Universidad de Oviedo

Programa de Doctorado “Investigación en Cáncer”

Implicación de las quimiocinas CCL2 y CCL5 en modelos de dolor neoplásico experimental

Tesis Doctoral

Marta Pevida López
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Firma

Índice de abreviaturas

AINE: antiinflamatorio no esteroideo

AMPA: ácido α-amino-3-hidroxi-5-metil-4-isoxazolpropiónico

AMPc: adenosín monofosfato cíclico

ASIC: acid-sensing ion channels.

B_1, B_2: receptor de bradicinina tipo 1 y 2

CB_1, CB_2: receptores cannabinoides tipo 1 y 2

CGRP: péptido relacionado con el gen de la calcitonina

COX: ciclooxigenasa

COX-2: ciclooxigenasa tipo 2

DEG/ENaC: familia de las degenerinas y de los canales de Na+ epiteliales

DRG: ganglios de la raíz dorsal

EP_1: receptor de la PGE_2

ERK: cinasas reguladas por señales extracelulares

FMRI: functional magnetic resonance imaging

GABA: ácido gamma aminobutírico

$GABA_A$, $GABA_B$: receptores GABA tipo A y B

GDNF: factor neurotrópico derivado de células gliales

GIRK: canales de K^+ de rectificación acoplados a proteínas G

GFAP: glial fibrillary acidic protein

GLT-1: glutamate transporter type 1

GLAST: glutamate-aspartate transporter

GPCR: G-protein coupled receptors

IASP: International Association for the Study of Pain

IB4: isolectina B4

iba-1: ionized calcium binding adaptor molecule 1

IEFN: fibras nerviosas intraepidérmicas

IL-1β: interleucina 1β

IL-2, 4, 6: interleucinas 2,4, y 6

IFN-γ: interferón gamma IFN-γ

IP_3K: cinasa del inositol trifosfato

KCNK: two-pore domain potassium channels

LPS: lipopolisacárido

MAPK: proteínas cinasas activadas por mitógenos

mGluR: receptor metabotrópico de glutamato acomplado a proteínas G

NF-κB: factor nuclear Kb

NGF: factor de crecimiento nervioso

NK1: receptor de neurocinina 1

NMDA: N-metil D-aspartato

OPG: osteoprotegerina

$P2X_3$, $P2X_2$, $P2X_4$: receptor purinérgico $P2X_3$, $P2X_2$, $P2X_4$

PGE_2: prostaglandina E_2

PKA: proteína cinasa A

PKC: proteína cinasa C

PLC: fosfolipasa C

PLCβ: fosgolipasa C beta

RANK: receptor activador del Factor nuclear κB

RANKL: ligando del Receptor Activador del Factor Nuclear κ B

RUNX1: runt-related transcription factor 1

RT-PCR: reacción en cadena de la polimerasa cuantitativa (o a tiempo real)

SNC: Sistema nervioso central

SNP: Sistema nervioso periférico

SP: sustancia P

TLR: Toll-like receptors

TNF-α: factor de necrosis tumoral

TrkA: receptor tirosina cinasa A

TRP: receptor de potencial transitorio

TRPA1: transient receptor potential ankyrin 1

TRPM8: transient receptor potential melastatin 8

TRPV1: transient receptor potential vanilloid 1

TRPV2: transient receptor potential vanilloid 2

TRPV3: transient receptor potential vanilloid 3

TRPV4: transient receptor potential vanilloid 4

TTX: tetrodotoxina

WDR: neuronas convergentes o de amplio rango dinámico (wide dynamic range)

Índice

1. Introducción

1.1 CONCEPTO DE DOLOR. PROCESAMIENTO DE LAS SEÑALES NOCICEPTIVAS

Según la IASP (International Association for the Study of Pain), el dolor se define como una experiencia desagradable, en lo sensorial y lo emocional, que se asocia a una lesión tisular real o posible, o que se define en función de dicha lesión. El dolor no solo comprende una dimensión sensorial sino también componentes emocionales y cognitivos estableciéndose un proceso realmente complejo ya que cada persona percibe esa sensación de manera diferente. No debe confundirse el dolor, emoción totalmente subjetiva, con el concepto de **nocicepción** que se refiere al conjunto de procesos implicados en la detección y procesamiento de los estímulos que componen la experiencia dolorosa (Loeser y Treede, 2008). En el ámbito de la experimentación animal se estudian los aspectos relativos a la nocicepción ya que los animales no son capaces de expresar una respuesta dolorosa.

1.1.1 Transmisión de señales nociceptivas en la periferia

Los nociceptores constituyen una población heterogénea de neuronas periféricas que detectan y transmiten estímulos nociceptivos térmicos, mecánicos o químicos (Basbaum y Jessel, 2000). Son neuronas pseudounipolares con un terminal central y otro periférico que proceden de un tronco axonal común y que pueden tanto recibir como enviar información (Basbaum et al., 2009; Dubin y Patapoutian, 2010). En un nociceptor puede distinguirse una terminación nerviosa periférica que detecta los estímulos e inicia los potenciales de acción, un axón que los conduce, un soma que se encuentra localizado en los ganglios de la raíz dorsal (DRG) o en el ganglio del trigémino, y un terminal central que penetra en el asta dorsal de la médula espinal para hacer sinapsis con las neuronas nociceptivas de segundo orden.

Las nociceptores pueden clasificarse en función del grado de mielinización y de la sección de sus axones en dos grandes grupos: Aδ y C. Los nociceptores Aδ están formados por fibras mielinizadas, de diámetro medio (1-5 μm) y con una velocidad de conducción rápida (5-30 m/s) relacionada con la detección del dolor bien localizado, dolor rápido o primer dolor. Los nociceptores C que forman el mayor grupo de neuronas nociceptivas primarias del sistema nervioso periférico (SNP) (Woolf y Ma, 2007) están constituidos por fibras no mielinizadas de diámetro pequeño (0,3-1,5 μm), conducción lenta (0,5-2 m/s) y median el dolor lento, peor localizado, también llamado “segundo dolor”. Son mayoritariamente polimodales (Dubin y Patapoutian, 2010), y pueden a su vez clasificarse según sus propiedades neuroquímicas en fibras peptidérgicas y no peptidérgicas. Las fibras peptidérgicas liberan neuropéptidos como la sustancia P (SP) y el péptido relacionado con el gen de la calcitonina (CGRP), y además, expresan el receptor tirosina cinasa A (TrkA) que responde al factor de crecimiento nervioso

(NGF). Las fibras no peptidérgicas, expresan receptores purinérgicos P2X3, el receptor del factor neurotrópico derivado de células gliales (GDNF), la proteína RUNX1 (runt-related transcription factor 1) y la mayoría de ellas muestran un marcaje positivo frente a isolectina B4 (IB4) (Chen et al., 2006; Woolf y Ma, 2007; Basbaum et al., 2009).

Por otro lado, las neuronas periféricas con fibras mielinizadas de tipo Aβ que responden a estímulos mecánicos inocuos o de baja intensidad no se comportan por regla general como nociceptores pero en determinadas condiciones de daño tisular pueden participar en la transmisión nociceptiva como se comenta más adelante.

Cuando el nociceptor recibe un estímulo externo de características apropiadas, se inicia el proceso de transducción que transforma este estímulo en potenciales de acción. Para que el nociceptor sea excitado es necesario que se genere una despolarización de la membrana, se alcance el potencial umbral y se inicie el potencial de acción que conduzca la información hasta la médula espinal. En este proceso participan tres clases de proteínas presentes en la superficie de los nociceptores: receptores ionotrópicos, receptores metabotrópicos acoplados a proteínas G (G-protein coupled receptors, GPCR) y receptores con actividad tirosina cinasa (Gold y Gebhart, 2010).

Los receptores ionotrópicos son canales iónicos de membrana permeables al Na^+ y Ca^{2+} cuya activación excita directamente al nociceptor. Cabe destacar que no parece que exista selectividad entre cada tipo concreto de estímulo (térmico, químico o mecánico) y una familia particular de receptores, sino que cada tipo de receptor ionotrópico puede ser activado por estímulos diferentes. El grupo más conocido de receptores ionotrópicos es la familia de los TRP (transient receptor potential) que está compuesta por al menos 30 tipos diferentes. El primero que se ha descrito es el TRPV1 (transient receptor potential vanilloid 1). Este canal iónico se expresa en los nociceptores y no en otras neuronas periféricas (Schaible et al., 2011), y se activa por estímulos térmicos superiores a 43°C, por acidificación del medio extracelular y por la capsaicina (Caterina et al., 1999). Se ha descrito cómo ratones que carecen de este receptor, presentan menor capacidad de responder a estímulos térmicos y a capsaicina (Caterina et al., 2000). En segundo lugar, los receptores TRPV2 (transient receptor potential vanilloid 2) presentes en los nociceptores Aδ se activan tanto frente a estímulos térmicos, a temperaturas superiores a 52°C, como mecánicos, respondiendo a cambios en la presión osmótica celular (Caterina et al., 1999; Muraki et al., 2003). Los canales TRPV3 (transient receptor potential vanilloid 3) y TRPV4 (transient receptor potential vanilloid 4) son activados por temperaturas moderadas (a partir de 31°C) (Chung et al., 2005). Adicionalmente, los canales TRPV4 juegan un papel importante en la mecanotransducción ya que al igual que los TRPV2, responden a cambios de presión osmótica (Güler et al., 2002). Posteriormente, se

describieron dos receptores TRP que pueden ser activados por frío, TRPM8 (transient receptor potential melastatin 8) y TRPA1 (transient receptor potential ankyrin 1). Los canales TRPM8 son sensibles a temperaturas inferiores a 25°C y se ha descrito cómo ratones que carecen de este receptor, presentan menor capacidad de responder estímulos fríos (Bautista et al., 2007). Además, los canales TRPM8 también son sensibles a sustancias químicas como el mentol (Peier et al., 2002). Los receptores TRPA1 son activados por umbrales térmicos inferiores a 18°C y también por sustancias químicas como la formalina, el aceite de mostaza y el mentol, aunque en este caso, la concentración necesaria para la estimulación es relativamente superior a la utilizada en la activación de los canales TRPM8 (Story et al., 2003). Diversos estudios sugieren un papel contradictorio de los receptores TRPA1 en la transducción de estímulos mecánicos. Así, mientras que algunos autores postulan que no se producen cambios aparentes en el umbral de detección de estímulos mecánicos en animales que carecen de este receptor (Bautista et al., 2006; Petrus et al., 2007), un trabajo reciente describe cómo ratones knockout para TRPA1 muestran respuestas modificadas ante una estimulación mecánica (Kwan et al., 2009).

Los receptores ASIC (acid-sensing ion channels) pertenecientes a la familia de las degenerinas y de los canales de Na^+ epiteliales (DEG/ENaC), responden a descensos de pH y/o liberación de protones al medio extracelular como consecuencia de un daño tisular o estrés metabólico. Entre ellos, destacan los ASIC 3 que además de en los nociceptores se expresan en fibras que inervan el músculo esquelético y cardiaco (Basbaum et al., 2009). La participación de estos canales en la mecanotransducción no está totalmente aclarada. Así como en algunos trabajos se determina que los canales ASIC2 y 3 no intervienen de forma significativa en la transducción de estímulos mecánicos (Drew et al., 2004; Roza et al., 2004), estudios posteriores sugieren la participación de estos canales en la transducción mecánica (Mogil et al., 2005; Corey, 2006; Borzan et al., 2010).

Otro grupo de receptores ionotrópicos en los que se ha centrado la atención en los últimos años son los activados por nucleótidos, entre los que destacan los receptores purinérgicos $P2X_2$ y $P2X_3$ (Schaible et al., 2011). Su activación se produce por la unión del ATP que liberan las células dañadas de la piel al medio extracelular en respuesta a una estimulación mecánica o a la presencia de inflamación (Linley et al., 2010). Se ha demostrado la participación de los receptores purinérgicos en la transducción de estímulos térmicos y mecánicos y su implicación en el desarrollo de hiperalgesia (Malin et al., 2008; Oliveira et al., 2009).

Por último, es importante mencionar la familia de canales de potasio, KCNK (two-pore domain potassium channels), entre los que se encuentran los receptores KCNK2 y KCNK4 que

se perfilan como posibles candidatos en la transducción de determinados estímulos como la presión y la temperatura (Basbaum et al., 2009). Estos canales se expresan en los nociceptores C y se ha descrito cómo ratones carentes de los mismos muestran dificultades en la percepción de estímulos térmicos y mecánicos (Alloui et al., 2006; Noel et al., 2009). Por otro lado, se ha sugerido la posible implicación del canal KCNK18 en la mecanotransducción ya que podría ser un regulador importante de la excitabilidad neuronal ante estímulos nocivos o inocuos táctiles (Basbaum et al., 2009).

Como se había mencionado anteriormente, al margen de los canales iónicos en la activación de los nociceptores también participan receptores metabotrópicos acoplados a proteínas G (G-protein coupled receptors, GPCR). Sobre ellos actúan factores liberados desde las células no neuronales que se encuentran en el propio tejido lesionado o en sus alrededores como basófilos, mastocitos, macrófagos, queratinocitos y fibroblastos e incluso factores liberados desde los nociceptores activados. Actúan mayoritariamente generando un aumento en la respuesta y un descenso en el umbral de detección durante un daño tisular. Este fenómeno se conoce como **sensibilización periférica** y es el responsable de la **hiperalgesia primaria** que se detecta en los tejidos lesionados, donde estímulos de baja intensidad pueden producir dolor.

Los GPCR con especial interés en el campo nociceptivo son los que se encuentran acoplados a las proteínas G_s y G_q. Los primeros, estimulan a la adenilato ciclasa incrementando los niveles de AMPc y provocando la consiguiente activación de la proteína cinasa A (PKA). Los sensibilizadores que actúan por medio de esta vía son principalmente las prostaglandinas y también otros como la noradrenalina, la serotonina y el neuropéptido CGRP. Por ejemplo, la prostaglandina E_2 (PGE_2) mediante la unión al receptor EP_1 induce la sensibilización de los receptores TRPV1 y el consiguiente descenso del umbral de detección de estímulos térmicos (Moriyama et al., 2005; Rukwied et al., 2007; Basbaum et al., 2009). En segundo lugar se encuentran los receptores acoplados a proteínas G_q, capaces de estimular la fosfolipasa C de tipo beta (PLCβ) produciendo liberación de calcio intracelular y activación de proteína cinasa C (PKC) (Stone y Molliver, 2009). Los principales mediadores que utilizan esta vía de señalización son la bradicinina, la histamina o la serotonina. Uno de los sensibilizadores más conocidos, la bradicinina, ejerce su acción uniéndose a dos tipos de receptores, el receptor de bradicinina tipo 2 (B_2), que se expresa de forma constitutiva, y el receptor de bradicinina tipo 1 (B_1), cuya expresión y liberación se induce en estados inflamatorios (Dray y Perkins, 1993). Secundariamente a la estimulación de estos receptores, el incremento de calcio en el interior de la célula induce la sensibilización del nociceptor como consecuencia de la activación de receptores TRPA1 y TRPV1 (Wang et al., 2008; Hamza et al., 2010). Además la bradicinina

también puede provocar la sensibilización de los nociceptores mediante la liberación de prostaglandinas (Mayer et al., 2007). A este grupo de receptores acoplados a proteínas G_q pertenecen también los receptores de quimiocinas (Kuang et al., 1996). Puesto que en esta tesis se estudian aspectos relacionados con la participación de las quimiocinas y sus receptores en el dolor canceroso, se realiza más adelante una descripción detallada sobre su implicación en la nocicepción.

Por último, los receptores con actividad tirosina cinasa se activan por la unión de péptidos como las neurotrofinas y citocinas. Los mediadores que pertenecen a estas familias están regulados al alza en situaciones patológicas y la unión a sus receptores provoca la sensibilización del nociceptor. El representante más estudiado de las neurotrofinas es el NGF cuya expresión se encuentra aumentada en los tejidos lesionados e induce la sensibilización de los nociceptores mediante su unión al receptor TrkA presente en fibras C peptidérgicas. Esta unión inicia una cascada de activación de segundos mensajeros tales como la fosfolipasa C (PLC), proteínas cinasas activadas por mitógenos (MAPK) y la cinasa del inositol trifosfato (IP_3K), que promueven el incremento de expresión de moléculas que participan en la nocicepción como pueden ser la SP y el CGRP, bradicinina, receptores de tipo TRPV1 o purinérgicos P2X y canales Na_v 1.8. Este proceso desencadena un incremento en la excitabilidad de los nociceptores y un descenso en el umbral de detección del estímulo doloroso (Gold y Gebhart, 2010). Además, está ampliamente documentado el papel de las citocinas en el desarrollo y mantenimiento de la respuesta inflamatoria. Así, en el lugar de la lesión se liberan proteínas tales como IL-1β (interleucina 1beta), IL-2 (interleucina 2), IL-6 (interleucina 6) o TNF-α (factor de necrosis tumoral), que favorecen el desarrollo de hiperalgesia e inflamación (Basbaum et al., 2009).

En cuanto a la conducción del potencial de acción generado desde el nociceptor hasta la médula espinal, debe señalarse el papel fundamental ejercido por los canales de Na^+ dependientes de voltaje (Na_v). Se ha descrito una gran variedad de canales, expresados tanto en neuronas nociceptivas como no nociceptivas. En mamíferos se conocen nueve isoformas diferentes de canales Na_v, de Na_v 1.1 a Na_v 1.9 (Catterall et al., 2005). Los Na_v presentes en los nociceptores pueden a su vez dividirse en función de su sensibilidad a la tetrodotoxina (TTX), neurotoxina que bloquea los canales de Na^+ dependientes de voltaje. Así los canales Na_v 1.1, 1.6 y 1.7 son sensibles a TTX mientras que los canales Na_v 1.8 y 1.9 son resistentes a la misma (Schaible et al., 2011; Basbaum et al., 2009). Algunos canales como los Na_v 1.7, se encuentran regulados al alza en el dolor inflamatorio y ratones que carecen de este canal presentan respuestas atenuadas a estímulos nociceptivos en presencia de inflamación (Nassar et al., 2004). Además también se ha descrito que diversos factores inflamatorios como NGF,

endotelinas, serotonina o TNF-α pueden producir un incremento de la transmisión de señales a través de los canales Na_v 1.8 (Gold, 2008). Estos canales han sido propuestos como dianas farmacológicas sobre las que bloqueantes selectivos podrían actuar mostrando una eficacia antinociceptiva relevante (Bulaj et al., 2006; Kers et al., 2012).

Junto con estos mecanismos periféricos, tanto a nivel de la médula espinal como en áreas supraespinales, diversos mediadores y receptores contribuyen de forma muy significativa a la generación y transmisión del estímulo doloroso. Se comentan a continuación algunos aspectos fundamentales en la transmisión nociceptiva a nivel del sistema nervioso central (SNC).

1.1.2 Transmisión de señales nociceptivas en la médula espinal

La siguiente "estación" donde tiene lugar el procesamiento de la señal dolorosa es la médula espinal. Como se ha descrito con anterioridad, los nociceptores son neuronas pseudounipolares cuyo terminal central penetra en el asta dorsal de la médula espinal. Las fibras Aδ y C proyectan principalmente sobre las láminas I y II, y en menor medida sobre láminas profundas (Todd, 2010), mientras que las fibras Aβ, que responden al tacto, penetran en las láminas III, IV y V (Maslany et al, 1992). Estas aferencias primarias realizan sinapsis principalmente con dos tipos de neuronas espinales que tienen distinta distribución anatómica, y, por tanto reciben diferentes proyecciones. En las láminas más superficiales (I-II), las neuronas nociceptivas específicas recogen la información de fibras Aδ y C exclusivamente respondiendo, por tanto, a estímulos nociceptivos. El otro grupo de neuronas son las convergentes o neuronas de amplio rango dinámico (WDR, wide dynamic range), localizadas principalmente en la lámina V. Éstas reciben información nociceptiva mediante las fibras Aδ y C e información no nociceptiva a través de fibras Aβ.

La transmisión de señales nociceptivas en la médula espinal es muy compleja, y en ella participan numerosos neurotransmisores excitadores e inhibidores. El glutamato es el neurotransmisor excitador por excelencia en el SNC, su presencia es imprescindible para la transmisión nociceptiva y actúa fundamentalmente a través de 3 tipos de receptores: receptores AMPA (ácido α-amino-3-hidroxi-5-metil-4-isoxazolpropiónico), receptores NMDA (N-metil D-aspartato) y receptores metabotrópicos de glutamato acoplados a proteínas G (mGluR) presentes en las neuronas postsinápticas del asta dorsal de la médula espinal. La conducción de la información nociceptiva en respuesta a un estímulo agudo leve deriva de la unión del glutamato, y en menor medida del aspartato, a los receptores AMPA generando la despolarización rápida de la membrana postsináptica y el incremento de la concentración de Na^+ intracelular en las neuronas espinales. Este mecanismo permite la transmisión de

potenciales de acción a neuronas nociceptivas supraespinales (Basbaum et al., 2009). Los receptores NMDA se encuentran bloqueados en condiciones fisiológicas normales por los iones Mg^{2+} presentes en el tejido nervioso y como se describe más adelante, es necesaria una despolarización mantenida de la membrana para inhibir este bloqueo, permitir su activación y secundariamente favorecer la entrada de Ca^{2+} en el interior de la célula (D'Mello y Dickenson, 2008). En último lugar, los mGluR intervienen en la transmisión de la señal dolorosa mediante despolarizaciones lentas de membrana, ya que favorecen la excitabilidad neuronal a través de la producción de segundos mensajeros (Valerio et al., 1997; Nakanishi et al., 1998).

Por otro lado, la sustancia P es, posiblemente, el neurotransmisor peptídico más conocido en la transmisión nociceptiva y se libera tras el estímulo doloroso en las sinapsis de la médula espinal desde los nociceptores peptidérgicos junto con otros neuropéptidos como el CGRP y el neuropéptido Y. El 80% de las neuronas espinales presentes en la lámina I expresan el receptor neurocinina 1 (NK1) de la SP. Tras la estimulación de los receptores NK1, se produce una despolarización más lenta de la membrana que la llevada a cabo a través de los receptores AMPA y NMDA, ya que los receptores NK1 se encuentran acoplados a proteína G_q y los mecanismos de transducción son más complejos y no tan inmediatos (Sakurada et al., 2005). Estas neuronas que expresan NK1 envían sus proyecciones a áreas supraespinales como el tálamo, la sustancia gris periacueductal y el área parabranquial (D'Mello y Dickenson, 2008).

Ante una estimulación nerviosa prolongada a nivel periférico, se produce una liberación continua de mediadores en la médula espinal como consecuencia del incremento potenciales de acción. Esta situación genera la liberación masiva de neurotransmisores (glutamato y aspartato) y péptidos (SP, CGRP, neuropéptido Y) cuya unión a los receptores en las neuronas postsinápticas origina una despolarización mantenida de la membrana (Henry, 1976). Esta despolarización permite la activación de los receptores NMDA debido al desplazamiento de los iones Mg^{2+} y como consecuencia de su activación se produce la entrada de Na^{+} y Ca^{2+} al interior de la neurona estimulada. El incremento de Ca^{2+} citosólico permite la activación de numerosas enzimas como la óxido nítrico sintetasa que induce la síntesis de óxido nítrico (Kitto et al., 1992). Además el aumento del Ca^{2+} facilita la activación de diversas vías de señalización donde participan proteínas como las cinasas activadas por la unión del calcio a la calmodulina (Malinow et al., 1989) o cinasas reguladas por señales extracelulares (ERK) (Xia et al., 1996), que inducen cambios postranscripcionales y de expresión génica. También se produce incremento en la expresión del gen de la ciclooxigenasa de tipo 2 (COX-2) que induce la síntesis de prostaglandinas y proteínas como la IL-1β, IL-4 y TNF- α (Voscopoulos y Lema, 2010). La regulación al alza de la expresión de la COX-2 y la óxido nítrico sintetasa, incrementa

los niveles espinales de óxido nítrico y prostaglandinas que a su vez se comportan como mensajeros retrógrados favoreciendo la liberación de más cantidad de glutamato y la activación de un mayor número de receptores NMDA. Esta situación favorece la excitabilidad neuronal dando lugar a una etapa de hipersensibilidad muy corriente en los estados de dolor crónico (Cury et al., 2011; Voscopoulos y Lema, 2010).

Este estado de hipersensibilidad en el sistema nervioso central, que conduce al descenso del umbral y al aumento de la actividad de las neuronas nociceptivas espinales, conlleva un tipo de plasticidad sináptica que se conoce como **sensibilización central** (D'Mello y Dickenson, 2008). La sensibilización central contribuye a que la estimulación de áreas no dañadas situadas alrededor de la lesión original produzca dolor, esto es lo que se conoce como **hiperalgesia secundaria**. Además, en este contexto, fibras aferentes Aβ que responden a estímulos inocuos, son capaces de activar neuronas nociceptivas espinales dando lugar al fenómeno conocido como **alodinia** (Basbaum et al., 2009; Schaible et al., 2011) La alodinia se define por la IASP como la respuesta nociceptiva desencadenada tras la aplicación de un estímulo no nociceptivo (Loeser y Treede, 2008).

En nuestro caso resulta de especial interés la implicación de las quimiocinas en el procesamiento de la señal dolorosa a nivel espinal. Estudios previos muestran cómo algunas quimiocinas (CX3CL1, CCL2, CCL5 y CCL3) inducen el desarrollo de hiperalgesia y alodinia en diferentes modelos de experimentación animal, demostrando su participación en la sensibilización central mediante la acción directa sobre sus receptores (Milligan et al., 2004; Dansereau et al., 2008; Baamonde et al., 2011; Benamar et al., 2008; Kiguchi et al., 2010a). Una descripción más detallada sobre sus efectos en la sensibilización central se realiza más adelante.

Junto con mediadores excitadores, en la nocicepción espinal también participan transmisores inhibidores, entre los que destacan los opioides y los cannabinoides. Los receptores opioides se expresan abundantemente en las láminas superficiales del asta dorsal de la médula, tanto a nivel presináptico como postsináptico, participando de modo muy importante en la modulación espinal de las señales nociceptivas. Sobre las neuronas postsinápticas espinales el estímulo de receptores opioides inhibe la apertura de los canales de Ca^{2+} regulados por voltaje y facilita la apertura de canales de K^+ reduciendo por tanto la excitabilidad neuronal y la propagación de los potenciales de acción (Zollner y Stein, 2007). A nivel espinal los receptores de tipo μ se encuentran mayoritariamente asociados a los canales de rectificación interna tipo K_{ir} acoplados a proteínas G (GIRK: canales de K^+ de rectificación acoplados a proteínas G) (Marker et al., 2005) y se ha descrito la disminución del efecto analgésico producido por la morfina en animales carentes de canales GIRK (Ikeda et al.,

2000). Estos receptores también están presentes en los DRG (Vadivelu et al., 2011) mediando analgesia opioide en el dolor inflamatorio (Endres-Becker et al., 2007) y en el dolor neuropático (Lee et al., 2011).

Adicionalmente, los receptores cannabinoides tipo 1 y 2 (CB_1 y CB_2), están presentes en los DRG, médula espinal y en diferentes áreas cerebrales, como el hipocampo y el cerebelo (Ameri, 1999; Morgan et al., 2009; Curto-Reyes et al., 2010; Cridge et al., 2013). Su estímulo se relaciona con la inhibición de la adenilato ciclasa y los canales de Ca^{2+} controlados por voltaje, así como con la activación de canales de K^+, favoreciendo la hiperpolarización y la reducción de neurotansmisores en el espacio presináptico (Cridge et al., 2013). Otros transmisores inhibidores son los aminoácidos glicina y el ácido gamma-aminobutírico (GABA), liberados por las interneuronas inhibitorias glicinérgicas y gabérgicas, ampliamente distribuidas por las áreas más superficiales de la médula espinal, especialmente en las láminas I a III (Melzack y Wall, 1965). En condiciones normales, el GABA provoca la entrada de Cl^- a través de su unión con los receptores $GABA_A$ y la salida de iones K^+ al unirse a los $GABA_B$, que conlleva a la hiperpolarización de las neuronas nociceptivas, disminuyendo así su excitabilidad (D'Mello y Dickenson, 2008).

1.1.3 Participación de las células gliales en la transmisión nociceptiva espinal

La glía constituye el conjunto de células inmunes que residen en el SNC, cuyas funciones más relevantes estaban clásicamente relegadas al sustento y la defensa de la población neuronal. En los últimos años, la contribución de la microglía y la astroglía al mantenimiento y desarrollo del dolor inflamatorio y/o neuropático se está convirtiendo en un campo de investigación muy atractivo.

En general, la microglía parece participar en el desarrollo de las fases tempranas del dolor crónico mientras que la astroglía ejerce un papel importante en el inicio del dolor agudo y el mantenimiento del dolor crónico (Smith, 2010; Chiang et al., 2012). La microglía, que en condiciones normales se encuentra en un estado de quiescencia, es activada rápidamente tras un daño tisular o infección (Schomberg y Olson, 2012), hipertrofiándose y aumentando su densidad celular (Smith, 2010). Se han descrito numerosos receptores y mediadores que participan en este proceso, entre los que destaca el papel de dos quimiocinas, la CX3CL1 y la CCL2. Tras un daño nervioso periférico aumenta la liberación de CX3CL1 (fractalkina) desde las neuronas y astrocitos de la médula espinal. Esta quimiocina, a su vez, estimula receptores CX3CR1 cuya expresión está aumentada en la superficie externa de las células de microglía en esta situación patológica (Schomberg y Olson, 2012). En cuanto a la CCL2, se ha descrito que tras su administración intratecal en roedores, la densidad de microglía aumenta y se produce el

desarrollo de alodinia, siendo estos fenómenos inhibidos por la administración espinal de un anticuerpo anti-CCL2 (Thacker et al., 2009). Otro mediador implicado en este proceso, es el interferón gamma (IFN-γ), del que se ha demostrado su participación en el dolor neuropático (Tsuda et al., 2009). Las familias de receptores TLR (Toll-like receptors) y P2X también participan en la activación de la microglía. En general, los receptores TLR desempeñan un papel fundamental en la respuesta inmune ya que responden a moléculas habitualmente presentes en los organismos patógenos (Smith, 2010; Basbaum, 2009). En concreto, el receptor TLR4, cuyo activador más conocido es el LPS (lipopolisacárido) presente por ejemplo en la mayoría de bacterias Gram -, se expresa exclusivamente en las células de microglía, provocando la activación de NF-κB (factor nuclear κB) y la liberación de citocinas pro-inflamatorias (Smith, 2010). Los receptores purinérgicos expresados en las células de microglía se activan por la unión del ATP liberado por las neuronas espinales que estimula, por ejemplo, los receptores P2X4 que se encuentran aumentados exclusivamente en células microgliales tras un daño nervioso periférico y cuya inhibición previene el desarrollo de alodinia (Smith, 2010). Como consecuencia de la activación microglial aumenta la expresión de proteínas de membrana como iba-1 (ionized calcium binding adaptor molecule 1) utilizada como marcador y se produce un incremento de mediadores celulares, como TNF-α, IL-1β o IL- 6 que actúan sobre receptores presentes en las neuronas espinales y en la propia microglía favoreciendo la sensibilización central (Schomberg y Olson, 2012).

Los astrocitos también se encuentran activados en la médula espinal tanto en situación de dolor agudo (Sweitzer et al., 1999) como crónico (Honoré et al., 2000b). Esta activación astroglial en las láminas superficiales y profundas de la médula espinal refuerza la idea de su implicación en el procesamiento de la señal nociceptiva y desarrollo de hipersensibilidad. En el dolor crónico, este hecho ocurre en general de forma más tardía que la activación microglial, y conlleva la proliferación de los propios astrocitos, el desarrollo de cambios morfológicos, y la expresión de marcadores de activación astroglial como las proteínas GFAP (glial fibrillary acidic protein) o S100β (Svensson y Brodin, 2010).

El glutamato es uno de los mediadores que promueve la participación de la astroglía en la nocicepción ya que en la superficie de estas células están presentes receptores AMPA y NMDA (Svensson y Brodin, 2010). Además, los astrocitos expresan receptores ionotrópicos como el P2X y metabotrópicos como P2Y que pueden ser activados por el ATP liberado por las neuronas espinales o por ellos mismos dando lugar a un incremento en los niveles de Ca2+ intracelular (Hald, 2009; Svensson y Brodin, 2010). Otros mediadores que participan en este proceso pueden ser la SP, ya que los receptores NK1 están presentes en los astrocitos (Too et al., 1994), o el péptido CGRP que mediante la unión a su receptor favorece la liberación de

citocinas incrementando la sensibilización espinal (Svensson y Brodin, 2010). Por último, tanto la quimiocina CCL2 como la CX3CL1 están implicadas en la activación de la astroglía al liberarse desde las neuronas espinales en respuesta a un daño nervioso o inflamación. Así, se ha descrito que ratones carentes del receptor CCR2 muestran una menor activación de las células gliales en condiciones de daño neuropático (Abbadie et al., 2003) o que el bloqueo del receptor CX3CR1 reduce la activación de los astrocitos en un modelo de artritis en rata, aunque no se descarta que este efecto sea mediado indirectamente por la acción de esta proteína en la microglía (Sun et al., 2007).

