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SIGMA-1 RECEPTORS CONTROL IMMUNE-DRIVEN PERIPHERAL OPIOID ANALGESIA DURING INFLAMMATION

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“Investigar es ver lo que todo el mundo ha visto,

y pensar lo que nadie más ha pensado”.

“Investigate is to see what everyone has seen,

and think what no one else has thought "

Albert Szent-Györgyi

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RESUMEN

1. Antecedentes, hipótesis y objetivos

1.1. Antecedentes

Existe la necesidad de nuevos analgésicos con mecanismos de acción diferentes a los actuales (Kissin, 2010). El receptor sigma-1 es una proteína chaperona neuromoduladora que modifica la función de varios receptores y canales iónicos implicados en los procesos de neurotransmisión (Su et al., 2016), y ha sido recientemente el foco de un exahustivo estudio preclínico para establecerlo como una nueva diana farmacológica para el tratamiento del dolor (Zamanillo et al., 2013; Vela et al., 2015).

Aunque la inhibición del receptor sigma-1 no influye en el dolor agudo inducido por estímulos térmicos o mecánicos (p.ej., Chien y Pasternak 1994, de la Puente et al 2009, Entrena et al 2009b, Marrazzo et al., 2011, Romero et al., 2012, Sánchez-Fernández et al., 2013), es capaz de aliviar tanto la segunda fase del dolor inducido por formalina como la hipersensibilidad sensorial de origen neuropático, mediando en estos efectos la atenuación del proceso de sensibilización central (Kim et al., 2006, Roh et al., 2008, de la Puente et al. 2009; Nieto et al., 2012, Romero et al., 2012). Estos hallazgos indican que los receptores sigma-1 juegan un papel importante en el procesamiento del dolor a nivel central. Entre los antagonistas selectivos sigma-1, los mejor caracterizados son el BD-1063 y el S1RA (Zamanillo et al., 2013). Este último compuesto ha superado los ensayos clínicos de fase I demostrando su seguridad y tolerabilidad en personas sanas (Abadias et al. 2013), y ha sido evaluado recientemente en ensayos clínicos de fase II, siendo su indicación principal el tratamiento del dolor neuropático (Vaqué et al., 2016).

Otra indicación potencial de este antagonista sigma-1, el S1RA, es el incremento de la analgesia inducida por los fármacos opioides (Vela et al., 2015). La potenciación de la antinocicepción opioide mediante el antagonismo del receptor sigma-1, fue descrita a principios de los años 90 (Chien y Pasternak, 1993). En estudios posteriores se observó que este efecto del antagonismo sigma-1 se puede producir a nivel central (Mei y Pasternak, 2002), y más recientemente, se ha visto que también ocurre a nivel periférico (Sánchez-Fernández et al., 2013 y 2014). De hecho, esta potenciación de la analgesia opioide por la inhibición de los receptores sigma-1 es muy sensible al efecto

de la naloxona metiodida (Sánchez-Fernández et al., 2014), un derivado cuaternario del antagonista opioide naloxona que es incapaz de penetrar a nivel central (Menéndez et al., 2005, Sevostianova et al., 2005, Parenti et al., 2012), lo que indica la participación de los receptores opioides periféricos en el efecto de los antagonistas sigma-1. Esta potenciación opioide periférica es congruente con la mayor densidad de receptores sigma-1 existentes en el ganglio de la raíz dorsal (DRG) comparado con varias áreas a centrales, tales como el asta dorsal de la médula espinal y la sustancia gris periaqueductal (Sánchez-Fernández et al., 2014). Además, los receptores sigma-1 en el DRG se localizan selectivamente en neuronas sensoriales y no en las células gliales (Mavlyutov et al., 2016). Actualmente se sabe que los receptores sigma-1 pueden formar un complejo macromolecular con los receptores opioides, inhibiendo tónicamente el funcionamiento de los mismos, y que el antagonismo del receptor sigma-1 puede impedir esta inhibición tónica a la que están sometido dichos receptores opioides por parte del receptor sigma-1, potenciando de esta manera la analgesia opioide (Kim Et al., 2010, Rodríguez-Muñoz et al., 2015). Sin embargo, aunque la capacidad del antagonismo sigma-1 para potenciar el efecto analgésico de los fármacos opioides es clara, el papel fisiológico o fisiopatológico de los receptores sigma-1 en la modulación opioide sigue siendo un enigma.

El dolor inflamatorio es uno de los principales tipo de dolor patológico (p.ej., Scholz y Woolf 2002, Woolf 2004). Al inicio de este proyecto de Tesis Doctoral se desconocía si los receptores sigma-1 podrían participar en este tipo de dolor. A diferencia del dolor neuropático, el dolor inflamatorio se caracteriza por un aumento más pronunciado de la capacidad de respuesta de los nociceptores (sensibilización periférica) por la estimulación de mediadores inflamatorios liberados en el foco de la inflamación (véase Scholz y Woolf 2002, Ji 2004, Latremoliere y Woolf 2009, Patapoutian et al., 2009). El dolor inflamatorio puede ser inducido experimentalmente por la administración intraplantar (i.pl.) de sustancias tales como la carragenina, y se caracteriza por el desarrollo de hiperalgesia a estímulos tanto mecánicos como caloríficos (Sandkühler 2009, Cobos y Portillo-Salido 2013), siendo estos tipos de hipersensibilidad sensorial el resultado de mecanismos que se solapan sólo parcialmente (Latremoliere y Woolf 2009, Dubin y Patapoutian 2010). La hiperalgesia inflamatoria es debida, al menos en parte, a la liberación de algógenos químicos producidos por las células inmunes que se

infiltran en el tejido inflamado (Ji et al., 2014). Es interesante señalar que las células inmunes, además de producir estos algógenos químicos, son capaces de producir péptidos opioides endógenos (POEs), siendo el más destacado la β-endorfina, la cual se sintetiza a partir de la pro-opiomelanocortina (POMC) (Hua y Cabot, 2010). Sin embargo, a pesar del potencial analgésico de estos POEs, la inflamación conlleva habitualmente el desarrollo de dolor. Se desconoce si los receptores sigma-1 reducen los efectos antinociceptivos de los POEs periféricos durante la inflamación, facilitando por este mecanismo el dolor inflamatorio.

1.2. Hipótesis y objetivos

Teniendo en cuenta estos antecedentes, la **hipótesis principal** de esta Tesis Doctoral fue que la inhibición del receptor sigma-1 podría aliviar la hiperalgesia inflamatoria mediante la desinhibición de las acciones periféricas de los POEs de origen inmune. Si este fuera el caso, sería un mecanismo de analgesia totalmente novedoso que podría expandir el potencial terapéutico de los antagonistas sigma-1.

Para probar esta hipótesis, nuestro **primer objetivo** fue evaluar si la inhibición del receptor sigma-1 alivia la hiperalgesia inflamatoria, y determinar la contribución de los receptores sigma-1 periféricos a este efecto. Para ello, estudiamos los efectos de la inactivación del receptor sigma-1, en ratones knockout sigma-1 (KO sigma-1), y de la administración sistémica (subcutánea) y local (intraplantar) de dos antagonistas selectivos del receptor sigma-1, el BD-1063 y el S1RA, en la hiperalgesia mecánica y térmica inflamatoria inducida por carragenina. Para estudiar si la inhibición del receptor sigma-1 produjera efectos antiinflamatorios que pudieran contribuir al alivio de la hiperalgesia, evaluamos la influencia de la inhibición de este receptor sobre el edema inducido por carragenina.

El **segundo objetivo** de esta Tesis Doctoral fue determinar si la activación de los receptores opioides contribuye a los efectos antihiperalgésicos inducidos por el antagonismo sigma-1, y estudiar si la acción de los opioides periféricos está involucrada en este proceso. Para ello, evaluamos si los efectos antihiperalgésicos del BD-1063 y el S1RA podrían ser revertidos tanto por el antagonista opioide naloxona, como por su análogo de acción periférica naloxona metiodida.

Nuestro **tercer objetivo** fue estudiar el papel de las células inmunes en el dolor inflamatorio inducido por carragenina. Con este fin, inhibimos la infiltración de las células inmunes en el foco inflamatorio (mediante la administración *in vivo* de un anticuerpo específico), para evaluar así su participación en la hiperalgesia inflamatoria y en el desarrollo del edema.

Nuestro **cuarto objetivo** fue determinar si las células inmunes producen β-endorfina (como ejemplo de un POE relevante) en el foco inflamatorio. Para ello, caracterizamos mediante citometría de flujo (FACS) las poblaciones de células inmunes existentes en el foco inflamatorio usando marcadores específicos de dichas células, y luego determinamos si estas células expresan ARNm para la POMC. Además, evaluamos los niveles de β-endorfina en la pata inflamada mediante un inmunoensayo enzimático de fluorescencia, y estudiamos si la eliminación de las células inmunes del foco inflamatorio disminuye los niveles de este POE.

El **quinto objetivo** de esta Tesis Doctoral fue determinar si los efectos antihiperalgésicos sensibles a la naloxona inducidos por el antagonismo sigma-1 durante la inflamación, dependen de la presencia de POEs de origen inmune. Para cumplir este objetivo, hemos estudiado si los efectos antihiperalgésicos inducidos por el antagonismo sigma-1 se ven afectados por la neutralización de los POEs en la pata inflamada (administrando localmente un anticuerpo que reconoce la mayoría de estos péptidos opioides), así como por la reducción de la infiltración inmune en el lugar de la inflamación.

Finalmente, nuestro **sexto objetivo** fue estudiar si el el efecto analgésico inducido por los antagonistas sigma-1 involucra las acciones de los POEs de origen inmune en otro modelo de dolor (diferente a la inflamación inducida por carragenina). Para ello, determinamos la infiltración inmune tras la administración de formalina intraplantar, evaluamos el efecto analgésico del S1RA en el dolor inducido por este irritante químico, y si este es sensibles al antagonismo opioide.

2. Materiales y métodos

2.1. Animales de experimentación

Todos los experimentos se hicieron en ratones hembras de la cepa CD1 de genotipo salvaje (Charles River, Barcelona, Spain) y KO sigma-1 (Laboratorios Esteve, Barcelona, Spain) generados en un fondo genético CD1 (Entrena et al., 2009b). Todo el trabajo realizado con animales se hizo de acuerdo con las normas institucionales (Comité de Ética de Experimentación Animal de la Universidad de Granada, España) y con las normas internacionales (Directiva 2010/63 del Consejo de la Unión Europea).

2.2. Inflamación inducida por carragenina

La inflamación de la pata se indujo mediante la inyección intraplantar de 50 µl de una solución de λ-carragenina (1% en peso/volumen en solución salina) (Sigma-Aldrich, Madrid, España), como se describió en trabajos previos (Posadas et al., 2004). La solución de carragenina se preparó cada día antes de realizar el experimento, y se inyectó en el tejido subcutáneo de la superficie de la planta de la pata trasera derecha usando una microjeringa Hamilton de TLL 1710 (Teknokroma, Barcelona, España) con una aguja de calibre $30^{1/2}$. Como control utilizamos una inyección i.pl. del mismo volumen de suero salino fisiológico (NaCl 0,9%).

2.3. Administración de fármacos y anticuerpos

Los antagonistas sigma-1 utilizados fueron el BD-1063 (Tocris Cookson Ltd., Bristol, United Kingdom) y el S1RA (suministrado por Laboratorios Esteve, Barcelona, Spain), ambos considerados selectivos para los receptores sigma-1 (Sánchez-Fernández et al., 2017), y como agonista selectivo se usó el PRE-084 (Tocris Cookson) (Cobos et al., 2008). Los antagonistas opioides utilizados fueron el clorohidrato de naloxona y su análogo cuaternario naloxona metiodida, carente de penetrabilidad central (Menéndez et al., 2005) (ambos de Sigma-Aldrich). Para controlar la especificidad del efecto de los antagonistas opioides, se usó el antiinflamatorio no esteroideo (AINE) ibuprofeno (de Sigma-Aldrich), como estándar de un analgésico no opioide, y la morfina (suministrada

por Laboratorios Esteve, Barcelona, Spain), como prototipo de un analgésico opioide. El corticosteroide prednisolona (Sigma-Aldrich S.A., Madrid, España) fue usado como control de fármaco antiinflamatorio. Todos los fármacos se disolvieron en suero salino fisiológico. Para estudiar el efecto de los tratamientos sistémicos, se administraron los fármacos por vía subcutánea (s.c.) en un volumen de 5 ml/kg en la zona interescapular. Para el estudio de los efectos locales de los fármacos, estos se administraron intraplantarmente (i.pl.) en un volumen de 20 µl.

Para bloquear el efecto de los péptidos opioides endógenos producidos durante el proceso inflamatorio, se inyectó i.pl. 20 µl del anticuerpo 3-E7 (MAB5276, Merck Millipore, Madrid, España), el cual reconoce la secuencia consenso *N*-terminal de la mayoría de los POEs (Rittner et al., 2001). Para inhibir la infiltración de neutrófilos en el foco inflamatorio, se administró por vía intraperitoneal (i.p.) el anticuerpo anti-Ly6G (BE0075-1, Bio X Cell, West Lebanon, NH, EE.UU.), en un volume de 10 mL/kg (Wang et al., 2012). Como control de los efectos de los anticuerpos, usamos suero salino e isotipos no reactivos: PP102 (Merck Millipore) como control de los efectos del 3-E7, y BE0089 (Bio X Cell) como control de los efectos del Ly6G.

2.4. Procedimientos generales para la evaluación comportamental y para los tratamientos con fármacos y anticuerpos

Lo primero que se hizo fue realizar un curso temporal de la hiperalgesia y el edema inducido por la administración de carragenina (usando los métodos descritos en los apartados 2.5, 2.6 y 2.7 de este capítulo). Para la evaluación del curso temporal de la hiperalgesia térmica y del desarrollo del edema, se realizó una medida basal antes de la inyección de carragenina o su solvente, y posteriormente se hicieron medidas repetidas cada hora hasta un máximo de 7 horas. Para el estudio del curso temporal de la hiperalgesia mecánica se usaron animales diferentes para cada tiempo evaluado, debido a que las medidas repetidas en este test sensibilizaban la respuesta del animal. Debido a los datos obtenidos en los cursos temporales, se escogió el tiempo de 3 h para la evaluación del edema y de la hiperalgesia mecánica y térmica en la inflamación aguda, aunque en algunos grupos se evaluó la hiperalgesia a los 5 días para el estudio

de la inflamación sostenida producida por carragenina (ver apartado "3. Resultados y Discusión" de este capítulo, para una información más detallada).

Los antagonistas sigma-1, el ibuprofeno y la morfina (o su solvente) se administraron por vía s.c. 30 min antes de su evaluación. Para evaluar el efecto de la asociación de estos fármacos con el agonistas sigma-1 o con los antagonistas opioides, estos se administraron por vía s.c. 5 min antes que los antagonistas sigma-1, el ibuprofeno o la morfina.

Para el estudio local del efecto producido por el bloqueo del receptor sigma-1, se inyectaron los antagonistas sigma-1 por vía i.pl. 30 min antes de su evaluación. Para estudiar el efecto de la administración local del PRE-084 y el 3-E7 (o sus controles) sobre el efecto antihiperalgesico producido por la administración s.c. de los antagonistas sigma-1, estos se inyectaron en la planta de la pata inmediatamente despúes de la administración sistémica del BD-1063 o S1RA (o el solvente). El anticuerpo Ly6G o su isotipo no reactivo, fueron administrados intraperitonealmente 24 h antes de la inducción de la inflamación.

Cada animal fue evaluado sólo en un solo test comportamental, y no fue incluido en más de un grupo de tratamiento.

2.5. Evaluación de la hiperalgesia mecánica

La estimulación mecánica se aplicó en la pata trasera derecha con un analgesímetro de presión (modelo 37215, Ugo-Basile, Varese, Italia) siguiendo el protocolo descrito previamente (Nakamura y Ferreira, 1988; Menéndez et al., 2005; Sánchez-Fernández et al., 2013), con leves modificaciones. Los ratones fueron suavemente inmovilizados, y se les aplicó un estímulo mecánico romo con una intensidad constante de 100 g sobre la superficie dorsal de la pata trasera, y se registró el tiempo de latencia de la respuesta de forcejeo. Cada animal se evaluó dos veces dejando un intervalo de 1 minuto entre estimulaciones, y el valor medio de las dos medidas se registró como la latencia de forcejeo del animal.

2.6. Evaluación de la hiperalgesia térmica

La hiperalgesia térmica se evaluó mediante el test de Hargreaves (Hargreaves et al., 1988) siguiendo el protocolo descrito previamente (Nieto et al., 2008), con leves modificaciones. Los ratones se habituaron durante 2 h en los habitáculos de evaluación. Estos habitáculos se colocaron sobre un suelo de vidrio atemperado a 30ºC. Después de la habituación, se registró el tiempo de retirada de la pata al ser estimulada con un haz de luz a 85mW/cm^2 (analgesímetro de calor de IITC, CA, USA). Cada ratón se evaluó tres veces, y se realizó el promedio de las medidas. Entre cada medida se dejó al menos 30 s, y se utilizó un tiempo de corte de 20 s en cada evaluación.

2.7. Evaluación del edema

El volumen de la pata trasera se midió con un pletismómetro (Ugo-Basile, Varese, Italia). La pata trasera se colocó en un sistema de vasos comunicantes rellenos con una solución conductora (hexametilentetramina 0,5%) y el volumen desplazado se midió con una resolución de 10 µl. El volumen del edema se determinó por la diferencia entre los valores obtenidos antes y después de la inyección i.pl. de carragenina o su solvente. Para minimizar las variaciones en las medidas dentro del mismo animal, antes de la primera medición se marcó con un rotulador la zona de la pata justo por debajo de donde acaba el pelo, y se usó como referencia para las mediciones posteriores. Para el estudio del efecto de los fármacos en el desarrollo del edema inflamatorio se siguieron dos pautas: 1) la administración 30 min antes de la evaluación, y 2) una administración preventiva 5 min antes de la inyección de carragenina. En todos los casos, los efectos de los fármacos (o del KO sigma-1) se evaluaron 3 horas tras la administración de carragenina.

2.8. Dolor inducido por formalina

El test de la formalina se realizó como se ha descrito previamente (Cendán et al., 2005 a y b). Se inyectó la solución de formalina (20 µl, al 2,5%) de manera i.pl. en la pata trasera derecha, utilizando para ello una microjeringa Hamilton con una aguja de calibre $30^{1/2}$. Inmediatamente después, el ratón se colocó en un cilindro de vidrio y se observó durante un período de 45 min (dividido en períodos de 5 min cada uno), midiéndose el tiempo de lamido o mordisqueo de la pata inyectada. La primera fase de dolor inducida por la formalina se registró durante los primeros 5 minutos, y la segunda fase entre los minutos 15 y 35 tras la inyección de la formalina. Las respuestas dolorosas en la segunda fase alcanzaron un pico máximo entre los 20 y 25 min, tiempo en el que se recogieron las muestras de pata para su posterior análisis mediante FACS, para determinar el contenido de células inmunes presentes en la pata.

2.9. Niveles de β-endorfina en la pata

Los niveles de β-endorfina se determinaron con un kit de inmunoensayo enzimático de fluorescencia (Phoenix Pharmaceuticals, Karlsruhe, Alemania). Pare ello, la pata fue cortada a la altura del tobillo y los huesos fueron extraidos, para utilizar sólo el tejido blando, el cual se homogeneizó en 1 ml de tampón RIPA que contiene 0,05% de inhibidores de proteasa (P8340, Sigma-Aldrich) y 0,1% de inhibidores de fosfatasa (P0044, Sigma-Aldrich), y se utilizó el protocolo recomendado en las instrucciones del fabricante.

2.10. FACS

Se extrajo el tejido blando de la planta de la pata y se digirió con colagenasa/ADNasa durante 1 h a 37ºC (1 mg/ml de colagenasa IV y 0,1% de ADNasa I, Worthington, Lakewood, NJ, EE.UU.). Después, las muestras se filtraron (tamaño de poro de 70 µm), y se incubaron durante 1 hora con anticuerpos que reconocen el marcador de células hematopoyéticas CD45 (14-0451-85, clon 30-F11; eBioscience, Vienna, Austria), el marcador de neutrófilos Ly6G (127602, clon 1A8 , Biolegend, Viena, Austria), y el

marcador de células mieloides CD11b (101243, clon M1/70; Biolegend, Vienna, Austria). La población de macrófagos/monocitos se determinó usando la siguiente combinación de marcadores: células CD45+CD11b+Ly6G-. Todos los anticuerpos se usaron a una concentración de 2 µg/ml. La viabilidad celular se determinó con DAPI (Sigma-Aldrich). Antes y después de la incubación con los anticuerpos, las células se lavaron tres veces en tampón para FACS (FCS al 1% en PBS), y finalmente las muestras se midieron con un citómetro de flujo BD LSRII (BD Biosciences, San José, CA, EUA) y los datos se analizaron con el software FlowJo (Treestar, Ashland, OR, EE.UU.). Las células sin marcaje de DAPI se eliminaron del análisis, para analizar sólo las células vivas.

2.11. PCR

Los neutrófilos y los macrófagos/monocitos purificados mediante FACs fueron depositados directamente en una solución de Trizol. Para extraer todo el ARN celular se utilizó el método de fenol-cloroformo. El ARN total se incubó con las enzimas necesarias para la transcripción inversa (iScript Reverse Transcription Supermix, BioRad, CA, EE.UU.). Se añadieron los cebadores a la mezcla de enzimas SYBR Green (iQ ™ SYBR® Green Supermix, BioRad), y se amplificó el transcrito que codifica para la POMC usando un Sistema a Tiempo Real (CFX Connect, Biorad). Las reacciones se realizaron durante 50 ciclos, y la especificidad se determinó mediante el análisis de la correspondiente curva de fusión. La determinación del tamaño de los productos de la PCR se determinó mediante una electroforesis en gel de agarosa al 2%. Los cebadores en sentido directo e inverso (diseñados usando el NCBI Primer Blast) para la POMC que usamos fueron: 5'-TAGCGGGAGAGAAAGCCGAG-3' y 5'TAGCGGGAGAGAAAGCCGAG-3' (tamaño esperado de banda de 165 pb).

2.12. Análisis estadístico

Cuando se compararon varias medias, el análisis estadístico se realizó con un análisis de la varianza de una o dos vías (ANOVA) dependiendo del experimento, seguido del test de Bonferroni, excepto en la Figura 18C donde se utilizó una prueba t de Student

de medidas independientes. Las ANOVA se realizaron con el programa SigmaPlot 12.0 (Systat Software Inc., San José, CA, EE.UU.).

3. Resultados y discusión

3.1. La inhibición del receptor sigma-1 revierte la hiperalgesia inflamatoria aguda: papel de los receptores sigma-1 periféricos

3.1.1. Curso temporal del edema y de la hiperalgesia mecánica y térmica inducida por carragenina

Después de la administración de la carragenina en ratones salvajes se realizó un curso temporal para el estudio del edema y de la hiperalgesia mecánica y térmica. En el estudio del edema, el volumen de la pata aumentó significativamente desde el primer tiempo evaluado (1 h), alcanzando un pico máximo a las 3 h y permaneciendo elevado durante todos los tiempos observados (hasta 7 h). Además, estos animales desarrollaron tanto hiperalgesia mecánica como térmica, las cuales fueron evidentes a las 2 h y alcanzando su máximo a las 3 h desde la administración de carragenina. Una vez alcanzado el máximo de la hiperalgesia mecánica, se mantuvo durante todo los tiempos evaluados (7 h); sin embargo, la hiperalgesia térmica desapareció gradualmente, y a las 6 h los animales mostraron una latencia de respuesta similar a los valores previos al tratamiento con carragenina. Los animales tratados con el solvente de la carragenina no manifestaron hiperalgesia mecánica ni térmica, y tampoco desarrollaron edema.

Por lo tanto, a pesar de que la carragenina produjo tanto hiperalgesia mecánica como térmica, sus cursos temporales fueron diferentes. Estas diferencias podrían ser debidas a que los mecanismos de producción de ambos tipos de hipersensibilidad sensorial difieren (Latremoliere y Woolf, 2009; Dubin y Patapoutian, 2010).

Dado que la hipersensibilidad tanto a los estímulos mecánicos como térmicos se desarrolló completamente a las 3 h, se usó este tiempo para evaluar el efecto del

bloqueo farmacológico (antagonistas sigma-1) o genético (KO) de los receptores sigma-1.

3.1.2. Efecto de la administración sistémica de los fármacos sigma-1 en la hiperalgesia inflamatoria y el dolor nociceptivo

La administración s.c. del BD-1063 o del S1RA revertió por completo la hiperalgesia mecánica y térmica producida por la inflamación. Sin embargo, la potencia de este efecto antihiperalgésico varió dependiendo tanto del fármaco usado, como del tipo de estimulación sensorial aplicada. El BD-1063 mostró una mayor potencia comparado con el S1RA en la reversión de ambos tipos de hiperalgesia. Dado que se ha descrito que el BD-1063 muestra una afinidad mayor por el receptor sigma-1 que el S1RA, esto podría explicar estas diferencias en la potencia antihiperalgésicas de ambos fármacos (Nieto et al., 2012). Ambos fueron más potentes revertiendo la hiperalgesia mecánica que la térmica, lo que podría indicar que el bloqueo del receptor sigma-1 no actúa por igual en las vías sensoriales para ambos tipos de estímulo.

Las dosis de los antagonistas sigma-1 capaces de revertir por completo la hiperalgesia mecánica y térmica, no mostraton ningún efecto sobre los animales sin inflamación. La ausencia de efecto de los antagonistas sigma-1 en el dolor nocieptivo son congruentes con estudios previos (Entrena et al., 2009a and b; Nieto et al., 2012; Romero et al., 2012). El agonista PRE-084 no modificó la respuesta al estímulo mecánico o térmico en animales con o sin inflamación, pero sí fue capaz de revertir de manera dosis dependiente los efectos antihiperalgésicos producidos por los antagonistas sigma-1, lo cual sugiere que los receptores sigma-1 median los efectos antihiperalgésicos inducidos por el BD-1063 y el S1RA, tanto frente al estímulo mecánico como térmico.

A diferencia de los ratones salvajes, los KO sigma-1 no desarrollaron hiperalgesia mecánica, lo cual concuerda con los resultados obtenidos al administrar los antagonistas sigma-1. Sin embargo, en la hiperalgesia térmica encontramos discrepancias entre los efectos inducidos por los antagonistas sigma-1 y los observados en los ratones KO sigma-1, puesto que los mutantes sí desarrollaron hiperalgesia térmica y en un grado similar al de los ratones salvajes. El patrón de respuesta de los

ratones salvajes y KO sigma-1 de este estudio es muy similar al descrito en el dolor neuropático, ya que los ratones KO sigma-1 no desarrollan hipersensibilidad frente a estímulos mecánicos (de la Puente et al., 2009; Nieto et al., 2012), mientras que mostraban la misma hiperalgesia térmica que los ratones salvajes (de la Puente et al., 2009). Estos resultados encontrados tanto en dolor inflamatorio como en dolor neuropático, ponen de manifiesto la diferencia existente entre el bloqueo genético o farmacológico del receptor sigma-1 en la hiperalgesia térmica. Además, se ha descrito que el bloqueo farmacológico del receptor sigma-1 es capaz de potenciar el efecto de la morfina y de otros agonistas opioides en el dolor nociceptivo inducido por estímulos térmicos y mecánicos, sin embargo los KO sigma-1 muestran una clara potenciación del efecto de los opioides en el dolor nociceptivo mecánico pero no cuando el estímulo es de origen térmico (Sánchez-Fernández et al., 2013 y 2014; Vidal-Torres et al., 2013). Estos resultados sugieren de manera indirecta que en los ratones KO sigma-1 existen mecanismos compensatorios en las vías de dolor para los estímulos térmicos (y no para las de los estímulos mecánicos). Las diferencias entre los efectos de la inhibición genética y farmacológica que mostramos en este estudio, no son una característica única del receptor sigma-1, ya que se han descrito discrepancias similares en otros estudios de dolor enfocados a otras dianas (por ejemplo, Petrus et al., 2007, Bonin et al., 2011), o incluso en otros campos diferentes al estudio del dolor (por ejemplo, Guscott et al. 2005, Voss et al., 2010). Por lo tanto, debe tenerse sumo cuidado con las conclusiones obtenidas de los estudios en los cuales la única herramienta disponible para estudiar una posible diana terapéutica es el uso de un ratón mutante. La discrepancia entre el desarrollo de hiperalgesia térmica y mecánica en los ratones KO sigma-1 sugiere nuevamente que la modulación de ambos tipos de hiperalgesia por los receptores sigma-1 no es absolutamente idéntica. Independientemente de la naturaleza de los posibles mecanismos adaptativos responsables de estas discrepancias entre el desarrollo de hiperalgesia térmica y mecánica en los ratones KO sigma-1, estos ratones mutantes pueden usarse para evaluar la especificidad de los fármacos, comprobando si aún muestran actividad cuando su diana farmacológica está ausente (Petrus et al., 2007; Vidal-Torres et al., 2013, González-Cano et al., 2013). Nuestros resultados indican que las dosis de los antagonistas sigma-1, BD-1063 y S1RA, que abolían por completo la hiperalgesia térmica en ratones salvajes, no produjeron

ningún efecto antihiperalgésico en los animales KO sigma-1, lo que sugiere que ambos antagonistas sigma-1 ejercen su efecto mediante sus acciones en el receptor sigma-1.

3.1.3. Efecto de la administración local de los fármacos selectivos sigma-1 en la hiperalgesia inflamatoria y en el dolor nociceptivo

Para evaluar si los receptores sigma-1 son capaces de modular la hiperalgesia inflamatoria actuando en la periferia, administramos los fármacos sigma-1 por vía i.pl..

La administración i.pl. del PRE-084 en la pata con o sin inflamación, no tuvo ningún efecto en la hiperalgesia mecánica ni térmica. Sin embargo, cuando se adminsitró en la pata inflamada fue capaz de revertir el efecto antihiperalgesico de los antagonistas sigma-1 BD-1063 y S1RA adminsitrados de manera sistémica. Para descartar un posible efecto sistémico del PRE-084, este se inyectó en la pata contralateral a la inflamación, y no modificó el efecto del BD-1063 ni del S1RA. Estos resultados demuestran que el agonista PRE-084 está actuando únicamente en el lugar de la inyección.

Para estudiar los efectos del bloqueo farmacológico del receptor sigma-1 en la periferia, se inyectaron los antagonistas BD-1063 y S1RA en la pata inflamada. Ambos antagonistas revirtieron de manera dosis dependiente la hiperalgesia mecánica. Este efecto local de los antagonistas sigma-1 fue revertido por la administración sistémica del agonista PRE-084. Sin embargo, cuando estos fármacos se administraron en la pata contralateral a la inflamación, no modificaron la hiperalgesia mecánica de los animales, lo que indica que los efectos de estos fármacos se producían en lugar de la administración.

En la hiperalgesia térmica, la administración local del S1RA obtuvo los mismos resultados que el descrito anteriormente para la hiperalgesia mecánica. Además, no modificó la hiperalgesia térmica de los ratones KO sigma-1 con inflamación, lo que refuerza la especificidad de su acción. Por el contrario, el BD-1063 no se comportó de la misma manera ya que no pudo revertir la hiperalgesia térmica de los animales salvajes inyectados con carragenina. Sorprendentemente, este fármaco incluso provocó hiperalgesia térmica en los animales sin inflamación cuando se inyecto en la

pata. Este efecto doloroso producido por el BD-1063 también se observó en los ratones KO sigma-1, lo cual indica que no se debe a un efecto sobre el receptor sigma-1, sino a otro mecanismo ajeno a él. A pesar de que el BD-1063 es antagonista sigma-1 estándar, su selectividad ha sido descrita en base a una batería de tan sólo 11 dianas diferentes (Matsumoto et al., 1995 and 2001). Por el contrario, el S1RA ha probado su selectividad frente a más de 170 dianas diferentes (Romero, et al., 2012), y como describimos en los resultados comentados anteriormente, sus efectos revierten con el agonista sigma-1 PRE-084 y están ausentes en el ratón KO sigma-1. Por lo tanto, estos resultados en su conjunto sugieren que los efectos del BD-1063 podrían deberse a acciones inespecíficas ajenas a su interacción con el receptor sigma-1, mientas que los efectos del S1RA parecen ser selectivos mediante su acción sobre estos receptores.

La importancia de nuestros resultados sobre el papel de los receptores sigma-1 en la hiperalgesia inflamatoria subyace en que todos los estudios previos sobre la función de estos receptores en el dolor tónico/crónico se han atribuido a sus efectos a la modulación de la sensibilización central (Cendán et al., 2005a y b, Entrena et al., 2009a y b, Kim et al., 2006, Roh et al., 2008 y 2011, de la Puente et al. , 2009), y el posible papel de los receptores sigma-1 periféricos en los procesos de sensibilización ha pasado desapercibido. La presencia de estos receptores en el DRG (Sánchez-Fernández et al., 2014, Bangaru et al., 2013), y con una densidad mucho mayor que en diversas áreas centrales relacionadas con el dolor, incluyendo el asta dorsal de la médula espinal (Sánchez-Fernández et al., 2014), apoyan la importancia del papel de los receptores sigma-1 periféricos en el procesamiento del dolor. La activación (fosforilación) de las quinasas relacionadas con la señalización extracelular (ERK, de sus siglas en inglés) es un proceso clave en la sensibilización de las vías del dolor, tanto a nivel central como periférico (Latremoliere y Woolf, 2009; Ji, 2004) y se ha descrito que el aumento de la ERK1/2 fosforilada (pERK1/2) en el asta dorsal de la médula espinal durante el dolor neuropático podía ser atenuado por la inhibición del receptor sigma-1 (de la Puente et al., 2009; Nieto et al., 2012). Sin embargo, no observamos ningún aumento aparente en los niveles de la pERK1/2 en el asta dorsal de la médula espinal o en el DRG tras la administración de carragenina. Esto no sólo indica que hay mecanismos diferentes que intervienen en la hipersensibilidad sensorial entre los

modelos de dolor inflamatorio y neuropático, como se ha mencionado anteriormente (véase Scholz y Woolf, 2002; Ji, 2004; Patapoutian et al., 2009; Latremoliere y Woolf, 2009), sino que también los mecanismos implicados en la modulación de estos tipos de dolor por los receptores sigma-1 también podrían ser diferentes, lo cual merece un estudio con mayor profundidad.

