, the immune infiltrate in mice with inflammation extended to the heel and was prominent

throughout the entire study period, as was mechanical allodynia in this area of the paw (in contrast to the recovery of the synovial membrane and grip strength). The persistent immune infiltrate in the heel may contribute to the long-lasting tactile hypersensitivity we detected in our mice, as it is known that immune cells play a pivotal role in inflammatory cutaneous allodynia (Ghasemlou et al., 2015). These results suggest that the histological alterations which drive functional deficits and tactile allodynia may differ. We did not detect either observable histological or sensory alterations in the paw pad of mice with periarticular CFA-induced inflammation. Of note regarding this observation is that mice gripped the metal bar connected to the force transducer with this part of the paw, and therefore, the deficits seen in grip strength in animals with inflammation cannot be ascribed to alterations in paw pad sensitivity (as mice do not develop allodynia in this area) but is instead likely to be the result of movement or tension in the inflamed joint during gripping.

Pain states can curtail body weight gain of rodents (Blackburn-Munro, 2004) and their exploratory activity, particularly their vertical activity (rearing) when the lower limbs are injured (reviewed by Cobos and Portillo-Salido, 2013). We found that mice with joint inflammation, despite their marked grip strength deficits, did not show alterations in body weight gain or exploratory activity. These results indicate that grip strength deficits are a more sensitive indicator of functional alterations than other pain-related outcomes.

To test whether the decrease in grip strength was related to pain, we investigated the effects of orally administered analgesics used for clinical treatment in humans. The NSAIDs ibuprofen and celecoxib, as well as acetaminophen, induced a significant but limited recovery from grip strength deficits. However, the opioids tramadol and oxycodone were able to completely reverse the functional deficit induced by joint inflammation. The higher efficacy of opioids in comparison to NSAIDs or acetaminophen in the recovery of grip strength is in agreement with the analgesic efficacy of these drugs in human patients, according to the WHO analgesic ladder (Sarzi-Puttini et al., 2012); therefore the alterations in grip strength during joint inflammation in our experimental animals are largely attributable to pain. This link between pain and grip strength deficits in mice agrees with the known correlation between pain and disability according to the

same outcome measure in patients with joint pain (e.g. Fraser et al., 1999; Overend et al., 1999). Sensitivity to analgesic treatment differed between grip strength recovery and mechanical allodynia: the former was a more sensitive indicator of the effects of analgesic drugs than the latter. To the best of our knowledge, the different sensitivities of tactile hypersensitivity and grip strength deficits to the effects of drugs have not been explored in earlier research that used grip strength as a pain outcome measure. The greater sensitivity of grip strength compared to tactile allodynia in reflecting the effects of drug-induced analgesia is in agreement with previous studies with other measures of physical functioning in rodents, such as changes in weight bearing in the injured paw, exploratory locomotion, burrowing behavior or wheel running (Andrews et al., 2012; Cobos et al., 2012; Huntjens et al., 2009; Matson et al., 2007). Therefore, the greater sensitivity to drug-induced analgesia may be an inherent quality of these types of more "natural" pain measures, as previously suggested (Cobos and Portillo-Salido, 2013; de la Puente et al., 2015). This might be particularly relevant for analgesic drug discovery, because many new potentially interesting compounds are discarded based on their lack of efficacy on tactile allodynia, although they might ameliorate functional measures of pain which currently are not routinely evaluated.

Importantly, none of the analgesics we tested modified grip strength in animals without inflammation, and therefore the recovery of grip strength in mice with joint inflammation was not due to nonspecific drug effects on normal grip strength.

Drugs may inhibit pain-like reflexes due to sedative and motor effects, resulting in false-positive results. Here we show that baclofen induced a marked increase in the von Frey threshold in mice with inflammation, and its effect was similar to that of the third-step opioid oxycodone. However, we found that "antiallodynic" doses of this drug also decreased grip strength in mice with and without inflammation. This suggests that the effects induced by baclofen on inflammatory mechanical allodynia are due to this drug's known motor impairment and sedative effects (Dario and Tomei, 2004) rather than to a true analgesic effect. Therefore, grip strength and von Frey threshold are not affected in the same way by the same confounders. Whereas drug-induced motor impairment might lead to a false analgesic-like effect in the von Frey test, it would not be expected to have this effect in tests of grip strength as a surrogate measure of pain. For analgesic

drug discovery it is essential to determine whether drugs induce signs of toxicity. Grip strength has been used in humans to test drug-induced toxicity (Savilampi et al., 2014) it is classically used to assess neurotoxicity in rodents (Meyer et al., 1979) and is even included in the Irwin screen (Irwin, 1968; Mattsson, 1996) which is ingrained in the pharmaceutical industry as the first tier of preclinical testing to detect drug-induced neurotoxic effects (Moser, 2011). Therefore, this outcome measure can be used to detect both drug-induced analgesia and toxicity, which is undoubtedly advantageous to determine the therapeutic index of drugs being tested during preclinical development.

The different responses of grip strength deficits and tactile allodynia to analgesic treatment, together with the previously noted differences in their time-courses, support the notion that different mechanisms are involved in the appearance of mechanical allodynia and joint pain-induced functional disability. In fact, we show that the nonselective TRP antagonist ruthenium red was able to abolish inflammatory tactile allodynia but without ameliorating grip strength deficits. Our results using ruthenium red agree with its previously reported effects on tactile allodynia from diverse etiology (Cui et al., 2014; Qu et al., 2016; Shinoda et al., 2008). Ruthenium red is a widely used TRP antagonist (e.g. St Pierre et al., 2009), although it also blocks other channels, including the mechanosensitive Piezo channels (Coste et al., 2012). Therefore, although our results indicate that tactile allodynia and grip strength deficits are not affected in the same way by this compound (and hence suggest that their mechanisms differ), this does not definitively link TRP channels to the effects we observed.

We then targeted the function of TRPV1+ neurons, which are known to express several TRP channels (Julius, 2013). In our study the ablation of these TRPV1-expressing sensory neurons did not alter the normal mechanical threshold, but completely attenuated tactile allodynia, which is consistent with the known pivotal role of TRPV1 neurons in cutaneous pain hypersensitivity during inflammatory arthritis (Borbély et al., 2015). However, we found no effect on joint inflammation-induced grip strength deficits, indicating that TRV1-expressing neurons are not responsible for the functional deficits observed. Grip strength has been previously used as an indicator of pain-induced disability in osteoarthritic pain (Chandran et al., 2009; Honore et al., 2009). It has been reported that TRPV1 antagonism is able to ameliorate grip strength deficits in rodents

with experimental osteoarthritis (Honore et al., 2009). Osteoarthritis has been classically considered a “noninflammatory arthritis” (e.g. Haroon et al., 2016), and although it is currently believed that osteoarthritis has an inflammatory component (Sokolove and Lepus, 2013), this is not as prominent as in our CFA-treated mice. Therefore, the differences between our findings and previous reports might be due to differences in the type of joint pain explored.

TRPV1 is present in nearly all unmyelinated (C-type) peptidergic neurons in the mouse DRG (Cavanaugh et al., 2011), but is almost absent from A-neurons and virtually absent from C-nonpeptidergic nociceptors (e.g. Cavanaugh et al., 2011; Niiyama et al., 2007). Therefore, other nociceptive neurons different from C-peptidergic nociceptors, such as Aδ or C-nonpeptidergic neurons (which express little or no TRPV1) (e.g. Niiyama et al., 2007), might contribute to grip strength deficits during joint inflammation. An alternative explanation is that joint tissues are also innervated by proprioceptors, which under painful conditions might lead to pain via central mechanisms (e.g. Mapp, 1995). Regardless of the exact mechanism involved in grip strength deficits during joint inflammation, the differential effects of ruthenium red and the ablation of TRPV1-expressing neurons on tactile allodynia and functional disability during joint inflammation strongly support the notion that their biological mechanisms differ.

3. MODULATION OF MORPHINE-INDUCED ANTINOCICEPTIVE EFFECTS AND TOLERANCE BY SIGMA-1 RECEPTORS: STUDIES ON NOCICEPTIVE HEAT PAIN AND ON TACTILE ALLODYNIA AND GRIP STRENGTH DEFICITS DURING JOINT INFLAMMATION

3.1. MATERIAL AND METHODS

3.1.1. Experimental animals

Female CD1 mice (Charles River, Barcelona, Spain) were used in all experiments. Animals weighing 25–30 g were tested randomly throughout the estrous cycle. Mice were housed in colony cages with free access to food and water prior to the experiments, and were kept in temperature- and light-controlled rooms (22 ± 2°C, and light–dark cycle of 12 h). The experiments were performed during the light phase (from 9:00 AM to 3:00 PM). Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, Granada, Spain), regional (Junta de Andalucía, Spain) and international standards (European Communities Council directive 2010/63). All mice were used in only one experimental procedure (heat nociception, von Frey testing or grip strength measurement).

3.1.2. CFA-induced periarticular inflammation

To induce the inflammation, mice were injected periarticularly with complete Freund's adjuvant (CFA) (Sigma-Aldrich, Madrid, Spain) according to the previously described method (Montilla-García et al., 2017). Briefly, CFA was subcutaneously administered around the tibiotarsal joint in two separate injections to the inner and outer side of the joint, in a volume of 15 µL/injection (30 µL/paw) to obtain homogeneous inflammation. Control animals received the same volume of sterile physiological saline (0.9% NaCl) using the same procedure. Injections were performed using a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a 30½-gauge needle under isoflurane anesthesia (IsoVet®, B. Braun, Barcelona, Spain). Behavioral evaluations on inflamed mice were performed two days after CFA or saline administration, since we have

previously reported that at this time there was the peak of both tactile allodynia and grip strength deficits (Montilla-García et al., 2017).

3.1.3. Drugs and drug administration

We used the opioid agonist morphine (supplied by the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health, Spain). S1RA (4-[2-[[5-methyl-1-(2-naphtalenyl)1H-pyraol-3-yl]oxy]ethyl] morpholine hydrochloride) (DC Chemicals, Shanghai, China) was used as a selective sigma-1 antagonist (Cobos et al., 2008; Romero et al., 2012). The dose of this sigma-1 antagonist used in the present study (80 mg/kg) was high enough to induce a maximal effect in several pain models (Nieto et al., 2012; Sánchez-Fernández et al., 2013 and 2014; Tejada et al., 2014 and 2017). PRE-084 ([2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride]) (Tocris Cookson Ltd.) was used as a selective sigma-1 agonist (Hayashi and Su, 2004; Cobos et al., 2008). All drugs used were dissolved in sterile physiological saline (0.9% NaCl); the solution of PRE-084 was appropriately alkalinized with NaOH. To evaluate the effects of systemic treatments, drugs were injected subcutaneously (s.c.) into the interscapular zone in a volume of 5 mL/kg. When the effect of the association of two or more drugs was tested, each drug was injected into a different area of the interscapular zone.

In the experiments on the acute effects of systemic morphine alone or associated with S1RA, morphine was s.c injected at 30 min before the behavioral evaluation, and S1RA immediately before morphine injection. When PRE-084 was used to reverse the effect of S1RA, it was s.c. injected 5 min before S1RA.

Morphine tolerance was induced using a 3-day cumulative dosage regimen consisting of twice daily s.c. injections (b.i.d.) at 9:30 a.m. and 9:30 p.m., starting on day 1 at 9:30 a.m. The individual doses were 30 mg/kg (a.m.) and 45 mg/kg (p.m.) on day 1, 60 mg/kg (a.m.) and 80 mg/kg (p.m.) on day 2, and 100 mg/kg (a.m.) and 120 mg/kg (p.m.) on day 3. To avoid tissue lesions by repeated injections, morphine administration was rotated in each of the four quadrants of the back of the mice.

To study whether S1RA administration was able to rescue morphine antinociception from tolerance once it was fully developed, on day 4, after carrying out the tolerance protocol, mice were randomized to receive s.c. a test dose of morphine (4 mg/kg on tactile allodynia, and 8 mg/kg on heat nociception and grip strength) alone or associated with S1RA (80 mg/kg, s.c. for both tests), and then the behavioral evaluation was performed (Fig. 1, Protocol I). Similarly to the protocol used to explore the acute effect of morphine alone and the influence of S1RA on the effects of this opioid, morphine was administered immediately after S1RA, 30 min before the behavioral evaluation. PRE-084 was administered 5 min before S1RA.

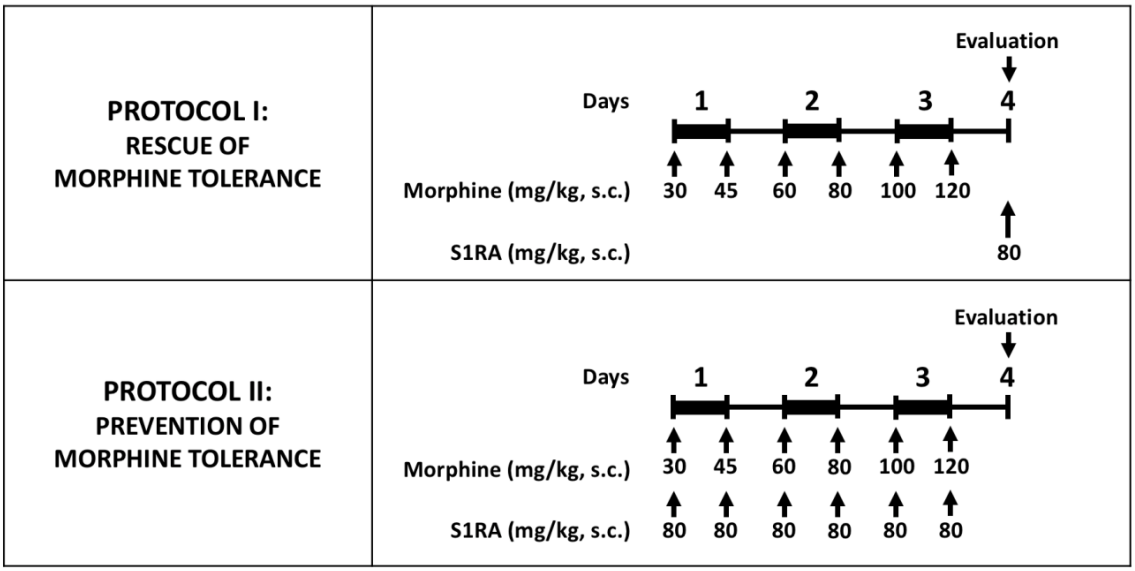

Fig. 1. Experimental protocols used to investigate the effect of S1RA on morphine tolerance. Morphine tolerance was induced by a 3 days cumulative dosage regimen using the subcutaneous doses of morphine indicated in the Figure. The first row shows the protocol used to test the effects of S1RA on the rescue of morphine effect from tolerance once it was fully developed. The second row shows the protocol to study the effect of S1RA on the prevention of the development of morphine tolerance. Drugs or their solvent (saline) were administered subcutaneously (s.c.). In all cases, "Evaluation" indicates the time at which the evaluation of heat nociception, von Frey threshold and grip strength took place, which was always at day 4 after the first morphine administration. On the evaluation day all animals received a dose of morphine (4 or 8 mg/kg, sc., depending on the experiment performed; see text for details) 30 min before evaluating their pain response.

To study whether S1RA was able to prevent the development of morphine tolerance, mice were given S1RA (b.i.d. 80 mg/kg, s.c.) immediately before each morphine injection

during the induction of analgesic tolerance (Fig. 1, Protocol II). For each set of injections, each administration was performed in different areas of the interscapular zone using a rotating protocol. Behavioral testing was performed at day 4 using the same doses of morphine described in the paragraph above.

Injections with the solvent of the drugs (saline) were used in all cases as a control.

3.1.4. Measurement of heat nociception (unilateral hot plate)

Heat nociception was assessed as previously described (Menendez et al., 2002; Montilla-García et al., 2018). The plantar side of the stimulated hindpaw was placed on the surface of a thermal analgesiometer (Model PE34, Series 8, IITC Life Science Inc., Los Angeles, USA) previously set at 55 ± 1 °C until the animal showed a paw withdrawal response. The latency in seconds from paw stimulation to the behavioral response was measured with a digital chronometer. Only a clear unilateral withdrawal of the paw was recorded as the nociceptive response. We avoided simultaneous heat stimulations in both hindpaws by placing the plantar side of the tested hindpaw on the hot plate while the other hindpaw was placed on filter paper (off the hot plate) during observations (see Supplementary Video 2, which demonstrates the procedure used to measure heat nociception). Evaluations were done twice alternately in each hindpaw at intervals of 1 min between each stimulation. A 50-s cut-off was used for each measurement to prevent tissue damage. The mean value of the two averaged measurements for each hindpaw was used to analyze the effects of the treatments.

3.1.5. Measurement of von Frey threshold

Tactile allodynia to a punctate stimulus was determined using the methods described in our previous study (Montilla-García et al., 2017). Briefly, animals were acclimated for 2 h in methacrylate test compartments (7.5 cm wide × 7.5 cm long × 15 cm high) placed on an elevated mesh-bottomed platform, to provide access to the plantar surface of the

hindpaws. The von Frey stimulations were performed in the heel, since using our CFA injection protocol inflammation and tactile allodynia are prominent in this area (Montilla-García et al., 2017). A logarithmic series of calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA), with bending forces that ranged from 0.02 to 1.4 g, were applied using the up-down paradigm (Chaplan et al., 1994), starting with the 0.6 g filament. Filaments were applied two times for 2–3 s, with inter-application intervals of at least 30 s to avoid sensitization to the mechanical stimuli. The response to the filament was considered positive if immediate licking/biting, flinching or rapid withdrawal of the stimulated paw was observed.

3.1.6. Measurement of grip strength

Grip strength was measured with a computerized grip strength meter (Model 47200, Ugo-Basile, Varese, Italy) according to the previously described method (Montilla-García et al., 2017). To measure grip strength in the hindpaws of the mice, the experimenter held the mouse gently by the base of the tail, allowing the animal to grasp the metal bar of the grip strength meter with its hindpaws. The metal bar is connected to a force transducer that automatically recorded the peak forc e of each measurement in grams (g). Hindlimb grip strength in each mouse was measured in triplicate. To prevent mice from gripping the metal bar with their forepaws during the recording, the animals were first allowed to grasp a wire mesh cylinder with their forepaws (see Supplemental Video 3, which demonstrates the procedure used to measure hindlimb grip strength). Basal grip strength values were recorded for each animal as the average of two determinations on different days before the administration of CFA or saline. This value was considered as 100% of grip strength and used as a reference for subsequent determinations.

3.1.7. Data analysis

The data were analyzed with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA). One-way, two-way, or two-way repeated-measures analysis of variance

(ANOVA), were used depending on the experiment; a Student-Newman-Keuls post-hoc test was done in all cases. The differences between means were considered significant when the *P* value was below 0.05.

3.2. RESULTS

3.2.1. Modulation by S1RA of morphine-induced antinociception to heat stimulus

The paw withdrawal latency to n ociceptive heat stimulus in mice without inflammation was short, l ess than 5 s (Fig. 2A). Morphine administration (2-8 mg/kg, s.c.) induced dose-dependent robust antinocicep tive effects, reaching values of about 30 s at the highest dose tested (Fig. 2A).

In contradistinction to morphine, the administration of the selective sigma-1 antagonist S1RA (80 mg/kg, s.c.) failed to alter the response latency of mice to nociceptive heat stimulus (Fig. 2B). However, when we associated this dose of S1RA with the administration of morphine 4 mg/kg (s.c.) we found a marked increase in the response latency of the mice (Fig. 2B). S1RA was also able to increase the antinociceptive effect induced by morphine 2 mg/kg (s.c.), but to a lower extent (data not shown). We also evaluated the effects of the sigma-1 agonist PRE-084 (40 mg/ kg, s.c.) on heat antinociception induced by association of S1RA with morphine, and found that treatment with the sigma-1 agonist abolished the S1RA-induced potentiation of morphine antinociception (Fig. 2B). These results support the selectivity of the effects induced by S1RA. Therefore, S1RA enhances the antinociceptive effect of morphine to heat stimulus through sigma-1 inhibition.

These results are summarized in Table 1 (see page 139).

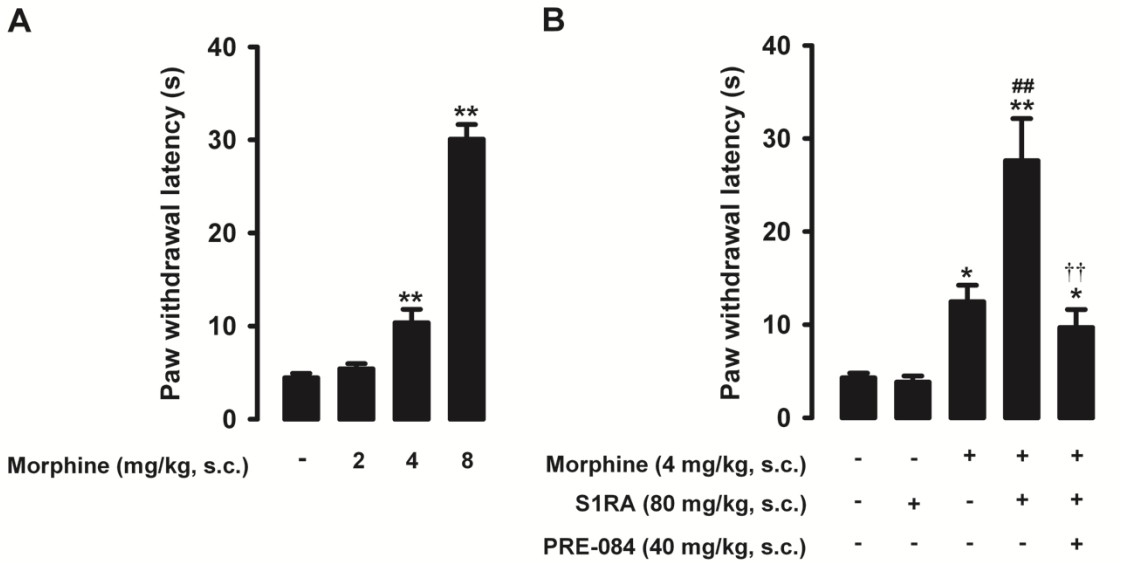

Fig. 2. Effects of morphine alone or associated with S1RA on nociceptive pain induced by heat stimulation. The results represe nt the paw withdrawal latency during stimulation of the hindpaws with 55 ºC. (A) Effect of the subcutaneous (s.c.) administratio n of several doses of morphine (2-8 mg/kg) or its vehicle. (B) Effect of the s.c. administration of morphine (4 mg/kg) , S1RA (80 mg/kg) or their vehicle; morphine (4 mg/kg) + S1RA (80 mg/kg), and the association of these drugs with PRE-084 (40 mg/kg) or its vehicle. (A and B) Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. (A) Statistically significant differenc es between the values obtained in animals treated with morphine or its vehicle: **P < 0.01 (one-way ANOVA followed by Student–Newman–Keuls test). (B) Statistically significant differences between the values obtained in animals treated with: morphine or its vehicle (*P < 0.05, **P < 0.01); morphine + S1RA or its vehicle ($^{\#\#}$P < 0.01); and morphine + S1RA associated with PRE-084 or its vehicle (††P < 0.01) (one-way ANOVA followed by Student–Newman–Keuls test).

3.2.2. Modulation by S1RA of the tolerance to the antinociceptive effect of morphine to heat stimulus

Animals were rendered tolerant to morphine by a 3-days escalating dosage regimen (see Fig. 1). Control non-tolerant mice were treated with the vehicle of morphine. On day 4, we show that non-tolerant mice showed a marked increase in the response latency induced by a morphine dose shown in the section above to induce obvious antinociception (8 mg/kg, s.c.) (Fig. 3A, black bars). However, morphine-tolerant mice show a marked reduction of the effect induced by this morphine dose (29.01 ± 3.67 and 9.25 ± 1.95 s, for paw withdrawal latency in non-tolerant and tolerant mice in response

to morphine, respectively) (Fig. 3A). Once animals were rendered tolerant to morphine, we associated the administration of S1RA (80 mg/kg, s.c.) to morphine (8 mg/kg, s.c.) (according to Protocol I in Fig. 1), and we found that the response latency increased to a level similar to that found in control non-tolerant mice, indicating that S1RA was able to rescue morphine antinociception from tolerant animals (Fig. 3A). The administration of PRE-084 (40 mg/kg, s.c.) completely abolished the increase in the antinociceptive effect of morphine induced by S1RA in morphine-tolerant mice, achieving values close to those found in tolerant mice treated with morphine alone the day of the experiment (Fig. 3A).