Además, como ocurre con las células de microglía, los astrocitos activados liberan gran cantidad de factores como es el caso de algunas citocinas que, mediante modificaciones postraslacionales y la regulación al alza de receptores, canales iónicos o enzimas, excitan o sensibilizan las neuronas espinales participando en la modulación de la percepción nociceptiva (Van Eldik y Zimmer, 1987; Guo et al., 2007; Jourdain et al., 2007).

1.1.4 Estructuras supraespinales implicadas en la transmisión nociceptiva

Las neuronas nociceptivas que envían información a áreas cerebrales se encuentran principalmente en la lámina I y V y en menor medida en la lámina II de la médula espinal (Todd, 2010). Estas neuronas forman parte de múltiples proyecciones entre las que destacan los tractos espinotalámico y espinoreticular, que conectan con numerosas zonas cerebrales que integran la información nociceptiva. De estas estructuras se envía la información a las áreas somatosensoriales de la corteza cerebral (Basbaum et al., 2009; D'Mello y Dickenson, 2008).

Los estudios más recientes referidos a la transmisión nociceptiva supraespinal han sido realizados fundamentalmente en ratas mediante técnicas electrofisiológicas y técnicas de imagen como FMRI (functional magnetic resonance imaging) que han permitido delimitar las estructuras que participan en la percepción del dolor en humanos (Apkarian et al., 2011). Debido a la subjetividad y complejidad que abarca la experiencia dolorosa, no existe una única área cortical que funcione como centro del dolor sino que se habla de una estructura no definida denominada "matriz del dolor" (D'Mello y Dickenson, 2008). De un modo somero ésta incluye dos componentes neuroanatómicos, por una parte las áreas somatosensoriales primarias y secundarias y el tálamo encargados de los aspectos sensoriales y discriminativos y por otra, las partes anteriores de la ínsula, córtex cingulado anterior y córtex prefrontal implicados en los aspectos afectivos y cognitivos (Basbaum et al., 2009).

1.2 EL DOLOR EN EL ÁMBITO DEL CÁNCER

El tratamiento del dolor en el cáncer constituye una estrategia fundamental en la terapia paliativa de dicho proceso. Aproximadamente, un 70-90% de los pacientes con cáncer avanzado experimentan síntomas dolorosos (Honoré et al., 2000a; Mantyh, 2006; Mercadante, 2013) relacionados con la progresión del tumor y/o el fallo del tratamiento analgésico (Laird et al., 2008). Además de las manifestaciones dolorosas derivadas de la presencia del tumor, el propio tratamiento quimioterápico también puede ocasionar dolor. Así, entre el 20 y el 40% de los pacientes desarrollan dolor neuropático como consecuencia del tratamiento con fármacos antineoplásicos (Mantyh, 2006; Wolf et al., 2008; Bennett, 2010; Pachman et al., 2011). Este dolor derivado de la administración de fármacos antineoplásicos es de tipo neuropático y puede conllevar incluso el abandono del tratamiento (Wang et al., 2012a). Sus manifestaciones clínicas más frecuentes son las disestesias y parestesias en pies, manos, brazos y piernas (Wolf et al., 2008).

Uno de los tipos de dolor neoplásico más frecuentes y a su vez más difíciles de tratar es el dolor oncológico de origen óseo. Éste se puede manifestar en pacientes con tumores primarios como es el caso de sarcomas y algunos tumores hematológicos o bien secundariamente a la presencia de metástasis de diversos tumores primarios como los de próstata, mama y pulmón (Honoré et al., 2000a; Mundy, 2001; Buga y Sarria 2012). Se ha descrito que hasta el 83% de los pacientes con metástasis óseas sufren dolor (Smith, 2011) siendo un contexto patológico complejo donde la presencia de la metástasis conlleva una remodelación del sistema óseo que lleva a fracturas, hipercalcemia y anemia (Mundy, 2001; Jiménez-Andrade et al., 2010; Mantyh, 2006) que pueden reducir notablemente el estado funcional, la calidad de vida y la supervivencia del paciente (Montiel-Ruiz et al., 2013). El dolor generado por un tumor óseo se describe como un síntoma presente de forma continuada y constante, que incrementa su intensidad gradualmente a medida que avanza el desarrollo de la enfermedad y relativamente resistente al tratamiento farmacológico (Mercadante, 2013). Además, durante la progresión y el crecimiento del tumor es frecuente la aparición espontánea del dolor irruptivo, caracterizado por episodios intermitentes de dolor extremo incluso en presencia de tratamiento analgésico (Zeppetella et al., 2011). El mayor problema de este dolor es su aparición repentina, manifestándose incluso varias veces al día, por lo es de difícil control, provocando una reducción en la calidad de vida de los pacientes (Jiménez-Andrade et al., 2010).

Como es bien conocido, el tratamiento del dolor canceroso en la clínica se suele hacer siguiendo las directrices marcadas por la Organización Mundial de la Salud sobre el tratamiento

farmacológico del dolor (World Health Organization, 1990; 1996; 2006). Los fármacos incluídos en la escalera analgésica son los antiinflamatorios no esteroideos (AINE) para el tratamiento del dolor leve a moderado (escalón 1), los opiáceos débiles combinados con AINE si el dolor permanece o aumenta (escalón 2) y los opiáceos potentes para el tratamiento del dolor intenso (escalón 3). Éstos, junto con otras terapias adjuvantes como los bisfosfonatos, corticosteroides o radioterapia pueden ser suficientes para controlar el dolor, aunque aproximadamente un 25% de los pacientes permanece refractario al tratamiento analgésico establecido (Smith, 2011). El empleo crónico de AINE y opiáceos conlleva efectos adversos, especialmente gástricos y vasculares en el caso de los AINE, y depresión respiratoria y estreñimiento en el caso de los opiáceos, lo que puede limitar su utilidad especialmente teniendo en cuenta que los procesos cancerosos se pueden cronificar en muchos casos (Mantyh, 2006; Jiménez-Andrade et al., 2010). El reconocimiento de la falta de respuesta al tratamiento analgésico disponible o de la presencia de efectos adversos que limitan el empleo crónico de estos analgésicos en los pacientes oncológicos, junto con el desconocimiento de las bases fisiopatológicas del dolor producido en el contexto de cáncer constituyen las razones básicas que han promovido el desarrollo de modelos de dolor neoplásico experimental.

1.2.1. El dolor neoplásico en animales de experimentación

El desarrollo de modelos de dolor neoplásico experimental ha contribuido al conocimiento de los cambios neuroquímicos inducidos en el dolor canceroso así como al hallazgo de nuevas dianas farmacológicas que pueden resultar útiles para mejorar su tratamiento. Debido a que la mayoría de los tumores dolorosos son de naturaleza ósea, la mayor parte de los trabajos se basan en la inoculación de células tumorales en hueso. El primer modelo descrito se basa en la administración en la cavidad medular del fémur de células de fibrosarcoma NCTC 2472 en ratones (Schwei et al., 1999). Posteriormente se realizaron modificaciones en las que se inocularon las células en diferentes localizaciones como el calcáneo (Wacnik et al., 2001), la tibia (Menéndez et al., 2003) o el húmero (Wacnik et al., 2003; Zhao et al., 2004). Estas células tumorales producen lesiones osteolíticas debido a la acumulación y activación de osteoclastos, induciendo cambios neurobiológicos en función del desarrollo del tumor así como respuestas nociceptivas en los ratones. Posteriormente, se han generado nuevos tumores dolorosos en roedores en los que se inoculan diferentes líneas celulares. En general, la inoculación de células tumorales se realizó en los huesos que forman parte de las extremidades inferiores por la mayor facilidad que ello supone para medir la reactividad a estímulos nociceptivos en ensayos de comportamiento, así como para determinar alteraciones en la locomoción vinculadas al desarrollo del tumor. Los principales modelos de

dolor neoplásico desarrollados mediante la inoculación de células en hueso en ratones y ratas se recogen en las tablas 1 y 2 respectivamente.

Tabla 1. Resumen de los modelos murinos de dolor neoplásico experimental.

TIPO CELULAR	LUGAR IMPLANTE	CEPA DE RATÓN	REFERENCIA
NCTC 2472 (Fibrosarcoma)	Fémur	C3H/HeJ	Schwei et al., 1999
	Calcáneo	C3H/HeJ	Wacnik et al., 2001
	Tibia	C3H/HeJ	Menéndez et al., 2003
	Húmero	C3H/HeJ	Wacnik et al., 2003 Zhao et al, 2004
B6 (Melanoma)	Calcáneo	B6C3fe/1	Wacnik et al., 2001
	Húmero		Wacnik et al., 2003
B16-F10 (Melanoma)	Fémur	C3H/SCID	Sabino et al., 2003
	Tibia	C57BL/6	Curto-Reyes et al., 2008
B16-BL6 (Melanoma)	Intraplantar	C57BL/6	Sasamura et al., 2002
C26 Colon (Adenocarcinoma)	Fémur	C3H/SCID	Sabino et al., 2003
HaC-1 (Hepatocarcinoma)	Intraplantar	C3H/HeJ	Seong et al., 2004
4T1 (Cáncer de mama)	Fémur	BALB/cAnNCr	Goblirsch et al., 2006
AC1 (Cáncer de próstata)	Fémur	Atímicos	Halvorson et al., 2005
SCC (Carcinoma oral de células escamosas)	Intraplantar	*Foxn1*^{nu}	Schmidt et al., 2007
LCC (Carcinoma pulmonar de Lewis)	Fémur	C57BL/6	Isono et al., 2011

Tabla 2. Resumen de los modelos de dolor neoplásico experimental desarrollados en ratas.

TIPO CELULAR	LUGAR IMPLANTE	CEPA DE RATA	REFERENCIA
MRMT-1 (Carcinoma glandular de mama)	Tibia	Sprague-Dawley	Medhurst et al., 2002
AT- 3.1 (Cáncer de próstata)	Tibia	Copenhagen	Zhang et al., 2005a
Walker 256 (Carcinoma glandular de mama)	Tibia	Wistar	Mao-Ying et al., 2006
	Intraplantar	Wistar	Brigatte et al., 2007
MLL (Cáncer de prostata)	Fémur	Copenhagen	De Ciantis et al., 2010
AT3B (Cáncer de próstata)	Tibia	Wistar	Muralidharan et al., 2013

Para valorar la sensibidad nociceptiva de la pata afectada por el tumor se han empleado diferentes métodos como la prueba de la placa caliente (Wacnik et al., 2001; Menéndez et al., 2003), el test de presión sobre la pata (Schwei et al., 1999; Honoré et al., 2000b; Wacnik et al., 2001; Zhang et al., 2005a), y los filamentos de von Frey (Honoré et al., 2000b; Medhurst et al., 2002), utilizadas para evaluar la medida de la hiperalgesia térmica, la hiperalgesia mecánica y la alodinia mecánica respectivamente. Por otro lado, para tratar de valorar episodios espontáneos e intermitentes de dolor intenso asociados al movimiento, se realizan pruebas comúnmente utilizadas en otros modelos de dolor inflamatorio o neuropático como pueden ser el "flinching" (movimiento de alzada breve, espontáneo e intermitente del miembro afectado por el tumor) , el "guarding" (alzada mantenida y recogida del miembro afectado) (Halvorson et al., 2005; Goblirsch et al., 2006), "licking" (lamido del miembro afectado) (Sasamura et al., 2002; Brigatte et al., 2007) o el control de la capacidad deambulatoria del animal (Schwei et al., 1999; Wacnik et al., 2001; Menéndez et al., 2003).

Junto a la medida de la respuesta a estímulos nociceptivos o la presencia de dolor espontáneo, se han descrito numerosas modificaciones neuroquímicas debidas a la inoculación de las células tumorales en los animales de experimentación. Aunque los animales inoculados con células tumorales presentan en general una mayor reactividad a estímulos dolorosos, las alteraciones espinales descritas en relación mediadores/receptores que influyen en la nocicepción no parecen que ofrezcan un patrón único. Ello parece depender del tipo de células tumorales inoculadas, así como de aspectos temporales, es decir, de en qué momento de evolución del tumor se realizan los estudios. Por otra parte, establecer una diferenciación con otros tipos de dolor como es el inflamatorio o el neuropático parece dificultoso, seguramente porque en un proceso neoplásico conviven en distinto grado, además de las células tumorales que pueden secretar mediadores que estimulen la nocicepción, células inflamatorias así como un componente neuropático por la posible compresión e infiltración nerviosa. Finalmente, en estos modelos de dolor localizados en el hueso, hay que considerar que la destrucción ósea conlleva la presencia de microfracturas que también contribuyen a la reactividad dolorosa presente en estos animales.

Los resultados inmunohistoquímicos relativos a la inervación de las patas de los roedores, muestran una pérdida de fibras sensoriales que expresan CGRP en la región tumoral aunque posteriormente se demostró que las células tumorales producen una destrucción también de fibras nociceptivas no peptidérgicas, adquiriendo todas ellas una apariencia fragmentada y discontinua en las zonas más centrales del tejido tumoral (Peters et al., 2005; Mantyh, 2006). Además, a nivel de los DRG, los receptores TRPV1 se encuentran regulados al alza tras la administración de células tumorales (Niiyama et al., 2007) y lo mismo ocurre con

numerosos mediadores liberados por células inflamatorias y cancerosas como PGE_2 (Sabino et al., 2002) o IL-1 (Lewis et al., 2006; Baamonde et al., 2007). En cambio, no se observaron cambios significativos en la expresión de marcadores clásicos como SP, IB4 y neuropéptido Y (Honoré et al., 2000b).

En la médula espinal se describió inicialmente un aumento en la expresión de c-Fos, utilizado a menudo como marcador medular de la recepción de estímulos nociceptivos, en las láminas I, II y V (Schwei et al., 1999) así como del péptido hiperalgésico dinorfina (Schwei et al., 1999). Con posterioridad, estudios realizados en éste y otros modelos demostraron la regulación al alza de diversos receptores como los CCR2, P2X4 (Vit et al., 2006) y TLR4 (Lan et al., 2010) o mediadores hipernociceptivos como la IL-1β (Baamonde et al., 2007; Zhang et al., 2008) o el TNF- α (Geis et al., 2010). Asimismo, en la médula espinal se ha detectado astrogliosis en los segmentos medulares que reciben las aferencias nociceptivas tumorales en los modelos murinos basados en la inoculación de células de fibrosarcoma y células de cáncer de colon (Schwei et al., 1999; Honoré et al., 2000b; Hald et al., 2009a) en la cavidad medular del fémur pero no en el caso de la implantación de células de melanoma (Sabino et al., 2003). En contraste, la respuesta de la microglía al desarrollo tumoral parece menos uniforme y no se ha detectado un aumento significativo tras la inoculación de células NCTC 2472 en algunos estudios (Schwei et al., 1999) pero sí en otros con las mismas células (Vit et al., 2006) o con otras líneas celulares (Zhang et al., 2005a).

1.2.1.1. Farmacología del dolor neoplásico experimental

Durante estos años se ha estudiado la eficacia analgésica de numerosos fármacos en estos modelos de dolor neoplásico. Aunque algunos de ellos son usados frecuentemente en la clínica, en su mayoría se trata de fármacos sin uso comercial de los que se pretende evaluar su potencial utilidad en esta situación.

De este modo, ha sido estudiada la eficacia de múltiples estrategias farmacológicas dirigidas a reducir la actividad de nociceptores, neuronas espinales o centros cerebrales implicados en la integración nociceptiva. La administración sistémica o local de morfina redujo las respuestas dolorosas en varios de estos modelos de dolor (Honoré et al., 2000b; Wacnik et al., 2001; Sasamura et al., 2002; Medhurst et al., 2002; Menéndez et al., 2003). Sin embargo, contrariamente a lo esperado, un trabajo describió cómo la administración crónica de morfina puede producir un aumento de los síntomas nociceptivos así como la destrucción de hueso en el modelo de fibrosarcoma murino (King et al., 2007). Estos resultados supusieron una llamada de atención acerca de la necesidad de comparar estudios entre sí y tratar de evaluar los efectos de los fármacos en diferentes modelos de dolor neoplásico y en distintas especies

(Kontinen y Kalso, 2007). Otros derivados opioides como el fentanilo y el sulfentanilo (El Mouedden y Meert, 2005; El Mouedden y Meert, 2007) se mostraron también eficaces. Junto al sistema opioide, los cannabinoides constituyen uno de los principales sistemas inhibidores en el control de la nocicepción tanto a nivel periférico como central. En relación con el dolor canceroso, la administración de agonistas no selectivos de receptores CB1 y CB2 (Kehl et al., 2003; Hald et al., 2008; Cui et al., 2011; Uhelski et al., 2013), selectivos CB_1 (Hamamoto et al., 2007; Furuse et al., 2009) o CB_2 (Curto-Reyes et al., 2010; Gu et al., 2011; Lozano-Ondoua et al., 2013) ha demostrado ser efectiva para reducir los síntomas nociceptivos asociados a procesos neoplásicos óseos.

Se ha estudiado asimismo la actividad analgésica de un buen número de fármacos que actúan inhibiendo los nociceptores bien a través del bloqueo de receptores activadores o por inhibir la acción de moléculas sensibilizadoras. Respecto a la primera posibilidad, resultan particularmente interesantes los resultados obtenidos con agonistas y antagonistas de los receptores TRPV1 (Ghilardi et al., 2005; Menéndez et al., 2006; Niiyama et al 2009; Nguyen et al., 2010), que están regulados al alza en los DRG de ratones con sarcoma femoral (Niiyama et al., 2007) o los receptores P2X3 sobre los que actúa el ATP (González-Rodríguez et al., 2009; Kaan et al., 2010). En relación con el bloqueo de la sensibilización, ha resultado eficaz tanto el mecanismo clásico de inhibición de la síntesis de eicosanoides como el antagonismo de algunos receptores sobre los que actúan moléculas sensibilizadoras que, en la mayoría de los casos se encuentran sobreexpresadas a nivel tumoral. El efecto antinociceptivo de los AINE se observó tras la administración de inhibidores no selectivos de la enzima ciclooxigenasa (COX) (Saito et al., 2005; Vit et al., 2006) así como tras el tratamiento crónico (Sabino et al., 2002; Fox et al., 2004; Mao-Ying et al., 2008), pero no agudo (Medhurst et al., 2002; Fox et al., 2004) de inhibidores selectivos de la COX-2. Respecto al efecto derivado del bloqueo de receptores sobre los que actúan moléculas sensibilizadoras, diferentes síntomas de dolor canceroso experimental fueron atenuados por antagonistas de receptores B_1 de bradicinina (Sevcik et al., 2005b), receptores de tipo A para endotelina (Yuyama et al., 2004; Peters et al., 2004; Hamamoto et al., 2008), receptores TrkA sobre los que actúa el NGF (Halvorson et al., 2005; Sevcik et al., 2005a; Jiménez-Andrade et al., 2011; Ghilardi et al., 2010; Bloom et al., 2011) receptores de TNF-α (Geis et al., 2010) o receptores de IL-1beta (Baamonde et al 2007; Zhang et al 2008).

Otras estrategias ensayadas fueron dirigidas a inhibir principalmente la neurotransmisión a nivel espinal. En ese contexto se enmarca la gabapentina, un fármaco frecuentemente utilizado en el tratamiento del dolor neuropático y a veces como coadyuvante en pacientes con dolor canceroso, que fue capaz de reducir el comportamiento nociceptivo asociado a la

locomoción (Peters et al., 2005). Además, su administración aguda inhibió la hiperalgesia térmica y la alodinia mecánica descritas como consecuencia del proceso tumoral (Menéndez et al., 2008; Muralidharan et al., 2013). También ejerce efectos antinociceptivos en ratones inoculados con células tumorales la ketamina (Saito et al., 2006) que actúa inhibiendo la activación de neuronas espinales mediante el bloqueo de receptores NMDA y ha sido empleada ocasionalmente en clínica en el tratamiento de procesos de dolor canceroso (Bredlau et al., 2013). En relación con la médula espinal, resulta de interés la eficacia demostrada por inhibidores de células gliales, tanto astroglia como microglía (Liu et al., 2012; Mao-Ying et al., 2012; Wang et al., 2012b; Bu et al., 2014).

Además de haberse explorado todas estas posibilidades relacionadas con la transmisión nociceptiva, también se estudiaron los efectos de la administración de fármacos capaces de proteger del daño óseo actuando sobre la remodelación del hueso. En la clínica, el grupo más destacado es el de los bisfosfonatos. La administración del ácido zoledrónico (Walker et al., 2002), alendronato (Sevcik et al., 2004), ibandronato (Halvorson et al., 2008) o risedronato (Hald et al., 2009b) en diferentes modelos de dolor experimental por inoculación de células tumorales se tradujo en una disminución del dolor espontáneo y un descenso en los cambios neuroquímicos observados en el SNC y SNP así como una reducción del número de osteoclastos y un descenso en la resorción ósea. Otro abordaje farmacológico que puede modificar el proceso resortivo óseo es la administración de osteoprotegerina (OPG), una glicoproteína clave en la regulación del remodelado óseo a través de la unión con el RANKL (ligando del receptor activador del NF-κB), presente en los osteoblastos, que evita su unión al receptor RANK (receptor activador del NF-κB) presente en los osteoclastos. Como consecuencia de este proceso, se bloquea la diferenciación del osteoclasto a su estado maduro y por consiguiente, se reduce la degradación de la matriz ósea. Como podía esperarse, la administración de OPG disminuyó eficazmente los síntomas dolorosos, la actividad osteoclástica y los cambios neuroquímicos que aparecen en los modelos de dolor neoplásico (Honoré et al., 2000c; Luger et al., 2001). Ello sugiere que esta estrategia podría ser una alternativa importante para paliar el dolor en tumores óseos y, de hecho, un anticuerpo anti-RANKL denominado denosumab se ha comercializado recientemente para el tratamiento de pacientes con metástasis óseas (Rolfo et al., 2014).

1.2.2 Modelos experimentales de dolor neuropático por la administración de fármacos antineoplásicos.

Como se ha comentado anteriormente, la administración de algunos fármacos antineoplásicos induce el desarrollo de neuropatías periféricas. Entre estos compuestos

destacan fármacos clásicos como los taxanos (paclitaxel y docetaxel), derivados del platino (cisplatino, oxaliplatino, carboplatino), alcaloides de la vinca (vincristina y vinblastina) y fármacos de introducción más reciente como bortezomib y lenolidamida (Wolf et al., 2008; Wang et al., 2012a). La aparición de la neuropatía suele ocurrir tras la administración de dosis repetida del antineoplásico (Ta et al., 2013; Naguib et al., 2012), aunque también se han descrito casos en los que aparecen síntomas hipernociceptivos tras una administración única (Authier et al., 2000; Gauchan et al., 2009; Hidaka et al., 2009). Los síntomas más característicos son la alodinia mecánica, la hiperalgesia al calor y/o la hiperalgesia y alodinia al frio (Dina et al., 2001; Weng et al., 2003; Ling et al., 2007). Este cuadro parece derivar fundamentalmente de los efectos neurotóxicos que producen los antineoplásicos en células de DRG y axones periféricos, ya que la mayoría de ellos no atraviesan la barrera hematoencefálica (Wang et al., 2012a). En estas neuronas parecen provocar daño en los microtúbulos, alteraciones del transporte axonal, interrupción de la función mitocondrial o incluso daños directos sobre el DNA, ocasionando la degeneración de los nervios periféricos (Authier et al., 2009). Las características de la neuropatía evocada por antitumorales depende del tipo de fármaco. Así, la administración de compuestos derivados del platino (oxaliplatino, cisplatino, carboplatino) provoca una neuropatía puramente sensorial y en cambio, tras la administración de taxanos o alcaloides de la vinca la neuropatía puede ser sensorial y motora (Authier et al., 2009).

Puesto que uno de los trabajos incluidos en esta tesis se ha realizado con paclitaxel, se describen a continuación algunos aspectos de la neuropatía producida por este antineoplásico en animales de experimentación. Se suele asumir que la neuropatía producida por el paclitaxel se debe a su acción citotóxica sobre los microtúbulos neuronales necesarios para el transporte axonal (Smith et al., 2004). Se ha podido observar un descenso del grado de mielinización del nervio ciático, nervios subcutáneos y neuronas de la médula espinal (Cavaletti et al., 1995; Authier et al., 2000), la reducción del calibre de axones de las neuronas presentes en los DRG (Cliffer et al., 1998), pérdida de fibras nerviosas intraepidérmicas (IEFN) (Liu et al., 2010) y un descenso generalizado en la velocidad de conducción nerviosa (Cavaletti et al., 1995; Cliffer et al., 1998). Secundariamente al daño periférico inducido por el paclitaxel, a nivel espinal la expresión de numerosas citocinas pro-inflamatorias como TNF-α, IL-1β o IL-6 se encuentra elevada (Burgos et al., 2012). Además también se ha descrito la activación de células gliales a este nivel, aunque hay autores que describen tanto la activación de la microglía como de la astroglía (Naguib et al., 2012; Burgos et al., 2012), otros trabajos describen la activación solamente en relación a la microglía (Peters et al., 2007) o a la astroglía (Zhang et al., 2012a), quizás derivado del empleo de distintos protocolos experimentales. El aumento de la

sensibilidad nociceptiva se ha podido comprobar mediante diferentes pruebas de comportamiento. Así, se ha descrito hiperalgesia térmica (Polomano et al., 2001; Pascual et al., 2005; Matsumoto et al., 2006; Burgos et al., 2012; Peters et al., 2007) y mecánica (Authier et al., 2000; Polomano et al., 2001; Hidaka et al., 2009), así como alodinia mecánica (Smith et al., 2004; Pascual et al., 2005; Liu et al., 2010; Naguib et al., 2012) y alodinia al frío (Polomano et al., 2001; Nieto et al., 2008). Sin embargo, este antineoplásico no produce alteraciones físicas en los animales de experimentación (Polomano et al., 2001; Smith et al., 2004; Matsumoto et al., 2006; Hidaka et al., 2009; Pascual et al., 2005; Burgos et al., 2012) aunque en alguna ocasión se ha descrito la pérdida de peso o la aparición de alopecia como consecuencia del tratamiento con paclitaxel (Cavaletti et al., 1995; Peters et al., 2007).

En la práctica clínica, no existe un tratamiento farmacológico analgésico de referencia (Pachman et al., 2011) para contrarrestar el dolor secundario a la neuropatía producida por paclitaxel. Algunos de los fármacos ensayados en humanos que han mostrado eficacia en este sentido, han sido el baclofeno (Barton et al., 2011), la venlafaxina (Ozdogan et al., 2004) o la pregabalina (Saif et al., 2010) mientras que otros como la gabapentina, utilizada en la práctica clínica habitual (Pachman et al., 2011), no parece que sea efectiva para el tratamiento de esta patología (Rao et al., 2007).

Experimentalmente, se ha ensayado el efecto de diferentes fármacos analgésicos sobre la hipernocicepción provocada por paclitaxel. Opiáceos como la morfina (Flatters y Bennett, 2004; Pascual et al., 2010), la metadona (Pascual et al., 2010) o el tramadol (Xiao et al., 2008), AINE como el inhibidor selectivo COX-2 etodolac (Ito et al., 2012), antidepresivos como la amitriptilina (Xiao et al., 2008) y otros fármacos de uso clínico como son la ketamina (Pascual et al., 2010) o la gabapentina (Matsumoto et al., 2006; Xiao et al., 2007) atenúan la neuropatía periférica inducida por paclitaxel. Otros abordajes más novedosos con fármacos no incluídos en la terapéutica como son los antagonistas de receptores de bradicinina B_1 (Costa et al., 2011), antagonistas de receptores TRPV1 (Chen et al., 2011; Hara et al., 2013), antagonistas del receptor de IL-1β (Ledeboer et al., 2007), agonistas de receptores cannabinoides (Pascual et al., 2005; Naguib et al., 2012; Burgos et al., 2012), inhibidores de la activación células gliales (Liu et al., 2010; Zhang et al., 2012a) o incluso TTX (Nieto et al., 2008) también han mostrado su eficacia en modelos de dolor neuropático experimental por paclitaxel.

En nuestro trabajo, hemos estudiado el posible efecto antinociceptivo derivado del empleo de antagonistas de los receptores de quimiocinas CCR1 y CCR2 en ratones tratados con paclitaxel. Dado que constituye el primer estudio que relaciona las quimiocinas con la producción de dolor tras el tratamiento con un antineoplásico, no existen trabajos relacionados que se puedan comentar al respecto. Tras haber comentado en la sección 1.1 de esta

introducción algunos aspectos sobre el dolor en general y cuestiones sobre dolor neoplásico en la sección 1.2, en el apartado siguiente se describen los principales aspectos relativos al papel de las quimiocinas en la nocicepción.

1.3 LAS QUIMIOCINAS

La familia de las quimiocinas está constituida por un numeroso grupo de proteínas de peso molecular relativamente bajo (7-14 kD) clasificadas en función de la presencia de residuos cisteína en su región N-terminal. Siguiendo este criterio, se considera la existencia de 4 clases distintas de quimiocinas: las de tipo CC (presentan dos residuos de cisteína adyacentes), las CXC (un aminoácido separa las dos cisteínas), las CX3C (las dos primeras cisteínas están separadas por 3 aminoácidos) y las XC (existe una sola cisteína en el extremo amino-terminal). Aunque la mayoría de quimiocinas tienen un nombre original, otorgado cuando se describieron inicialmente, su nomenclatura se ha ido sistematizando con el tiempo, añadiendo al código anterior una letra (L, de ligando) y un número. Por ejemplo, la CCL2 corresponde a la quimiocina inicialmente llamada MCP-1 (monocyte chemoattractant protein-1) y la bautizada como MIP-1α (macrophage inflammatory protein -1- alpha) corresponde a la CCL3 en la denominación sistemática. Pese a que la denominación sistemática va ganando terreno a la original (y es la que se empleará en esta tesis doctoral), existen algunas quimiocinas cuyo nombre original sigue siendo ampliamente utilizado como es el caso de la fractalkina (CX3CL1) (White y Wilson, 2008).

Las quimiocinas ejercen sus efectos sobre receptores que se han clasificado con la misma clave inicial, en función del tipo de quimiocinas que actúa sobre ellos, pero seguidos de la letra R, de receptor. Algunos ejemplos pueden ser: CCR1, CCR2 o CX3CR1. Así pues, se reconoce la existencia de 11 tipos de receptores CC (CCR1-CCR11), 7 receptores CXC (CXCR1-CXCR7), 1 receptor CX3C (CX3CR1) y 1 receptor XC (XCR1). Añade mayor complejidad el hecho de que no siempre existe selectividad de unión entre una quimiocina y un receptor sino que varios receptores pueden ser estimulados por distintas quimiocinas y muchas quimiocinas pueden, a su vez, activar diferentes receptores (Charo y Ransohoff, 2006). Existe una excepción que confirma la regla y ésta es la unión exclusiva del CX3CL1 (fractalkina) al receptor CX3CR1 (D'Haese et al., 2010). Todos los receptores de quimiocinas están formados por 7 dominios transmembrana que están acoplados a proteínas G_q (Kuang et al., 1996). En su transducción están implicadas numerosas cascadas de segundos mensajeros como la vía de la cinasa del inositol trifosfato y de la proteina cinasa C (Murphy, 1994; Bajetto et al., 2002; Cartier et al., 2005). La función biológica inicial de las quimiocinas se relacionó con su capacidad para

atraer leucocitos desde la sangre hacia tejidos dañados, por ello reciben el nombre de citocinas "quimiotácticas", considerando que ejercen un papel fundamental en los procesos inflamatorios. Posteriormente, además de su implicación en la respuesta inflamatoria aguda, se ha descrito su mediación en la patogénesis de muchos procesos inflamatorios crónicos (Charo y Ransohoff, 2006) o en procesos neurológicos que se acompañan de reacciones inmunitarias, como la esclerosis múltiple (Ubogu et al, 2006). Además de la participación de estas proteínas en la inflamación, se ha demostrado el papel de los receptores de quimiocinas en funciones tan importantes como el desarrollo cerebral, la curación de heridas, la angiogénesis y por último en el mantenimiento y desarrollo de estados dolorosos (Zhang y Oppenheim, 2005; Gao y Ji, 2010).

1.3.1 Papel de las quimiocinas en la transmisión nociceptiva

La implicación de estas proteínas en la nocicepción representa un campo de investigación relativamente reciente. Por ello no existe todavía una visión completa sobre qué quimiocinas, mediante qué receptores y en qué condiciones pueden participar en la transmisión de las señales nociceptivas. Las quimiocinas se pueden sintetizar y liberar desde las neuronas periféricas de los DRG así como desde las neuronas localizadas en el asta dorsal de la médula espinal. Además, se ha descrito la presencia de receptores de quimiocinas a lo largo de las vías nociceptivas, a nivel periférico y a nivel central (Qin et al, 2005). Es importante destacar que la expresión de quimiocinas no se limita a neuronas sino que aparecen también en otros tipos celulares implicados en el desarrollo y mantenimiento del dolor crónico. Ejemplos de ello son las células de Schwann periféricas donde la CCL2 está regulada al alza (Fischer et al, 2008), o a nivel central, la células gliales. Tanto la astroglía como la microglía expresan algunos receptores de quimiocinas (CCR1, CCR2, CCR5, CXC3R o CX3CR1) cuando son activadas (Abbadie et al, 2003; Tanuma et al, 2006; Eltayeb et al, 2007; Gao et al., 2009).