3.1.4. Efecto de la inhibición del receptor sigma-1 en el edema inflamatorio

Estudiamos si la inhibición del receptor sigma-1 era capaz de disminuir el edema inflamatorio. Como indicador de la formación del edema, medimos en incremento del volumen de la pata inyectada con carragenina (o su solvente).

La administración intraplantar del solvente de la carragenina no aumentó el volumen de la pata ni en ratones salvajes ni en los KO sigma-1. Por el contrario, la carragenina produjo un marcado edema en la pata inyectada de los ratones salvajes, y esto no se redujo significativamente en los ratones mutantes. El bloqueo farmacológico del receptor sigma-1 por el BD-1063 o S1RA en ratones salvajes tampoco disminuyó el edema inducido por la carragenina, independientemente de si los fármacos se administraron de manera preventiva (5 min antes de la inyección i.pl. de carragenina) o después de la inducción de la inflamación (30 min antes de la evaluación). Sin embargo, el tratamiento preventivo con la prednisolona (10 mg/kg, s.c.), usado como control de los efectos de un fármaco con actividad antiinflamatoria conocida, abolió completamente el edema inducido por carragenina. Puesto que la inhibición del receptor sigma-1 no revirtió el edema inducido por la carragenina, los efectos antihiperalgesicos observados por la inhibición de este receptor no se explican por un efecto antiedematoso, y sugieren que la inhibición sigma-1 en el foco inflamatorio influye en el procesamiento del dolor y no en la inflamación.

3.2. El mecanismo: los receptores sigma-1 controlan la analgesia opioide periférica de origen inmune durante la inflamación

3.2.1. El efecto del antagonismo sigma-1 en la hiperalgesia inflamatoria aguda es sensible al antagonismo opioide periférico

El efecto antihiperalgesico del BD-1063 y del S1RA, fue revertido por la administración de los antagonistas opioides naloxona y naloxona metiodida. Para comprobar la especificidad de los efectos inducidos por estos dos antagonistas opioides, estudiamos la asociación de naloxona y naloxona metiodida al opioide prototipo morfina y al analgésico sin actividad opioide ibuprofeno. Los antagonistas opioides fueron capaces de revertir el efecto de la morfina, pero no el del ibuprofeno, lo que confirma que naloxona y naloxona metiodida no revierten el efecto de los fármacos que no poseen acción opioide.

La naloxona es un antagonista opioide que ejerce su acción tanto a nivel central como periférico, ya que es capaz de atravesar la barrera hematoencefálica, cosa que no puede hacer la naloxona metiodida, por lo que el efecto de esta última se limita sólo a la periferia (Menéndez et al., 2005). Puesto que el efecto de los antagonistas sigma-1 administrados por vía sistémica fue revertido completamente por la administración de naloxona metiodida, esto demuestra la importancia del papel periférico de los receptores sigma-1 en el dolor inflamatorio. De hecho, la administración del antagonista sigma-1 S1RA en el foco inflamatorio revirtió por completo la hiperalgesia mecánica y térmica, y este efecto pudo ser abolido tanto por la administración del agonista sigma-1 PRE-084, como por el antagonista opioide periférico naloxona metiodida.

Para determinar si la inhibición de los efectos antihiperalgésicos de los antagonistas sigma-1 por el PRE-084 o por la naloxona metiodida es de naturaleza competitiva o no competitiva, se realizó una curva dosis respuesta de los efectos del BD-1063 y del S1RA en presencia de dosis constantes de PRE-084 o naloxona metiodida (Rang et al., 2007). La asociación PRE-084 con el BD-1063 o con el S1RA indujo un desplazamiento hacia la

derecha de la curva dosis-respuesta de los antagonistas sigma-1, sin alterar su efecto máximo. Por el contrario la asociación de la naloxona metiodida indujo una disminución marcada del techo de acción de los antagonistas sigma-1 a pesar de incrementar las dosis de estos últimos. Estos resultados indican que hay una interacción competitiva entre el PRE-084 y los antagonistas sigma-1, y una interacción no competitiva entre estos antagonistas sigma-1 y la naloxona metiodida.

A pesar de que la naloxona metiodida es capaz de abolir el efecto antihiperalgesico del BD-1063 y del S1RA, esta no se une al receptor sigma-1 (Sánchez-Fernández et al., 2014), y por otro lado, estos los antagonistas sigma-1 no poseen afinidad por los receptores opioides (Romero et al., 2012; Sánchez-Fernández et al., 2013). Sin embargo, el receptor sigma-1 se puede unir al receptor opioide formando un complejo macromolecular, produciendo una inhibición tónica de su función, y la inhibición del receptor sigma-1 se traduce en un incremento de la señalización y analgesia opioide (Kim et al., 2010; Rodríguez-Muñoz et al., 2015). Por lo tanto, una posible explicación a la reversión del efecto de los antagonistas sigma-1 por la naloxona metiodida, es que el antagonista opioide esté inhibiendo la acción de los receptores opioides periféricos, impidiendo así la acción de los agonistas opioides endógenos (cuya acción es potenciada por el antagonismo del receptor sigma-1) producidos en el foco inflamatorio. Esta hipótesis implica necesariamente que durante la inflamación se debe producir un incremento en la producción de POEs en el foco inflamatorio, y que estos sean susceptibles de ser modulados por los receptores sigma-1.

3.2.2. El antagonismo sigma-1 y los péptidos opioides endógenos durante la inflamación aguda

Se investigó la acción de los POEs sobre el efecto antihiperalgesico de los antagonistas sigma-1. Este estudio se realizó mediante la administración de un anticuerpo monoclonal (clon 3-E7) en el foco inflamatorio, el cual neutraliza la acción de los POEs al reconocer la secuencia pan-opioide (Tyr-Gly-Gly-Phe de la región *N*-terminal) presente en la mayoría de los POEs (Rittner et al., 2001). La inyección local de este anticuerpo no modificó el tiempo de la latencia de respuesta en animales con o sin

inflamación, sin embargo, eliminó el efecto antihiperalgésico de los antagonistas sigma-1 tanto frente al estímulo mecánico como térmico. Como control se usó un isotipo no reactivo del anticuerpo 3-E7, el cual no indujo ningún cambio en la respuesta de los animales con o sin inflamación, ni revirtió el efecto antihiperalgesico de los antagonistas sigma-1. Este efecto del anticuerpo 3-E7 sólo tuvo lugar a nivel local, pues cuando se administró en la pata contralateral a la inflamación, los antagonistas sigma-1 siguieron conservando su efecto antihiperalgesico en la pata inflamada. Estos resultados ponen de manifiesto que los efectos antihiperalgésicos de los antagonistas sigma-1 son debidos a la acción de los POEs presentes en el foco inflamatorio.

Nuestros datos muestran por primera vez que la inhibición tónica de la analgesia opioide periférica por los receptores sigma-1 es producida fisiológicamente durante un proceso inflamatorio, limitando la capacidad de los POEs para producir analgesia opioide endógena, y facilitando de esta manera el dolor inflamatorio.

3.2.3. Neutrófilos y péptidos opioides endógenos

Quisimos buscar la fuente responsable de sintetizar los POEs causantes del efecto antihiperalgesico inducido por el bloqueo del receptor sigma-1. Se sabe que las células inmunes se acumulan en el foco inflamatorio, y que son capaces de producir y liberar POEs (Rittner and Stein, 2005; Hua and Cabot, 2010). Mediante FACS, usando marcadores específicos, determinamos que a las 3 h desde la administración de la carragenina, la mayoría de las células hematopoyéticas eran neutrófilos (aproximadamente un 70% de las células hematopoyéticas), aunque también se encontraron macrófagos/monocitos, pero en menor cantidad (en torno al 10%). Puesto que la mayoría de las células inmunes eran neutrófilos, y que además se sabe que estos producen POEs (Sahbaie et al., 2012), determinamos si los neutrófilos en nuestras condiciones eran capaces de producir β-endorfina. Determinamos la presencia del ARNm que codifica para la pro-opiomelanocortina (POMC) en neutrófilos purificados mediante FACS, que es el precursor de la β-endorfina (Hua and Cabot, 2016). La proteína Ly6G es exclusiva de neutrófilos, y es crucial para la migración y

reclutamiento de estas células (Wang et al., 2012). Esta proteína puede ser bloqueada *in vivo* por la administración sistémica de un anticuerpo, el anti-Ly6G (Wang et al., 2012). En este estudio mostramos que la administración del anti-Ly6G es capaz de inhibir de manera dosis dependiente el número de neutrófilos en el foco inflamatorio hasta su completa eliminación, sin variar el número de macrófagos/monocitos. Por el contrario, el isotipo del Ly6G no modificó los niveles de las células inmunes. Se observó, que los animales con inflamación tenían concentraciones más altas de β-endorfina en el lugar de la inflamación; y que la administración del anti-Ly6G redujo los niveles de este POE de manera dosis dependiente, mientras que los animales tratados con su isotipo no manifestaron ningún cambio en los niveles de β-endorfina en la pata. Estos resultados vinculan la presencia de neutrófilos al contenido en β-endorfina en el foco inflamatorio durante la inflamación aguda. Se cree que la β-endorfina es el POE de producción inmune más relevante (Hua and Cabot, 2010), fue esto por lo que evaluamos los niveles de este POE concreto en nuestras condiciones experimentales. Sin embargo, los leucocitos también son capaces de producir encefalinas y dinorfinas (Rittner and Stein, 2005), los cuales también podrían jugar un papel relevante en nuestros resultados. A raíz de estos datos, pensamos que el efecto antihiperalgesico de los antagonistas sigma-1 (sensible a la naloxona) podría ser debido a los POEs sintetizados por los neutrófilos durante la inflamación aguda.

3.2.4. Neutrófilos, edema, hiperalgesia y receptores sigma-1

Aunque los neutrófilos (y otras células inmunes) producen POEs, y estos pudieran tener cierto potencial analgésico durante la inflamación (Hua and Cabot, 2010), estas células también son capaces de producir dolor debido a la síntesis y liberación de algógenos químicos (Ji et al., 2014), además de participar en el desarrollo del edema (Wang et al., 2014). De hecho, el edema es capaz de incrementar la presión de las terminaciones de las fibras nociceptivas, lo cual también promueve el dolor inflamatorio (Julius y Basbaum, 2001). Por ello, estudiamos el efecto de los neutrófilos sobre la hiperalgesia y el edema. La administración del anti-Ly6G (que como se describe en la subsección anterior, impide la infiltración neutrofílica), fue capaz de revertir el edema de manera dosis dependiente, aunque sólo parcialmente y a dosis

altas, mientras que su isotipo no tuvo ningún efecto sobre el volumen de la pata. Estas dosis altas que revirtieron parcialmente el edema, lograron abolir la hiperalgesia mecánica inducida por carragenina, pero no se observó ninguna disminución en la hiperalgesia térmica.

Las neuronas sensoriales periféricas están especializadas en la detección de estímulos específicos, por lo que los mecanismos de nocicepción para estímulos térmicos y mecánicos no son idénticos (Juslius y Basbaum, 2001). Nuestros datos sugieren que los neutrófilos pueden participar en el desarrollo de la hiperalgesia mecánica mediante la formación del edema, con el consiguiente aumento de la estimulación de los nociceptores sensibles a la presión; sin embargo, estas células inmunes son prescindibles para el desarrollo de hiperalgesia térmica, que debe ser inducida por algógenos químicos producidos por otras fuentes.

Posteriormente, estudiamos el papel de los neutrófilos sobre el efecto antihiperalgesico de los antagonistas sigma-1 durante la inflamación aguda inducida por carragenina. Para ello se usó una dosis intermedia de Ly6G que reducía de manera significativa el número de neutrófilos, pero que no modificaba el edema ni la respuesta comportamental en animales con y sin inflamación frente a estímulos mecánicos y térmicos. Esta dosis de anti-Ly6G fue capaz de eliminar los efectos antihiperalgésicos del BD-1063 y del S1RA tanto frente a estímulos térmicos como mecánicos, mientras que dosis altas de su control de isotipo no alteraron las respuestas frente a estas estimulaciones sensoriales. Estos datos en conjunto, muestran que los efectos antihiperalgésicos sensibles a naloxona inducidos por los antagonistas sigma-1 durante la inflamación aguda, requieren de la presencia de POEs producidos por los neutrófilos (que constituyen la mayor parte del infiltrado inmune) en el foco inflamatorio.

Aunque no estudiamos el subtipo de receptor opioide implicado, se sabe que los receptores sigma-1 pueden modular la analgesia producida por los agonistas μ, δ o κ (revisado por Sánchez-Fernández et al., 2017). Teniendo en cuenta que las células inmunes producen una amplia variedad de POEs (Rittner et al., 2001; Brack et al., 2004; Labuz et al 2006; Sauer et al., 2014; Wang et al. 2014) con afinidades diferentes por los distintos tipos de receptores opioides (Rónai et al., 2009; Pasternak and Pan, 2013; Liu et al., 2017), es probable que haya subtipos diferentes de receptores

opioides implicados en los efectos antihiperalgésicos de los antagonistas sigma-1. Por todo ello, podríamos especular que la mayor potencia de los antagonistas sigma-1 observada en la reversión de la hiperalgesia mecánica frente a la térmica, pudiera estar determinada por las diferencias previamente descritas en la expresión de los distintos subtipos de receptores opioides en las aferentes primarias sensibles a estímulos mecánicos y térmicos (Scherrer et al., 2009), y a su interacción con sus respectivos ligandos peptídicos.

3.2.5. El efecto de los antagonistas sigma-1 en la inflamación sostenida es sensible a naloxona

Puesto que los tipos de células inmunes predominantes varían con la evolución de la inflamación (Rittner et al., 2001), se buscó determinar si los efectos antihiperalgésicos de los antagonistas sigma-1 sensibles a la naloxona se mantenía cuando las células mieloides predominantes durante la inflamación eran diferentes a los neutrófilos. Determinamos que cinco días después de la administración de carragenina, la presencia de los neutrófilos en el foco inflamatorio era prácticamente nula, mientras que los niveles de macrófagos/monocitos aumentaron considerablemente, siendo en esta situación los más abundantes (sobre el 70% de las células hematopoyéticas). Al igual que ocurría en los neutrófilos, en macrófagos/monocitos también encontramos ARNm que codifica para la POMC. Estos datos son congruentes con los estudios previos que indican que todas las subpoblaciones de células inmunes son capaces de producir POEs (Rittner and Stein, 2005), y que la fuente principal de estos péptidos en los diferentes estadíos de inflamación varía dependiendo de los distintos linajes leucocitarios predominantes en cada momento (Rittner et al., 2001). Bajo estas condiciones de inflamación sostenida, los animales manifestaron una hiperalgesia mecánica marcada que pudo ser revertida por el BD-1063 y el S1RA. Este efecto de los antagonistas sigma-1 pudo ser revertido a su vez por la administración del PRE-084 y por el antagonista opioide periférico naloxona metiodida. Estos datos indican que tanto los receptores sigma-1 como los receptores opioides periféricos están implicados en el efecto antihiperalgésico del bloqueo sigma-1 durante la inflamación sostenida, y

demuestran que la modulación de la analgesia opioide periférica de origen inmune por los receptores sigma-1 no está limitada a la inflamación neutrofílica.

3.2.6. Efecto del antagonismo sigma-1 en el dolor inducido por formalina

También evaluamos si el efecto antihiperalgésico del antagonismo sigma-1 en otros modelos de dolor es mediado, o no, por la acción de los POEs de origen inmune. El S1RA disminuyó de manera dosis dependiente la segunda fase del dolor inducido por formalina, como ha sido descrito previamente para este y otros antagonistas sigma-1 (Zamanillo et al., 2013). Sin embargo, la administración de antagonistas opioides o del anti-Ly6G no modificó los efectos antinociceptivos del S1RA, y curiosamente, en el pico de máxima respuesta dolorosa (20-25 min), la formalina fue incapaz de reclutar neutrófilos en la pata inyectada. Estos resultados sugieren que el antagonismo sigma-1 necesita de la presencia de células inmunes que contengan POEs para poder producir su efecto dependiente de la activación opioide. Por lo tanto, teniendo en cuenta estos datos obtenidos en el modelo de dolor inducido por formalina, este no es el único mecanismo de acción de los antagonistas sigma-1 para aliviar el dolor. Nuestros resultados son congruentes con los estudios previos que muestran que el efecto de varios antagonistas sigma-1 no selectivos (haloperidol y sus metabolitos) en modelos comportamentales donde existe una sensibilización central prominente (como la segunda fase del dolor inducido por formalina o la hipersensibilidad mecánica secundaria inducida por capsaicina), no son revertidos por la naloxona (Cendán et al., 2005a, Entrena et al., 2009b).

El receptor sigma-1 es una chaperona regulada por ligandos que participa en la neurotransmisión del dolor a través de múltiples vías (Zamanillo et al., 2013). Aunque aquí hemos demostrado que el mecanismo de acción de este receptor durante la inflamación se produce a través de la modulación de los POEs liberados por las células inmunes en el foco inflamatorio, esta no es necesariamente la única forma en la que la inhibición del receptor sigma-1 produce analgesia en otros modelos. Se necesitan más

estudios para caracterizar los mecanismos implicados en los distintos tipos de dolor donde los antagonistas sigma-1 tienen efecto analgésico.

4. Conclusiones

4.1. Conclusiones específicas

1. Los receptores sigma-1 desempeñan un papel fundamental en la hiperalgesia inflamatoria, y el bloqueo de este receptor en el tejido inflamado es suficiente para eliminar por completo la hiperalgesia. Sin embargo, la inhibición del receptor sigma-1 no altera el edema inflamatorio, lo que sugiere que los receptores sigma-1 modulan el procesamiento del dolor y no la inflamación.

2. Los efectos antihiperalgésicos del antagonismo del receptor sigma-1 dependen de la actividad de los receptores opioides periféricos.

3. Las células inmunes son responsables (al menos en parte) de la hiperalgesia inflamatoria, y de la formación del edema durante la inflamación.

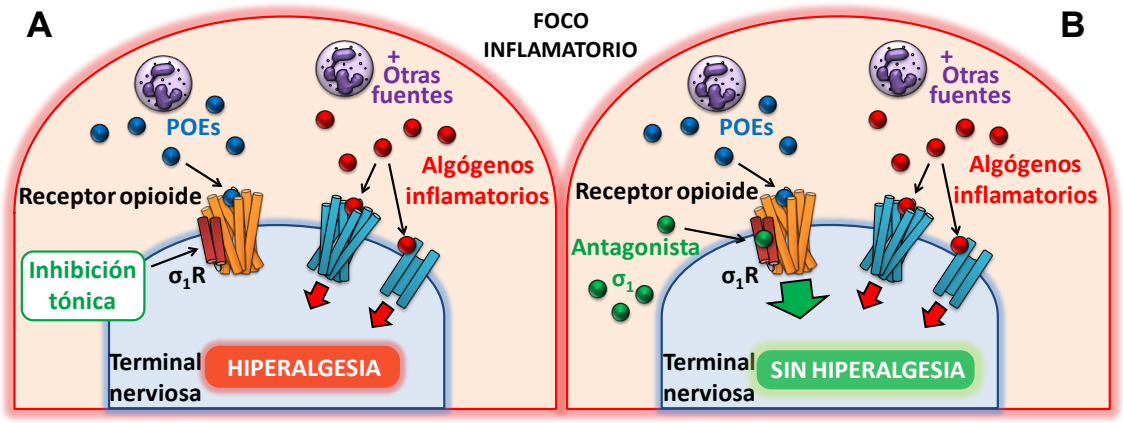
4. Las células inmunes presentes en el lugar de la inflamación producen péptidos opioides endógenos (β-endorfina).

5. Los efectos antihiperalgésicos del antagonismo del receptor sigma-1 dependen de la presencia de péptidos opioides endógenos producidos por las células inmunes presentes en el foco inflamatorio.

6. En los modelos de dolor en los que no se recluta un número significativo de células inmunes (como durante el dolor inducido por formalina), la inhibición del receptor sigma-1 alivia el dolor mediante mecanismos independientes de la modulación opioide.

4.2. Conclusión general

Los receptores sigma-1 periféricos constituyen un freno biológico a la analgesia opioide de origen inmune durante un proceso inflamatorio, donde las células inmunes y otras fuentes productoras de algógenos químicos producen dolor. Este freno biológico a la analgesia opioide puede ser eliminado farmacológicamente por los antagonistas sigma-1, los cuales promueven la analgesia opioide en el foco inflamatorio, mediante la desinhibición del efecto de los péptidos opioides endógenos de origen inmune. Este mecanismo (que se resume en la figura siguiente) maximiza el potencial analgésico de los leucocitos que se acumulan de forma natural en foco inflamatorio, siendo un mecanismo totalmente diferente al de los analgésicos convencionales. Los hallazgos de esta Tesis Doctoral sugieren que los antagonistas sigma-1 merecen un estudio más exhaustivo como analgésicos potenciales para el tratamiento del dolor inflamatorio.



Propuesta del mecanismo de acción del efecto del antagonismo sigma-1 en la hiperalgesia inflamatoria. (*A*) Las células inmunes (por ejemplo, los neutrófilos) que se infiltran en la pata inflamada (junto con otras fuentes) liberan algógenos químicos que sensibilizan los nociceptores, pero también son capaces de sintetizar péptidos opioides endógenos (POEs). Estos POEs de origen inmune no son capaces de revertir el dolor inflamatorio, debido a la inhibición tónica del funcionamiento opioide por los receptores sigma-1 (σ_1R). El equilibrio entre los efectos de los algógenos químicos y los POEs, está desplazado hacia la producción de la hiperalgesia característica de la inflamación. (*B*) Los antagonistas sigma-1 liberan a los receptores opioides de la inhibición tónica inducida por los receptores sigma-1, potenciando los efectos de los POEs de origen inmune y produciendo un efecto antihiperalgésico opioide durante la inflamación.

INTRODUCTION

1. PAIN AND PAIN DURING INFLAMMATION

1.1. Pain: Villain or Hero?

Pain is a disabling symptom of many diseases, and pain control is one of the most pursued therapeutic priorities. Pain is a subjective experience, difficult to define accurately even though we all know what it means. The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain has a dual purpose, since it can instruct us to avoid the current and future sources of potential tissue damage, but also after injury it facilitates the recovery of the injured tissue by promoting avoidance behaviors (Cobos and Portillo-Salido, 2013). Therefore, pain is not a villain itself but it could be considered to be a hero, as this highly conserved evolutionary mechanism is aimed to help to the survival of the organism. However, when pain persists and becomes chronic, it loses its protective aim and can seriously affect the quality of life of patients.

Pain can be classified depend on its duration (acute or chronic), intensity (from low to high) and etiology (nociceptive, inflammatory or neuropathic) (Scholz and Woolf, 2002). As the aim of this PhD project is to study the role of sigma-1 receptors on inflammatory pain, we will briefly summarize in this section the pain pathways and then we will focus on the mechanisms of inflammatory pain.

1.2. The pain pathways: an overview

Normally, pain perception is produced only at pressures and temperatures high enough to injure (or potentially injure) tissues, as well as by toxic molecules and inflammatory mediators. These high threshold stimuli are detected by specialized peripheral sensory neurons which are called nociceptors (Dubin and Patapoutian, 2010). Therefore, the pathways that send the sensory information from the skin to supraspinal structures depend on the type of stimulus. Light mechanical stimulation stimulates low-threshold mechanoreceptors (Aβ-fibers) whereas noxious stimulus activates nociceptive fibers (C- and Aδ-fibers). Aβ-fibers have specialized endings to detect specific types of nonnoxious mechanical stimuli, and transmit the information at high speed due to their large myelin sheath around their axons (Hall, 2011a). In

contrast C- and Aδ-fibers have simply free endings (Gardner et al., 2000), and show a lower speed conduction than Aβ-fibers. Whereas Aδ-fibers have a thin myelin sheath around their axons C-fibers are unmyelinated, which makes them the slowest conducting fibers (Djouhri and Sawson, 2004; Woolf and Ma, 2007). Since Aδ-fibers conduct faster than C-fibers, drive the first pain sensation, whereas the longer latency of the second pain sensation is consistent with the activation of the slower conducting C-fibers (Schepers and Ringkamp, 2009). Some subsets of C- and Aδ-fibers can be exclusively activated by thermal or mechanical stimuli, whereas others can respond to both types of sensory stimuli. In most cases, if a nerve fiber is responsive to both heat and mechanical stimuli, it will be activated by chemical stimuli as well (Davis et al., 1993). In addition to the coding of noxious information, C-fibers also conduct the nonpainful thermal information (Peier et al., 2002; Benham et al., 2003). All this sensory specificity is possible thanks to the differential expression of specific transducers which can be activated by mechanical, thermal (heat or cold) or chemical stimuli (Gardner et al., 2000; Scholz and Woolf, 2002; Benham et al., 2003; Lewin and Moshourab, 2004; Hall, 2011a). Some of these transducers are shown in Figure 1.

The somas of all these primary afferents are located in the dorsal root ganglia (DRG), from where they project to the spinal cord dorsal horn. C- and Aδ-neurons project to the second order neurons at laminae I and II, while the Aβ-fibers synapse in deeper laminae (Rexed, 1952). The ascending pathways of nociceptive and nonnociceptive neurons differ, since whereas the nonnoxious mechanical stimulation reaches the thalamus through the dorsal columns, noxious stimuli reaches the thalamus by the anterolateral system (Willis, 2007; Hall, 2011a). The thalamus then sends the information to the somatosensory cortex which is involved in the processing of somatic sensory information (Purves et al., 2006; Hall, 2011b). In addition, several structures from the limbic system such as the amygdala and hypothalamus receive projections from the cerebral cortex and other areas, which add the emotional interpretation to the sensory information (e.g. unpleasantness in response to a noxious stimulus) (Chen et al., 2008).

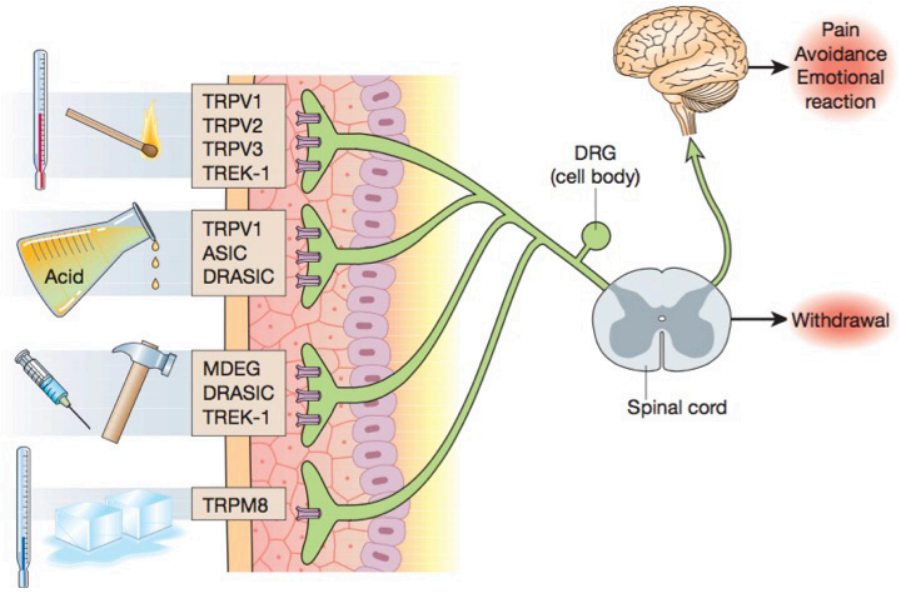


Figure 1. Activation of nociceptors and the ascending pain pathway. The different noxious stimuli (chemical, mechanical or thermal) are transformed into electrical signal by specific transducers. Then, this signal is conducted by nerve fibers to the dorsal root ganglion (DRG), where the somas of peripheral sensory neurons reside. DRG neurons then send projections to the spinal dorsal horn which in turn sends projections to supraspinal estructures (taken from Scholz and Woolf 2002).

Pain pathways are not a one direction road to supraspinal structures, since there is a descending system able to modulate pain transmission. The first reports on a central modulation of pain were made by Melzack R and Wall P (Melzack and Wall, 1965). They found that stimulation of low-threshold mechanoreceptors was able to activate inhibitory interneurons at the dorsal horn to inhibit the input of nociceptive activation. This theory was named as the gate control theory, since these interneurons acted as a "gate" allowing or not pain transmission. This initial model suffered modifications to include facilitatory interneurons which also play a pivotal role on pain modulation (Woolf, 2011; Pereira and Lerner, 2017). This "gate" was much later shown to be modulated by descending supraspinal fibers which synapse with the modulatory interneurons and hence controlling the nociceptive input. The descending pathway of pain is 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). Cerebral cortex projects to amygdala and other structures of the limbic system, which in turn send projections to three brainstem areas: the periacueductal 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 norepinephrine and serotonin, which inhibit or decrease painful ascending information at the dorsal horn (Heinricher et al., 1994; Benarroch, 2008; Ossipov et al., 2010) (Figure 2).

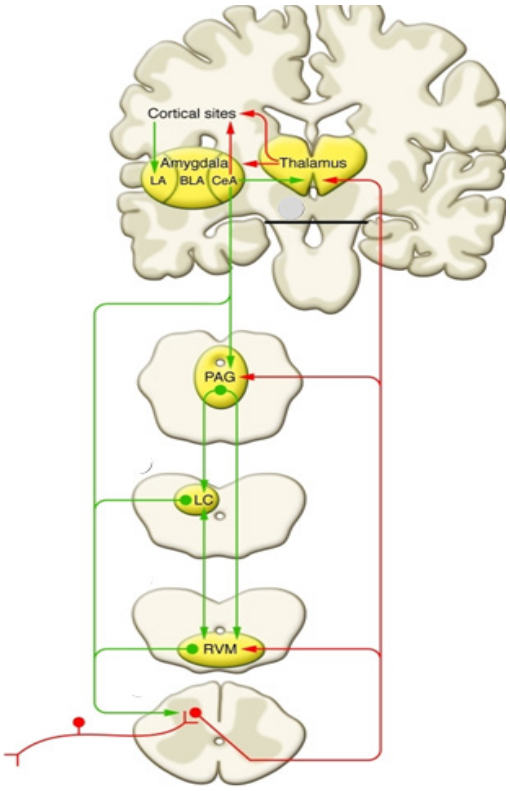


Figure 2. The descending modulatory pain pathways. Limbic system structures receive projections from the cerebral cortex and synapse with the periaqueductal gray (PAG), locus ceruleous (LC) and the rostral ventromedial medulla (RVM), located at the brainstem. These brainstem areas project to the spinal cord releasing inhibitory neurotrasnmitters to inhibit the conduction of the painful information (taken from Ossipov et al., 2010, with modifications).

1.3. Inflammatory pain

Inflammatory pain is one of the major types of clinical pain (Scholz and Woolf 2002; Woolf 2004). Inflammation itself is a defensive mechanism aimed to the repair and healing of the damaged tissue. The inflammatory process involves a series of molecular, cellular and vascular phenomena in response to aggressions of different nature (e.g. infections or injury), and is characterized by the appearance of the cardinal signs of the inflammation. The first four signs were first established by Aulus Cornelius Celsus more than 2.000 years ago, and were: heat, redness, swelling, and pain (Lawrence et al., 2002). Much later loss of function, which is also related to pain (Cobos and Portillo-Salido, 2013), was added by Galen in the second century A.D. as the fifth cardinal sign. These cardinal signs are showed in Figure 3.

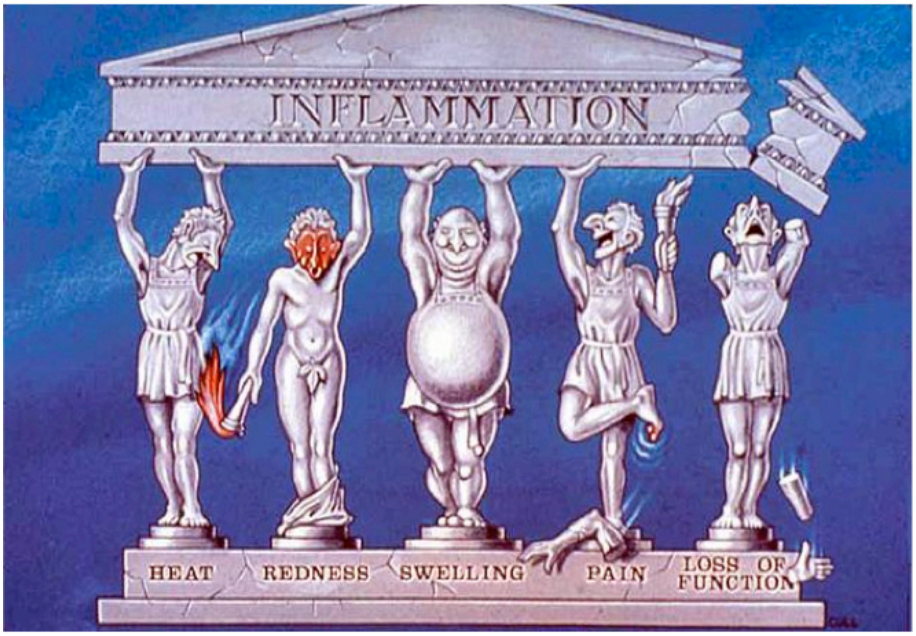


Figure 3. Cardinal sings of the inflammation. From left to right: heat, redness, swelling, pain and loss of function (taken from Lawrence, 2002).