We also tested whether the repeated administration of S1RA (80 mg/kg, s.c.) before each dose of morphine during the 3-days morphine administration (according to Protocol II in Fig. 1) would have a preemptive effect on the development of morphine tolerance to its effect on nociceptive heat pain. When the effect of morphine (8 mg/kg, s.c.) was tested on day 4, without any further administration of S1RA, the animals showed a reduced morphine effect (Fig. 3B). These results indicate that tolerance to its antinociceptive effect to heat stimulus was still developed in spite to repeated preemptive S1RA administration.

Therefore, sigma-1 receptor inhibition by S1RA was able to restore the antinociceptive effect of morphine to heat stimulus in mice tolerant to this opioid, but S1RA preemptive treatment failed to affect the development of tolerance to the effect of morphine on nociceptive heat pain.

These results are summarized in Table 1 (see page 139).

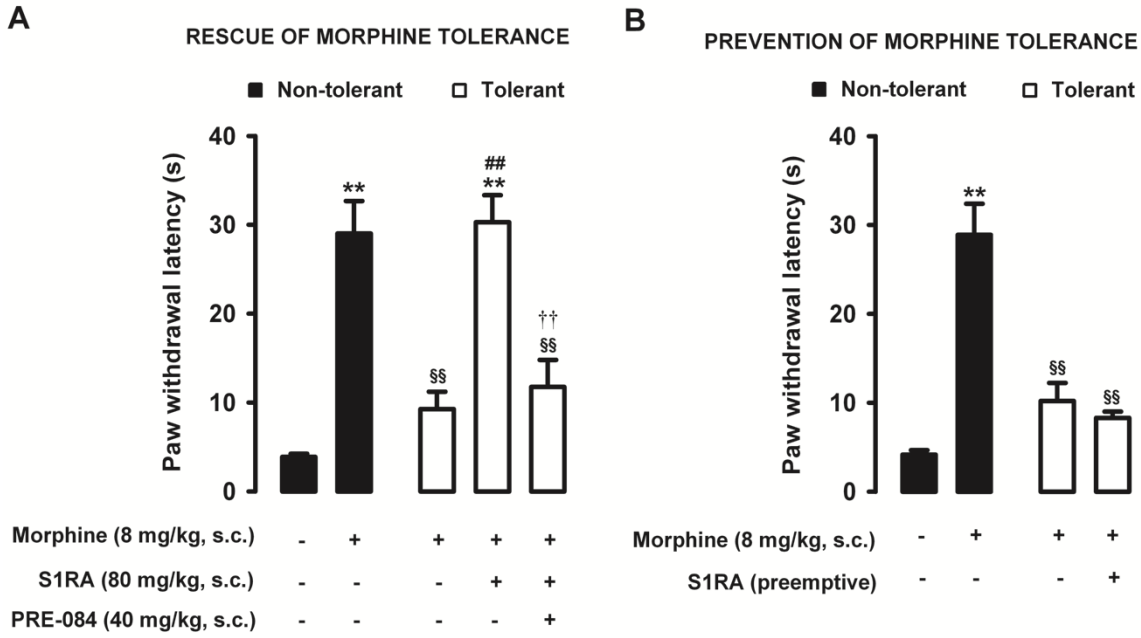

Fig. 3. Modulation by S1RA of the tolerance to the effect of morphine on nociceptive pain due to heat stimulation. The results represent the paw withdrawal latency during stimulation of the hindpaws with 55 ºC. (A) Rescue of mo rphine tolerance by S1RA: Animals were repeatedly treated during 3 days with morphine (tolerant, white bars) or its v ehicle (non-tolerant, black bars), according to Protocol I in Fig. 1. The day of evaluation (day 4) mice were treated subcutaneously (s.c.) with morphine (8 mg/kg) or its vehicle, morphine (8 mg/kg) + S1RA (80 mg/kg) or its vehicle, and the combination of these drugs with PRE-084 (40 mg/kg) or its vehicle. (B) Prevention of morphine tolerance by S1RA: Animals were treated s.c. with S1RA (80 mg/kg) or its vehicle immediately before each dose of morphine (tolerant, white bars) during the induction of morphine tolerance, according to Protocol II in Fig. 1. Control mice (non-tolerant, black bars) received the vehicles of morphine and S1RA during 3 days, according to Protocol II in Fig. 1. The evaluation day (day 4) tolerant and non-tolerant mice were treated only with morphine (8 mg/kg, s.c.) or its vehicle. (A and B) Each bar and vertical line represents the mean ± SEM of the values obtained in 8-10 animals. (A and B) Statistically significant differences between the values obtained in mice treated with morphine or its vehicle (**$P < 0.01$), and between the values obtained in tolerant and non-tolerant animals treated with morphine the day of the evaluation ($^{§§}P < 0.01$) (two-way ANOVA followed by Student–Newman–Keuls test). (A) Statistically significant differences in tolerant mice between the values obtained in mice treated the day of evaluation with: morphine + S1RA or its vehicle ($^{##}P < 0.01$); morphine + S1RA associated with PRE-084 or its vehicle ($^{††}P < 0.01$) (two-way ANOVA followed by Student–Newman–Keuls test). (B) There were no statistically significant differences in the effect of morphine on the evaluation day between the values obtained from mice treated with S1RA or its vehicle during the induction of morphine tolerance (two-way ANOVA followed by Student–Newman–Keuls test).

3.2.3. Modulation by S1RA of the antiallodynic effect of morphine during inflammation

Mice administered with CFA around the tibiotarsal joint showed a marked decrease in their mechanical threshold in the heel, denoting the presence of tactile allodynia (0.73 ± 0.05 g and 0.06 ± 0.01 g for mice without and with inflammation, respectively) (Fig. 4A). Joint inflammation did not induce alterations in the von Frey threshold in the non-inflamed area (pad) of the paw (data not shown), indicating that the sensory alterations appeared to be restricted to the inflamed area. Morphine administration (1-4 mg/kg, s.c.) induced a dose-dependent antiallodynic effect in animals with inflammation, achieving a full recovery of the normal mechanical threshold at the highest dose tested (Fig. 4A). Morphine effect was more prominent in tactile allodynia than on nociceptive heat pain, since the doses needed to induce significant effects were lower (compare Figs. 2A and 4A).

The administration of S1RA alone (80 mg/kg, s.c.) did not ameliorate inflammatory tactile allodynia (Fig. 4B). However, the association of this dose of S1RA with a low dose of morphine (1 mg/kg, s.c.), which was also devoid of antiallodynic effect *per se*, resulted in a marked increase of the mechanical threshold of mice with inflammation, achieving values similar to those from non-inflamed mice (Fig 4B). Similar to the results found in heat nociception, the administration of the sigma-1 agonist PRE-084 (40 mg/kg, s.c.) abolished the potentiation induced by S1RA of the antiallodynic effec t of morphine (Fig. 4B), supporting the selectivity of the effects induced by S1RA. Therefore, S1RA enhances the antiallodynic effect of morphine through sigma-1 inhibition. These results are summarized in Table 1 (see page 139).

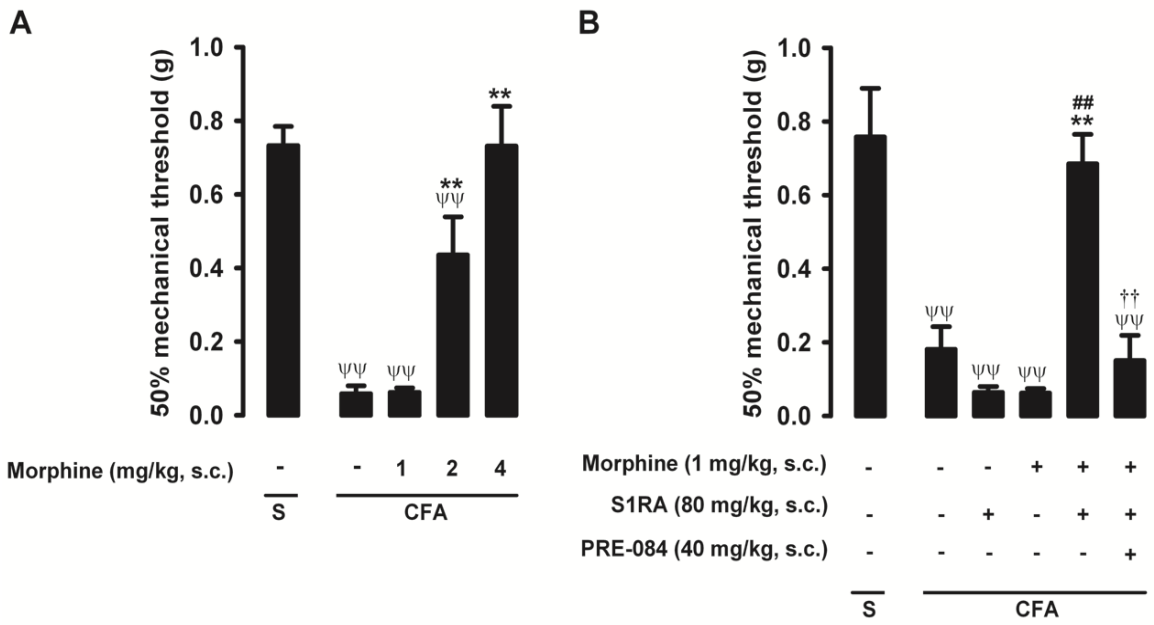

Fig. 4. Effects of morphine alone or associated with S1RA on inflammatory mechanical allodynia. The results represent the 50% mechanical threshold (determined with von Frey filaments) of mice treated periarticularly with CFA or saline (S). (A) Effect of the subcutaneous (s.c.) administration of several doses of morphine (1-4 mg/kg) o r its vehicle. (B) Effect of the s.c. administration of morphine (1 mg/kg), S1RA (80 mg/kg) or their vehicle; m orphine (1 mg/kg) + S1RA (80 mg/kg), and the association of these drugs with PRE-084 (40 mg/kg) or its vehicle. (A and B) Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. (A and B) Statistically significant differences between the values obtained in: animals with and without inflammation ($^{\Psi\Psi}P < 0.01$); animals treated with morphine or its vehicle ($^{**}P < 0.01$) (one-way ANOVA followed by Student–Newman–Keuls test). (B) Statistically significant differences between the values obtained in animals with inflammation treated with: morphine + S1RA or its vehicle ($^{\#\#}P < 0.01$); morphine + S1RA associated with PRE-084 or its vehicle ($^{\dagger\dagger}P < 0.01$) (one-way ANOVA followed by Student–Newman–Keuls test).

3.2.4. Modulation by S1RA of the tolerance to the antiallodynic effect of morphine during inflammation

As described above for heat nociception, animals were rendered tolerant to morphine by a 3-days escalating dosage regimen (Fig. 1, Protocol I), whereas control non-tolerant mice received morphine vehicle. On day 4, non-tolerant mice with inflammation showed a full reversion of inflammation-induced tactile allodynia induced by the acute administration of morphine 4 mg/kg (s.c.) (Fig. 5A, black bars).

However, morphine-tolerant mice showed a marked reduction in the antiallodynic effect induced by the same morphine dose (0.73 ± 0.09 and 0.27 ± 0.11 g, for the mechanical threshold in non-tolerant and tolerant mice with inflammation in response

to morphine, respectively) (Fig. 5A). Once animals were rendered tolerant to morphine, we associated the administration of S1RA (80 mg/kg, s.c.) to morphine (4 mg/kg, s.c.) (according to Protocol I in Fig. 1), and we found that the mechanical threshold increased to a level similar to that found in non-tolerant mice with inflammation treated with this opioid drug (Fig. 5A). These results indicate that S1RA was able to rescue morphine antiallodynic effect from tolerant animals. The administration of PRE-084 (40 mg/kg, s.c.) completely abolished the effect of S1RA in morphine-treated tolerant mice, achieving values close to those found in tolerant mice treated with morphine alone the day of the experiment (Fig. 5A).

We also tested whether the repeated administration of S1RA (80 mg/kg, s.c.) before each dose of morphine during the 3-days morphine administration would have a preemptive effect on the development of morphine tolerance to its antiallodynic effect (according to Protocol II in Fig. 1). When the effect of morphine (4 mg/kg, s.c.) was tested on day 4, without any further administration of S1RA, the animals showed a reduced morphine effect (Fig. 5B). These results indicate that tolerance to the antiallodynic effect of morphine was developed in spite of S1RA preemptive repeated administration.

Therefore, similar to the results found on heat nociception, sigma-1 receptor inhibition by S1RA was able to restore the morphine-induced antiallodynic effects in mice with inflammation tolerant to this opioid, but S1RA preemptive treatment failed to affect the development of tolerance to the antiallodynic effect of morphine.

These results are summarized in Table 1 (see page 139).

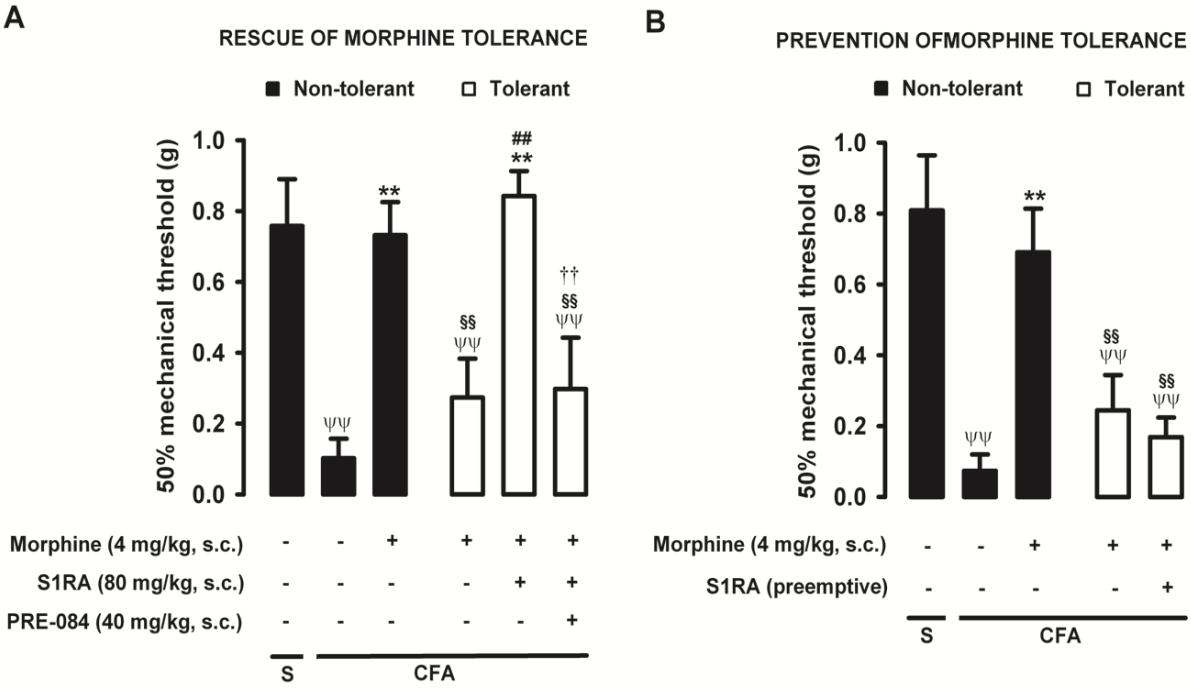

Fig. 5. Modulation by S1RA of the tolerance to the effect of morphine on inflammatory mechanical allodynia. The results represent the 50% mechanical threshold (determined with von Frey filaments) of mice treated periarticularly with CFA or saline (S). (A) Rescue of morphine tolerance by S1RA: Animals were repeatedly treated during 3 days with morphine (tolerant, white bars) or its vehicle (non-tolerant, black bars), according to Protocol I in Fig. 1. The day of evaluation (day 4) mice were treated subcutaneously (s.c.) with morphine (4 mg/kg) or its vehicle, the combination of morphine (4 mg/kg) and S1RA (80 mg/kg) or its vehicle, and the combination of these drugs with PRE-084 (40 mg/kg) or its vehicle. (B) Prevention of morphine tolerance by S1RA: Animals were treated s.c. with S1RA (80 mg/kg) or its vehicle immediately before each dose of morphine (tolerant, white bars) during the induction of morphine tolerance, according to Protocol II in Fig. 1. Control mice (non-tolerant, black bars) received the vehicles of morphine and S1RA during 3 days, according to Protocol II in Fig. 1. The evaluation day (day 4), tolerant and non-tolerant mice were treated s.c. only with morphine (4 mg/kg) or its vehicle. (A and B) Each bar and vertical line represents the mean ± SEM of the values obtained in 8-10 animals. (A and B) Statistically significant differences between the values obtained in: mice with and without inflammation that receive the same treatment ($^{\Psi\Psi}P < 0.01$); tolerant and non-tolerant animals treated with morphine the day of the evaluation ($^{\S\S}P < 0.01$); mice treated with saline or morphine the day of evaluation ($^{**}P < 0.01$) (two-way ANOVA followed by Student–Newman–Keuls test). (A) Statistically significant differences in tolerant mice between the values obtained in animals treated the evaluation day with: morphine + S1RA or its vehicle ($^{\#\#}P < 0.01$); morphine + S1RA associated with PRE-084 or its vehicle ($^{\dagger\dagger}P < 0.01$) (two-way ANOVA followed by Student–Newman–Keuls test). (B) There were no statistically significant differences in the effect of morphine on the evaluation day between mice treated with S1RA or its vehicle during the induction of morphine tolerance (two-way ANOVA followed by Student–Newman–Keuls test).

3.2.5. *Absence of modulation by S1RA of morphine-induced recovery of grip strength deficits during inflammation*

Mice periarticularly administered with saline showed grip strength values close to the 100% of their basal values, whereas mice with CFA-induced joint inflammation showed a decrease in their grip strength values of about half of their baseline measurement (Fig. 6A, first two bars). Morphine a dministration (2-8 mg/kg, s.c.) induced a dose-dependent recovery of grip strength deficits in animals with inflammation, achieving a full recovery of normal grip strength values at the highest dose tested (Fig. 6A). Morphine was less potent to reverse grip strength deficits than to inhibit tactile allodynia during inflammation, and both endpoints were more sensitive to morphine than nociceptive heat pain (compare Figs. 2A, 4A and 6A).

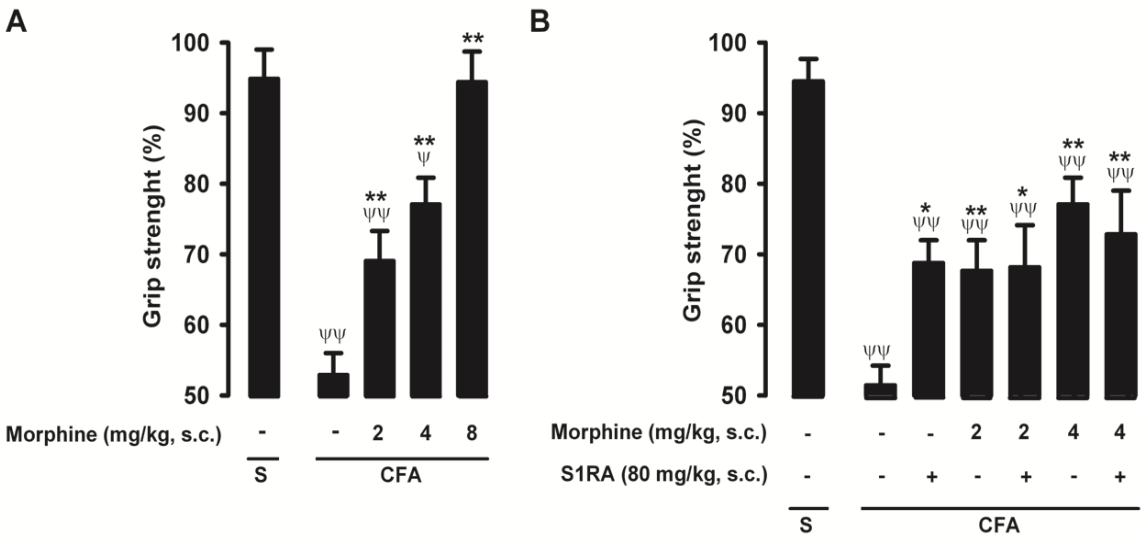

Fig. 6. Effects of morphine alone or associated with S1RA on grip strength deficits induced by inflammation. The results represent the grip strength values (expressed as the averaged % of the basal value of each individual mouse before the periarticular injection) of mice treated periarticularly with CFA or saline (S), (A) Effect of the subcutaneous (s.c.) administration of several doses of morphine (2-8 mg/kg) or its vehicle. (B) Effect of the s.c. administration of morphine (2 and 4 mg/kg), S1RA (80 mg/kg) or their vehicle, and the association of morphine (2 and 4 mg/kg) with S1RA (80 mg/kg). (A and B) Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. (A and B) Statistically significant differences between the values obtained in mice with and without inflammation: $^{\Psi}P < 0.05$, $^{\Psi\Psi}P < 0.01$; and between animals with inflammation treated with morphine or its vehicle: $*P < 0.05$, $**P < 0.01$ (one-way ANOVA followed by Student–Newman–Keuls test). (B) There were no statistically significant differences between the values obtained in mice treated with morphine + S1RA or its vehicle (one-way ANOVA followed by Student–Newman–Keuls test).

The administration of S1RA alone (80 mg/kg, s.c.) induced a slight but significant increase in the grip strength values of mice with inflammation (Fig. 6B). In contrast to the results found on heat nociception and on inflammatory tactile allodynia, S1RA administration was unable to enhance the effect of morphine administered at either 2 or 4 mg/kg (s.c.) (Fig. 6B). To further confirm the lack of effect of S1RA on morphine effect in the grip strength of mice with inflammation, we performed a time-course of drug effects. The association of S1RA with morphine did not alter the effect of this opioid drug when administered at 2 mg/kg (s.c.), at any time-point tested between 30-180 min after drug administration (Fig. 7A).