Numerosos estudios apoyan el papel de las quimiocinas en el dolor agudo. En pruebas de comportamiento se ha descrito cómo éstas pueden producir hipernocicepción en animales de experimentación. Un trabajo pionero en esta línea de investigación describe el efecto hiperalgésico de la IL-8 (actualmente considerada la quimiocina CXCL8) (Cunha et al., 1991), resultados que fueron corroborados por Oh y cols (2001). También se demostraron los efectos nociceptivos directos de CCL2, CCL3, CCL5, CCL22, CXCL12 o CX3CL1 (Abbadie, 2005; White y Wilson, 2008), relacionados con el estímulo de los receptores correspondientes expresados en los DRG. Además también a nivel periférico, se ha demostrado que determinadas quimiocinas, como CCL2 y CCL3, pueden sensibilizar los nociceptores facilitando las respuestas de los receptores TRPV1 y TRPA1 (Zhang et al, 2005b; Jung et al, 2008). Es

importante destacar también cómo algunas quimiocinas como la CCL2 regula al alza la excitabilidad de los canales Na_v 1.8 (Belkouch et al. 2011) y otras como la CCL5 inducen el incremento de calcio intracelular en los DRG (Bolin et al., 1998; Bhangoo et al., 2007). En el SNC, se ha observado que algunas quimiocinas son hipernociceptivas cuando se administran a nivel espinal, como es el caso de CCL2, CCL3 o CX3CL1 (Milligan et al., 2004; Tanaka et al., 2004; Kiguchi et al., 2010a; Dansereau et al. 2008; Baamonde et al., 2011) o bien cuando se administran en la sustancia gris periacueductal, como ocurre con CCL5 (Benamar et al., 2008).

El papel de las quimiocinas en dolor crónico parece aún más importante que en dolor agudo (Abbadie et al., 2009) y como se comenta a continuación su función hipernociceptiva ha sido estudiada en diferentes tipos de dolor crónico. La participación de estas proteínas en el dolor producido por un daño neuropático ha sido ampliamente estudiada (White y Wilson, 2008). Se ha descrito que tras una lesión neuropática, se produce una regulación al alza de diferentes receptores de quimiocinas como pueden ser CCR2, CCR5, CXCR4, y CXCR3, además de la liberación de sus correspondientes ligandos endógenos en las neuronas de los DRG afectados por el daño (White et al., 2007). Debido a ello, puede darse la situación en que estas quimiocinas liberadas por las neuronas sensitivas actúen sobre un número elevado de receptores generándose un sistema de amplificación que contribuya de modo importante a la hiperexcitabilidad de los nociceptores. La participación de la quimiocina CCL2 y su receptor, CCR2, en el procesamiento de las señales nociceptivas tanto en periferia como a nivel central es particularmente importante en el dolor neuropático (White et al., 2005; Jeon et al., 2011). En concordancia con esta información, se ha descrito cómo ratones que carecen del receptor CCR2, reducen sus respuestas nociceptivas tras un daño en el nervio (Abbadie et al., 2003). La fractalkina (CX3CL1) y su receptor (CX3CR1), al igual que CCL4 participan también de forma relevante en la génesis del dolor neuropático (Staniland et al., 2010; Saika et al., 2012). Además otras quimicionas como CCL3 (Kim et al., 2011; Kiguchi et al., 2010a; Kiguchi et al., 2010b) y CCL5 (Benamar et al., 2008; Liou et al., 2012) participan en la modulación nociceptiva tras una lesión neuropática. Ambas se unen al receptor CCR1, el cual se encuentra regulado al alza en esta situación (Kiguchi et al., 2010a; Kiguchi et al., 2010b; Knerlich-Lukoschus et al., 2011).

Las quimiocinas también participan en la génesis del dolor tras el daño tisular producido por un proceso inflamatorio, aunque la información recogida en la bibliografía sobre esta premisa es más limitada. Se ha descrito cómo ratones sin el receptor CCR2 presentan menos síntomas nociceptivos en un modelo de dolor inflamatorio (Miller et al., 2012), así como una regulación al alza de la quimiocina CCL2 en los DRG de animales con inflamación mientras que no se han detectado cambios de esta proteína a nivel espinal (Jeon et al., 2008).

Respecto a la implicación de las quimiocinas en el dolor neoplásico experimental, antes de comenzar con nuestro estudio solamente dos trabajos habían mostrado información sobre la participación de la CCL2 en la hipernocicepción tumoral. Khasabova y cols. (2007), describen la liberación de esta quimiocina desde un fibrosarcoma inducido por inoculación células NCTC 2472 en el calcáneo. Además también muestran la presencia de cambios neuroquímicos en las neuronas periféricas que parecen estar vinculados al aumento en los niveles de CCL2, como el incremento de la subunidad $\alpha_2\delta$-1 de canales de Ca^{2+} dependientes de voltaje o de receptores TRPV1. El otro estudio, realizado en ratones inoculados con las mismas células NCTC 2472 en el humero, muestra un ligero aumento en la densidad de receptores CCR2 espinales (Vit et al., 2006). Aunque en ambos trabajos se señalaba la posible contribución de las quimiocinas al dolor tumoral, no existía ningún estudio de comportamiento que comprobara esta posibilidad.

La ausencia de datos sobre el papel jugado por la quimiocinas tanto en el dolor neoplásico experimental como en el dolor neuropático producido por antineoplásicos, nos animó a orientar el trabajo recogido en esta tesis hacia el estudio de diferentes aspectos relacionados con la implicación de CCR2 y CCR1 en estos ámbitos. Como se comenta más adelante, durante la realización de esta tesis, se han publicado tres trabajos relacionados con la implicación de las quimiocinas CCL2 y CCL5 en un modelo de dolor debido a la inoculación de células tumorales derivadas de una línea de cáncer de mama en ratas (Hu et al., 2012; Hu et al., 2013; Hang et al., 2013). Junto con los datos recogidos en esta tesis, estos resultados comienzan a perfilar la relevancia funcional de las quimiocinas en este contexto experimental.

2. Justificación y Objetivos

Durante estos últimos años, la investigación básica sobre dolor oncológico ha experimentado un cierto auge debido al desarrollo de modelos experimentales en roedores. En su mayor parte, estos modelos se han centrado en el estudio del dolor secundario a la presencia de procesos neoplásicos óseos, lo que parece justificado dado que la afectación ósea está muy frecuentemente asociada a la aparición de síntomas dolorosos y tiene además elevada incidencia (Mercadante, 2013). De hecho, el hueso es uno de los sitios más comunes donde se localizan las metástasis (Rubens, 1998; Montiel-Ruiz et al., 2013) y se ha calculado que al menos un tercio de los pacientes con cáncer en estado avanzado puede sufrir metástasis óseas en el transcurso de la enfermedad (Thurlimann y de-Stoutz, 1996). El dolor asociado a los procesos cancerosos se trata de forma eficaz en la mayoría de los casos con los analgésicos actualmente disponibles. Sin embargo, en el 10% de los pacientes el tratamiento farmacológico no resulta eficaz (Meuser et al., 2001) y por otra parte, la deseable y cada vez más frecuente, cronificación de muchos procesos cancerosos hace que el empleo prolongado de estos fármacos pueda verse limitado por la aparición de efectos adversos (IASP, 2009). A través de los estudios realizados con estos modelos de dolor óseo de origen neoplásico se ha avanzado de un modo importante en el conocimiento de los mecanismos que generan y mantienen el dolor canceroso óseo, lo que puede resultar fundamental para promover el desarrollo de nuevas estrategias terapeúticas.

Las quimiocinas constituyen un sistema de mediadores que, además de su papel previamente reconocido en situaciones de inflamación (Charo y Ransohoff, 2006), se han implicado más recientemente en la nocicepción, especialmente en el campo del dolor neuropático (White et al., 2007; White y Wilson, 2008). Su localización y funcionalidad tanto en los ganglios de la raíz dorsal (DRG) (Jung et al., 2009) como en el sistema nervioso central (SNC), especialmente en la médula espinal (Gao et al., 2009), así como su expresión aumentada en muchos casos tras neuropatía (Jeon et al., 2011; Kiguchi et al., 2010a; Knerlich-Lukoschus et al., 2011) hacen de este sistema una diana atractiva para estudiar su implicación en el dolor relacionado con el cáncer.

A raíz de la incorporación al Instituto de Oncología del Principado de Asturias (IUOPA) en el año 2000, nuestro grupo del laboratorio de Farmacología de la Universidad de Oviedo se ha dedicado al estudio de la hipernocicepción de origen tumoral. A nivel experimental, el dolor tumoral resulta un problema complejo ya que en él entran en juego muchas variables como el tipo de células tumorales, la mayor o menor presencia de células inflamatorias, el posible desarrollo de una neuropatía acompañante o las lesiones que se producen en el propio hueso sobre el que asientan las células tumorales que pueden conducir a microfracturas o incluso fracturas (Mantyh, 2006). Considerando la diversidad de mecanismos que pueden participar en

estas situaciones, hemos tratado de no ceñirnos exclusivamente al uso de un único modelo experimental para lo que hemos desarrollado dos tipos de tumores óseos que producen diferentes lesiones. El modelo utilizado inicialmente está basado en la inoculación en la cavidad medular de la tibia de células de fibrosarcoma NCTC 2472 a ratones C3H/He (Menéndez et al., 2003), lo que provoca fundamentalmente lesiones osteolíticas. Los ratones inoculados presentan alodinia mecánica dos semanas tras la inoculación, hiperalgesia mecánica a las tres semanas, e hiperalgesia térmica a las cuatro semanas (Menéndez et al., 2003; Menéndez et al., 2005; Baamonde et al., 2006). Junto a este modelo, hemos trabajado también con ratones C57BL/6 inoculados en la tibia con células B16-F10, procedentes de una línea tumoral de melanoma que generan lesiones en las que se observa actividad osteogénica junto a la osteolítica (Curto-Reyes et a., 2008). Las alteraciones comportamentales, hiperalgesia y alodinia, aparecen antes que en el caso anterior y se pueden medir ya a partir de la primera semana tras la inoculación de las células tumorales (Curto-Reyes et al., 2008).

Al lado de estos modelos de dolor debidos a la presencia de procesos cancerosos, en el contexto de cáncer también es relevante el dolor derivado del tratamiento con antineoplásicos. La administración de determinados antineoplásicos como los taxanos, alcaloides de la vinca, cisplatino o bortezomib se asocian a dolor neuropático, que puede incluso llegar a ser un factor limitante de su uso clínico (Argyriou et al., 2008; Wolf et al., 2008). Con la intención de encontrar estrategias útiles para contrarrestar esta neuropatía, también se han desarrollado modelos animales en los que se miden fundamentalmente la hiperalgesia o la alodinia tras la administración de estos antineoplásicos (Dina et al., 2001; Weng et al., 2003; Ling et al., 2007; Ta et al., 2013; Naguib et al., 2012).

En este trabajo se plantea la **hipótesis** de que **el bloqueo de los receptores de quimiocinas de tipo CCR2 y CCR1 podría contrarrestar las manifestaciones nociceptivas en animales con procesos tumorales o tratados con antineoplásicos**. En base a esta idea, se diseñaron experimentos relacionados con la quimiocina CCL2, el principal ligando de los receptores CCR2 (Abbadie et al., 2003) así como con otro grupo de quimiocinas capaces de estimular los receptores CCR1, como son la CCL3 y CCL5, de las que, a diferencia del caso anterior no existe mucha bibliografía previa en relación con la modulación de la nocicepción (Eijkelkamp et al., 2010; Kim et al., 2011; Liou et al., 2012).

En el momento del diseño del trabajo solamente existía algún dato relacionado con el dolor neoplásico experimental en dos publicaciones previas: la descripción de la presencia aumentada de la quimiocina CCL2 en un modelo dolor derivado de la inoculación de células NCTC 2472 en el calcáneo en ratones C3H (Khasabova et al., 2007) así como la expresión

aumentada del receptor sobre el que actúa esta quimiocina CCL2, CCR2, en la médula espinal de ratones también inoculados con células NCTC 2472 (Vit et al., 2006) pero no existía ningún estudio funcional de comportamiento en el que se demostrase la eficacia del bloqueo de los CCR2 para contrarrestar las manifestaciones nociceptivas en estos ratones. Por otra parte, ninguna publicación relacionaba la presencia de quimiocinas con el dolor neuropático producido tras la administración de antineoplásicos. En base a estos datos y a las consideraciones anteriormente expuestas, nuestros ***objetivos*** fueron los siguientes:

1. Valorar el posible efecto antinociceptivo derivado del bloqueo de los CCR2 presentes en la zona tumoral en ratones inoculados con células tumorales NCTC 2472 y B16-F10, así como su posible relación con modificaciones en los niveles de CCL2.

2. Analizar el papel del sistema CCR2/CCL2 a nivel espinal en las reacciones hipernociceptivas medidas en ratones inoculados con células NCTC 2472 y especialmente su relación con la glía espinal, que resulta activada en esta situación experimental.

3. Evaluar el efecto antinociceptivo derivado de la administración de antagonistas de receptores CCR2 y CCR1 en un modelo de dolor neuropático basado en la administración sistémica de paclitaxel en ratones así como su relación con la presencia de quimiocinas y con la activación de las células gliales de la médula espinal.

4. Ensayar la eficacia del bloqueo de receptores CCR1 para producir efectos antihiperalgésicos y antialodínicos en los dos modelos murinos de dolor neoplásico basados en la inoculación intratibial de células NCTC 2472 o de células B10-F10, tratando de relacionar los posibles efectos con variaciones en los niveles de las quimiocinas, CCL3 y CCL5, capaces de activar estos receptores.

3. Artículos

3.1 Artículo 1:

Pevida M, González-Rodríguez S, Lastra A, Hidalgo A, Menéndez L, Baamonde A. **CCL2 released at tumoral level contributes to the hyperalgesia evoked by intratibial inoculation of NCTC 2472 but not B16-F10 cells in mice**. Naunyn-Schmiedeberg's Arch Pharmacol 385:1053-1061. 2012.

OBJETIVO

Valorar el posible efecto antinociceptivo derivado del bloqueo de los CCR2 presentes en la zona tumoral en ratones inoculados con células tumorales NCTC 2472 y B16-F10, así como su posible relación con modificaciones en los niveles de CCL2.

MÉTODOS

- Ratones C3H/He inoculados con células NCTC 2472 y ratones C57BL/6 inoculados con células B16-F10 en la cavidad medular de la tibia.
- ELISA (cuantificación de los niveles de la quimiocina CCL2).
- Test de la placa caliente unilateral (medida de la hiperalgesia térmica).
- Test de presión sobre la pata (modificación del test Randall-Selitto para la medida de la hiperalgesia mecánica).
- Test de von Frey (medida de la alodinia mecánica).

RESULTADOS Y CONCLUSIONES

- El desarrollo tumoral produce un incremento local de los niveles de la quimiocina CCL2 tras la inoculación intratibial de células de fibrosarcoma NCTC 2472 pero no de células de melanoma B16-F10.
- El bloqueo de los receptores CCR2 periféricos inhibe la hiperalgesia mecánica o térmica pero no la alodinia de origen tumoral en ratones inoculados con células de fibrosarcoma NCTC 2472 pero no con células de melanoma B16-F10. La quimiocina CCL2 endógena participa en la hipernocicepción tumoral evocada en ratones que reciben la inoculación intratibial de células de fibrosarcoma NCTC 2472.

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ORIGINAL ARTICLE

CCL2 released at tumoral level contributes to the hyperalgesia evoked by intratibial inoculation of NCTC 2472 but not B16-F10 cells in mice

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Abstract The participation of the chemokine CCL2 (monocyte chemoattractant protein-1) in inflammatory and neuropathic pain is well established. Furthermore, the release of CCL2 from a NCTC 2472 cells-evoked tumor and its involvement in the upregulation of calcium channel α2δ1 subunit of nociceptors was demonstrated. In the present experiments, we have tried to determine whether the increase in CCL2 levels is a common property of painful tumors and, in consequence, the administration of a chemokine receptor type 2 (CCR2) antagonist can inhibit tumoral hypernociception. CCL2 levels were measured by ELISA in the tumoral region of mice intratibially inoculated with NCTC 2472 or B16-F10 cells, and the antihyperalgesic and antiallodynic effects evoked by the administration of the selective CCR2 antagonist RS 504393 were assessed. Cultured NCTC 2472 cells release CCL2 and their intratibial inoculation evokes the development of a tumor in which CCL2 levels are increased. Moreover, the systemic or peritumoral administration of RS 504393 inhibited thermal and mechanical hyperalgesia, but not mechanical allodynia evoked after the inoculation of these cells. Thermal hyperalgesia was also inhibited by the peritumoral administration of a neutralizing CCL2 antibody. In contrast, no change in CCL2 levels was observed in mice inoculated with B16-F10 cells, and RS 504393 did not inhibit the hypernociceptive reactions evoked by their intratibial inoculation. The peripheral release of CCL2 is involved in the development of thermal and mechanical hyperalgesia, but not mechanical allodynia evoked by the inoculation of NCTC 2472 cells, whereas this chemokine seems unrelated to the hypernociception induced by B16-F10 cells.

Keywords Bone cancer-induced pain · Hyperalgesia · Allodynia · CCL2 · CCR2 antagonists · Mouse · NCTC 2472 cells

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Introduction

CCL2, also called monocyte chemoattractant protein-1 (MCP-1), is a member of the CC group of chemokines that, apart from its involvement in monocyte/macrophage chemotaxis, plays an important role in the modulation of nociception in pathological settings (Abbadie et al. 2009). As occurs with the majority of chemokines, CCL2 does not act exclusively on a unique type of receptors, its main effects being evoked through the activation of chemokine receptor type 2 (CCR2) (Abbadie et al. 2003).

Different experimental data illustrate a complex modulation of nociceptive processing by the CCL2/CCR2 system both at peripheral and spinal level. Thus, the ability of CCL2 to provoke neural sensitization in the spinal cord has been demonstrated by electrophysiological recordings of spinal lamina II neurons (Gao et al. 2009) and also by behavioral experiments in which hypernociception occurs when CCL2 is directly administered into the spinal cord of rodents (Tanaka et al. 2004; Dansereau et al. 2008; Baamonde et al. 2011).

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More directly related with the present experiments is the involvement of CCL2 in the peripheral processing of nociceptive signals. A role for CCL2 as peripheral sensitizer is supported by the induction of mechanical (Bogen et al. 2009) and thermal (Qin et al. 2005) hyperalgesia following its administration into the rat paw. CCL2 can sensitize nociceptors through several mechanisms, such as the stimulation of phospholipase C and protein kinase C together with the release of calcium (Qin et al. 2005), the transactivation of capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1) (Jung et al. 2008), or the enhancement of Nav1.8 channels excitability (Belkouch et al. 2011). Interestingly, CCL2-inmunoreactive inflammatory cells are detected in inflamed tissues (Ogura et al. 2010), and the increased presence of this chemokine has also been demonstrated in dorsal root ganglia (DRG) of rats inflamed with complete Freund's adjuvant (Jeon et al. 2008). Supporting the relevance of these findings, a possible correlation between CCL2 gene expression in inflamed tissue and pain intensity perceived in patients with oral inflammation has been proposed (Wang et al. 2009). Besides, CCL2 also plays a prominent role in the reaction that appears after nerve injury. Both CCL2 and CCR2 can be upregulated at DRG neurons in neuropathic settings (White et al. 2005; Jeon et al. 2011), and it has been further described that CCL2 upregulation seems to be related to the presence of TNFα (Jeon et al. 2011) or IL-1β (White et al. 2005). In accordance with these studies, mice lacking CCR2 show decreased nociceptive behaviors after neuropathic injury (Abbadie et al. 2003).

Our knowledge related to the involvement of CCL2 in painful neoplastic processes is more limited. It seems likely that CCL2 could participate in tumoral hypernociception since its release has been detected from different types of human tumors, some of which are associated with painful symptoms such as pancreatic cancer (Chehl et al. 2009) or bone metastases derived from prostate or breast neoplastic processes (Shirotake et al. 2012; Soria et al. 2011). Experimentally, Khasabova et al. (2007) have demonstrated an increase in CCL2 concentrations at tumoral level in mice inoculated with NCTC 2472 fibrosarcoma cells into the calcaneus. In these studies, the participation of CCL2 in the induction of phenotypic changes that can lead to hyperalgesia, such as the increase in the expression of $\alpha_2\delta$-1 subunit of calcium channels in nociceptors, was also shown (Khasabova et al. 2007). Although these data seem to indicate that CCL2 could play a role in the development of tumoral hyperalgesia, the possible antihyperalgesic effect evoked by CCR2 antagonists has not been assessed in experimental models of neoplastic hypernociception. Thus, in the present experiments, we study whether CCL2 concentrations are increased at tumoral level and DRG in two models of murine bone cancer-induced pain. The intratibial inoculation of NCTC fibrosarcoma 2472 cells in C3H/He mice induces osteolytic lesions accompanied by thermal and mechanical hyperalgesia as well as mechanical allodynia that can be measured at weeks 4 and 2, respectively (Menéndez et al. 2003: Baamonde et al. 2007). Besides, the inoculation of B16-F10 melanoma cells induces rapid changes in bone (osteoid formation with osteolytic lesions) together with thermal and mechanical hyperalgesia as well as mechanical allodynia during the first week after their inoculation (Curto-Reyes et al. 2008). In these tumor-bearing mice, we further explored whether the administration of RS 504393, a selective CCR2 antagonist, can inhibit bone cancer-evoked hypernociception.

Methods

Animals

Experiments were performed in 5- to 6-week-old C3H/He and C57BL/6 male mice bred in the Animalario de la Universidad de Oviedo (Reg. 33044 13A), maintained on a 12-h dark–light cycle with free access to food and water. Experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain) and according to the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann 1983). Each animal was used only once. Behavioral experiments were performed between 15:00 and 20:00 in a thermostated (21 °C) and noise-isolated room. The experimenter was not systematically unaware about the treatment received by each mouse. In order to minimize experimental bias, mice were randomly assigned to solvent or drug treatment, taking care that animals treated with solvent and with different doses of drug were tested in every session.

Cell inoculation

NCTC 2472 cells (American Type Culture Collection, ATCC) were cultured in NCTC 135 medium (Sigma) containing 10 % horse serum (Sigma), passaged weekly according to ATCC guidelines. At confluence, cells were detached by scraping, centrifuged at 400 × g for 10 min and the remaining pellet suspended in PBS (Menéndez et al. 2003). B16-F10 melanoma cells (American Type Culture Collection) were cultured in DMEM (Gibco) enriched with 10 % fetal calf serum (FCS, Gibco). When cells were preconfluent, they were treated with trypsin/EDTA (0.05/0.02 %) and detached. The trypsin/EDTA solution was recovered, neutralized with DMEM supplemented with 10 % FCS and centrifuged at 400 × g for 10 min (Curto-Reyes et al. 2008).

For surgical procedures, anesthesia was induced by spontaneous inhalation of 3 % isoflurane (Isoflo®, Esteve) and maintained by administering 1.5 % isoflurane in oxygen through a breathing mask. A suspension of 10^5 cells in

5 μl of PBS was injected into the right tibial medullar cavity, and after applying acrylic glue (Hystoacril®, Braun) on the tibial plateau incised area, surgery was finished with a stitch of the skin. Control mice received the inoculation of 10^5 cells previously killed by quickly freezing them three times without cryoprotection. According to the appropriate timing to reach maximal hypernociception, mechanical allodynia was assessed 2 weeks after the inoculation of NCTC 2472 cells and thermal and mechanical hyperalgesia 4 weeks after cell implantation (Menéndez et al. 2003). In mice that received intratibial B16-F10 cells, experiments were performed 1 week after inoculation (Curto-Reyes et al. 2008).

Drugs

The CCR2 receptor antagonist, RS 504393 (Tocris), or its corresponding solvent (5 % DMSO at the highest concentration used) was subcutaneously administered under the fur of the neck in a volume of 10 ml/kg 30 min before testing.

RS 504393 or its solvent, a goat anti-mouse CCL2 antibody (R&D, AF-479-NA) or an anti-mouse goat antiserum diluted in saline at the same IgG concentration than the anti-CCL2 antibody (Sigma) were peritumorally administered. The cross-reactivity of this antibody against other mouse chemokines such as MCP-5, MIP 1α, MIP 1β, MIP 1γ, MIP-2, MIP 3β, and RANTES is less than 0.01 %. For peritumoral administration, drugs were injected subcutaneously over the tibial tumor mass in a volume of 100 μl. Injections in the left, contralateral, paw were performed in the same region of the limb which was, in this case, free of tumor.

Behavioral studies

Unilateral hot plate test

Thermal withdrawal latencies were measured by the unilateral hot plate test (Menéndez et al. 2003). Briefly, mice were gently restrained and the plantar side of the tested paw placed on the hot plate surface (50.5 °C for C3H/He mice and 49.5 °C for B16-F10) as previously described (Curto-Reyes et al. 2010). Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at 2-min intervals and the mean of two measures was considered. A cut-off of 20 s was established.

Paw pressure test

Mechanical withdrawal latencies were measured by a previously described adaptation of the Randall–Selitto method (Menéndez et al. 2005), in which a constant pressure stimulus is used. Mice were gently restrained and a pressure of 450 g was applied to their hindpaws with a Ugo Basile 7200 apparatus until a struggle reaction appears. The measurements of the withdrawal latencies of each hindpaw were made separately and alternately at 2-min intervals and the mean of two measures made in each hindpaw was considered. A 60-s cut-off was established in order to prevent tissue damage.

von Frey test

Mechanical allodynia was assessed by applying von Frey filaments (Stoelting) to the plantar side of the paws as previously reported (Baamonde et al. 2007). Mice were placed on a wire mesh platform and allowed for habituation for 20 min. The von Frey filaments 2.44, 2.83, 3.22, 3.61, 4.08, and 4.56 were used, and, starting with the 3.61 filament, six measurements were taken in each animal randomly starting by the left or right paw. Based on the "up and down" method (Chaplan et al. 1994), the observation of a positive response (lifting, shaking, or licking of the paw) was followed by the application of the immediate thinner filament or the immediate thicker one if the response was negative. The 50 % response threshold was calculated using the following formula: 50 % g threshold = $(10^{Xf + \kappa\delta})$ / 10,000; where Xf is the value of the last von Frey filament applied; κ is a correction factor based on pattern of responses (from the Dixon's calibration table); and δ is the mean distance in log units between stimuli (here, 0.4).

Enzyme-linked immunosorbent assay (ELISA)

CCL2 levels were measured in NCTC 2472 and B16-F10 cell culture medium before and after the incubation of cells during 4 days in flasks with 15 ml when cells reached confluence. Experiments were also performed in tissue homogenates prepared from hind limbs or DRG either ipsilateral or contralateral to the inoculated paw of mice receiving live or killed tumoral cells. Once harvested, media and tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Hind limbs were denuded from ankle to knee, including bone and soft tissue, and individually homogenized. Homogenates of DRG were prepared in pools of L3–L5 DRG obtained from six animals (18 DRG per homogenate). The buffer used consisted 0.1 M Tris, 0.15 M NaCl, 0.5 % CTAB (Fluka), and a protease inhibitor (1 tablet/50 ml buffer, Roche Diagnostics). Limbs were homogenized in a volume of 3 μl/mg with a Polytron PT-MR3100 (Kinematica) and DRG in a volume of 24 μl by using a Minicraft MB130. Next, homogenates were centrifuged at 15,000 × g for 15 min at 4 °C, and protein concentration of supernatants was measured by a BCA protein assay (Pierce) according to the manufacturer's protocol.

The level of CCL2 was measured with a commercially available sandwich enzyme-linked immunosorbent assay (R&D Systems, DuoSet® Mouse CCL2/JE/MCP-1). Following the instructions of the manufacturer, plates (R&D

Systems) were coated overnight at room temperature with an antibody specific for mouse CCL2. In order to achieve a value in the range of the standard curve of CCL2, protein quantities added to the wells for the different homogenates were 100 μg for limbs and 70 μg for DRG in a final volume of 100 μl. After washing, a 2-h incubation period was performed with a second biotinylated anti-mouse CCL2 antibody and followed by a 20 min incubation period with streptavidin-peroxidase (HRP). After washing to remove all the unbound enzyme, color was developed by adding a stabilized chromogen (tetramethylbenzidine: H_2O_2, 1:1) and the reaction was terminated with a stop solution (2 N H_2SO_4). The intensity of the colored product was quantified spectrophotometrically at 450 nm substracting the readings obtained at 570 nm in order to correct optical background of plates.

Values obtained from culture media and limbs came from four to five independent measures performed in duplicate and in the case of DRG, from five independent data.

Statistical analysis

The mean values and the corresponding standard errors were calculated for each ELISA measurement or behavioral assay. The values of CCL2 levels obtained in culture media were compared by a Student's *t* test and those obtained from tissue homogenates were compared by an initial one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Thermal and mechanical withdrawal latencies were compared by the Student's *t* test for grouped values when the latency obtained in the ipsilateral paw was compared with that obtained in the contralateral one or by a one-way ANOVA followed by the Dunnett's *t*- when groups that received different doses of RS 504393 were compared with the solvent-treated one. Threshold values obtained by the von Frey test in ipsilateral and contralateral paws were compared by the *U* Mann–Whitney's test. In all cases, the level of significance was set at $P<0.05$.

Results

Measurement of CCL2 levels in the culture medium of NCTC 2472 cells and in samples coming from limbs and DRG of mice intratibially inoculated with NCTC 2472

In order to determine if tumoral cells can release CCL2, the concentration of this chemokine was measured by ELISA in the culture medium before and after NCTC 2472 cell incubation. Low CCL2 levels were present in the medium before its contact with tumoral cells, whereas an important increase (eight times) was found 4 days after culture when cells reached confluence (Fig. 1a).

In homogenates prepared from the tibial region obtained from ipsilateral and contralateral limbs of C3H/He mice 4 weeks after the intratibial inoculation of either live or killed NCTC 2472 cells, the levels of CCL2 were also measured. Similar low [CCL2] were obtained in homogenates prepared both from limbs of mice inoculated with killed cells and from the contralateral paw of osteosarcoma-bearing mice. In contrast, the concentrations measured in homogenates coming from tumor-bearing paws were about 12 times higher (Fig. 1b).

No change in the levels of CCL2 measured in homogenates prepared from L3–L5 DRG was detected when comparing those obtained in the ipsilateral side with those of the contralateral ones in mice inoculated with killed or live NCTC 2472 cells (Fig. 1c).

Measurement of CCL2 levels in the culture medium of B16-F10 cells and in samples coming from limbs and DRG of mice intratibially inoculated with B16-F10

No change was detected in the concentration of CCL2 detected in culture medium before and after the growth of B16-F10 cells (Fig. 2a). Also, similar CCL2 levels were found when homogenates of the tibial region of mice inoculated with killed or live B16-F10 cells 1 week before were compared (Fig. 2b). The values obtained in homogenates prepared with ipsilateral or contralateral DRG coming from mice inoculated with killed or live B16-F10 cells were also indistinguishable (Fig. 2c).

The administration of the CCR2 antagonist RS 504393 or a CCL2 antibody inhibits NCTC 2472-evoked thermal hyperalgesia

The intratibial inoculation of live NCTC 2472 cells 4 weeks before evoked a thermal hyperalgesic reaction. This thermal hyperalgesia was dose-dependently inhibited 30 min after the s.c. administration of the selective CCR2 antagonist RS 504393 (0.3–3 mg/kg). The dose of 0.3 mg/kg did not modify thermal withdrawal latencies, a partial antihyperalgesic effect was observed after the administration of 1 mg/kg, and a complete inhibition of osteosarcoma-induced hyperalgesia was attained when 3 mg/kg of RS 504393 was administered (Fig. 3a). The administration of the highest dose did not modify thermal latencies in mice inoculated with killed NCTC 2472 cells (data not shown).

In order to elucidate whether the analgesic effect evoked by RS 504393 in tumor-bearing mice could be peripherally mediated, RS 504393 was locally administered at tumoral level. A dose-dependent antihyperalgesic effect was again observed 30 min after the peritumoral injection of 3–30 μg of the CCR2 antagonist (Fig. 3b). In contrast, the administration of the maximal dose in the contralateral paw did not modify thermal withdrawal latencies in the tumor-bearing paws (not shown), supporting the involvement of locally-produced antihyperalgesic effects.