As previously mentioned pain is part of the defensive mechanism of our organism in response to injury which aids to the healing of the damaged tissue. However, when inflammatory pain is too severe or when it becomes chronic as in nonresolving inflammation, it can seriously affect the quality of life of patients and needs to be treated (Woolf, 2004; Giacomelli et al., 2015). Inflammatory pain is characterized by a pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the milieu of inflammatory mediators released at the inflamed site that reduce the pain threshold. This leads to ongoing pain and hyperalgesia (Scholz and Woolf, 2002; Ji, 2004; Patapoutian et al., 2009; Latremoliere and Woolf, 2009). In addition to peripheral sensitization, inflammatory pain is also contributed by central sensitization (Woolf, 2011; Nieto et al., 2016; Meacham et al., 2017). However, it is thought that the peripheral component is more relevant than the central contribution to this particular type of pain, which is in contrast to the predominant central contribution over peripheral sensitization of other clinically relevant pain types, such as neuropathic pain (Phillips and Clauw, 2011).

During the inflammatory process, the injured cells and other resident cells release algogenic mediators which sensitize nociceptors. Resident and recruited immune cells

participate on the repair of the damaged tissue but also on inflammatory pain by releasing a variety of inflammatory mediators. See Figure 4 for a schematic representation of some of the most important algogenic chemicals released during inflammation. In this section we will briefly summarize all these processes occurring at the site of inflammation.

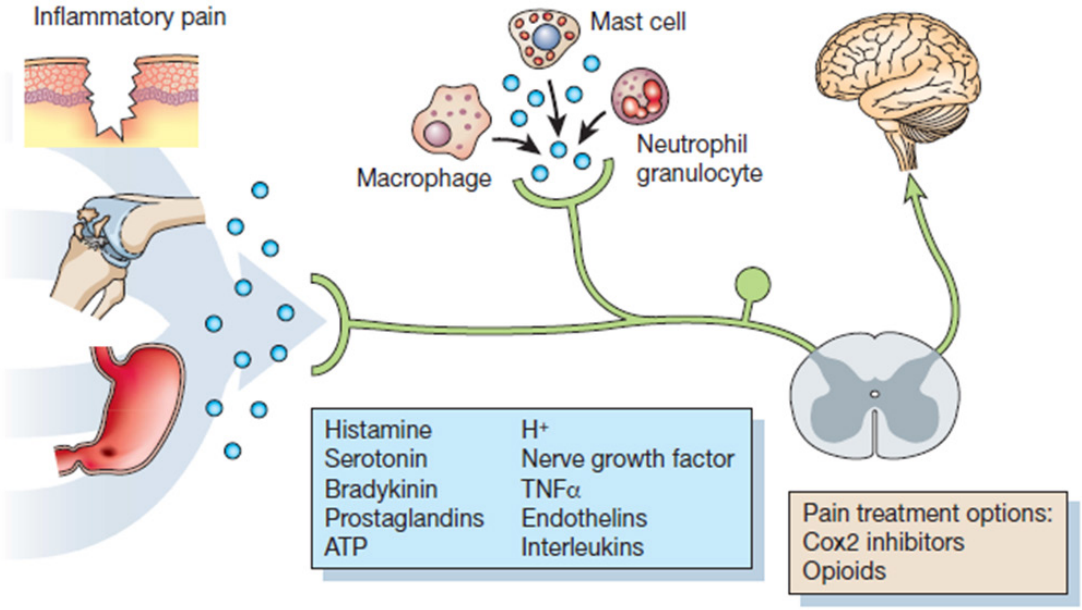


Figure 4. The algogenic chemicals produced during inflammation or tissue injury. Several algogenic chemicals are produced by tissue resident or recruited immune cells. These compounds have specific receptors in peripheral sensory neurons, which become sensitized (Scholz and Woolf 2002).

1.3.1. Role of nonimmune cells in inflammatory pain

One of the first signals which alert our nociceptive system of the tissue damage is the release of protons and ATP by injured cells (Millan, 1999; Moalem et al.; 2005). These signals activate distinct pathways on C-nociceptors. Protons induce the depolarization and sensitization of these nociceptive fibers by a local pH fall. The acidity of the inflammatory microenvironment is detected by chemoelectrical transducers that belong to two families of ion channels: transient receptor potential (TRP) channels, mainly the TRP vanilloid-1 (V1), and acid-sensing ion channels (ASICs), mainly ASIC3 (Deval et al., 2010). ATP released by the injured tissue exerts a pronociceptive action acting mainly on P2X3 receptors, inducing neuronal depolarization by a Ca^{2+}-dependent mechanism (King et al., 1997; Millan et al., 1999; Molliver et al., 2005).

Activated platelets also play an important role on peripheral sensitization, since they can release serotonin (5-HT) and histamine (Dray, 1995; Millan, 1999; Sommer, 2004) in the inflamed area. 5-HT acts on C-fibers by activating 5-HT_{2A} and 5-HT_3 receptors (Abbott et al., 1996; Tokunaga et al., 1998; Ernberg et al., 2000; Sommer, 2004). 5-HT_3 receptors are ligand-gated cation channels permeable to sodium, potassium and calcium leading to neuronal excitation (Thompson and Lummis, 2006). On the other hand, 5-HT_{2A} are G-protein coupled receptors (GPCRs), which activate Gαq signal transduction and thereby stimulating the phospholipase C (PLC)-protein kinase C (PKC) pathway (Abbott et al., 1996; Sommer, 2004; Fisher et al., 2010). Histamine can bind to H1 and H2 receptors which are present on nociceptive fibers (Pinho-Ribeiro et al., 2017). Both H1 and H2 receptors belong to the GPCR superfamily, but whereas H1 receptors are coupled to Gαq activating the PLC-PKC pathway (Brown et al., 2002; Lu et al., 2016), the H2 subtype is coupled to Gαs and thereby increasing intracellular cAMP concentration leading to the activation of protein kinase A (PKA) (Traiffor et al., 1992; Sakhalkar et al., 2005).

NGF is another of the main peripheral sensitizers. This neurotrophin acts by both neurotrophic tyrosine kinase receptor A (TrkA) and p75 neurotrophin receptor (p75NTR) pathways (Bannister et al., 2017). NGF levels increase in the inflamed tissue due to a large number of inflammatory mediators which promote its production in mast cells but also in neurons, epithelial, endothelial or connective tissue cells (Kidd and Urban, 2001; Minnone et al., 2017).

Bradykinin is one of the most powerful algogenic mediators (Dray and Perkins, 1993; Dray, 1997). This peptide is mainly produced in activated endothelial cells by the kinin-forming cascade (Joseph et al., 2002). Bradykinin activates two types of GPCRs termed B1 and B2 receptors. Although both types of B receptors participate on inflammatory hyperalgesia, it is though that the B1 subtype, which is largely upregulated after inflammation or injury (Chen and Johnson, 2007), plays a more important role on this than B2 receptors. Both B1 and B2 receptors are located in primary sensory neurons and signal through the PLC-PKC pathway (Burgess et al., 1989; Dray et al., 1992; Levine et al., 1993; Fischer et al., 2010). Moreover, bradykinin can induce the release of prostaglandins, cytokines and nitric oxide (NO) from the vascular endothelium,

neurons or immune cells, and all of them induce nociceptor sensitization (Dray and Perkins, 1997; Ma and Quirion, 2008; Fischer et al., 2010; Petho and Reeh, 2012). Endothelins also contribute to inflammatory pain. These peptides can be produced by a variety of cell types, including endothelial cells (Khodorova et al., 2009) and interact to ET_A receptors located in C- and Aδ-nociceptors which through PLC-PKC pathway promotes nociceptor sensitization (Khodorova et al., 2009).

In all these former cases, activation of kinases in peripheral sensory neurons induces the phosphorilation of cation channels, such as TRPV1, promoting Ca^{2+} influx which leads to neuronal sensitization/depolarization (Bhave et al., 2003; Mohapatra and Nau, 2005; Brackley et al., 2017).

In addition to the production of algogenic substances, endothelial cells have other prominent role on the development of inflammatory pain. Endothelial cells lining the vessel wall and are connected by cell-to-cell adherens, gap and tight junctions, which regulate vascular permeability (Wallez and Huber, 2008). During inflammation, in response to edemagenic agonists such as thrombin, prostaglandin E2, histamine, serotonin or bradykinin, these cell-to-cell junctions are disrupted, increasing the permeability of the vascular endothelium and promoting the formation of the inflammatory edema (Majno and Palade, 1961; Joris et al., 1972; Simionesu et al., 1978; Nagy et al., 2008; Ashina et al., 2015). In turn, the inflammatory edema exerts a mechanical pressure on nociceptors which also contributes to inflammatory pain (Julius and Basbaum, 2001).

In addition to the release of algogenic chemicals and playing a pivotal role on the formation of the inflammatory edema, endothelial cells are also responsible for the extravasation (also called diapedesis) of the immune cells to the inflamed site, which will be described below.

1.3.2. Immune cell extravasation

In the absence of lesions, most leukocytes are patrolling inside blood vessels, and a few of them are tissue resident leukocytes (such as mast cells and resident macrophages). During the inflammatory process, the number of white blood cells in the inflamed site highly increases and most of them arrive from the blood flow. In this process the activated vascular endothelium plays the major role. Activated endothelial cells express both chemokines and a number of adhesion proteins in their surface, which are recognized by patrolling leukocytes in the blood (Kolaczkowska and Kubes, 2013). Once leukocytes from the blood recognize these adhesion proteins, a complex process starts leading to leukocyte extravasation to the inflamed site. This process occurs in different steps: tethering, rolling, adhesion, crawling and transmigration through the wall of vessels (Kolaczkowska and Kubes, 2013) (see Figure 5).

Inflammatory mediators such as tumor necrosis factor (TNF), histamine, IL-1, IL-6, leukotrienes and others, which can be produced by activated tissue resident sentinel macrophages and mast cells (Italiani and Boraschi, 2014; Kolaczkowska and Kubes, 2013) trigger the expression of adhesion molecules in the endothelial cells (Watson et al., 1996; Brown et al., 2001; Granger and Senchenkova, 2010). These adhesion molecules include CAMs (cell adhesion molecules) and selectins (among others). These molecules bind to specific proteins expressed in the membrane of leukocytes, and are important for each of the steps of the process leading to leukocyte extravasation. Table 1 shows some of these molecules expressed in endothelial cells and leukocytes and their involvement on tethering, rolling, adhesion, crawling and transmigration.

The binding of the adhesion molecules expressed on endothelial cells to their partners on leukocytes leads to the tethering of free flowing leukocytes. This binding decreases leukocyte speed on the blood, causing them to roll through the endothelium wall. This rolling favors the binding of CAMs on the endothelial cells (which are the ligands of integrins) to the integrins on the leukocytes, leading to the adhesion endothelium-leukocyte till these cells are fully arrested. More CAMs and integrins are then recruited to conform the firm adhesion phase (Granger and Senchenkova, 2010; Kolaczkowska and Kubes, 2013;).

Step of leukocyte extravasation	Molecules on endothelium	Molecules on leukocyte
Tethering	P-selectin	PSGL-1
	E-selectin	PSGL-1, ESL1, CD44
	PSGL1, GLyCAM	L-selectin
Rolling	ICAM 1	LFA1 (β2-integrin)
	E-selectin	PSGL1, ESL1, CD44
Adhesion	ICAM-1	LFA1 (β2-integrin)
	VCAM-1	VLA4 (β2-integrin)
Crawling	ICAM-1	MAC1
Transmigration	ICAM-1,2	MAC1
	VCAM-1	VLA4
	PECAM-1	PECAM-1
	JAMA	LFA1
	JAMB	VLA4
	JAMC	MAC1

Table 1. Proteins involved in the different steps of the process leading to leukocyte extravasation. The table shows the main molecules expressed in endothelial cells and leukocytes involved in each step of leukocyte extravasation. Some of these molecules are expressed in both endothelial cells and leukocytes. See Ley et al., 2007; Kolaczkowska and kobes, 2013; Gerhardt and Ley, 2015 for references.

Apart from the proteins showed in Table 1, leukocytes have specific proteins important for the extravasation process. As an example, Ly6C is present in monocytes/macrophages but also in neutrophils, whereas the structurally related protein Ly6G is exclusively expressed in neutrophils. Both proteins have a close relationship with β2-integrins and play an important role in the adhesion phase of these leukocytes (Wang et al., 2012; Nourshargh and Alon, 2014). These molecules are that important for the adhesion of leukocytes, that their blockade by an antibody with affinity for both Ly6C and Ly6C abolish both macrophage and neutrophil extravasation to the inflamed tissue (Schumak et al., 2015), whereas selective blockade of Ly6G abolish selectively the neutrophil extravasation (Hickey, 2012; Wang et al., 2012). This is illustrated in Figure 5.

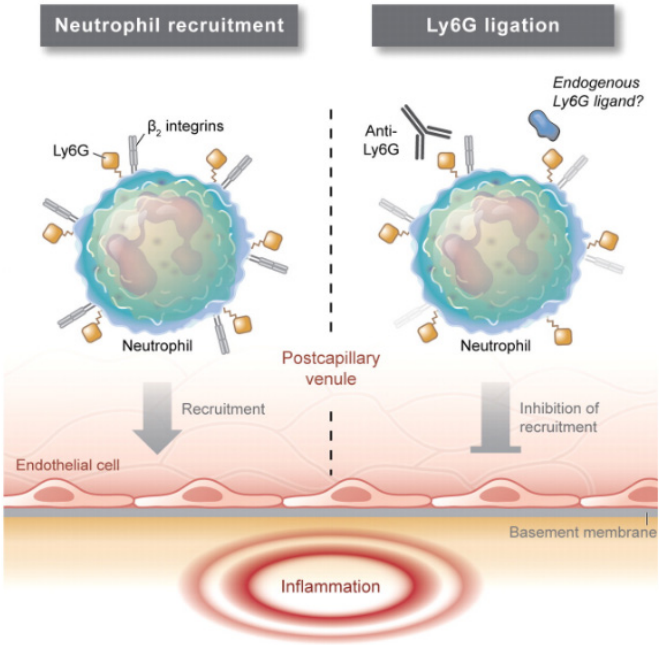


Figure 5. The blockade of Ly6G by an anti-Ly6G antibody blocks neutrophil recruitment. Both Ly6G and β2-integrins, are located close to each other and the blockade of Ly6G by a selective antibody prevents neutrophil recruitment (taken from Hickey, 2012).

Once the leukocytes are firmly adhered to the endothelium, the crawling phase begins. In this phase, leukocytes get ready for the transmigration process which is defined as the crossing of the leukocytes through the vascular endothelium to reach the damaged tissue (Kolaczkowska and Kubes, 2013; Nourshargh and Alon, 2014). The previously commented disruption of cell-to-cell junctions in endothelial cells by inflammatory mediators (see section above) plays a pivotal role on the transmigration process, as most immune cells cross the endothelium between endothelial cells (paracellular transmigration). In fact, leukocytes act in conjunction to the inflammatory mediators from other cell types to eliminate the cell-to-cell junctions between endothelial cells (Ley et al., 2007; Kolaczkowska and Kubes, 2013; Gerhardt and Ley, 2015). However, a small percentage of leukocytes can cross endothelial cells through them rather than between them (transcellular transmigration) (Sorokin, 2010; Kolaczkowska and Kubes, 2013). All processes leading to leukocyte extravasation are schematically summarized in Figure 6.

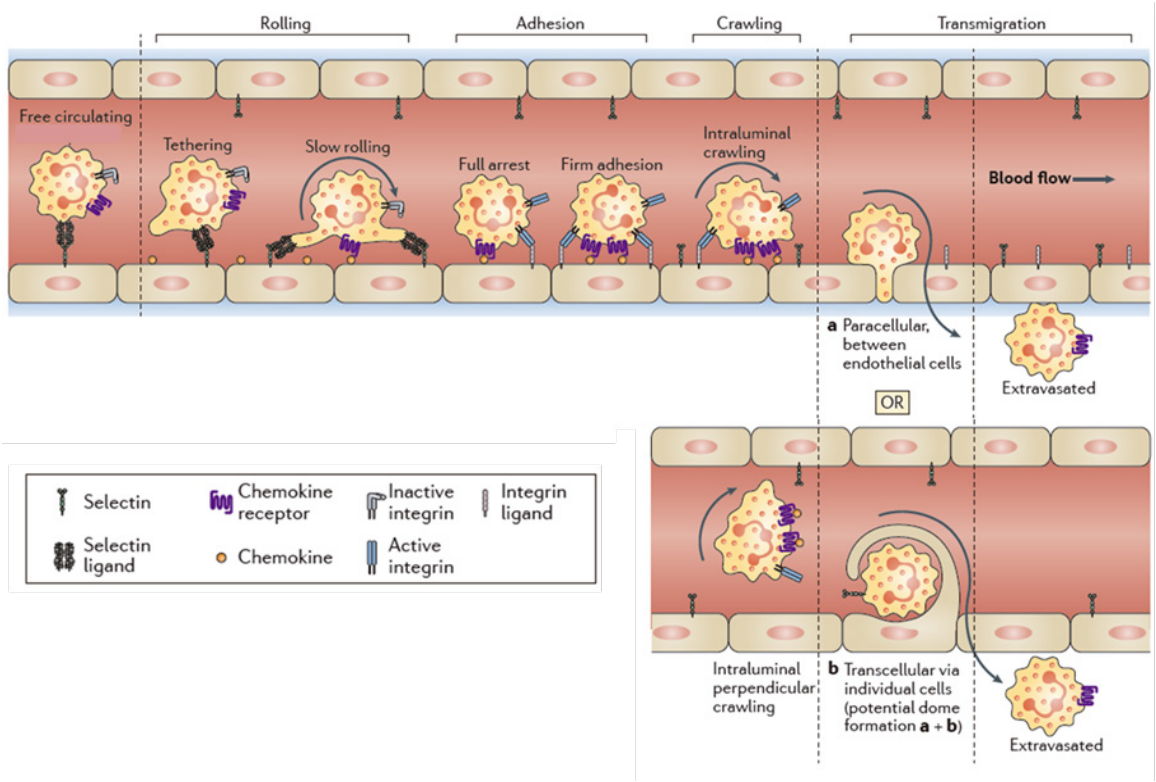


Figure 6. Schematic representation of leukocyte extravasation. This process begin with the tethering where the selectins and their ligands bind the leukocyte to the endothelium, decrease its circulating rate and roll it by the endothelium (rolling). This later step allows the binding of integrins to the endothelial cell which express integrin ligands, leading to the full arrest and firm adhesion of the leukocyte. Finally, after the intraluminal crawling the leukocyte transmigrates to the inflamed site (taken from Kolaczkowska and Kubes, 2013, with modifications).

Once the immune cells passed through the endothelium, leukocytes must penetrate the basement membrane to reach the site of injury or infection, and this is a rate-limiting step in the extravasation process (Sorokin, 2010). Leukocytes can produce and secrete proteases which cleave type IV collagen and laminin α5 which are key components of the basement membrane. In addition, the filopodia of leukocytes also exert a mechanical force helping them to cross through the basement membrane (Sorokin, 2010). When the extravasated leukocytes finally reach the interstitial fluid, they migrate along a chemotactic gradient towards the injured site (Sorokin, 2010; Nitzsche et al., 2017).

There are differences in the temporal pattern of the recruitment of the different types of immune cells. During acute inflammation (within hours), the first cells recruited are neutrophils (Rittner et al., 2001; Ghasemlou et al., 2015). If the inflammation does not

resolve, then the infiltration of monocytes is promoted to the inflamed site, which differentiate to macrophages by the presence of inflammatory mediators in the environment (Mahdavian et al., 2011). Simultaneously, the number of neutrophils in the inflamed site decrease (Rittner et al., 2001; Ghasemlou et al., 2015). These myeloid cells constitute a large portion of the innate immune response. At later phases of the inflammatory process, lymphocytes (both T and B cells) which are part of the adaptive immune response heavily infiltrate in the injured tissue, to constitute the majority of the immune infiltrate (Rittner at al., 2001; Hoebe et al., 2004).

1.3.3. Role of immune cells in inflammatory pain

In noninjured conditions, or immediately after injury, just a few resident immune cells are present in the tissue. Although these resident immune cells might play a role on inflammatory pain (as described in the Section 1.3.3), after injury there is a marked proliferation of tissue resident myeloid cells which together to the marked recruitment of leukocytes from the blood enormously increase the number of immune cells present in the inflamed site. It is then when leukocytes play their most important role on inflammatory pain.

Immune cells that infiltrate the inflamed tissue produce and release algogenic chemicals that participate in the sensitization of nociceptors; thus, immune cells promote pain during inflammation (e.g. Ji et al., 2014; Pinho-Ribeiro et al., 2017). However, these immune cells can also produce endogenous opioid peptides (EOPs) (e.g. Hua and Cabot, 2010). Therefore, their role in pain is complex. In this section we will summarize the most relevant current literature on the role of the immune system on pain during inflammation.

Not only endothelial cells express ICAM-1, but peripheral nerve terminals are also able to express this protein (Hua, 2016). The expression of neuronal ICAM-1 facilitates that a portion of leukocytes present in the inflammatory site physically interact with the nerve endings to facilitate a direct cross-talk between immune cells and peripheral sensory neurons, and therefore modulating nociceptive neurotransmission (Hua, 2016). See Figure 7.

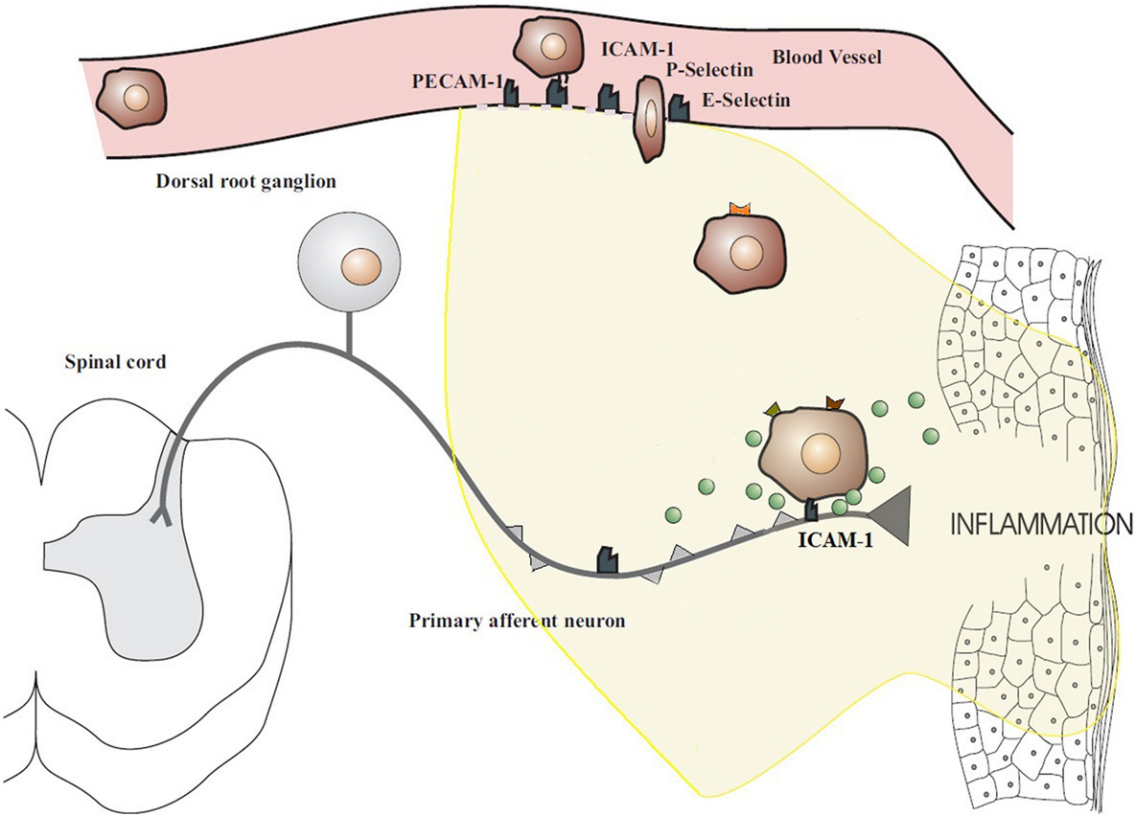


Figure 7. Neuron-immune cross-talk is facilitated by the expression of ICAM-1 by peripheral nerve terminals. Leukocytes reaching the inflamed site after extravasation can physically contact nerve fibers which express ICAM-1 (taken from Hua, 2016, with modifications).

1.3.3.1. Proalgesic role of immune cells

Immune cells are major sources of several pain sensitizers including NGF, TNF, IL-1 β, IL-6, histamine, and several arachidonic acid derivatives such as PGE2, 5,6-epoxyeicosatrienoic acid (5,6-EET), hydroxyicosatetraenoic acid (HETE), among many others (reviewed by Ji et al., 2014).

This milieu of algogenic chemicals is able to sensitize nociceptors by different mechanisms. For instance NGF acts trhough TrkA pathway, and histamine thought its specific receptors, and both are able to sensitize nociceptors (as described in the Section 1.3.1 of this Chapter). TNF, IL-1β, IL-6 and PGE2 can be produced by a variety of cells, but there is no doubt that immune cells are major producers of these chemicals (Stein and Gordon, 1991; Zhang and An, 2007; Kawabata, 2011; Italiani and Boraschi, 2014; Menaldo et al., 2017). TNF receptors (TNFR1 and TNFR2) are present in both immune cells and neurons (Zhang and An, 2007), and their inhibition attenuates inflammatory pain (Zhang et al., 2011). The mechanism of IL-1β to promote pain is

complex, since this cytokine in addition to induce the synthesis of several peripheral sensitizers, it directly acts on neurons by modulating tetrodotoxin-resistant Na^+ channels. This results on an enhanced conduction of the nociceptive imput (Binshtok et al., 2008). It was reported that IL-1β can also be produced by nociceptors (Copray et al., 2001), which might aid to the immune-neuronal crosstalk during chronic pain. IL-6 exerts its effect by activating IL-6 specific receptors (IL-6R). IL-6R can be anchored to the membrane or in soluble form. In this later state they can activate neurons via the transducer glycoprotein 130 (gp130) promoting TRPV1 sensitization (Andratsch et al., 2009). PGE2 acts on eicosanoid-prostanoid receptors (EPs), which are GPCRs relatively widely distributed. EP1 and EP4 are located on peripheral sensory neurons, and their activation leads to the phosphorilation of cation channels, including TRPV1, P2X3 and the voltage-dependent Ca^{2+} channel Cav3.2 (Kawabata, 2011). Other arachidonic acid derivatives such as 5,6-EET and HETE are also able to sensitize TRP channels (Sisignano et al., 2012; Ji et al., 2014).

Although the main source during inflammation of extracellular ATP and H^+ are the injured cells, some immune cells (neutrophils) have been described to also release ATP (Burnstock and Boeynaems, 2014) and H^+ (van Zwieten et al., 1981), which might contribute to nociceptor sensitization by actions on neuronal cation channels (Ji et al., 2014).

The release of these algogenic chemicals by immune cells, together to those produced by nonimmune cells (described in the Section 1.3.3 of this Chapter) are the main responsible of the enhanced nociceptor sensitization during inflammation. Interestingly, nociceptors activated by peripheral sensitizers can release neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) (Ji et al., 2014; Pinho-Ribeiro et al., 2017), which are the responsible of neuron-induced vasodilatation and increased permeability of the vascular endothelium, a process called “neurogenic inflammation” (Ji et al., 2014; Pinho-Ribeiro et al., 2017). These neuropeptides are also pain-inducers by their own right acting on nociceptors (O'Connor et al., 2004; Benemei et al., 2009), and also induce the release of algogenic chemicals by immune cells, establishing a loop between the secretion of neuropeptides and inflammatory algogens (Rosa and Fantozzi, 2013; Ji et al., 2014) (see Figure 8). Therefore,

inflammatory pain is the complex result of a milieu of inflammatory mediators from different sources which act together by diverse mechanisms to promote neuronal activity (see Figure 8).

There are some studies focused on the role of specific immune cell types on pain. The pronociceptive role of activated macrophages is out of any doubt. They release a wide variety of proinflammatory cytokines including IL-1β and IL-6 (Wang et al., 2014), and are the major source of TNF (Stein and Gordon, 1991). In fact, macrophage depletion has an ameliorative effect on inflammatory pain hypersensitivity (Ghasemlou et al., 2015). Mast cells are a major source of histamine and they also produce 5-HT (Fisher et al, 2010), which are both well known peripheral sensitizers (as commented in the Section 1.3.1 of this Chapter). Although their role in acute inflammatory pain is clear (Oliveira et al., 2011), their role in chronic inflammatory pain is questioned (Lopes et al., 2017). Neutrophils are known to promote the formation of inflammatory edema (Wang et al., 2012), and also produce several chemical algogens which induce pain sensitization (Cunha et al., 2008; Kolaczkowska and Kube, 2013). The role of neutrophils on inflammatory pain has also been subject of debate. While in some studies these immune cells appear to play a key pronociceptive role (e.g. Bennett et al., 1998; Cunha et al., 2008), other recent study do not find differences after neutrophil depletion (Ghasemlou et al., 2015). Fewer studies have been focused on the role of T cells on pain. Although T cells clearly play a role on the development or maintenance of neuropathic pain (reviewed by Pinho-Ribeiro et al., 2017), their role on inflammatory pain is less clear (Ghasemlou et al., 2015; Baddack-Werncke et al., 2017). Therefore, although there is overwhelming evidence on the production of peripheral sensitizers by a variety of immune cells, more studies are needed to clarify the specific contribution of each immune cell type to inflammatory pain hypersensitivity.

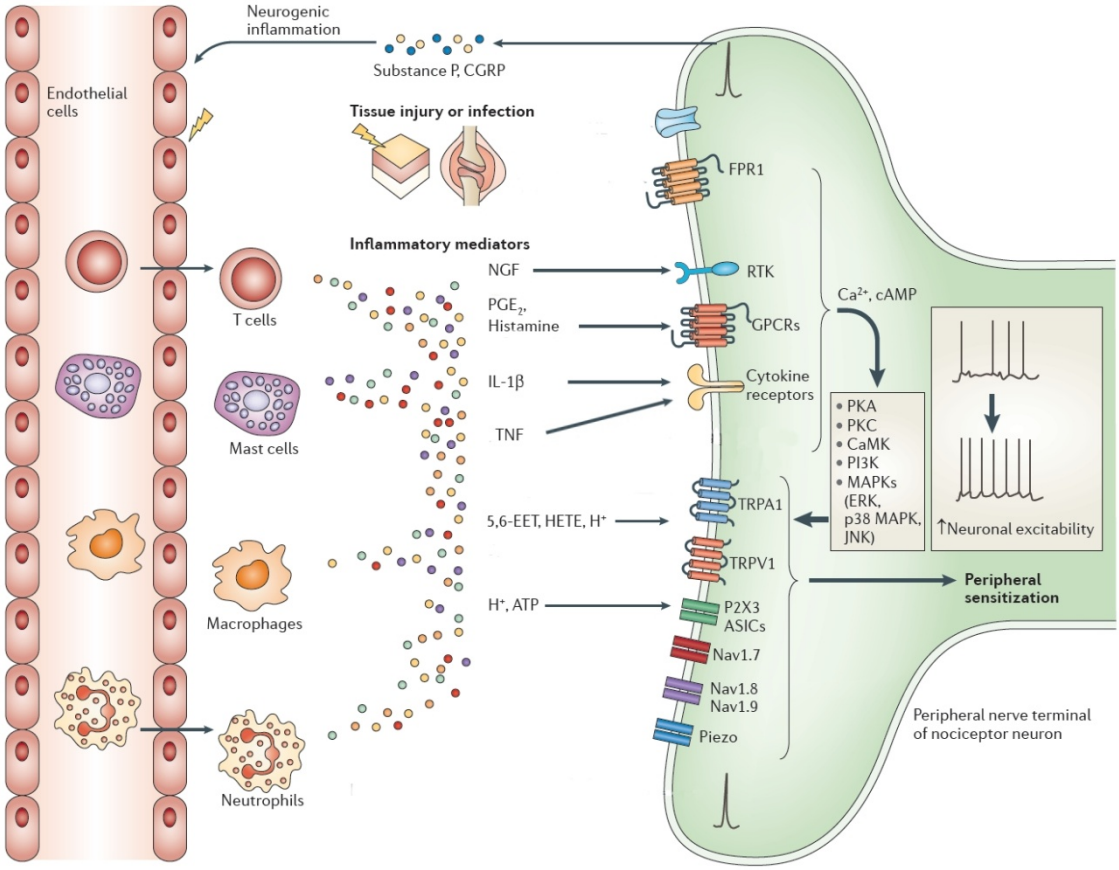


Figure 8. Major algogenic chemicals released by immune cells and the release of neuropeptides by peripheral sensory neurons. Leukocytes reaching the inflamed site release a wide variety of chemicall algogens which interact with their specific receptors to promote neuronal hyperexcitability. In turn, sensitized neurons release neuropeptides which promote neurogenic inflammation (taken from Ji et al., 2014, with modifications).