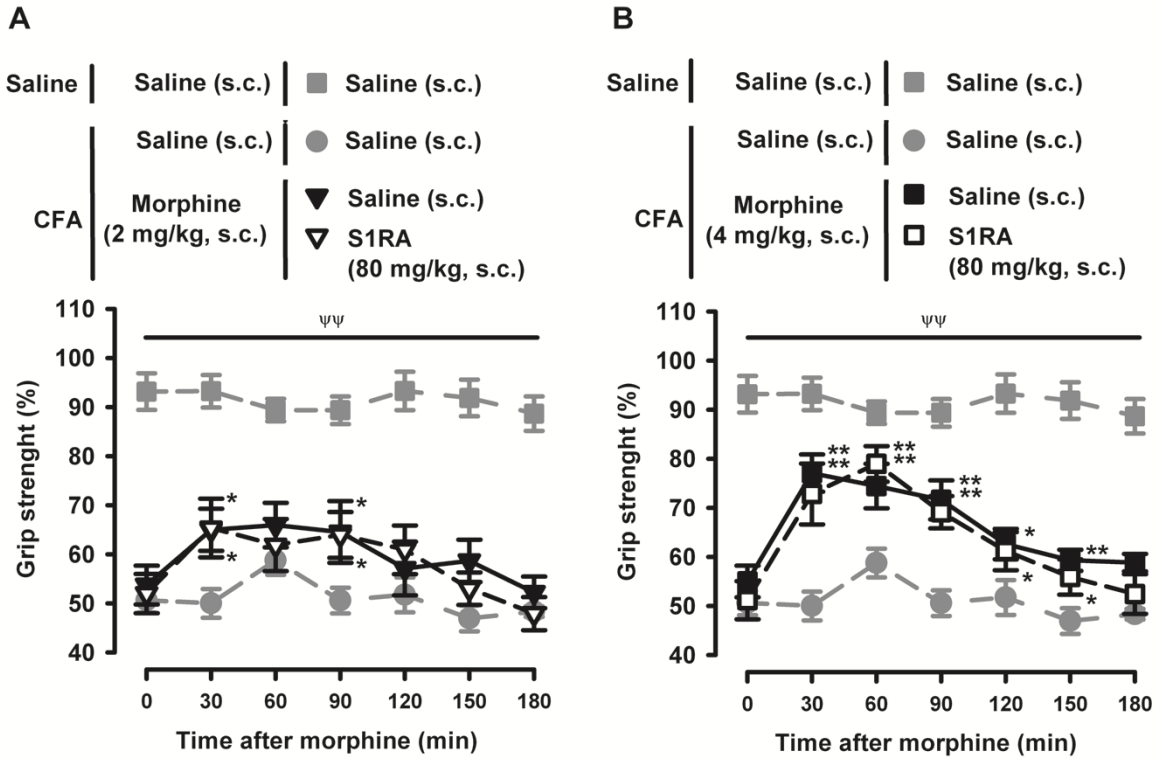

Fig. 7. Time-course of the effects of morphine and its association with S1RA on grip strength deficits induced by inflammation. The results represent the grip strength values (expressed as the % of the basal value of each individual mouse before the periarticular injection of mice) treated periarticularly with CFA or saline. (A) Time-course of the effects of the subcutaneous (s.c.) administration of morphine 2 mg/kg associated with S1RA (80 mg/kg) or its vehicle. (B) Time-course of the effects of the s.c. administration of morphine 4 mg/kg associated with S1RA (80 mg/kg) or its vehicle. (A and B) Each point and vertical line represents the mean ± SEM of values obtained in 8-10 animals. (A and B) Statistically significant differences between the values from mice with inflammation treated with the drug or vehicle at each time-point: **P* < 0.05, ***P* < 0.01. There were no statistically significant differences between the values from mice treated with morphine associated with S1RA or its vehicle at any time-point tested (two-way repeated measures ANOVA followed by Student–Newman–Keuls test).

Similar results were found when we tested the effects of the association of S1RA with morphine 4 mg/kg (s.c.) (Fig. 7B).

Therefore, in contradistinction to the potentiation by S1RA of morphine-induced heat antinociception and the antiallodynic effects in mice with inflammation, S1RA is unable to modify morphine effects on grip strength deficits induced by joint inflammation.

These results are summarized in Table 1 (see page 139).

3.2.6. Modulation by S1RA of the tolerance to morphine-induced recovery of grip strength deficits during inflammation

Animals were rendered tolerant to morphine by the same procedure described in previous sections (Fig.1), whereas control non-tolerant mice received morphine vehicle. Non-tolerant mice with inflammation showed a marked reversion of grip strength deficits in response to the acute administration of morphine 8 mg/kg (s.c.) (Fig. 8A, black bars). However, morphine-tolerant mice did not show significant effects on grip strength by the same morphine dose (8 mg/kg, s.c.) whose effect remained at about half of the baseline grip strength values (83.95 ± 4.8 and 57.81 ± 4.2 % of baseline grip strength values in non-tolerant and tolerant mice with inflammation in response to morphine, respectively) (Fig. 8A).

Once animals were rendered tolerant to morphine, we associated the administration of S1RA (80 mg/kg, s.c.) to morphine (8 mg/kg, s.c.) (according to Protocol I in Fig. 1), and we found that grip strength increased up to values close to 80% of baseline measurements (Fig. 8A). Therefore, as for nociceptive heat pain and inflammatory allodynia, S1RA was able to rescue morphine effect on grip strength from tolerant animals. The administration of PRE-084 (40 mg/kg, s.c.) completely abolished the effect of S1RA in morphine-treated tolerant mice, achieving values close to those found in tolerant mice treated with morphine alone the day of the experiment (Fig. 8A), supporting the selectivity of the effects induced by S1RA.

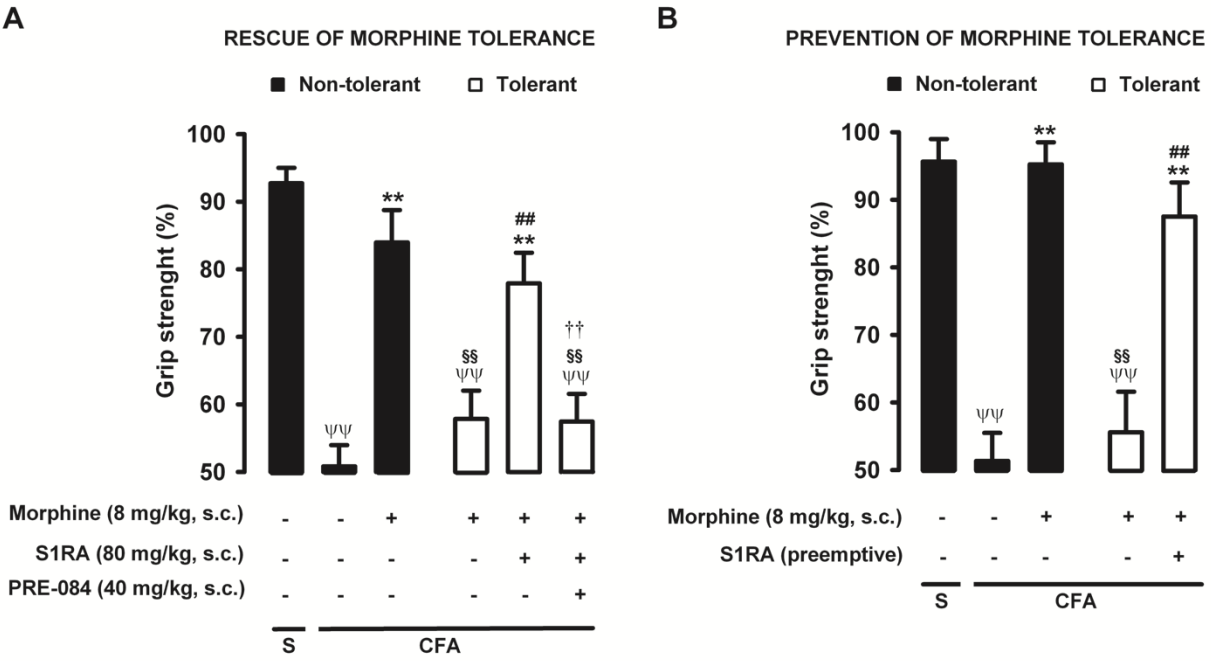

Fig. 8. Modulation by S1RA of the tolerance to the effect of morphine on grip strength deficits induced by inflammation. The results represent the grip strength values (expressed as the averaged % of the basal value of each individual mouse before the periarticular injection) of mice treated periarticularly with CFA or saline (S), (A) Rescue of morphine tolerance by S1RA: Animals were repeatedly treated during 3 days subcutaneously (s.c.) with morphine (tolerant, white bars) or its vehicle (non-tolerant, black bars), according to Protocol I in Fig. 1. The day of evaluation (day 4) mice were treated s.c. with morphine (8 mg/kg) or its vehicle; morphine (8 mg/kg) + S1RA (80 mg/kg) or its vehicle, and the combination of these drugs with PRE-084 (40 mg/kg) or its vehicle. (B) Prevention of morphine tolerance by S1RA: animals were treated s.c. with S1RA (80 mg/kg) or its vehicle immediately before each dose of morphine (tolerant, white bars) during the induction of morphine tolerance, according to Protocol II in Fig. 1. Control mice (non-tolerant, black bars) received the vehicles of morphine and S1RA during 3 days, according to Protocol II in Fig. 1. The evaluation day (day 4), tolerant and non-tolerant mice were treated only with morphine (8 mg/kg, s.c.) or its vehicle. (A and B) Each bar and vertical line represents the mean ± SEM of the values obtained in 8-10 animals. (A and B) Statistically significant differences between the values obtained in: mice with and without inflammation that receive the same treatment ($^{\Psi\Psi}P < 0.01$); tolerant and non-tolerant animals treated with morphine the day of the experiment ($^{\S\S}P < 0.01$); mice treated with saline or morphine the day of the behavioral testing ($^{**}P < 0.01$); tolerant mice treated with S1RA or its vehicle either the day of the evaluation or as a preemptive treatment ($^{\#\#}P < 0.01$) (two-way ANOVA followed by Student–Newman–Keuls test). (A) Statistically significant differences between the values obtained in tolerant mice treated the day of evaluation with morphine + S1RA associated with PRE-084 or its solvent: $^{\dagger\dagger}P < 0.01$ (two-way ANOVA followed by Student–Newman–Keuls test).

We also tested whether the repeated administration of S1RA (80 mg/kg, s.c.) during the 3-days morphine administration (according to Protocol II in Fig. 1) would have a preemptive effect on the development of morphine tolerance to its effect on grip

strength deficits. When the effect of morphine (8 mg/kg, s.c.) was tested the evaluation day, without any further administration of S1RA, mice showed a robust effect in response to morphine (Fig. 8B). These results indicate that S1RA was able to successfully prevent morphine tolerance in this particular outcome, in contradistinction to the results on nociceptive heat pain and inflammatory tactile allodynia shown in the preceding sections.

Therefore, the administration of S1RA, either when morphine tolerance was fully developed or during the induction of tolerance, was able to preserve morphine effect on grip strength deficits in mice with joint inflammation.

These results are summarized in Table 1.

Table 1. Summary of the main results obtained in this study of the role of sigma-1 receptors on nociceptive heat pain and inflammation-induced tactile allodynia and grip strength deficits

Pain state	Stimulus	Sensitivity to morphine	Sensitivity to S1RA	Potentiation of morphine by S1RA	Effect of S1RA on morphine tolerance	
					Rescue of morphine tolerance	Prevention of morphine tolerance
Nociceptive	Heat	+	-	+	+	-
Inflammatory	von Frey	+++	-	+	+	-
	Grip strength	++	+	-	+	+

S1RA was always administered subcutaneously (s.c.) at 80 mg/kg. The doses of s.c. morphine used to test the potentiation of its effect by S1RA varied according to the sensitivity to the opioid for each test (1 mg/kg for von Frey testing in mice with inflammation and 2-4 mg/kg for nociceptive heat pain and grip strength deficits induced by inflammation), as well as for the evaluation of analgesic tolerance (4 mg/kg for von Frey testing in mice with inflammation and 8 mg/kg for nociceptive heat pain and grip strength deficits induced by inflammation).

3.3. DISCUSSION

We studied and compared the effects of morphine, S1RA and their association in three different pain measures: nociceptive heat pain, inflammatory tactile allodynia and grip strength deficits induced by inflammation. In addition, we studied the effects of S1RA on morphine tolerance in these three different measures.

In this study we show that morphine was able to induce analgesic-like effects in nociceptive heat pain, inflammatory tactile allodynia and grip strength deficits induced by inflammation. However, the sensitivity to this opioid drug varied depending on the endpoint examined. Inflammatory allodynia or grip strength deficits induced by inflammation were more sensitive to morphine effects than nociceptive heat pain. These results agree with the widely reported increase in opioid effects in cutaneous sensory hypersensitivity during inflammation (reviewed in Stein et al., 2009), and indicate that the enhancement of opioid effects during inflammation is a feature shared by the functional deficit associated to this pathological condition. Interestingly, we previously compared the pharmacology of grip strength deficits and tactile allodynia during inflammation using several drugs from different pharmacological groups, including opioids (oxycodone and tramadol), non-steroidal anti-inflammatory drugs (ibuprofen and celecoxib), and acetaminophen (Montilla-García et al., 2017). With the exception of oxycodone, which showed a similar potency in reversing tactile allodynia and grip strength deficits, all other known analgesic drugs showed effects at lower doses in functional deficits than on cutaneous hypersensitivity (Montilla-García et al., 2017). Here we show that morphine was less potent in reversing grip strength deficits than tactile allodynia, which support that the pharmacological sensitivity of both outcomes during inflammation is not identical. Differences between the analgesic sensitivity of standard cutaneous measures of pain and pain-induced functional deficits are not limited to grip strength, as they have been previously described for other outcomes aimed to test pain interference on physical function such as inflammation-induced weight bearing differences or wheel running depression (reviewed in Cobos and Portillo-Salido, 2013).

We also tested the effects of S1RA (in the absence of morphine administration) on nociceptive heat pain, inflammatory tactile allodynia and grip strength induced by inflammation. S1RA did not alter nociceptive heat pain, as previously described for this and other sigma-1 antagonists (e.g. Chien and Pasternak, 1994 and 1995; Tejada et al., 2014 and 2017). We also show that S1RA administration failed to induce any effect on inflammatory tactile allodynia. These results are in contrast to previous studies in which sigma-1 antagonism was reported to ameliorate inflammatory hypersensitivity (reviewed in Gris et al., 2015; Tejada et al., 2018). In the previous study which use CFA as the inflammatory compound, the dose used to induce inflammation was considerably lower than the one used in the present study (20 *vs* 30 µl in former and present studies) (Gris et al., 2014, and present study). We needed to use a higher dose of CFA in our study since lower doses of this compound do not induce enough decrease in grip strength to reliably assess for the effects of analgesic drugs, as we previously reported (Montilla-García et al., 2017). Therefore, our experimental conditions might be too restrictive to be overcome by the antiallodynic effects induced by sigma-1 antagonism. In spite of the absence of effect of S1RA on inflammatory tactile allodynia, here we show that this sigma-1 antagonist was able to partially ameliorate grip strength deficits induced by inflammation, which again indicates that the sensitivity to drug effects of tactile allodynia and grip strength deficits differ. We have previously shown that both nociceptive heat pain and tactile allodynia during inflammation in our experimental conditions are sensitive to the *in vivo* ablation of transient receptor potential vanilloid (TRPV1)–expressing neurons by resiniferatoxin (Montilla-García et al., 2017 and 2018). However, grip strength deficits during inflammation are insensitive to resiniferatoxin treatment (Montilla-García et al., 2017). These results indicate that the neurobiological mechanisms of grip strength deficits during inflammation and the behavioral tests of cutaneous sensitivity explored here differ. Therefore, the effect of S1RA on grip strength deficits of mice with inflammation might be due to sigma-1 actions in other pain pathways not related to those involved in heat nociceptive pain or inflammatory tactile allodynia.

In this study we show that the systemic administration of S1RA was able to enhance morphine antinociception to heat stimulus. These results are apparently contradictory

with our previous study which shows that the local peripheral administration of this sigma-1 antagonist was unable to potentiate opioid antinociception (Montilla-García et al., 2018). Since central sigma-1 inhibition is known to markedly increase opioid antinociception to a heat stimulus (Pan et al., 1998; Mei and Pasternak, 2002, 2007), it could be concluded that the enhancement of opioid antinociception to heat stimulus by systemic sigma-1 antagonism should occur at the central but not at the peripheral level. We show that S1RA markedly potentiated the antiallodynic effect of morphine on mice with inflammation. To our knowledge this is the first report showing that a sigma-1 antagonist enhances the effect of an opioid drug in a pathological pain model. The enhancement of morphine effects on nociceptive heat pain and inflammatory tactile allodynia by S1RA were abolished by the administration of the sigma-1 agonist PRE-084, which argues in favor of an action mediated by sigma-1 receptors on these effects induced by S1RA. In spite of the obvious increase of morphine effects in nociceptive heat pain and inflammatory tactile allodynia commented above, and to the higher sensitivity of grip strength deficits to detect the effects of S1RA when administered alone (in the absence of morphine), we show that S1RA was not able to potentiate morphine effects on grip strength deficits induced by inflammation, which further reinforces that the neurobiology of grip strength deficits is different from that of cutaneous pain.

We also explored the modulation of morphine analgesic tolerance by S1RA, as this opioid effect is a substantial drawback for the use of opioid analgesics (Morgan and Christie, 2011). We found that when S1RA was administered to morphine-tolerant mice, it was able to rescue morphine effect on nociceptive pain to heat stimulus, in agreement with previous reports using this type of stimulus (Vidal-Torres et al., 2013; Rodríguez-Muñoz et al., 2015b). We also show that S1RA was able to rescue morphine effects on inflammatory tactile allodynia and grip strength deficits induced by inflammation. The rescue of morphine tolerance by this sigma-1 antagonist in the three outcomes examined was abolished by the administration of PRE-084, which again argues in favor of an action mediated by sigma-1 receptors on the effects induced by S1RA. It has been suggested that sigma-1 antagonism both potentiates opioid analgesia and rescues morphine analgesia from tolerance by decreasing the inhibitory actions of *N*-methyl-*D*-aspartate receptor (NMDAR) activity on μ-opioid receptors (Rodríguez-Muñoz et al.,

2015a and b). Since S1RA failed to enhance morphine effect on grip strength deficits during inflammation in non-tolerant mice but it was able to successfully rescue from morphine tolerance, this suggests that the mechanisms for opioid potentiation by sigma-1 receptors appear to be dissociated from the rescue of opioid analgesia, at least in this particular outcome.

To study whether the administration of S1RA was able to prevent the development of morphine tolerance, we administered this sigma-1 antagonist during the escalating dosage regimen of morphine (but not in the day of the behavioral evaluation). We show that S1RA failed to prevent the tolerance to morphine effects on nociceptive heat stimulus, as previously reported (Vidal-Torres et al., 2013). In addition, sigma-1 antagonism also failed to prevent tolerance to the antiallodynic effect of morphine during inflammation, but surprisingly, S1RA completely prevented the development of morphine tolerance to its effects on grip strength deficits. There have been reported other long-lasting effects of sigma-1 drugs, specifically induced by the sigma-1 antagonists S1RA and BD-1063, which prevent paclitaxel-induced neuropathic pain (Nieto et al., 2012 and 2014), and the sigma-1 agonist PRE-084, which promotes pain sensitization far beyond its expected half-life (Entrena et al., 2016). Sigma-1 drugs are known to alter the translocation of neurotransmitter receptors such as NMDAR to the plasma membrane, with the consequent modulation of their excitatory actions (Pabba et al., 2014). These plastic changes might outlast the presence of the drug (either sigma-1 agonists or antagonists) in the organism, so the biological half-life of the drug's effects may be longer than the half-life of the drug in the organism, which might explain the longer-lasting actions of S1RA. However, it remains unclear why this long-lasting effect of S1RA only affects grip strength deficits ant not heat nociception or inflammatory tactile allodynia. The mechanisms of cutaneous sensitivity are extensively explored, but further studies are needed to unveil the mechanisms of pain-induced functional impairment, and they might not be fully overlapping (Cobos and Portillo-Salido, 2013; Montilla-García et al., 2017; Negus et al., 2018). Similarly, the mechanisms for opioid analgesic tolerance might also depend on the measure explored. Regardless of the exact mechanisms involved in the differential results obtained in grip strength deficits and the

other two pain measures explored in the present study, in the light of our results it is clear that they are not fully equivalent.

Although von Frey testing, the most widely behavioral test currently used in preclinical pain research, is undoubtedly useful to detect sensory alterations in neuropathic patients (e.g. Bennett, 2001; Bouhassira et al., 2005; Moharic et al., 2012), the use of von Frey filaments in other human pain conditions such as during rheumatic diseases is virtually absent. Therefore, although von Frey testing has been established as the standard of preclinical pain testing, it is not an extended pain measure in patients with non-neuropathic chronic painful diseases. On the other hand, grip strength has been widely and routinely evaluated for decades in rheumatology as a functional measure in patients with joint inflammation (e.g. Bijlsma et al., 1987; Lee, 2013; Pincus and Callahan,1992), and it is known to correlate to pain (Callahan et al., 1987; Fraser et al., 1999; Overend et al., 1999). In fact, one set of consensus-based recommendations advocates measuring physical function as one of the main outcomes in clinical trials of pain treatments (Dworkin et al., 2008). Taking into account the differences in the modulation of opioid analgesia and tolerance by sigma-1 receptors in grip strength deficits and the measures of cutaneous sensitivity explored in the present study we believe it is worth to include measures of physical functioning in the standard repertoire of behavioral tests in preclinical laboratories, to approach the human pain phenotype to preclinical pain research.

We conclude that sigma-1 receptors play a pivotal role on the control of morphine analgesia and tolerance (although in a manner dependent on the type of painful stimulus explored). These findings might have important therapeutic implications for the use of sigma-1 antagonists as opioid adjuvants. In addition, the results obtained using grip strength deficits as a surrogate pain measure are not equivalent to those found when exploring the standard measures of cutaneous sensitivity. Further studies are needed to fully understand the mechanisms of pain interference on physical function.

CONCLUSIONS

SPECIFIC CONCLUSIONS

1. Clinically relevant μ-opioid analgesics (morphine, buprenorphine and oxycodone), in the absence of sigma-1 receptor inhibition, produce modality-specific antinociception since they induce prominent systemic and peripheral antinociceptive effects to heat stimulus, but little or no antinociceptive effects to mechanical stimulation.

2. Peripheral sigma-1 receptors play a key role in the modality-specific peripheral effects of opioid analgesics, since opioid antinociception to mechanical stimulus is limited by peripheral sigma-1 tonic inhibitory actions, whereas peripheral opioid antinociception to heat stimulus (produced in TRPV1-expressing neurons) is not.

3. Grip strength deficits during joint inflammation in mice are largely attributable to pain, since they are inhibited by known analgesics in a way consistent with their analgesic efficacy in humans (ameliorative effects of opioids are higher than those induced by acetaminophen or NSAIDs).

4. The neurobiology of inflammatory tactile allodynia and grip strength deficits differ, since these two outcomes develop with distinct time-courses, and respond differentially to TRP antagonism and to the ablation of TRPV1-expressing neurons (which abolished tactile allodynia without altering grip strength deficits).

5. The modulation by sigma-1 receptor of morphine-induced antinociception and tolerance on standard measures of cutaneous sensitivity is different than in pain-induced functional deficit, since systemic sigma-1 antagonism:

    - Potentiates the antinociceptive effect of morphine to heat stimulus and the effects on inflammatory tactile allodynia induced by this opioid, but it does not potentiate morphine effects on grip strength deficits induced by joint inflammation.
    - Rescues morphine antinociception from tolerance on all three pain measures evaluated (nociceptive pain to heat stimulus, inflammatory tactile allodynia and grip strength deficit induced by inflammation). However, it only prevents the development of morphine tolerance on grip strength deficit.

GENERAL CONCLUSION

Sigma-1 receptors play a pivotal role on the control of morphine-induced antincociception and tolerance, although in a manner dependent on the type of painful stimulus explored (mechanical *vs* heat nociceptive stimulus and cutaneous sensitivity *vs* pain-induced functional disability). These findings might have important therapeutic implications for the use of sigma-1 antagonists as opioid adjuvants.