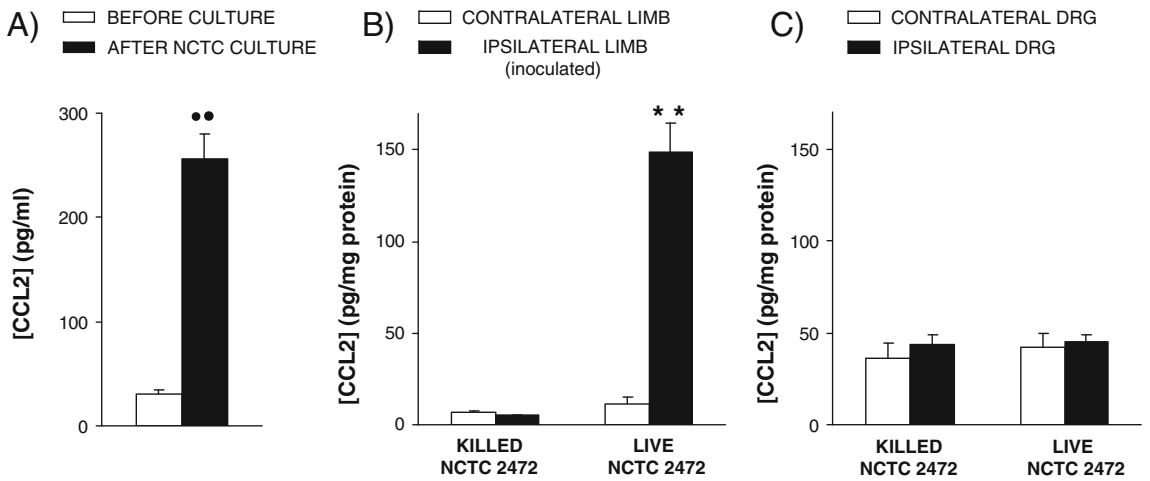

Fig. 1 CCL2 levels measured by ELISA. **a** [CCL2] in NCTC 2472 culture medium in the absence of cells and after cell confluence. **b** and **c** CCL2 levels measured in homogenates of hindpaws (**b**) or of L3-L5 DRG (**c**) of mice intratibially inoculated 4 weeks before with 10^5 live or killed NCTC 2472 cells. Means and their corresponding standard errors are represented (n=4-5). *Double black circles* indicate $P<0.01$ compared with the values obtained before culture. *Double asterisks* indicate $P<0.01$ compared with contralateral limbs and limbs inoculated with killed cells, Newman–Keuls test

Since CCL2 is one of the main endogenous chemokines that bind CCR2, we have assessed the effect of neutralizing this chemokine. The peritumoral administration of a CCL2-anti-mouse antibody (4 μg) completely blocked thermal hyperalgesic responses induced in these tumor-bearing mice (Fig. 3c), supporting the role of this chemokine. The administration of this dose of antibody showed no effect in mice inoculated with killed NCTC 2472 cells (not shown).

The administration of the CCR2 antagonist RS 504393 inhibits mechanical hyperalgesia, but not mechanical allodynia, evoked by the inoculation of NCTC 2472 cells

A decrease in withdrawal latencies was measured in the paw pressure test 4 weeks after the intratibial inoculation of NCTC 2472 cells. This mechanical hyperalgesia was completely inhibited 30 min after the administration of RS 504393 either subcutaneously (3 mg/kg; Fig. 4a) or peritumorally (30 μg; Fig. 4b). The administration of 3 mg/kg of RS 504393 to mice inoculated with killed NCTC 2472 or the administration of 30 μg in the contralateral paw of tumor-bearing mice did not modify withdrawal latencies in the paw pressure test (data not shown).

Mechanical allodynia measured by the von Frey test appears in mice inoculated 2 weeks before with NCTC 2472 cells. The lowered threshold measures obtained in the von Frey test in inoculated mice were not modified by the s.c. administration of RS 504393 (3 mg/kg; Fig. 4c), thus mechanical allodynia being unaffected by the blockade of CCR2 receptors.

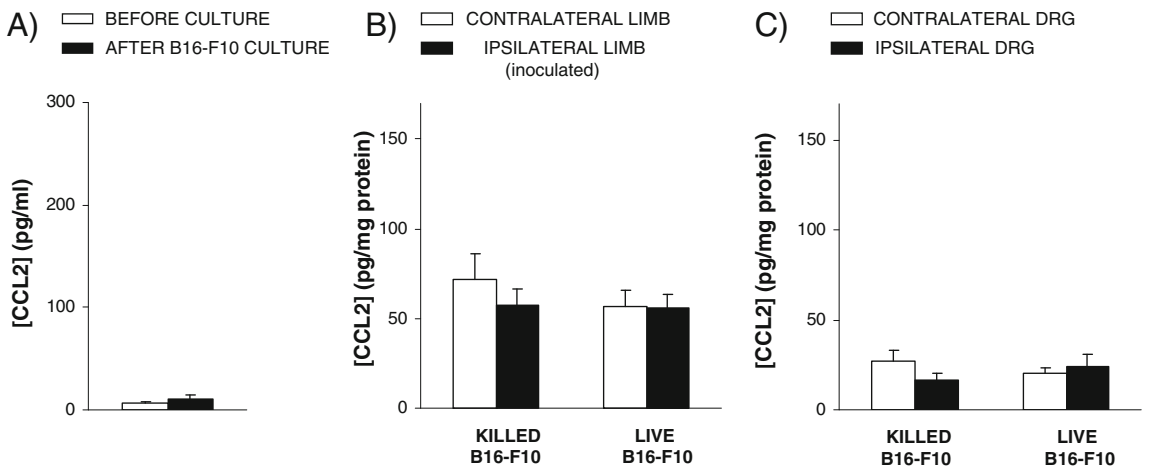

Fig. 2 CCL2 levels measured by ELISA. **a** [CCL2] in B16-F10 culture medium in the absence of cells and after cell confluence. **b** and **c** CCL2 levels measured in homogenates of hind paws (**b**) or of L3–L5 DRG (**c**) of mice intratibially inoculated 4 weeks before with 10^5 live or killed B16-F10. Means and their corresponding standard errors are represented (n=4–5)

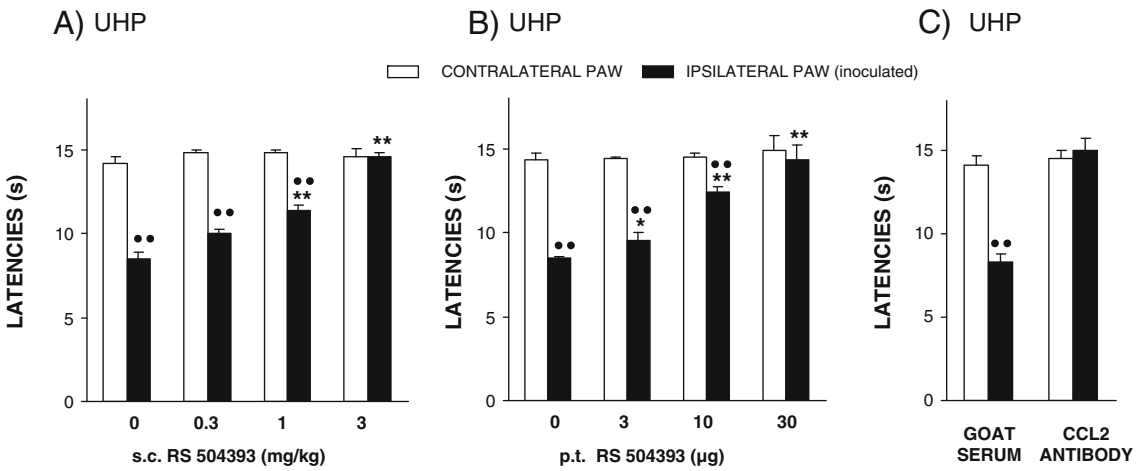

Fig. 3 Effects of RS 504393 or a CCL2 antibody on thermal withdrawal latencies measured in the unilateral hot plate test (*UHP*) in mice intratibially inoculated with live NCTC 2472 cells. In **a,** RS 504393 (0–3 mg/kg) was subcutaneously (*s.c.*) administered 30 min before. In **b** and **c**, RS 504393 (3–30 µg) or the CCL2 antibody (4 µg) were peritumorally (*p.t.*) administered into the inoculated paw 30 min before. Means and corresponding standard errors are represented (n= 5-6). *Double black circles* indicate $P<0.01$ compared with its corresponding, contralateral, left paw, Student's t test; *Single asterisk* indicates $P<0.05$; *double asterisks* indicate $P<0.01$ compared with solvent-treated group, Dunnett's t test

The administration of the CCR2 antagonist RS 504393 does not modify the hyperalgesia or allodynia evoked by the inoculation of B16-F10 cells

Thermal and mechanical hyperalgesia as well as mechanical allodynia can be measured in mice inoculated 1 week before with B16-F10 cells. A slight inhibition of thermal hyperalgesia appeared after the administration of the maximal dose of RS 504393 assayed subcutaneously (3 mg/kg), remaining withdrawal latency values significantly lower than those obtained in the contralateral paw (Fig. 5a). A similar result was obtained after the local administration of RS 504393 over the tumoral mass since only the maximal dose assayed (30 µg) induced an antihyperalgesic effect although withdrawal latencies remained significantly lower than those obtained in the contralateral non-injured paw (Fig. 5b). As shown in Figs. 5c and d, the low mechanical withdrawal latencies and mechanical threshold values obtained in tumor-bearing paws remained unaffected after the systemic administration of RS 504393 (3 mg/kg) 30 min before. In all cases, thermal and mechanical latencies as well as mechanical thresholds remained unaltered in mice inoculated with B16-F10 killed cells (data not shown).

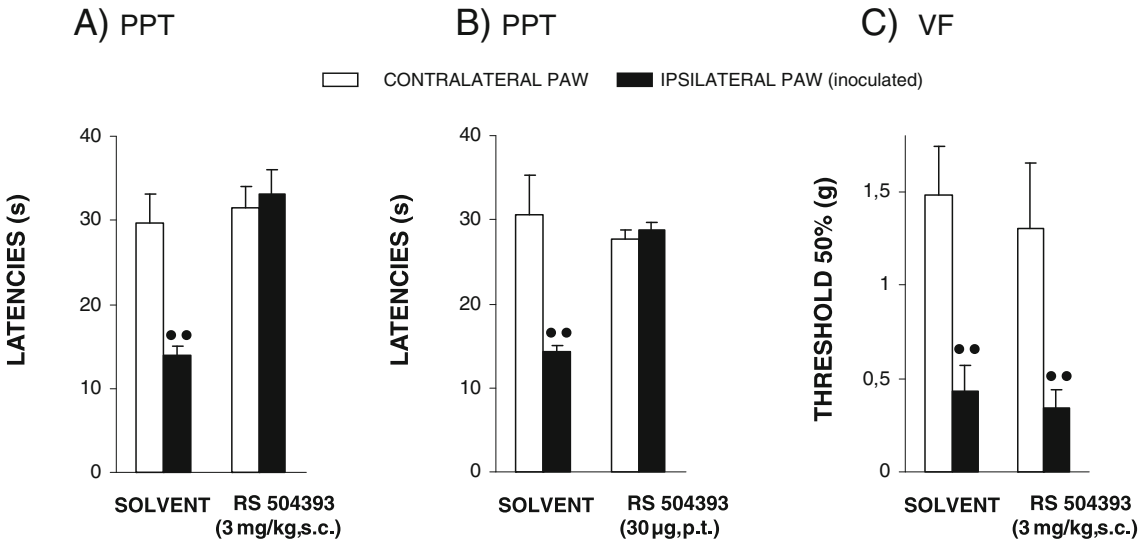

Fig. 4 Effects of RS 504393 in the paw pressure test (*PPT*) and von Frey (*VF*) test in mice intratibially inoculated with live NCTC 2472 cells. Inhibition of mechanical hyperalgesia after subcutaneous (*s.c.* 3 mg/kg, **a**) or peritumoral (*p.t.* 30 µg, **b**) administration of RS 504393. **c** Absence of antiallodynic effect after the s.c. administration of RS 504393 (3 mg/kg, **c**). Means and corresponding standard errors are represented (n=5–7). *Double black circles* indicate $P<0.01$ compared with its corresponding left paw, Student's t test (**a**, **b**) or Mann–Whitney's U test (**c**)

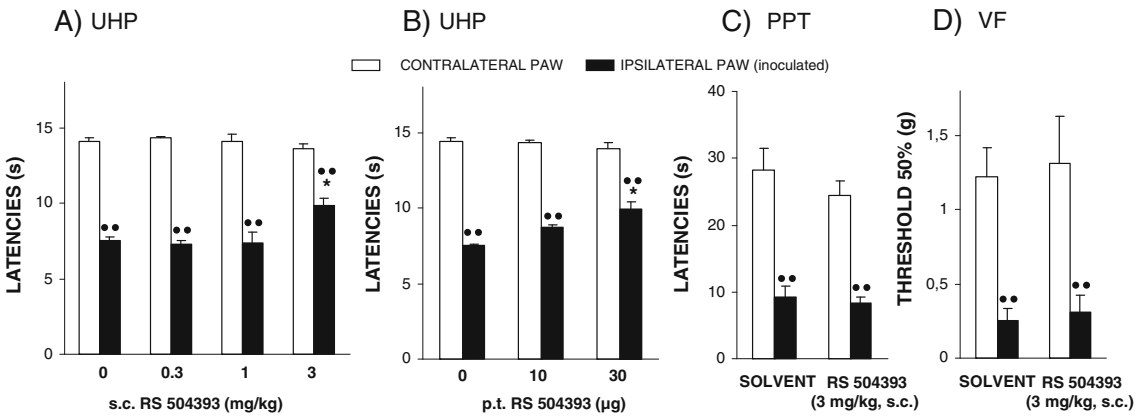

Fig. 5 Effects of RS 504393 in mice intratibially inoculated with live B16-F10 cells. In **a** and **b** RS 504393 was tested in the unilateral hot plate test (*UHP*) after its subcutaneous (*s.c.*; 0–3 mg/kg, A) or peritumoral (*p.t.*, 10 and 30 μg, **b**) administration. In **c** and **d**, the effects produced by RS 504393 in the paw pressure test (*PPT*) and von Frey (*VF*) tests after its subcutaneous administration (3 mg/kg) are shown. Means and corresponding standard errors are represented (n=5–7). *Double black circles* indicate $P<0.01$ compared with its corresponding left paw, Student's t test (**a**, **b**, **c**) or Mann–Whitney's U test (**d**); *double asterisks* indicate $P<0.01$ compared with solvent-treated group, Dunnett's t test

Discussion

The present study shows that the chemokine CCL2 can be released in particular bone cancer-induced pain processes in which CCR2 receptor antagonists can exert an antihyperalgesic effect. Our results confirm previous data showing that CCL2 is released from NCTC 2472 cells in culture (Khasabova et al. 2007; Schiller et al. 2009) and in vivo from the osteosarcoma developed after their intraosteal inoculation (Khasabova et al. 2007). Moreover, behavioral results obtained after the administration of the CCR2 antagonist RS 504393 demonstrate that CCL2 released at tumoral level plays a hyperalgesic role in osteosarcoma-bearing mice. In contrast, the measurement of CCL2 released from melanoma B16-F10 cells in culture as well as from tumors due to their intratibial inoculation indicates that the production of CCL2 by these cells is under detection limits either in vitro or in vivo, and, accordingly, the administration of RS 504393 is almost ineffective in this case.

Initially, the results obtained with NCTC 2472 cells ratify that these cells can release CCL2 in culture and that this property is maintained in vivo after their inoculation into the medullar cavity of the tibia since CCL2 levels are also augmented in tumoral tissue. As increasingly characterized, a cross-talk exists in the injured tissue, between pronociceptive cytokines, peptides, and modulators derived from tumoral and immune cells, as well as from osteoclasts (Stösser et al. 2011). In fact, it has been demonstrated that the release of CCL2 by NCTC 2472 cells is potentiated when these tumoral cells are cocultured with bone, especially if its hematopoietic system is conserved (Schiller et al. 2009). Furthermore, particular mediators involved in the nociceptive reactions measured in this bone cancer-induced model of pain could also contribute to the release of CCL2. This could be the case for the hematopoietic-released factors (Schweizerhof et al. 2009) that evoke the release of CCL2 in other types of tumors (Owen et al. 2007), IL-1β (Baamonde et al. 2007) whose presence can activate the CCL2 gene in synoviocites (Ogura et al. 2010), or TNFα (Constantin et al. 2008) which is able to induce the synthesis of CCL2 in fibroblasts from colorectal liver metastases (Mueller et al. 2010). In any case, our results demonstrate that while CCL2 levels increase about eight times when NCTC 2472 cells are kept in culture, a higher increase of CCL2 levels (of about 12 times) is detected in the tumoral tissue, where fibrosarcoma cells grow in the vicinity of bone and immune cells. This augmentation in CCL2 levels is similar to that described after the calcaneus administration of these cells (Khasabova et al. 2007). Apart from increasing at tumoral level, CCL2 could also be upregulated at DRG cells, as described in neuropathic (Tanaka et al. 2004; White et al. 2005; Jung et al. 2008) or inflammatory settings (Jeon et al. 2008). However, no increase in CCL2 levels was observed in DRG ipsilateral to the tibia inoculated with NCTC 2472 cells, representing this fact a difference with the above mentioned models. Thus, it may be concluded that in this model of bone cancer, sensitive neurons do not contribute to the augmentation of peripheral CCL2 and that its main source comes from the interplay between bone, tumoral, and immune cells.

In order to test whether the augmented presence of CCL2 measured in mice inoculated with NCTC 2472 cells could induce hyperalgesic responses, the effect of RS 5044393, a selective antagonist of CCR2 (Mirzadegan et al. 2000), was tested. The fact that both systemic and peritumoral administration of RS 504393 dose-dependently antagonized osteosarcoma-evoked thermal and mechanical hyperalgesia demonstrates that CCL2 is acting as hyperalgesic at tumoral level. Although the possibility that CCL2 can evoke hyperalgesic effects at spinal cord cannot be discarded, the complete inhibition of thermal and mechanical hyperalgesia obtained when CCR2 receptors are blocked at tumoral level demonstrates the involvement of peripheral CCR2 in neoplastic

hypernociception. Furthermore, the antihyperalgesic effect obtained after the peritumoral administration of a mouse antibody against CCL2 chemokine strongly suggests that this endogenous chemokine could be responsible for this peripheral CCR2-mediated hyperalgesia.

In contrast with the antihyperalgesic effects obtained by blocking CCR2 receptors, mechanical allodynia due to tumoral development remained unaffected after CCR2 inhibition, thus indicating that the efficacy of an analgesic drug to inhibit distinct pain symptoms can be different. In this sense, previous studies from our laboratory described that when opioid or cannabinoid receptors are activated exclusively at the periphery, these drugs evoke antihyperalgesic, but not antiallodynic responses (Curto-Reyes et al. 2008; Curto-Reyes et al. 2010). The fact that peripheral mechanisms are more effective to inhibit hyperalgesia than allodynia both in the mentioned cases and in the present experiments seems to reflect the more important involvement of spinal mechanisms, such as central sensitisation, in the establishment of allodynia (Kim et al. 2012).

In order to explore if the involvement of peripheral CCL2 could be a general property of bone cancer pain, we have performed experiments with mice receiving the intratibial inoculation of B16-F10 melanoma cells. Although in clinical settings, bone metastases are not frequently associated to melanoma in comparison with prostate or breast tumors, the use of this experimental model offers the possibility of exploring the pharmacological properties of analgesic drugs when the underlying bone modifications due to the presence of cancer cells are different than those of the osteosarcoma model (Curto-Reyes et al. 2008). Previous data related to the release of CCL2 from human melanoma tumoral cells (Li et al. 2009) suggested the possibility that B16-F10 cells could release CCL2. However, demonstrating that the release of CCL2 is not a general property of tumoral cells able to evoke bone painful tumors, B16-F10 cells did not produce this chemokine in culture or after intratibial inoculation. In agreement with the lack of increased CCL2 tumoral levels in mice inoculated with B16-F10 cells, RS 504393 was almost ineffective against thermal hyperalgesia and completely inactive against mechanical hyperalgesia and allodynia. Although the fact that B16-F10 cells do not induce an increase in CCL2 levels in the tumoral paw could suggest that the reactivity to nociceptive stimuli in these melanoma-bearing mice should be lower than that measured in NCTC 2472-inoculated mice, a similar degree of thermal and mechanical hypernociception appears in mice inoculated either with NCTC 2472 or B16-F10 cells (Baamonde et al. 2007; Curto-Reyes et al. 2008).

Globally, the present results demonstrate that the peripheral release of CCL2 is involved in thermal and mechanical hyperalgesia, but not mechanical allodynia evoked by the intraosteal inoculation of NCTC 2472 cells, whereas hypernociceptive symptoms produced after the inoculation of B16-F10 melanoma cells are CCL2-independent. This indicates that CCL2 is not a hyperalgesic mediator involved in all types of painful tumors and suggests that CCR2 antagonists could be useful to reduce hyperalgesia only in the particular type of tumors in which CCL2 is involved.

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3.2 Artículo 2:

Pevida M, González-Rodríguez S, Lastra A, García-Súarez O, Hidalgo A, Menéndez L, Baamonde A. **Involvement of spinal chemokine CCL2 in the hyperalgesia evoked by bone cancer in mice: a role for astroglia and microglia.** Cellular and Molecular Neurobiology. Oct 10 [Epub ahead of print] 2013.

OBJETIVO

Analizar el papel del sistema CCR2/CCL2 a nivel espinal en las reacciones hipernociceptivas medidas en ratones inoculados con células NCTC 2472 y especialmente su relación con la glía espinal, que resulta activada en esta situación experimental.

MÉTODOS

- Ratones C3H/He inoculados con células NCTC 2472 en la cavidad medular de la tibia.
- Test de la placa caliente unilateral (medida de la hiperalgesia térmica).
- ELISA (cuantificación de los niveles de la quimiocina CCL2 en la médula espinal y los DRG).
- Western blot de receptores CCR2 en médula espinal.
- PCR cuantitativa (RT-PCR) para medir los niveles de mRNA de CCL2 en DRG.
- Inmunohistoquímica (estudio de la activación microglial y astroglial y de la posible expresión de CCL2 en astrocitos o microglía de animales con tumor).

RESULTADOS Y CONCLUSIONES

- El bloqueo de receptores CCR2 espinales inhibe la hiperalgesia térmica observada en ratones con tumor.
- El desarrollo tumoral induce un aumento en los niveles de CCL2 en la médula espinal pero no en los DRG. La expresión de los CCR2 espinales no varía.
- La microglia y astroglía espinal se activan en respuesta al desarrollo tumoral. Ambos tipos celulares expresan CCL2 en animales con tumor sugiriendo su posible implicación en la liberación de dicha quimiocina. Además, la propia CCL2 participa en la activación de astroglía, pero no de microglía, en los ratones con tumor.

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ORIGINAL RESEARCH

Involvement of Spinal Chemokine CCL2 in the Hyperalgesia Evoked by Bone Cancer in Mice: A Role for Astroglia and Microglia

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Abstract The hypernociceptive role played by the chemokine CCL2, and its main receptor, CCR2, in pathological settings is being increasingly recognized. We aimed to characterize the involvement of spinal CCL2 in the hyperalgesia due to the intratibial inoculation of fibrosarcoma NCTC 2472 cells in mice. The intrathecal (i.t.) administration of the CCR2 antagonist RS 504393 (1–3 μg) or an anti-CCL2 antibody inhibited tumoral hyperalgesia. No change in the expression of spinal CCR2 was detected by western blot, whereas immunohistochemical experiments demonstrated increased CCL2 staining at the superficial laminae of the spinal cord ipsilateral to the tumor. This spinal CCL2 does not seem to be released from nociceptors since CCL2 mRNA and CCL2 levels in DRGs, as measured by RT-PCR and ELISA, remain unmodified in tumor-bearing mice. In contrast, immunohistochemical assays demonstrated the spinal up-regulations of GFAP and Iba-1, respective markers of astroglia and microglia, and the expression of CCL2 in both types of glial cells at the superficial laminae of the spinal cord of tumor-bearing mice. Finally, since CCL2 could induce astroglial or microglial activation, we studied whether the blockade of CCR2 could inhibit the increased spinal glial expression. GFAP, but not Iba-1, up-regulation was reduced in tumor-bearing mice treated for 3 days with i.t. RS 504393, indicating that spinal CCL2 acts as an astroglial activator in this setting. The participation at spinal level of CCL2/CCR2 in tumoral hypernociception, together with its previously described involvement at periphery, makes attractive the modulation of this system for the alleviation of neoplastic pain.

Keywords Bone cancer-induced pain · Mice · CCL2 · CCR2 · Glial cells

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Introduction

The chemokine (C–C motif) ligand 2 (CCL2), also called monocyte chemoattractant protein-1 (MCP-1), is a member of the CC group of chemokines that acts as the main endogenous agonist of the chemokine receptor type 2 (CCR2) (Abbadie et al. 2003). Apart from its involvement in monocyte/macrophage chemotaxis, CCL2 plays an important role in the modulation of nociception (Abbadie et al. 2009), and accordingly, the expression of CCR2 has been demonstrated in dorsal root ganglia (DRGs) (Jung et al. 2009) and spinal cord in neurons (Gao et al. 2009), microglia (Abbadie et al. 2003), and astroglia (Knerlich-Lukoschus et al. 2008). Confirming its functional relevance, it has been proven that the administration of CCL2 to experimental animals either at the periphery (Abbadie et al. 2003) or at the spinal cord (Tanaka et al. 2004; Dansereau et al. 2008; Baamonde et al. 2011) evokes hypernociceptive behavioral reactions.

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The participation of CCL2 in the peripheral or central processing of nociceptive signals is particularly important in pathological situations, as demonstrated in inflammatory (Jeon et al. 2008; Ogura et al. 2010), neuropathic (White et al. 2005; Jeon et al. 2011; Pevida et al. 2013) or neoplastic (Khasabova et al. 2007; Pevida et al. 2012; Hu et al. 2012, 2013) experimental settings. In accordance, neuropathic or inflammatory hypernociception is importantly reduced in mice in which the expression of CCR2 is deleted (Abbadie et al. 2003; Miller, et al. 2012). At spinal level, the mechanisms involved in the amplification of nociceptive signals evoked by CCL2 involve neural and glial processes. Thus, it has been reported that the application of CCL2 to laminae II neurons of the spinal cord leads to a direct neural sensitization through the enhancement of AMPA and NMDA receptor activity (Gao et al. 2009). Furthermore, the presence of CCL2 at the spinal cord can also contribute to the activation of glial cells, particularly microglia (Thacker et al. 2009), thus promoting the release of several pronociceptive mediators that can provoke neural sensitization (Watkins et al. 2001), one of which could be CCL2 itself (Old and Malcangio 2012). These processes seem to be particularly relevant after a neuropathic injury, a case in which, besides the release of CCL2 from activated glial cells (Gao et al. 2009, 2010), it has been observed that injured nociceptors can become an additional source of CCL2 into the spinal cord (Thacker et al. 2009; Zhang and De Koninck 2006; Van Steenwinckel et al. 2011).

Previous data suggest that CCL2 and CCR2 are also involved in cancer, and particularly in the hypernociception due to neoplastic processes. Thus, the release of CCL2 has been demonstrated from different types of human tumors, some of which are associated with painful symptoms, such as pancreatic cancer (Chehl et al. 2009) or bone metastases derived from prostate or breast tumors (Shirotake et al. 2012; Soria et al. 2011). In accordance, increased CCL2 concentrations have been measured at tumoral level in mice inoculated with NCTC 2472 fibrosarcoma cells into the calcaneus (Khasabova et al. 2007) or the tibia (Pevida et al. 2012). Functionally, it has also been demonstrated in this murine model that the peripheral administration of a CCR2 antagonist can inhibit bone cancer-induced thermal hyperalgesia (Pevida et al. 2012). Related to the spinal cord, an increase in CCR2 expression has been measured by immunohistochemical methods after the inoculation of NCTC 2472 cells (Vit et al. 2006). More recently, the alteration of spinal CCR2 and CCL2 levels in response to the development of a tibial tumor due to the inoculation of mammary carcinoma cells has been studied in rats (Hu et al. 2012, 2013). In this model, increases in the spinal expressions of CCL2 and CCR2 together with a partial antiallodynic effect after the spinal administration of an anti-CCL2 antibody were described (Hu et al. 2012; 2013).

The present study was designed to characterize the involvement of spinal CCL2 and CCR2 in the model of murine osteosarcoma produced after the intratibial inoculation of NCTC 2472 cells. We have assessed whether the intrathecal (i.t.) administration of a selective CCR2 antagonist or an anti-CCL2 antibody can inhibit osteosarcoma-evoked thermal hyperalgesia. After demonstrating this behavioral effect, we have studied the expression of CCR2 or CCL2 at the spinal cord in response to the tibial tumoral presence and explored if spinal glial cells could be involved in the role played by CCL2 in tumoral hyperalgesia.

Methods

Animals

Experiments were performed in 5–6-week-old C3H/He mice bred in the Animalario of the Universidad de Oviedo (Reg. 33044 13A), housed six per cage with a bedding of sawdust and maintained on a 12-h dark–light cycle with free access to food and water. All the experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain). Each animal was used only once.

Cell Inoculation

NCTC 2472 cells (American Type Culture Collection, ATCC) were cultured in NCTC 135 medium (Sigma) containing 10 % horse sera (Sigma), passaged weekly according to ATCC guidelines. At confluence, cells were detached by scraping, centrifuged at 400 g for 10 min, and the remaining pellet was suspended in PBS (Menéndez et al. 2003).

For surgical procedures, anesthesia was induced by spontaneous inhalation of 3 % isoflurane (Isoflo®, Esteve) and maintained by administering 1.5 % isoflurane in oxygen through a breathing mask. A suspension of 10^5 cells in 5 µl of PBS was injected into the right tibial medullar cavity and after applying acrylic glue (Hystoacril®, Braun) on the tibial plateau incised area, surgery was finished with a stitch of the skin. Control mice received the inoculation of 10^5 cells previously killed by quickly freezing them three times without cryoprotection. According to the appropriate timing to reach maximal hypernociception, thermal hyperalgesia was assessed 4 weeks after the inoculation of NCTC 2472 cells (Menéndez et al. 2003).

Drugs and Drug Administration

As previously described (Menéndez et al. 2002) for i.t. injections, a lumbar cut was made, and the tip of a

30-gauge needle inserted in a Hamilton microsyringe was introduced at the level of L_5–L_6 to inject a volume of 5 µl. The CCR2 receptor antagonist, RS 504393 (Tocris), was solved in 5 % DMSO at the highest concentration used, and minocycline hydrochloride (Sigma), L-2-aminoadipic acid (L-AA, Sigma) and the goat polyclonal anti-CCL2 antibody (R&D AF-479-NA) were solved in saline. Following the information supplied by the providers, this anti-CCL2 antibody shows less than 0.01 % cross-reactivity with other molecules that could interfere with nociceptive modulation such as rmMCP-5, rmMIP-1 alpha, rmMIP-1 beta, and rmMIP-1 gamma or rmRANTES. In all cases, control animals received an injection of the corresponding solvents and, for the goat antibody, goat IgG (Sigma) dissolved in saline was used as control.

Unilateral Hot Plate Test

As previously described (Menéndez et al. 2002), mice were gently restrained, and the plantar side of the tested paw was placed on a hot plate (IITC Life Science) set to 49.8 °C. Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at two minute-intervals and the mean of two measures was considered. A cut-off of 20 s was established. All the behavioral experiments were performed between 15:00 and 20:00 in a thermostatized (21 °C) and noise-isolated room.

Western Blot

Mice inoculated with live or killed NCTC 2472 cells were anesthetized with 5 % isoflurane (Isoflo®, Esteve) and decapitated. The vertebral column was sectioned at thoracic and sacral levels and extracted by flushing 3–5 ml of ice-cold saline through the spinal cavity with a syringe and L_2-L_6 segments were selected, frozen immediately in liquid nitrogen, and stored at −80 °C until use. Next, spinal tissue was homogenized in ice-cold buffer containing 60 mM Tris–HCl (pH 7.4), 10 % glycerol, 80 mM sodium dodecyl sulfate (SDS) and protease inhibitors (1 tablet/50 ml buffer, Roche Diagnostics) in a volume of 6 µl/mg of tissue and then centrifuged (120 g, 10 min, 4 °C). The supernatant obtained was centrifuged again (26,000 g, 20 min, 4 °C), collected and conserved at −80 °C until its further use.

Protein concentrations were determined by a BCA protein assay (Pierce) according to the manufacturer's protocol. As previously described (Curto-Reyes et al. 2010), the volume of homogenate corresponding to 20 µg of protein was vigorously mixed with the volume of sample buffer (200 mM Tris–HCl at pH 6.8, 0.02 % bromophenol blue, 8 % mercaptoethanol, 40 % glycerol, 8 % SDS) necessary to obtain 30 µl, placed in an eppendorf tube and heated at 100 °C for 5 min. After this, samples were run on a 10 % SDS-PAGE gel at 90 V during 90 min. Samples were then transferred onto a nitrocellulose membrane (Bio-Rad) at 4 °C during 90 min using 100 V. The membrane was blocked in Tris buffered saline-Tween (TBST, Tris 10 mM, NaCl 150 mM, Tween 20 0.1 %) with 5 % non-fat milk for 90 min at RT, washed with TBST and incubated overnight at 4 °C with goat polyclonal anti-CCR2 receptor (1:5,000; Santa Cruz Biotechnology, sc-6228). After incubation, the membrane was washed with TBST and incubated with the secondary antibody (donkey anti-goat IgG-HRP, 1:40,000 Santa Cruz Biotechnology) dissolved in TBST containing 0.1 % non-fat milk for 90 min. After final washes, labeled CCR2 receptor protein was detected by enhanced chemiluminescence detection autoradiography using Immobilon™ Western chemiluminiscent HRP substrate kit (Millipore) according to the manufacturer's protocol. The molecular weight standards used were pre-stained SDS-PAGE from BioRad (Ref. 161-0318). The localization of CCR2 corresponds to a band with a molecular weight higher than 42 kDa, the value most often described for CCR2 (Gosselin et al. 2005) and seems more compatible with the previously reported 50 kDa glycosylated form of CCR2 (Preobrazhensky et al. 2000). Immune reaction intensity was determined by computer-assisted densitometry (ImageJ, NIH) on exposed LS film (Kodak X-Omat).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed protein of 35 kDa, was also measured by western blotting using a polyclonal rabbit anti-GAPDH antibody (1:30,000; Sigma) revealed with a goat anti-rabbit IgG peroxidase conjugate secondary antibody (1:40,000; Sigma).