1.3.3.2. Immune cells and peripheral opioid analgesia

Neutrophils, macrophages and lymphocytes have all been described to produce EOPs (Rittner et al., 2001; Brack et al., 2004; Labuz et al 2006; Sauer et al., 2014; Wang et al. 2014), and it has been described that the main source of EOPs in the inflamed tissue comes from immune cells (e.g. Przewlocki et al., 1992; Rittner et al., 2001). However, as the predominant immune cell types vary with the time course of the inflammation (as described in the Section 1.3.2 of this Chapter), distinct leukocyte lineages are responsible for EOPs production in the inflamed site at different stages of inflammation (Rittner et al., 2001). Interestingly, opioid peptide expression is increased in immune cells from inflamed tissue compared to quiescent leukocytes, indicating that active immune cells enhance the synthesis of EOPs (Kapitzke et al., 2005). It has been described that immune cells can produce several EOPs, including β-endorphin,

enkephalins, dynorphins, and endomorphins (Rittner et al., 2001; Mousa et al., 2002; Kapitzke et al., 2005; Labuz et al 2006). Each EOP is synthesized by different precursors. Proopiomelanocortin (POMC), proenkephalin and prodynorphin are the precursors for β-endorphin, enkephalins and dynorphins, respectively (Mousa, 2003; Kapitzke et al., 2005). The biosynthetic pathway for endomorphin-1 and 2 or even the gene/es encoding for these peptides still remain unknown (Rónai et al., 2009). All these EOPs except endomorphins share the so-called "pan-opioid sequence" of 4 aminoacids (Tyr-Gly-Gly-Phe) at the *N* terminus (Pasternak and Pan, 2013), as shown in Table 2.

Table 2. Sequences of relevant mammalian endogenous opioid peptides (the "pan-opioid sequence" is labeled in bold)

β-endorphin	**Tyr-Gly-Gly-Phe**-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Dynorphin A	**Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Dynorphin B	**Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
Leu-enkephalin	**Tyr-Gly-Gly-Phe**-Leu
Met-enkephalin	**Tyr-Gly-Gly-Phe**-Met
Endomorphin-1	Tyr-Pro-Trp-Phe-NH2
Endomorphin-2	Tyr-Pro-Phe-Phe-NH2

Each EOP has a distinct selectivity profile for opioid receptors. While dynorphins have higher affinity for κ opioid receptors, and enkephalins for δ opioid receptors, β-endorphin binds preferentially to μ opioid receptors (Pasternak and Pan, 2013), and the two endomorphins are highly selective μ agonists (Rónai et al., 2009; Liu et al., 2017). All opioid receptors are GPCRs, and after stimulation with an endogenous or exogenous opioid agonist, they signal through inhibitory Gα, although the precise subtype of inhibitory Gα depends on the agonist tested (Sánchez-Blázquez et al., 2001). Activation of these inhibitory Gα subunits leads to a decrease in intracellular cAMP production (Al-Hasani and Bruchas, 2001). The activated GβϒY subunits play a role in the modulation of ion channels. These proteins induce a reduction in P/Q-type,

N-type, and L-type Ca^{2+} currents (Al-Hasani and Bruchas, 2001). In addition, GβϒY subunits stimulate the opening of the potassium channels $K_{ir}3$. Although the effects of μ agonists on $K_{ir}3$ channels are clear, it is not that feasibly demonstrate for κ or δ opioid receptors (Ocaña et al., 2004). See Fig. 10 for a schematic representation of the basic mechanism of opioid receptor activation.

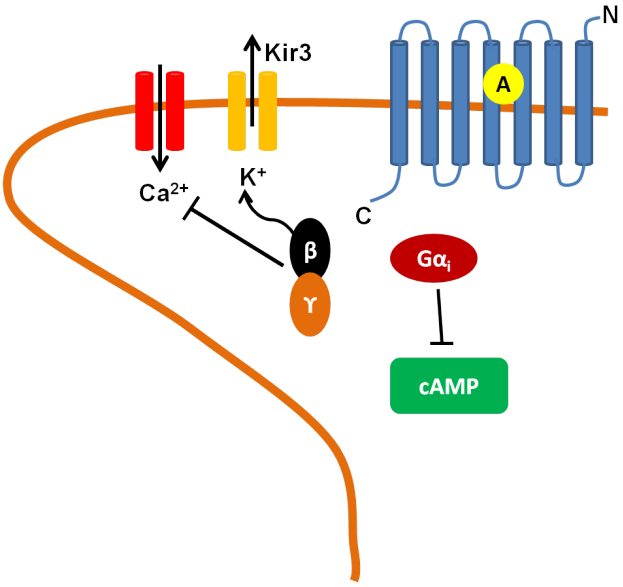


Figure 9. Basic mechanisms of opioid receptor activation. Agonism on μ, δ or κ opioid receptors triggers the activity of inhibitory Gα subunits (Gαi) which decrease cAMP production. On the other hand, GβϒY subunits inhibit the activity of Ca^{2+} currents and activate $K_{ir}3$ potassium channels.

Although opioid analgesia is classically thought to be primarily produced at central levels (Greenwood-Van Meerveld and Standifer, 2008; Joshi et al., 2008; Thomas et al., 2008; Khalefa et al., 2012; Ringkamp and Raja, 2012), opioid receptors are also expressed on peripheral sensory neurons (Stein, 2003; Stein and Machelska, 2011; Sullivan et al., 2016), and in fact, opioid drugs are able to attenuate the excitability of peripheral nociceptor terminals, the release of neuropeptides (such as substance P or CGRP) from peripheral sensory nerve terminals with the consequent decrease in neurogenic inflammation, as well as the propagation of action potentials in peripheral nerves (reviewed by Stein, 2013). During peripheral inflammation there is an increase in the synthesis and axonal transport of opioid receptors from DRG neurons to the peripheral terminals (Stein and Zöllner, 2009; Stein, 2013). In addition, low pH (which is indeed produced during inflammation) can increase opioid agonist efficacy by increasing the efficiency of the coupling between opioid receptors with G proteins (Selley et al. 1993; Vetter et al. 2006). Also, inflammation induces the sprouting of sensory nerve terminals and disruption of the perineurial barrier, thus enhancing the

access of opioid agonists to their receptors (Rittner et al., 2012). All these events contribute to the increase of the peripheral effects of opioid agonists during inflammation.

In spite of this enhancement of peripheral opioid effects during inflammation and the previously commented presence of endogenous opioid peptides produced by the immune cells at the inflamed site, inflammation still leads to pain. This means that the activity of the EOPs at the inflamed site is not enough to counterbalance the effects of the myriad of proalgesic agents released during inflammation. One of the reasons for this limited activity of EOPs at the inflamed site is the rapid enzymatic iactivation of EOPs by aminopeptidases, such as aminopeptidase N and neutral endopeptidase, which are expressed by both leukocytes and peripheral nerves (Barnes et al., 1991; Lendeckel, et al., 2000). In fact, the decrease of the endopeptidase activity at the inflamed site leads to opioid-dependant antihyperalgesic effects (Schreiter et al., 2012). Another way to promote immune cell driven peripheral opioid antinociception is by enhancing EOP release. This can be achieved by the administration of corticotrophin-releasing factor (CRF), which stimulates secretion of EOPs from immune cells in a receptor-specific and calcium-dependent manner (Schaëfer et al. 1994; Cabot et al. 1997, 2001). CRF is a key molecule produced in response to emotional stress (Ohmura and Yoshioka, 2009), and in fact, stress itself is able to induce opioid-dependant antihyperalgesic effects at the inflamed site (Schäfer et al., 1996; Robertson et al., 2008; Parikh et al., 2011).

In conclusion, although the actions of EOPs from immune origin are not normally able to counteract pain during inflammation, they have a clear analgesic potential that can be shown by strategies aimed to decrease their degradation or promoting their release. It would be interesting to test whether modulators of opioid receptors would be able to maximize the analgesic potential of immune cells during inflammation.

1.4. Limitations of current analgesics: the need for a new class of analgesic drugs

The most widely used analgesics used to treat inflammatory pain conditions are nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen and opioid drugs (Blondell et al., 2013; Gupta and Ba, 2016; Helander et al., 2017). Both NSAIDs and acetaminophen have a limited analgesic efficacy and therefore they are used (as a single therapy) in the first step of the WHO analgesic ladder (Blondell et al., 2013). Opioids are used in the second and third step of the WHO analgesic ladder, generally associated to NSAIDs or acetaminophen (Caraceni et al., 2012; Mercadante, 2015). NSAIDs, in particular nonselective cyclooxygenase (COX)-inhibitors are known to induce a number of side effects including gastrointestinal erosions and renal insufficiency, which are mostly dependent on COX-1 inhibition (Süleyman et al., 2007). Selective COX-2 inhibitors induce a lower rate of those adverse events induced by nonselective COX inhibitors, but their repeated administration may result in thrombotic cardiovascular problems, which can be life-threatening and therefore limit their use (Mukherjee, 2002). Although acetaminophen has a better safety profile than NSAIDs, it does not have anti-inflammatory properties, which is a disadvantage over NSAIDs (Simmons et al., 2000). Opioid drugs have a higher analgesic efficacy than NSAIDs or acetaminophen but they induced a high number of side effects which impact the quality of life of patients on opioid therapy. These side effects include nausea, constipation, sedation, respiratory depression, tolerance and dependence, among many others (Al-Hasani and Bruchas, 2011). Therefore, there is a need for the development of novel analgesic medications with new mechanisms of action. In this sense, sigma-1 receptors, which will be described in detail in the next chapter, have been established recently as a promising target for analgesic drug development.

2. SIGMA-1 RECEPTORS: FROM AN OPIOID RECEPTOR SUBCLASS TO A MOLECULAR CHAPERONE INVOLVED IN PAIN MODULATION

2.1. The discovery of sigma receptors and the identification of the sigma receptor subtypes

Sigma-1 receptors were first described by Martin and coworkers in 1976 when they attempted to explain the psychotomimetic actions of (±)-SKF-10,047 (*N*-allylnormetazocine) and other racemic benzomorphans. These early studies classified sigma receptors as a subclass of opioid receptors (Martin et al., 1976). The complex pharmacology of (±)-SKF-10,047 was the responsible for this early confusion, as further studies showed that (-)-SKF-10,047 was a κ opioid agonist and its effects could be reversed by the opioid antagonist naloxone. In contrast, the (+)-isomer does not have affinity for any opioid receptor subtype, and binds to a different binding site which retained the designation of sigma receptors (Matsumoto et al., 2003; Cobos et al., 2008; Zamanillo et al., 2013).

Later, further studies found that sigma receptors could be classified into two subtypes, based on their pharmacological profile, which were designated as sigma-1 and sigma-2 (Quirion et al., 1992). In addition to their differences regarding their pharmacology it was found that they also differed in their molecular mass (29 kDa for sigma-1 and 18-21.5 kDa for sigma-2) (Hellewell and Bowen 1990). The sigma-1 receptor was cloned for the first time in guinea pig (Hanner et al., 1996), and later in others species such as mice, rats and human (Pan et al., 1998). The identification of the sigma-2 receptor was done much later, and it has been very recently identified as TMEM97, a protein involved in cholesterol homeostasis (Alon et al., 2017).

The cloning of the sigma-1 receptor helped to progress in the understanding of its molecular structure, and showed the sigma-1 receptor as a single polypeptide, constituted by 223 amino acids with a high homology between species (Seth et al., 2001), and without homology with any mammalian protein. These data indicated that sigma-1 receptors constituted a new molecular entity distinct to any other known protein. Moreover, the cloning of these receptors made possible to design anti-sense oligodeoxynucleotides to investigate sigma-1 receptors function (Pan et al., 1998; Mei

and Pasternak, 2002 and 2007), and the development of sigma-1 knockout mice (Langa et al., 2003), which are important tools to improve our knowledge on σ_1 receptor functions.

2.2. Anatomical distribution of sigma-1 receptors

The sigma-1 receptor is found in anatomical important areas for pain control in both the central and peripheral nervous systems. At the central nervous system, these receptors are located in supraspinal sites related to pain modulation such as the locus coeruleus (LC), periacueductal gray matter (PAG) and rostroventral medulla (RVM), as well as in the spinal cord dorsal horn (Roh et al., 2008; Zamanillo et al., 2013 and Sánchez-Fernández et al., 2014). In the peripheral areas, this receptor is found in the dorsal root ganglion (DRG) (Sánchez-Fernández et al., 2014) specifically in the soma of peripheral sensory neurons (Bangaru et al., 2013; Mavlyutov et al., 2016), and along the nerve in Schwann cells (Palacios et al., 2004). It is worth noting that the expression of sigma-1 receptors is much higher in the DRG than in any central area tested (Sánchez-Fernández et al., 2014), and therefore pointing to a prominent role of sigma-1 receptors on pain modulation at the peripheral level.

In addition to pain-related areas, sigma-1 receptors are also located in areas related to memory and in structures of the limbic system, where it plays a role on cognition and mood states (Bermack and Debonnel, 2005; Maurice et al., 2006). This receptor is not located exclusively in nervous tissue, but it can be found in other organs such as liver, spleen, kidney, gastrointestinal tract and heart, but its function in these areas is less well studied (Bowen, 2000; Stone et al., 2006; Bhuiyan and Fukunaga, 2011).

2.3. Sigma-1 receptor structure, subcellular location and its chaperoning function

Early bioinformatic studies indicated that sigma-1 receptor presented only a single transmembrane domain (Hanner et al., 1996; Dussossoy et al., 1999), but deeper more recent studies showed the existence of two transmembrane domains (reviewed in Chu

and Ruoho, 2016). However, the structure of sigma-1 receptors is still under debate, since a recent report suggests that sigma-1 receptors follow a trimeric structure with a single transmembrane domain of each protomer (Schmidt et al., 2016), remaining to the first proposed structure. Therefore, a consensus regarding the exact structure of sigma-1 receptors is still far to be reached. However, it is clear that sigma-1 receptors do not resemble to any other known families of receptors such as G-protein coupled receptors (GPCRs) or ionotropic receptors. In fact, it is known that sigma-1 receptors have a chaperone domain close the C-terminus (Hayashi and Su, 2007), which confers to this receptor its peculiar neuromodulatory actions as it will be described below.

At subcellular levels, this receptor is found in multiple membranes such as nuclear and plasma membranes, and at particularly high levels at the endoplasmic reticulum membrane (Alonso et al., 2000; Hayashi and Su 2004), where it locates in mitochondrion-associated endoplasmic reticulum membranes (MAM) (Hayashi and Su 2007; Hayashi et al., 2011). In stress situations, the sigma-1 receptor is translocated from the endoplasmic reticulum to other areas of the cell, such as the plasmalemmal area within the extended endoplasmic reticulum reticular network, or to the plasma membrane itself (Su et al., 2010), where it can physically interact with several protein targets to modify their function (Su et al., 2010; Kourrich et al., 2012). Intracellular Ca^{2+} regulates the interaction between sigma-1 receptors and their protein targets (Rodríguez-Muñoz et al., 2015a), so sigma-1 receptors act as a Ca^{2+} sensor to modulate neuronal physiology. The protein targets of sigma-1 receptors which has been more deeply studied are the GPCR μ-opioid receptor and ionotropic receptor *N*-methyl-*D*-aspartate receptor (NMDAR) (reviewed by Sánchez-Fernández et al., 2017), although there has been described a number of additional protein partners for sigma-1 receptors, such as the GPCRs dopamine D1 and D2 receptors, cannabinoid receptor 1 (CB1R), and serotonin receptors 1A and 2A, and the ion channels voltage-dependent K+ channels (Kv1.2, Kv1.3, Kv1.4 and Kv1.5), L-type voltage-dependent Ca2+ channels (VDCC) (but not other types of VDCC), acid-sensing ion channels of the 1a subtype (ASIC1a) and the ionotropic receptors $GABA_A$ receptors (reviewed by Sánchez-Fernández et al., 2017) (Figure 10). Taking into account the numerous protein partners of sigma-1 receptors that have been already described (and the ones that are probably

still unknown), it is not surprising that sigma-1 receptors play an important role on neurotransmission.

Interestingly, some of these proteins not only associate to sigma-1 receptors, but can also interact between themselves to act coordinately. For example, NMDARs and sigma-1 receptors can form a macromolecular complex together with μ-opioid receptors, to regulate the function of the latter (as it will described in more detail in the Section 2.5. of this Chapter).

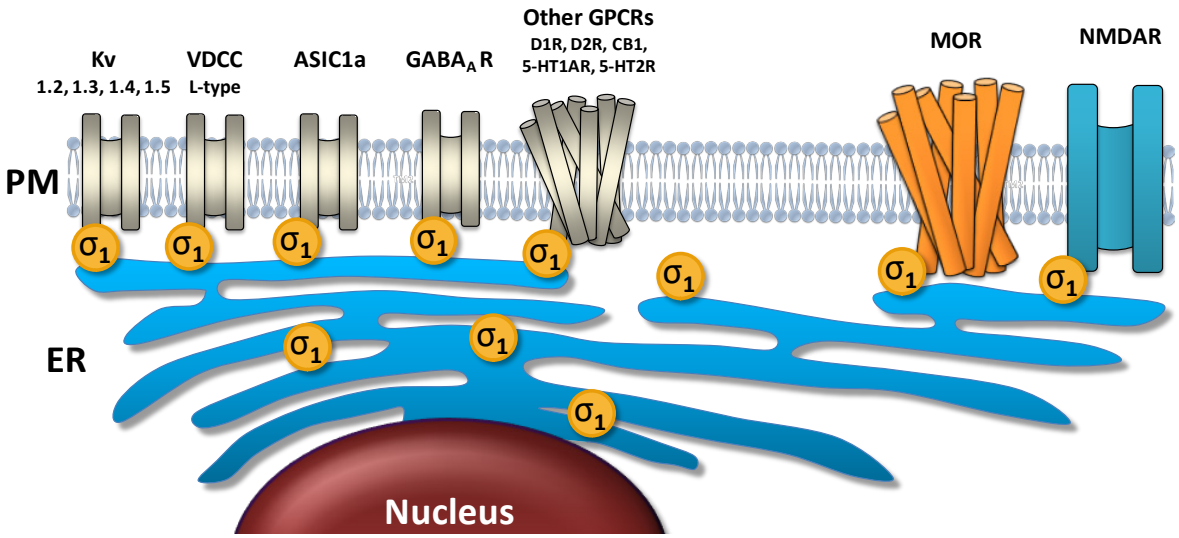


Figure 10. Main protein targets of sigma-1 receptors. Sigma-1 receptors located in the endoplasmic reticulum (ER) reticular network can modulate through protein–protein interactions several receptors and channels in the plasma membrane (PM). The two best known protein targets of sigma-1 receptors are the *N*-methyl-*D*-aspartate receptor (NMDAR) and the μ-opioid receptor (MOR), and are represented at a larger scale to draw the attention of the readers. Other protein partners of sigma-1 receptors at the PM include voltage-gated K+ channels (Kv1.2, 1.3, 1.4, 1.5), L-type voltage-dependent calcium channels (VDCC), acid-sensing ion channels of the 1a subtype (ASIC1a), $GABA_A$ receptors ($GABA_AR$), and other G-protein-coupled receptors (GPCRs) such as D1R and D2R, CB1R, 5-HT1AR and 5-HT2AR (see text for details). Taken from Sánchez-Fernández et al., 2017 with some modifications.

2.4. Sigma-1 receptor pharmacology

Despite that sigma-1 receptors were initially classified as a subtype of opioid receptors because of the mixed pharmacology of (±)-SKF-10,047 (as previously mentioned), it is now known that sigma-1 receptors do not bind prototypic opioid agonists including morphine, fentanyl, oxycodone, tramadol or buprenorphine, nor the prototypic opioid

antagonist naloxone or its quaternary derivative naloxone methiodide (Sánchez-Fernández et al., 2014), although it can bind with high affinity both enantiomers of phenazocine (Prezzavento et al., 2017), an old opioid agonist which is not currently in clinical use (Conaghan et al., 1966; Hopton, 1971). However, in spite of that sigma-1 receptors do not bind most opioid drugs, they bind with high affinity a large list of compounds from different pharmacological classes and therapeutic uses such as antipsychotics (haloperidol), antitussives (carbetapentane or dextromethorphan), antidepressants (fluvoxamine, sertraline), drugs for the treatment of neurodegenerative disorders (amantadine, donepezil), and drugs of abuse (cocaine and methamphetamine) among many others (reviewed by Cobos et al., 2008; Almansa and Vela, 2014).

Although all the above mentioned drugs show affinity for sigma-1 receptors, this is not their main pharmacological target. There are prototypic selective drugs available to study sigma-1 receptor function. These include the agonists (+)-pentazocine and PRE-084 and the antagonists NE-100, BD-1047, BD-1063 and S1RA (Cobos et al., 2008; Romero et al. 2012). This latter compound has been proved its selectivity for sigma-1 receptors on a panel of 170 targets (Romero et al., 2012), and has being recently evaluated in phase II clinical trials for a primary indication for neuropathic pain treatment and as an adjuvant to opioid analgesia (Vaqué et al., 2016), after successful positive phase I studies demonstrated its acceptable safety and tolerability in healthy people (Abadias et al., 2013).

In addition to these synthetic compounds, there has been proposed some endogenous ligands for sigma-1 receptors, such as the neurosteroids pregnenolone, degydroepiandrosterone, their sulphate esters, and progesterone (Cobos et al., 2008) and the natural hallucinogen *N*,*N*-dimethyltryptamine (Fontanilla et al., 2009).

2.5. Role of sigma-1 receptors on acute nociceptive pain: modulation of opioid antinociception and opioid-induced side effects

In the early 1990s Chien and Pasternak found that the nonselective sigma-1 antagonist haloperidol (by then one of the few tools available to study sigma receptors) was able to increase morphine antinociception (Chien and Pasternak 1993, 1994 and 1995). These were the first evidences for a role of sigma-1 in pain. Later studies using selective sigma-1 antagonists and antisense oligodeoxynucleotides against sigma-1 receptor sequence clearly showed that sigma-1 inhibition leads to an increase in morphine antinociception (reviewed by Sánchez-Fernández et al., 2017). In addition, the enhancement of morphine analgesia by sigma-1 inhibition was shown to be extensive to several other μ-opiopids on clinical use, such as fentanyl, oxycodone, buprenorphine or tramadol (Vidal-Torres et al., 2013; Sánchez-Fernández et al., 2014). Although these μ-opiopid drugs do not show affinity for sigma-1 receptors (as previously mentioned), both enantiomers of phenazocine can bind to both μ-opioid receptors and to sigma-1 receptors, and these compounds are mixed opioid agonists/sigma-1 antagonists (Prezzavento et al., 2017). In fact, the analgesic effects induced by phenazocine enantiomers are due to the joint actions of μ-opioid/sigma-1 activities present in the same molecules (Prezzavento et al., 2017). In addition to the modulation of μ-opioid drugs, sigma-1 receptors are known to be able to modulate the analgesic effects induced by κ and δ opioid agonists (reviewed in Zamanillo et al., 2013; Sánchez-Fernández et al., 2017). Importantly, although sigma-1 inhibition clearly enhanced opioid antinociception, it did not modify acute nociceptive pain in the absence of opioid drugs (de la Puente et al., 2009; Entrena et al., 2009b; Nieto et al., 2012; Sánchez-Fernández et al., 2013 and 2014), and therefore the potentiation of opioid antinociception was not due to summative effects of sigma-1 inhibition-opioid agonism, but it was due to synergistic effects on both targets.

Since opioid analgesia is classically thought to be primarily produced at central levels in both humans and rodents (Greenwood-Van Meerveld and Standifer, 2008; Joshi et al., 2008; Thomas et al., 2008; Khalefa et al., 2012; Ringkamp and Raja, 2012), further studies were conducted to study the anatomical location of opioid modulation by sigma-1 receptors, in particular at central levels. It was found that sigma-1 inhibition

by intracerebroventricular administration of antisense oligodeoxynucleotides or pharmacological antagonists increased opioid antinociception (Pan et al., 1998; Mei and Pasternak, 2002; Rodríguez-Muñoz et al., 2015b). In addition, sigma-1 inhibition in RVM led to a robust increase in the analgesic effect of morphine, indicating that sigma-1 receptors play a prominent role on the descending modulatory pain pathways (Mei and Pasternak, 2007). More recent studies from our research group showed that sigma-1 receptors also control peripheral opioid antinociception, since local peripheral sigma-1 antagonism was able to enhance the antinociception induced by opioid drugs, and these effects were reversed by the peripherally-restricted opioid antagonist naloxone methiodide (Sánchez-Fernández et al., 2013 and 2014).

Interestingly, sigma-1 receptor knockout mice replicated the effects obtained with the pharmacological inhibition of sigma-1 receptors under nociceptive mechanical stimulation (Sánchez-Fernández et al., 2013 and 2014), but these mutant animals did not show enhanced opioid antinociception to noxious thermal stimulus (Vidal-Torres et al., 2013). However, sigma-1 antagonists were devoid of effect on sigma-1 knockout mice under both experimental situations (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013), indicating the selectivity of the pharmacological approach. These data indicate that compensatory mechanisms might be developing in thermal pain pathways in the mutant mice.

Taking altogether these findings, it can be assured that sigma-1 receptors constitute a biological brake to the antinociception induced by opioid drugs acting both centrally and peripherally. Of note regarding all these previous studies, although the modulation of opioid antinociception by sigma-1 receptors is clear, up to now all research has been done in models of acute nociceptive pain. Therefore, it would be interesting to test whether this modulation still occurs when the nociceptive system is sensitized during pathological pain.

In addition to opioid antinociception, the possible modulation of opioid-induced side effects by sigma-1 receptors has been also tested. These opioid side effects include tolerance, dependence, and constipation, which are clinically relevant and limit opioid use (Dhingra et al., 2013). In contradistinction to the increased opioid antinociception by sigma-1 inhibition, neither sigma-1 antagonism nor sigma-1 knockout induced

enhanced opioid-induced side effects (Sánchez-Fernández et al., 2013 and 2014; Vidal-Torres, 2013). Therefore, these receptors are currently considered to be a promising pharmacological target to increase the therapeutic index of opioids.

The molecular mechanism of opioid modulation by sigma-1 receptors has been recently unveiled. μ-opioid receptors and NMDARs physically interact, together with the histidine triad nucleotide-binding protein 1 (HINT1). When μ-opioid receptors are activated by an opioid agonist, NMDARs became activated through protein kinase C γ (PCKγ) activity. This NMDAR activation leads to an increase in the Ca^{2+} influx, which in turn facilitates the interaction of sigma-1 receptors with NMDARs blocking the inhibitory action of Ca^{2+}–calmodulin on NMDARs, and therefore NMDARs remain in active form. Ca^{2+}–calmodulin is also able to decrease μ-opioid receptor signaling by the activation of calmodulin-dependent kinase II (CaMKII). In the presence of a sigma-1 antagonist, the association between sigma-1 receptors and μ-opioid receptors is facilitated, transferring HINT1 to NMDARs. When NMDARs do not bind sigma-1 receptors (as they bind HINT1 instead), they are more susceptible to the inhibitory effect of CaM. This finally results in the increase of μ-opioid receptor signalling by the decrease in the inhibitory influence of NMDARs over the μ-opioid receptors (Rodríguez-Muñoz et al., 2015a and b). In other words, sigma-1 receptors facilitate the action of NMDARs decreasing the function of opioids, whereas sigma-1 antagonists inhibit the action of this biological brake to the opioid functioning, with the resulting potentiation of opioid analgesia. This process is summarized in Figure 11.

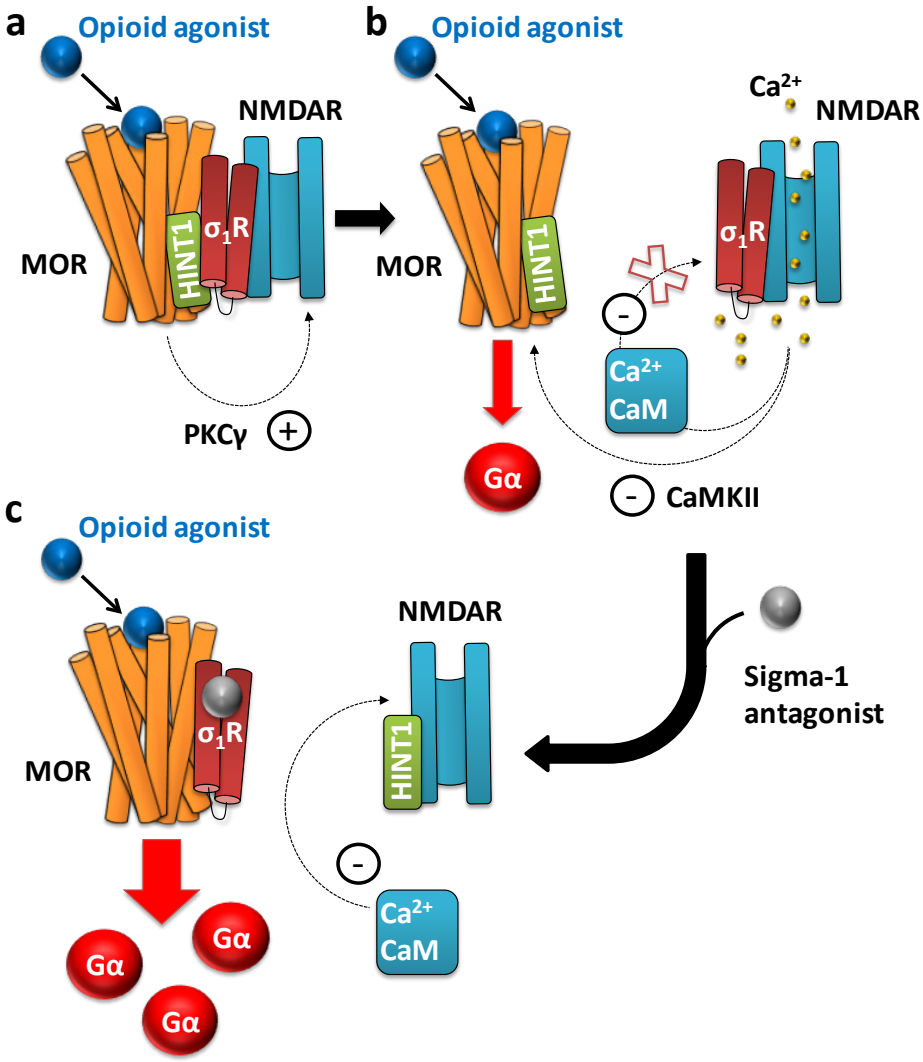


Figure 11. Mechanism of action of sigma-1 antagonists to enhance opioid antinociception. a) The opioid agonist activates μ-opioid receptors (MOR) leading to activation of NMDARs by PKCγ pathway. b) NMDAR activation increases Ca^{2+} influx, facilitating the association of sigma-1 receptors (σ_1R) to NMDARs, which in turn inhibits the binding of Ca^{2+}-Calmodulin (CaM) to NMDARs. Ca^{2+}-CaM activates calmodulin-dependent kinase II (CaMKII) which inhibits MOR. c) sigma-1 antagonists stabilize the association between σ_1R and MOR, promoting the association between histidine triad nucleotide-binding protein 1 (HINT1) and NMDARs. When NMDARs are free from the association with sigma-1 receptors, Ca^{2+}-CaM can interact with NMDARs to inhibit their actions, which results on the increase in MOR signalling (taken from Sánchez-Fernández et al., 2017).

Although this mechanism has been demonstrated at the molecular level with the μ-opioid receptor, it is likely that a similar process occurs with κ or δ opioid receptors, as sigma-1 inhibition also results in enhanced antinociception by selective agonists of these two other opioid subtypes, as previously mentioned.

It is worth noting that the differential modulation of sigma-1 receptors on opioid analgesia and side effects suggests that the complex formed by μ-opioid receptors/NMDAR/sigma-1 receptors might be present in neurons involved in pain pathways but not in other neuronal types. However, further studies are needed to fully understand the effects of sigma-1 receptors on opioid modulation.

2.6. Modulation of tonic and chronic pain by sigma-1 receptors

Although sigma-1 inhibition do not modify acute nociceptive pain to either thermal or mechanical stimuli (in the absence of opioid drugs) (as commented in the previous section), there is overwhelming evidence that it is able to ameliorate pain states when the nociceptive system is sensitized, as it will be described below.

The first experiments were done using the chemical irritant formalin. Formalin induces a biphasic pain response: an initial acute pain induced by direct nociceptor activation (first phase), continued by a tonic longer response (second phase), which is (at least partially) due to central sensitization (Le Bars et al., 2001). Sigma-1 receptor antagonism was able to abolish these biphasic pain responses, although the potency of sigma-1 antagonists to ameliorate the second phase was considerably higher than for the first phase (Cendán et al., 2005b;Romero et al., 2012; Díaz et al., 2013; Gomez-Soler et al., 2014; Lan et al., 2014; Vidal-Torres et al., 2014). Similar results were obtained in sigma-1 knockout mice, indicating the specificity of the analgesic effects of sigma-1 antagonism in this pain model (Cendán et al., 2005a).

The behavioral effect of sigma-1 antagonism in formalin-induced pain is accompanied by a decreased in the phosphorylation the NR1 subunit of NMDA receptors, which plays a key role on central sensitization (Kim et al., 2006). Moreover, sigma-1 antagonism in the spinal cord increased noradrenalin levels, and α2-adrenoreceptor antagonism reversed the effect of sigma-1 antagonism, indicating that the modulation of the descending noradrenergic system plays a role in the effects of sigma-1 inhibition in this model (Vidal-Torres et al, 2014). Furthermore, the local administration of sigma-1 antagonists into the formalin-injected paw also decreased formalin-induced pain (Vidal-Torres et al., 2014), indicating that in addition to the modulation of central sensitization, sigma-1 receptors can modulate pain transmission peripherally.