ABBREVIATIONS

LIST OF ABBREVIATIONS

AC	Adenylate cyclase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BD-1047	*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide
BD-1063	1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride
B.C.	Before Christ
CaM	Ca^{++}–calmodulin
cAMP	3′-5′ cyclic adenosine monophosphate
CaMKII	Calmodulin-dependent kinase II
CCI	Chronic constriction injury
CFA	Complete Freud's adjuvant
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRM I	Cholesterol recognition motif I
CRM II	Cholesterol recognition motif II
DAMGO	[D-Ala2, N-MePhe4, Gly-ol]-enkephalin
DHEA	Dehydroepiandrosterone
DMT	*N*,*N*-dimethyltryptamine

DOR	δ-opioid receptor
DPDPE	[D-Pen2, D-Pen5]enkephalin
DRG	Dorsal root ganglion
Enk	Enkephalins
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GRKs	G-protein receptor kinases
GxxxG	Canonical membrane-imbedded dimerization sequence
H	Helical region
HINT1	Histidine triad nucleotide-binding protein 1
HPMC	Hydroxypropyl meth- ylcellulose
5-HT	Serotonin
IB4	Isolectin B4
ICSS	Intracranial self-stimulation
i.c.v.	Intracerebroventricular
IL-1β	Interleukin 1 beta
i.p.	Intraperitoneal
i.pl.	Intraplantar
IP_3	Inositol 1,4,5- trisphosphate
JNK2	c-Jun *N*-terminal kinase 2
KO	Knockout
LC	Locus coeruleus
LTM	Low-threshold mechanoreceptors

MAM	Mitochondria-associated endoplasmic reticulum membrane
MAPKs	Mitogen-activated protein kinases
MIAs	Mechanically insensitive afferents
MOR	μ-opioid receptor
Mrgprd	Mas-related G-protein-coupled receptor D
MR200	(+)-methyl (1R,2S)-2-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate
MSAs	Mechanically sensitive afferents
N/OFQ	Nociceptin/orphanin FQ
NalBzoH	Naloxone benzoylhydrazone
NE	Norepinephrine
NeuN	Neuronal nuclei
NE-100	*N,N*-dipropyl-2-[4-methoxy-3-(2- phenylethoxy)phenyl]ethylamine hydrochloride
NF-200	Neurofilament-200
NMDA	*N*-methyl-*D*-aspartate
NMDAR	*N*-methyl-*D*-aspartate receptor
NSAID	Nonsteroidal anti-inflammatory drug
ORL-1	Opioid receptor like-1
PAN	Primary afferent nociceptor
PAG	Periaqueductal grey matter
PKCγ	Protein kinase C γ
pERK 1/2	Diphosphorylated ERKs 1 and 2
PKA	Protein kinase A

p.o.	Orally
PRE-084	[2-(4-morpholinethyl) 1- phenylcyclohexanecarboxylate hydrochloride]
QST	Quantitative sensory testing
RR	Ruthenium red
RTX	Resiniferatoxin
RVM	Rostral ventromedial medulla
s.c.	Subcutaneous
SC	Spinal cord
S1RA	4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride
SBDL1	Steroid binding domain-like 1
SBDL2	Steroid binding domain-like 2
SP	Substance P
STT	Spinothalamic tract
σ receptor	Sigma receptor
σ_1 receptor	Sigma-1 receptor
T	Pain transmission neuron
TM1	Transmembrane domain 1
TM2	Transmembrane domain 2
TNF-α	Tumor necrosis factor alpha
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid type 1
WT	Wild-type

REFERENCES

REFERENCES

Abadias,M., Escriche,M., Vaque,A., Sust,M., Encina,G., 2013. Safety, tolerability and pharmacokinetics of single and multiple doses of a novel sigma-1 receptor antagonist in three randomized phase I studies. Br.J.Clin.Pharmacol. 75, 103-117.

Abbott,F.V., Melzack,R., Leber,B.F., 1982. Morphine analgesia and tolerance in the tail-flick and formalin tests: dose-response relationships. Pharmacol.Biochem.Behav. 17, 1213-1219.

Abdelaziz,D.M., Abdullah,S., Magnussen,C., Ribeiro-da-Silva,A., Komarova,S.V., Rauch,F., Stone,L.S., 2015. Behavioral signs of pain and functional impairment in a mouse model of osteogenesis imperfecta. Bone 81, 400-406.

Akintola,T., Raver,C., Studlack,P., Uddin,O., Masri,R., Keller,A., 2017. The grimace scale reliably assesses chronic pain in a rodent model of trigeminal neuropathic pain. Neurobiol.Pain 2, 13-17.

Al-Hasani,R., Bruchas,M.R., 2011. Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology 115, 1363-1381.

Alon,A., Schmidt,H.R., Wood,M.D., Sahn,J.J., Martin,S.F., Kruse,A.C., 2017. Identification of the gene that codes for the sigma2 receptor. Proc.Natl.Acad.Sci.U.S.A 114, 7160-7165.

Alonso,G., Phan,V., Guillemain,I., Saunier,M., Legrand,A., Anoal,M., Maurice,T., 2000. Immunocytochemical localization of the sigma(1) receptor in the adult rat central nervous system. Neuroscience 97, 155-170.

Altarifi,A.A., Negus,S.S., 2015. Differential tolerance to morphine antinociception in assays of pain-stimulated vs. pain-depressed behavior in rats. Eur.J.Pharmacol. 748, 76-82.

Altarifi,A.A., Yuan,Y., Zhang,Y., Selley,D.E., Negus,S.S., 2015. Effects of the novel, selective and low-efficacy mu opioid receptor ligand NAQ on intracranial self-stimulation in rats. Psychopharmacology (Berl) 232, 815-824.

Allouche,S., Noble,F., Marie,N., 2014. Opioid receptor desensitization: mechanisms and its link to tolerance. Front Pharmacol. 5, 280.

Amata,E., Rescifina,A., Prezzavento,O., Arena,E., Dichiara,M., Pittala,V., Montilla-García,A., Punzo,F., Merino,P., Cobos,E.J., Marrazzo,A., 2018. (+)-Methyl (1R,2S)-2-{[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropa necarboxylate [(+)-MR200] Derivatives as Potent and Selective Sigma Receptor Ligands: Stereochemistry and Pharmacological Properties. J.Med.Chem. 61, 372-384.

Andrews,N., Legg,E., Lisak,D., Issop,Y., Richardson,D., Harper,S., Pheby,T., Huang,W., Burgess,G., Machin,I., Rice,A.S., 2012. Spontaneous burrowing behaviour in the rat is reduced by peripheral nerve injury or inflammation associated pain. Eur.J.Pain 16, 485-495.

Angeby-Moller,K., Berge,O.G., Hamers,F.P., 2008. Using the CatWalk method to assess weight-bearing and pain behaviour in walking rats with ankle joint monoarthritis induced by carrageenan: effects of morphine and rofecoxib. J.Neurosci.Methods 174, 1-9.

Antolín,F., Buxó,R., Jacomet,S., Navarrete,V., Saña,M., 2014. An integrated perspective on farming in the early Neolithic lakeshore site of La Draga (Banyoles, Spain). Environmental Archaeology 19, 241-255.

Arendt-Nielsen,L., Schipper,K.P., Dimcevski,G., Sumikura,H., Krarup,A.L., Giamberardino, M.A., Drewes,A.M., 2008. Viscero-somatic reflexes in referred pain areas evoked by capsaicin stimulation of the human gut. Eur.J.Pain 12, 544-551.

Authier,S., Arezzo,J., Delatte,M.S., Kallman,M.J., Markgraf,C., Paquette,D., Pugsley,M.K., Ratcliffe,S., Redfern,W.S., Stevens,J., Valentin,J.P., Vargas,H.M., Curtis,M.J., 2016. Safety pharmacology investigations on the nervous system: An industry survey. J.Pharmacol.Toxicol.Methods 81, 37-46.

Bangaru,M.L., Weihrauch,D., Tang,Q.B., Zoga,V., Hogan,Q., Wu,H.E., 2013. Sigma-1 receptor expression in sensory neurons and the effect of painful peripheral nerve injury. Mol.Pain 9, 47.

Bardoni,R., Tawfik,V.L., Wang,D., Francois,A., Solorzano,C., Shuster,S.A., Choudhury,P., Betelli,C., Cassidy,C., Smith,K., de Nooij,J.C., Mennicken,F., O'Donnell,D., Kieffer,B.L., Woodbury,C.J., Basbaum,A.I., MacDermott, A.B., Scherrer,G., 2014. Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. Neuron 81, 1312-1327.

Baron,R., 2000. Capsaicin and nociception: from basic mechanisms to novel drugs. Lancet 356, 785-787.

Barrett,J.E., 2015. The pain of pain: challenges of animal behavior models. Eur.J.Pharmacol. 753, 183-190.

Basbaum,A.I., Bautista,D.M., Scherrer,G., Julius,D., 2009. Cellular and molecular mechanisms of pain. Cell 139, 267-284.

Basbaum,A.I. and Jessell,T.M.,2000. The perception of pain. In: Principles of Neural Science. 4th ed. Kandel ER, Schwartz JH and Jessell TM eds. McGraw-Hill Interamericana. Madrid.

Bederson,J.B., Fields,H.L., Barbaro,N.M., 1990. Hyperalgesia during naloxone-precipitated withdrawal from morphine is associated with increased on-cell activity in the rostral ventromedial medulla. Somatosens. Mot.Res. 7, 185-203.

Beissner,F., Brandau,A., Henke,C., Felden,L., Baumgartner,U., Treede,R.D., Oertel,B.G., Lotsch,J., 2010. Quick discrimination of A(delta) and C fiber mediated pain based on three verbal descriptors. PLoS One 5.

Ben-Bassat, J., Peretz, E., Sulman,F.G., 1959. Analgesimetry and ranking of analgesic drugs by the receptacle method. Arch.Int.Pharmacodyn.Ther. 122, 434-447.

Benarroch,E.E., 2008. Descending monoaminergic pain modulation: bidirectional control and clinical relevance. Neurology 71, 217-221.

Benham,C.D., Gunthorpe,M.J., Davis,J.B., 2003. TRPV channels as temperature sensors. Cell Calcium 33, 479-487.

Bennett,G.J., Xie,Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87-107.

Bennett,M., 2001. The LANSS Pain Scale: the Leeds assessment of neuropathic symptoms and signs. Pain 92, 147-157.

Benyamin,R., Trescot,A.M., Datta,S., Buenaventura,R., Adlaka,R., Sehgal,N., Glaser,S.E., Vallejo,R., 2008. Opioid complications and side effects. Pain Physician 11, S105-S120.

Bhuiyan,M.S., Fukunaga,K., 2011. Targeting sigma-1 receptor signaling by endogenous ligands for cardioprotection. Expert.Opin. Ther.Targets. 15, 145-155.

Bigliardi-Qi,M., Sumanovski,L.T., Buchner,S., Rufli,T., Bigliardi,P.L., 2004. Mu-opiate receptor and Beta-endorphin expression in nerve endings and keratinocytes in human skin. Dermatology 209, 183-189.

Bijlsma,J.W., Huber-Bruning,O., Thijssen,J.H., 1987. Effect of oestrogen treatment on clinical and laboratory manifestations of rheumatoid arthritis. Ann.Rheum.Dis. 46, 777-779.

Blackburn-Munro,G., 2004. Pain-like behaviours in animals - how human are they? Trends Pharmacol.Sci. 25, 299-305.

Bon,K., Lichtensteiger,C.A., Wilson,S.G., Mogil,J., 2003. Characterization of cyclophosphamide cystitis, a model of visceral and referred pain, in the mouse: species and strain differences. J.Urol. 170, 1008-1012.

Bonica,J.J., 1990. Anatomic and physiologic basis of nociception and pain. In: The Management of Pain. 4th ed. Fissheman S, Ballantyne and Rathmell JP eds. Lippincott Williams & Wilkins. Philadelphia.

Bonin,R.P., Labrakakis,C., Eng,D.G., Whissell,P.D., De,K.Y., Orser,B.A., 2011. Pharmacological enhancement of delta-subunit-containing GABA(A) receptors that generate a tonic inhibitory conductance in spinal neurons attenuates acute nociception in mice. Pain 152, 1317-1326.

Borbély,E., Botz,B., Bolcskei,K., Kenyer,T., Kereskai,L., Kiss,T., Szolcsanyi,J., Pinter,E., Csepregi,J.Z., Mocsai,A., Helyes,Z., 2015. Capsaicin-sensitive sensory nerves exert complex regulatory functions in the serum-transfer mouse model of autoimmune arthritis. Brain Behav.Immun. 45, 50-59.

Bouhassira,D., Attal,N., Alchaar,H., Boureau,F., Brochet,B., Bruxelle,J., Cunin,G., Fermanian,J., Ginies,P., Grun-Overdyking,A., Jafari-Schluep,H., Lanteri-Minet,M., Laurent,B., Mick,G., Serrie,A., Valade,D., Vicaut,E., 2005. Comparison of pain syndromes associated with nervous or somatic lesions and development of a new neuropathic pain diagnostic questionnaire (DN4). Pain 114, 29-36.

Bowen,W.D., 2000. Sigma receptors: recent advances and new clinical potentials. Pharm.Acta Helv. 74, 211-218.

Brennan,T.J., Vandermeulen,E.P., Gebhart,G.F., 1996. Characterization of a rat model of incisional pain. Pain 64, 493-501.

Brooks,J., Tracey,I., 2005. From nociception to pain perception: imaging the spinal and supraspinal pathways. J.Anat. 207, 19-33.

Bruna,J., Videla,S., Argyriou,A.A., Velasco,R., Villoria,J., Santos,C., Nadal,C., Cavaletti,G., Alberti,P., Briani,C., Kalofonos,H.P., Cortinovis,D., Sust,M., Vaque,A., Klein,T., Plata-Salaman,C., 2018. Efficacy of a Novel Sigma-1 Receptor Antagonist for Oxaliplatin-Induced Neuropathy: A Randomized, Double-Blind, Placebo-Controlled Phase IIa Clinical Trial. Neurotherapeutics. 15, 178-189.

Brune,S., Pricl,S., Wunsch,B., 2013. Structure of the sigma1 receptor and its ligand binding site. J.Med.Chem. 56, 9809-9819.

Bryden,L.A., Nicholson,J.R., Doods,H., Pekcec,A., 2015. Deficits in spontaneous burrowing behavior in the rat bilateral monosodium iodoacetate model of osteoarthritis: an objective measure of pain-related behavior and analgesic efficacy. Osteoarthritis. Cartilage. 23, 1605-1612.

Bura,A.S., Guegan,T., Zamanillo,D., Vela,J.M., Maldonado,R., 2013. Operant self-administration of a sigma ligand improves nociceptive and emotional manifestations of neuropathic pain. Eur.J.Pain 17, 832-843.

Burma,N.E., Leduc-Pessah,H., Fan,C.Y., Trang,T., 2017. Animal models of chronic pain: Advances and challenges for clinical translation. J.Neurosci.Res. 95, 1242-1256.

Buvanendran,A., Kroin,J.S., Kerns,J.M., Nagalla,S.N., Tuman,K.J., 2004. Characterization of a new animal model for evaluation of persistent postthoracotomy pain. Anesth.Analg. 99, 1453-1460.

Calhoun,G., Wang,L., Almeida,L.E., Kenyon,N., Afsar,N., Nouraie,M., Finkel,J.C., Quezado,Z.M., 2015. Dexmedetomidine ameliorates nocifensive behavior in humanized sickle cell mice. Eur.J.Pharmacol. 754, 125-133.

Calvino,B., Grilo,R.M., 2006. Central pain control. Joint Bone Spine 73, 10-16.

Callahan,L.F., Brooks,R.H., Summey,J.A., Pincus,T., 1987. Quantitative pain assessment for routine care of rheumatoid arthritis patients, using a pain scale based on activities of daily living and a visual analog pain scale. Arthritis Rheum. 30, 630-636.

Castany,S., Gris,G., Vela,J.M., Verdu,E., Boadas-Vaello,P., 2018. Critical role of sigma-1 receptors in central neuropathic pain-related behaviours after mild spinal cord injury in mice. Sci.Rep. 8, 3873.

Cavanaugh,D.J., Chesler,A.T., Braz,J.M., Shah,N.M., Julius,D., Basbaum,A.I., 2011. Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. J.Neurosci. 31, 10119-10127.

Cavanaugh,D.J., Lee,H., Lo,L., Shields,S.D., Zylka,M.J., Basbaum,A.I., Anderson,D.J., 2009. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc.Natl.Acad.Sci.U.S.A 106, 9075-9080.

Cendán,C.M., Pujalte,J.M., Portillo-Salido,E., Baeyens,J.M., 2005a. Antinociceptive effects of haloperidol and its metabolites in the formalin test in mice. Psychopharmacology (Berl) 182, 485-493.

Cendán,C.M., Pujalte,J.M., Portillo-Salido,E., Montoliu,L., Baeyens,J.M., 2005b. Formalin-induced pain is reduced in sigma(1) receptor knockout mice. Eur.J.Pharmacol. 511, 73-74.

Cervero,F., Laird,J.M.A., 2009. Spinal Mechanisms of visceral pain and hyperalgesia. In Malcangio M. Synaptic Plasticity in Pain. New York: Springer. pp: 289–306.

Cobos,E.J., Entrena,J.M., Nieto,F.R., Cendán,C.M., Del Pozo,E., 2008. Pharmacology and therapeutic potential of sigma(1) receptor ligands. Curr.Neuropharmacol. 6, 344-366.

Cobos,E.J., Ghasemlou,N., Araldi,D., Segal,D., Duong,K., Woolf,C.J., 2012. Inflammation-induced decrease in voluntary wheel running in mice: a nonreflexive test for evaluating inflammatory pain and analgesia. Pain 153, 876-884.

Cobos,E.J., Portillo-Salido,E., 2013. "Bedside-to-Bench" Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. Curr.Neuropharmacol. 11, 560-591.

Commons,D., Greenwood,K.M., Anderson,R.A., 2016. A Preliminary Investigation into Worry about Mental Health: Development of the Mental Health Anxiety Inventory. Behav.Cogn Psychother. 44, 347-360.

Costantino,C.M., Gomes,I., Stockton,S.D., Lim,M.P., Devi,L.A., 2012. Opioid receptor heteromers in analgesia. Expert.Rev.Mol.Med. 14, e9.

Coste,B., Xiao,B., Santos,J.S., Syeda,R., Grandl,J., Spencer,K.S., Kim,S.E., Schmidt,M., Mathur,J., Dubin,A.E., Montal,M., Patapoutian,A., 2012. Piezo proteins are pore-forming subunits of mechanically activated channels. Nature 483, 176-181.

Croci,T., Zarini,E., 2007. Effect of the cannabinoid CB1 receptor antagonist rimonabant on nociceptive responses and adjuvant-induced arthritis in obese and lean rats. Br.J.Pharmacol. 150, 559-566.

Crottes,D., Guizouarn,H., Martin,P., Borgese,F., Soriani,O., 2013. The sigma-1 receptor: a regulator of cancer cell electrical plasticity? Front Physiol 4, 175.

Cui,Y.Y., Xu,H., Wu,H.H., Qi,J., Shi,J., Li,Y.Q., 2014. Spatio-temporal expression and functional involvement of transient receptor potential vanilloid 1 in diabetic mechanical allodynia in rats. PLoS.One. 9, e102052.

Curtis,M.J., Bond,R.A., Spina,D., Ahluwalia,A., Alexander,S.P., Giembycz,M.A., Gilchrist,A., Hoyer,D., Insel,P.A., Izzo,A.A., Lawrence,A.J., MacEwan,D.J., Moon,L.D., Wonnacott,S., Weston,A.H., McGrath,J.C., 2015. Experimental design and analysis and their reporting: new guidance for publication in BJP. Br.J.Pharmacol. 172, 3461-3471.

Curto-Reyes,V., Juárez,L., García-Perez,E., Fresno,M.F., Hidalgo,A., Menéndez,L., Baamonde,A., 2008. Local loperamide inhibits thermal hyperalgesia but not mechanical allodynia induced by intratibial inoculation of melanoma cells in mice. Cell Mol.Neurobiol. 28, 981-990.

Chandran,P., Pai,M., Blomme,E.A., Hsieh,G.C., Decker,M.W., Honore,P., 2009. Pharmacological modulation of movement-evoked pain in a rat model of osteoarthritis. Eur.J.Pharmacol. 613, 39-45.

Chaplan,S.R., Bach,F.W., Pogrel,J.W., Chung,J.M., Yaksh,T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J.Neurosci.Methods 53, 55-63.

Charlton,J.J., Allen,P.B., Psifogeorgou,K., Chakravarty,S., Gomes,I., Neve,R.L., Devi,L.A., Greengard,P., Nestler,E.J., Zachariou,V., 2008. Multiple actions of spinophilin regulate mu opioid receptor function. Neuron 58, 238-247.

Chen,H.I., Lin,L.C., Yu,L., Liu,Y.F., Kuo,Y.M., Huang,A.M., Chuang,J.I., Wu,F.S., Liao,P.C., Jen,C.J., 2008. Treadmill exercise enhances passive avoidance learning in rats: the role of down-regulated serotonin system in the limbic system. Neurobiol.Learn.Mem. 89, 489-496.

Chen,S.R., Pan,H.L., 2006. Loss of TRPV1-expressing sensory neurons reduces spinal mu opioid receptors but paradoxically potentiates opioid analgesia. J.Neurophysiol. 95, 3086-3096.

Chen,Y., Willcockson,H.H., Valtschanoff,J.G., 2009. Vanilloid receptor TRPV1-mediated phosphorylation of ERK in murine adjuvant arthritis. Osteoarthritis.Cartilage. 17, 244-251.

Chien,C.C., Pasternak,G.W., 1993. Functional antagonism of morphine analgesia by (+)-pentazocine: evidence for an anti-opioid sigma 1 system. Eur.J.Pharmacol. 250, R7-R8.

Chien,C.C., Pasternak,G.W., 1994. Selective antagonism of opioid analgesia by a sigma system. J.Pharmacol.Exp.Ther. 271, 1583-1590.

Chien,C.C., Pasternak,G.W., 1995. (-)-Pentazocine analgesia in mice: interactions with a sigma receptor system. Eur.J. Pharmacol. 294, 303-308.

Chien,C.C., Pasternak,G.W., 1995. Sigma antagonists potentiate opioid analgesia in rats. Neurosci.Lett. 190, 137-139.

Chiu,I.M., Barrett,L.B., Williams,E.K., Strochlic,D.E., Lee,S., Weyer,A.D., Lou,S., Bryman,G.S., Roberson,D.P., Ghasemlou,N., Piccoli,C., Ahat,E., Wang,V., Cobos,E.J., Stucky,C.L., Ma,Q., Liberles,S.D., Woolf,C.J., 2014. Transcriptional profiling at whole population and single cell levels reveals somatosensory neuron molecular diversity. Elife. 3.

Choi,S.R., Roh,D.H., Yoon,S.Y., Kang,S.Y., Moon,J.Y., Kwon,S.G., Choi,H.S., Han,H.J., Beitz,A.J., Oh,S.B., Lee,J.H., 2013. Spinal sigma-1 receptors activate NADPH oxidase 2 leading to the induction of pain hypersensitivity in mice and mechanical allodynia in neuropathic rats. Pharmacol.Res. 74, 56-67.

Choi,S.R., Roh,D.H., Yoon,S.Y., Kwon,S.G., Choi,H.S., Han,H.J., Beitz,A.J., Lee,J.H., 2016. Astrocyte sigma-1 receptors modulate connexin 43 expression leading to the induction of below-level mechanical allodynia in spinal cord injured mice. Neuropharmacology 111, 34-46.

Choi,Y., Yoon,Y.W., Na,H.S., Kim,S.H., Chung,J.M., 1994. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. Pain 59, 369-376.

Christie,M.J., Connor,M., Vaughan,C.W., Ingram,S.L., Bagley,E.E., 2000. Cellular actions of opioids and other analgesics: implications for synergism in pain relief. Clin.Exp.Pharmacol.Physiol 27, 520-523.

Chu,U.B., Ruoho,A.E., 2016. Biochemical Pharmacology of the Sigma-1 Receptor. Mol.Pharmacol. 89, 142-153.

D'Amour, F.E., Smith,D.L., 1941. A method for determining loss of pain sensation. J. PharmacoL Exp. Ther. 72, 74-79.

Dale,R., Stacey,B., 2016. Multimodal Treatment of Chronic Pain. Med.Clin.North Am. 100, 55-64.

Dario,A., Tomei,G., 2004. A benefit-risk assessment of baclofen in severe spinal spasticity. Drug Saf 27, 799-818.