Five independent samples were analyzed in mice inoculated with killed or live NCTC 2472 cells. Results are reported as the ratio of optical densities of the CCR2 receptor and GAPDH by normalizing the amount of CCR2 receptor to the immunoreactivity of GAPDH.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA experiments were performed in tissue homogenates prepared from lumbar spinal segments or DRGs of mice intratibially inoculated 4 weeks before with live or killed NCTC 2472 cells. L_2-L_6 lumbar segments were harvested and conserved as described in the previous section. Ipsilateral or contralateral L_3-L_5 DRGs were isolated from mice inoculated with killed or live NCTC 2472 cells, based on a previous study that describes the innervation of the tibial region (Ivanusic et al. 2009), and immediately frozen in liquid nitrogen.

In experiments with spinal tissue, each sample was drawn from a single animal, whereas in the case of DRGs,

each sample was from a pool of 18 DRGs coming from six mice. Spinal cord segments were weighed and homogenized in a volume of 6 µl of buffer per mg of tissue, and DRG samples were homogenized in 24 µl of buffer, using in both cases, a handy homogenizer (Minicraft MB130®). The buffer used consisted in 0.1 M Tris, 0.15 M NaCl, 0.5 % CTAB (Fluka), and a protease inhibitor (1 tablet/50 ml buffer, Roche Diagnostics). Next, homogenates were centrifuged at 15,000 g for 15 min at 4 °C and protein concentration of supernatants measured by a BCA protein assay (Pierce) by means of a spectrophotometer (Nanodrop 2000C, Thermoscientific).

The level of CCL2 was measured with a commercially available ELISA kit (R&D Systems, DuoSet®). Plates (R&D Systems) were coated overnight at room temperature with an antibody specific for mouse CCL2. In order to achieve a value in the range of the standard curve of CCL2, a protein quantity of 50 µg of the spinal cord homogenate or of 70 µg of the DRGs homogenate was added to the wells in a final volume of 100 µl. After washing, a 2-h incubation period was allowed with a second biotinylated anti-mouse CCL2 antibody that was followed by a 20-min incubation period with streptavidin–peroxidase. Plates were washed to remove the unbound enzyme, color was developed by adding a stabilized chromogen (tetramethylbenzidine: H_2O_2, 1:1), and the reaction was terminated with a stop solution (2 N H_2SO_4). The intensity of the colored product was quantified using a spectrophotometer (µQuant, Bio-Tek Instruments) at 450 nm, subtracting the readings obtained at 570 nm to correct optical background of plates. Values obtained from spinal cords came from six independent measures performed in duplicate. In the case of DRGs, due to the low weight of samples, duplicates were not possible, and values are obtained from six single independent data.

Immunohistochemical Assays

Lumbar spinal cord enlargement was obtained as described in previous sections and immediately immersed for 24 h in 4 % formaldehyde diluted in PBS 0.1 M at 4 °C. After fixation, spinal cords were cryoprotected by 12–24-h immersion in 15 % sucrose, dissolved in PBS 0.01 M, and some 24 h afterward in 30 % sucrose at 4 °C. 30-µm-thick sections were obtained using a freezing microtome (Mikrom HM430) and serially collected on gelatin-coated slides (Super-Frost® Plus, Menzel–Glaser).

Sections were initially incubated in cold acetone (Prolabo) for 10 min, rinsed during 30 min in PBS (0.01 M), and further incubated at 4 °C overnight in a humid chamber with primary antibodies. The primary antibodies used were a polyclonal rabbit glial fibrillary acid protein (GFAP) antibody (DakoCytomation; 1:500 in 0.01 M PBS) to detect astrocytes; a polyclonal rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) antibody (Wako; 1:250 in 0.01 M PBS) to detect microglia and a rabbit anti-rat polyclonal anti-CCL2 antibody (Torrey-Pines; 1:200 in 0.01 M PBS). Before the incubation with the Iba-1 primary antibody, sections were immersed for 30 min in 0.01 M PBS containing 0.1 % Triton X-100. After overnight incubation, sections were rinsed for 30 min in 0.01 M PBS, incubated for 1.5 h at room temperature in a dark humid chamber with green Alexa fluor® 488-conjugated goat anti-rabbit IgG (Life Technologies; 1:250 in 0.01 M PBS); washed for 30 min in 0.01 M PBS; and, finally, mounted and cover slipped using Fluoromount-G® (Southern Biotech). As a procedural control, primary antibodies were omitted and replaced by PBS, and under these conditions, no staining occurred.

Since the intensity of the immunoreaction can slightly change on a daily basis, to minimize variations, samples coming from mice submitted to the different treatments were simultaneously processed every experimental day and also analyzed together. Immunostained tissue sections were imaged using a BX61 Olympus microscope with a 4x/0.16NA objective and, in some particular cases, using a 10x/0.40NA objective.

Images were acquired with a CCD camera Olympus DP-70 using blue-excitation filter (BP470-490) and processed using the software Olympus DP-controller 1.2.1.108 and Olympus DP-manager 1.2.1.107. The exposures used each day to acquire photographs were the same for control and experimental groups.

As previously reported (Pevida et al. 2013), quantitative analysis of the immunofluorescence was performed by measuring the percentage of green immunoreactive surface for GFAP, Iba-1, or CCL2 using a computer-assisted imaging software analysis system (Image J version 1.43u, NIH). Staining intensities were examined in different regions of the lumbar spinal cord corresponding to laminae I-II, III-IV, V, X, and ventral horn. For fluorescence quantification, two members of the team blinded to mouse treatment established the adequate threshold to detect the more intense fluorescence coming from specific immunoreactive cells subtracting inespecific background. Next, the immunoreactive surface was measured by means of Image J version 1.43u, NIH by a third person also in a blinded fashion. For each experiment, measurements came from at least five different mice in which the five slices with the greater staining were selected.

For double-immunolabeling assays simultaneously addressed against CCL2 and GFAP or CCL2 and Iba-1, the antibodies used to detect GFAP and Iba-1 were different from those mentioned above, since antibodies generated in animals other than rabbits, in which the CCL2 antibody was produced, were necessary. In the CCL2-GFAP assay,

the procedure was initiated with the incubation overnight in a humid chamber at 4 °C with a mixture of the antibodies against CCL2 (rabbit polyclonal; Torrey-Pines, 1:200 in 0.01 M PBS) and GFAP (mouse monoclonal, Cell Signaling, 1:100 in 0.01 M PBS). For the double-staining CCL2-Iba-1, sections were initially washed with 0.01 M PBS containing 0.1 % Triton X-100 and then incubated for 24 h at room temperature with the antibody for Iba-1 (goat polyclonal; Santa Cruz, 1:50 in 0.01 M PBS).

On the next day, the antibody against CCL2 (rabbit polyclonal; Torrey-Pines, 1:200 in 0.01 M PBS) was added, and sections were further incubated overnight at room temperature with the combination of both primary antibodies. Next, sections corresponding to the CCL2-GFAP assay were first incubated for 90 min with green Alexa fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, 1:250 in 0.01 M PBS) to reveal CCL2 staining, and after 30 min in 0.01 M PBS, they were further incubated for a 90-min period with Texas red-conjugated goat anti-mouse IgG (Molecular Probes; 1:100) to detect GFAP immunoreaction. In the CCL2-Iba-1 assay, sections were incubated, as before, with Alexa fluor 488-conjugated goat anti-rabbit IgG (90 min), and then, after 30 min in 0.01 M PBS, with red Alexa fluor 546-conjugated rabbit anti-goat IgG (Molecular Probes; 1:200) for 90 min. Finally, sections were washed for 30 min in PBS and mounted. Double staining was detected using a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg GmbH) with a Leica DM IRE2 automatic fluorescence inverted microscope. A 488-nm Krypton/Argon laser line was used to excite Alexa fluor 488, and the excitation of Alexa fluor 546 or Texas-red was produced with a Helium/Neon laser line of 543 nm. For obtaining overlapping stacks in Z-plane, 6–8 images at 1.7 µm were acquired with an objective 40× 1.25 PL APO, and the more detailed pictures of isolated astroglial or microglial cells in a single stack were obtained with an objective 63× 1.4 PL AP, with a zoom of 1.72× and 2.65× respectively.

Real-Time Polymerase Chain Reaction (RT-PCR)

L_3-L_5 DRGs coming from mice inoculated with live or killed NCTC 2472 cells were obtained as described in previous sections, frozen in liquid nitrogen, and conserved at −80 °C. Total mRNA was extracted using Total RNA Kit I (Omega) following the manufacturer's protocol. RNA integrity was estimated using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer.

First strand cDNA was synthesized with random primers from the whole amount of the isolated RNA using a commercial kit for reverse transcription (Qiagen Iberia). Next, real-time PCR was performed in each sample by means of specific primers for the mouse gene of CCL2 chemokine and for the mouse housekeeping gene β-actin, used as control to normalize RNA input. Reactions were conducted on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Duplicates of five independent samples were analyzed in each group. Data were analyzed by the 2-ΔΔCt method (Livak et al. 2001) using as control condition the expression level of CCL2 in the samples from contralateral DRGs of mice inoculated with killed NCTC cells. Primer pairs for mouse CCL2 and β-actin (Invitrogen) were as follows: CCL2 forward 5′ GAAGGAATG GGTCCAGACAT 3′ and reverse 5′ ACGGGTCAACTT CACATTCA 3′ (Schiller et al. 2009); β-actin: forward 5′ GCAGCTCCTTCGTTGCCGGT 3′ and reverse 5′ TACA GCCCGGGGAGCATCGT3′ (NCBI accession number NM_007393.3).

Statistical Analysis

Mean values and their corresponding standard errors were calculated for behavioral assays, western blots, ELISA measurements, immunohistochemical image analysis and RT-PCR data. Thermal withdrawal latencies were compared by a Student's *t* test when two groups were considered, while comparisons among several groups that received different doses of a drug or studied at different times were done by an initial one-way analysis of variance (ANOVA) followed by the Dunnett's *t* test. The values related to the expression of CCR2 protein measured by western blot, the immunoreactive surface obtained when labeling with GFAP, Iba-1, or CCL2, the levels of CCL2 measured by ELISA, or the mRNA CCL2 expression measured by RT-PCR in mice inoculated with killed or live NCTC 2472 cells were compared by means of a Student's *t* test. The criterion for statistical significance was $P < 0.05$.

Results

Thermal Hyperalgesia Evoked After the Inoculation of NCTC 2472 Cells is Inhibited by the i.t. Administration of the CCR2 Receptor Antagonist RS 504393 or an Anti-CCL2 Antibody

As previously described (Menéndez et al. 2003), thermal hyperalgesia can be measured in C3H/He mice 4 weeks after the intratibial administration of NCTC 2472 cells. This tumoral hyperalgesia was dose-dependently inhibited 30 min after the i.t. administration of the selective CCR2 antagonist RS 504393 (1–3 µg), being the maximal dose ineffective to evoke analgesia when administered to mice inoculated with killed tumoral cells (Fig. 1a). Furthermore, osteosarcoma-evoked hyperalgesia was also completely

inhibited when 1.5 μg of an anti-CCL2 antibody were i.t. administered 30 min before. This dose of antibody did not modify thermal latencies when administered to mice inoculated with killed NCTC 2472 (Fig. 1b).

CCR2 Receptors are not Up-Regulated in the Spinal Cord of Tumor-Bearing Mice

The expression of spinal CCR2 receptors was assessed by western blot in lumbar spinal cord homogenates of mice inoculated either with killed or live NCTC 2472 cells. As shown in the examples of Fig. 2a, a band corresponding to about 50 kD, compatible with the molecular weight of the glycosylated form of CCR2, was labeled by the CCR2 receptor antibody. The intensities of this band were similar in both groups of mice, and the ratios of the optical densities obtained for CCR2 and the constitutive protein GAPDH used as control showed that the expression of CCR2 at the spinal cord does not change in response to tumor development (Fig. 2b).

Tumor Development Evokes an Increased Presence of Spinal CCL2 Detected by Immunohistochemical but not by ELISA Methods

The measurement by ELISA of CCL2 content present in homogenates of lumbar spinal cord did not reveal differences due to tumor development. Thus, in homogenates coming from the spinal cord of mice inoculated with killed NCTC 2472 cells, CCL2 level was 41.5 ± 4.9 pg/mg protein, and in homogenates prepared from tumor-bearing mice, the concentration measured was 42.9 ± 6.4 pg/mg protein (Fig. 3a).

In contrast, when the presence of CCL2 in the spinal cord was quantified by immunohistochemical methods, higher CCL2 expression was found in tumor-bearing mice. A stained area present at the more superficial spinal cord laminae of the dorsal horn, approximately corresponding to laminae I-II, was visualized in mice inoculated with either live or killed NCTC 2472 cells. However, this immunoreaction was more marked in the ipsilateral side of the spinal cord of mice inoculated with live NCTC 2472 cells (Fig. 3b, c). Thus, when the % of CCL2 immunoreactive

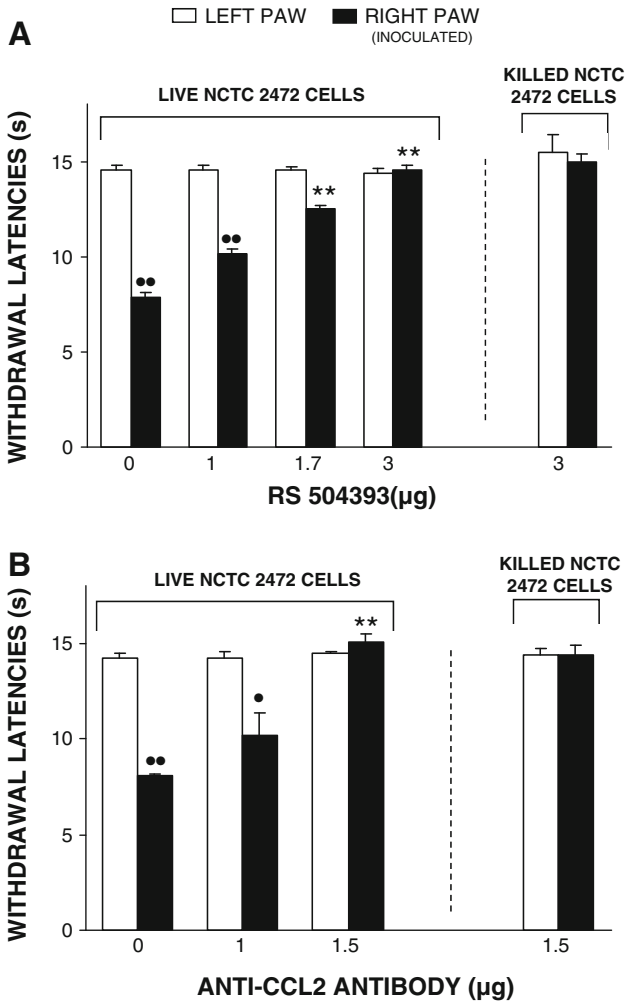

Fig. 1 Inhibition of osteosarcoma-induced thermal hyperalgesia after the i.t. administration of RS 504393 (1–3 μg, **a**) or an anti-CCL2 antibody (1–1.5 μg, **b**). In both cases, the lack of effect on the withdrawal latencies of the maximal dose assayed in mice inoculated with killed tumoral cells is also represented at the *right hand* side of the graphs. Means ($n = 5$–7) and their SEM are represented. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ compared with its corresponding, contralateral, left paw, Student's t test; $^{**}P < 0.01$ compared with solvent-treated group, Dunnett's t test

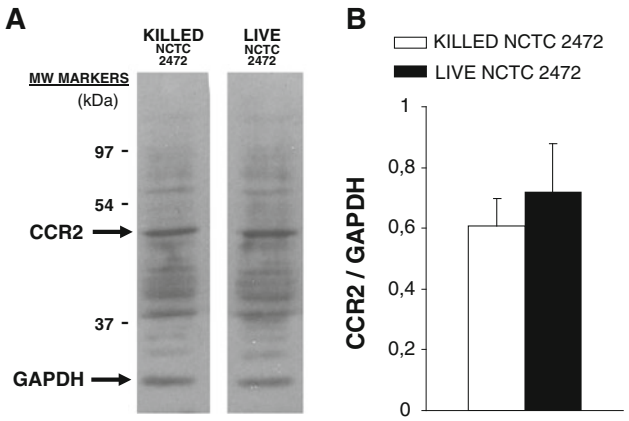

Fig. 2 Expression of CCR2 measured by western blot in the spinal cord of mice intratibially inoculated with NCTC 2472 cells. **a** Representative lanes of western blots for CCR2 receptor expression performed in lumbar spinal cord homogenates of mice inoculated with live or killed NCTC 2472 cells. Molecular weight markers are indicated at the *left hand* side, and the bands for CCR2 and GAPDH are indicated with *arrows*. **b** Fold change in CCR2 receptor protein estimated by using its corresponding GAPDH as endogenous control. Means ($n = 5$) and their SEM are represented. GAPDH, glyceraldehyde-3-phosphate dehydrogenase

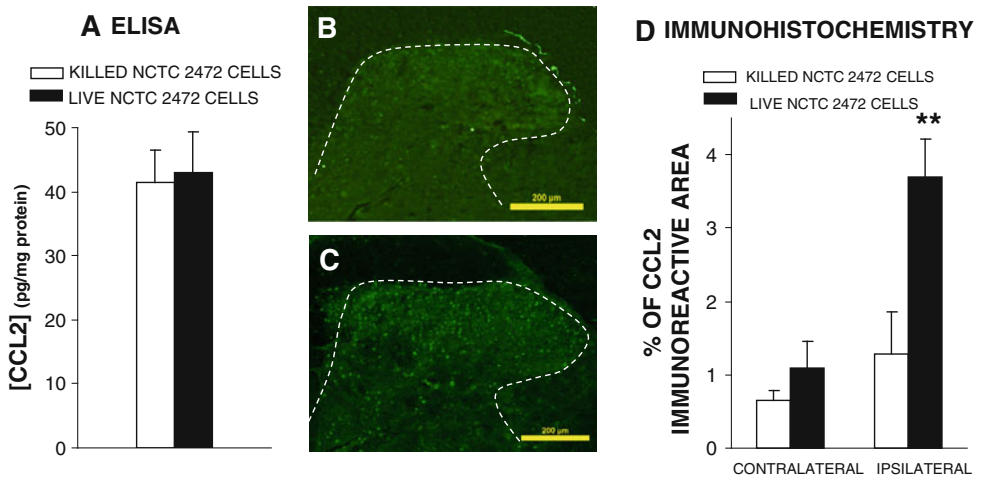

Fig. 3 CCL2 protein in lumbar spinal cord of mice intratibially inoculated with NCTC 2472 cells. **a** CCL2 content measured by ELISA in lumbar spinal cord homogenates of mice inoculated either with killed or live NCTC 2472 cells ($n = 6$). Representative examples of the immunohistochemical expression of CCL2 in the ipsilateral dorsal horn of mice inoculated either with killed (**b**) or live (**c**) NCTC 2472 cells; *scale bar*, 200 µm. **d** % of CCL2 immunoreactive area measured in the superficial laminae of the ipsilateral and contralateral dorsal horns of mice inoculated either with killed ($n = 5$) or live ($n = 6$) NCTC 2472 cells. *Bars* correspond to means and their SEM. $^{**}P < 0.01$, Student's *t* test

surface was quantified, the value obtained in both sides of the spinal cord of mice inoculated with killed cells and in the contralateral side of mice inoculated with live cells was very similar, in the range of 1 % of the measured area, whereas the stained area in the superficial laminae of the ipsilateral spinal cord of mice receiving the intratibial injection of live tumoral cells significantly augmented (3.7 % of the measured area; Fig. 3d).

The fact that CCL2 up-regulation only occurs in a small surface of the spinal cord could help to understand the discrepant results obtained by ELISA and immunohistochemical methods. We have observed in some digitalized images of lumbar spinal sections that the surface corresponding to laminae I-II only represents about a 3 % of the total spinal area. Thus, it seems conceivable that this localized increase in CCL2 can become undetectable when measured in complete spinal cord homogenates, as occurs in ELISA assays.

CCL2 is not Up-Regulated in DRGs Neurons but it is Expressed in Astrocytes and Microglial Cells of Tumor-Bearing Mice

We have designed experiments to explore in which type of cells related to nociception CCL2 could be expressed in osteosarcoma-bearing mice. We have initially examined if CCL2 could be up-regulated in the soma of sensory neurons that connect the tumoral region with the dorsal horn of the corresponding segments of the spinal cord. The measurement in L_3-L_5 DRGs of CCL2 mRNA expression by RT-PCR assays reveals that there are statistically no significant changes in CCL2 mRNA levels found in contralateral and ipsilateral DRGs obtained from mice inoculated with either killed or live NCTC 2472 cells (Fig. 4a). Furthermore, ELISA assays showed that the content of CCL2 protein is similar in the contralateral or ipsilateral DRGs of mice inoculated 4 weeks before with killed or live NCTC cells, with the values obtained in all cases being around 40 pg/mg of protein (Fig. 4b).

We have next performed immunohistochemical assays to study whether CCL2 detected in the spinal cord of cancer-bearing mice could be expressed in glial cells. We have initially studied if, as expected, spinal astroglia and microglia are activated in response to tumor development, by quantifying the immunoreactive surface stained with an astroglial (glial fibrillary acidic protein, GFAP) and a microglial (Ionized calcium-binding adapter molecule 1, Iba-1) marker. A slight GFAP immunoreaction appeared in the spinal cord of mice inoculated with killed cells (Fig. 5a), whereas this staining appears clearly enhanced in mice inoculated with live NCTC 2472 cells throughout the entire gray matter of the spinal cord ipsilateral to tumor development (Fig. 5b). A regional analysis indicates that mice inoculated with live tumoral cells show significant increases in the % of GFAP immunoreactive surface at laminae I-II, laminae III-IV, lamina V, lamina X, and ventral horn of the ipsilateral side (Fig. 5c). In the contralateral side, significant increases were also observed at lamina X of tumor-bearing mice (Fig. 5c).

Also, Iba-1 immunoreactive surface was increased in the ipsilateral side of osteosarcoma-bearing mice (Fig. 6a, b).

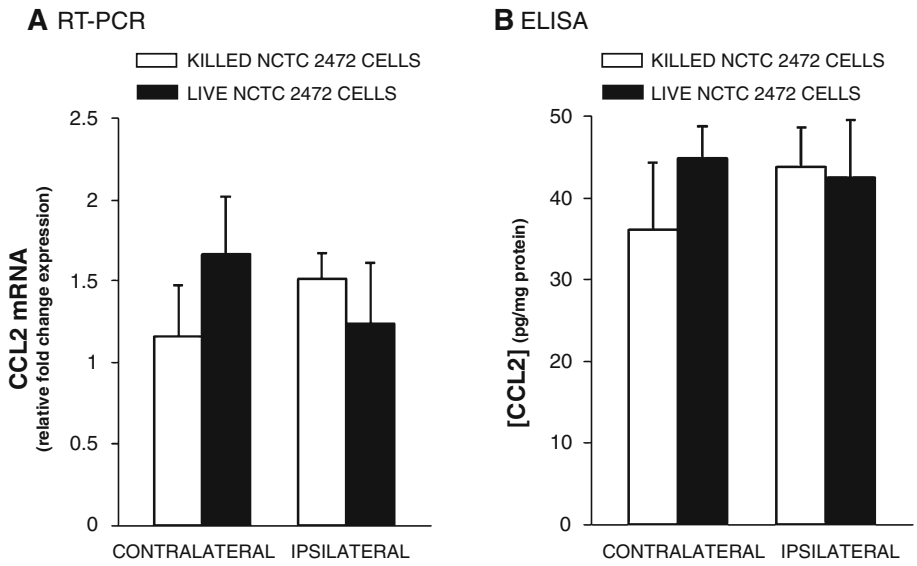

Fig. 4 Expressions of CCL2 mRNA and CCL2 protein levels in DRGs of mice intratibially inoculated with NCTC 2472 cells. **a** Relative expression levels of CCL2 mRNA determined by real-time PCR in ipsilateral or contralateral L3-L5 DRGs coming from mice inoculated either with killed or live NCTC 2472 cells ($n = 5$ per group). The expression of β-actin mRNA was used as internal control. **b** CCL2 content measured by ELISA in L3-L5 DRGs homogenates of mice inoculated either with killed or live NCTC 2472 cells ($n = 6$)

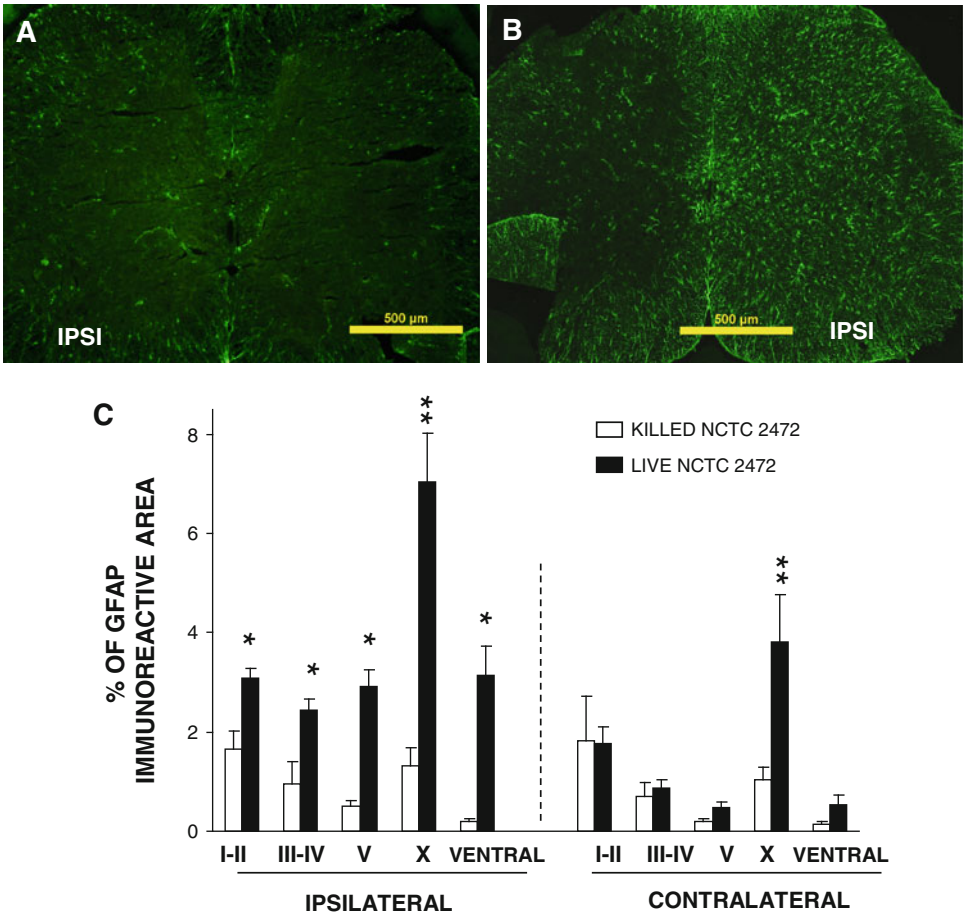

Fig. 5 Immunohistochemical staining obtained with an antibody against the astroglial marker GFAP (glial fibrillary acid protein) in the lumbar spinal cord of mice inoculated with either killed or live NCTC 2472 cells. **a** Representative example of GFAP immunoreactivity in a section of the lumbar spinal cord of a mouse inoculated with killed NCTC 2472 cells. IPSI: ipsilateral; *scale bar*, 500 µm. **b** Representative example of GFAP immunoreactivity in a section of the lumbar spinal cord of a mouse inoculated with live NCTC 2472 cells. IPSI: ipsilateral; *scale bar*, 500 µm. **c** % of GFAP immunoreactive area measured in *five regions* of the ipsilateral and contralateral spinal cords of mice inoculated either with killed ($n = 5$) or live ($n = 8$) NCTC 2472 cells. *Bars* correspond to means and their SEM. $*P < 0.05$, $**P < 0.01$, Student's *t* test

Thus, a slight stained area was observed in both sides of the spinal cord of mice inoculated with killed cells and in the contralateral side of mice inoculated with live NCTC 2472 cells. A significant increase of the % of Iba-1 immunostaining appeared in laminae I-II and III-IV of the ipsilateral side of the spinal dorsal horn of osteosarcoma-bearing mice compared with mice receiving the inoculation of killed cells (Fig. 6c). The tendency was not significant at deeper laminae of the ipsilateral side or at the contralateral one (Fig. 6c).

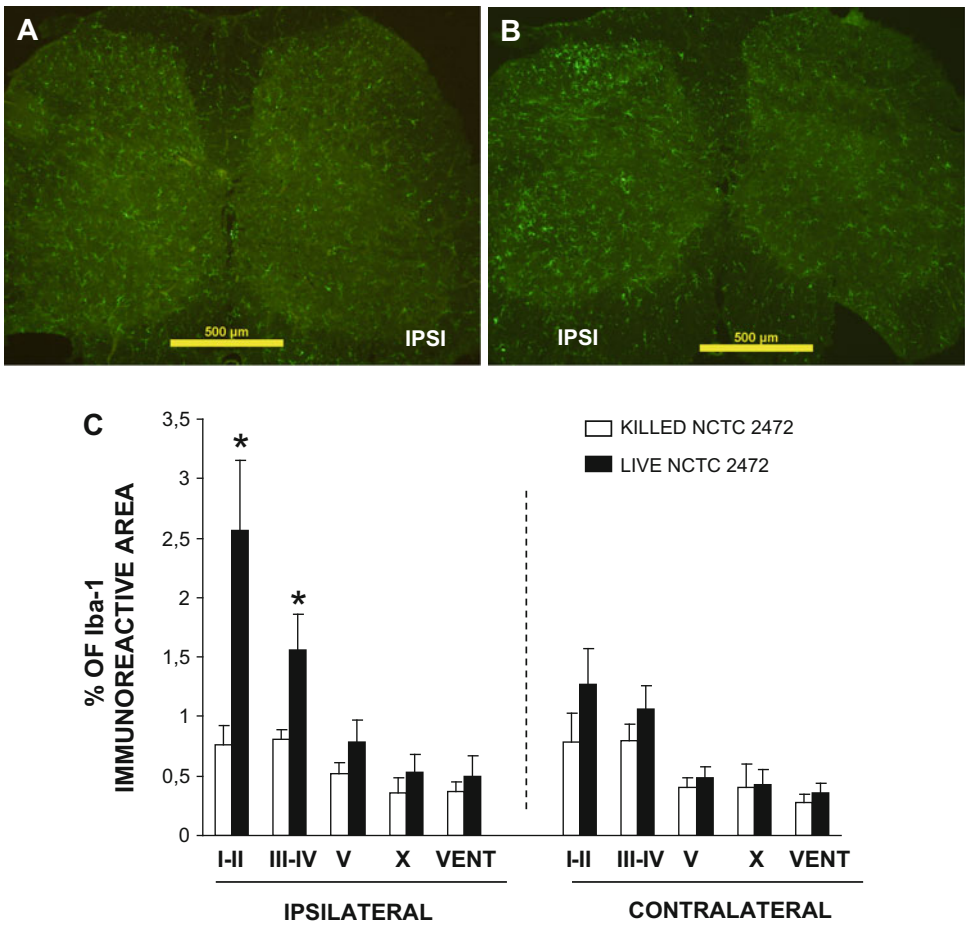

Fig. 6 Immunohistochemical staining obtained with an antibody against the microglial marker Iba-1 (ionized calcium-binding adaptor molecule 1) in the lumbar spinal cord of mice inoculated with either killed or live NCTC 2472 cells. **a** Representative example of Iba-1 immunoreactivity in a section of the lumbar spinal cord of a mouse inoculated with killed NCTC 2472 cells. IPSI: ipsilateral; *scale bar*, 500 µm. **b** Representative example of Iba-1 immunoreactivity in a section of the lumbar spinal cord of a mouse inoculated with live NCTC 2472 cells. IPSI: ipsilateral; *scale bar*, 500 µm. **c** % of Iba-1 immunoreactive area measured in *five regions* of the ipsilateral and contralateral spinal cords of mice inoculated either with killed ($n = 6$) or live ($n = 6$) NCTC 2472 cells. *Bars* correspond to means and their SEM. $^*P < 0.05$, Student's *t* test

In order to accomplish our aim of elucidating if CCL2 expression occurs in astroglial or microglial cells, we have studied the putative colocalization of CCL2 with GFAP or Iba-1 in osteosarcoma-bearing mice with a double immunostaining approach. In the experiments performed with anti-GFAP and anti-CCL2 antibodies, a projection in the z-plane of several stacks shows the presence of both markers in several astrocytes (Fig. 7a). The colocalization of CCL2 and GFAP was demonstrated when analyzing the double staining in a single stack plane (Fig. 7b). A similar result was obtained in experiments performed with anti-CCL2 and anti-Iba-1 antibodies, in which the presence of CCL2 and Iba-1 in microglial cells was observed in z-plane projections of several stacks (Fig. 7c) and the colocalization was confirmed when studying the staining in a single stack plane (Fig. 7d).

The i.t. Administration of the CCR2 Antagonist RS 504393 Inhibits Astroglial Activation in the Superficial Laminae of the Spinal Cord in Tumor-Bearing Mice

Since our data indicate that 4 weeks after the intratibial inoculation of NCTC 2472 spinal glial cells are activated and that CCL2 is up-regulated in the spinal cord of these mice, and previous reports describe the ability of CCL2 to activate glial cells, we hypothesized that CCL2 could participate in tumoral hypernociception through glial activation.