Other different chemical irritant used was capsaicin. The intradermal administration of this algogenic chemical induces a strong C-fiber activation leading to the development of mechanical hypersensitivity in the normal skin surrounding the injection site (named area of secondary hypersensitivity), which is due to central sensitization (Baron, 2000). The interest of this model is that it is considered to be a surrogate model of neuropathic pain, since it is predictive of the efficacy of drugs on this type of pain (Gottrup et al., 2004; Entrena et al., 2009a and b). Capsaicin-induced secondary hypersensitivity was abolished by sigma-1 antagonism and in sigma-1 knockout mice (Entrena et al., 2009a and b; Romero et al., 2012), and conversely, sigma-1 agonism enhanced the sensitizing effects of capsaicin (Entrena et al., 2016).

Importantly, the effect of sigma-1 antagonism on formalin-induced pain and capsaicin-induced secondary hypersensitivity was not reversed by the administration of the opioid antagonist naloxone (Cendán et al., 2005b; Entrena et al., 2009 b; Vidal-Torres et al., 2014). Therefore, the ameliorative effects of sigma-1 inhibition in these pain models are not due to the modulation of the opioidergic system. In fact, up to now there are no reports on the modulation of the endogenous opioidergic system by sigma-1 receptors on any type of pain.

Capsaicin administration is not only used as a somatic model of pain, but is also used as a visceral pain model when administering the chemical irritant into the colon (Laird et al., 2001; Schmidt et al., 2004). In this latter case, capsaicin administration induces an initial acute pain followed by referred hyperalgesia (sensory gain in the abdominal skin) which has been also reported in both humans (Schmidt et al., 2004) and rodents (Laird et al., 2001). Sigma-1 antagonism inhibited both the acute pain and referred hyperalgesia induced by the intracolonic administration of this compound (González-Cano et al., 2013).

Further studies were carried out in neuropathic pain. The majority of the studies were done after mechanical injury of the sciatic nerve. Sigma-1 inhibition was able to fully ameliorate mechanical and cold allodynia, as well as heat hyperalgesia induced by peripheral nerve injury (Romero et al., 2012; Díaz et al., 2012; Bura et al., 2013; Moon et al., 2013, 2014 and 2015; Choi et al., 2013; Espinosa-Juarez et al., 2017a and b). In addition, the selective sigma-1 antagonist S1RA was shown to ameliorate neuropathic

anhedonia (Bura et al., 2013), which is a key symptom of the neuropathic pain phenotype (Cobos and Portillo-Salido, 2013). These behavioral changes are accompanied by the decrease of known markers of central sensitization in the spinal cord, such as the phosphorylation of the NR1 subunit of NMDA receptors (Roh et al., 2008), the phosphorylation of extracellular signal-regulated kinase (ERK1/2) (de la Puente et al., 2009), and the production of reactive oxygen species (Choi et al., 2013) Furthermore, sigma-1 receptor antagonism also decreases the activation of spinal cord astrocytes, which are known to play a key role on central sensitization (Moon et al., 2013, 2014 and 2015). All these data indicate a pivotal role of sigma-1 receptors on the development of the prominent central sensitization which is produced after nerve injury.

In addition, to these studies on mechanically-induced nerve injury, there has been reported that sigma-1 inhibition is also effective on the neuropathic pain induced by the antineoplastic paclitaxel. Interestingly, sigma-1 antagonism was not only able to reverse neuropathic hypersensitivity once the neuropathy was developed but it was also able to prevent its development when administered during the antineoplastic administration (Nieto et al., 2012 and 2014). The attenuation in neuropathic pain behaviour was accompanied by a decrease in the phosphorilation of ERK1/2 in the spinal cord dorsal horn (Nieto et al., 2012) indicating again the importance of sigma-1 receptors on central sensitization. Interestingly, sigma-1 knockout mice showed a decrease in paclitaxel-induced mitochondrial abnormalities (as a sign of the toxicity induced by the taxane) in myelinated A-fibers (Nieto et al., 2014). These results indicate that not only central sigma-1 receptors are important for paclitaxel-induced neuropathic pain but that it has also a prominent neuroprotective role on primary afferent neurons.

At the beginning of the research work of this PhD project there were no publications on the role of sigma-1 receptors on inflammatory pain, but there are currently published a few studies on this topic. The first studies about the role of sigma-1 receptors on inflammatory pain were published almost simultaneously in 2014 (Parenti et al., 2014a and b; Gris et al., 2014) together with part of this PhD project (Tejada et al., 2014). In those studies it was shown that sigma-1 inhibition ameliorates

inflammatory hyperalgesia. However, the mechanism by which sigma-1 receptors modulate inflammatory pain has not been published till much recently (Tejada et al., 2017) and constituted the main experimental results obtained in this PhD project.

RATIONALE, HYPOTHESIS AND GOALS

1. Rationale

There is a need for new analgesics with innovative mechanisms of action (Kissin, 2010). The sigma-1 receptor is a neuromodulatory chaperone protein (Hayashi and Su 2007; Cobos et al. 2008; Zamanillo et al. 2013), which modifies the function of several receptors and channels important in neurotransmission (Su et al., 2016), and has been recently the focus of intense preclinical research as a new pharmacological target for pain treatment (Zamanillo et al., 2013; Vela et al., 2015).

Although sigma-1 receptor inhibition does not influence acute pain induced by thermal or mechanical stimuli (e.g., Chien and Pasternak 1994; De la Puente et al. 2009; Entrena et al. 2009b; Marrazzo et al. 2011; Romero et al. 2012; Sánchez-Fernández et al. 2013), it ameliorates the second phase of formalin-induced pain as well as neuropathic hypersensitivity by inhibiting central sensitization (Kim et al., 2006; Roh et al. 2008; de la Puente et al. 2009; Nieto et al. 2012; Romero et al. 2012). These findings indicate an important role for these receptors in central pain processing. Among the selective sigma-1 antagonists, the best characterized are BD-1063 and S1RA (Zamanillo et al., 2013). The latter compound has being recently evaluated in phase II clinical trials with a primary indication for neuropathic pain treatment (Vaqué et al., 2016), after successful positive phase I studies demonstrated its acceptable safety and tolerability in healthy people (Abadias et al., 2013).

A further potential indication for the sigma-1 antagonist S1RA is the enhancement of opioid analgesia (Vela et al., 2015). The potentiation of opioid antinociception by sigma-1 antagonism was described in the early 1990s (Chien and Pasternak, 1993). Later studies showed that the enhancement of opioid antinociception by sigma-1 antagonism is produced at central levels (Mei and Pasternak, 2002). In addition, we recently reported that peripheral sigma-1 inhibition was also able to enhance opioid antinociception (Sánchez-Fernández et al., 2013 and 2014), and this was very sensitive to naloxone methiodide (Sánchez-Fernández et al., 2014), a quaternary derivative of the opioid antagonist naloxone lacking of central penetrability (Menéndez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012), indicating the participation of peripheral opioid receptors on these effects. The marked potentiation of opioid

antinociception by peripheral sigma-1 antagonism is consistent with the higher density of sigma-1 receptors in the dorsal root ganglion (DRG) than in several central areas, such as the spinal cord dorsal horn and the periaqueductal gray matter (Sánchez-Fernández et al., 2014). Moreover, these receptors in the DRG are selectively located in sensory neurons and not in glial cells (Mavlyutov et al., 2016). It is now known that sigma-1 receptors can form a macromolecular complex with opioid receptors, tonically inhibiting receptor functioning, and that sigma-1 antagonism can protect opioid receptors from the tonic inhibitory effects of sigma-1 receptors, thus enhancing opioid analgesia (Kim et al., 2010, Rodríguez-Muñoz et al., 2015). However, although the ability of sigma-1 antagonism to potentiate the analgesic effects of opioid drugs is clear, the physiological or pathophysiological role of sigma-1 receptors in opioid modulation remain unknown.

Inflammatory pain is a major type of clinical pain (e.g., Scholz and Woolf 2002; Woolf 2004). At the beginning of the research work of this PhD project there were no publications on the role of sigma-1 receptors on this type of pain. In contrast to neuropathic pain, inflammatory pain is characterized by a more pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the milieu of inflammatory mediators released at the inflammation site (see Scholz and Woolf 2002; Ji 2004; Patapoutian et al. 2009; Latremoliere and Woolf 2009, for reviews). Inflammatory pain can be experimentally induced by the intraplantar administration of agents such as carrageenan (Sandkühler 2009; Cobos and Portillo-Salido 2013), and is characterized by both mechanical and heat hyperalgesia-types of hypersensitivity that result from only partially overlapping mechanisms (Latremoliere and Woolf 2009; Dubin and Patapoutian 2010). The development of inflammation-induced hyperalgesia is thought to be contributed by algogenic chemicals released by immune cells that infiltrate the inflamed tissue (Ji et al., 2014). Interestingly, all endogenous opioid peptides (EOPs), as well as mRNA transcripts encoding their precursor proteins, have been identified within immune cells, with β-endorphin from pro-opiomelanocortin (POMC) being the most prominent one (Hua and Cabot, 2010). However, despite the analgesic potential of these EOPs, the end result of inflammation is usually pain. It is unknown whether sigma-1 receptors curtail the antinociceptive

effects of peripheral EOPs during inflammation and thereby facilitate inflammatory pain.

2. Hypothesis and goals

Taking into account these antecedents, the **main hypothesis** of this Doctoral Thesis was that sigma-1 inhibition might ameliorate inflammatory hyperalgesia by the disinhibition of the peripheral actions of EOPs from immune origin. If this was the case, it would constitute an innovative mechanism of analgesia that might expand the therapeutic potential of sigma-1 antagonists.

To test this hypothesis, our **first goal** was to evaluate whether sigma-1 inhibition ameliorates inflammatory hyperalgesia and to determine the contribution of peripheral sigma-1 receptors to this effect. To this end, we studied the effects of sigma-1 receptor inactivation (in sigma-1 knockout mice) and of the systemic (subcutaneous) and local (intraplantar) administration of the selective sigma-1 receptor antagonists BD-1063 and S1RA, on mechanical and thermal inflammatory hyperalgesia induced by carrageenan. To control for possible antiinflammatory effects of sigma-1 inhibition that might contribute to the amelioration of hyperalgesia, we also tested the influence of sigma-1 inhibition on carrageenan-induced edema.

The **second goal** of this Doctoral Thesis was to determine whether the activation of opioid receptors contributes to the antihyperalgesic effects induced by sigma-1 antagonism, and to study whether peripheral opioid actions are involved in this process. In order to do that we tested whether the antihyperalgesic effects of BD-1063 and S1RA could be reversed by both the opioid antagonist naloxone and its peripherally-restricted analog naloxone methiodide.

Our **third goal** was to evaluate the participation of immune cells on carrageenan-induced inflammatory pain. To this end, we tested the effects of the reduction of the immune cell load at the inflamed site (by the *in vivo* administration of a specific antibody) in inflammatory hyperalgesia, as well as in the development of the inflammatory edema.

Our **fourth goal** was to test whether immune cells produce β-endorphin (as an example of a prominent EOP) at the inflamed site. To achieve this goal we characterized by fluorescence-activated cell sorting (FACS) the populations of immune cells at the inflamed site using cell-specific markers, and we then purified them to test whether these cells express POMC mRNA. We also determined β-endorphin levels in the inflamed paw by a fluorescent enzyme immunoassay, and tested whether immune cell depletion is able to decrease the levels of this EOP.

The **fifth goal** of this Doctoral Thesis was to determine whether the naloxone-sensitive antihyperalgesic effects induced by sigma-1 antagonism during inflammation depend on the presence of EOPs from immune origin. To fulfill this goal we tested whether the antihyperalgesic effects induced by sigma-1 antagonism are affected by the neutralization of the actions of EOPs at the inflamed paw (by administering locally an antibody which recognizes most opioid peptides), and by the reduction of the immune cell load at the site of the inflammation.

Finally, our **sixth goal** was to evaluate whether the ameliorative effects of sigma-1 antagonism in other pain model also involve the actions of EOPs of immune origin. To achieve this goal, we determined the immune cell load in the paw after formalin injection, and tested the effects of S1RA on formalin-induced pain and whether these effects are sensitive to opioid antagonism.

MATERIALS AND METHODS

1. Experimental animals

All experiments were done in female wild-type (WT) (Charles River, Barcelona, Spain) and sigma-1 knockout (KO) (Laboratorios Esteve, Barcelona, Spain) CD-1 mice, weighing 25-30 g. The knockout mice were generated on a CD-1 background as previously described (Entrena et al., 2009b). All mice were housed in a temperature-controlled room (22 ± 2 C) with an automatic 12-hour light/dark cycle (08:00–20:00 h). They were acclimated to our facilities during at least 4 days before the testing took place. The animals were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water *ad libitum.* The experiments were done during the light phase (9:00–15:00 h), and at random times throughout the estrous cycle. All work with the animal was carried out in accordance with institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council directive 86/609).

2. Carrageenan-induced inflammation

Paw inflammation was induced by the intraplantar (i.pl.) injection of 50 µL of a λ-carrageenan solution (1% weight/vol in saline) (Sigma-Aldrich, Madrid, Spain), as previously described (Posadas et al., 2004). Carrageenan solutions were freshly prepared the day of the experiments, and injected into subcutaneous tissue of the plantar surface of the right hindpaw with a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) and a $30^{1/2}$-gauge needle. As a control we used i.pl. injections of saline.

3. Drugs and antibodies for *in vivo* administration

The sigma-1 receptor antagonists used were BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride; Tocris Cookson Ltd., Bristol, United Kingdom) and S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)1H-pyrazol-3-yl] oxy] ethyl] morpholine hydrochloride; kindly supplied by Laboratorios Esteve, Barcelona, Spain). Both sigma-1 antagonists are considered to be selective for sigma-1 receptors (Entrena et al., 2009a; Sánchez-Fernández et al., 2013). PRE-084 (2-[4-morpholinethyl]1-

phenylcyclohexanecarboxylate hydrochloride; Tocris Cookson) was used as a selective sigma-1 receptor agonist (Cobos et al., 2008). The opioid antagonists used were centrally penetrant naloxone hydrochloride and its peripherally-restricted analog naloxone methiodide (Menéndez et al., 2005) (both from Sigma-Aldrich). The nonsteroidal antiinflammatory drug (NSAID) ibuprofen sodium salt (from Sigma-Aldrich) was used as a standard nonopioid analgesic, and the opioid agonist morphine (supplied by Laboratorios Esteve) was used a standard opioid analgesic. The corticosteroid prednisolone (Sigma-Aldrich S.A., Madrid, Spain) was used as a control antiinflammatory drug. All drugs were dissolved in sterile physiological saline (NaCl 0.9%). The solutions of the sigma-1 ligands were appropriately alkalinized with NaOH. To study the effects of systemic treatments, drug solutions (5 mL/kg) were administered s.c. into the interscapular zone. To test the effect of local drug treatments, drug solutions (20 µL) were administered i.pl.

To block the effects of the endogenous opioid peptides produced during the inflammatory process, 20 µL of a solution containing 3-E7 monoclonal antibody, which recognizes the pan-opioid sequence Tyr-Gly-Gly-Phe at the *N*-terminus of most EOPs (Rittner et al., 2001) (MAB5276; Merck Millipore, Madrid, Spain), was administered i.pl. An anti-Ly6G antibody (BE0075-1; Bio X Cell, West Lebanon, NH, USA) was administered intraperitoneally (10 mL/kg) to inhibit neutrophil infiltration (Wang et al., 2012). Saline injections and the administration of nonreactive isotype antibodies (obtained from the same providers as the active antibodies) were used as a control for the effects of 3-E7 (PP102; Merck Millipore) or anti-Ly6G (BE0089; Bio X Cell).

4. General procedures for behavioral assays and drug/antibody treatments in the evaluation of inflammatory hyperalgesia

We initially tested the time-course of carrageenan-induced hyperalgesia and edema (using the methods described in the Sections 5, 6 and 7 of this Chapter). For the time course of thermal hyperalgesia, mice were tested before and repeatedly after carrageenan (or saline) injection each hour for up to 7 h. To record the time-course of carrageenan-induced mechanical hyperalgesia, a different group of mice was tested for each time-point evaluated, since we observed that response latency decreased

progressively in untreated mice when tested repeatedly (data not shown). Then, most experiments were performed during acute (3 h) inflammation, although we also tested some experimental groups during sustained (5 days) inflammation induced by carrageenan.

The sigma-1 receptor antagonists, ibuprofen or morphine (or their solvent) were injected s.c. 30 min before the application of the mechanical or thermal stimulus. To test for the effects of the association of these drugs with PRE-084, naloxone or naloxone methiodide (or their solvent), the sigma-1 agonist, or the opioid antagonists (or solvent) were injected s.c. 5 min before the administration of the sigma-1 antagonist, ibuprofen or morphine.

To test for the effects of the local administration of sigma-1 antagonists, the drugs were injected i.pl. 30 min before the behavioral evaluation. To test for the effects of the local administration of PRE-084 or 3-E7 antibody (or solvent/isotype controls) on the antihyperalgesia induced by systemic sigma-1 antagonists, they were injected i.pl. immediately after the s.c. administration of the sigma-1 antagonist solution (or their solvent). Anti-Ly6G or its isotype control were injected 24 h before inflammation was induced.

Each animal was evaluated in only one pain test, and received a single dose of one sigma-1 antagonist (or its solvent) either alone or associated with PRE-084.

5. Evaluation of mechanical hyperalgesia (paw pressure)

The animals were placed in the experimental room for a 1-h acclimation period before starting the experiments. Mechanical stimulation was applied to the right hindpaw with an Analgesimeter (Model 37215, Ugo-Basile, Varese, Italy) as previously described (Nakamura and Ferreira, 1988; Menéndez et al., 2005; Sánchez-Fernández et al., 2013), with slight modifications. Briefly, the mice were gently restrained and a blunt cone-shaped paw-presser was applied at a constant intensity of 100 g to the dorsal surface of the hindpaw until the animal showed a struggle response. The struggle latency was measured with a chronometer. The test was done twice with a 1-min interval between stimulations, and the mean value of the two trials was recorded as the animal's struggle latency.

6. Evaluation of thermal hyperalgesia (Hargreaves test)

Heat hyperalgesia was tested with the Hargreaves method (Hargreaves et al., 1988) with slight modifications, as previously reported (Nieto et al., 2008). Briefly, the mice were habituated for 2 h in individual opaque evaluation chambers placed on a glass floor at 30 °C. After habituation, a beam of radiant heat was focused to the plantar surface of the right hindpaw with a plantar test apparatus (IITC, CA, USA) until a withdrawal response occurred, and the latency to withdrawal response was recorded. Intensity of the light was adjusted at the start of the experiments to 85 mW/cm^2 with an I.R. Heat-Flux Radiometer (Model 37300, Ugo-Basile), and this intensity was not changed throughout the experiments. With this intensity of stimulation, average baseline latency in naïve animals was about 11 s. Each mouse was tested three times, and the latencies were averaged for each animal. At least 30 s were allowed between consecutive measurements. A cut-off latency time of 20 s was used in each measurement.

7. Determination of carrageenan-induced paw edema

Hindpaw volume was measured with a plethysmometer (Ugo-Basile, Varese, Italy). The hindpaw was placed in a conductive solution (hexamethylenetetramine 0.5%) and the displaced volume was measured to a resolution of 10 µL. The volume of edema was determined by the difference between the values obtained before and after the i.pl. injection of carrageenan or its solvent. To minimize variations in the readings within each animal, the junction of hairy and glabrous skin was marked with a pen before the first measurement, and used as a reference for subsequent determinations.

To elucidate the time-course of the development of carrageenan-induced edema, values were recorded before and hourly up to 7 h after the i.pl. administration of carrageenan or saline in the right hindpaw. To test for possible antiedematous effects of sigma-1 inhibition, we tested carrageenan-induced edema in sigma-1 KO mice and in WT mice treated systemically (s.c.) with the sigma-1 antagonists BD-1063, S1RA or their solvent. Prednisolone was used in these experiments as a control antiinflammatory drug. Paw volume was determined before and 3 h after carrageenan administration, since at this time the increase in paw volume was maximal (see Figure

1*A* for details). We followed two different protocols to test the effects of drugs on carrageenan-induced edema. One set of mice was treated with the sigma-1 antagonists 30 min before the evaluation, to compare the results with those obtained in measures of hyperalgesia. In a different set of mice the drugs were given preemptively 5 min before carrageenan injection, to facilitate the detection of the possible antiedematous effects of the drugs tested.

8. Formalin-induced Pain

The formalin test was performed as previously described (Cendán et al., 2005a). Formalin solution (20 µL, 2.5 %) was injected was injected i.pl. in the right hindpaw, using a Hamilton microsyringe with a $30^{1/2}$-gauge needle. Immediately thereafter, the mouse was put into a glass cylinder and observed. The time spent licking or biting the injected paw during the 45 min period after the injection (divided into periods of 5 min each) was measured. The first phase of formalin-induced pain was recorded for 0–5 min after the injection. The second phase of pain was recorded for 15–35 min after injection. Pain-like responses in the second phase peaked at 20–25 min (see Results and Discussion for details), and paw samples were collected at this time for FACS analysis to determine the immune cell content of the paw tissue.

9. β-endorphin levels in the paw

The paw was excised at the ankle and the bones were removed. Soft tissue was homogenized in 1 mL RIPA buffer containing 0.05% protease inhibitors (P8340; Sigma-Aldrich) and 0.1% phosphatase inhibitors (P0044; Sigma-Aldrich). β-endorphin levels were determined with a fluorescent enzyme immunoassay kit (Phoenix Pharmaceuticals, Karlsruhe, Germany) according to the manufacturer's instructions.

10. FACS analysis

Plantar tissue was dissected and digested with collagenase/DNAse for 1 h at 37 °C (1 mg/mL collagenase IV and 0.1% DNAse I; Worthington, Lakewood, NJ, USA). Samples were filtered (pore size 70 µm), and incubated with antibodies recognizing the hematopoietic cell marker CD45 (14-0451-85, clone 30-F11; eBioscience, Vienna,

Austria), the neutrophil-specific marker Ly6G (127602, clone 1A8; Biolegend, Vienna, Austria), and the myeloid marker CD11b (101243, clone M1/70; Biolegend, Vienna, Austria) for 1 h on ice. The population of macrophages/monocytes, was determined by using the following markers: CD45+CD11b+Ly6G- cells. All antibodies were used at a concentration of 2 μg/mL. Cell viability was determined with DAPI (Sigma-Aldrich). Before and after incubation with the antibodies, the cells were washed three times in 1% FCS/PBS (FACS buffer). Samples were acquired with a BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed with FlowJo software (Treestar, Ashland, OR, USA). Cells were pre-gated in a DAPI-negative population to analyze only living cells.

11. PCR analysis

FACS–purified neutrophils and macrophages/monocytes were sorted directly into Trizol, and total RNA was extracted using phenol-chloroform method. Equal amounts of total RNA were added to the reverse transcriptase enzyme mix (iScript Reverse Transcription Supermix, BioRad, CA, USA). Templates, and primers were added to SYBR Green enzyme mix (iQ™ SYBR® Green Supermix, BioRad), and transcripts encoding pro-opiomelanocortin (POMC) and 18S ribosomal RNA (18srRNA) (as an internal standard) were amplified using Real Time System (CFX Connect, Biorad). Reactions were run for 50 cycles, and the specificity was determined by subsequent melting curve analysis. PCR reactions were run on 2% agarose gel for determination of product size. Following forward and reverse primers (NCBI Primer Blast) were used for amplification: 5'-GGCCGTTCTTAGTTGGTGGAGCG-3' and 5'-CTGAACGCCACTTGTCCCTC-3' for 18srRNA (predicted band size 133bp), 5'-TAGCGGGAGAGAAAGCCGAG-3' and 5' TAGCGGGAGAGAAAGCCGAG-3' for POMC (predicted band size 165bp).

12. Data analysis

When several means were compared, statistical analysis was carried out with one-way or two-way analysis of variance (ANOVA) depending on the experiment, followed by a Bonferroni post-hoc test, except in Figure 18*C* where an unpaired Student's *t*-test was used. ANOVAs were performed with the SigmaPlot 12.0 program (Systat Software Inc.,

San Jose, CA, USA).

The dose-response curves of the antihyperalgesic effects of drugs were estimated with the equation for a sigmoid plot. To compare the effects of drugs on mechanical and thermal hyperalgesia, the degree of antihyperalgesia was calculated as the percentage increase in the latency to response to mechanical or thermal stimulation in drug-treated inflamed mice over vehicle-treated inflamed mice. The value in the latter group was considered as 0%, and the value in non-inflamed mice treated with the vehicle of the drugs was considered 100% of possible recovery. The dose of drug that produced 50% of the maximal effect against carrageenan-induced hyperalgesia (ED_{50}), and the maximum recovery induced by each drug (E_{max}) were calculated from the dose-response curves with nonlinear regression analysis of the equation for a sigmoid plot. Parameters obtained from the sigmoid plots and their standard errors were calculated as the best-fit values ± standard errors of regression; when required, drug potencies were compared by analyzing the goodness-of-fit of different models that had the same or different ED_{50} values. To test whether the E_{max} values of the drugs were statistically distinguishable from the hypothetical 100% recovery value (maximum efficacy) in mechanical and thermal hyperalgesia, we compared the goodness-of-fit of the dose-response curves with and without constraining the E_{max} values to 100%. Snedecor's *F* test was used for all comparisons of parameters from nonlinear regressions, using the GraphPad Prism 5.00 program (GraphPad Software Inc., San Diego, CA, USA).

The differences between values either after ANOVAs or in Snedecor's *F* tests were considered significant when the *p* value was below 0.05.

RESULTS AND DISCUSSION

1. SIGMA-1 RECEPTOR INHIBITION REVERSES ACUTE INFLAMMATORY HYPERALGESIA IN MICE: ROLE OF PERIPHERAL SIGMA-1 RECEPTORS

1.1. Time-course of carrageenan-induced edema, mechanical hyperalgesia and thermal hyperalgesia

We evaluated the time-course of the development of inflammatory edema and hyperalgesia to either a mechanical or thermal stimulus after carrageenan administration in WT mice. Carrageenan injection significantly increased paw volume from the first time-point evaluated (1 h), and this effect persisted throughout the evaluation period (up to 7 h), peaking at 3 h (Figure 1*A*). However, saline (the vehicle used for carrageenan) was rapidly absorbed after its i.pl. administration, producing no significant increase in paw volume at any time-point.

The animals showed a progressive reduction in the time to response to either mechanical pressure or radiant heat (i.e. mechanical and thermal hyperalgesia, respectively). Hyperalgesia was apparent at 2 h and peaked at 3 h after carrageenan administration for both sensory modalities (Figs. 1*B* and *C*, respectively). Once maximal mechanical hyperalgesia had been reached, it was maintained throughout the time-course (7 h); however, thermal hyperalgesia gradually disappeared, and at 6 h after carrageenan administration the animals showed latencies in the response to radiant heat that did not differ significantly from those in untreated animals (time 0). Saline administration (i.pl.) did not modify the response to either mechanical or thermal stimuli at any time-point tested, in comparison to noninjected animals (Figs. 1*B* and *C*).

Therefore, although carrageenan induced both mechanical and thermal inflammatory hyperalgesia, the time-course of the development of the sensory hypersensitivity differed depending on the type of the stimulation. These differences might be attributable to the known differences in the mechanisms for the production of these types of sensory hypersensitivity (Latremoliere and Woolf, 2009; Dubin and Patapoutian, 2010).

Since hypersensitivity to both mechanical and thermal stimuli was fully developed at 3 h after carrageenan administration, the effects of sigma-1 drugs or knockout (KO) shown in this chapter of “Results and Discussion” were assessed at this time.

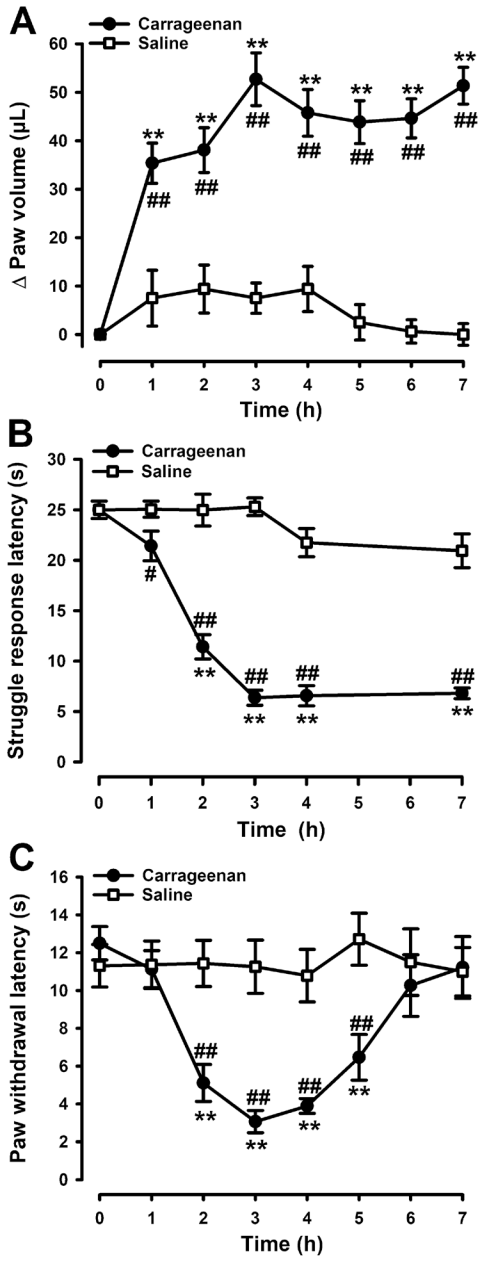


Figure 1. Time-course of carrageenan-induced edema and hyperalgesia to mechanical and thermal stimulation in wild-type mice. The paw volume (*A*), the latency to struggle response evoked by paw pressure (*B*) and the latency to hindpaw withdrawal in response to radiant heat (*C*) were recorded in naïve mice (time 0) and at several time-points after the intraplantar injection of carrageenan or its solvent (saline). Each animal was evaluated in only one test. Each point and vertical line represents the mean ± SEM of the values obtained in the injected paw of 8–10 animals. Statistically significant differences between the values obtained in carrageenan- and solvent-treated animals: $^{**}p < 0.01$; and between the values obtained on time 0 and subsequent evaluations after the injection of carrageenan or its solvent: $\#P < 0.05$, $\#\#P < 0.01$ (two-way repeated measures ANOVA for *A* and *C*, and two-way ANOVA for *B*; Bonferroni post hoc test was used for all comparisons).

1.2. Effects of systemic (subcutaneous) administration of selective sigma-1 drugs on inflammatory hyperalgesia and nociceptive pain

The s.c. administration of BD-1063 or S1RA dose-dependently reversed inflammatory mechanical and thermal hyperalgesia, increasing the response latencies in mice that were stimulated in the carrageenan-injected paw with either pressure or heat (Figure 2A and *B*, respectively). The potencies of these antihyperalgesic effects depended both on the drug tested and the type of sensory stimulation applied. ED_{50} values for the reversion of mechanical and heat hyperalgesia by BD-1063 were 4.2 ± 0.1 and 6.1 ± 0.1

mg/kg, respectively. ED_{50} values of S1RA to reverse mechanical and heat hyperalgesia were 8.5 ± 0.4 and 33.1 ± 2.1 mg/kg, respectively. ED_{50} values were significantly lower ($p < 0.01$) for BD-1063 in comparison to S1RA for either type of sensory stimulation. The higher potency of BD-1063 in comparison to S1RA in the reversion of carrageenan-induced hyperalgesia might be related with its previously reported higher affinity for sigma-1 receptors (Nieto et al., 2012). Both compounds were significantly more potent ($p < 0.01$) in reversing mechanical than thermal hyperalgesia (*F* test). This might indicate that sigma-1 antagonism do not act identically in mechanical and thermal pain pathways.

Although mechanical hyperalgesia was more sensitive to sigma-1 antagonism, both drugs were able to increase the response latencies to either sensory stimuli in inflamed mice up to values close (non-significantly different) to those in non-inflamed control mice (Figure 2*A* and *B*). Therefore, the E_{max} values of BD-1063 and S1RA approached maximum efficacy and did not differ significantly from 100% of the antihyperalgesic effect for either sensory modality (*F* test). Hence, treatment with these sigma-1 antagonists fully reversed both mechanical and thermal inflammatory hyperalgesia.

The range of systemic doses of the sigma-1 antagonists which reversed inflammatory hyperalgesia is similar or even lower (in particular for mechanical hyperalgesia) than the doses previously reported to be active against neuropathic hypersensitivity (Nieto et al., 2012; Díaz et al., 2012; Romero et al., 2012).

Doses of these selective sigma-1 antagonists able to fully reverse mechanical (BD-1063 8 mg/kg and S1RA 32 mg/kg) or thermal (BD-1063 16 mg/kg and S1RA 64 mg/kg) hyperalgesia were unable to alter the response latency to mechanical pressure or radiant heat in non-inflamed mice (Figure 3*A* and *B*, left panels), indicating that they did not induce mechanical or thermal antinociception in the absence of inflammation. These findings are consistent with previously reported data showing that sigma-1 antagonism does not alter nociceptive pain to either mechanical or thermal stimuli (Entrena et al., 2009a and b; Nieto et al., 2012; Romero et al., 2012).