Davis,M.P., 2015. Sigma-1 receptors and animal studies centered on pain and analgesia. Expert.Opin.Drug Discov. 10, 885-900.

de la Puente,B., Nadal,X., Portillo-Salido,E., Sánchez-Arroyos,R., Ovalle,S., Palacios,G., Muro,A., Romero,L., Entrena,J.M., Baeyens,J.M., López-García,J.A., Maldonado,R., Zamanillo,D., Vela,J.M., 2009. Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. Pain 145, 294-303.

de la Puente,B., Romero-Alejo,E., Vela,J.M., Merlos,M., Zamanillo,D., Portillo-Salido,E., 2015. Changes in saccharin preference behavior as a primary outcome to evaluate pain and analgesia in acetic acid-induced visceral pain in mice. J.Pain Res. 8, 663-673.

Decosterd,I., Woolf,C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87, 149-158.

Delgado-García,G., Rodríguez-Návarez,C., Estañol,B., 2016. On roman philonium. Gac Med Mex. 152, 749-753.

Deseure,K., Hans,G., 2018. Orofacial neuropathic pain reduces spontaneous burrowing behavior in rats. Physiol Behav. 191, 91-94.

Deuis,J.R., Dvorakova,L.S., Vetter,I., 2017. Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol.Neurosci. 10, 284.

Díaz,J.L., Cuberes,R., Berrocal,J., Contijoch,M., Christmann,U., Fernández,A., Port,A., Holenz,J., Buschmann,H., Laggner,C., Serafini,M.T., Burgueno,J., Zamanillo,D., Merlos,M., Vela,J.M., Almansa,C., 2012. Synthesis and biological evaluation of the 1-arylpyrazole class of sigma(1) receptor antagonists: identification of 4-{2-[5-methyl-1-(naphthalen-2-yl)-1H-pyrazol-3-yloxy]ethyl}morpholine (S1RA, E-52862). J.Med.Chem. 55, 8211-8224.

Díaz,J.L., Christmann,U., Fernández,A., Luengo,M., Bordas,M., Enrech,R., Carro,M., Pascual,R., Burgueno,J., Merlos,M., Benet-Buchholz,J., Ceron-Bertran,J., Ramirez,J., Reinoso,R.F., Fernández de Henestrosa,A.R., Vela,J.M., Almansa,C., 2013. Synthesis and biological evaluation of a new series of hexahydro-2H-pyrano[3,2-c]quinolines as novel selective sigma1 receptor ligands. J.Med.Chem. 56, 3656-3665.

Díaz,J.L., Christmann,U., Fernández,A., Torrens,A., Port,A., Pascual,R., Álvarez,I., Burgueno,J., Monroy,X., Montero,A., Balada,A., Vela,J.M., Almansa,C., 2015. Synthesis and structure-activity relationship study of a new series of selective sigma(1) receptor ligands for the treatment of pain: 4-aminotriazoles. J.Med.Chem. 58, 2441-2451.

Drewes,A.M., Schipper,K.P., Dimcevski,G., Petersen,P., Gregersen,H., Funch-Jensen,P., Arendt-Nielsen,L., 2003. Gut pain and hyperalgesia induced by capsaicin: a human experimental model. Pain 104, 333-341.

Dubin,A.E., Patapoutian,A., 2010. Nociceptors: the sensors of the pain pathway. J Clin Invest 120.

Dubuisson,D., Dennis,S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161-174.

Dumas,E.O., Pollack,G.M., 2008. Opioid tolerance development: a pharmacokinetic/pharmacodynamic perspective. AAPS.J. 10, 537-551.

Dworkin,R.H., Turk,D.C., Wyrwich,K.W., Beaton,D., Cleeland,C.S., Farrar,J.T., Haythornthwaite,J.A., Jensen,M.P., Kerns,R.D., Ader,D.N., Brandenburg,N., Burke,L.B., Cella,D., Chandler,J., Cowan,P., Dimitrova,R., Dionne,R., Hertz,S., Jadad,A.R., Katz,N.P., Kehlet,H., Kramer,L.D., Manning,D.C., McCormick,C., McDermott,M.P., McQuay,H.J., Patel,S., Porter,L., Quessy,S., Rappaport,B.A., Rauschkolb,C., Revicki,D.A., Rothman,M., Schmader,K.E., Stacey,B.R., Stauffer,J.W., von,S.T., White,R.E., Witter,J., Zavisic,S., 2008. Interpreting the clinical importance of treatment outcomes in chronic pain clinical trials: IMMPACT recommendations. J.Pain 9, 105-121.

Entrena,J.M., Cobos,E.J., Nieto,F.R., Cendán,C.M., Baeyens,J.M., Del Pozo,E., 2009a. Antagonism by haloperidol and its metabolites of mechanical hypersensitivity induced by intraplantar capsaicin in mice: role of sigma-1 receptors. Psychopharmacology (Berl) 205, 21-33.

Entrena,J.M., Cobos,E.J., Nieto,F.R., Cendán,C.M., Gris,G., Del Pozo,E., Zamanillo,D., Baeyens,J.M., 2009b. Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice. Pain 143, 252-261.

Entrena,J.M., Sánchez-Fernandez,C., Nieto,F.R., González-Cano,R., Yeste,S., Cobos,E.J., Baeyens,J.M., 2016. Sigma-1 Receptor Agonism Promotes Mechanical Allodynia After Priming the Nociceptive System with Capsaicin. Sci.Rep. 6, 37835.

Espinosa-Juarez,J.V., Jaramillo-Morales,O.A., López-Muñoz,F.J., 2017a. Haloperidol Decreases Hyperalgesia and Allodynia Induced by Chronic Constriction Injury. Basic Clin.Pharmacol.Toxicol. 121, 471-479.

Espinosa-Juarez,J.V., Jaramillo-Morales,O.A., Navarrete-Vazquez,G., Melo-Hernández, L.A., Deciga-Campos,M., López-Muñoz,F.J., 2017b. N-(2-morpholin-4-yl-ethyl)-2-(1naphthyloxy)acetamide inhibits the chronic constriction injury-generated hyperalgesia via the antagonism of sigma-1 receptors. Eur.J.Pharmacol. 812, 1-8.

Ewan,E.E., Martin,T.J., 2014. Differential suppression of intracranial self-stimulation, food-maintained operant responding, and open field activity by paw incision and spinal nerve ligation in rats. Anesth.Analg. 118, 854-862.

Fallon,M.T., Albert,L.E., McQuade,R., Rossetti,S., Sanchez,R., Sun,W., Wright,S., Lichtman,A.H., Kornyeyeva,E., 2017. Sativex oromucosal spray as adjunctive therapy in advanced cancer patients with chronic pain unalleviated by optimized opioid therapy: two double-blind, randomized, placebo-controlled phase 3 studies. Br.J.Pain 11, 119-133.

Farquhar-Smith,W.P., 2008. Anatomy, physiology and pharmacology of pain. Pain. 9, 3- 7.

Fields,H.L., 1992. Is there a Facilitating Component to Central Pain Modulation?. APS Journal. 1, 139-141.

Fields,H., 2004. State-dependent opioid control of pain. Nat.Rev.Neurosci. 5, 565-575.

Fioravanti,B., Vanderah,T.W., 2008. The ORL-1 receptor system: are there opportunities for antagonists in pain therapy? Curr.Top.Med.Chem. 8, 1442-1451.

Fischer,B.D., Adeyemo,A., O'Leary,M.E., Bottaro,A., 2017. Animal models of rheumatoid pain: experimental systems and insights. Arthritis Res.Ther. 19, 146.

Flórez,J., 2008. Anatomic and physiologic basis of nociception and pain. In Farmacología Humana. 5th ed. Flórez J, Aermijo JA, Mediavilla A eds. Elsevier, Barcelona, España.

Fontanilla,D., Johannessen,M., Hajipour,A.R., Cozzi,N.V., Jackson,M.B., Ruoho,A.E., 2009. The hallucinogen N,N-dimethyltryptamine (DMT) is an endogenous sigma-1 receptor regulator. Science 323, 934-937.

Forte,B.L., Slosky,L.M., Zhang,H., Arnold,M.R., Staatz,W.D., Hay,M., Largent-Milnes,T.M., Vanderah,T.W., 2016. Angiotensin-(1-7)/Mas receptor as an antinociceptive agent in cancer-induced bone pain. Pain 157, 2709-2721.

Franchini,S., Battisti,U.M., Prandi,A., Tait,A., Borsari,C., Cichero,E., Fossa,P., Cilia,A., Prezzavento,O., Ronsisvalle,S., Arico,G., Parenti,C., Brasili,L., 2016. Scouting new sigma receptor ligands: Synthesis, pharmacological evaluation and molecular modeling of 1,3-dioxolane-based structures and derivatives. Eur.J.Med.Chem. 112, 1-19.

Fraser,A., Vallow,J., Preston,A., Cooper,R.G., 1999. Predicting 'normal' grip strength for rheumatoid arthritis patients. Rheumatology.(Oxford) 38, 521-528.

Fujiwara,M., Iwata,M., Inoue,T., Aizawa,Y., Yoshito,N., Hayashi,K., Suzuki,S., 2017. Decreased grip strength, muscle pain, and atrophy occur in rats following long-term exposure to excessive repetitive motion. FEBS Open.Bio 7, 1737-1749.

García-Larrea,L., Bastuji,H., 2017. Pain and consciousness.Prog.Neuropsychopharmacol .Biol.Psychiatry. S0278-5846, 30510-30519.

Garzón,J., Rodríguez-Munoz,M., Sánchez-Blázquez,P., 2008. Do pharmacological approaches that prevent opioid tolerance target different elements in the same regulatory machinery? Curr.Drug Abuse Rev. 1, 222-238.

Gilron,I., Jensen,T.S., Dickenson,A.H., 2013. Combination pharmacotherapy for management of chronic pain: from bench to bedside. Lancet Neurol. 12, 1084-1095.

Goldberg,D.S., McGee,S.J., 2011. Pain as a global public health priority. BMC.Public Health 11, 770.

Gomes,I., Jordan,B.A., Gupta,A., Trapaidze,N., Nagy,V., Devi,L.A., 2000. Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. J.Neurosci. 20, RC110.

Gómez-Soler,M., Fernández-Dueñas,V., Portillo-Salido,E., Pérez,P., Zamanillo,D., Vela,J.M., Burgueno,J., Ciruela,F., 2014. Predicting the antinociceptive efficacy of sigma(1) receptor ligands by a novel receptor fluorescence resonance energy transfer (FRET) based biosensor. J.Med.Chem. 57, 238-242.

González-Cano,R., Merlos,M., Baeyens,J.M., Cendán,C.M., 2013. sigma1 receptors are involved in the visceral pain induced by intracolonic administration of capsaicin in mice. Anesthesiology 118, 691-700.

Goudie-DeAngelis,E.M., Abdelhamid,R.E., Nunez,M.G., Kissel,C.L., Kovacs,K.J., Portoghese,P.S., Larson,A.A., 2016. Modulation of musculoskeletal hyperalgesia by brown adipose tissue activity in mice. Pain 157, 2561-2570.

Grace,P.M., Strand,K.A., Maier,S.F., Watkins,L.R., 2014. Suppression of voluntary wheel running in rats is dependent on the site of inflammation: evidence for voluntary running as a measure of hind paw-evoked pain. J.Pain 15, 121-128.

Grau,J.W., Huie,J.R., Lee,K.H., Hoy,K.C., Huang,Y.J., Turtle,J.D., Strain,M.M., Baumbauer,K.M., Miranda,R.M., Hook,M.A., Ferguson,A.R., Garraway,S.M., 2014. Metaplasticity and behavior: how training and inflammation affect plastic potential within the spinal cord and recovery after injury. Front Neural Circuits. 8, 100.

Greenwood-Van,M.B., Standifer,K.M., 2008. Methylnaltrexone in the treatment of opioid-induced constipation. Clin.Exp. Gastroenterol. 1, 49-58.

Gregoire,S., Michaud,V., Chapuy,E., Eschalier,A., Ardid,D., 2012. Study of emotional and cognitive impairments in mononeuropathic rats: effect of duloxetine and gabapentin. Pain 153, 1657-1663.

Gregory,N.S., Harris,A.L., Robinson,C.R., Dougherty,P.M., Fuchs,P.N., Sluka,K.A., 2013. An overview of animal models of pain: disease models and outcome measures. J.Pain 14, 1255-1269.

Gris,G., Cobos,E.J., Zamanillo,D., Portillo-Salido,E., 2015. Sigma-1 receptor and inflammatory pain. Inflamm.Res. 64, 377-381.

Gris,G., Merlos,M., Vela,J.M., Zamanillo,D., Portillo-Salido,E., 2014. S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in the carrageenan and complete Freund's adjuvant models in mice. Behav.Pharmacol. 25, 226-235.

Gris,G., Portillo-Salido,E., Aubel,B., Darbaky,Y., Deseure,K., Vela,J.M., Merlos,M., Zamanillo,D., 2016. The selective sigma-1 receptor antagonist E-52862 attenuates neuropathic pain of different aetiology in rats. Sci.Rep. 6, 24591.

Groer,C.E., Schmid,C.L., Jaeger,A.M., Bohn,L.M., 2011. Agonist-directed interactions with specific beta-arrestins determine mu-opioid receptor trafficking, ubiquitination, and dephosphorylation. J.Biol.Chem. 286, 31731-31741.

Guingamp,C., Gegout-Pottie,P., Philippe,L., Terlain,B., Netter,P., Gillet,P., 1997. Mono-iodoacetate-induced experimental osteoarthritis: a dose-response study of loss of mobility, morphology, and biochemistry. Arthritis Rheum. 40, 1670-1679.

Guo,A., Vulchanova,L., Wang,J., Li,X., Elde,R., 1999. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur.J.Neurosci. 11, 946-958.

Guscott,M., Bristow,L.J., Hadingham,K., Rosahl,T.W., Beer,M.S., Stanton,J.A., Bromidge,F., Owens,A.P., Huscroft,I., Myers,J., Rupniak,N.M., Patel,S., Whiting,P.J., Hutson,P.H., Fone,K.C., Biello,S.M., Kulagowski,J.J., McAllister,G., 2005. Genetic knockout and pharmacological blockade studies of the 5-HT7 receptor suggest therapeutic potential in depression. Neuropharmacology 48, 492-502.

Haberstock-Debic,H., Kim,K.A., Yu,Y.J., Von,Z.M., 2005. Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. J.Neurosci. 25, 7847-7857.

Haley,J.E., Dickenson,A.H., 2016. Evidence for spinal N-methyl-d-aspartate receptor involvement in prolonged chemical nociception in the rat. Brain Res. 1645, 58-60.

Hall,J.E., 2011a. Sensibilidades Somáticas: I. Organización general, las sensaciones táctil y posicional. In Tratado de Fisiología Médica. Hall JE eds. Elsevier. Barcelona.

Hall,J.E., 2011b. Sensibilidades Somáticas: II. Dolor, cefalea y sensibilidad térmica. In Tratado de Fisiología Médica. Hall JE eds. Elsevier. Barcelona.

Hanner,M., Moebius,F.F., Flandorfer,A., Knaus,H.G., Striessnig,J., Kempner,E., Glossmann,H., 1996. Purification, molecular cloning, and expression of the mammalian sigma1-binding site. Proc.Natl.Acad.Sci.U.S.A 93, 8072-8077.

Hardy,J.D., Wolff,H.G., Goodell,H., 1940. Studies on pain. a new method for measuring pain threshold: observations on spatial summation of pain. J.Clin.Invest 19, 649-657.

Hargreaves,K., Dubner,R., Brown,F., Flores,C., Joris,J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32, 77-88.

Haroon,M., Rafiq Chaudhry,A.B., Fitzgerald,O., 2016. Higher Prevalence of Metabolic Syndrome in Patients with Psoriatic Arthritis: A Comparison with a Control Group of Noninflammatory Rheumatologic Conditions. J.Rheumatol. 43, 463-464.

Hayashi,T., Fujimoto,M., 2010. Detergent-resistant microdomains determine the localization of sigma-1 receptors to the endoplasmic reticulum-mitochondria junction. Mol.Pharmacol. 77, 517-528.

Hayashi,T., Su,T.P., 2003. Intracellular dynamics of sigma-1 receptors (sigma(1) binding sites) in NG108-15 cells. J.Pharmacol.Exp.Ther. 306, 726-733.

Hayashi,T., Su,T.P., 2004. Sigma-1 receptor ligands: potential in the treatment of neuropsychiatric disorders. CNS.Drugs 18, 269-284.

Hayashi,T., Su,T.P., 2007. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. Cell 131, 596-610.

Hayhurst,C.J., Durieux,M.E., 2016. Differential Opioid Tolerance and Opioid-induced Hyperalgesia: A Clinical Reality. Anesthesiology 124, 483-488.

Hellewell,S.B., Bowen,W.D., 1990. A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. Brain Res. 527, 244-253.

Hendiani,J.A., Westlund,K.N., Lawand,N., Goel,N., Lisse,J., McNearney,T., 2003. Mechanical sensation and pain thresholds in patients with chronic arthropathies. J.Pain 4, 203-211.

Hervera,A., Negrete,R., Leanez,S., Martín-Campos,J.M., Pol,O., 2011. Peripheral effects of morphine and expression of mu-opioid receptors in the dorsal root ganglia during neuropathic pain: nitric oxide signaling. Mol.Pain 7, 25.

Hill,R., 2000. NK1 (substance P) receptor antagonists--why are they not analgesic in humans? Trends Pharmacol.Sci. 21, 244-246.

Hillhouse,T.M., Negus,S.S., 2016. Effects of the noncompetitive N-methyl-d-aspartate receptor antagonists ketamine and MK-801 on pain-stimulated and pain-depressed behaviour in rats. Eur.J.Pain 20, 1229-1240.

Hojo,M., Sudo,Y., Ando,Y., Minami,K., Takada,M., Matsubara,T., Kanaide,M., Taniyama,K., Sumikawa,K., Uezono,Y., 2008. mu-Opioid receptor forms a functional heterodimer with cannabinoid CB1 receptor: electrophysiological and FRET assay analysis. J.Pharmacol.Sci. 108, 308-319.

Honore,P., Chandran,P., Hernandez,G., Gauvin,D.M., Mikusa,J.P., Zhong,C., Joshi,S.K., Ghilardi,J.R., Sevcik,M.A., Fryer,R.M., Segreti,J.A., Banfor,P.N., Marsh,K., Neelands,T., Bayburt,E., Daanen,J.F., Gomtsyan,A., Lee,C.H., Kort,M.E., Reilly,R.M., Surowy,C.S., Kym,P.R., Mantyh,P.W., Sullivan,J.P., Jarvis,M.F., Faltynek,C.R., 2009. Repeated dosing of ABT-102, a potent and selective TRPV1 antagonist, enhances TRPV1-mediated analgesic activity in rodents, but attenuates antagonist-induced hyperthermia. Pain 142, 27-35.

Hsieh,Y.L., Chiang,H., Lue,J.H., Hsieh,S.T., 2012a. P2X3-mediated peripheral sensitization of neuropathic pain in resiniferatoxin-induced neuropathy. Exp.Neurol. 235, 316-325.

Hsieh,Y.L., Chiang,H., Tseng,T.J., Hsieh,S.T., 2008. Enhancement of cutaneous nerve regeneration by 4-methylcatechol in resiniferatoxin-induced neuropathy. J.Neuropathol.Exp.Neurol. 67, 93-104.

Hsieh,Y.L., Lin,C.L., Chiang,H., Fu,Y.S., Lue,J.H., Hsieh,S.T., 2012b. Role of peptidergic nerve terminals in the skin: reversal of thermal sensation by calcitonin gene-related peptide in TRPV1-depleted neuropathy. PLoS.One. 7, e50805.

Huang,W., Calvo,M., Karu,K., Olausen,H.R., Bathgate,G., Okuse,K., Bennett,D.L., Rice,A.S., 2013. A clinically relevant rodent model of the HIV antiretroviral drug stavudine induced painful peripheral neuropathy. Pain 154, 560-575.

Huang,Y., Zheng,L., Halliday,G., Dobson-Stone,C., Wang,Y., Tang,H.D., Cao,L., Deng,Y.L., Wang,G., Zhang,Y.M., Wang,J.H., Hallupp,M., Kwok,J., Chen,S.D., 2011. Genetic polymorphisms in sigma-1 receptor and apolipoprotein E interact to influence the severity of Alzheimer's disease. Curr.Alzheimer Res. 8, 765-770.

Huggins,J.P., Smart,T.S., Langman,S., Taylor,L., Young,T., 2012. An efficient randomised, placebo-controlled clinical trial with the irreversible fatty acid amide hydrolase-1 inhibitor PF-04457845, which modulates endocannabinoids but fails to induce effective analgesia in patients with pain due to osteoarthritis of the knee. Pain 153, 1837-1846.

Hughes,J., Kosterlitz,H.W., Leslie,F.M., 1975. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antogonist potencies of narcotic analgesics. Br.J.Pharmacol. 53, 371-381.

Huntjens,D.R., Spalding,D.J., Danhof,M., Della Pasqua,O.E., 2009. Differences in the sensitivity of behavioural measures of pain to the selectivity of cyclo-oxygenase inhibitors. Eur.J.Pain 13, 448-457.

Inoue,K., Tsuda,M., 2018. Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. Nat.Rev.Neurosci. 19, 138-152.

Inoue,M., Ueda,H., 2000. Protein kinase C-mediated acute tolerance to peripheral mu-opioid analgesia in the bradykinin-nociception test in mice. J.Pharmacol.Exp. Ther. 293, 662-669.

Irwin,S., 1968. Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia. 13, 222-257.

Ivanavicius,S.P., Ball,A.D., Heapy,C.G., Westwood,F.R., Murray,F., Read,S.J., 2007. Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: increased expression of ATF-3 and pharmacological characterisation. Pain 128, 272-282.

Jeong,Y.C., Son,J.S., Kwon,Y.B., 2015. The spinal antinociceptive mechanism determined by systemic administration of BD1047 in zymosan-induced hyperalgesia in rats. Brain Res.Bull. 119, 93-100.

Jirkof,P., Cesarovic,N., Rettich,A., Nicholls,F., Seifert,B., Arras,M., 2010. Burrowing behavior as an indicator of post-laparotomy pain in mice. Front Behav.Neurosci. 4, 165.

Jirkof,P., Leucht,K., Cesarovic,N., Caj,M., Nicholls,F., Rogler,G., Arras,M., Hausmann,M., 2013. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. Lab Anim 47, 274-283.

Joshi,S.K., Mikusa,J.P., Weaver,B., Honore,P., 2008. Morphine and ABT-594 (a nicotinic acetylcholine agonist) exert centrally mediated antinociception in the rat cyclophosphamide cystitis model of visceral pain. J.Pain 9, 146-156.

Julius,D., 2013. TRP channels and pain. Annu.Rev.Cell Dev.Biol. 29, 355-384.

Julius,D., Basbaum,A.I., 2001. Molecular mechanisms of nociception. Nature 413, 203-210.

Kandasamy,R., Calsbeek,J.J., Morgan,M.M., 2017a. Analysis of inflammation-induced depression of home cage wheel running in rats reveals the difference between opioid antinociception and restoration of function. Behav.Brain Res. 317, 502-507.

Kandasamy,R., Lee,A.T., Morgan,M.M., 2017b. Depression of home cage wheel running is an objective measure of spontaneous morphine withdrawal in rats with and without persistent pain. Pharmacol.Biochem.Behav. 156, 10-15.

Kaplan,H., Fields,H.L., 1991. Hyperalgesia during acute opioid abstinence: evidence for a nociceptive facilitating function of the rostral ventromedial medulla. J.Neurosci. 11, 1433-1439.

Kehl,L.J., Fairbanks,C.A., 2003. Experimental animal models of muscle pain and analgesia. Exerc.Sport Sci.Rev. 31, 188-194.

Kehl,L.J., Trempe,T.M., Hargreaves,K.M., 2000. A new animal model for assessing mechanisms and management of muscle hyperalgesia. Pain 85, 333-343.