We initially aimed to confirm that astroglial and microglial activation secondary to tumor development could participate in tumoral thermal hyperalgesia. Thus, we assessed the antihyperalgesic effect of the i.t. administration of the astroglial inhibitor L-AA and the microglial inhibitor minocycline. As shown in Fig. 8, the i.t. administration of 10 nmol of L-AA inhibited osteosarcoma-evoked thermal hyperalgesia for at least 6 h, and a complete inhibition was measured for at least 3 h after the i.t. administration of 10 nmol of minocycline.

Once it was confirmed that spinal astroglial and microglial activation participate in tumoral hyperalgesia evoked by NCTC 2472 cells, we have studied if the activation of spinal astroglia or microglia could be a consequence of CCR2 stimulation. With this aim, GFAP and Iba-1 immunostaining were measured in osteosarcoma-bearing mice receiving five i.t. administrations of RS 504393 distributed in 2 days (48, 36, 24, 12, and 1 h before sacrifice). As shown in Fig. 9a–c, the immunoreactive area

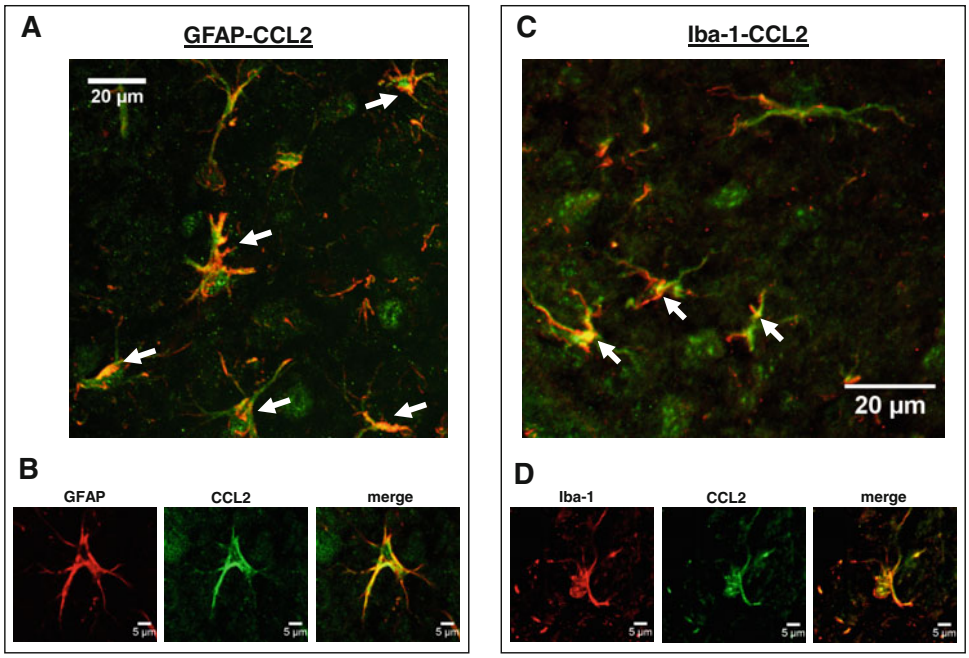

Fig. 7 Colocalization of CCL2 with GFAP and Iba-1 in the spinal cord of mice intratibially inoculated with NCTC 2472 cells. **a** Image of the double staining with anti-CCL2 and anti-GFAP antibodies in the spinal cord of mice inoculated with live NCTC 2472 cells obtained as a stack in the z-plane of 8 pictures at 1,7 µm. CCL2 staining appears in *green*, GFAP in *red* and double labeling in *yellow*. *White arrows* mark astrocytes showing double staining. *Scale bar* 20 µm. B) Images obtained from a single stack of an astrocyte labeled with anti-CCL2 and anti-GFAP antibodies. *Scale bar*, 5 µm. C) Image of the double staining with anti-CCL2 and anti-Iba-1 antibodies in the spinal cord of mice inoculated with live NCTC 2472 cells obtained as a stack in the z-plane of 6 pictures at 1,7 µm. CCL2 staining appears in *green*, Iba-1 in *red* and double labeling in *yellow*. *White arrows* mark microglial cells showing double staining. *Scale bar* 20 µm. **d** Images obtained from a single stack of a microglial cell labeled with anti-CCL2 and anti-Iba-1 antibodies. *Scale bar*, 5 µm

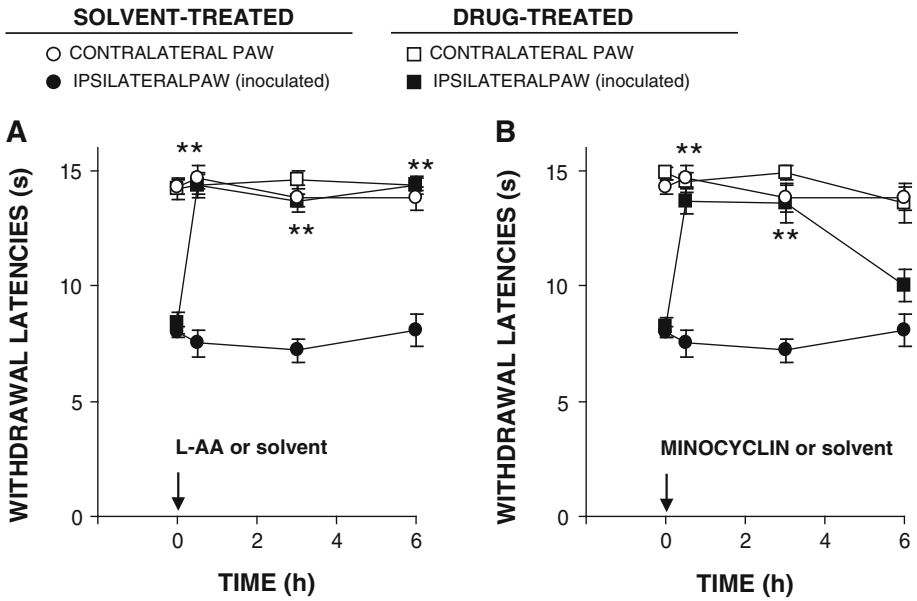

Fig. 8 Inhibition of osteosarcoma-induced thermal hyperalgesia after the i.t. administration of the astroglial inhibitor L-aminoadipate (L-AA; 10 nmol; **a**) or the microglial inhibitor minocyclin (10 nmol; **b**). In both experiments, thermal withdrawal latencies were measured before and 0.5, 3, and 6 h after the administration of the drugs or its respective solvent. Means ($n = 5$–7) and their SEM are represented. $^{**}P < 0.01$, compared with the respective latency value obtained at time 0, Dunnett's *t* test

stained by GFAP in the ipsilateral superficial laminae of the dorsal horn was significantly reduced in mice treated with RS 504393. In contrast, astroglial labeling remained unchanged at laminae III-IV or lamina V (Fig. 9a–c). When Iba-1 immunostaining was measured following the same experimental approach, the immunoreactive area in tumor-bearing mice was not modified by the administration of RS 504393 (Fig. 9d–f).

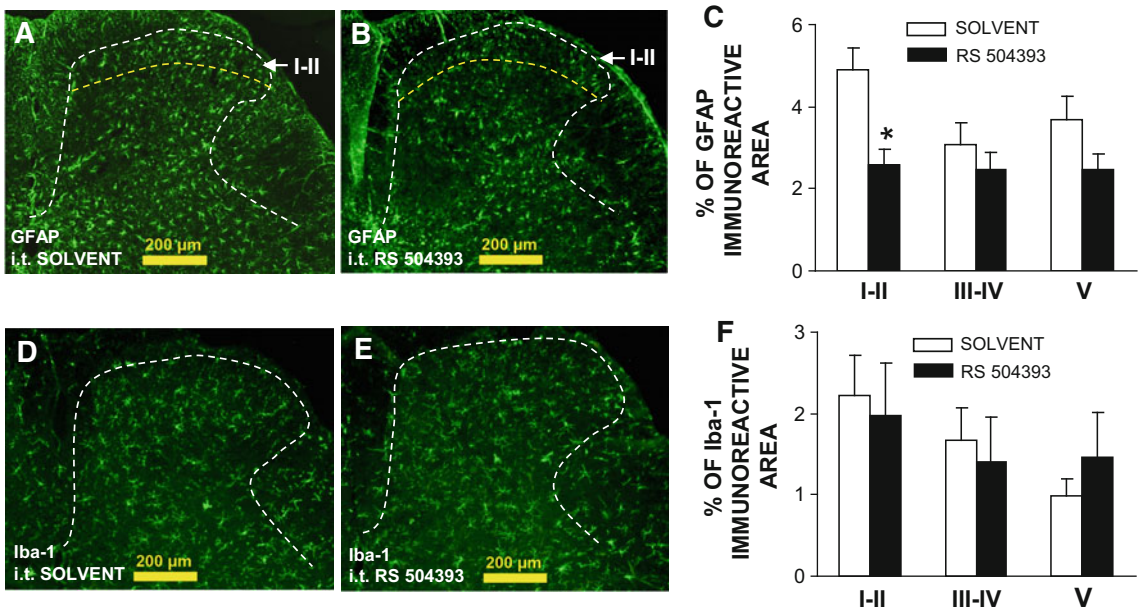

Fig. 9 Immunohistochemical staining obtained with an anti-GFAP (**a**–**c**) or an anti-Iba-1 (**d**–**f**) antibody in the spinal cord of mice inoculated with live NCTC 2472 cells and receiving 5 i.t. administrations of 3 µg of RS 504393 ($n = 5$) or solvent ($n = 4$) every 12 h during the 2 previous days. **a**–**b** Representative examples of GFAP immunoreactivity in the ipsilateral dorsal horn of mice treated either with solvent (**a**) or RS 504393 (**b**); *scale bar*, 200 µm. **c** % of GFAP immunoreactive area measured in *three regions* of the ipsilateral dorsal horn of mice inoculated with NCTC 2472 cells and treated either with i.t. solvent or RS 504393. *Bars* correspond to means and their SEM. $^*P < 0.05$, Student's *t* test. **d**–**e** Representative examples of Iba-1 immunoreactivity in the ipsilateral dorsal horn of mice treated either with solvent (**d**) or RS 504393 (**e**); *scale bar*, 200 µm. **f** % of Iba-1 immunoreactive area measured in *three regions* of the ipsilateral dorsal horn of mice inoculated with NCTC 2472 cells and treated either with i.t. solvent or RS 504393. *Bars* correspond to means and their SEM. $^*P < 0.05$, Student's *t* test

Discussion

We describe here that thermal hyperalgesia due to the intratibial inoculation of NCTC 2472 cells can be inhibited by blocking CCR2 at the spinal cord or by neutralizing spinal CCL2 with a specific antibody. In mice inoculated with these tumoral cells, CCL2 is up-regulated at the superficial laminae of the spinal cord and expressed in microglia and astroglia. In addition, our results show that the activation of CCR2 contributes to astrocyte, but not microglial, activation in cancer-bearing mice.

Thermal hyperalgesia induced in mice inoculated with NCTC 2472 cells was dose-dependently inhibited after the i.t. administration of the selective CCR2 antagonist RS 504393 (Mirzadegan et al. 2000) or of an anti-CCL2 antibody. It has been previously described that the i.t. administration of the CCR2 antagonist AZ889 produces analgesia in a neuropathic model in rats (Serrano et al. 2010) and that the spinal administration of an anti-CCL2 antibody evokes antinociceptive effects in mice suffering nerve injury (Gao et al. 2009) or in rats with an experimental surgical incision (Peters et al. 2010). Related to bone cancer, our data point in the same direction as a recent study, performed in rats intratibially inoculated with mammary Walker 256 cells, in which the i.t. administration of an anti-CCL2 antibody produced a partial antiallodynic effect (Hu et al. 2012).

In order to explore whether the behavioral effects induced by the CCR2 antagonist or the CCL2 antibody might depend on an enhanced presence of CCR2 or CCL2 at spinal level following tumor development, we performed western blot, ELISA, and immunohistochemical experiments. In contrast with the increase of spinal CCR2 described in mice inoculated with NCTC 2472 (Vit et al. 2006) or rats inoculated with Walker 256 cells (Hu et al. 2012, 2013), our data show a similar expression of CCR2 in mice inoculated with live or killed NCTC 2472 tumoral cell. This result suggests that the behavioral effects evoked by the CCR2 antagonist or the CCL2 antibody are not due to the up-regulation of CCR2 in the spinal cord. We checked the possibility that the development of the tibial sarcoma could lead to an increase in the expression of CCL2 at the spinal cord by performing ELISA and immunohistochemical experiments. Although CCL2 concentrations obtained by ELISA in spinal homogenates from mice inoculated with killed or live tumoral cells were similar, the more detailed information obtained with immunohistochemical methods revealed a significant increase in the expression of CCL2 at the external laminae of the spinal dorsal horn ipsilateral to the neoplastic injury.

Since increases of CCL2 levels at tumoral level have been previously reported in mice inoculated with NCTC 2472 cells by using this ELISA method (Pevida et al. 2012), it seems likely that the limited territory of the spinal cord at which the up-regulation of CCL2 occurs could explain why the increase of CCL2 becomes undetectable in ELISA experiments performed in homogenates of the whole L_2-L_6 spinal segments. An up-regulation of spinal CCL2 has also been described in several models of neuropathic pain (Zhang et al. 2006; Van Steenwinckel et al. 2011; Zhang et al. 2012) and in the above mentioned rat model of bone cancer-evoked pain (Hu et al. 2012, 2013), thus suggesting that this can be a common event involved in spinal hyperalgesia in neuropathic and neoplastic settings.

Once it was established that spinal CCL2 levels are increased, we have intended to determine if, as reported after nerve injury, this chemokine could be released into the spinal cord from nociceptors (Thacker et al. 2009; Zhang et al. 2006; Van Steenwinckel et al. 2011) or from glial cells (Gao et al. 2009, 2010). Neither CCL2 mRNA measured in RT-PCR assays nor CCL2 protein levels measured by ELISA were altered in DRGs of mice affected by the tumor, suggesting that this chemokine is not released from nociceptors at this stage of tumor development.

Next, we assessed the possibility that CCL2 up-regulation could be related to astroglial or microglial cells. Initially (Honore et al. 2000), described astroglial activation throughout the entire gray matter, but not microgliosis, 21 days after intrafemoral inoculation of NCTC 2472 cells. A further study also described consistent astroglial activation but only a very subtle activation of spinal microglial cells located at the superficial layers of the dorsal horn at day 8, but not at day 14 after the inoculation of NCTC 2472 cells (Hald et al. 2009). Contrasting with these data, Vit et al. (Vit et al. 2006) found that the inoculation of NCTC 2472 cells in both humeri of female mice evokes astroglial and microglial activations in the spinal cord 18 days after inoculation. In our conditions, we observed a pronounced increase in the immunoreactive area marked with the GFAP antibody at laminae I-II, III-IV, V, Xs and ventral horn ipsilateral to the tumor. Besides, microglial expression, measured as Iba-1 immunoreactive surface, was significantly increased in laminae I-II and III-IV of the ipsilateral spinal cord. Furthermore, the inhibition of tumoral hyperalgesia after the i.t. administration of the astroglial inhibitor L-AA or the microglial inhibitor minocycline demonstrates the involvement of astroglial and microglial cells in this form of neoplastic pain, as previously demonstrated in other experimental models (Mao-Ying et al. 2012; Wang et al. 2012).

In order to determine whether CCL2 could be expressed in astroglial and microglial cells in the spinal cord of tumor-bearing mice, we designed double-staining immunohistochemical assays. In our experiments, we have observed that immunostaining with the anti-CCL2 antibody can be colocalized together with that obtained with anti-GFAP and anti-Iba-1 antibodies, suggesting that CCL2 could be released into the spinal cord of osteosarcoma-bearing mice from both types of glial cells. The ability of astrocytes to express and release CCL2 has been previously demonstrated following neuropathic injury (Gao et al. 2009; 2010) and the instauration of CCL2-dependent persistent pain symptoms after the direct spinal administration of activated astrocytes in mice remarks the relevance of CCL2 released from astrocytes in hypernociception (Gao et al. 2010; Zhang et al. 2012). In relation to microglial cells, CCL2 expression has been previously detected in the hippocampus in response to neuronal injury (Babcock et al. 2003) and an augmentation of CCL2 mRNA has been described in cultured cortical rat microglia in response to glucose exposition (Quan et al. 2011). The present results showing the expression of this chemokine in spinal microglia of mice inoculated with NCTC 2472 cells open the possibility that CCL2 released from spinal microglial cells could participate in bone cancer-induced pain. In any case, the presence of CCL2 in astroglia and microglia shown in our experiments does not discard the hypothesis that CCL2 involved in spinal hypernociception could also proceed from spinal neurons.

The possibility that glial cells, apart from being a probable source of CCL2 in tumor-bearing mice, could, in turn, be activated by CCL2 is supported by a previous report demonstrating that the hypernociception evoked by the spinal administration of CCL2 in mice can be antagonized by the i.t. administration of a microglial or an astroglial inhibitor (Baamonde et al. 2011). To ascertain whether the osteosarcoma-evoked spinal glial activation could be linked to CCR2 stimulation, we have studied if the i.t. administration of the CCR2 antagonist RS 504393 could alter the increased GFAP or Iba-1 immunostaining evoked by the tumor. The results obtained demonstrate that astroglial activation depends, at least in part, on CCR2 receptors. Thus, although GFAP staining was not completely inhibited after RS 504393 treatment, it was significantly reduced in the superficial layers of the spinal cord, a territory playing a prominent role in pain modulation, in which the presence of CCL2 has been demonstrated in our immunohistochemical experiments. In contrast, there was no difference in Iba-1 immunoreactivity between mice treated with RS 504393 or solvent, indicating that spinal microglial activation in osteosarcoma-bearing mice occurs independently of CCR2 receptor activation. There is a general agreement with respect to the involvement of CCL2 and CCR2 in the activation of microglia (Abbadie et al. 2003; Thacker, et al. 2009; Zhang and De Koninck. 2006; Zhang et al. 2007), whereas no general consensus exists with regard to the role

played by CCL2/CCR2 in astroglia. Besides the lack of expression of CCR2 on spinal astrocytes reported in some studies (Abbadie et al. 2003), the presence of CCR2 mRNA and CCR2 proteins has been demonstrated in rat brain astrocytes (Banisadr et al. 2002), cultured human fetal astrocytes (Andjelkovic et al. 2002)s or astroglial cells of rat spinal cord (Knerlich-Lukoschus et al. 2008). Furthermore, CCL2 can evoke functional responses in astrocytes, such as their migration (Heesen et al. 1996; Quinones et al. 2008) or, more related to nociception, the phosphorylation of ERK (Old and Malcangio 2012).

Overall, our results show that the development in mice of a tibial tumor following the inoculation of NCTC 2472 cells evokes an up-regulation of CCL2 in the external laminae of the spinal dorsal horn that contributes to tumoral hyperalgesia. The increase in the spinal concentration of this chemokine does not seem to depend on its release from nociceptors, but is probably related to its expression in astrocytes and microglial cells activated in response to tumor development. Furthermore, the reduction of tumor-induced astroglial activation at the superficial laminae of the spinal cord that occurs when CCR2 are blocked suggests that astroglial activation can be one of the mechanisms by which the stimulation of spinal CCR2 receptors leads to hyperalgesia in osteosarcoma-bearing mice. Our results add new information related to the involvement of spinal CCL2/CCR2 and glial cells in tumoral hypernociception and, these results together with former reports describing the relevance of peripheral CCL2 or CCR2 in tumoral hyperalgesia (Khasabova et al. 2007; Pevida et al. 2012; Hu et al. 2012; 2013), suggest that the pharmacological modulation of this system could be an interesting strategy for the control of neoplastic pain.

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Conflict of interest The authors declare that they have no conflict of interest

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3.3 Artículo 3:

Pevida M, Lastra A, Hidalgo A, Baamonde A, Menéndez L. **Spinal chemokine CCL2 and microglial activation are involved in paclitaxel – evoked cold hyperalgesia**. Brain Res. Bull. 95:21-27. 2013.

OBJETIVO

Evaluar el efecto antinociceptivo derivado de la administración de antagonistas de receptores CCR2 y CCR1 en un modelo de dolor neuropático basado en la administración sistémica de paclitaxel en ratones así como su relación con la presencia de quimiocinas y con la activación de las células gliales de la médula espinal.

MÉTODOS

- Ratones Swiss CD-1 tratados con paclitaxel (10mg/kg, s.c.).
- Test de la placa fría unilateral (medida de la hiperalgesia al frío, 0°C).
- ELISA (cuantificación de los niveles de la quimiocina CCL2).
- Inmunohistoquímica (estudio de la expresión de CCL2 y de la activación microglial y astroglial).

RESULTADOS Y CONCLUSIONES

- Mientras que la hiperalgesia al frío evocada por paclitaxel es independiente de la activación de receptores CCR1, la administración sistémica o espinal de un antagonista selectivo de receptores CCR2 reduce dicha hiperalgesia de un modo dependiente de la dosis.
- La quimiocina CCL2 participa en el desarrollo de la hiperalgesia al frío tras la administración de paclitaxel, ya que se produce un incremento de los niveles espinales de la misma en los ratones tratados con el antineoplásico y los síntomas hipernociceptivos se eliminan tras la administración espinal de un anticuerpo anti-CCL2 o un antagonista de CCR2.
- La administración de paclitaxel provoca la activación de microglía pero no de astroglía espinal y la presencia de CCL2 parece involucrada en la activación microglial, ya que ésta se inhibe tras la administración de un anticuerpo anti-CCL2.

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Research report

Spinal CCL2 and microglial activation are involved in paclitaxel-evoked cold hyperalgesia

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ABSTRACT

The antineoplastic paclitaxel induces a sensory neuropathy that involves the spinal release of neuroinflammatory mediators and activation of glial cells. Although the chemokine CCL2 can evoke glial activation and its participation in neuropathic pain has been demonstrated in other models, its involvement in paclitaxel-evoked neuropathy has not been previously explored. Paclitaxel-evoked cold hypernociception was assessed in mice by the unilateral cold plate test and the effects on cold hyperalgesia of the CCR2 antagonist RS 504393, the CCR1 antagonist J113863, the microglial inhibitor minocycline or an anti-CCL2 antibody were tested. Furthermore, ELISA measurements of CCL2 concentration and immunohistochemical assays of Iba-1 and GFAP, markers of microglial and astroglial cells respectively, were performed in the lumbar spinal cord.

Cold hypernociception measured 3 days after the administration of paclitaxel (10 mg/kg) was inhibited by the s.c. (0.3–3 mg/kg) or i.t. (1–10 μg) administration of RS 504393 but not of J113863 (3–30 mg/kg). CCL2 levels measured by ELISA in the lumbar spinal cord were augmented in mice treated with paclitaxel and the i.t. administration of an anti-CCL2 antibody completely suppressed paclitaxel-evoked cold hyperalgesia, strongly suggesting that CCL2 is involved in the hypernociception evoked by this taxane. Besides, the implication of microglial activation is supported by the increase in the immunolabelling of Iba-1, but not GFAP, in the spinal cord of paclitaxel-treated mice and by the inhibition of cold hyperalgesia produced by the i.t. administration of the microglial inhibitor minocycline (1–10 nmol). Finally, the neutralization of spinal CCL2 by the i.t. administration of a selective antibody for 3 days almost totally inhibited paclitaxel-evoked microglial activation.

In conclusion, our results indicate that paclitaxel-evoked cold hypernociception depends on the activation of CCR2 due to the spinal release of CCL2 and the subsequent microglial activation.

1. Introduction

Paclitaxel is an antineoplastic agent isolated from the pacific yew tree (*Taxus brevifolia*) used in the management of different solid tumours (Le and Bast, 2011). As occurs with other taxanes, paclitaxel induces a sensory neuropathy sometimes with "glove and stocking" distribution (Quasthoff and Hartung, 2002; Dougherty et al., 2004). Although the incidence of paclitaxel-induced neuropathy has not been widely explored, it has been recently reported that up to 60% of patients treated with this drug can develop diverse degrees of sensory neuropathy (Ghoreishi et al., 2012). Patients suffering neuropathy after treatment with paclitaxel usually report severe burning pain in response to the application of cold stimuli that are innocuous in healthy volunteers (Dougherty et al., 2004), thus reflecting cold allodynia. This type of disturbing adverse effect can appear even after the first cycle of chemotherapy (Dougherty et al., 2004; Argyriou et al., 2008) and generally increases in subsequent cycles (Dougherty et al., 2004), being an important cause of dose reduction or discontinuation of treatment (Argyriou et al., 2008; Wolf et al., 2008).

One of the mechanisms involved in the neuropathy evoked by paclitaxel seems related to its main antitumoural cytotoxic action, based on the disruption of neural microtubules and the alteration of axonal transport (De Brabander et al., 1981; Rowinsky et al., 1988; Jaggi and Singh, 2012). Other peripheral events, such as increased calcium release, TRPV1 phosphorylation, activation of caspases/calpains or proinflammatory cytokine upregulation together with central mechanisms can participate in the hypernociception induced by paclitaxel (Jaggi and Singh, 2012; Wang et al., 2012). At the spinal cord, the involvement of inflammatory mediators such as TNF-α or IL-1β (Burgos et al., 2012) and the activation of glial cells have been demonstrated following its administration (Peters et al., 2007; Cata et al., 2008; Liu et al., 2010; Burgos et al., 2012).

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Chemokines constitute a large family of peptidic molecules directly involved in the establishment of painful symptoms present in pathological settings (Gao and Ji, 2010; Kiguchi et al., 2012; Old and Malcangio, 2012). Several chemokines as CCL2 can amplify nociceptive processing after its release from different sources. Thus, apart from its release from injured nociceptors (Thacker et al., 2009; Van Steenwinckel et al., 2011), the ability of glial cells to release CCL2 has been also demonstrated. This is the case for hippocampal microglia in response to neuronal injury (Babcock et al., 2003) or for cultured astrocytes in response to several stimuli (Hinojosa et al., 2011). Interestingly, it has been demonstrated that the spinal expression of CCL2 (monocyte chemoattractant protein 1, MCP-1) in neurons and astroglial cells (White et al., 2005; Thacker et al., 2009; White et al., 2009; Gao et al., 2009; Zhang et al., 2012b) and its release into the dorsal horn from nociceptors (Thacker et al., 2009; Van Steenwinckel et al., 2011) is increased secondary to a neuropathic injury. One important mechanism by which CCL2 evokes hypernociception is the activation of spinal microglial cells (Abbadie et al., 2003; Zhang et al., 2007; Thacker et al., 2009; Baamonde et al., 2011). Related to CCR2, the main receptors on which CCL2 acts, their increased expression in neurons and microglial cells has been demonstrated in neuropathic states (Abbadie et al., 2003; Gao et al., 2009; Zhang et al., 2012b) and an important reduction of neuropathic painful reactions has been reported after their deletion (Abbadie et al., 2003).

Since the role of spinal CCL2 in the production of hypernociceptive symptoms evoked by paclitaxel has not been previously studied, we study here whether the blockade of CCR2 receptors modify paclitaxel-evoked cold hypernociception, if spinal endogenous CCL2 could underlie it and, finally, if the putative effects evoked by CCL2 could be mediated through spinal glial activation.

2. Methods

2.1. Animals

CD-1 Swiss male mice 8–10 week old bred in the Animalario of the Universidad de Oviedo (Reg. 33044 13A), maintained on a 12 h dark–light cycle with free access to food and water were used. Experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain).

2.2. Drugs and drug administration

Paclitaxel (Tocris) was initially dissolved in Cremophor EL/ethanol (1:1, 6 mg/ml) and further diluted to 1 mg/ml in saline. Administration was performed subcutaneously (s.c.) under the fur of the neck in a final volume of 10 ml/kg. Effects were usually tested 3 days after administration.

The CCR2 receptor antagonist RS 504393 (Tocris), its corresponding solvent (5% DMSO in saline at the highest concentration used), the CCR1 antagonist, J113863 (Tocris) or its corresponding solvent (10% DMSO in distilled water at the highest concentration used) were s.c. administered 30 min before testing. RS 504393 or its solvent, a goat anti-mouse CCL2 antibody (R&D, AF-479-NA), goat IgG (Sigma), minocycline hydrochloride (Sigma) or saline were intrathecally (i.t.) administered 30 min before testing in a volume of 5 μl.

For i.t. injections, mice were slightly anaesthetized with 5% isoflurane (Isoflo®, Esteve), a lumbar cut was made and the tip of a 30 gauge needle inserted in a Hamilton microsyringe was introduced at the level of L_5–L_6 to inject the drug, as previously described (Menéndez et al., 2002).

2.3. Unilateral cold plate test

Paw withdrawal latencies from a cold plate (IITC Life Science) set at 0 °C were obtained. Measures were taken following a similar procedure to that previously described by the unilateral hot plate test (Menéndez et al., 2002) when hot is used as the nociceptive stimulus. Briefly, mice were gently restrained by the fur of the neck and, in order to obtain independent measures of the withdrawal latency of each paw, only one paw was set on the cooled surface at each time. As previously described, the withdrawal latency was measured and, after a 2-min interval the procedure was repeated by lining the other paw on the surface. Since in this case it is expected that modifications of withdrawal latencies should occur similarly in both paws, the mean of both measures was finally considered. A theoretical cut-off of 30 s was established, although in no case latencies in solvent treated mice were greater than 20 s. Since the stimulus applied by exposing the plantar side of the paw to a cold plate at 0 °C did evoke nociceptive, withdrawal, responses in solvent-treated mice, we have preferred to describe the decrease in cold withdrawal latencies as cold hyperalgesia rather than cold allodynia.

When using a cold stimulus, we have observed two main differences related to the studies performed with the hot plate. Firstly, it was necessary to dry frequently the water condensation that appears on the plate surface. Secondly, we have observed in previous experiments that, in contrast with the hot plate test in which latencies are rather stable upon repetition, a tendency to the decrease of latencies occurred when repeated cold plate measurements were performed the same day in the same mouse. For this reason, only a single measure was performed in each paw.

2.4. Immunohistochemical assays

Mice were anaesthetized with 5% isoflurane (Isoflo®, Esteve) and then decapitated. The vertebral column was sectioned at thoracic and sacral levels and the lumbar spinal cord enlargement was removed and fixed 24 h in 4% formaldehyde at 4 °C. After fixation, spinal cords were cryoprotected by 24 h immersion in 15% sucrose and some 24 h further in 30% sucrose at 4 °C. Spinal cord was cut in 30 μm thick sections using a freezing microtome (Mikrom HM430) and these were serially collected on gelatin coated slides (Super-Frost® Plus, Menzel –Glaser).

Sections were initially incubated in cold acetone (Prolabo) for 10 min, rinsed during 30 min in PBS (0.01 M) and further incubated at 4 °C overnight in a humid chamber with primary antibodies. For identification of astroglial cells, the polyclonal rabbit anti-glial fibrillary acid protein (GFAP) antibody (DakoCytomation) was used diluted 1:500 in 0.01 M PBS and for identification of microglial cells, the polyclonal rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) antibody (Wako) was diluted 1:250 in 0.01 M PBS. After overnight incubation, sections were rinsed for 30 min in 0.01 M PBS and incubated 1 h at room temperature in a dark humid chamber with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Life Technologies), diluted 1:250 in 0.01 M PBS. Sections were finally washed 30 min in 0.01 M PBS, mounted and cover slipped using Fluoromount-G® (Southern Biotech). As a procedural control, primary antibodies were omitted and replaced by PBS and under these conditions no staining occurred.

Immunostained tissue sections were imaged using a BX61 Olympus microscope with a 4×/0.16NA objective and, in some particular cases a 10×/0.40NA objective. For quantification, images were acquired with a CCD camera Olympus DP-70 using blue-excitation filter (BP470-490) and processed using the software Olympus DP-controller 1.2.1.108 and Olympus DP-manager 1.2.1.107. Quantitative analysis of the immunofluorescence was performed by measuring the percentage of green immunoreactive surface for GFAP or Iba-1 with a computer-assisted imaging software analysis system (Image J version 1.43u; NIH). Staining intensities were examined in the complete dorsal and ventral horns, and the laminae I-II. For each experiment, measurements came from at least five different mice in which the five more immunoreactive slices in the side of the spinal cord showing the greater staining were used.

2.5. Enzyme-linked immunosorbent assay (ELISA)

CCL2 levels were measured in tissue homogenates prepared from lumbar spinal cords of mice treated 3 days before with paclitaxel (10 mg/kg) or solvent. Mice were anaesthetized with 5% isoflurane (Isoflo®, Esteve) and then decapitated. The vertebral column was sectioned at thoracic and sacral levels and the lumbar cord enlargement was harvested, frozen immediately in liquid nitrogen and stored at −80 °C until use. Next, the tissue was weighed, and homogenized in a volume of 6 μl of buffer per mg of tissue using a handy homogenizer (Minicraft MB130®). The buffer used consisted in 0.1 M Tris, 0.15 M NaCl, 0.5% CTAB (Fluka) and a protease inhibitor (1 tablet/50 ml buffer, Roche Diagnostics). Next, homogenates were centrifuged at 15,000 × *g* for 15 min at 4 °C and protein concentration of supernatants measured by a BCA protein assay (Pierce) by using a spectrophotometer (Nanodrop 2000C, Thermoscientific).