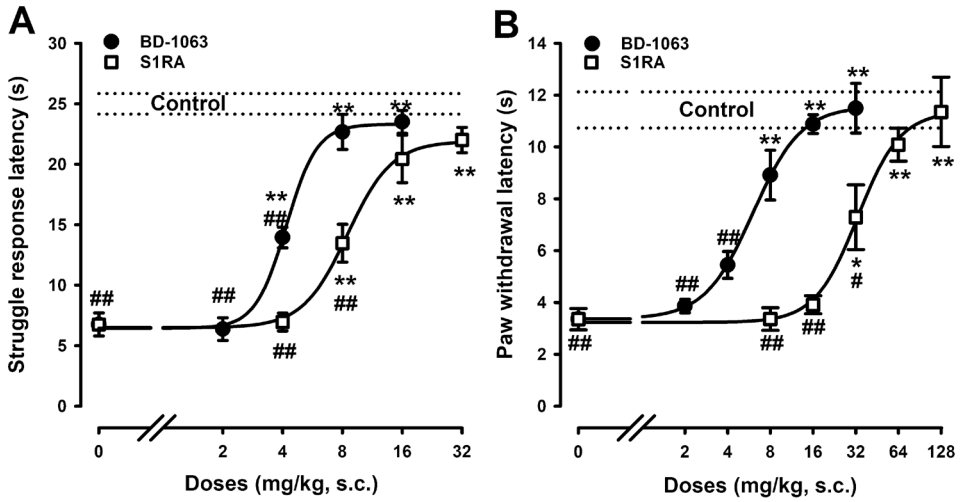


Figure 2. Dose-response curves of the effects induced by the systemic administration of sigma-1 receptor antagonists BD-1063 or S1RA on mechanical and thermal inflammatory hyperalgesia in wild-type mice. The results represent the latency to struggle response evoked by paw pressure (*A*), and the latency to hindpaw withdrawal in response to radiant heat (*B*) in mice treated intraplantarly with carrageenan, and given several doses of BD-1063, S1RA or their solvent (dose 0) subcutaneously (s.c.). Mice were tested (in the paw injected with carrageenan or its solvent) 3 h after the intraplantar injection, and drugs or their solvent were administered 30 min before the evaluation. Each animal was evaluated in only one behavioral pain test and received only one dose of the drug tested (or their solvent). Each point and vertical line represents the mean ± SEM of values obtained in 8-10 animals. The dashed lines (control) represent the mean ± SEM in non-inflamed mice (injected intraplantarly with saline) treated s.c. with the vehicle of the drugs. Statistically significant differences between the values obtained in drug-treated and vehicle-treated groups given either an intraplantar injection of carrageenan (dose 0): **P* < 0.05; ***P* < 0.01, or saline (control): #*P* < 0.05; ##*P* < 0.01 (one-way ANOVA followed by Bonferroni test).

In contrast to the effect of sigma-1 antagonists, the selective sigma-1 agonist PRE-084 (16 mg/kg, s.c.) was unable to alter the latency to response in non-inflamed or inflamed mice stimulated with either pressure or heat (Figure 3*A* and *B*, respectively). A much higher dose of PRE-084 (64 mg/kg ,s.c.) also failed to alter the responses to either type of sensory stimulation in non-inflamed mice (data not shown). However, PRE-084 (2-16 mg/kg) completely abolished, in a dose-dependent manner, the effect on mechanical and thermal hyperalgesia of BD-1063 and S1RA (Figure 3*A* and *B*, right panel), arguing for the involvement of sigma-1 receptors in the antihyperalgesic effects of both drugs.

Therefore, BD-1063 and S1RA fully reversed inflammatory mechanical and thermal hyperalgesia in a PRE-084 sensitive manner, and without affecting nociceptive pain. These results are summarized in Table 1.

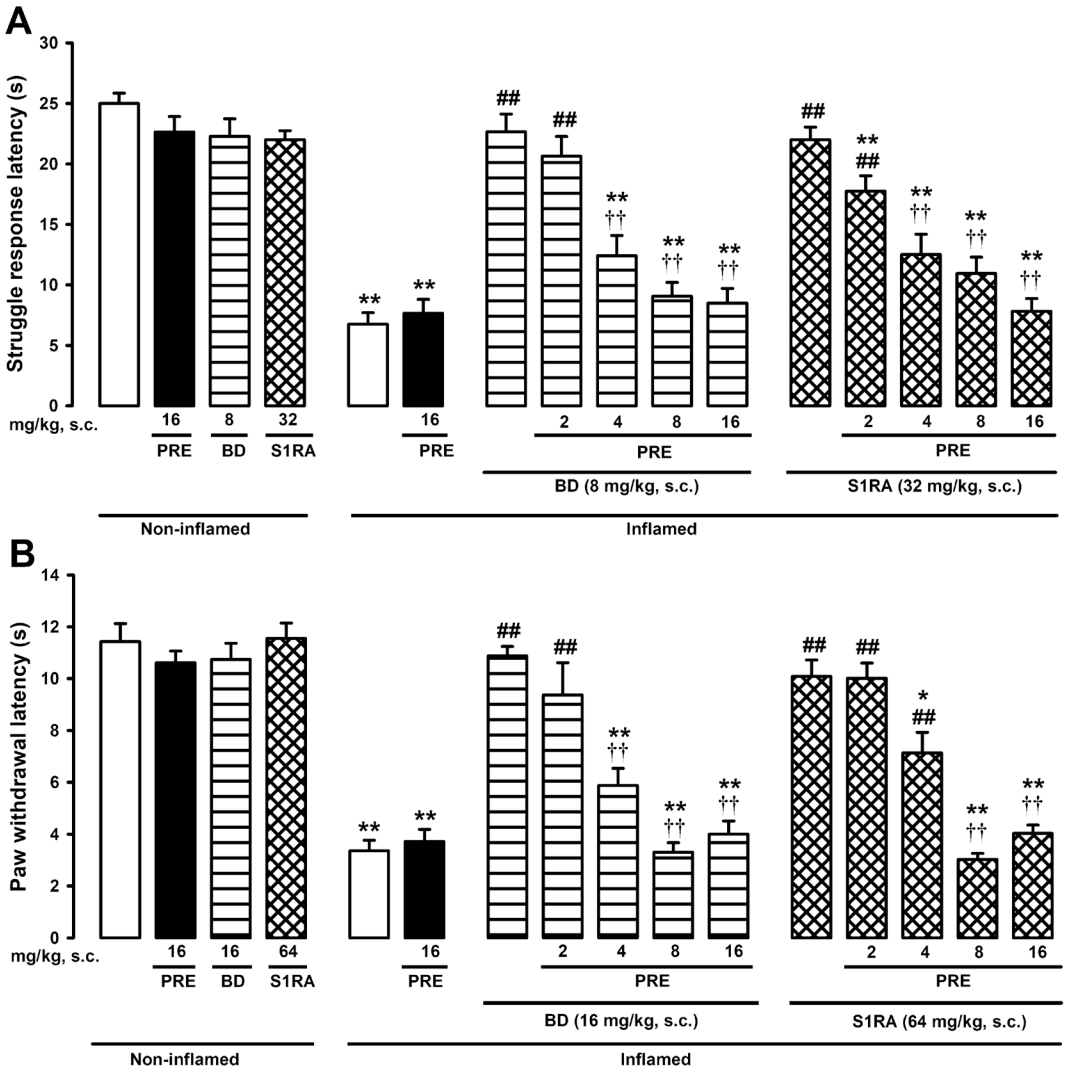


Figure 3. Effects of the systemic administration of sigma-1 antagonists BD-1063 (BD) or S1RA, administered alone or associated with PRE-084 (PRE), on the behavioral responses to mechanical and thermal stimulation in non-inflamed and inflamed wild-type mice. The results represent the latency to struggle response evoked by paw pressure (*A*), and the latency to hindpaw withdrawal in response to radiant heat (*B*) in mice treated intraplantarly with carrageenan (inflamed) or saline (non-inflamed), and then given subcutaneously (s.c.) BD, S1RA or PRE alone, or the association of the sigma-1 antagonists with PRE. Mice were tested (in the paw injected with carrageenan or its solvent) 3 h after the intraplantar injection. The sigma-1 antagonist solution or saline was administered 30 min before the evaluation, and PRE or its solvent was injected 5 min before the former. 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 8-10 animals. Statistically significant differences between the values obtained in non-inflamed mice treated with the solvent of the drugs and inflamed mice treated with drugs or their solvent: **P* <*P* < 0.05, ***P* <*P* < 0.01; between the values obtained in inflamed mice treated with an sigma-1 antagonist and animals treated with its solvent: ##*P* < 0.01; and between the values obtained in inflamed mice treated with an sigma-1 antagonist alone or associated with PRE: ††*P* < 0.01. There were no statistically significant differences between the values obtained in non-inflamed mice treated with the sigma-1 drug or its solvent (one-way ANOVA followed by Bonferroni test).

In contrast to WT mice, sigma-1-KO mice showed no appreciable change in the latency to struggle response to mechanical stimulation of the inflamed hindpaw (Figure 4*A*), indicating that they did not develop mechanical hyperalgesia, as would be expected in the light of the full reversion of mechanical hyperalgesia by sigma-1 pharmacological blockade in WT mice. However, we found an apparent discrepancy between the abolishment of heat hypersensitivity in WT treated with sigma-1 antagonists and the normal development of heat hyperalgesia in sigma-1–KO mice, since sigma-1-KO mice developed inflammatory thermal hypersensitivity to the same degree as WT animals, with a clear decrease in the response latency when stimulated in the inflamed hindpaw with heat (Figure 4*B*).

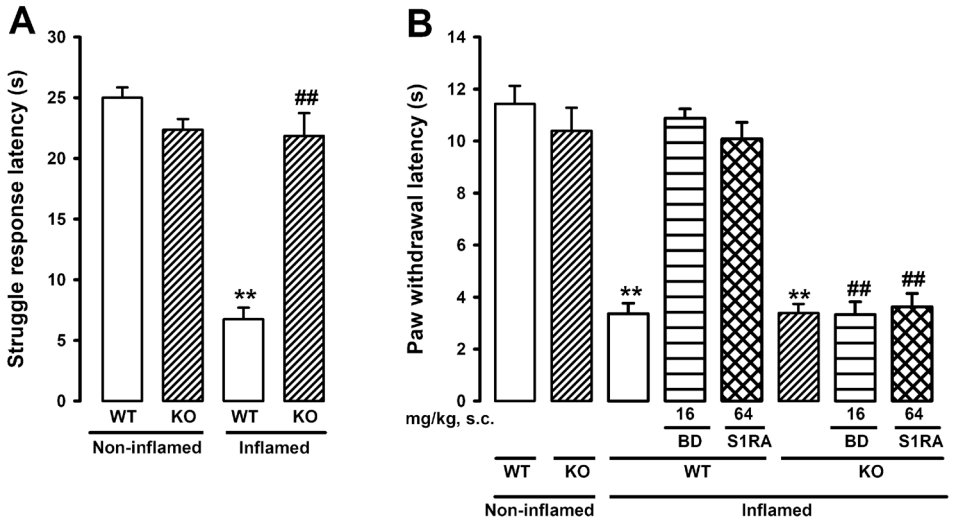


Figure 4. Comparison of mechanical and heat hyperalgesia induced by carrageenan in wild-type (WT) and sigma-1 receptor knockout (sigma-1-KO) mice, and effects of the systemic administration of BD-1063 (BD) or S1RA on thermal hyperalgesia in WT and sigma-1-KO mice. The results represent the latency to struggle response evoked by paw pressure (*A*), and the latency to hindpaw withdrawal in response to radiant heat (*B*) in WT and sigma-1-KO mice treated intraplantarly with carrageenan (inflamed) or saline (non-inflamed). Mice were tested (in the paw injected with carrageenan or its solvent) 3 h after the intraplantar injection, and the sigma-1 antagonist solution or saline was administered subcutaneously (s.c.) 30 min before the evaluation (where indicated). Each animal was evaluated in only one pain test and received only one drug treatment. Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in inflamed and non-inflamed animals of the same genotype: $^{**}P < 0.01$; and between the values obtained in WT and sigma-1-KO mice under the same experimental conditions: $\#\#P < 0.01$ (one-way ANOVA followed by Bonferroni test).

The pattern of response of WT and sigma-1 KO mice in models of neuropathic pain mimics that found in the present study with a model of acute inflammation. In neuropathic pain studies, sigma-1 KO mice showed an absolute lack of mechanical and cold allodynia whereas WT animals showed both (de la Puente et al., 2009; Nieto et al., 2012); however, sigma-1 KO mice showed heat hyperalgesia to the same extent as WT mice (de la Puente et al., 2009). Interestingly, in models of neuropathic pain, systemically administered sigma-1 antagonists inhibited not only mechanical and cold allodynia (Nieto et al., 2012; Romero et al., 2012) but also heat hyperalgesia (Díaz et al., 2012; Romero et al., 2012), a finding which also mimics the results of the present study and highlights the difference between the effect of genetic and pharmacological blockade of sigma-1 receptors in heat hyperalgesia. In addition, it has been reported that sigma-1 antagonists increase morphine-induced antinociception against mechanical and thermal stimuli in WT mice, whereas in sigma-1 KO mice the antinociceptive effect of morphine and other opioid agonists is enhanced against mechanical but not thermal stimuli (Sánchez-Fernández et al., 2013 and 2014; Vidal-Torres et al., 2013). These results indirectly suggest that compensatory mechanisms develop in the heat pain pathways of sigma-1-KO mice. Conflicting results between the pharmacological and genetic inhibition of targets other than sigma-1 receptors were also found in previous pain studies (e.g. Petrus et al., 2007; Bonin et al., 2011) and in fields other than pain research (e.g. Guscott et al., 2005; Voss et al., 2010). Therefore, this appears to be a general concern in experiments with knockout animals rather than a particularity of sigma-1–KO mice, and indicates that caution should be used in drawing conclusions when a mutant mouse strain is the only tool available to study a putative therapeutic target. The differential development of thermal and mechanical hyperalgesia in sigma-1–KO mice also suggests that the mechanisms involved in the modulation of each type of inflammatory hyperalgesia by sigma-1 are not absolutely identical, supporting also the previously commented differences in the potency of both sigma-1 antagonists tested in reversing mechanical or heat hyperalgesia.

Regardless of the exact mechanisms involved in the absence of a pain phenotype in knockout mice, they can be used to evaluate drug specificity by testing whether drugs still show activity when their pharmacological target is absent (Petrus et al., 2007;

Vidal-Torres et al., 2013; González-Cano et al., 2013). Using this approach, we found that the s.c. doses of BD-1063 and S1RA (16 and 64 mg/kg, respectively) that induced a maximal antihyperalgesic effect to heat stimulation in inflamed WT mice had no such antihyperalgesic effect in sigma-1-KO mice (Figure 4*B*). The inactivity of both sigma-1 antagonists in mice lacking sigma-1 receptors, at doses that were highly efficacious in WT mice, strongly suggests the involvement of on-target mechanisms in their effects on thermal hyperalgesia.

The results are summarized in Table 1

1.3. Effects of local (intraplantar) administration of selective sigma-1 drugs on inflammatory hyperalgesia and nociceptive pain

To test whether sigma-1 receptors were able to modulate inflammatory hyperalgesia acting on the periphery, we tested the effects of i.pl. injections of sigma-1 drugs.

The local administration of PRE-084 (75 µg/paw, equivalent to 211.9 nmol) in the hindpaw of either non-inflamed or inflamed mice did not alter their behavioral responses to mechanical stimulation (Figure 5*A*). However, when PRE-084 was administered i.pl. in the carrageenan-injected paw of inflamed mice, it completely abolished the effects of the systemic (s.c.) administration of BD-1063 or S1RA (which were injected at doses that exert maximal mechanical antihyperalgesia, i.e. 8 mg/kg and 32 mg/kg, respectively) (Figure 5*A*). To test for possible systemic effects of the i.pl. administration of PRE-084, we injected it in the non-inflamed paw of inflamed mice, and evaluated the animals' response to mechanical stimulation in the carrageenan-injected paw. Under these conditions, PRE-084 did not alter the mechanical antihyperalgesic effects of BD-1063 or S1RA (Figure 5*B*).

The i.pl. administration of PRE-084 (75 µg/paw) was also unable to alter the behavioral response of non-inflamed or inflamed mice to thermal stimuli (Figure 5*C*), but completely abolished the antihyperalgesic effect on thermal hyperalgesia of the s.c. administration of BD-1063 and S1RA (both administered at doses that fully reversed thermal hypersensitivity, i.e. 16 and 64 mg/kg, respectively) (Figure 5*C*). The effect of

PRE-084 administered i.pl. was observed exclusively in the injected paw: both sigma-1 antagonists (administered s.c.) still induced maximum thermal antihyperalgesia in the inflamed paw when PRE-084 was injected in the non-inflamed paw (Figure 5*D*). This suggests that blockade of peripheral sigma-1 receptors in the inflamed site is involved in the antihyperalgesic effects induced by the sigma-1 antagonists.

Therefore, local treatment with the sigma-1 agonist PRE-084 in the inflamed (but not in the non-inflamed) paw abolished the ameliorative effects of systemically administered sigma-1 antagonists on inflammatory hyperalgesia, without altering nociceptive pain. These results are summarized in Table 1.

To test whether the pharmacological blockade of peripheral sigma-1 receptors located in the inflamed site would elicit antihyperalgesic effects, we administered BD-1063 and S1RA i.pl. in the carrageenan-injected paw. The local administration of either BD-1063 (25-75 µg/paw, equivalent to 72.2-216.7 nmol) or S1RA (75-125 µg/paw, equivalent to 200.6-334.3 nmol) in the inflamed paw dose-dependently and fully reversed inflammatory mechanical hyperalgesia, increasing the response latencies in the inflamed paw subjected to paw pressure (Figure 6*A*, right panel). The antihyperalgesic effects induced by BD-1063 and S1RA were fully reversed by PRE-084 (16 mg/kg, s.c.) (Figure 6*A*, right panel). In contrast to the prominent effects on mechanical hyperalgesia induced by the local administration of these sigma-1 antagonists, they had no mechanical antinociceptive effects in the absence of inflammation (Figure 6*A*, left panel). To test for possible systemic effects of the i.pl. administration of the highest doses used of BD-1063 and S1RA (75 and 125 µg/paw, respectively), we injected the drugs in the non-inflamed paw and evaluated the response to a mechanical stimulus in the carrageenan-injected paw. Under these conditions both drugs failed to alter carrageenan-induced mechanical hyperalgesia (Figure 6*B*).

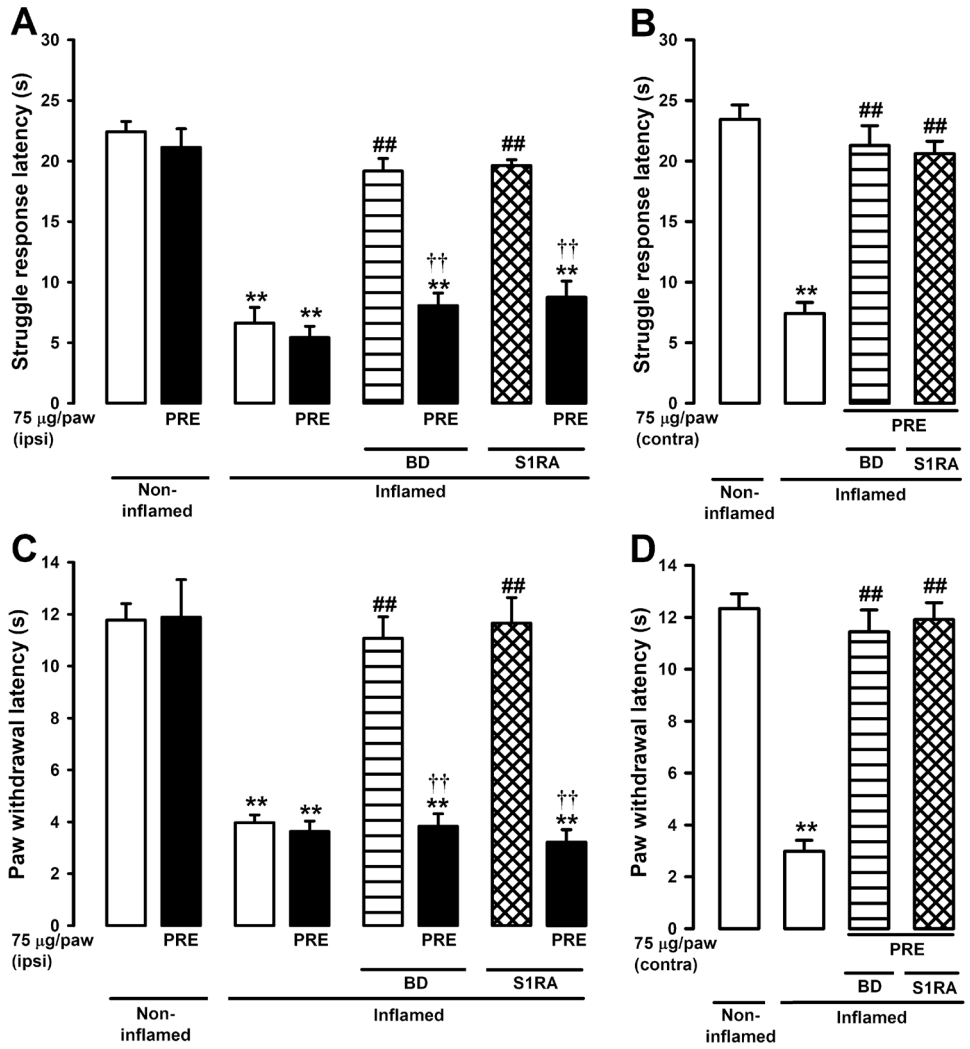


Figure 5. Effects of the systemic administration of the sigma-1 antagonists BD-1063 (BD) or S1RA, alone or associated with the intraplantar (i.pl.) injection of PRE-084 (PRE), on the behavioral responses to mechanical and thermal stimulation in non-inflamed and inflamed wild-type mice. The results represent the latency to struggle response evoked by paw pressure in mice injected i.pl. with carrageenan (inflamed) or saline (non-inflamed), and treated subcutaneously (s.c.) with sigma-1 antagonists (BD 8 mg/kg, S1RA 32 mg/kg) or their solvent (*A* and *B*), and the latency to hindpaw withdrawal in response to radiant heat in inflamed and non-inflamed mice treated s.c. with sigma-1 antagonists (BD 16 mg/kg, S1RA 64 mg/kg) or their solvent (*C* and *D*). Mice were also treated i.pl. with PRE or its solvent in the same paw (ipsi) as carrageenan or saline (*A* and *C*), or in the paw contralateral (contra) to carrageenan (*B* and *D*). All mice received sensory stimulation in the paw into which carrageenan or its solvent was injected 3 h after administration. The sigma-1 antagonist solution was administered 30 min before the evaluation, and PRE was injected immediately after the sigma-1 antagonist. 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 8-10 animals. Statistically significant differences between the values obtained in non-inflamed mice treated with the solvent of the drugs and inflamed mice treated with drugs or their solvent: $^{**}P < 0.01$; between the values obtained in mice treated with the sigma-1 antagonists and those treated with their solvent: $^{\#\#}P < 0.01$; and between the values obtained in mice treated with an sigma-1 antagonist alone or associated with PRE in the inflamed paw: $^{\dagger\dagger}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

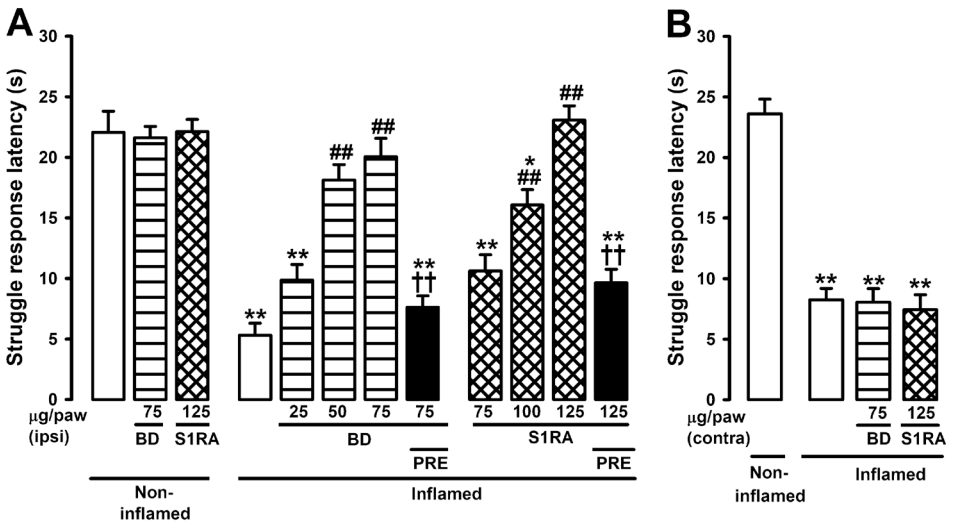


Figure 6. Effect of the local administration of BD-1063 (BD) or S1RA on the behavioral responses to mechanical stimulation of non-inflamed and inflamed wild-type mice. The results represent the latency to struggle response evoked by paw pressure in mice injected intraplantarly (i.pl.) with carrageenan (inflamed) or saline (non-inflamed). Mice were treated i.pl. with BD, S1RA or their solvent in the same paw (ipsi) as carrageenan or saline (*A*); or in the paw contralateral (contra) to carrageenan or its solvent (*B*). Some animals were pretreated subcutaneously with PRE-084 (PRE, 16 mg/kg) or its solvent before the i.pl. administration of BD or S1RA (*A*). All mice received sensory stimulation in the paw injected with carrageenan or its solvent 3 h after their administration. The sigma-1 antagonist solution was administered 30 min before the evaluation, and PRE was injected 5 min before the former. Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in non-inflamed mice treated with the solvent of the drugs and inflamed mice treated with drugs or their solvent: $*P < 0.05$, $**P < 0.01$; between the values obtained in inflamed mice treated with the sigma-1 antagonists and those treated with their solvent: $\#\#P < 0.01$; and between the values obtained in mice treated in the inflamed paw with an sigma-1 antagonist alone or associated with PRE: $\dagger\dagger P < 0.01$. There were no statistically significant differences between the values obtained in inflamed mice when the sigma-1 antagonist or its solvent was injected in the paw contralateral to carrageenan (one-way ANOVA followed by Bonferroni test).

Therefore, treatment with sigma-1 antagonists in the inflamed (but not in the non-inflamed) paw induced maximal mechanical antihyperalgesic effects, which were sensitive to PRE-084. However, the same treatments failed to alter mechanical nociceptive pain. These results are summarized in Table 1.

In contrast to the effects of the local administration of BD-1063 in mechanical hyperalgesia, the i.pl. administration of BD-1063 (75 μg) was unable to modify carrageenan-induced thermal hypersensitivity (Figure 7). In fact, this drug unexpectedly induced thermal hyperalgesia when injected i.pl. in non-inflamed WT

mice (Figure 7). Importantly, BD-1063-induced hyperalgesia was also detected in non-inflamed sigma-1-KO mice (Figure 7), which indicates that this effect cannot be attributed to the interaction of BD-1063 with sigma-1 receptors, and therefore is exerted by other mechanisms.

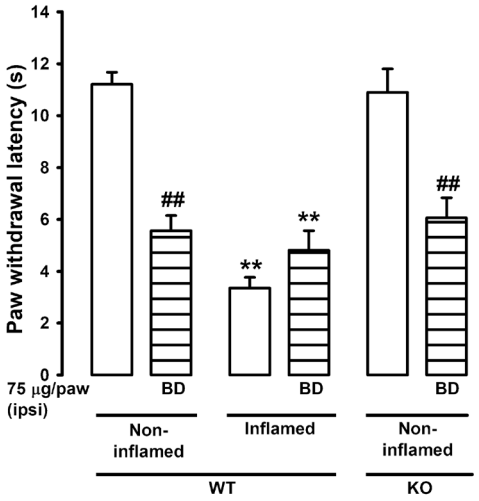


Figure 7. Effect of local administration of BD-1063 (BD) on the behavioral response to heat stimuli in non-inflamed and inflamed wild-type (WT) mice, and in non-inflamed sigma-1 receptor knockout (sigma-1-KO) mice. The results represent the latency to hindpaw withdrawal in response to radiant heat of mice intraplantarly (i.pl.) injected with carrageenan or saline (inflamed and non-inflamed mice, respectively), followed by the administration of BD (75 µg/paw) or saline. All injections and sensory stimulations were performed in the right hindpaw. Animals were evaluated 3 h after the administration of carrageenan or its solvent, and received BD or its solvent 30 min before the evaluation. Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in non-inflamed mice of both genotypes treated with the sigma-1 antagonist and animals treated with their solvent: ##$P < 0.01$; between the values obtained in non-inflamed mice treated with the solvent of the drugs and inflamed mice treated with BD or its solvent: **$P < 0.01$. There were no statistically significant differences between the values obtained in inflamed WT mice treated with BD and those treated with its solvent (one-way ANOVA followed by Bonferroni test).

However, S1RA (100-150 µg/paw, equivalent to 267.5-401.2 nmol) induced marked reversion of thermal hyperalgesia when administered in the inflamed paw of WT mice (Figure 8*A*, right panel). The antihyperalgesic effect of S1RA was fully reversed by PRE-084 (16 mg/kg, s.c.) and was not observed in sigma-1-KO mice (Figure 8*A*, right panel), suggesting that S1RA acts on sigma-1 receptors to inhibit heat hyperalgesia. As we found for systemic treatment with this sigma-1 antagonist, higher doses of S1RA injected i.pl. were needed to reverse thermal than mechanical hyperalgesia (compare Figures 6*A* and 8*A*). Despite the effects of the i.pl. administration of S1RA on

inflammatory thermal hyperalgesia, it did not modify the response to thermal stimulation of non-inflamed mice of either genotype (Figure 8*A*, left panel). We also tested for possible systemic effects of the i.pl. administration of the highest dose of S1RA (150 µg/paw) by injecting it into the non-inflamed paw, and found that it did not alter thermal hyperalgesia in the inflamed paw (Figure 8*B* and *C*). These results are summarized in Table 1.

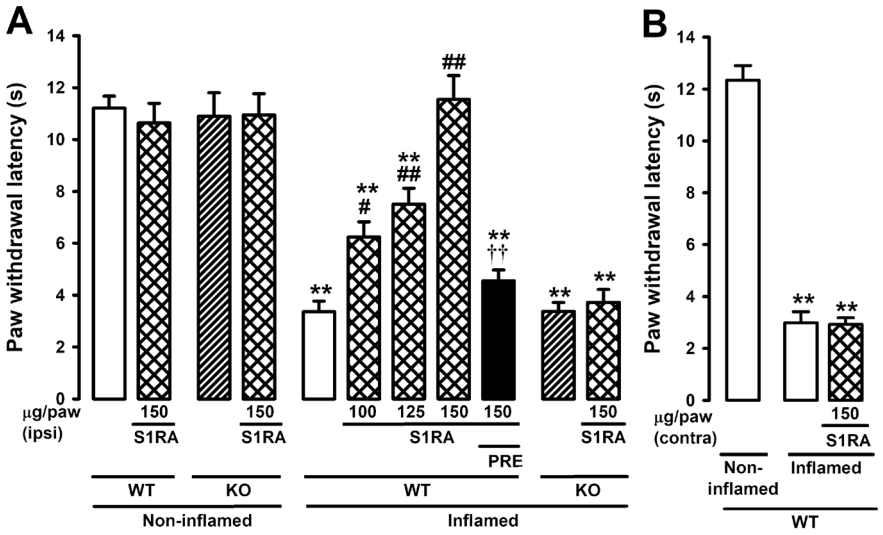


Figure 8. Effect of the local administration of S1RA on the behavioral responses to thermal stimulation of non-inflamed and inflamed wild-type (WT) and sigma-1 receptor knockout (sigma-1-KO) mice. The results represent the latency hindpaw withdrawal in response to radiant heat in mice injected intraplantarly (i.pl.) with carrageenan (inflamed) or saline (non-inflamed). Mice were treated i.pl. with BD, S1RA or their solvent in the same paw (ipsi) as carrageenan or saline (**a**); or in the paw contralateral (contra) to carrageenan or its solvent (**b**). Some animals were pretreated subcutaneously with PRE-084 (PRE, 16 mg/kg) or its solvent before the i.pl. administration of S1RA (**a**). All mice received sensory stimulation in the paw injected with carrageenan or its solvent 3 h after their administration. The sigma-1 antagonist solution was administered 30 min before the evaluation, and PRE was injected 5 min before the former. Each animal received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in non-inflamed mice treated with the solvent of the drugs and inflamed mice treated with S1RA or its solvent: $^{**}P < 0.01$; between the values obtained in inflamed mice treated with S1RA and those treated with its solvent: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; and between the values obtained in mice treated in the inflamed paw with S1RA alone or associated with PRE: $^{\dagger\dagger}P < 0.01$. There were no statistically significant differences between the values obtained in inflamed (sigma-1-KO) mice when treated in the inflamed paw with S1RA or its solvent, and between the values from inflamed WT mice when S1RA or its solvent was injected in the paw contralateral to carrageenan (one-way ANOVA followed by Bonferroni test).