Khademi,H., Kamangar,F., Brennan,P., Malekzadeh,R., 2016. Opioid Therapy and its Side Effects: A Review. Arch.Iran Med. 19, 870-876.

Khalefa,B.I., Shaqura,M., Al-Khrasani,M., Furst,S., Mousa,S.A., Schafer,M., 2012. Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. Eur.J.Pain 16, 690-705.

Khanna,I.K., Pillarisetti,S., 2015. Buprenorphine - an attractive opioid with underutilized potential in treatment of chronic pain. J.Pain Res. 8, 859-870.

Khansari,M., Sohrabi,M., Zamani,F., 2013. The Useage of Opioids and their Adverse Effects in Gastrointestinal Practice: A Review. Middle East J.Dig.Dis. 5, 5-16.

Kim,F.J., Kovalyshyn,I., Burgman,M., Neilan,C., Chien,C.C., Pasternak,G.W., 2010. Sigma 1 receptor modulation of G-protein-coupled receptor signaling: potentiation of opioid transduction independent from receptor binding. Mol.Pharmacol. 77, 695-703.

Kim,H.W., Kwon,Y.B., Roh,D.H., Yoon,S.Y., Han,H.J., Kim,K.W., Beitz,A.J., Lee,J.H., 2006. Intrathecal treatment with sigma1 receptor antagonists reduces formalin-induced phosphorylation of NMDA receptor subunit 1 and the second phase of formalin test in mice. Br.J.Pharmacol. 148, 490-498.

Kim,S.H., Chung,J.M., 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50, 355-363.

King,T., Ossipov,M.H., Vanderah,T.W., Porreca,F., Lai,J., 2005. Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance? Neurosignals. 14, 194-205.

Kirkpatrick,D.R., McEntire,D.M., Hambsch,Z.J., Kerfeld,M.J., Smith,T.A., Reisbig,M.D., Youngblood,C.F., Agrawal,D.K., 2015. Therapeutic Basis of Clinical Pain Modulation. Clin.Transl.Sci. 8, 848-856.

Kissin,I., 2010. The development of new analgesics over the past 50 years: a lack of real breakthrough drugs. Anesth.Analg. 110, 780-789.

Kitaichi,K., Chabot,J.G., Moebius,F.F., Flandorfer,A., Glossmann,H., Quirion,R., 2000. Expression of the purported sigma(1) (sigma(1)) receptor in the mammalian brain and its possible relevance in deficits induced by antagonism of the NMDA receptor complex as revealed using an antisense strategy. J.Chem.Neuroanat. 20, 375-387.

Kolesnikov,Y., Pasternak,G.W., 1999. Topical opioids in mice: analgesia and reversal of tolerance by a topical N-methyl-D-aspartate antagonist. J.Pharmacol.Exp.Ther. 290, 247-252.

Kolesnikov,Y.A., Chereshnev,I., Pasternak,G.W., 2000. Analgesic synergy between topical lidocaine and topical opioids. J.Pharmacol.Exp.Ther. 295, 546-551.

Koppert,W., Likar,R., Geisslinger,G., Zeck,S., Schmelz,M., Sittl,R., 1999. Peripheral antihyperalgesic effect of morphine to heat, but not mechanical, stimulation in healthy volunteers after ultraviolet-B irradiation. Anesth.Analg. 88, 117-122.

Kumar,A., Kaur,H., Singh,A., 2018. Neuropathic Pain models caused by damage to central or peripheral nervous system. Pharmacol.Rep. 70, 206-216.

Kuner,R., Flor,H., 2016. Structural plasticity and reorganisation in chronic pain. Nat.Rev.Neurosci. 18, 20-30.

Kupers,R.C., Gybels,J.M., 1993. Electrical stimulation of the ventroposterolateral thalamic nucleus (VPL) reduces mechanical allodynia in a rat model of neuropathic pain. Neurosci.Lett. 150, 95-98.

Kuraishi,Y., Hirota,N., Satoh,M., Takagi,H., 1985. Antinociceptive effects of intrathecal opioids, noradrenaline and serotonin in rats: mechanical and thermal algesic tests. Brain Res. 326, 168-171.

Kwilasz,A.J., Negus,S.S., 2012. Dissociable effects of the cannabinoid receptor agonists Delta9-tetrahydrocannabinol and CP55940 on pain-stimulated versus pain-depressed behavior in rats. J.Pharmacol.Exp.Ther. 343, 389-400.

Lääveri,T., Sterne,J., Rombo,L., Kantele,A., 2016. Systematic review of loperamide: No proof of antibiotics being superior to loperamide in treatment of mild/moderate travellers' diarrhoea. Travel.Med.Infect.Dis. 14, 299-312.

Lan,Y., Chen,Y., Cao,X., Zhang,J., Wang,J., Xu,X., Qiu,Y., Zhang,T., Liu,X., Liu,B.F., Zhang,G., 2014. Synthesis and biological evaluation of novel sigma-1 receptor antagonists based on pyrimidine scaffold as agents for treating neuropathic pain. J.Med.Chem. 57, 10404-10423.

Langa,F., Codony,X., Tovar,V., Lavado,A., Gimenez,E., Cozar,P., Cantero,M., Dordal,A., Hernandez,E., Perez,R., Monroy,X., Zamanillo,D., Guitart,X., Montoliu,L., 2003. Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. Eur.J.Neurosci. 18, 2188-2196.

Langford,D.J., Bailey,A.L., Chanda,M.L., Clarke,S.E., Drummond,T.E., Echols,S., Glick,S., Ingrao,J., Klassen-Ross,T., Lacroix-Fralish,M.L., Matsumiya,L., Sorge,R.E., Sotocinal,S.G., Tabaka,J.M., Wong,D., van den Maagdenberg,A.M., Ferrari,M.D., Craig,K.D., Mogil,J.S., 2010. Coding of facial expressions of pain in the laboratory mouse. Nat.Methods 7, 447-449.

Larsen,J.J., Arnt,J., 1985. Reduction in locomotor activity of arthritic rats as parameter for chronic pain: effect of morphine, acetylsalicylic acid and citalopram. Acta Pharmacol.Toxicol.(Copenh) 57, 345-351.

Latremoliere,A., Woolf,C.J., 2009. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J.Pain 10, 895-926.

Lau,W., Dykstra,C., Thevarkunnel,S., Silenieks,L.B., de Lannoy,I.A., Lee,D.K., Higgins,G.A., 2013. A back translation of pregabalin and carbamazepine against evoked and non-evoked endpoints in the rat spared nerve injury model of neuropathic pain. Neuropharmacology 73, 204-215.

Laux-Biehlmann,A., Boyken,J., Dahllof,H., Schmidt,N., Zollner,T.M., Nagel,J., 2016. Dynamic weight bearing as a non-reflexive method for the measurement of abdominal pain in mice. Eur.J.Pain 20, 742-752.

Lavand'homme,P., Steyaert,A., 2017. Opioid-free anesthesia opioid side effects: Tolerance and hyperalgesia. Best.Pract.Res.Clin.Anaesthesiol. 31, 487-498.

Lawson,J.J., McIlwrath,S.L., Woodbury,C.J., Davis,B.M., Koerber,H.R., 2008. TRPV1 unlike TRPV2 is restricted to a subset of mechanically insensitive cutaneous nociceptors responding to heat. J.Pain 9, 298-308.

Lazenka,M.F., Freitas,K.C., Henck,S., Negus,S.S., 2017. Relief of Pain-Depressed Behavior in Rats by Activation of D1-Like Dopamine Receptors. J.Pharmacol.Exp.Ther. 362, 14-23.

Lazenka,M.L., Moerke,M.J., Townsend,E.A., Freeman,K.B., Carroll,F.I., Negus,S.S., 2018. Dissociable effects of the kappa opioid receptor agonist nalfurafine on pain/itch-stimulated and pain/itch-depressed behaviors in male rats. Psychopharmacology (Berl) 235, 203-213.

Le Bars,D., Gozariu,M., Cadden,S.W., 2001. Animal models of nociception. Pharmacol.Rev. 53, 597-652.

Lee,Y., Pai,M., Brederson,J.D., Wilcox,D., Hsieh,G., Jarvis,M.F., Bitner,R.S., 2011. Monosodium iodoacetate-induced joint pain is associated with increased phosphorylation of mitogen activated protein kinases in the rat spinal cord. Mol.Pain 7, 39.

Lee,Y.C., 2013. Effect and treatment of chronic pain in inflammatory arthritis. Curr.Rheumatol.Rep. 15, 300.

Lefkowitz,R.J., Shenoy,S.K., 2005. Transduction of receptor signals by beta-arrestins. Science 308, 512-517.

Legakis,L.P., Bigbee,J.W., Negus,S.S., 2018. Lack of paclitaxel effects on intracranial self-stimulation in male and female rats: comparison to mechanical sensitivity. Behav.Pharmacol. 29, 290-298.

Leitl,M.D., Potter,D.N., Cheng,K., Rice,K.C., Carlezon,W.A., Jr., Negus,S.S., 2014. Sustained pain-related depression of behavior: effects of intraplantar formalin and complete freund's adjuvant on intracranial self-stimulation (ICSS) and endogenous kappa opioid biomarkers in rats. Mol.Pain 10, 62.

Lewter,L.A., Fisher,J.L., Siemian,J.N., Methuku,K.R., Poe,M.M., Cook,J.M., Li,J.X., 2017. Antinociceptive Effects of a Novel alpha2/alpha3-Subtype Selective GABAA Receptor Positive Allosteric Modulator. ACS Chem.Neurosci. 8, 1305-1312.

Linhart,O., Obreja,O., Kress,M., 2003. The inflammatory mediators serotonin, prostaglandin E2 and bradykinin evoke calcium influx in rat sensory neurons. Neuroscience 118, 69-74.

Lolignier,S., Amsalem,M., Maingret,F., Padilla,F., Gabriac,M., Chapuy,E., Eschalier,A., Delmas,P., Busserolles,J., 2011. Nav1.9 channel contributes to mechanical and heat pain hypersensitivity induced by subacute and chronic inflammation. PLoS.One. 6, e23083.

Lorenz,J., Hauck,M., 2010. Supraspinal mechanisms of pain and nociception. In Bonica‟s Management of Pain. 4th ed. Fissheman S, Ballantyne and Rathmell JP eds. Lippincott Williams & Wilkins. Philadelphia.

Luk,J., Lu,Y., Ackermann,A., Peng,X., Bogdan,D., Puopolo,M., Komatsu,D.E., Tong,S., Ojima,I., Rebecchi,M.J., Kaczocha,M., 2018. Contribution of diacylglycerol lipase beta to pain after surgery. J.Pain Res. 11, 473-482.

Llorca-Torralba,M., Borges,G., Neto,F., Mico,J.A., Berrocoso,E., 2016. Noradrenergic Locus Coeruleus pathways in pain modulation. Neuroscience 338, 93-113.

Macht,D.I., Herman,N.B., Levy,C.S., 1915. A Quantitative Study of Cutaneous Analgesia Produced by Various Opium Alkaloids. Proc.Natl.Acad.Sci.U.S.A 1, 582-585.

Manzhulo,I.V., Ogurtsova,O.S., Lamash,N.E., Latyshev,N.A., Kasyanov,S.P., Dyuizen,I.V., 2015. Analgetic effect of docosahexaenoic acid is mediated by modulating the microglia activity in the dorsal root ganglia in a rat model of neuropathic pain. Acta Histochem. 117, 659-666.

Mao,J., 2009. Translational pain research: achievements and challenges. J.Pain 10, 1001-1011.

Mao,J., Gold,M.S., Backonja,M.M., 2011. Combination drug therapy for chronic pain: a call for more clinical studies. J.Pain 12, 157-166.

Mapp,P.I., 1995. Innervation of the synovium. Ann.Rheum.Dis. 54, 398-403.

Mark,M.D., Herlitze,S., 2000. G-protein mediated gating of inward-rectifier K+ channels. Eur.J.Biochem. 267, 5830-5836.

Marrazzo,A., Parenti,C., Scavo,V., Ronsisvalle,S., Scoto,G.M., Ronsisvalle,G., 2006. In vivo evaluation of (+)-MR200 as a new selective sigma ligand modulating MOP, DOP and KOP supraspinal analgesia. Life Sci. 78, 2449-2453.

Martin,J.M., Jessel,T.M., 2000. Anatomy of the Somatic Sensory System. In Principles of Neural Science 4th ed. Kandel ER, Schwartz JH and Jessell TM eds. McGraw-Hill Interamericana. Madrid.

Martin,T.J., Kahn,W.R., Eisenach,J.C., 2005. Abdominal surgery decreases food-reinforced operant responding in rats: relevance of incisional pain. Anesthesiology 103, 629-637.

Martin,T.J., Zhang,Y., Buechler,N., Conklin,D.R., Eisenach,J.C., 2005. Intrathecal morphine and ketorolac analgesia after surgery: comparison of spontaneous and elicited responses in rats. Pain 113, 376-385.

Martin,W.R., Eades,C.G., Thompson,J.A., Huppler,R.E., Gilbert,P.E., 1976. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J.Pharmacol.Exp.Ther. 197, 517-532.

Martins,I., Tavares,I., 2017. Reticular Formation and Pain: The Past and the Future. Front Neuroanat. 11, 51.

Matson,D.J., Broom,D.C., Carson,S.R., Baldassari,J., Kehne,J., Cortright,D.N., 2007. Inflammation-induced reduction of spontaneous activity by adjuvant: A novel model to study the effect of analgesics in rats. J.Pharmacol.Exp.Ther. 320, 194-201.

Matsumoto,R.R., Liu,Y., Lerner,M., Howard,E.W., Brackett,D.J., 2003. Sigma receptors: potential medications development target for anti-cocaine agents. Eur.J.Pharmacol. 469, 1-12.

Maurice,T., Su,T.P., 2009. The pharmacology of sigma-1 receptors. Pharmacol.Ther. 124, 195-206.

Mavlyutov,T.A., Duellman,T., Kim,H.T., Epstein,M.L., Leese,C., Davletov,B.A., Yang,J., 2016. Sigma-1 receptor expression in the dorsal root ganglion: Reexamination using a highly specific antibody. Neuroscience 331, 148-157.

Mavlyutov,T.A., Epstein,M.L., Andersen,K.A., Ziskind-Conhaim,L., Ruoho,A.E., 2010. The sigma-1 receptor is enriched in postsynaptic sites of C-terminals in mouse motoneurons. An anatomical and behavioral study. Neuroscience 167, 247-255.

McCoy,E.S., Taylor-Blake,B., Zylka,M.J., 2012. CGRPalpha-expressing sensory neurons respond to stimuli that evoke sensations of pain and itch. PLoS.One. 7, e36355.

McGlone,F., Reilly,D., 2010. The cutaneous sensory system. Neurosci.Biobehav.Rev. 34, 148-159.

McRoberts,J.A., Ennes,H.S., Marvizon,J.C., Fanselow,M.S., Mayer,E.A., Vissel,B., 2011. Selective knockdown of NMDA receptors in primary afferent neurons decreases pain during phase 2 of the formalin test. Neuroscience 172, 474-482.

Meacham,K., Shepherd,A., Mohapatra,D.P., Haroutounian,S., 2017. Neuropathic Pain: Central vs. Peripheral Mechanisms. Curr.Pain Headache Rep. 21, 28.

Mehta,N., O'Connell,K., Giambrone,G.P., Baqai,A., Diwan,S., 2016. Efficacy of methylnaltrexone for the treatment of opiod-induced constipation: a meta-analysis and systematic review. Postgrad.Med. 128, 282-289.

Mei,J., Pasternak,G.W., 2002. Sigma1 receptor modulation of opioid analgesia in the mouse. J.Pharmacol.Exp.Ther. 300, 1070-1074.

Mei,J., Pasternak,G.W., 2007. Modulation of brainstem opiate analgesia in the rat by sigma 1 receptors: a microinjection study. J.Pharmacol.Exp.Ther. 322, 1278-1285.

Melief,E.J., Miyatake,M., Carroll,F.I., Beguin,C., Carlezon,W.A., Cohen,B.M., Grimwood,S., Mitch,C., Rorick-Kehn,L.M., Chavkin,C., 2011. Duration of action of a broad range of selective kappa opioid receptor antagonists is positively correlated with c-Jun N-Terminal kinase-1 activation. Mol Pharmacol. 80, 920-929.

Menéndez,L., Juarez,L., García,E., García-Suárez,O., Hidalgo,A., Baamonde,A., 2006. Analgesic effects of capsazepine and resiniferatoxin on bone cancer pain in mice. Neurosci.Lett. 393, 70-73.

Menéndez,L., Lastra,A., Hidalgo,A., Baamonde,A., 2002. Unilateral hot plate test: a simple and sensitive method for detecting central and peripheral hyperalgesia in mice. J.Neurosci.Methods 113, 91-97.

Menéndez,L., Lastra,A., Meana,A., Hidalgo,A., Baamonde,A., 2005. Analgesic effects of loperamide in bone cancer pain in mice. Pharmacol.Biochem.Behav. 81, 114-121.

Mercadante,S., 2017. The role of tapentadol as a strong opioid in cancer pain management: a systematic and critical review. Curr.Med.Res.Opin. 33, 1965-1969.

Merlos,M., Romero,L., Zamanillo,D., Plata-Salaman,C., Vela,J.M., 2017. Sigma-1 Receptor and Pain. Handb.Exp.Pharmacol. 244, 131-161.

Meuser,T., Giesecke,T., Gabriel,A., Horsch,M., Sabatowski,R., Hescheler,J., Grond,S., Palmer,P.P., 2003. Mu-opioid receptor mRNA regulation during morphine tolerance in the rat peripheral nervous system. Anesth.Analg. 97, 1458-1463.

Meyer,O.A., Tilson,H.A., Byrd,W.C., Riley,M.T., 1979. A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav.Toxicol. 1, 233-236.

Meyer,R.A., Ringkamp,M., Campbell,J.N., Raja,S.N. 2008. Peripheral mechanisms of cutaneous nociception. In: Wall and Melzack's Textbook of Pain. 5th ed. Saunders ed. Churchill Livingstone Elsevier. London.

Michael,G.J., Priestley,J.V., 1999. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J.Neurosci. 19, 1844-1854.

Millan,M.J., 2002. Descending control of pain. Prog.Neurobiol. 66, 355-474.

Miller,L.L., Leitl,M.D., Banks,M.L., Blough,B.E., Negus,S.S., 2015. Effects of the triple monoamine uptake inhibitor amitifadine on pain-related depression of behavior and mesolimbic dopamine release in rats. Pain 156, 175-184.

Miller,L.L., Picker,M.J., Schmidt,K.T., Dykstra,L.A., 2011. Effects of morphine on pain-elicited and pain-suppressed behavior in CB1 knockout and wildtype mice. Psychopharmacology (Berl) 215, 455-465.

Milligan,G., 2005. Opioid receptors and their interacting proteins. Neuromolecular.Med. 7, 51-59.

Mills,C.D., Grady,J.J., Hulsebosch,C.E., 2001. Changes in exploratory behavior as a measure of chronic central pain following spinal cord injury. J.Neurotrauma 18, 1091-1105.

Miranda,H.F., Lopez,J., Sierralta,F., Correa,A., Pinardi,G., 2001. NSAID antinociception measured in a chemical and a thermal assay in mice. Pain Res.Manag. 6, 190-196.

Mishra,S.K., Hoon,M.A., 2010. Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. Mol.Cell Neurosci. 43, 157-163.

Miyatake,R., Furukawa,A., Matsushita,S., Higuchi,S., Suwaki,H., 2004. Functional polymorphisms in the sigma1 receptor gene associated with alcoholism. Biol.Psychiatry 55, 85-90.

Mogil,J.S., 2009. Animal models of pain: progress and challenges. Nat.Rev.Neurosci. 10, 283-294.

Mogil,J.S., Crager,S.E., 2004. What should we be measuring in behavioral studies of chronic pain in animals? Pain 112, 12-15.

Mogil,J.S., Graham,A.C., Ritchie,J., Hughes,S.F., Austin,J.S., Schorscher-Petcu,A., Langford,D.J., Bennett,G.J., 2010. Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. Mol.Pain 6, 34.

Moharic,M., Vidmar,G., Burger,H., 2012. Sensitivity and specificity of von Frey's hairs for the diagnosis of peripheral neuropathy in patients with type 2 diabetes mellitus. J.Diabetes Complications 26, 319-322.

Montilla-García,Á., Perazzoli,G., Tejada,M.A., González-Cano,R., Sánchez-Fernández,C., Cobos,E.J., Baeyens,J.M., 2018. Modality-specific peripheral antinociceptive effects of mu-opioid agonists on heat and mechanical stimuli: Contribution of sigma-1 receptors. Neuropharmacology 135, 328-342.

Montilla-García,Á., Tejada,M.A., Perazzoli,G., Entrena,J.M., Portillo-Salido,E., Fernández-Segura,E., Cañizares,F.J., Cobos,E.J., 2017. Grip strength in mice with joint inflammation: A rheumatology function test sensitive to pain and analgesia. Neuropharmacology 125, 231-242.

Moon,J.Y., Choi,S.R., Roh,D.H., Yoon,S.Y., Kwon,S.G., Choi,H.S., Kang,S.Y., Han,H.J., Kim,H.W., Beitz,A.J., Oh,S.B., Lee,J.H., 2015. Spinal sigma-1 receptor activation increases the production of D-serine in astrocytes which contributes to the development of mechanical allodynia in a mouse model of neuropathic pain. Pharmacol.Res. 100, 353-364.

Morgan,D., Carter,C.S., DuPree,J.P., Yezierski,R.P., Vierck,C.J., Jr., 2008. Evaluation of prescription opioids using operant-based pain measures in rats. Exp.Clin.Psychopharmacol. 16, 367-375.

Morgan,M.M., Christie,M.J., 2011. Analysis of opioid efficacy, tolerance, addiction and dependence from cell culture to human. Br.J.Pharmacol. 164, 1322-1334.

Morris,V.H., Cruwys,S.C., Kidd,B.L., 1997. Characterisation of capsaicin-induced mechanical hyperalgesia as a marker for altered nociceptive processing in patients with rheumatoid arthritis. Pain 71, 179-186.

Moser,V.C., 2011. Functional assays for neurotoxicity testing. Toxicol.Pathol. 39, 36-45.

Munro,G., Lopez-Garcia,J.A., Rivera-Arconada,I., Erichsen,H.K., Nielsen,E.O., Larsen,J.S., Ahring,P.K., Mirza,N.R., 2008. Comparison of the novel subtype-selective GABAA receptor-positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. J.Pharmacol.Exp.Ther. 327, 969-981.

Muralidharan,A., Kuo,A., Jacob,M., Lourdesamy,J.S., Carvalho,L.M., Nicholson, J.R., Corradini,L., Smith,M.T., 2016. Comparison of Burrowing and Stimuli-Evoked Pain Behaviors as End-Points in Rat Models of Inflammatory Pain and Peripheral Neuropathic Pain. Front Behav.Neurosci. 10, 88.

Nakazato-Imasato,E., Tanimoto-Mori,S., Kurebayashi,Y., 2009. Effect of mexiletine on dynamic allodynia induced by chronic constriction injury of the sciatic nerve in rats. J.Vet.Med.Sci. 71, 991-994.

Nee,J., Zakari,M., Lembo,A.J., 2015. Current and emerging drug options in the treatment of diarrhea predominant irritable bowel syndrome. Expert.Opin.Pharmacother. 16, 2781-2792.

Negus,S.S., 2013. Expression and treatment of pain-related behavioral depression. Lab Anim (NY) 42, 292-300.

Negus,S.S., 2018. Addressing the Opioid Crisis: The Importance of Choosing Translational Endpoints in Analgesic Drug Discovery. Trends Pharmacol.Sci. 39, 327-330.

Negus,S.S., Neddenriep,B., Altarifi,A.A., Carroll,F.I., Leitl,M.D., Miller,L.L., 2015. Effects of ketoprofen, morphine, and kappa opioids on pain-related depression of nesting in mice. Pain 156, 1153-1160.