The level of CCL2 was measured with a commercially available ELISA (R&D Systems, DuoSet®). Plates (R&D Systems) were coated overnight at room temperature with an antibody specific for mouse CCL2. In order to achieve a value in the range of the standard curve of CCL2, a protein quantity of 10 μg of the spinal cord homogenate was added to the wells in a final volume of 100 μl. After washing, a 2 h incubation period was allowed with a second biotinylated anti-mouse CCL2 antibody that was followed by a 20 min incubation period with streptavidin-peroxidase. Plates were washed to remove the unbound enzyme, colour was developed by adding a stabilized chromogen (tetramethylbenzidine:H_2O_2, 1:1) and the reaction was terminated with a stop solution (2 N H_2SO_4). The intensity of the coloured product was quantified with a spectrophotometer (μQuant; Bio-Tek Instruments) at 450 nm subtracting the readings obtained at 570 nm in order to correct optical background of plates.

The values obtained came from samples of 4 solvent-treated and 4 paclitaxel-treated mice performed in duplicate. These mice were exclusively used in ELISA experiments and were not the same than those used in behavioural or immunohistochemical experiments.

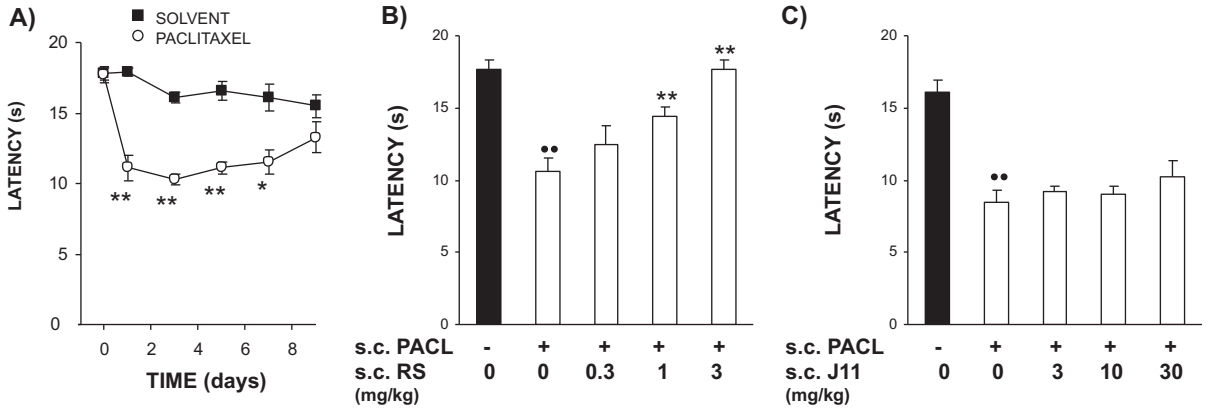

Fig. 1. Effects of CCR2 or CCR1 antagonists on paclitaxel-evoked cold hypernociception. (A) Time course of the effects of the s.c. administration of 10 mg/kg of paclitaxel in mice measured by the unilateral cold plate test ($n = 7$ per group). (B) Dose-dependent inhibition of paclitaxel (PACL)-evoked cold hypernociception after the s.c. administration of the CCR2 antagonist RS 504393 (RS, 0.3–3 mg/kg) ($n = 5$–6 per group). (C) Lack of effect of the CCR1 antagonist J113863 (J11, 3–30 mg/kg; s.c.) on paclitaxel (PACL)-evoked cold hypernociception ($n = 5$–8 per group). Means and their S.E.M. are represented. ••$P < 0.01$, comparing paclitaxel- and solvent-treated mice not receiving RS 504393 or J113863, Student's t-test. **$P < 0.01$ comparisons between paclitaxel-treated mice receiving solvent or different doses of RS 504393, Dunnett's t-test.

2.6. Statistical analysis

Mean values and their corresponding standard errors were calculated for behavioural assays, immunohistochemical image analysis or ELISA measurement. Cold withdrawal latencies were compared by a Student's t-test when two groups were considered. The comparisons of thermal latencies among several groups were done by an initial one-way analysis of variance (ANOVA) followed by either the Dunnett's t-test when groups received different doses of a drug or the Newman–Keuls test when groups received different drug treatments. The values related to the immunoreactive surface obtained when labelling with GFAP or Iba-1 as well as those of CCL2 levels measured by ELISA in paclitaxel- and solvent-treated mice were compared by a Student's t-test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Inhibition of paclitaxel-evoked cold hyperalgesia by the blockade of CCR2 but not CCR1 receptors

The effect of the s.c. administration of 10 mg/kg of paclitaxel on cold sensitivity was assessed by the unilateral cold plate (0 °C). Withdrawal latencies of solvent-treated mice were about 16–18 s, whereas those measured in paclitaxel-treated mice from day 1 to day 7 were about 10–11 s (Fig. 1A). In further behavioural experiments, we have studied cold hyperalgesia at day 3 after paclitaxel administration.

In order to test whether chemokine CCR2 receptors could be involved in paclitaxel-evoked cold hyperalgesia, the effect of the s.c. administration of RS 504393, a selective CCR2 antagonist, was assayed at day 3 after paclitaxel administration. As shown in Fig. 1B, a dose-dependent inhibition of cold hypernociception was obtained 30 min after the administration of RS 504393 (0.3–3 mg/kg). In contrast, paclitaxel-evoked cold hyperalgesia remained unmodified in mice that received the administration of the selective CCR1 receptor antagonist J113863 (3–30 mg/kg, 30 min before testing) (Fig. 1C).

3.2. Involvement of spinal CCR2 receptors and endogenous chemokine CCL2 in paclitaxel-evoked cold hyperalgesia

As shown in Fig. 2A, the i.t. administration of RS 504393 (1–10 μg) 30 min before testing dose-dependently inhibited paclitaxel-evoked cold hyperalgesia, supporting the participation of spinal CCR2 receptors in the development of this type of hypernociception. In order to test if the release of the endogenous

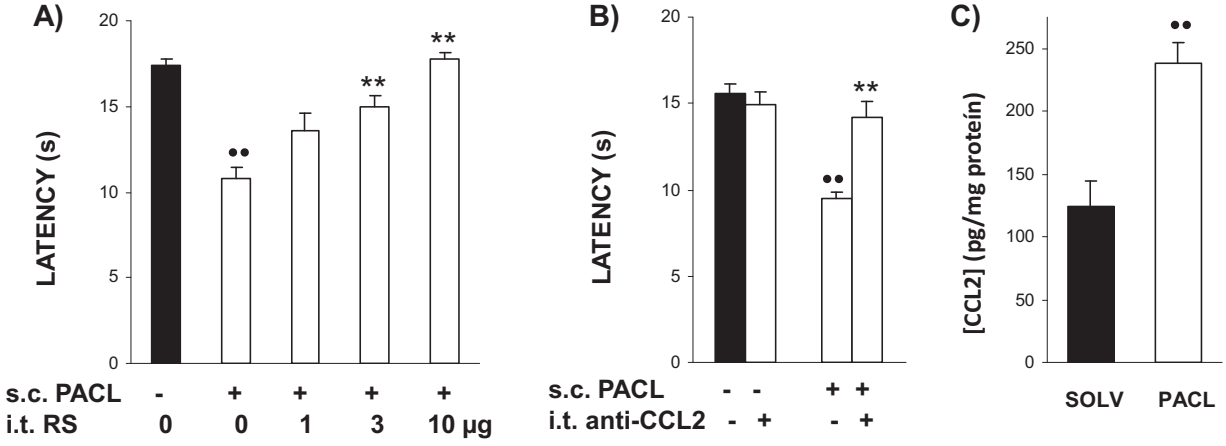

Fig. 2. Inhibition of paclitaxel-evoked hypernociception after the i.t. administration of a CCR2 antagonist or an anti-CCL2 antibody and increased spinal levels of CCL2 in paclitaxel-treated mice. Inhibition of paclitaxel (PACL)-evoked cold hypernociception after the i.t. administration of RS 504393 (RS, 1–10 μg; $n = 6$–7 per group (A)) or an anti-CCL2 antibody (1 μg; $n = 7$–9 per group (B)). In (A), mice of the control group received the s.c and i.t. administration of the corresponding solvents. In (B), mice of the control group received the s.c. administration of the paclitaxel solvent and the i.t. administration of goat IgG. Means and their S.E.M. are represented. ••$P < 0.01$, compared to solvent-treated mice, Student's t-test. **$P < 0.01$ comparisons of paclitaxel-treated mice receiving solvent with those treated with RS 504393 (Dunnett's t-test (A)) or an anti-CCL2 antibody (Student's t-test (B)). (C) Increase of CCL2 content measured by ELISA in spinal cord homogenates of mice treated with paclitaxel ($n = 7$–9). ••$P < 0.01$, compared to solvent-treated mice, Student's t-test.

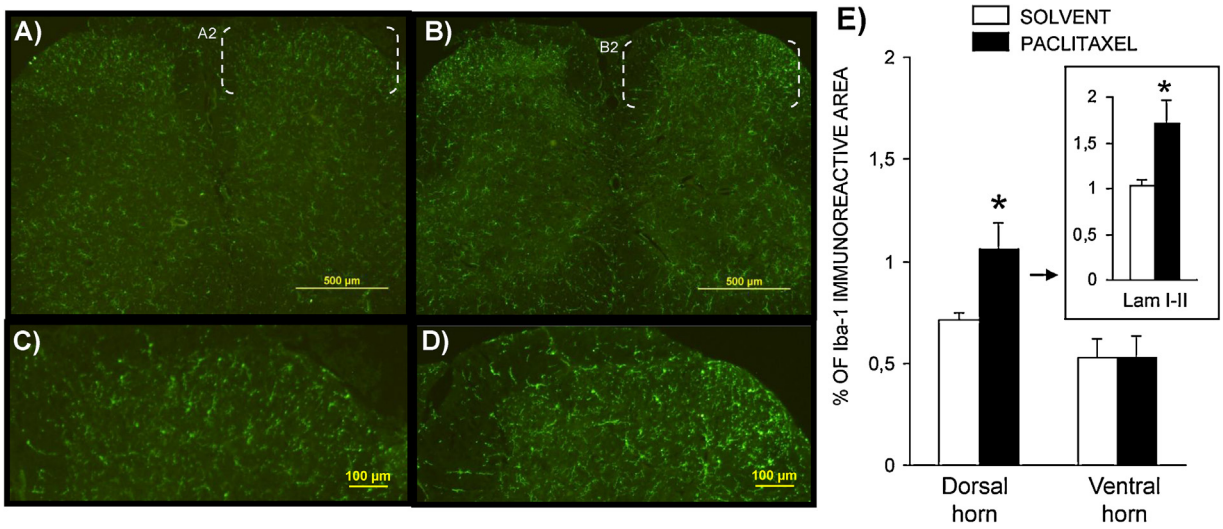

Fig. 3. Increase of Iba-1 immunoreactive area in the lumbar spinal cord of mice treated with paclitaxel. Representative examples of the immunohistochemical expression of Iba-1 (4×) in solvent- or paclitaxel-treated mice are shown in (A) and (B). In (C) and (D) amplification (10×) of the superficial laminae of the ipsilateral dorsal horn, including laminae I and II, corresponding to the signalled areas of (A and B). In (E) % of immunoreactive area measured in the dorsal and ventral horns of mice treated with solvent ($n = 5$) or paclitaxel ($n = 6$) is shown in the main graph and the same parameter measured in laminae I and II of the dorsal cord is represented in the inset. Bars correspond to means and their S.E.M. $^*P < 0.05$, Student's t-test.

chemokine CCL2 at the spinal cord could be responsible for CCR2 activation, we assayed the effect produced by the i.t. administration of 1 μg of an anti-CCL2 antibody on paclitaxel-induced cold hyperalgesia. The administration 30 min before testing of the anti-CCL2 antibody, but not of goat IgG, completely abolished paclitaxel-evoked cold hyperalgesia (Fig. 2B), thus indicating that CCL2 released at the spinal cord is involved in this process.

ELISA experiments were performed to directly assess whether the treatment with paclitaxel evokes an increase of CCL2 levels at the spinal cord. As represented in Fig. 2C, a spinal concentration of 123.6 ± 21 pg/mg protein was detected in solvent-treated mice, whereas 237.9 ± 17.1 pg/mg protein in paclitaxel-treated mice, representing a 90% increase of CCL2.

3.3. Activation of microglial but not astroglial cells in the spinal cord of paclitaxel-treated mice

We have determined by immunohistochemical studies if microglial or astroglial activation occurs in the spinal cord of mice 3 days after receiving 10 mg/kg of paclitaxel. The mean immunoreactive area stained by the microglial marker antibody anti-Iba-1 was significantly increased when measured in the whole dorsal horn of the spinal cord of mice treated with paclitaxel (Fig. 3A and B) whereas no difference was obtained in the Iba-1 labelling obtained in the ventral horn of paclitaxel- and solvent-treated mice (Fig. 3E). In particular, immunoreactivity was specially increased in the superficial laminae (I and II) of spinal cord and significant differences between paclitaxel- and solvent-treated mice were again obtained (Fig. 3C and D).

The immunoreactive surface stained with the antibody used for measuring astroglial activation, GFAP, was slightly increased in the dorsal horn of some paclitaxel-treated mice when compared to solvent-treated ones. However, this tendency was not consistent and the final analysis did not reveal a significant increase in GFAP immunoreaction in response to paclitaxel treatment in none of the regions analyzed (dorsal and ventral horns as well as superficial layers of the dorsal horn) (Fig. 4).

3.4. Inhibition of paclitaxel-evoked cold hyperalgesia after the i.t. administration of minocycline and reduction of paclitaxel-evoked spinal Iba-1 labelling by the i.t. administration of an anti-CCL2 antibody

In order to establish if cold hyperalgesia measured in paclitaxel-treated mice could be related to microglial activation, the effect of the spinal administration of the microglial inhibitor minocycline was assessed. As shown in Fig. 5A, the i.t. administration of minocycline (1–10 nmol, 30 min before) dose-dependently inhibited paclitaxel-evoked cold hyperalgesia, demonstrating the involvement of microglial activation.

To elucidate if microglial activation evoked by paclitaxel could be a consequence of the presence of CCL2, we have studied whether the neutralization of CCL2 by the i.t. administration of an anti-CCL2 antibody could avoid paclitaxel-evoked microglial activation. Paclitaxel-treated mice received 4 i.t. administrations of either an anti-CCL2 antibody (1 μg) or the same amount of goat IgG used as control. Injections were performed at days 0 (simultaneously to paclitaxel administration), 1, 2 and 3, being mice sacrificed 30 min after the last i.t. injection. As represented in Fig. 5B–D, the microglial activation evoked by paclitaxel in the dorsal horn and, particularly, in the superficial laminae was almost completely suppressed in mice receiving the anti-CCL2 antibody.

4. Discussion

Our results show that the levels of the chemokine CCL2 are increased in the spinal cord of mice treated with paclitaxel and that this chemokine, acting through CCR2 receptors, can stimulate microglial cell activity and participate in the hypernociceptive response to cold stimuli present in mice treated with this antineoplastic drug.

Paclitaxel-induced hypernociception has been demonstrated in laboratory animals in which this drug is administered following different schedules (Burgos et al., 2012; Hidaka et al., 2009; Liu et al., 2010; Naguib et al., 2012; Peters et al., 2007; Zhang et al., 2012a). We show here that a single administration of 10 mg/kg of paclitaxel in mice evokes cold hyperalgesia measured by using a 0 °C cold plate. The instauration of this effect was detected as

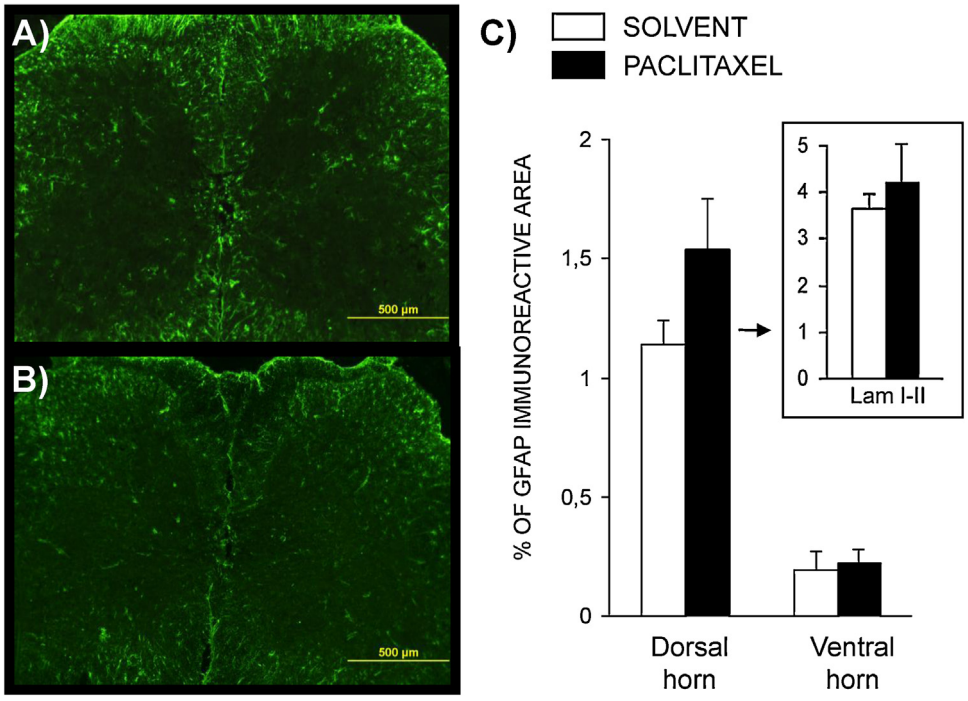

Fig. 4. Mice treated with paclitaxel do not show a significant increase of GFAP immunoreactive area in the lumbar spinal cord. Representative examples of the immunohistochemical expression of GFAP (4×) in solvent- or paclitaxel-treated mice are shown in (A) and (B), respectively. In (C), the % of immunoreactive area measured in the dorsal and ventral horns of mice treated with solvent ($n = 5$) or paclitaxel ($n = 7$) is shown in the main graph and the same parameter measured in laminae I and II of the dorsal horn, is represented in the inset. Bars correspond to means and their S.E.M.

early as 1 day after paclitaxel administration and lasted for 7 days, in accordance with a previous report that described hyperalgesia and allodynia in mice treated with this dose regimen of paclitaxel (Hidaka et al., 2009). Supporting the relevance of this observation, it has been described that sensory symptoms may occur in clinical settings within 24–72 h following the first administration of single high doses of paclitaxel (Argyriou et al., 2008).

The systemic administration of the selective antagonist of CCR2, RS 504393 (Mirzadegan et al., 2000), dose-dependently inhibited paclitaxel-evoked cold hyperalgesia, suggesting that endogenous chemokines that bind CCR2 participate in the establishment of this hypernociceptive symptom. In contrast, although previous data demonstrated the involvement of the endogenous CCR1 agonist, CCL3, in neuropathic pain (Kiguchi et al., 2010), the lack of effect of the CCR1 antagonist, J113863, seems to indicate that endogenous chemokines that bind to these receptors are not involved in paclitaxel-evoked cold hypernociception. Furthermore, when the CCR2 antagonist RS 504393 was i.t. administered at doses similar to those used by other authors to block neuropathic pain (Zhang et al., 2012b), paclitaxel-evoked cold hypernociception was also

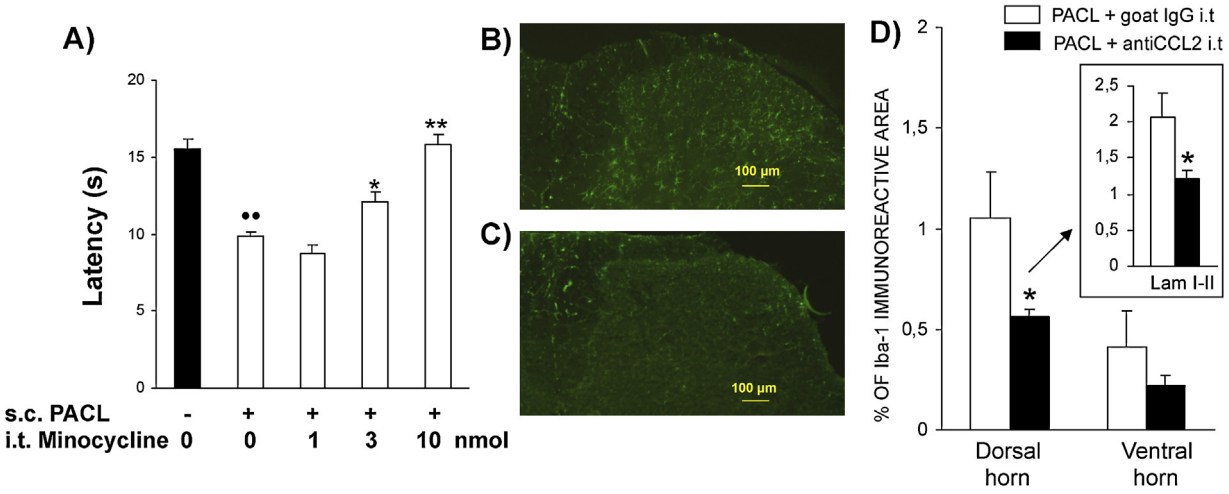

Fig. 5. Minocycline inhibits paclitaxel-evoked cold hypernociception and the neutralization of spinal CCL2 with an antibody avoids paclitaxel-evoked microglial activation. (A) Inhibition of paclitaxel-evoked cold hypernociception 30 min after i.t. administration of minocycline (1–10 nmol). Means ($n = 4$–5 per group) and their S.E.M. are represented. ••$P < 0.01$, comparing paclitaxel- and solvent-treated mice not receiving minocycline, Student's t-test. *$P < 0.05$, **$P < 0.01$ comparisons between paclitaxel-treated mice receiving solvent or minocycline, Dunnett's t-test. In (B and C), representative examples of Iba-1 immunoreactivity in the spinal cord of paclitaxel (PACL)-treated mice receiving 4 i.t. injections of either a goat IgG (B) or an anti-CCL2 antibody (1 µg, C) 0, 24 and 48 and 72 h after paclitaxel. In (D), mean of % of immunoreactive area measured in the dorsal and ventral horns of paclitaxel-treated mice receiving the CCL2 antibody ($n = 5$) or goat IgG ($n = 5$) is shown in the main graph and the same parameter measured in laminae I and II of the dorsal cord is represented in the inset. Bars correspond to means and their S.E.M. *$P < 0.05$, Student's t-test.

dose-dependently inhibited. This result indicates that, in addition to the important role that CCR2 can play in the establishment of neuropathic pain when acting at peripheral level (White et al., 2005), CCR2 receptors present at spinal level also participate in paclitaxel-evoked neuropathy.

Since CCL2 is one of the main endogenous CCR2 agonists (Ransohoff et al., 2007) and its release at the spinal cord has been demonstrated in other models of neuropathic injury (Thacker et al., 2009; Van Steenwinckel et al., 2011), we have measured by ELISA whether paclitaxel administration leads to augmented spinal concentrations of CCL2. A 90% increase in spinal CCL2 levels was found in mice treated with this antineoplastic drug. The functional involvement of spinal CCL2 in this type of cold hyperalgesia was confirmed by the complete inhibition obtained after the i.t. administration of an anti-CCL2 antibody at a dose similar than that previously used in other "in vivo" assays (Gao et al., 2010). Thus, our data strongly suggest that paclitaxel administration evokes an increase of CCL2 levels and that the binding of this chemokine to CCR2 leads to cold hyperalgesia. Based on the poor penetration through the blood-brain barrier of this antineoplastic (Glantz et al., 1995) it seems likely that, as occurs in other neuropathic processes, the injury produced by paclitaxel in peripheral nerves could trigger CCL2 release at the spinal cord.

Considering that the amplification of spinal nociceptive processing evoked by the CCL2/CCR2 system is often associated with glial activation (Calvo and Bennett, 2012; Schomberg and Olson, 2012), we have initially studied if glial immunostaining could be observed in the spinal cord after the administration of this antineoplastic drug. Although the activation of glial cells have been previously demonstrated in rats treated with paclitaxel, results are not completely uniform perhaps reflecting the heterogeneity of paclitaxel treatment schedules and the time elapsed after its administration. Peters et al. (2007) described that the administration of paclitaxel (2×18 mg/kg) evokes a clear-cut increase in microglial immunoreaction without significant astrocyte activation 10 days after paclitaxel administration. Further studies performed 4 weeks after the administration of paclitaxel described either the activation of microglial and astroglial cells after the administration of 4×1 mg/kg (Burgos et al., 2012; Naguib et al., 2012) or the exclusive activation of astroglia when administering 4×2 mg/kg (Zhang et al., 2012a). Thus, it has been previously suggested (Calvo and Bennett, 2012) that low doses of paclitaxel (4×2 mg/kg) did not evoke microglial activation (Zhang et al., 2012a; Zheng et al., 2011), whereas high doses evoke more intense staining (Peters et al., 2007).

Our results obtained in mice a single dose of paclitaxel (10 mg/kg) show a significant increase of Iba-1 but not of GFAP immunoreactivity 3 days after its administration. These data are rather similar to those reported by Peters et al. (2007), since we detect a significant increase in microglial activation and a non significant tendency towards the enhancement of GFAP immunoreactivity. However, in contrast with Peters et al. (2007), that found the greatest microglial activation in laminae III-VI, we have detected Iba-1 immunoreactivity preferentially at the superficial laminae of the dorsal horn. Although it could be initially thought that the absence of astroglial activation could be related to the early time at which we perform the study (3 days after administration), it has been previously shown in rats that GFAP expression can be already up-regulated in astrocytes 4 h after the administration of the first dose of paclitaxel (Zhang et al., 2012a).

The functional involvement of spinal microglial cell activation in paclitaxel-induced cold hyperalgesia is supported by the dose-dependent inhibition induced after the i.t. administration of the microglial inhibitor minocycline at doses previously shown to block hypernociception in mice with a neuropathic process (Narita et al., 2006). Previous reports have described the antinociceptive effect evoked by systemic minocycline in paclitaxel-treated rats and ascribed its antinociceptive effect to a peripheral immunomodulatory action of the drug (Cata et al., 2008; Liu et al., 2010). We show here that this microglial inhibitor can also exert direct spinal antinociceptive effects in mice treated with paclitaxel, as previously proposed in rats (Burgos et al., 2012).

Since CCL2 has been previously shown to induce the activation of microglia (Zhang et al., 2007; Thacker et al., 2009), and our immunostaining data demonstrated the activation of microglial cells in the superficial laminae of the spinal cord where the release of CCL2 has been described in other neuropathic settings (Jeon et al., 2009), we have studied if the effect of CCL2 on paclitaxel hyperalgesia could be related to microglial activation. With this aim, we have studied if the immunostaining of Iba-1 that appears in the superficial laminae of the spinal cord of paclitaxel-treated mice could be modified after the i.t. administration of an anti-CCL2 antibody. Although the antihyperalgesic effect evoked by the anti-CCL2 antibody was detected only 30 min after its i.t. administration, we have performed these immunohistochemical experiments after 4 repeated injections of this antibody for 3 days. The main reason to explain this difference is that, although the inhibition of microglial involvement in hyperalgesia occurs immediately after its neutralization with the anti-CCL2 antibody, it can be thought that maintained morphological alterations (cell proliferation or shape changes) that occur during microglial activation (Watkins et al., 2007) could be more permanent only disappearing after a sustained inhibition of CCL2. In these immunohistochemical experiments, we have observed a very important reduction of Iba-1 labelled surface measured in the superficial laminae of the spinal cord of paclitaxel-treated mice receiving the spinal administration of the anti-CCL2 antibody. Thus, when considering that the microglial inhibition evoked by minocycline abolished paclitaxel-induced cold hypernociception and that CCL2 neutralization prevents the microglial activation evoked by paclitaxel, it seems conceivable that the hyperalgesia evoked by this antineoplastic drug could be due to the spinal release of CCL2 and the subsequent activation of microglia.

Globally, our results suggest that acute paclitaxel can evoke in mice the release of CCL2 that, acting through CCR2, leads to the activation of microglia at the spinal cord, being this process crucial for the establishment of paclitaxel-evoked cold hypernociception. In accordance, it might be proposed that the modulation of the CCL2/CCR2 system could be a useful strategy to inhibit some symptoms related to paclitaxel-evoked neuropathy.

Conflict of interest

The authors state that they have no conflict of interest.

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3.4 Artículo 4:

Pevida M, Lastra A, Meana A, Hidalgo A, Baamonde A, Menéndez L. **The chemokine CCL5 induces CCR1-mediated hyperalgesia in mice inoculated with NCTC 2472 tumoral cells**. Neuroscience 259:113-125. 2013.

OBJETIVO

Ensayar la eficacia del bloqueo de receptores CCR1 para producir efectos antihiperalgésicos y antialodínicos en los dos modelos murinos de dolor neoplásico basados en la inoculación intratibial de células NCTC 2472 o de células B10-F10, tratando de relacionar los posibles efectos con variaciones en los niveles de las quimiocinas, CCL3 y CCL5, capaces de activar estos receptores.

MÉTODOS

- Ratones C3H/He inoculados con células NCTC 2472 y ratones C57BL/6 inoculados con células B16-F10 en la cavidad medular de la tibia.
- Test de la placa caliente unilateral (medida de la hiperalgesia térmica).
- Test de presión de la pata (modificación del test Randall-Selitto para la medida de la hiperalgesia mecánica).
- Test de Von-Frey (medida de la alodinia mecánica).
- ELISA (cuantificación de los niveles de la quimiocinas CCL3 y CCL5).

RESULTADOS Y CONCLUSIONES

- El bloqueo de receptores CCR1 periféricos inhibe la hiperalgesia mecánica o térmica pero no la alodinia de origen tumoral en ratones inoculados con células de fibrosarcoma NCTC 2472. Estos efectos no se observan en ratones inoculados con células B16-F10.
- La inoculación intratibial de células de fibrosarcoma NCTC 2472 pero no células B16-F10 provoca un incremento en los niveles de CCL5 a nivel tumoral pero no espinal. En contraste, el desarrollo tumoral no conduce a ninguna modificación de las concentraciones de CCL3.
- En ensayos de comportamiento, se observa que la administración de un anticuerpo anti-CCL5 a nivel tumoral evita la hiperalgesia tumoral evocada tras la inoculación intratibial de células NCTC 2472. Esta CCL5 aumentada participa en la hiperalgesia tumoral a través de la estimulación de los CCR1 ya que el bloqueo de otros receptores a los que se puede unir, CCR5, no evita este comportamiento nociceptivo.

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THE CHEMOKINE CCL5 INDUCES CCR1-MEDIATED HYPERALGESIA IN MICE INOCULATED WITH NCTC 2472 TUMORAL CELLS

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Abstract—Although the expression of the chemokine receptor CCR1 has been demonstrated in several structures related to nociception, supporting the nociceptive role of chemokines able to activate it, the involvement of CCR1 in neoplastic pain has not been previously assessed. We have assayed the effects of a CCR1 antagonist, J113863, in two murine models of neoplastic hyperalgesia based on the intratibial injection of either NCTC 2472 fibrosarcoma cells, able to induce osteolytic bone injury, or B16-F10 melanoma cells, associated to mixed osteolytic/osteoblastic bone pathological features. The systemic administration of J113863 inhibited thermal and mechanical hyperalgesia but not mechanical allodynia in mice inoculated with NCTC 2472 cells. Moreover, in these mice, thermal hyperalgesia was counteracted following the peritumoral (10–30 μg) but not spinal (3–5 μg) administration of J113863. In contrast, hyperalgesia and allodynia measured in mice inoculated with B16-F10 cells remained unaffected after the administration of J113863. The inoculation of tumoral cells did not modify the levels of CCL3 at tumor or spinal cord. In contrast, although the concentration of CCL5 remained unmodified in mice inoculated with B16-F10 cells, increased levels of this chemokine were measured in tumor-bearing limbs, but not the spinal cord, of mice inoculated with NCTC 2472 cells. Increased levels of CCL5 were also found following the incubation of NCTC 2472, but not B16-F10, cells in the corresponding culture medium. The intraplantar injection of CCL5 (0.5 ng) to naïve mice evoked thermal hyperalgesia prevented by the coadministration of J113863 or the CCR5 antagonist, D-Ala-peptide T-amide (DAPTA), demonstrating that CCL5 can induce thermal hyperalgesia in mice through the activation of CCR1 or CCR5. However, contrasting with the inhibitory effect evoked by J113863, the systemic administration of DAPTA did not prevent tumoral hyperalgesia. Finally, the peritumoral administration of an anti-CCL5 antibody completely inhibited thermal hyperalgesia evoked by the inoculation of NCTC 2472 cells.

Key words: bone cancer-induced pain, mouse, hyperalgesia, CCR1, CCL5, J113863.