Although BD-1063 is considered to be a prototypical sigma-1 antagonist, the selectivity of this drug was tested only in a panel of 11 receptors (Matsumoto et al., 1995 and 2001), and therefore additional actions in targets related to thermal sensitivity cannot be ruled out. In contrast, S1RA was recently shown to exhibit exquisite selectivity for sigma-1 receptors in a panel comprising 170 targets (Romero, et al., 2012), and was able to abolish thermal hyperalgesia when administered locally in the inflamed site. This effect was mediated through sigma-1 inhibition, since it was reversed by PRE-084 and was absent in sigma-1-KO mice. Importantly, the effects induced by the administration of sigma-1 drugs (agonist or antagonists) in the inflamed paw were mediated locally, since injection of these drugs in a distant site (the paw contralateral to the inflammation) was devoid of effect.

The possibility of targeting peripheral sigma-1 receptors to ameliorate inflammatory hyperalgesia is of particular importance, since all previous studies of the role of sigma-1 receptors in tonic/chronic pain ascribed the ameliorative effects of sigma-1 inhibition to the decreased amplification of pain signaling within the spinal cord (central sensitization) (Cendán et al., 2005a and b; Entrena et al., 2009a and b; Kim et al., 2006; Roh et al., 2008 and 2011; de la Puente et al., 2009; Nieto et al., 2012; Romero et al., 2012), and overlooked the possible role of peripheral sigma-1 receptors on peripheral sensitization. In support of a role for peripheral sigma-1 receptors in pain processing, it was reported that they are present in the DRG (Sánchez-Fernández et al., 2014; Bangaru et al., 2013), and at much higher density than in pain-related central areas, including the dorsal spinal cord (Sánchez-Fernández et al., 2014). The activation (phosphorylation) of extracelular signaling-related kinases (ERK) is a key process involved in the sensitization of both central and peripheral pain pathways (Latremoliere and Woolf, 2009; Ji, 2004), and we have found that the increase in pERK1/2 in the dorsal spinal cord during neuropathy was attenuated by sigma-1 receptor inhibition (de la Puente et al., 2009; Nieto et al., 2012). However, we observed no apparent increase in pERK1/2 levels in either the spinal cord dorsal horn or DRG after carrageenan administration (data not shown). This not only indicates that different mechanisms are involved in sensory hypersensitivity in experimental models of inflammatory and neuropathic pain, as previously reported (see Scholz and Woolf,

2002; Ji, 2004; Patapoutian et al., 2009; Latremoliere and Woolf, 2009, for reviews), but also that the mechanisms involved in the modulation of neuropathic and inflammatory pain by sigma-1 receptors might also be different and worth further exploration.

1.4. Effects of sigma-1 receptor inhibition on inflammatory edema

We also evaluated whether sigma-1 inhibition reduced inflammatory edema by testing changes in paw volume in response to carrageenan injected i.pl. in sigma-1-KO mice and WT mice treated with sigma-1 antagonists.

The i.pl. administration of saline (vehicle for carrageenan) did not appreciably increase paw volume in either WT or sigma-1-KO mice. Carrageenan injection produced marked edema in the injected hindpaw of WT mice, and this was not significantly attenuated in sigma-1-KO mice (Figure 9). Pharmacological sigma-1 blockade by BD-1063 (16 mg/kg, s.c.) or S1RA (64 mg/kg, s.c.) in WT mice was unable to alter carrageenan-induced edema, regardless of whether the drugs were administered as preemptive treatment (i.e. 5 min before the i.pl. injection) (Figure 9) or after carrageenan was injected but 30 min before the paw volume was recorded (data not shown). However, preemptive treatment with the control antiinflamatory drug prednisolone (10 mg/kg, s.c.) fully abolished the edema produced by the inflammatory agent in WT mice (Figure 9). Since sigma-1 inhibition was unable to reverse the edema induced by carrageenan, antiedematous effects of sigma-1 inhibition do not account for the antihyperalgesic effects observed, and suggest that pain processing is modulated by sigma-1 inhibition rather than inflammation.

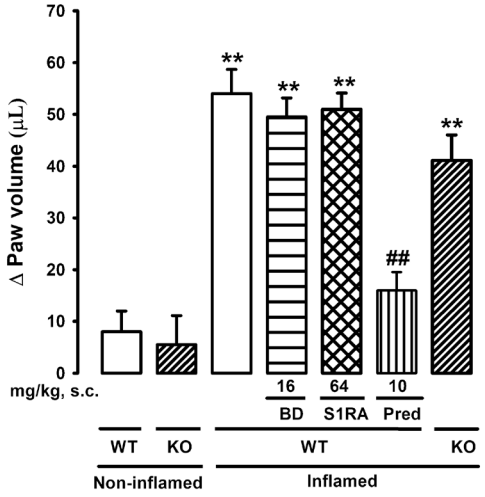


Figure 9. Effects of sigma-1 inhibition on carrageenan-induced paw edema. The results represent the increase in paw volume of the injected paw in wild-type (WT) and sigma-1 receptor knockout (sigma-1-KO) mice treated intraplantarly with carrageenan (inflamed) or saline (non-inflamed). Mice were tested 3 h after the intraplantar injection. BD-1063 (BD), S1RA, prednisolone (Pred) or their solvent were administered subcutaneously (s.c.) to WT mice 5 min before the intraplantar injection of carrageenan. Each bar and vertical line represent the mean ± SEM of at least 8 mice. Statistically significant differences between the values in inflamed and non-inflamed mice of the same genotype: $^{**}P < 0.01$; and between inflamed WT mice treated with prednisolone or its solvent: $^{\#\#}P < 0.01$. There were no statistically significant differences between the values obtained in inflamed WT mice treated with an sigma-1 antagonist or its solvent, or between inflamed WT and sigma-1-KO mice, and neither between non-inflamed WT mice and carrageenan-treated WT mice injected with Pred (one way ANOVA followed by Bonferroni test).

Table 1 Summary of the experimental approaches and the main results obtained in this part of the study on the role of sigma-1 receptors on acute inflammatory hyperalgesia. The figures that show the results for different experiments are indicated.

				Effect on inflammatory hyperalgesia			
Genotype	**σ_1 antagonist**		**σ_1 agonist (PRE-084)**	**Mechanical (paw pressure)**		**Thermal (radiant heat)**	
Wild-type	BD-1063	s.c.	-	Antihyperalgesia	Figure 2a	Antihyperalgesia	Figure 2b
			s.c.	Reversion of antihyperalgesia	Figure 3a	Reversion of antihyperalgesia	Figure 3b
			i.pl.	Reversion of antihyperalgesia	Figure 5a	Reversion of antihyperalgesia	Figure 5c
		i.pl.	-	Antihyperalgesia	Figure 6a	No effect[a]	Figure 7
			s.c.	Reversion of antihyperalgesia	Figure 6a	Not tested[b]	-
	S1RA	s.c.	-	Antihyperalgesia	Figure 2a	Antihyperalgesia	Figure 2b
			s.c.	Reversion of antihyperalgesia	Figure 3a	Reversion of antihyperalgesia	Figure 3b
			i.pl.	Reversion of antihyperalgesia	Figure 5a	Reversion of antihyperalgesia	Figure 5c
		i.pl.	-	Antihyperalgesia	Figure 6a	Antihyperalgesia	Figure 8a
			s.c.	Reversion of antihyperalgesia	Figure 6a	Reversion of antihyperalgesia	Figure 8a
σ_1-KO	-	-	-	Antihyperalgesia	Figure 4a	Normal hyperalgesia[c]	Figure 4b
	BD-1063	s.c.	-	Not tested[d]	-	Abolishment of the antihyperalgesia induced by σ_1 antagonists	Figure 4b
	S1RA	s.c.	-	Not tested[d]	-		
		i.pl.	-	Not tested[d]	-	Abolishment of the antihyperalgesia induced by S1RA	Figure 8a

Drugs were administered systemically (subcutaneous, s.c.) or locally (i.pl.). The results shown for i.pl. treatments are only to those obtained when both sensory stimulation and drug administration were performed in the inflamed paw. No effect was observed on the behavioral responses to mechanical or thermal stimuli when animals were stimulated in the inflamed paw and drugs were administered in the non-inflamed paw. None of the experimental manipulations resulted in significant changes in the behavioral responses to mechanical or thermal stimuli in non-inflamed mice, with the exception of the i.pl. administration of BD-1063, which induced thermal hyperalgesia. See "Results and Discussion" for details.

[a] The unspecific thermal hyperalgesia induced by this treatment interferes with its possible antihyperalgesic effects.

2. THE MECHANISM: SIGMA-1 RECEPTORS CONTROL IMMUNE-DRIVEN PERIPHERAL OPIOID ANALGESIA DURING INFLAMMATION

2.1. The effects of sigma-1 antagonists on acute inflammatory hyperalgesia are sensitive to peripheral opioid antagonism

In agreement with the results showed in the preceding chapter, mice showed a significant mechanical and heat hyperalgesia 3 h after carrageenan-induced acute inflammation (Figure 10*A* and *B*, respectively). The systemic administration of sigma-1 antagonists BD-1063 and S1RA fully attenuated this inflammatory hyperalgesia to mechanical and thermal stimuli (Figure 10*A* and *B*), at doses that did not modify the latency to mechanical or thermal stimulation in mice without inflammation (Figure 11*A* and *B*). In contrast to the sigma-1 antagonists, the selective sigma-1 agonist PRE-084 had no effect on the response latency in mice without inflammation or in mice with inflammation stimulated with either pressure or heat (Figure 11*A* and *B*, respectively), but abolished the inhibitory effects of BD-1063 and S1RA on mechanical and thermal hyperalgesia (Figure 10*A* and *B*).

Interestingly, the ameliorative effects induced by BD-1063 or S1RA on inflammatory hyperalgesia to mechanical or thermal stimuli were also reversed by the opioid antagonists naloxone and naloxone methiodide (Figure 10*A* and *B*). To control for the specificity of the effects induced by these opioid antagonists, experiments in which antihyperalgesic effects were induced with the nonsteroidal anti-inflammatory drug ibuprofen and the opioid agonist morphine were conducted. Both ibuprofen and morphine induced dose-dependent antihyperalgesic effects to mechanical and thermal stimuli (Figure 12*A* and *B*). Naloxone and naloxone methiodide reversed the antihyperalgesic effects induced by morphine (at the same doses that reversed the effects of the sigma-1 antagonists), but had no effect on ibuprofen-induced antihyperalgesia (Figure 12*C* and *D*). These results indicate that the opioid antagonists used in this study are unable to reverse the analgesic effects induced by drugs without opioid action. To our knowledge, this is the first report of a reversion by opioid antagonism of the ameliorative effects of sigma-1 antagonists on pathological pain.

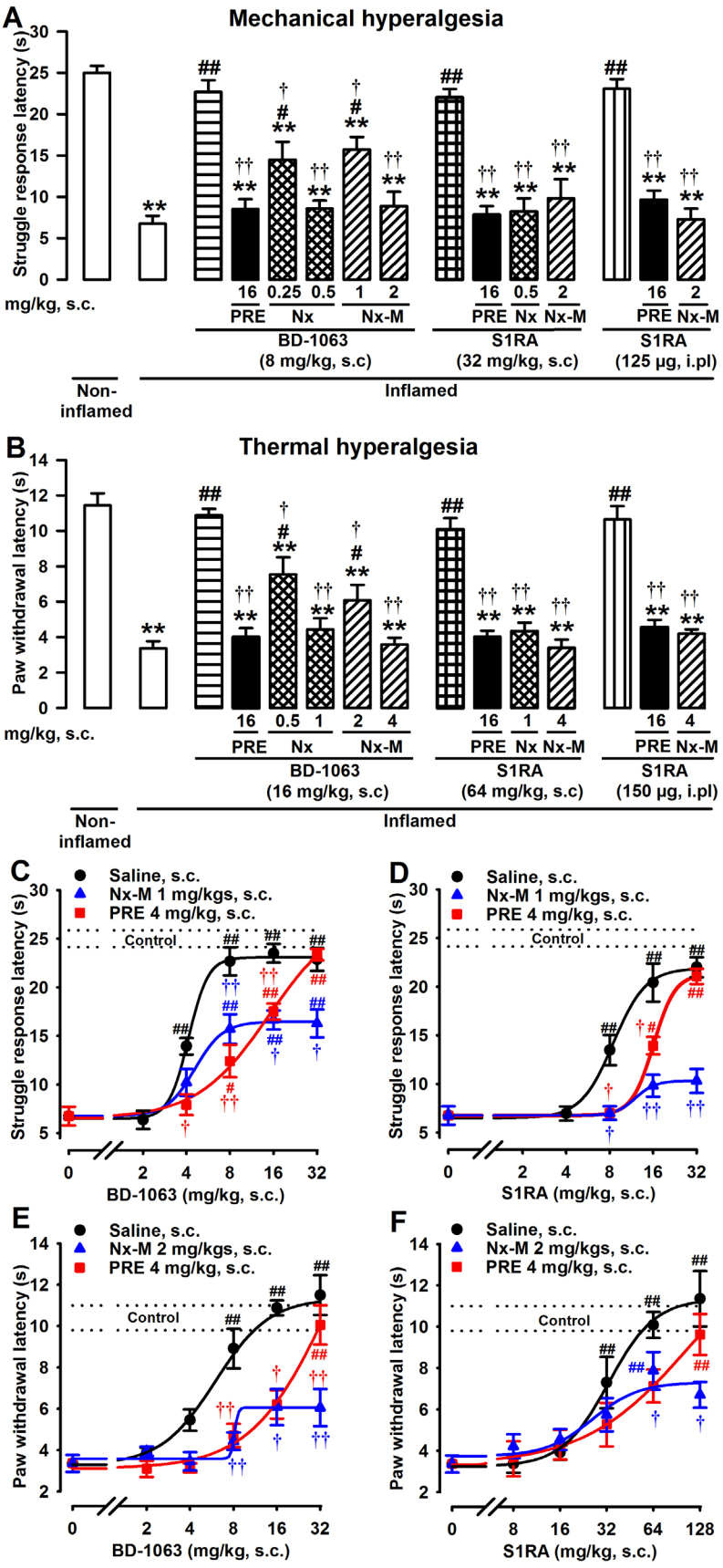


Figure 10. Reversion of the antihyperalgesic effects of sigma-1 antagonists by both opioid antagonism and sigma-1 agonism during acute inflammation. Mice were evaluated 3 h after intraplantar (i.pl.) injection with carrageenan (inflamed) or saline (non-inflamed). Sigma-1

antagonists were administered either subcutaneously (s.c.) or i.pl.; PRE-084 (PRE), naloxone (Nx) and naloxone methiodide (Nx-M) were administered s.c. Effects induced by a single dose of sigma-1 antagonists BD-1063 (BD) or S1RA on hyperalgesia to mechanical (*A*) or thermal (B) stimuli, and reversion by sigma-1 agonist PRE and opioid antagonists Nx and Nx-M. Dose-response curves of the effects induced by BD-1063 (*C*) and S1RA (*D*) on carrageenan-induced mechanical hyperalgesia, and by BD-1063 (*E*) and S1RA (*F*) on inflammatory thermal hyperalgesia. Sigma-1 antagonists were administered alone or with fixed doses of PRE or Nx-M, or solvent controls. The dashed lines (control) represent the mean ± SEM in mice without inflammation. Bars or points show means ± SEM from 8–10 animals. ***P*<0.01, mice without vs. mice with inflammation (for clarity these comparisons are omitted in panels *C*-*F*); #*P*<0.05, ##*P*<0.01 mice with inflammation treated with sigma-1 antagonists vs. mice with inflammation treated with solvent controls; †*P*<0.05, ††*P*<0.01, mice with inflammation treated with sigma-1 antagonist alone vs. mice with inflammation treated with PRE, Nx or Nx-M; one-way ANOVA followed by Bonferroni test.

In contrast to the known central penetrability of naloxone, naloxone methiodide (a quaternary derivative of naloxone) is unable to cross the blood–brain barrier, and is therefore a peripherally-restricted opioid antagonist (Menéndez et al., 2005). Naloxone methiodide fully reversed the antihyperalgesic effects induced by systemic sigma-1 antagonists, highlighting the importance of peripheral actions on the analgesic effects of these drugs. In fact, the intraplantar administration of S1RA to the inflamed site fully reversed the observed hyperalgesia to mechanical and thermal stimuli, and these effects were also reversed by PRE-084 and by the peripherally-restricted drug naloxone methiodide (Figure 10*A* and *B*).

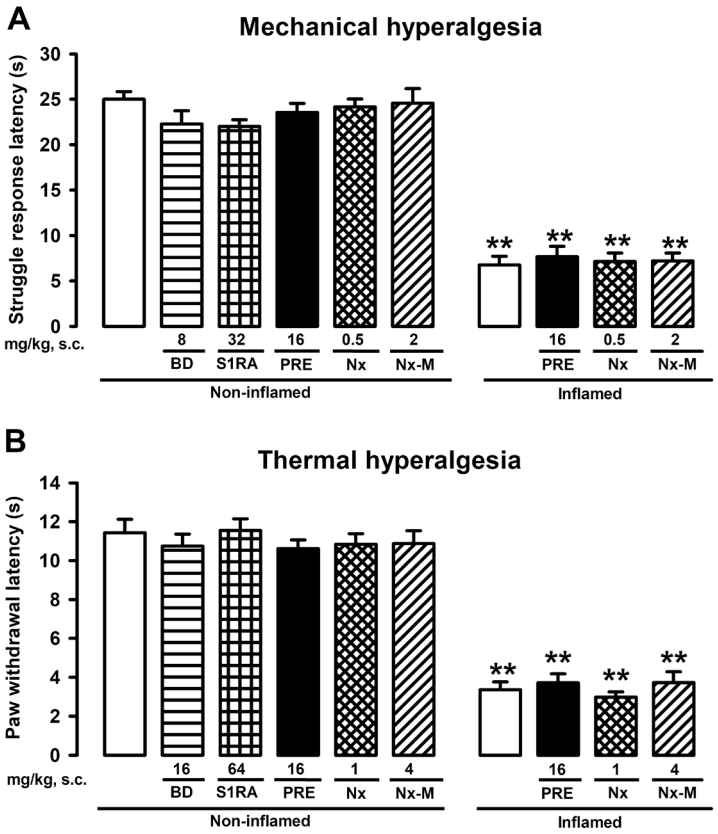


Figure 11. Absence of effects of sigma-1 agonism and opioid antagonism in mice with or without inflammation. Mice were injected intraplantarly (i.pl.) with carrageenan (inflamed) or saline (non-inflamed), and subcutaneously (s.c.) with sigma-1 agonist PRE-084, opioid antagonists naloxone and naloxone methiodide, or solvent controls, and subjected to mechanical (*A*) or thermal (*B*) stimulation. Bars show means ± SEM from 8–10 animals. ***P*<0.01, mice without vs. mice with inflammation; no significant differences between values from mice without inflammation treated with the drugs or their solvent, or between the values from mice with inflammation treated with the drugs or their solvent; one-way ANOVA followed by Bonferroni test.

To determine whether the inhibition by PRE-084 or naloxone methiodide of the antihyperalgesic effects induced by BD-1063 or S1RA on carrageenan-induced acute inflammation were of a competitive or noncompetitive nature, we tested whether the effects of fixed doses of PRE-084 or naloxone methiodide were overcome by increasing doses of sigma-1 antagonists (Rang et al., 2007). Inhibition by PRE-084 of the antihyperalgesic effects induced by BD-1063 or S1RA, to either mechanical (Figure 10*C* and *D*) or thermal (Figure 10*E* and *F*) stimuli, were fully overcome by increasing the dose of sigma-1 antagonists. This indicates a competitive interaction between PRE-084 and both sigma-1 antagonists. These results agree with the pharmacological profile of

these drugs, as it is known that PRE-084, BD-1063, and S1RA bind to sigma-1 receptors (Cobos et al., 2008; Romero et al., 2012).

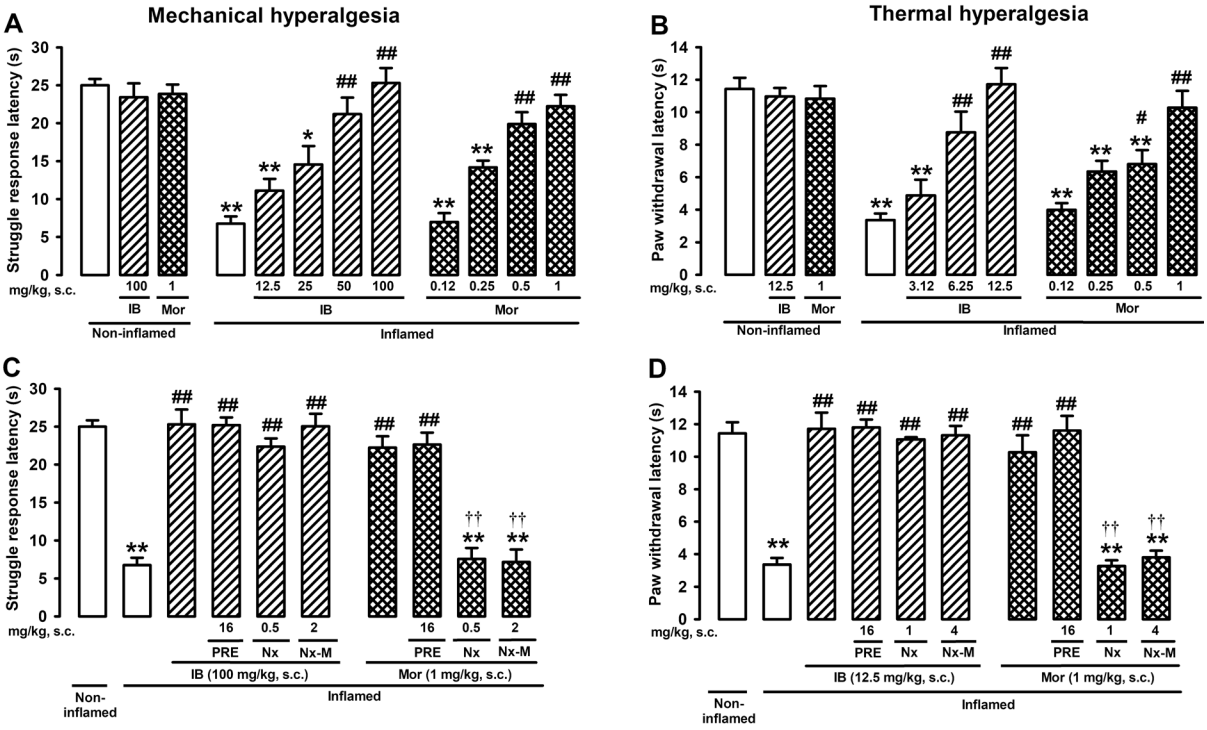


Figure 12. Opioid antagonism reverses the antihyperalgesic effect induced by morphine but not the effect induced by ibuprofen. Inhibitory effects of ibuprofen (IB) and morphine (Mor) on mechanical (*A*) and thermal (*B*) inflammatory hyperalgesia. Effects of sigma-1 agonist PRE-084 (PRE), or opioid antagonists naloxone (Nx) or naloxone methiodide (Nx-M) with IB or Mor on mechanical (*C*) or thermal (*D*) hyperalgesia. Mice were injected intraplantarly (i.pl.) with carrageenan (inflamed) or saline (non-inflamed), and subcutaneously (s.c.) with the drugs or solvent controls. Bars show means ± SEM from 8–10 animals. **P*<0.05, ***P*<0.01, mice without vs. mice with inflammation; #P<0.05, ##P<0.01, mice with inflammation treated with IB or Mor vs. mice with inflammation treated with solvent controls; ††*P*<0.01, mice treated with Nx or Nx-M vs. mice treated with solvent controls, among those mice with inflammation and treated with Mor; one-way ANOVA followed by Bonferroni test.

In contrast, increasing the dose of BD-1063 or S1RA was unable to overcome the inhibition by naloxone methiodide of the antihyperalgesic effects of these sigma-1 antagonists to mechanical (Figure 10*C* and *D*) or thermal (Figure 10*E* and *F*) stimuli, indicating that naloxone methiodide inhibits the effects of the sigma-1 antagonists in a noncompetitive manner. Naloxone methiodide does not bind to sigma-1 receptors (Sánchez-Fernández et al., 2014) and, conversely, the sigma-1 antagonists BD-1063 and

S1RA do not bind to opioid receptors (Romero et al., 2012; Sánchez-Fernández et al., 2013). However, sigma-1 receptors can form macromolecular complexes with opioid receptors to produce tonic inhibition of receptor functioning (Kim et al., 2010; Rodríguez-Muñoz et al., 2015). Sigma-1 antagonism is well known to increase opioid agonist-induced signaling, resulting in the potentiation of opioid analgesia by sigma-1 antagonists (Kim et al., 2010; Rodríguez-Muñoz et al., 2015). Therefore, a possible explanation for the sensitivity of sigma-1-mediated antihyperalgesic effects to opioid antagonism is that naloxone methiodide antagonizes peripheral opioid receptors, thereby impeding the action of endogenous opioid agonists produced at the site of inflammation (whose action is maximized by sigma-1 antagonism), resulting in noncompetitive inhibition of the antihyperalgesic effects induced by sigma-1 antagonism. This hypothesis necessarily implies that during inflammation, the production of EOPs that can be modulated by sigma-1 receptors is increased at the site of inflammation.

2.2. Sigma-1 antagonism and endogenous opioid peptides during acute inflammation

The effects of inhibiting the action of EOPs on the antihyperalgesic effects induced by sigma-1 antagonists were investigated. The actions of EOPs at the inflamed site were neutralized by local administration of the monoclonal antibody 3-E7, which recognizes the pan-opioid sequence Tyr-Gly-Gly-Phe at the *N*-terminus of most EOPs (Rittner et al., 2001). Intraplantar administration of 3-E7 (5 µg/paw) did not alter the behavioral responses to mechanical stimuli in mice with or without inflammation (Figure 13*A*), indicating that even if EOPs were present in the inflamed paw, the levels were not sufficient to alleviate hyperalgesia under inflammatory conditions. However, administration of 3-E7 into the inflamed paw abolished the antihyperalgesic effects in response to mechanical stimuli induced by systemic administration of BD-1063 or S1RA (Figure 13*A*). The same dose of an isotype control antibody did not alter the behavioral responses to mechanical stimuli in animals with or without inflammation regardless of whether they were treated or not with sigma-1 antagonists (Figure 13*A*). The effect of

3-E7 antibody on the antihyperalgesic effect induced by sigma-1 antagonism was seen exclusively in the injected paw: both BD-1063 and S1RA still induced maximal mechanical antihyperalgesia in the inflamed paw when the antibody was injected in the non-inflamed paw (Figure 13*B*). Identical results were seen for the effects of sigma-1 antagonists on thermal hyperalgesia: 3-E7 administration in the inflamed paw abolished the antihyperalgesic effects of BD-1063 and S1RA, without altering the responses to heat stimuli in control mice with or without inflammation, and the isotype control was without effect under all experimental conditions (Figure 13*C*). Injection of 3-E7 in the paw contralateral to the site of inflammation also had no effect on the antihyperalgesia induced by sigma-1 antagonists, indicating again a local effect of this antibody (Figure 13*D*). These results show that the antihyperalgesic effects of sigma-1 antagonists are due to the action of EOPs at the inflamed site.

Since the early 1990s sigma-1 antagonists have been known to potentiate the analgesic effects of opioid drugs, and it was therefore suggested that an anti-opioid sigma-1 system tonically inhibits opioid drug-induced analgesia in the central nervous system (Chien and Pasternak, 1993). Our data show for the first time that peripheral tonic inhibition of opioid analgesia is produced physiologically during inflammation by sigma-1 receptors, limiting the ability of EOPs to induce endogenous opioid analgesia, thereby facilitating inflammatory pain.

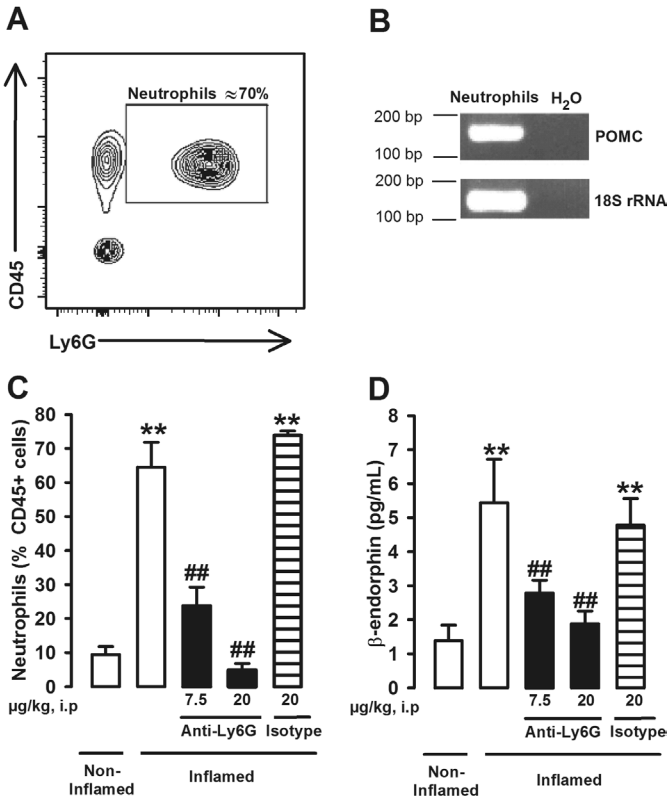


Figure 13. Endogenous opioid peptides (EOPs) at the inflamed site participate in the antihyperalgesic effects of sigma-1 antagonists. Mice were evaluated 3 h after intraplantar (i.pl.) injection with carrageenan (inflamed) or saline (non-inflamed). Animals were treated subcutaneously (s.c.) with sigma-1 antagonists (BD-1063 and S1RA) or solvent controls, and subjected to mechanical (*A* and *B*) or thermal stimulation (C and *D*). Mice were also treated i.pl. with 3-E7 anti-EOP monoclonal antibody, its isotype control, or solvent control in the same paw (ipsi) as carrageenan or saline (*A* and *C*), or in the paw contralateral (contra) to carrageenan (*B* and *D*). All mice received sensory stimulation in the paw into which carrageenan or its solvent was injected. Bars show means ± SEM from 8–10 animals. ***P*<0.01, mice without inflammation treated with the solvent of the drugs or antibodies vs. mice with inflammation; ##*P*<0.01, mice with inflammation treated with sigma-1 antagonists vs. mice with inflammation treated with solvent control; ††*P*<0.01, mice with inflammation treated with a sigma-1 antagonist alone vs. mice with inflammation treated with 3-E7 antibody in the inflamed paw; one-way ANOVA followed by Bonferroni test.

2.3. Neutrophils and endogenous opioid peptides

We wanted to identify the source of the endogenous opioid agonists responsible for the antihyperalgesic effects induced by sigma-1 antagonism. Immune cells are known to produce and secrete EOPs (Rittner and Stein, 2005; Hua and Cabot, 2010), and they naturally accumulate at inflamed sites. Fluorescence-activated cell sorting (FACS) with cell-specific markers in tissue from the inflamed paw was used to determine the

predominant types of hematopoietic cells (CD45+ cells) in the paw during carrageenan-induced acute inflammation. Neutrophils (CD45+Ly6G+ cells) constituted the majority (about 70%) of hematopoietic cells in the inflamed paws 3 h after carrageenan administration (Figure 14*A*), as expected in acute inflammation. Macrophages/monocytes (CD45+CD11b+Ly6G- cells) were also present but to a lesser extent (about 10% CD45+ cells) (Figure 15*A*). As neutrophils were the predominant type of myeloid cell in the inflamed paw, and β-endorphin is known to be produced by these immune cells (Sahbaie et al., 2012), we determined whether neutrophils were able to produce this EOP under our experimental conditions. We found that neutrophils express pro-opiomelanocortin (POMC) mRNA (Figure 14B), the precursor of β-endorphin (Hua and Cabot, 2016). Ly6G is selectively present in neutrophils, and is needed for migration and recruitment of these immune cells (Wang et al., 2012). The actions of Ly6G can be inhibited *in vivo* by the systemic administration of an anti-Ly6G antibody (Wang et al., 2012). The *in vivo* administration of anti-Ly6G antibody (7.5-20 μg) resulted in complete, dose-dependent inhibition of neutrophil infiltration in the inflamed paw, whereas the administration of its isotype control (20 μg) had no effect on neutrophil levels (Figure 14*C*). However, treatment with anti-Ly6G had no impact on macrophage/monocyte infiltration in the inflamed tissue (Figure 15*B*), indicating the specificity of this approach to reduce neutrophil levels. Mice were found to have increased β-endorphin in the inflamed paw, and *in vivo* administration of anti-Ly6G dose-dependently reduced the levels of this EOP, whereas the isotype control antibody had no effect (Figure 14*D*). These results mirrored the effects of neutrophil depletion in response to anti-Ly6G administration, and suggest that neutrophils contribute to the production of this EOP in carrageenan-induced acute inflammation. β-endorphin is thought to be the predominant EOP produced by immune cells (Hua and Cabot, 2010), and consequently here we tested its levels to exemplify that under our experimental conditions immune cells can produce EOPs. However, leukocytes have also been shown to produce enkephalins and dynorphins (Rittner and Stein, 2005), and they might also play a role in our results. We therefore hypothesize that EOP production by neutrophils may participate in the naloxone-sensitive antihyperalgesic effects of sigma-1 antagonists during acute inflammation.