Negus,S.S., Vanderah,T.W., Brandt,M.R., Bilsky,E.J., Becerra,L., Borsook,D., 2006. Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. J.Pharmacol.Exp.Ther. 319, 507-514.

Nencini,P., 1997. The rules of drug taking: wine and poppy derivatives in the ancient world. VI. Poppies as a source of food and drug. Subst.Use.Misuse. 32, 757-766.

Ness,T.J., Randich,A., 2010. Neonatal bladder inflammation alters activity of adult rat spinal visceral nociceptive neurons. Neurosci.Lett. 472, 210-214.

Nevins,M.E., Nash,S.A., Beardsley,P.M., 1993. Quantitative grip strength assessment as a means of evaluating muscle relaxation in mice. Psychopharmacology (Berl) 110, 92-96.

Nieto,F.R., Cendán,C.M., Cañizares,F.J., Cubero,M.A., Vela,J.M., Fernández-Segura,E., Baeyens,J.M., 2014. Genetic inactivation and pharmacological blockade of sigma-1 receptors prevent paclitaxel-induced sensory-nerve mitochondrial abnormalities and neuropathic pain in mice. Mol.Pain 10, 11.

Nieto,F.R., Cendán,C.M., Sánchez-Fernández,C., Cobos,E.J., Entrena,J.M., Tejada,M.A., Zamanillo,D., Vela,J.M., Baeyens,J.M., 2012. Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. J.Pain 13, 1107-1121.

Nieto,F.R., Clark,A.K., Grist,J., Hathway,G.J., Chapman,V., Malcangio,M., 2016. Neuron-immune mechanisms contribute to pain in early stages of arthritis. J.Neuroinflammation. 13, 96.

Nieto,M.M., Wilson,J., Walker,J., Benavides,J., Fournie-Zaluski,M.C., Roques,B.P., Noble,F., 2001. Facilitation of enkephalins catabolism inhibitor-induced antinociception by drugs classically used in pain management. Neuropharmacology 41, 496-506.

Niiyama,Y., Kawamata,T., Yamamoto,J., Omote,K., Namiki,A., 2007. Bone cancer increases transient receptor potential vanilloid subfamily 1 expression within distinct subpopulations of dorsal root ganglion neurons. Neuroscience 148, 560-572.

Nolan,M.W., Long,C.T., Marcus,K.L., Sarmadi,S., Roback,D.M., Fukuyama,T., Baeumer,W., Lascelles,B.D., 2017. Nocifensive Behaviors in Mice with Radiation-Induced Oral Mucositis. Radiat.Res. 187, 397-403.

Norn,S., Kruse,P.R., Kruse,E., 2005. [History of opium poppy and morphine]. Dan. Medicinhist.Arbog. 33, 171-184.

Nowoczyn,M., Marie,N., Coulbault,L., Hervault,M., Davis,A., Hanouz,J.L., Allouche,S., 2013. Remifentanil produces cross-desensitization and tolerance with morphine on the mu-opioid receptor. Neuropharmacology 73, 368-379.

O'Brien,J., Keaveny,J., Pollard,V., Nugent,L.E., 2017. Advancing Nursing Practice: Management of Neuropathic Pain With Capsaicin 8% Without Physician Supervision. Clin.Nurse Spec. 31, 157-162.

Ocana,M., Cendan,C.M., Cobos,E.J., Entrena,J.M., Baeyens,J.M., 2004. Potassium channels and pain: present realities and future opportunities. Eur.J.Pharmacol. 500, 203-219.

Ocaña,M., Del Pozo,E., Barrios,M., Baeyens,J.M., 1995. Subgroups among mu-opioid receptor agonists distinguished by ATP-sensitive K+ channel-acting drugs. Br.J.Pharmacol. 114, 1296-1302.

Okun,A., Liu,P., Davis,P., Ren,J., Remeniuk,B., Brion,T., Ossipov,M.H., Xie,J., Dussor,G.O., King,T., Porreca,F., 2012. Afferent drive elicits ongoing pain in a model of advanced osteoarthritis. Pain 153, 924-933.

Olausson,H., Cole,J., Rylander,K., McGlone,F., Lamarre,Y., Wallin,B.G., Kramer,H., Wessberg,J., Elam,M., Bushnell,M.C., Vallbo,A., 2008. Functional role of unmyelinated tactile afferents in human hairy skin: sympathetic response and perceptual localization. Exp.Brain Res. 184, 135-140.

Oliver,V.L., Thurston,S.E., Lofgren,J.L., 2018. Using Cageside Measures to Evaluate Analgesic Efficacy in Mice (Mus musculus) after Surgery. J.Am.Assoc.Lab Anim Sci. 57, 186-201.

Ossipov,M.H., Dussor,G.O., Porreca,F., 2010. Central modulation of pain. J Clin Invest 120.

Ossipov,M.H., Morimura,K., Porreca,F., 2014. Descending pain modulation and chronification of pain. Curr.Opin.Support.Palliat.Care 8, 143-151.

Overend,T.J., Wuori-Fearn,J.L., Kramer,J.F., MacDermid,J.C., 1999. Reliability of a patient-rated forearm evaluation questionnaire for patients with lateral epicondylitis. J.Hand Ther. 12, 31-37.

Pabba,M., Wong,A.Y., Ahlskog,N., Hristova,E., Biscaro,D., Nassrallah,W., Ngsee,J.K., Snyder,M., Beique,J.C., Bergeron,R., 2014. NMDA receptors are upregulated and trafficked to the plasma membrane after sigma-1 receptor activation in the rat hippocampus. J.Neurosci. 34, 11325-11338.

Pajak,A., Kostrzewa,M., Malek,N., Korostynski,M., Starowicz,K., 2017. Expression of matrix metalloproteinases and components of the endocannabinoid system in the knee joint are associated with biphasic pain progression in a rat model of osteoarthritis. J.Pain Res. 10, 1973-1989.

Palacios,G., Muro,A., Verdu,E., Pumarola,M., Vela,J.M., 2004. Immunohistochemical localization of the sigma1 receptor in Schwann cells of rat sciatic nerve. Brain Res. 1007, 65-70.

Palmer,C.P., Mahen,R., Schnell,E., Djamgoz,M.B., Aydar,E., 2007. Sigma-1 receptors bind cholesterol and remodel lipid rafts in breast cancer cell lines. Cancer Res. 67, 11166-11175.

Pan,Y.X., Mei,J., Xu,J., Wan,B.L., Zuckerman,A., Pasternak,G.W., 1998. Cloning and characterization of a mouse sigma1 receptor. J.Neurochem. 70, 2279-2285.

Paniagua,N., Giron,R., Goicoechea,C., Lopez-Miranda,V., Vela,J.M., Merlos,M., Martin Fontelles,M.I., 2017. Blockade of sigma 1 receptors alleviates sensory signs of diabetic neuropathy in rats. Eur.J.Pain 21, 61-72.

Parenti,C., Marrazzo,A., Arico,G., Cantarella,G., Prezzavento,O., Ronsisvalle,S., Scoto,G.M., Ronsisvalle,G., 2014a. Effects of a selective sigma 1 antagonist compound on inflammatory pain. Inflammation 37, 261-266.

Parenti,C., Marrazzo,A., Arico,G., Parenti,R., Pasquinucci,L., Ronsisvalle,S., Ronsisvalle,G., Scoto,G.M., 2014b. The antagonistic effect of the sigma 1 receptor ligand (+)-MR200 on persistent pain induced by inflammation. Inflamm.Res. 63, 231-237.

Park,K.M., Max,M.B., Robinovitz,E., Gracely,R.H., Bennett,G.J., 1995. Effects of intravenous ketamine, alfentanil, or placebo on pain, pinprick hyperalgesia, and allodynia produced by intradermal capsaicin in human subjects. Pain 63, 163-172.

Pasternak,G., Pan,Y.X., 2011. Mu opioid receptors in pain management. Acta Anaesthesiol.Taiwan. 49, 21-25.

Pasternak,G.W., 2014. Opioids and their receptors: Are we there yet? Neuropharmacology 76 Pt B, 198-203.

Pasternak,G.W., Pan,Y.X., 2013. Mu opioids and their receptors: evolution of a concept. Pharmacol.Rev. 65, 1257-1317.

Pasternak,G.W., Snyder,S.H., 1975. Identification of novel high affinity opiate receptor binding in rat brain. Nature 253, 563-565.

Patapoutian,A., Tate,S., Woolf,C.J., 2009. Transient receptor potential channels: targeting pain at the source. Nat.Rev.Drug Discov. 8, 55-68.

Pathan,H., Williams,J., 2012. Basic opioid pharmacology: an update. Br J Pain. 6, 11-16.

Peier,A.M., Reeve,A.J., Andersson,D.A., Moqrich,A., Earley,T.J., Hergarden,A.C., Story,G.M., Colley,S., Hogenesch,J.B., McIntyre,P., Bevan,S., Patapoutian,A., 2002. A heat-sensitive TRP channel expressed in keratinocytes. Science 296, 2046-2049.

Percie Du,S.N., Rice,A.S., 2014. Improving the translation of analgesic drugs to the clinic: animal models of neuropathic pain. Br.J.Pharmacol. 171, 2951-2963.

Pergolizzi,J., Boger,R.H., Budd,K., Dahan,A., Erdine,S., Hans,G., Kress,H.G., Langford,R., Likar,R., Raffa,R.B., Sacerdote,P., 2008. Opioids and the management of chronic severe pain in the elderly: consensus statement of an International Expert Panel with focus on the six clinically most often used World Health Organization Step III opioids (buprenorphine, fentanyl, hydromorphone, methadone, morphine, oxycodone). Pain Pract. 8, 287-313.

Pert,C.B., Snyder,S.H., 1973. Opiate receptor: demonstration in nervous tissue. Science 179, 1011-1014.

Petrus,M., Peier,A.M., Bandell,M., Hwang,S.W., Huynh,T., Olney,N., Jegla,T., Patapoutian,A., 2007. A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. Mol.Pain 3, 40.

Philippe,L., Gegout-Pottie,P., Guingamp,C., Bordji,K., Terlain,B., Netter,P., Gillet,P., 1997. Relations between functional, inflammatory, and degenerative parameters during adjuvant arthritis in rats. Am.J.Physiol 273, R1550-R1556.

Philpott,H.T., O'Brien,M., McDougall,J.J., 2017. Attenuation of early phase inflammation by cannabidiol prevents pain and nerve damage in rat osteoarthritis. Pain 158, 2442-2451.

Phillips,S.N., Fernando,R., Girard,T., 2017. Parenteral opioid analgesia: Does it still have a role? Best.Pract.Res.Clin.Anaesthesiol. 31, 3-14.

Piergentili,A., Amantini,C., Del,B.F., Giannella,M., Mattioli,L., Palmery,M., Perfumi,M., Pigini,M., Santoni,G., Tucci,P., Zotti,M., Quaglia,W., 2010. Novel highly potent and selective sigma 1 receptor antagonists related to spipethiane. J.Med.Chem. 53, 1261-1269.

Piesla,M.J., Leventhal,L., Strassle,B.W., Harrison,J.E., Cummons,T.A., Lu,P., Whiteside,G.T., 2009. Abnormal gait, due to inflammation but not nerve injury, reflects enhanced nociception in preclinical pain models. Brain Res. 1295, 89-98.

Pina,L.T.S., Gouveia,D.N., Costa,J.S., Quintans,J.S.S., Quintans-Junior,L.J., Barreto,R.S.S., Guimaraes,A.G., 2017. New perspectives for chronic pain treatment: a patent review (2010-2016). Expert.Opin.Ther.Pat 27, 787-796.

Pincus,T., Callahan,L.F., 1992. Rheumatology function tests: grip strength, walking time, button test and questionnaires document and predict longterm morbidity and mortality in rheumatoid arthritis. J.Rheumatol. 19, 1051-1057.

Pitzer,C., Kuner,R., Tappe-Theodor,A., 2016. EXPRESS: Voluntary and evoked behavioral correlates in neuropathic pain states under different housing conditions. Mol.Pain 12. 12, pii 1744806916656635.

Porreca,F., Ossipov,M.H., Gebhart,G.F., 2002. Chronic pain and medullary descending facilitation. Trends Neurosci. 25, 319-325.

Prezzavento,O., Arena,E., Sánchez-Fernández,C., Turnaturi,R., Parenti,C., Marrazzo,A., Catalano,R., Amata,E., Pasquinucci,L., Cobos,E.J., 2017. (+)-and (-)-Phenazocine enantiomers: Evaluation of their dual opioid agonist/sigma1 antagonist properties and antinociceptive effects. Eur.J.Med.Chem. 125, 603-610.

Priestley,J.V. 2009. Neuropeptides: Sensory Systems. In Synaptic Plasticity in Pain textbook. Malcangio Marzia ed. Springer. UK.

Purves,D., Augustine,G.J., Fitzpatrick,D., Hall,W.C., Lamantia,A.S., Mcnamara,J.O., Williams,S.M., 2006. Sistema Somatosensitivo. In Tratado de Neurociencia. 3th Ed. Purves D eds. Panamericana. Madrid.

Qu,Y.J., Zhang,X., Fan,Z.Z., Huai,J., Teng,Y.B., Zhang,Y., Yue,S.W., 2016. Effect of TRPV4-p38 MAPK Pathway on Neuropathic Pain in Rats with Chronic Compression of the Dorsal Root Ganglion. Biomed.Res.Int. 2016, 6978923.

Quirion,R., Bowen,W.D., Itzhak,Y., Junien,J.L., Musacchio,J.M., Rothman,R.B., Su,T.P., Tam,S.W., Taylor,D.P., 1992. A proposal for the classification of sigma binding sites. Trends Pharmacol.Sci. 13, 85-86.

Raehal,K.M., Schmid,C.L., Groer,C.E., Bohn,L.M., 2011. Functional selectivity at the mu-opioid receptor: implications for understanding opioid analgesia and tolerance. Pharmacol.Rev. 63, 1001-1019.

Raffa,R.B., Buschmann,H., Christoph,T., Eichenbaum,G., Englberger,W., Flores,C.M., Hertrampf,T., Kogel,B., Schiene,K., Strassburger,W., Terlinden,R., Tzschentke,T.M., 2012. Mechanistic and functional differentiation of tapentadol and tramadol. Expert.Opin.Pharmacother. 13, 1437-1449.

Ramaswamy,S., Chang,S., Mehta,V., 2015. Tapentadol--the evidence so far. Anaesthesia 70, 518-522.

Randall,L.O., Selitto,J.J., 1957. A method for measurement of analgesic activity on inflamed tissue. Arch.Int.Pharmacodyn.Ther. 111, 409-419.

Rau,K.K., McIlwrath,S.L., Wang,H., Lawson,J.J., Jankowski,M.P., Zylka,M.J., Anderson,D.J., Koerber,H.R., 2009. Mrgprd enhances excitability in specific populations of cutaneous murine polymodal nociceptors. J Neurosci 29, 8612–8619.

Rexed,B., 1952. The cytoarchitectonic organization of the spinal cord in the cat. J.Comp Neurol. 96, 414-495.

Reynolds,D.V., 1969. Surgery in the rat during electrical analgesia induced by focal brain stimulation. Science 164, 444-445.

Rice,D.A., McNair,P.J., Lewis,G.N., Dalbeth,N., 2015. The effects of joint aspiration and intra-articular corticosteroid injection on flexion reflex excitability, quadriceps strength and pain in individuals with knee synovitis: a prospective observational study. Arthritis Res.Ther. 17, 191.

Richardson,J.D., Vasko,M.R., 2002. Cellular mechanisms of neurogenic inflammation. J.Pharmacol.Exp.Ther. 302, 839-845.

Ringkamp,M., Raja,S.N., 2012. Dissecting the relative contribution of central versus peripheral opioid analgesia: are the analgesic and adverse effects of opioids inseparable? Eur.J.Pain 16, 621-623.

Ríos,C., Gomes,I., Devi,L.A., 2006. mu opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. Br.J.Pharmacol. 148, 387-395.

Rodríguez-Muñoz,M., Cortés-Montero,E., Pozo-Rodrigalvarez,A., Sánchez-Blázquez,P., Garzón-Nino,J., 2015a. The ON:OFF switch, sigma1R-HINT1 protein, controls GPCR-NMDA receptor cross-regulation: implications in neurological disorders. Oncotarget. 6, 35458-35477.

Rodriguez-Muñoz,M., Sánchez-Blázquez,P., Herrero-Labrador,R., Martinez-Murillo,R., Merlos,M., Vela,J.M., Garzón,J., 2015b. The sigma1 receptor engages the redox-regulated HINT1 protein to bring opioid analgesia under NMDA receptor negative control. Antioxid.Redox.Signal. 22, 799-818.

Rodríguez-Muñoz,M., Sánchez-Blázquez,P., Vicente-Sanchez,A., Bailón,C., Martin-Aznar,B., Garzón,J., 2011. The histidine triad nucleotide-binding protein 1 supports mu-opioid receptor-glutamate NMDA receptor cross-regulation. Cell Mol.Life Sci. 68, 2933-2949.

Roh,D.H., Choi,S.R., Yoon,S.Y., Kang,S.Y., Moon,J.Y., Kwon,S.G., Han,H.J., Beitz,A.J., Lee,J.H., 2011. Spinal neuronal NOS activation mediates sigma-1 receptor-induced mechanical and thermal hypersensitivity in mice: involvement of PKC-dependent GluN1 phosphorylation. Br.J.Pharmacol. 163, 1707-1720.

Roh,D.H., Kim,H.W., Yoon,S.Y., Seo,H.S., Kwon,Y.B., Kim,K.W., Han,H.J., Beitz,A.J., Na,H.S., Lee,J.H., 2008. Intrathecal injection of the sigma(1) receptor antagonist BD1047 blocks both mechanical allodynia and increases in spinal NR1 expression during the induction phase of rodent neuropathic pain. Anesthesiology 109, 879-889.

Romera,I., Montejo,A.L., Caballero,F., Caballero,L., Arbesu,J., Polavieja,P., Desaiah,D., Gilaberte,I., 2011. Functional impairment related to painful physical symptoms in patients with generalized anxiety disorder with or without comorbid major depressive disorder: post hoc analysis of a cross-sectional study. BMC.Psychiatry 11, 69.

Romero,L., Merlos,M., Vela,J.M., 2016. Antinociception by Sigma-1 Receptor Antagonists: Central and Peripheral Effects. Adv.Pharmacol. 75, 179-215.

Romero,L., Zamanillo,D., Nadal,X., Sánchez-Arroyos,R., Rivera-Arconada,I., Dordal,A., Montero,A., Muro,A., Bura,A., Segales,C., Laloya,M., Hernandez,E., Portillo-Salido,E., Escriche,M., Codony,X., Encina,G., Burgueno,J., Merlos,M., Baeyens,J.M., Giraldo,J., López-García,J.A., Maldonado,R., Plata-Salaman,C.R., Vela,J.M., 2012. Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization. Br.J.Pharmacol. 166, 2289-2306.

Ruoho,A.E., Chu,U.B., Ramachandran,S., Fontanilla,D., Mavlyutov,T., Hajipour,A.R., 2012. The ligand binding region of the sigma-1 receptor: studies utilizing photoaffinity probes, sphingosine and N-alkylamines. Curr.Pharm.Des 18, 920-929.

Ruscher,K., Wieloch,T., 2015. The involvement of the sigma-1 receptor in neurodegeneration and neurorestoration. J.Pharmacol.Sci. 127, 30-35.

Rutten,K., Gould,S.A., Bryden,L., Doods,H., Christoph,T., Pekcec,A., 2018. Standard analgesics reverse burrowing deficits in a rat CCI model of neuropathic pain, but not in models of type 1 and type 2 diabetes-induced neuropathic pain. Behav.Brain Res. 350, 129-138.

Rutten,K., Schiene,K., Robens,A., Leipelt,A., Pasqualon,T., Read,S.J., Christoph,T., 2014. Burrowing as a non-reflex behavioural readout for analgesic action in a rat model of sub-chronic knee joint inflammation. Eur.J.Pain 18, 204-212.

Sánchez-Blázquez,P., Gomez-Serranillos,P., Garzón,J., 2001. Agonists determine the pattern of G-protein activation in mu-opioid receptor-mediated supraspinal analgesia. Brain Res.Bull. 54, 229-235.

Sánchez-Fernández,C., 2014. Differential modulation by sigma-1 receptors of μ-opioid-induced antinociception and side effects: role of peripheral sigma-1 receptors. Doctoral Thesis. University of Granada, Spain.

Sánchez-Fernández,C., Entrena,J.M., Baeyens,J.M., Cobos,E.J., 2017. Sigma-1 Receptor Antagonists: A New Class of Neuromodulatory Analgesics. Adv.Exp.Med. Biol. 964, 109-132.

Sánchez-Fernández,C., Montilla-García,A., González-Cano,R., Nieto,F.R., Romero,L., Artacho-Cordón,A., Montes,R., Fernández-Pastor,B., Merlos,M., Baeyens,J.M., Entrena,J.M., Cobos,E.J., 2014. Modulation of peripheral mu-opioid analgesia by sigma1 receptors. J.Pharmacol.Exp.Ther. 348, 32-45.

Sánchez-Fernández,C., Nieto,F.R., González-Cano,R., Artacho-Cordón,A., Romero,L., Montilla-García,A., Zamanillo,D., Baeyens, J.M., Entrena,J.M., Cobos,E.J., 2013. Potentiation of morphine-induced mechanical antinociception by sigma(1) receptor inhibition: role of peripheral sigma(1) receptors. Neuropharmacology 70, 348-358.

Sandkuhler,J., 2009. Models and mechanisms of hyperalgesia and allodynia. Physiol Rev. 89, 707-758.

Sarzi-Puttini,P., Vellucci,R., Zuccaro,S.M., Cherubino,P., Labianca,R., Fornasari,D., 2012. The appropriate treatment of chronic pain. Clin.Drug Investig. 32 Suppl 1, 21-33.

Sato,T., Sakurada,S., Takahashi,N., Sakurada,T., Tan-No,K., Wako,K., Kisara,K., 1999. Contribution of spinal mu1-opioid receptors to morphine-induced antinociception. Eur.J.Pharmacol. 369, 183-187.

Savilampi,J., Ahlstrand,R., Magnuson,A., Geijer,H., Wattwil,M., 2014. Aspiration induced by remifentanil: a double-blind, randomized, crossover study in healthy volunteers. Anesthesiology 121, 52-58.

Sawynok,J., Liu,J., 2014. Contributions of peripheral, spinal, and supraspinal actions to analgesia. Eur.J.Pharmacol. 734, 114-121.

Scanzello,C.R., Goldring,S.R., 2012. The role of synovitis in osteoarthritis pathogenesis. Bone 51, 249-257.

Scott,D., Coleman,P.J., Mason,R.M., Levick,J.R., 2000. Interaction of intraarticular hyaluronan and albumin in the attenuation of fluid drainage from joints. Arthritis Rheum. 43, 1175-1182.

Scherer,P.C., Zaccor,N.W., Neumann,N.M., Vasavda,C., Barrow,R., Ewald,A.J., Rao,F., Sumner,C.J., Snyder,S.H., 2017. TRPV1 is a physiological regulator of mu-opioid receptors. Proc.Natl.Acad.Sci.U.S.A 114, 13561-13566.

Scherrer,G., Imamachi,N., Cao,Y.Q., Contet,C., Mennicken,F., O'Donnell,D., Kieffer,B.L., Basbaum,A.I., 2009. Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. Cell 137, 1148-1159.

Schmauss,C., Shimohigashi,Y., Jensen,T.S., Rodbard,D., Yaksh,T.L., 1985. Studies on spinal opiate receptor pharmacology. III. Analgetic effects of enkephalin dimers as measured by cutaneous-thermal and visceral-chemical evoked responses. Brain Res. 337, 209-215.

Schmidt,B., Hammer,J., Holzer,P., Hammer,H.F., 2004. Chemical nociception in the jejunum induced by capsaicin. Gut 53, 1109-1116.

Schmidt,H.R., Zheng,S., Gurpinar,E., Koehl,A., Manglik,A., Kruse,A.C., 2016. Crystal structure of the human sigma1 receptor. Nature 532, 527-530.

Scholz,J., Woolf,C.J., 2002. Can we conquer pain? Nat.Neurosci. 5 Suppl, 1062-1067.

Schott,E., Berge,O.G., Angeby-Moller,K., Hammarstrom,G., Dalsgaard,C.J., Brodin,E., 1994. Weight bearing as an objective measure of arthritic pain in the rat. J.Pharmacol.Toxicol.Methods 31, 79-83.

Schulz,S., Mayer,D., Pfeiffer,M., Stumm,R., Koch,T., Hollt,V., 2004. Morphine induces terminal μ-opioid receptor desensitization by sustained phosphorylation of serine-375. EMBO J. 23, 3282-3289.

Schwarz,S., Pohl,P., Zhou,G.Z., 1989. Steroid binding at sigma-"opioid" receptors. Science 246, 1635-1638.

Sehgal,N., Smith,H.S., Manchikanti,L., 2011. Peripherally acting opioids and clinical implications for pain control. Pain Physician 14, 249-258.

Sher,G.D., Mitchell,D., 1990. N-methyl-D-aspartate receptors mediate responses of rat dorsal horn neurones to hindlimb ischemia. Brain Res. 522, 55-62.

Shi,C., Das,V., Li,X., Kc,R., Qiu,S., Sullivan,I., Ripper,R.L., Kroin,J.S., Mwale,F., Wallace,A.A., Zhu,B., Zhao,L., van Wijnen,A.J., Ji,M., Lu,J., Votta-Velis,G., Yuan,W., Im,H.J., 2017. Development of an in vivo mouse model of discogenic low back pain. J.Cell Physiol. [Epub ahead of print]

Shinoda,M., Ogino,A., Ozaki,N., Urano,H., Hironaka,K., Yasui,M., Sugiura,Y., 2008. Involvement of TRPV1 in nociceptive behavior in a rat model of cancer pain. J.Pain 9, 687-699.

Shioda,N., Ishikawa,K., Tagashira,H., Ishizuka,T., Yawo,H., Fukunaga,K., 2012. Expression of a truncated form of the endoplasmic reticulum chaperone protein, sigma1 receptor, promotes mitochondrial energy depletion and apoptosis. J.Biol.Chem. 287, 23318-23331.

Shir,Y., Seltzer,Z., 1990. A-fibers mediate mechanical hyperesthesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of causalgiform pain disorders in rats. Neurosci.Lett. 115, 62-67.

Siegmund,E., Cadmus,R., Lu,G., 1957. A method for evaluating both non-narcotic and narcotic analgesics. Proc.Soc.Exp.Biol.Med. 95, 729-731.

Simon,E.J., 1973. In search of the opiate receptor. Am.J.Med.Sci. 266, 160-168.

Sisignano,M., Baron,R., Scholich,K., Geisslinger,G., 2014. Mechanism-based treatment for chemotherapy-induced peripheral neuropathic pain. Nat.Rev.Neurol. 10, 694-707.

Sokolove,J., Lepus,C.M., 2013. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. Ther.Adv.Musculoskelet. Dis. 5, 77-94.

Sotocinal,S.G., Sorge,R.E., Zaloum,A., Tuttle,A.H., Martin,L.J., Wieskopf,J.S., Mapplebeck,J.C., Wei,P., Zhan,S., Zhang,S., McDougall,J.J., King,O.D., Mogil,J.S., 2011. The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol.Pain 7, 55.

St,Pierre,M., Reeh,P.W., Zimmermann,K., 2009. Differential effects of TRPV channel block on polymodal activation of rat cutaneous nociceptors in vitro. Exp.Brain Res. 196, 31-44.

Stein,C., 1995. The control of pain in peripheral tissue by opioids. N.Engl.J.Med. 332, 1685-1690.

Stein,C., 2013. Opioids, sensory systems and chronic pain. Eur.J.Pharmacol. 716, 179-187.

Stein,C., Clark,J.D., Oh,U., Vasko,M.R., Wilcox,G.L., Overland,A.C., Vanderah,T.W., Spencer,R.H., 2009. Peripheral mechanisms of pain and analgesia. Brain Res.Rev. 60, 90-113.

Stein,C., Schafer,M., Machelska,H., 2003. Attacking pain at its source: new perspectives on opioids. Nat.Med. 9, 1003-1008.

Stevenson,G.W., Bilsky,E.J., Negus,S.S., 2006. Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. J.Pain 7, 408-416.

Stone,J.M., Arstad,E., Erlandsson,K., Waterhouse,R.N., Ell,P.J., Pilowsky,L.S., 2006. [123I]TPCNE--a novel SPET tracer for the sigma-1 receptor: first human studies and in vivo haloperidol challenge. Synapse 60, 109-117.

Stubbs,D., Krebs,E., Bair,M., Damush,T., Wu,J., Sutherland,J., Kroenke,K., 2010. Sex Differences in Pain and Pain-Related Disability among Primary Care Patients with Chronic Musculoskeletal Pain. Pain Med. 11, 232-239.

Su,T.P., Hayashi,T., Maurice,T., Buch,S., Ruoho,A.E., 2010. The sigma-1 receptor chaperone as an inter-organelle signaling modulator. Trends Pharmacol.Sci. 31, 557-566.

Sun,H., Shi,M., Zhang,W., Zheng,Y.M., Xu,Y.Z., Shi,J.J., Liu,T., Gunosewoyo,H., Pang,T., Gao,Z.B., Yang,F., Tang,J., Yu,L.F., 2016. Development of Novel Alkoxyisoxazoles as Sigma-1 Receptor Antagonists with Antinociceptive Efficacy. J.Med.Chem. 59, 6329-6343.

Sun,S., Xu,Q., Guo,C., Guan,Y., Liu,Q., Dong,X., 2017. Leaky Gate Model: Intensity-Dependent Coding of Pain and Itch in the Spinal Cord. Neuron 93, 840-853.

Suokas,A.K., Sagar,D.R., Mapp,P.I., Chapman,V., Walsh,D.A., 2014. Design, study quality and evidence of analgesic efficacy in studies of drugs in models of OA pain: a systematic review and a meta-analysis. Osteoarthritis. Cartilage. 22, 1207-1223.

Takizawa,R., Hashimoto,K., Tochigi,M., Kawakubo,Y., Marumo,K., Sasaki,T., Fukuda,M., Kasai,K., 2009. Association between sigma-1 receptor gene polymorphism and prefrontal hemodynamic response induced by cognitive activation in schizophrenia.Prog.Neuropsychopharmacol. Biol.Psychiatry 33, 491-498.

Tejada,M.A., Montilla-García,A., Cronin,S.J., Cikes,D., Sánchez-Fernández,C., González-Cano,R., Ruíz-Cantero,M.C., Penninger,J.M., Vela,J.M., Baeyens,J.M., Cobos,E.J., 2017. Sigma-1 receptors control immune-driven peripheral opioid analgesia during inflammation in mice. Proc.Natl.Acad.Sci. U.S.A 114, 8396-8401.

Tejada,M.A., Montilla-García,A., González-Cano,R., Bravo-Caparrós,I., Ruíz-Cantero,M.C., Nieto,F.R., Cobos,E.J., 2018. Targeting immune-driven opioid analgesia by sigma-1 receptors: Opening the door to novel perspectives for the analgesic use of sigma-1 antagonists. Pharmacol.Res. 131, 224-230.

Tejada,M.A., Montilla-García,A., Sánchez-Fernández,C., Entrena,J.M., Perazzoli,G., Baeyens,J.M., Cobos,E.J., 2014. Sigma-1 receptor inhibition reverses acute inflammatory hyperalgesia in mice: role of peripheral sigma-1 receptors. Psychopharmacology (Berl) 231, 3855-3869.

Terenius,L., 1973. Characteristics of the "receptor" for narcotic analgesics in synaptic plasma membrane fraction from rat brain. Acta Pharmacol.Toxicol.(Copenh) 33, 377-384.

Terenius,L., Wahlstrom,A., 1975. Search for an endogenous ligand for the opiate receptor. Acta Physiol Scand. 94, 74-81.

Tétényi,P., 2010. Opium Poppy (Papaver somniferum): Botany and Horticulture. Horticultural Reviews. In Jules Janick ed. John Wiley & Sons, Inc. USA.

Tetreault,P., Dansereau,M.A., Dore-Savard,L., Beaudet,N., Sarret,P., 2011. Weight bearing evaluation in inflammatory, neuropathic and cancer chronic pain in freely moving rats. Physiol Behav. 104, 495-502.

Thomas,J., Karver,S., Cooney,G.A., Chamberlain,B.H., Watt,C.K., Slatkin,N.E., Stambler,N., Kremer,A.B., Israel,R.J., 2008. Methylnaltrexone for opioid-induced constipation in advanced illness. N.Engl.J.Med. 358, 2332-2343.

Tilson,H.A., 1990. Behavioral indices of neurotoxicity. Toxicol.Pathol. 18, 96-104.

Todd,A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat.Rev.Neurosci. 11, 823-836.

Trang,T., Al-Hasani,R., Salvemini,D., Salter,M.W., Gutstein,H., Cahill,C.M., 2015. Pain and Poppies: The Good, the Bad, and the Ugly of Opioid Analgesics. J.Neurosci. 35, 13879-13888.

Tseng,L.F., Collins,K.A., Wang,Q., 1995. Differential ontogenesis of thermal and mechanical antinociception induced by morphine and beta-endorphin. Eur.J.Pharmacol. 277, 71-76.

Turner,J.A., Ersek,M., Kemp,C., 2005. Self-efficacy for managing pain is associated with disability, depression, and pain coping among retirement community residents with chronic pain. J.Pain 6, 471-479.

Tuttle,A.H., Molinaro,M.J., Jethwa,J.F., Sotocinal,S.G., Prieto,J.C., Styner,M.A., Mogil,J.S., Zylka,M.J., 2018. A deep neural network to assess spontaneous pain from mouse facial expressions. Mol.Pain 14, 1744806918763658.

Unruh,A.M., 1996. Gender variations in clinical pain experience. Pain 65, 123-167.

Urban,R., Scherrer,G., Goulding,E.H., Tecott,L.H., Basbaum,A.I., 2011. Behavioral indices of ongoing pain are largely unchanged in male mice with tissue or nerve injury-induced mechanical hypersensitivity. Pain 152, 990-1000.

Vadivelu,N., Huang,Y., Mirante,B., Jacoby,M., Braveman,F.R., Hines,R.L., Sinatra,R., 2013. Patient considerations in the use of tapentadol for moderate to severe pain. Drug Healthc.Patient.Saf 5, 151-159.

van Laarhoven,A.I., Kraaimaat,F.W., Wilder-Smith,O.H., van Riel,P.L., van de Kerkhof,P.C., Evers,A.W., 2013. Sensitivity to itch and pain in patients with psoriasis and rheumatoid arthritis. Exp.Dermatol. 22, 530-534.

van Niel,J.C., Schneider,J., Tzschentke,T.M., 2016. Efficacy of Full micro-Opioid Receptor Agonists is not Impaired by Concomitant Buprenorphine or Mixed Opioid Agonists/Antagonists - Preclinical and Clinical Evidence. Drug Res.(Stuttg) 66, 562-570.

Vela,J.M., Merlos,M., Almansa,C., 2015. Investigational sigma-1 receptor antagonists for the treatment of pain. Expert. Opin.Investig.Drugs 24, 883-896.

Vidal-Torres,A., de la Puente,B., Rocasalbas,M., Tourino,C., Bura,S.A., Fernández-Pastor,B., Romero,L., Codony,X., Zamanillo,D., Buschmann,H., Merlos,M., Baeyens,J.M., Maldonado,R., Vela,J.M., 2013. Sigma-1 receptor antagonism as opioid adjuvant strategy: enhancement of opioid antinociception without increasing adverse effects. Eur.J.Pharmacol. 711, 63-72.

Vidal-Torres,A., Fernández-Pastor,B., Carceller,A., Vela,J.M., Merlos,M., Zamanillo,D., 2014. Effects of the selective sigma-1 receptor antagonist S1RA on formalin-induced pain behavior and neurotransmitter release in the spinal cord in rats. J.Neurochem. 129, 484-494.

Vos,B.P., Strassman,A.M., Maciewicz,R.J., 1994. Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. J.Neurosci. 14, 2708-2723.

Voss,L.J., Melin,S., Jacobson,G., Sleigh,J.W., 2010. GABAergic compensation in connexin36 knock-out mice evident during low-magnesium seizure-like event activity. Brain Res. 1360, 49-55.

Vrinten,D.H., Hamers,F.F., 2003. 'CatWalk' automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. Pain 102, 203-209.

Wacnik,P.W., Kehl,L.J., Trempe,T.M., Ramnaraine,M.L., Beitz,A.J., Wilcox,G.L., 2003. Tumor implantation in mouse humerus evokes movement-related hyperalgesia exceeding that evoked by intramuscular carrageenan. Pain 101, 175-186.

Waldhoer,M., Bartlett,S.E., Whistler,J.L., 2004. Opioid receptors. Annu.Rev.Biochem. 73, 953-990.

Wang,C., Wang,H.G., Xie,H., Pitt,G.S., 2008. Ca2+/CaM controls Ca2+-dependent inactivation of NMDA receptors by dimerizing the NR1 C termini. J.Neurosci. 28, 1865-1870.

Wang,L.X., Wang,Z.J., 2003. Animal and cellular models of chronic pain. Adv.Drug Deliv.Rev. 55, 949-965.

Wegert,S., Ossipov,M.H., Nichols,M.L., Bian,D., Vanderah,T.W., Malan,T.P., Jr., Porreca,F., 1997. Differential activities of intrathecal MK-801 or morphine to alter responses to thermal and mechanical stimuli in normal or nerve-injured rats. Pain 71, 57-64.

Wermeling,D.P., 2015. Review of naloxone safety for opioid overdose: practical considerations for new technology and expanded public access. Ther.Adv.Drug Saf 6, 20-31.

Whistler,J.L., Enquist,J., Marley,A., Fong,J., Gladher,F., Tsuruda,P., Murray,S.R., Von,Z.M., 2002. Modulation of postendocytic sorting of G protein-coupled receptors. Science 297, 615-620.

Whistler,J.L., Von,Z.M., 1998. Morphine-activated opioid receptors elude desensitization by beta-arrestin. Proc.Natl.Acad.Sci.U.S.A 95, 9914-9919.

Whitehead,R.A., Lam,N.L., Sun,M.S., Sanchez,J., Noor,S., Vanderwall,A.G., Petersen,T.R., Martin,H.B., Milligan,E.D., 2017. Chronic Sciatic Neuropathy in Rat Reduces Voluntary Wheel-Running Activity With Concurrent Chronic Mechanical Allodynia. Anesth.Analg. 124, 346-355.

Whiteside,G.T., Harrison,J., Boulet,J., Mark,L., Pearson,M., Gottshall,S., Walker,K., 2004. Pharmacological characterisation of a rat model of incisional pain. Br.J.Pharmacol. 141, 85-91.

Williams,J.T., Ingram,S.L., Henderson,G., Chavkin,C., Von,Z.M., Schulz,S., Koch,T., Evans,C.J., Christie,M.J., 2013. Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol.Rev. 65, 223-254.

Willis,W.D., Jr., 2007. The somatosensory system, with emphasis on structures important for pain. Brain Res.Rev. 55, 297-313.

Wodarski,R., Delaney,A., Ultenius,C., Morland,R., Andrews,N., Baastrup,C., Bryden,L.A., Caspani,O., Christoph,T., Gardiner,N.J., Huang,W., Kennedy,J.D., Koyama,S., Li,D., Ligocki,M., Lindsten,A., Machin,I., Pekcec,A., Robens,A., Rotariu,S.M., VoB,S., Segerdahl,M., Stenfors,C., Svensson,C.I., Treede,R.D., Uto,K., Yamamoto,K., Rutten,K., Rice,A.S., 2016. Cross-centre replication of suppressed burrowing behaviour as an ethologically relevant pain outcome measure in the rat: a prospective multicentre study. Pain 157, 2350-2365.

Wolkerstorfer,A., Handler,N., Buschmann,H., 2016. New approaches to treating pain. Bioorg.Med.Chem.Lett. 26, 1103-1119.

Woodbury,C.J., Zwick,M., Wang,S., Lawson,J.J., Caterina,M.J., Koltzenburg,M., Albers,K.M., Koerber,H.R., Davis,B.M., 2004. Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J.Neurosci. 24, 6410-6415.

Woolfe,G., Macdonald,A.D., 1944.The evaluation of the analgesic action of pethidine hydrochloride (demerol). J.Pharmacol.Exp.Ther. 80, 300-307.

Yaksh,T.L., Woller,S.A., Ramachandran,R., Sorkin,L.S., 2015. The search for novel analgesics: targets and mechanisms. F1000Prime.Rep. 7, 56.

Yamamoto,W., Sugiura,A., Nakazato-Imasato,E., Kita,Y., 2008. Characterization of primary sensory neurons mediating static and dynamic allodynia in rat chronic constriction injury model. J.Pharm.Pharmacol. 60, 717-722.

Yassen,A., Olofsen,E., Dahan,A., Danhof,M., 2005. Pharmacokinetic-pharmacodynamic modeling of the antinociceptive effect of buprenorphine and fentanyl in rats: role of receptor equilibration kinetics. J.Pharmacol. Exp.Ther. 313, 1136-1149.

Zador,F., Kiraly,K., Varadi,A., Balogh,M., Feher,A., Kocsis,D., Erdei,A.I., Lacko,E., Zadori,Z.S., Hosztafi,S., Noszal,B., Riba,P., Benyhe,S., Furst,S., Al-Khrasani,M., 2017. New opioid receptor antagonist: Naltrexone-14-O-sulfate synthesis and pharmacology. Eur.J.Pharmacol. 809, 111-121.

Zamanillo,D., Romero,L., Merlos,M., Vela,J.M., 2013. Sigma 1 receptor: a new therapeutic target for pain. Eur.J.Pharmacol. 716, 78-93.

Zampieri,D., Vio,L., Fermeglia,M., Pricl,S., Wunsch,B., Schepmann,D., Romano,M., Mamolo,M.G., Laurini,E., 2016. Computer-assisted design, synthesis, binding and cytotoxicity assessments of new 1-(4-(aryl(methyl)amino)butyl)-heterocyclic sigma 1 ligands. Eur.J.Med.Chem. 121, 712-726.

Zhang,J., Cavanaugh,D.J., Nemenov,M.I., Basbaum,A.I., 2013. The modality-specific contribution of peptidergic and non-peptidergic nociceptors is manifest at the level of dorsal horn nociresponsive neurons. J.Physiol 591, 1097-1110.

Zhu,C.Z., Mills,C.D., Hsieh,G.C., Zhong,C., Mikusa,J., Lewis,L.G., Gauvin,D., Lee,C.H., Decker,M.W., Bannon,A.W., Rueter,L.E., Joshi,S.K., 2012. Assessing carrageenan-induced locomotor activity impairment in rats: comparison with evoked endpoint of acute inflammatory pain. Eur.J.Pain 16, 816-826.

Zhuo,M., Gebhart,G.F., 1992. Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. J.Neurophysiol. 67, 1599-1614.

Zwick,M., Davis,B.M., Woodbury,C.J., Burkett,J.N., Koerber,H.R., Simpson,J.F., Albers,K.M., 2002. Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J.Neurosci. 22, 4057-4065.

Zylka,M.J., Dong,X., Southwell,A.L., Anderson,D.J., 2003. Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. Proc.Natl.Acad.Sci.U.S.A 100, 10043-10048.

Zylka,M.J., Rice,F.L., Anderson,D.J., 2005. Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45, 17-25.

PUBLISHED ARTICLES

ARTICLES DIRECTLY RELATED TO THIS PHD THESIS

Montilla-García,Á.*, Tejada,M.A.*, Perazzoli,G., Entrena,J.M., Portillo-Salido,E., Fernández-Segura,E., Cañizares,F.J., Cobos,E.J., 2017. Grip strength in mice with joint inflammation: A rheumatology function test sensitive to pain and analgesia. Neuropharmacology 125, 231-242.

(*equal contribution)

Montilla-García,Á., Perazzoli,G., Tejada,M.A., González-Cano,R., Sánchez-Fernández,C., Cobos,E.J., Baeyens,J.M., 2018. Modality-specific peripheral antinociceptive effects of mu-opioid agonists on heat and mechanical stimuli: Contribution of sigma-1 receptors. Neuropharmacology 135, 328-342.

Montilla-García,Á., Tejada,M.A., Ruíz-Cantero,M.C., Yeste,S., Bravo-Caparrós,I., Zamanillo,D., Cobos,E.J., 2018. Modulation of morphine analgesia and tolerance by sigma-1 receptors: studies on nociceptive heat pain and on tactile allodynia and grip strength deficits during joint inflammation. Submitted.

Tejada,M.Á.*, **Montilla-García,Á.***, González-Cano,R., Bravo-Caparrós,I., Ruíz-Cantero,M.C., Nieto,F.R., Cobos,E.J., 2018.Targeting immune-driven opioid analgesia by sigma-1 receptors: Opening the door to novel perspectives for the analgesic use of sigma-1 antagonists. Pharmacol Res. 131, 224-230.

(*equal contribution)

OTHER ARTICLES

Tejada,M.Á.*, **Montilla-García,Á.***, Cronin,S.J., Cikes,D., Sánchez-Fernández,C., González-Cano,R., Ruíz-Cantero,M.C., Penninger,J.M., Vela,J.M., Baeyens,J.M., Cobos,E.J., 2017. Sigma-1 receptors control immune-driven peripheral opioid analgesia during inflammation in mice. Proc Natl Acad Sci U S A 114, 8396- 8401.

(*equal contribution)

Amata,E., Rescifina,A., Prezzavento,O., Arena,E., Dichiara,M., Pittalà,V., **Montilla-García,Á.**, Punzo,F., Merino,P., Cobos,E.J., Marrazzo,A.., 2018. (+)-Methyl (1R,2S)-2-{[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate [(+)-MR200] Derivatives as Potent and Selective Sigma Receptor Ligands: Stereochemistry and Pharmacological Properties. J Med Chem. 11, 372-384.

Luna-Sánchez,M., Díaz-Casado,E., Barca,E., Tejada,M.Á., **Montilla-García,Á.**, Cobos,E.J., Escames,G., Acuña-Castroviejo,D., Quinzii,C.M., López,L.C., 2015. The clinical heterogeneity of coenzyme Q10 deficiency results from genotypic differences in the Coq9 gene. EMBO Mol Med. 7, 670-687.

Tejada,M.Á., **Montilla-García,Á.**, Sánchez-Fernández,C., Entrena,J.M., Perazzoli,G., Baeyens,J.M., Cobos,E.J., 2014. Sigma-1 receptor inhibition reverses acute inflammatory hyperalgesia in mice: role of peripheral sigma-1 receptors. Psychopharmacology (Berl) 231, 3855-3869.

Sánchez-Fernández,C., **Montilla-García,Á.**, González-Cano,R., Nieto,F.R., Romero,L., Artacho-Cordón,A., Montes,R., Fernández-Pastor,B., Merlos,M., Baeyens,J.M., Entrena,J.M., Cobos,E.J., 2014. Modulation of peripheral μ-opioid analgesia by σ1 receptors. J Pharmacol Exp Ther. 348: 32-45.

Sánchez-Fernández,C., Nieto,F.R., González-Cano,R., Artacho-Cordón,A., Romero,L., **Montilla-García,Á.**, Zamanillo,D., Baeyens,J.M., Entrena,J.M., Cobos,E.J., 2013. Potentiation of morphine-induced mechanical antinociception by σ_1 receptor inhibition: role of peripheral σ_1 receptors. Neuropharmacology 70, 348-358.