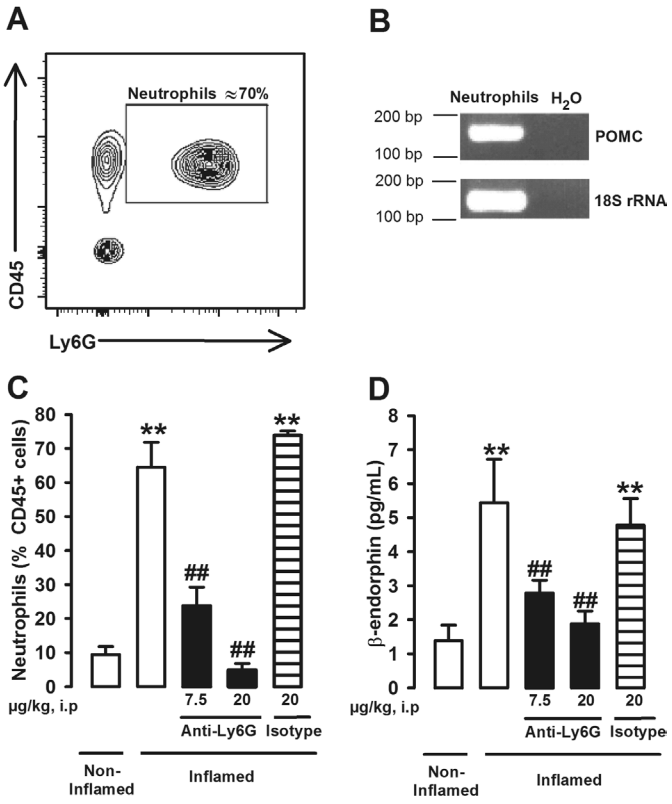


Figure 14. Neutrophils contribute to the production of β-endorphin in the carrageenan-injected paw during acute inflammation. (*A*) Representative FACS (fluorescence-activated cell sorting) diagram showing CD45+Ly6G+ cells from the inflamed paw, corresponding to neutrophils. (*B*) Real-time PCR products for pro-opiomelanocortin (POMC) mRNA and 18S ribosomal RNA (18S rRNA) as an internal standard (predicted band sizes 165bp and 133bp, respectively) from FACS-purified neutrophils. (*C*) Effects of *in vivo* treatment with anti-Ly6G on the population of neutrophils in the inflamed paw, determined by FACS. (*D*). Effects of *in vivo* treatment with anti-Ly6G on β-endorphin levels in the inflamed paw, measured by fluorescent enzyme immmunoassay. Mice were treated intraperitoneally (i.p.) with anti-Ly6G antibody, solvent control or isotype control antibody, and injected intraplantarly (i.pl.) with carrageenan (inflamed) or saline (non-inflamed) 3 h before obtaining the samples. Graphs show means ± SEM from n = 6–10 determinations. ***P*<0.01, mice without vs. mice with inflammation; ##*P*<0.01, mice with inflammation treated with anti-Ly6G vs. mice with inflammation treated with solvent control.

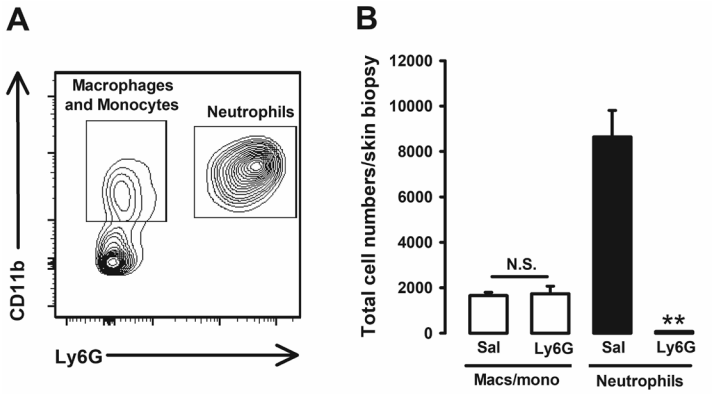


Figure 15. The *in vivo* treatment with anti-Ly6G decreases neutrophil load in the paw during carrageenan-induced acute inflammation without altering the number of macrophages/monocytes. (*A*) Representative FACS (fluorescence-activated cell sorting) diagram of CD45+ cell populations showing that neutrophils CD11b+Ly6G+ are the most abundant leukocytes in the inflamed paw 3 h after carrageenan injection, whereas macrophages/monocytes (CD11b+Ly6G-) constitute a minor population. (*B*) Effects of *in vivo* treatment with anti-Ly6G on neutrophils and macrophages/monocytes (macs/mono) in the inflamed paw. Mice were treated intraperitoneally with anti-Ly6G antibody or its solvent control, and injected intraplantarly with carrageenan 3 h before obtaining the samples. Graph show means ± SEM from n = 4 determinations. **P<0.01 number of neutrophils in mice treated with anti-Ly6G vs. solvent control. There were no statistical differences (N.S.) between the number of macrophages from mice treated with anti-Ly6G vs. solvent control; one-way ANOVA followed by Bonferroni test.

2.4. Neutrophils, edema, hyperalgesia and sigma-1 receptors

Although neutrophils (and other immune cells) can produce EOPs, and therefore may participate in decreasing pain during inflammation (Hua and Cabot, 2010), it is conventionally accepted that they promote pain by synthesizing and releasing algogenic chemicals (Ji et al., 2014), and also participate in the development of edema (Wang et al., 2014). In turn, edema can increase pressure on nociceptive nerve endings also involved in pain during inflammation (Julius and Basbaum, 2001). We therefore tested the effects of anti-Ly6G treatment on inflammatory edema and hyperalgesia. Carrageenan induced prominent edema 3 h after its administration, which was monitored as the increase in volume of the injected paw (Figure 16A). This edema was decreased in a dose-dependent manner by anti-Ly6G treatment, although only partly and at high doses (10–20 µg), whereas the isotype control antibody (20 µg) lacked effect (Figure 16*A*). These results are in agreement with previous reports, where high doses of anti-Ly6G antibody were needed to ameliorate inflammatory edema (Wang et

al., 2012). Neither treatment with anti-Ly6G nor the isotype control modified the response latencies in mice without inflammation subjected to mechanical or thermal stimuli (Figure 16*B* and *C*, respectively), indicating that neutrophils do not play a role in acute nociception to either type of stimulus. Treatment with anti-Ly6G, but not with the isotype control, increased the response latency to mechanical stimuli in mice with inflammation (Figure 16*B*) at doses that decreased edema, whereas neither anti-Ly6G or the isotype control antibody had an effect on thermal hyperalgesia at any dose tested (Figure 16*C*).

Peripheral sensory neurons are specialized in detecting specific sensory stimuli, and therefore the mechanisms for thermal and mechanical nociception are not fully overlapping (Juslius and Basbaum, 2001). Our data suggest that neutrophils may participate in the development of mechanical hyperalgesia by promoting edema, with a consequent increase in the stimulation of pressure-sensitive nociceptors, but that other sources of algogenic chemicals apart from these immune cells account for carrageenan-induced thermal hypersensitivity.

The influence of neutrophils on the antihyperalgesic effects induced by sigma-1 antagonists during carrageenan-induced acute inflammation was then explored. For these experiments, a submaximal dose of anti-Ly6G (7.5 µg), which was enough to markedly decrease neutrophil infiltration at the inflamed site without significantly altering inflammatory edema or the behavioral responses of mice with or without inflammation to mechanical or thermal stimuli (Figure 16*D* and *E*, respectively) was used. This dose of anti-Ly6G abolished the ameliorative effects of BD-1063 and S1RA on inflammatory mechanical and thermal hypersensitivity (Figure 16*D* and *E*, respectively), whereas a high dose of isotype control (20 µg) had no effect (Figure 16*D* and *E*). These results suggested that the observed effects were specific. Together, our findings show that the naloxone-sensitive antihyperalgesic effects induced by sigma-1 antagonism on carrageenan-induced acute inflammation require the presence of EOPs produced by neutrophils (which constitute the majority of the immune infiltrate) at the inflamed site.

Although in this research work we have not determined the specific opioid receptor subtypes involved in the actions mediated by sigma-1 receptors, it is known that sigma-1 receptors can modify the analgesic effects induced by μ, δ or κ agonists (Sánchez-Fernández et al., 2017). Taking into account that immune cells produce a wide variety of EOPs (Rittner et al., 2001; Brack et al., 2004; Labuz et al 2006; Sauer et al., 2014; Wang et al. 2014) with diverse preference for distinct opioid receptors subtypes (Rónai et al., 2009; Pasternak and Pan, 2013; Liu et al., 2017), it is likely that several opioid receptor subtypes are involved in the effects we observed. It is tempting to speculate that the higher potency of sigma-1 antagonists in reversing mechanical than thermal hyperalgesia is determined by the known differences in the expression of opioid receptor subtypes in mechano- and heat-sensitive primary afferents (Scherrer et al., 2009), and their interaction with their respective peptide ligands.

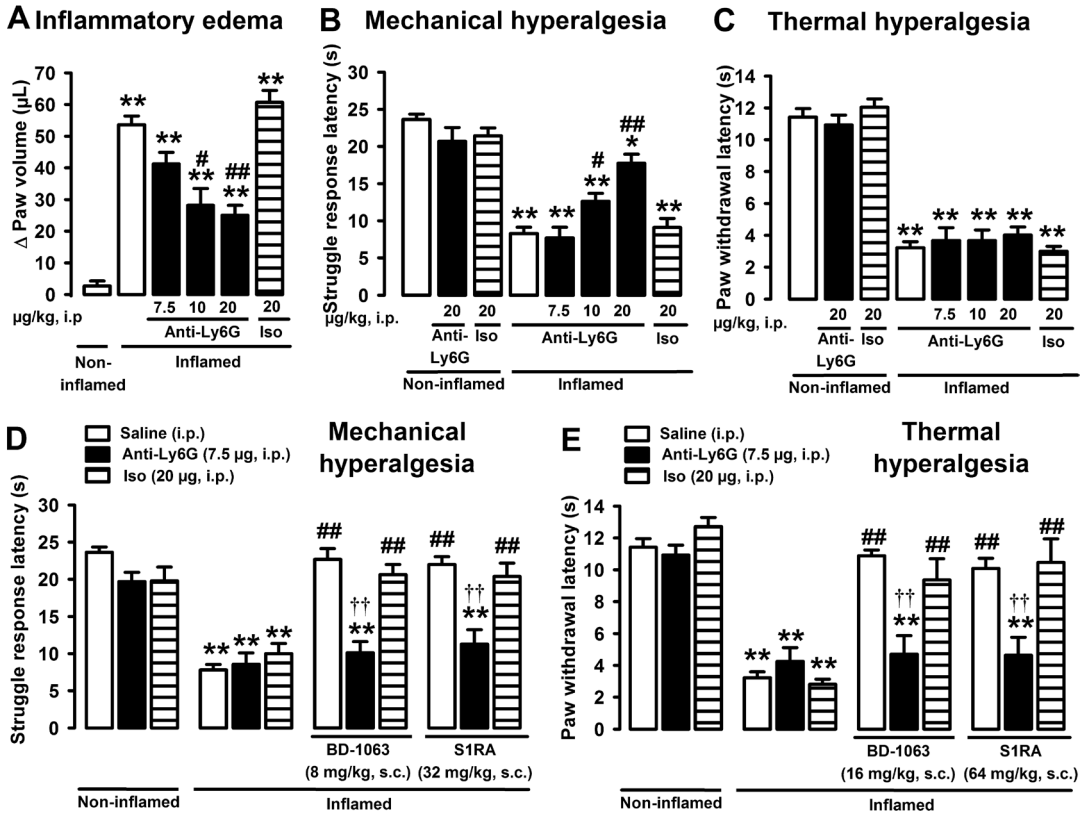


Figure 16. Neutrophils contribute to both inflammatory hyperalgesia and the antihyperalgesic effects of sigma-1 antagonists during acute inflammation. Effect of the administration of anti-Ly6G antibody on: inflammatory edema (*A*), mechanical hyperalgesia (*B*), thermal hyperalgesia (*C*), and on the effects of sigma-1 antagonists on mechanical (*D*) and thermal (*E*) hyperalgesia. Mice were treated intraperitoneally (i.p.) with anti-Ly6G, solvent control or isotype (iso) control, and injected intraplantarly (i.pl.) with carrageenan (inflamed) or saline (non-inflamed) 3 h before the evaluation (*A*-*E*). Mice were treated subcutaneously (s.c.) with sigma-1 antagonists (BD-1063 and S1RA) or solvent controls (*D* and *E*). Bars show means ± SEM from 8–10 animals. **P*<0.05, ***P*<0.01, mice without inflammation treated with solvent controls or antibodies vs. mice with inflammation; #*P*<0.05, ##*P*<0.01, mice with inflammation treated with anti-Ly6G or sigma-1 antagonists alone vs. mice with inflammation treated with solvent control; ††*P*<0.01, mice with inflammation treated with a sigma-1 antagonist alone vs. with inflammation treated with anti-Ly6G antibody; one-way ANOVA followed by Bonferroni test.

2.5. Naloxone-Sensitive effects of sigma-1 antagonists in sustained inflammation

As the predominant immune cell types vary with the time course of the inflammation (Rittner et al., 2001), we sought to determine whether the naloxone-sensitive antihyperalgesic effects of sigma-1 antagonists were preserved when the predominant myeloid cells during inflammation differ from neutrophils. The presence of neutrophils

5 days after carrageenan administration was almost negligible while the presence of macrophages/monocytes (CD11b+Ly6G- cells) was largely increased, constituting the majority (about 70%) of CD45+ cells (Figure 17*A*). Similar to neutrophils, macrophages/monocytes were found to express POMC mRNA (Figure 17*B*). These data are consistent with previous reports showing that all immune cell subpopulations produce EOPs (Rittner and Stein, 2005), and that distinct leukocyte lineages are the main source of these peptides at different stages of inflammation (Rittner et al., 2001). Under this sustained inflammatory condition, animals showed a prominent mechanical hyperalgesia which was reversed by the sigma-1 antagonists S1RA and BD-1063 (Figure 17*C*). The effects of these sigma-1 antagonists were abolished by both the sigma-1 agonist PRE-084 and the peripheral opioid antagonist naloxone methiodide (Figure 17*C*), indicating that both sigma-1 receptors and peripheral opioid receptors are involved in the effects induced by these drugs during sustained inflammation, and support that immune-driven peripheral opioid analgesia induced by sigma-1 antagonism is not limited to neutrophilic inflammation.

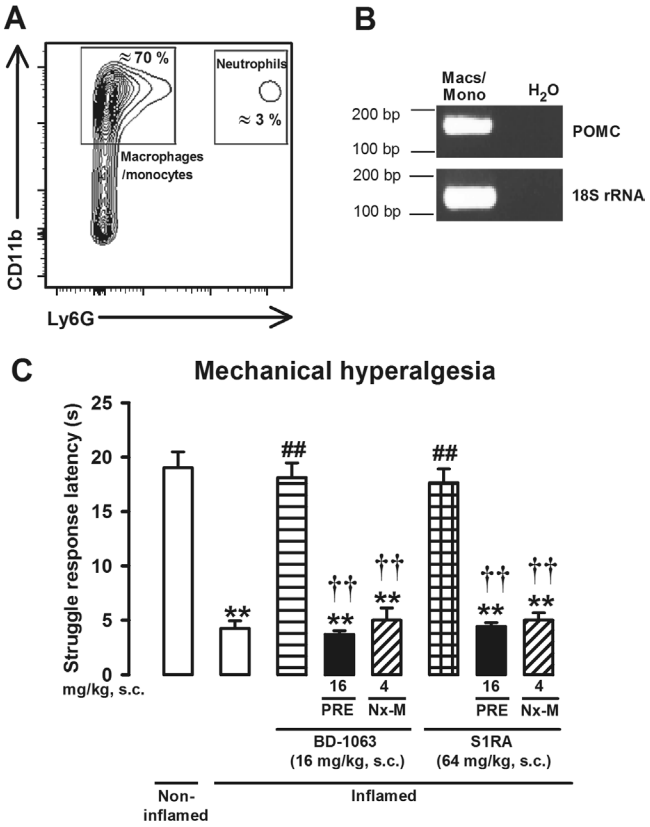


Figure 17. The peripheral opioid-dependent effects of sigma-1 antagonists are preserved during sustained inflammation. (*A*) Representative FACS (fluorescence-activated cell sorting) blot of CD45+ cell populations in the inflamed paw: CD11b+Ly6G- (macrophages/monocytes) and CD11b+Ly6G+ (neutrophils) (*B*) Real-time PCR products for pro-opiomelanocortin (POMC) mRNA and 18S ribosomal RNA (18S rRNA) as an internal standard (predicted band sizes 165bp and 133bp, respectively) from FACS-purified macrophages/monocytes (macs/mono). (*C*) Effects induced by the sigma-1 antagonists BD-1063 (BD) or S1RA on mechanical hyperalgesia. Mice were injected intraplantarly with carrageenan (inflamed) or saline (non-inflamed) 5 days before the evaluation, and administered subcutaneously (s.c.). with the sigma-1 antagonists, PRE-084 (PRE) and naloxone methiodide (Nx-M). Bars show means ± SEM from 8–10 animals. ***P*<0.01, mice without vs. mice with inflammation; ##*P*<0.01 mice with inflammation treated with sigma-1 antagonists vs. mice with inflammation treated with solvent controls; ††*P*<0.01, mice with inflammation treated with sigma-1 antagonist alone vs. mice with inflammation treated with PRE or Nx-M; one-way ANOVA followed by Bonferroni test.

2.6. Effects of sigma-1 antagonism on formalin-induced pain

We also tested whether the ameliorative effects of sigma-1 antagonism in other pain models also involve the actions of EOPs of immune origin. S1RA dose-dependently decreased the second phase of formalin-induced pain (Figure 18*A*), as previously described for this and other sigma-1 antagonists (Zamanillo et al., 2013). However, the administration of opioid antagonists or anti-Ly6G did not modify the antinociceptive

effects of S1RA (Figure 18*B*). Interestingly, at the peak of the nociceptive behaviors (20–25 min) formalin was unable to recruit neutrophils to the injected paw (Figure 18*C*). These results suggest that sigma-1 antagonism requires the presence of immune cells harboring EOPs to induce their opioid-dependent effects, but that this is not the only mechanism used by sigma-1 antagonists to ameliorate pain. Our results are consistent with previous findings that the ameliorative effects of nonselective sigma-1 antagonists (i.e., haloperidol and its metabolites) in behavioral models involving central sensitization (such as the second phase of formalin-induced pain or capsaicin-induced secondary mechanical hypersensitivity) are not reversed by naloxone (Cendán et al., 2005a; Entrena et al., 2009b).

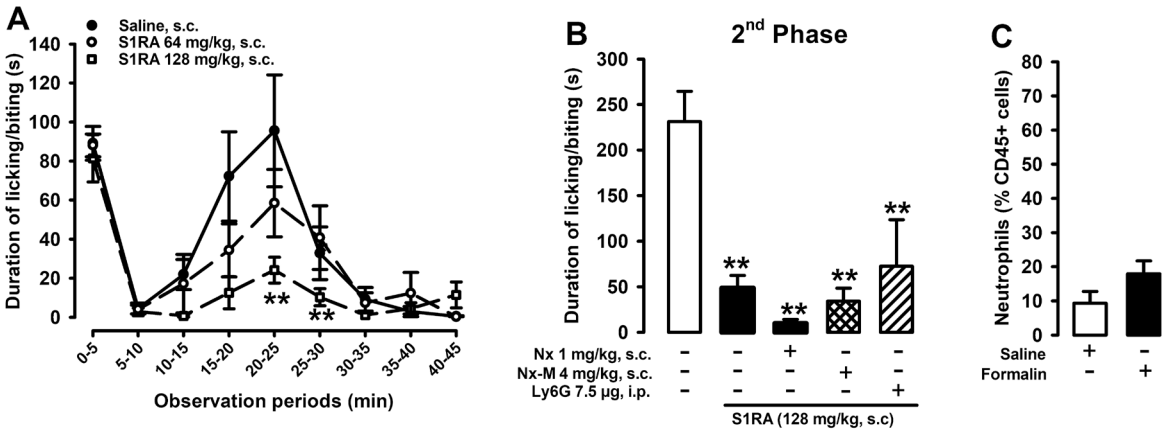


Figure 18. Endogenous opioid peptides of immune origin do not play a role in the antinociceptive effects induced by S1RA in the formalin test. (*A*) Time course of pain-like responses (duration of licking or biting the affected paw) induced by the intraplantar (i.pl.) injection of formalin (2.5%) in mice treated subcutaneously (s.c.) with S1RA or solvent control. **P*<0.05, ***P*<0.01, mice treated with S1RA vs. mice treated with solvent control; two-way repeated measures ANOVA followed by Bonferroni test. (*B*) Effects of administration of S1RA (s.c.) with the administration of naloxone (Nx; s.c.) or naloxone methiodide (Nx-M; s.c.), and administration of anti-Ly6G antibody (intraperitoneal [i.p.]) on the second phase of formalin-induced pain (15–35 min after injection). ***P*<0.01, mice treated with S1RA vs. mice treated with solvent control; no significant differences between mice treated with S1RA alone vs. mice treated with S1RA and other agents were observed; one-way ANOVA followed by Bonferroni test. (C) Effects of formalin on neutrophil levels (CD45+Ly6G+ cells) in the injected paw, determined by FACS (fluorescence-activated cell sorting). No significant differences between mice treated with formalin vs. mice treated with saline were observed; unpaired Student's *t*-test. Bars or points show means ± SEM from 8–10 animals.

The sigma-1 receptor is a ligand-regulated chaperone that participates in pain neurotransmission through multiple pathways (Zamanillo et al., 2013). Although we show here that the predominant mechanism of action of sigma-1 antagonists in ameliorating inflammatory hyperalgesia involves the modulation of EOPs from immune cells at the site of inflammation, this is not necessarily the case in all pain conditions. Further research is needed to fully characterize the mechanisms involved in the actions of sigma-1 antagonists in different types of pain.

CONCLUSIONS

1. Specific conclusions

1- Sigma-1 receptors play a pivotal role in inflammatory hyperalgesia, and blockade of sigma-1 receptor function in the inflamed tissue is sufficient to completely abolish hyperalgesia. However, sigma-1 inhibition does not alter inflammatory edema, which suggests that pain processing is modulated by sigma-1 inhibition rather than inflammation.

2- The antihyperalgesic effects of sigma-1 receptor antagonism depend on the activity of peripheral opioid receptors.

3- Immune cells are responsible (at least partially) of inflammatory hyperalgesia and edema formation during inflammation.

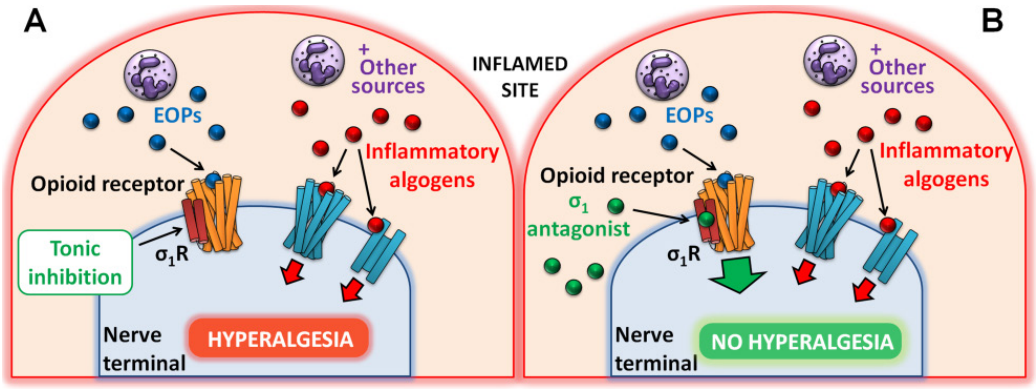
4- Immune cells present and the inflamed site produce endogenous opioid peptides (β-endorphin).

5- The antihyperalgesic effects of sigma-1 receptor antagonism depend on the presence of endogenous opioid peptides produced by immune cells at the inflamed site.

6- In pain states which do not induce significant immune cell recruitment (formalin-induced pain), sigma-1 inhibition ameliorates pain using mechanisms independent of opioid modulation.

2. General conclusion

Peripheral sigma-1 receptors constitute a biological brake to immune-driven opioid analgesia during inflammatory conditions in which immune cells and other sources of algogenic chemicals promote inflammatory pain. This biological brake to opioid antinociception can be released pharmacologically by sigma-1 antagonists, which promote opioid analgesia at the site of inflammation by the disinhibition of the effects of endogenous opioid peptides of immune origin. This mechanism (which is summarized in the figure below), maximizes the analgesic potential of immune cells that naturally accumulate in painful inflamed sites, and differs from that of conventional analgesics. The findings of this PhD project suggest that sigma-1 antagonists merit further research as potential agents for the treatment of inflammatory pain.



Proposed mechanism of action for the effects of sigma-1 antagonism on inflammatory hyperalgesia. (*A*) Immune cells (for instance neutrophils) infiltrating the inflamed paw (and other sources) release algogenic chemicals that sensitize nociceptors, but also endogenous opioid peptides (EOPs). These EOPs of immune origin do not relieve inflammatory pain, because of tonic inhibition of opioid functioning by sigma-1 receptors (σ_1R). The balance between the effects of algogenic chemicals and EOPs favors increased sensitivity to pain characteristic of inflammation. (*B*) Sigma-1 antagonists protect opioid receptors from the tonic inhibition induced by sigma-1 receptors, potentiating the effects of EOPs of immune origin and producing opioid-mediated antihyperalgesic effects during inflammation.

ABBREVIATIONS

ANOVA: analysis of variance

ASIC3: acid-sensing ion channels type 3

ASICs: acid-sensing ion channels

ATP: adenosine Triphosphate

B1 receptor: bradykinin receptor type 1

B2 receptor: bradykinin receptor type 2

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

Ca^{2+}: calcium

Ca^{2+}-CaM: calcium calmodulin

CaMKII: calmodulin-dependent kinase II

cAMP: 3´-5´cyclic adenosine monophosphate

cAMP: 3´-5´cyclic adenosine monophosphate

CAMs: cell adhesion molecules

Cav3.2: voltage-dependent Ca^{2+} channel type 3.2

CB1R: cannabinoid receptor type 1

CGRP: calcitonin gene-related peptide

Contra: contralateral

COX1: cyclooxygenase type 1

COX2: cyclooxygenase type 2

CRF: corticotrophin-releasing factor

D1R: dopamine 1 receptor

D2R: dopamine 2 receptor

DRG: dorsal root ganglion

E_{max}: Maximum effect

EOPs: endogenous opioid peptides

EP1: eicosanoid-prostanoid receptors type 1

EP4: eicosanoid-prostanoid receptors type 4

EPs: eicosanoid-prostanoid receptors

ERK: extracellular signal-regulated kinase

ERK1/2: extracellular signal-regulated kinase 1/2

ESL1: E-selectin ligand

ET_A: Endothelin receptor type A

FACS: fluorescence-activated cell sorting

$GABA_AR$: gamma aminobutyric acid receptor type A

GDP: guanosine diphosphate

GlyCAM: glycosylation-dependent cell adhesion molecule

gp130: glycoprotein 130

GPCRs: G-protein coupled receptors

H^+: proton

H1 receptor: histamine receptor type 1

H2 receptor: histamine receptor type 2

HETE: hydroxyicosatetraenoic acid

HINT1: histidine triad nucleotide-binding protein 1

i.p.: intraperitoneal

i.pl.: intraplantar

IASP: International Association for the Study of Pain

IB: ibuprophen

ICAM-1: intercellular adhesion molecule type 1

ICAM-2: intercellular adhesion molecule type 2

IL1: interleukin type 1

IL1β: interleukin type 1β

IL6: interleukin type 6

IL-6R: interleukin 6 specific receptors

Ipsi: ipsilateral

Iso: isotype

JAMA: junctional adhesión molecule type A

JAMB: junctional adhesión molecule type B

JAMC: junctional adhesión molecule type C

$K_{ir}3$: inwardly rectifying potassium channels type 3

KO: knockout

Kv: voltage-gated K+ channels

LC: locus coeruleus

LFA1: β2 integrin

Ly6C: marker of macrophage/monocyte and neutrophils

Ly6G: specific neutrophil marker

MAC1: β2 integrin

Macs: macrophages

MAM: mitochondrion-associated endoplasmic reticulum membrane

Mono: monocytes

Mor: morphine

MOR: μ-opioid receptor

Na^+: sodium

NADPH: nicotinamide adenine dinucleotide phosphate

Na_v: voltage-gated sodium channel

NE-100: *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride

NGF: nerve growth factor

NMDA: *N*-methyl-*D*-aspartate

NMDAR: *N*-methyl-*D*-aspartate receptor

NO: nitric oxide

NR1: subunit of NMDA receptors

NSAID: nonsteroidal anti-inflammatory drug

Nx: naloxone

Nx-M: naloxone methiodide

p75NTR: p75 neurotrophin receptor

PAG: periacueductal gray matter

PBS: phosphate buffer saline

PCKγ: protein kinase C γ

PCR: polymerase chain reaction

PECAM1: Platelet endothelial cell adhesion molecule

PGE2: Prostaglandin type E2

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

POMC: Pro-opiomelanocortin

PRE-084: [2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride]

PSGL-1: P-selectin glycoprotein ligand-1

RVM: rostroventral medulla

s.c.: subcutaneous

S1RA: 4-[2-[[5-methyl-1-(2-naphtalenyl)1H-pyraol-3-yl]oxy]ethyl] morpholine hydrochloride

SEM: standard error of the mean

T cells: Lymphocytes type T

TM1: transmembrane domain 1

TM2: transmembrane domain 2

TNF: tumor necrosis factor

TNFR1: tumor necrosis factor receptor type 1

TNFR2: tumor necrosis factor receptor type 2

TrkA: tyrosine kinase receptor A

TRP: transient receptor potential

TRPA1: transient receptor potential ankyrin 1

TRPV1: transient receptor potential vanilloid 1

VCAM-1: vascular cell adhesion molecule type 1

VDCC: L-type voltage-dependent calcium channels

VLA4: β1 integrin

WHO: World Health Organization

WT: wild-type

σ_1-KO: sigma-1 knockout

σ_1R: sigma-1 receptor

(±)-SKF-10,047: *N*-allylnormetazocine

5,6-EET: 5,6-epoxyeicosatrienoic acid

5-HT: serotonin

5-HT_{2A}R: serotonin receptor type 2A

5-HT_3R: serotonin receptor type 3

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The image of the neutrophil used in the cover was taken from:
http://www.wisegeek.com/what-is-a-neutrophil-granulocyte.htm#

LIST OF PUBLICATIONS

1. Published articles from this PhD Thesis

1. **Tejada MA**, Montilla-Garcia A, Cronin SJ, Cikes D, Sanchez-Fernandez C, Gonzalez-Cano R, Ruiz-Cantero MC, Penninger JM, Vela JM, Baeyens JM, Cobos EJ. 2017. Sigma-1 receptors control immune-driven peripheral opioid analgesia during inflammation in mice. Proc Natl Acad Sci U S A 114:8396-8401.
   Impact factor: 9.661 4/64 in the area Multidisciplinary Sciences

2. **Tejada MA**, Montilla-Garcia A, Sanchez-Fernandez C, Entrena JM, Perazzoli G, Baeyens JM, Cobos EJ. 2014. Sigma-1 receptor inhibition reverses acute inflammatory hyperalgesia in mice: role of peripheral sigma-1 receptors. Psychopharmacology (Berl) 231:3855-3869.
   Impact factor: 3.874 47/254 in the area Pharmacology & Pharmacy

3. Other published articles

1. Montilla-Garcia A*, **Tejada MA***, Perazzoli G, Entrena JM, Portillo-Salido E, Fernandez-Segura E, Canizares FJ, Cobos EJ. 2017. Grip strength in mice with joint inflammation: A rheumatology function test sensitive to pain and analgesia. Neuropharmacology 125:231-242.
   (*Equal contribution).
   Impact factor: 5.012 24/256 in the area Pharmacology & Pharmacy

2. Gonzalez-Cano R*, **Tejada MA***, Artacho-Cordon A*, Nieto FR, Entrena JM, Wood JN, Cendan CM. 2017. Effects of Tetrodotoxin in Mouse Models of Visceral Pain. Mar Drugs 15.
   (*Equal contribution).
   Impact factor: 3.503 13/60 in the area Chemistry, Medicinal

3. Mendivil-Perez M, Soto-Mercado V, Guerra-Librero A, Fernandez-Gil BI, Florido J, Shen YQ, **Tejada MA**, Capilla-Gonzalez V, Rusanova I, Garcia-Verdugo JM, Acuna-Castroviejo D, Lopez LC, Velez-Pardo C, Jimenez-Del-Rio M, Ferrer JM, Escames G. 2017. Melatonin enhances neural stem cell differentiation and engraftment by increasing mitochondrial function. J Pineal Res 63.
   Impact factor: 10.391 3/84 in the area Physiology

4. Hockley JR, Gonzalez-Cano R, McMurray S, **Tejada-Giraldez MA**, McGuire C, Torres A, Wilbrey AL, Cibert-Goton V, Nieto FR, Pitcher T, Knowles CH, Baeyens JM, Wood JN, Winchester WJ, Bulmer DC, Cendan CM, McMurray G. 2017. Visceral and somatic pain modalities reveal NaV 1.7-independent visceral nociceptive pathways. J Physiol 595:2661-2679.
   Impact factor: 4.739 9/84 in the area Physiology

5. Luna-Sanchez M, Diaz-Casado E, Barca E, **Tejada MA**, Montilla-Garcia A, Cobos EJ, Escames G, Acuna-Castroviejo D, Quinzii CM, Lopez LC. 2015. The clinical heterogeneity of coenzyme Q10 deficiency results from genotypic differences in the Coq9 gene. EMBO Mol Med 7:670-687.
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