INTRODUCTION

Besides the well-characterized role played by several chemokines on the activation and recruitment of leukocytes (Blanchet et al., 2012), there is also a large body of evidence showing their implication in the modulation of nociceptive processes, especially in neuropathic pain (Gosselin et al., 2008; Abbadie et al., 2009; White and Miller, 2010; Kiguchi et al., 2012). Accordingly, several chemokines, such as CCL2 (chemokine (C-C motif) ligand 2) , CCL3, CCL5 (chemokine (C-C motif) ligand 5), CXCL12 or CX3CL1 and the receptors where they bind to, are expressed at peripheral or central sites of the nervous system relevant for the transmission of nociceptive signals (Bhangoo et al., 2007; Old and Malcangio, 2012; Kiguchi et al., 2012; Réaux-Le Goazigo et al., 2013). The two chemokines for which the involvement in nociceptive processing is more clearly established are CCL2 (MCP-1, monocyte chemotactic protein-1) and CX3CL1 (fractalkine), which mainly act through their respective receptors CCR2 and CX3CR1. Previous reports describe that the blockade of these receptors can inhibit nociceptive behaviors measured in experimental models of pathological pain, such as inflammatory (Sun et al., 2007; Arms et al., 2013), neuropathic (Milligan et al., 2004; Thacker et al., 2009; Staniland et al., 2010; Pevida et al., 2013) or neoplastic (Khasabova et al., 2007; Pevida et al., 2012; Hu et al., 2012a,b, 2013).

Apart from CCR2 and CX3CR1, CCR1 (C-C chemokine receptor type 1) is another type of chemokine receptor whose profile supports its involvement in nociceptive modulation. This receptor is expressed in the dorsal root ganglia (DRG) (Zhang et al., 2005) and the spinal cord of rodents (Rajan et al., 2000; di Prisco et al., 2012) and is up-regulated in response to nerve (Kiguchi et al., 2010a) or spinal cord (Knerlich-Lukoschus et al., 2011b) injury. In addition, the involvement in nociceptive processing of chemokines

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Abbreviations: ANOVA, analysis of variance; CCL2, chemokine (C-C motif) ligand 2; CCL5, chemokine (C-C motif) ligand 5; CCR1, C-C chemokine receptor type 1; CCR5, C-C chemokine receptor type 5; DAPTA, D-Ala-peptide T-amide; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglia; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; MIP-1α, macrophage inhibitory protein-1α; PBS, phosphate-buffered saline; RANTES, regulated on activation normal T cell expressed and secreted; TRPV1, transient receptor potential vanilloid 1.

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that can activate CCR1 has also been described (Zhang et al., 2005; Eijkelkamp et al., 2010; Kiguchi et al., 2010a,b). As occurs with most chemokine receptors, several chemokines, such as CCL3 (macrophage inhibitory protein-1α, MIP-1α), CCL5 (regulated on activation normal T cell expressed and secreted, RANTES), CCL7, CCL8, CCL14, CCL15, CCL16 or CCL23 (Sarau et al., 1997; Saeki and Naya, 2003; Cheng and Jack, 2008) can bind to CCR1. Among them, only three molecules, namely CCL3, CCL5 and CCL7, have been related to pain modulation. However, considering that the report describing a hyperalgesic role for CCL7 is related to the activation of CCR2 (Imai et al., 2013), it might be proposed that CCL3 and CCL5 are the more likely candidates to participate in nociceptive processing through the activation of CCR1.

CCL3 is expressed in DRG neurons (Zhang et al., 2005), and its mRNA is detected at the spinal cord (Kiguchi et al., 2010a). Behaviorally, its direct administration either at periphery (Zhang et al., 2005; Eijkelkamp et al., 2010; Kiguchi et al., 2010b) or the spinal cord (Kiguchi et al., 2010a) evokes nociceptive reactions. Supporting its involvement in pathological pain, CCL3 expression is increased at DRG (Kim et al., 2011) and at the spinal cord of rodents with nerve or spinal injury (Kiguchi et al., 2010a; Knerlich-Lukoschus et al., 2011a,b). Moreover, the administration of an anti-CCL3 antibody can counteract neuropathic hypernociception (Kiguchi et al., 2010a).

Related to CCL5, several data support its involvement in nociception. Thus, CCL5 is expressed in DRG neurons (Bolin et al., 1998), where it induces transient intracellular calcium increases (Bolin et al., 1998; Oh et al., 2001; Bhangoo et al., 2007) and, behaviorally, it evokes hypernociceptive reactions after intradermal injection in rats (Oh et al., 2001). Furthermore, CCL5 mRNA has been detected in the spinal cord (Bolin et al., 1998; Jones et al., 2005), a level at which this chemokine can enhance glutamate exocytosis (di Prisco et al., 2012). At supraspinal structures, the intracisternal administration (Ahn et al., 2005) or the microinjection into the periaqueductal gray (Benamar et al., 2008) of CCL5 evokes hypernociceptive effects. Finally, it has been recently shown that neuropathic hypersensitivity diminishes in mice deficient in CCL5 (Liou et al., 2012) and that spinal CCL5 is involved in the development of mechanical hyperalgesia in rats intratibially inoculated with tumoral cells (Hang et al., 2013).

Based on these data supporting the involvement in neuropathic hypernociception of CCR1 or of some endogenous chemokine with high affinity for this receptor, we have considered the possibility that CCR1 could also participate in tumoral hyperalgesia. With this aim, we have assayed the effect of a selective CCR1 antagonist, J113863 (Naya et al., 2001), on the hyperalgesia evoked after the intratibial inoculation of NCTC 2472 fibrosarcoma or B16-F10 melanoma cells, two tumoral cell lines that induce, respectively, either osteoclastic (Schwei et al., 1999; Menéndez et al., 2003) or mixed osteoblastic/osteoclastic (Curto-Reyes et al., 2008) bone tumors in mice. Once demonstrated that the administration of J113863 can inhibit the hyperalgesia evoked by NCTC 2472 cells, we have intended to elucidate whether CCL3 or CCL5 were involved in CCR1 activation in this particular setting.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed in 5–6 week-old C57BL/6 and C3H/He male mice bred in the Animalario de la Universidad de Oviedo (Reg. 33044 13A), maintained on a 12-h dark–light cycle with free access to food and water. Experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain) and performed according to the European Commission Directive of 22 September 2010 (2010/63/EU). Each animal was used only once.

Cell inoculation

NCTC 2472 cells (American Type Culture Collection, ATCC) were cultured in NCTC 135 medium (Sigma) containing 10% horse serum (Sigma), passaged weekly according to ATCC guidelines. When cells were confluent, they were detached by scraping, centrifuged at 400*g* for 10 min and the remaining pellet suspended in phosphate-buffered saline (PBS) (Menéndez et al., 2003). B16-F10 melanoma cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, England) enriched with 10% fetal calf serum (FCS, Gibco). When cells were preconfluent, they were treated with trypsin/EDTA (0.05%/ 0.02%) and detached. The trypsin/EDTA solution was recovered, neutralized with DMEM supplemented with 10% FCS and centrifuged at 400*g* for 10 min (Curto-Reyes et al., 2008).

For surgical procedures, anesthesia was induced by spontaneous inhalation of 3% isoflurane (Isoflo®, Esteve) and maintained by administering 1.5% isoflurane in oxygen through a breathing mask. A suspension of 10^5 cells in 5 μl of PBS was injected into the right tibial medullar cavity and after applying acrylic glue (Hystoacril®, Braun, Tuttlingen, Germany) on the tibial plateau incised area, surgery was finished with a stitch of the skin. Control mice received the inoculation of 10^5 cells previously killed by quickly freezing them three times without cryoprotection.

We have previously characterized the time course of the instauration of different nociceptive parameters in mice receiving the intratibial inoculation of NCTC 2472 or B16-F10 cells. In these studies, we have observed that thermal hyperalgesia appears only 4 weeks after the intratibial inoculation of NCTC 2472 cells following an early period of opioid-mediated thermal hypoalgesia (Menéndez et al., 2003). In contrast, mechanical allodynia can be measured by von Frey filaments 2 weeks after inoculation (Baamonde et al., 2006) and mechanical hyperalgesia can be detected by a paw

pressure test from the third week after inoculation (Menéndez et al., 2005). Related to B16-F10 cells both thermal and mechanical hyperalgesia as well as mechanical allodynia can be already measured 1 week after cell inoculation (Curto-Reyes et al., 2008).

Based on these data, mechanical allodynia was assessed 2 weeks after inoculation of NCTC 2472 cells, and thermal or mechanical hyperalgesia 4 weeks after cell inoculation. In contrast, both hyperalgesia and allodynia were measured 1 week after B16-F10 cell inoculation.

Unilateral hot plate test

As previously described (Menéndez et al., 2002), mice were gently restrained and the plantar side of the tested paw placed on a hot plate (IITC Life Science, Woodland Hills, California, USA). The temperature of the plate was 49.8 °C for C3H/He mice and 48.5 °C for C57-BL/6 mice. Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at 2-min-intervals and the mean of two measures was considered. A cut-off of 20 s was established. All the behavioral experiments were performed between 15:00 and 20:00 in a thermostatized (21 °C) and noise-isolated room.

Paw pressure test

Mechanical withdrawal latencies were measured by a previously described adaptation of the Randall–Selitto method (Menéndez et al., 2005), in which a constant pressure stimulus is used. Mice were gently restrained and a pressure of 450 g was applied to their hindpaws with a Ugo Basile 7200 apparatus, until a struggle reaction appears. The measurements of the withdrawal latencies of each hindpaw were made separately and alternately at 2-min intervals and the mean of two measures made in each hindpaw was considered. A 60-s cut-off was established in order to prevent tissue damage.

von Frey test

Mechanical allodynia was assessed by applying von Frey filaments (Stoelting) to the plantar side of the paws as previously reported (Baamonde et al., 2006). Mice were placed on a wire mesh platform and 20 min were allowed for habituation. The von Frey filaments 2.44, 2.83, 3.22, 3.61, 4.08, 4.56 were used and, starting with the 3.61 filament, six measurements were taken in each animal randomly starting with the left or right paw. Based on the "up and down" method (Chaplan et al., 1994), the observation of a positive response (lifting, shaking or licking of the paw) was followed by the application of the immediate thinner filament or the immediate thicker one if the response was negative. The 50% response threshold was calculated using the following formula: 50% g threshold = $(10^{Xf+\kappa\delta})/10{,}000$; where Xf is the value of the last von Frey filament applied; κ is a correction factor based on pattern of responses (from the Dixon's calibration table); δ is the mean distance in log units between stimuli (here, 0.4).

Drugs and drug administration

The CCR1 antagonist, J113863 (Tocris, Bristol, England) was initially dissolved in 100% DMSO in a stock solution of 33.33 mg/ml and further dilutions were done in distilled water. The drug or its solvent was subcutaneously (s.c.), peritumorally, intraplantarly (i.pl.) or intrathecally (i.t.) administered. The CCR5 (C-C chemokine receptor type 5) antagonist, DAPTA (D-Ala-peptide T-amide, Tocris) was dissolved in distilled water and administered s.c. and i.pl. A rat anti-mouse CCL5 antibody (R&D) or the same quantity of IgG from rat serum (Sigma, St. Louis, Missouri, USA) was peritumorally administered diluted in saline. CCL5 (Prospec, Ness Ziona, Israel) diluted in saline was intraplantarly administered with J113863, DAPTA or their respective solvents.

The s.c. administration was performed under the fur of the neck in a volume of 10 ml/kg. For peritumoral administration, drugs were injected subcutaneously over the tibial tumor in a volume of 100 µl and injections in the contralateral paw (left paw) were performed in the same area of the limb but, in this case, without tumor mass. Intraplantar (i.pl.) and intrathecal (i.t.) injections were performed in mice anesthetized by spontaneous inhalation of 3% isoflurane (Isoflo®, Esteve, Maidenhead, Berkshire, England). I.pl. administrations consisted in the injection of 25 µl into the plantar side of the right hind paw. For i.t. injections, a small cut in the skin at the dorsal lumbar level was done, the tip of a 30-gauge needle inserted in a Hamilton microsyringe was introduced at the level of L_5–L_6 and, finally, a volume of 5 µl was injected (Gonzalez-Rodriguez et al., 2012). In all cases, drugs were administered 30 min before testing.

Enzyme-linked immunosorbent assay (ELISA)

CCL3 and CCL5 levels were measured in NCTC 2472 and B16-F10 cell culture medium before and after the incubation of cells during 4 days in flasks with 15 ml, when cells reached confluence. Experiments were also performed in tissue homogenates prepared from hind limbs ipsilateral or contralateral to the inoculated paw and in tissue homogenates coming from the spinal cord of mice receiving live or killed tumoral cells. Tissues were obtained at the times at which behavioral experiments were performed, that is, 4 weeks after the inoculation of NCTC 2472 cells and 1 week after inoculation of B16-F10 cells. Once harvested, media and tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use.

The ankle to knee denuded hind limbs, including bone and soft tissue as well as L_3–L_5 spinal cord segments were harvested and homogenized in a buffer consisting in 0.1 M Tris, 0.15 M NaCl, 0.5% CTAB (Fluka, Steinheim, Germany) and a protease inhibitor (1 tablet/50 ml buffer, Roche Diagnostics, Indianapolis, Indiana, USA). Limbs were homogenized in a volume of 3 µl/mg

with a Polytron PT-MR3100 (Kinematica, Littau, Switzerland) and spinal cord segments in a volume of 6 μl/mg by using a Minicraft MB730®. Next, homogenates were centrifuged at 15,000*g* for 15 min at 4 °C. Protein concentration of supernatants was measured by a BCA protein assay (Pierce) by using a spectrophotometer (Nanodrop 2000C, Thermoscientific, Wilmington, USA) according to the manufacturer's protocol.

Levels of CCL3 and CCL5 were measured with commercially available sandwich ELISAs (R&D, DuoSet® Mouse CCL3/MIP-1α and CCL5/RANTES, respectively). Following the instructions of the manufacturer, plates (R&D) were coated overnight at room temperature with an antibody specific for mouse CCL3 or CCL5. A volume of 100 μl of sample homogenates or of culture media were added into wells. In order to achieve a value in the range of the standard CCL3 or CCL5 curves, protein quantities added to the wells for the homogenates were 100 μg in all cases, either limbs or spinal cord segments. After washing, a 2-h incubation period was performed with a second biotinylated anti-mouse CCL3 or anti-mouse CCL5 antibody and followed by a 20-min incubation period with streptavidin–peroxidase (HRP). After washing to remove all the unbound enzyme, color was developed by adding a stabilized chromogen (tetramethylbenzidine: H_2O_2, 1:1) and the reaction was terminated with a stop solution (2 N H_2SO_4). The intensity of the colored product was quantified spectrophotometrically at 450 nm substracting the readings obtained at 570 nm in order to correct optical background of the plates.

Values obtained from culture media, limbs and spinal cords came from at least four independent samples performed in duplicate.

Statistical analysis

The mean values and the corresponding standard errors were calculated for each ELISA measurement or behavioral assay. The values of the levels of CCL3 and CCL5 obtained in culture media and spinal cord homogenates were compared by a Student's *t* test and those obtained from hind limb homogenates were compared by an initial one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Thermal and mechanical withdrawal latencies were compared by the Student's *t* test for grouped values when the latency obtained in the ipsilateral paw was compared with that obtained in the contralateral one. For the comparisons of thermal and mechanical withdrawal latencies among different groups, an initial one-way ANOVA was performed and was followed either by the Newman–Keuls test when groups received different treatments or by the Dunnett's *t* when groups that received different doses of J113863 or of CCL5 anti-mouse antibody were compared with the solvent-treated one. Threshold values obtained by the von Frey test in ipsilateral and contralateral paws were compared by the *U* Mann–Whitney's test. In all cases, the level of significance was set at $P < 0.05$.

RESULTS

The CCR1 antagonist J113863 inhibits thermal hyperalgesia evoked by the intratibial inoculation of NCTC 2472 cells acting at tumoral level

Thermal hyperalgesia measured 4 weeks after the intratibial inoculation of NCTC 2472 cells was dose-dependently inhibited 30 min after the s.c. administration of the selective CCR1 antagonist J113863 (3–30 mg/kg). The dose of 3 mg/kg did not modify thermal withdrawal latencies, a partial antihyperalgesic effect was observed after the administration of 10 mg/kg and a complete inhibition of tumoral hyperalgesia was attained when 30 mg/kg of J113863 were administered. The administration of the highest dose did not modify thermal latencies in mice inoculated with killed NCTC 2472 cells (Fig. 1A).

In order to elucidate whether the analgesic effect evoked by J113863 in mice inoculated with NCTC 2472 cells could occur on CCR1 receptors located at tumoral or spinal level, the antagonist was locally administered in the vicinity of the tumor or into the spinal cord. A dose-dependent antihyperalgesic effect was again observed 30 min after the peritumoral injection of 10–30 μg of J113863 (Fig. 1B) and, supporting the involvement of local mechanisms, the administration of 30 μg of J113863 in the contralateral paw did not modify thermal hyperalgesia in tumor-bearing mice. In addition, the administration of the highest dose of the antagonist did not modify thermal latencies in mice inoculated with killed NCTC 2472 cells (data not shown).

Contrasting with this peripheral antihyperalgesic effect, the i.t. administration of J113863 (3–5 μg) did not modify thermal withdrawal latencies in mice inoculated with NCTC 2472 cells (Fig. 1C). The effect induced by greater doses was not tested due to the appearance of motor side effects. Paralysis of both hind limbs was observed for more than 30 min in four out of six mice after the i.t. administration of 10 μg of J113863, making impossible the measurement of paw withdrawal latencies. Some mice receiving the i.t. administration of 5 μg of J113863 showed muscular flaccidity of the hind quarters that usually lasted 10–15 min, being the majority of mice completely recovered at testing time (30 min after administration). Only one mouse treated with 5 μg of J113863, that showed persistent flaccidity at this time, was excluded from the study.

Lack of effect of the CCR1 antagonist J113863 on thermal hyperalgesia evoked by the intratibial inoculation of B16-F10 cells

In contrast with the antihyperalgesic effect evoked by J113863 in mice inoculated with NCTC 2472 cells, thermal hyperalgesia measured 1 week after the intratibial inoculation of B16-F10 melanoma cells to C57BL/6 mice was not modified 30 min after the s.c. administration of 30 mg/kg (Fig. 2A), the peritumoral administration of 30 μg (Fig. 2B) or the i.t. administration of 5 μg of J113863 (Fig. 2C). As before, the effect of the i.t. administration of 10 μg of J113863

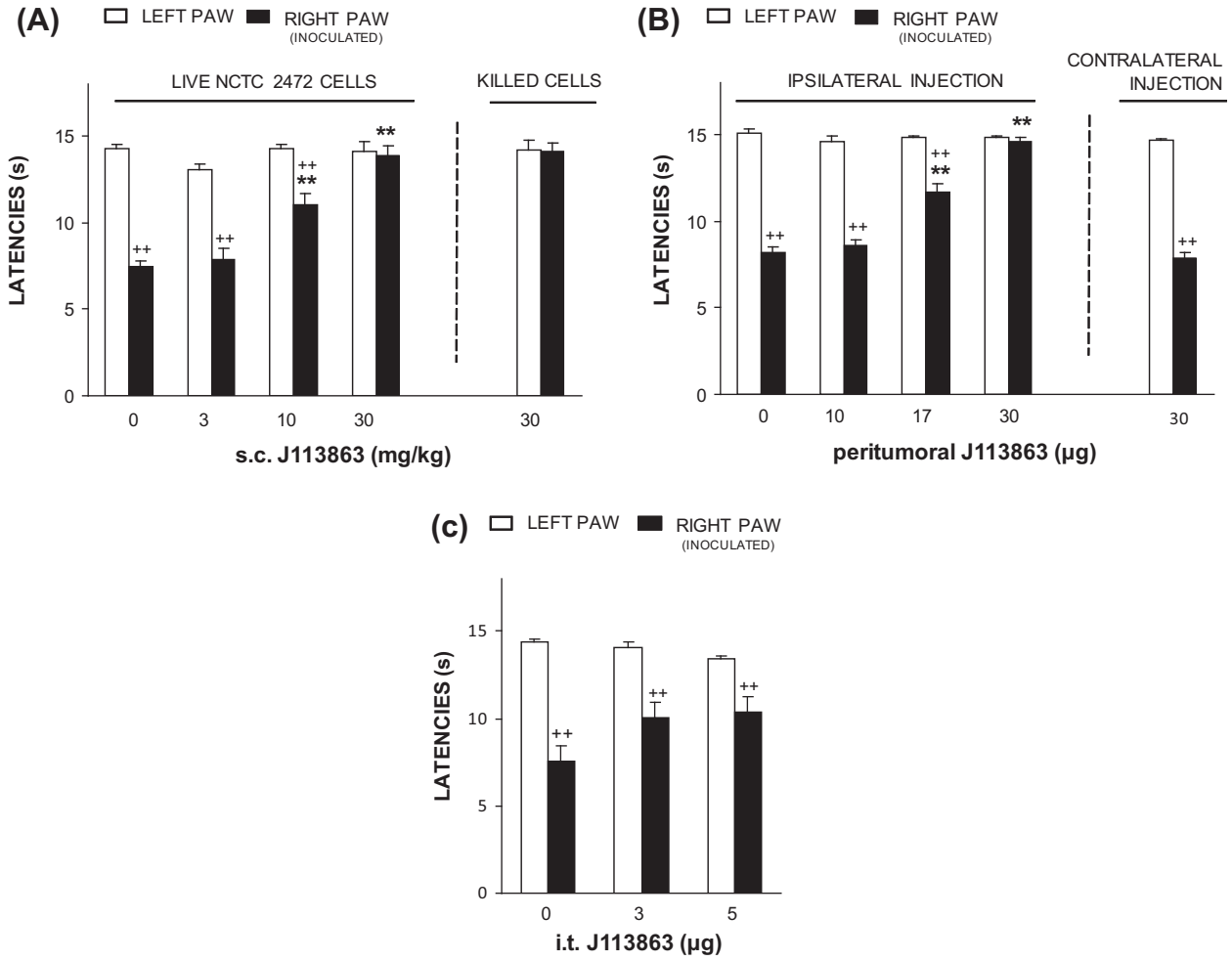

Fig. 1. (A) Inhibition of thermal hyperalgesia evoked by NCTC 2472 cells after the s.c. administration of J1138633 (3–30 mg/kg). The lack of effect on the withdrawal latencies of the maximal dose administered to mice inoculated with killed tumoral cells is represented at the right hand side of the graph. (B) Inhibition of thermal hyperalgesia evoked by NCTC 2472 cells after the peritumoral administration of J1138633 (10–30 µg). The lack of effect of the maximal dose assayed when it is administered in the contralateral paw is represented at the right hand side of the graph. (C) The i.t administration of J1138633 (3–5 µg) to mice inoculated with live NCTC 2472 cells did not modify tumoral hyperalgesia. Higher i.t. doses of J1138633 were not tested due to the appearance of motor side effects. Means (n = 5–6) and their S.E.M. are represented. $^{++}P < 0.01$ compared with its corresponding, contralateral, left paw, Student's t test; $^{**}P < 0.01$ compared with solvent-treated group, Dunnett's t test.

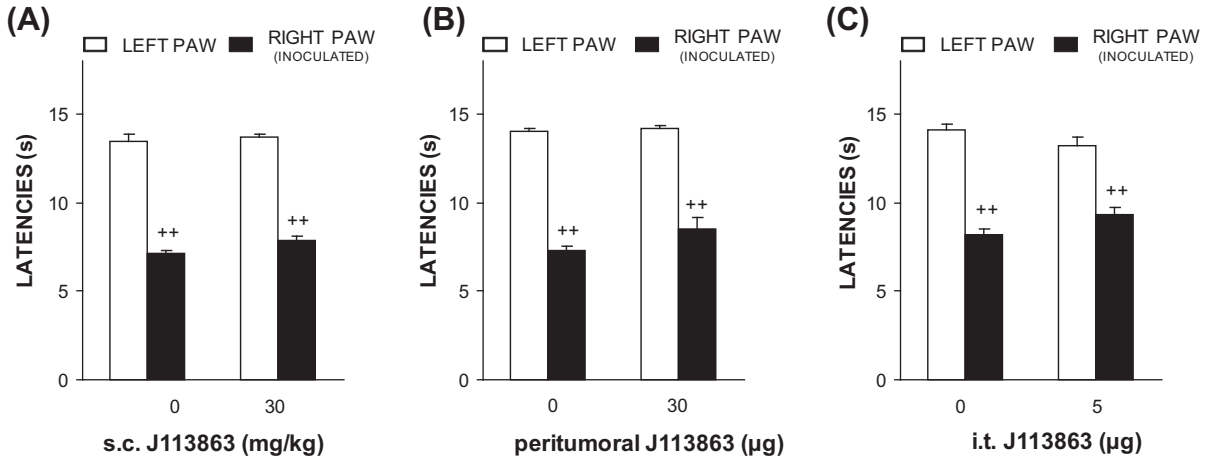

Fig. 2. Lack of effect of the s.c. (A; 30 mg/kg), peritumoral (B; 30 µg) or i.t. (C; 5 µg) administration of J1138633 on thermal hyperalgesia evoked in mice intratibially inoculated with B16-F10 cells. Means (n = 5–6) and their S.E.M. are represented. $^{++}P < 0.01$ compared with its corresponding, contralateral, left paw, Student's t test.

was not tested because this dose induced paralysis of hind limbs.

Effects of the systemic administration of the CCR1 antagonist J113863 on the mechanical hyperalgesia or allodynia evoked by the intratibial inoculation of NCTC 2472 or B16-F10 cells

Once established that the blockade of CCR1 inhibits thermal hyperalgesia evoked by the inoculation of NCTC 2472, but not B16-F10 cells, we have explored if the administration of J113863 can also inhibit mechanical hypernociception.

Mechanical hyperalgesia measured by the paw pressure test 4 weeks after the intratibial inoculation of NCTC 2472 cells was completely inhibited 30 min after the administration of 30 mg/kg of J113863 (Fig. 3A), the same dose that evoked a complete inhibition of thermal hyperalgesia in these mice. Contrasting with thermal or mechanical hyperalgesia, mechanical allodynia measured by the von Frey test 2 weeks after NCTC 2472 cell inoculation remained unaffected by the administration of the same dose of the CCR1 antagonist J113863 (Fig. 3B).

As occurred with thermal hyperalgesia, both mechanical hyperalgesia and mechanical allodynia evoked by the intratibial inoculation of B16-F10 cells were unaffected by the administration of 30 mg/kg of the CCR1 antagonists J113863 (Fig. 3C, D).

CCL3 concentrations are unaltered at tumoral or spinal level after the intratibial inoculation of NCTC 2472 or B16-F10 cells

CCL3 concentrations were measured by ELISA in homogenates prepared from the tibial region or the lumbar segments of the spinal cord of mice inoculated with either live or killed NCTC 2472 cells. The levels of CCL3 found in the tibial region of tumor-bearing paws were undistinguishable from those measured in their contralateral paws or in the paws of mice inoculated with killed cells (Fig. 4A). Furthermore, no change of CCL3 concentrations was detected in homogenates prepared from lumbar spinal cord segments of mice inoculated with killed or live NCTC 2472 cells (Fig. 4B).

Similar CCL3 amounts were also found when ELISA measurements were performed in homogenates coming from the tibial region (Fig. 4A) or the spinal cord

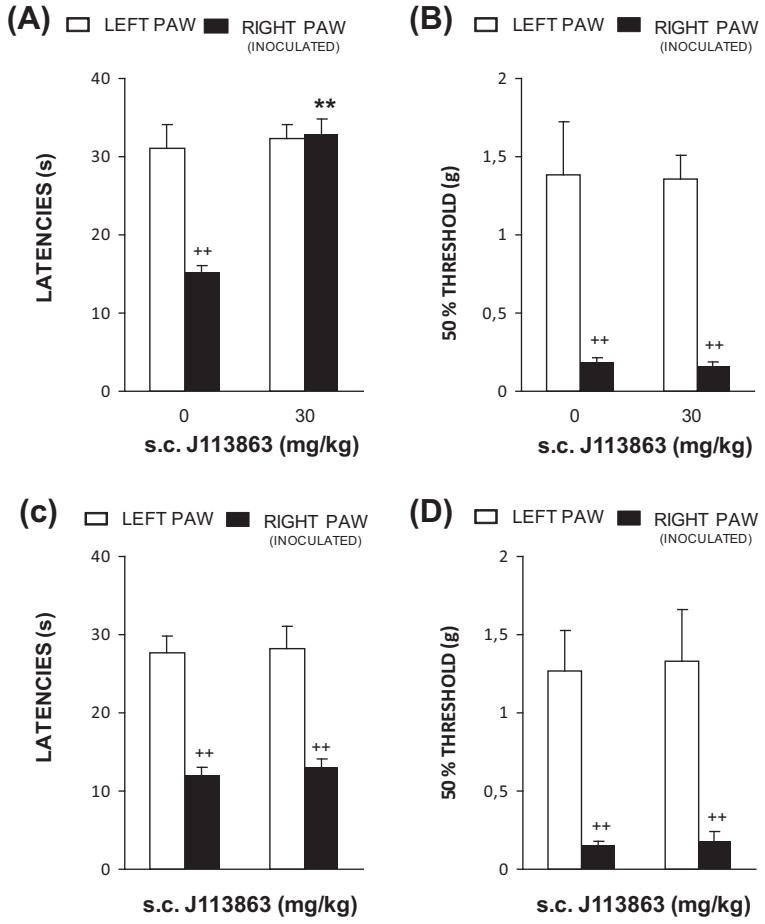

Fig. 3. Effect of the s.c. administration of J1138633 (30 mg/kg) on the mechanical hyperalgesia (A, C) or allodynia (B, D) measured in mice inoculated with NCTC 2472 cells after (A, B) and in mice intratibially inoculated with B16-F10 cells (C, D). Means (n = 5–6) and their S.E.M. are represented. $^{++}P < 0.01$ compared with its corresponding, contralateral, left paw, Student's t test (paw withdrawal latency) or U Mann–Whitney (50% threshold). $^{**}P < 0.01$ compared with solvent-treated group, Newman–Keuls test.

(Fig. 4B) of C57BL/6 mice inoculated with either killed or live B16-F10 cells.

When CCL3 concentrations were determined in the culture media used for NCTC 2472 or B16-F10 cells either before cell culture or after cells reached confluence, similar levels of CCL3 were found (Fig. 4C), suggesting that neither NCTC 2472 nor B16-F10 cells can release detectable amounts of CCL3 in culture.

CCL5 concentrations are increased at tumoral level after the intratibial inoculation of NCTC 2472 but not B16-F10 cells

Since our previous results suggested that CCR1 expressed at tumoral level are involved in the hyperalgesia measured in mice inoculated with NCTC 2472 cells and that CCL3 is not the endogenous agonist implicated, we have explored the possible involvement of CCL5, another endogenous chemokine that shows a high affinity for CCR1. CCL5 concentrations were measured by ELISA in homogenates of the tibial region obtained from the hind paws of C3H/He mice inoculated with either live or killed NCTC 2472 cells. Whereas similar CCL5 levels were obtained in homogenates prepared from both limbs of mice inoculated with killed cells, the concentration of CCL5 measured in homogenates coming from paws of mice inoculated with NCTC 2472 cells was about two times higher than those obtained in their contralateral paws (Fig. 5A). In contrast, similar CCL5 levels were obtained in homogenates prepared from lumbar spinal cord segments obtained from mice inoculated with killed or live cells (Fig. 5B).

The determination of CCL5 in the culture medium of NCTC 2472 cells revealed a significant increase after the presence of NCTC 2472 cells, thus indicating that NCTC 2472 cells in culture can release significant amounts of CCL5 (Fig. 5C).

Contrasting with the results obtained in mice receiving NCTC 2472 cells, no change in CCL5 levels were found in homogenates prepared from the limbs (Fig. 6A) or the lumbar spinal cord (Fig. 6B) of mice inoculated with live or killed B16-F10 cells. Moreover, although the concentration of CCL5 detected in the B16-F10 culture medium in the absence of tumoral cells was higher than the one measured in the NCTC 2472 medium, this concentration was not increased after the culture of B16-F10 cells (Fig. 6C).

CCL5 evokes peripheral hyperalgesia in mice inoculated with NCTC 2472 cells

Initially, in order to determine the possible hyperalgesic role played by CCL5 at peripheral level, a single dose of this chemokine was intraplantarly administered to naïve C3H/He mice. Thermal hyperalgesia was detected 30 min after the intraplantar administration of 0.5 ng of CCL5 (Fig. 7A). We have further assayed the effects on CCL5-evoked hyperalgesia of the blockade of two of the main receptors that can be activated by CCL5, such as CCR1 and CCR5. Either the coadministration of 1 μg of the CCR1 antagonist J113863 or of the CCR5 antagonist DAPTA together with CCL5 completely inhibited the antihyperalgesic effect evoked by this chemokine. The administration of the same doses of J113863 or DAPTA alone did not modify thermal withdrawal latencies (Fig. 7A).

Next, to ascertain whether CCL5 could participate in tumoral hyperalgesia, an anti-CCL5 antibody was peritumorally administered to mice inoculated with NCTC 2472 cells. The administration of the anti-CCL5 antibody (3–10 μg) dose-dependently inhibited thermal hyperalgesia in these mice (Fig. 7B). Withdrawal latencies remained unmodified after the administration of the same amount of IgG from rat serum to mice inoculated with live NCTC 2472 cells (not shown) or of the highest dose of anti-CCL5 antibody (10 μg) to mice inoculated with killed NCTC 2472 cells (Fig. 7B).

Overall, our results indicated that CCL5 can evoke hyperalgesia in naïve mice by activating either CCR1 or CCR5. Related to thermal hyperalgesia measured in mice inoculated with NCTC 2472 cells, data obtained

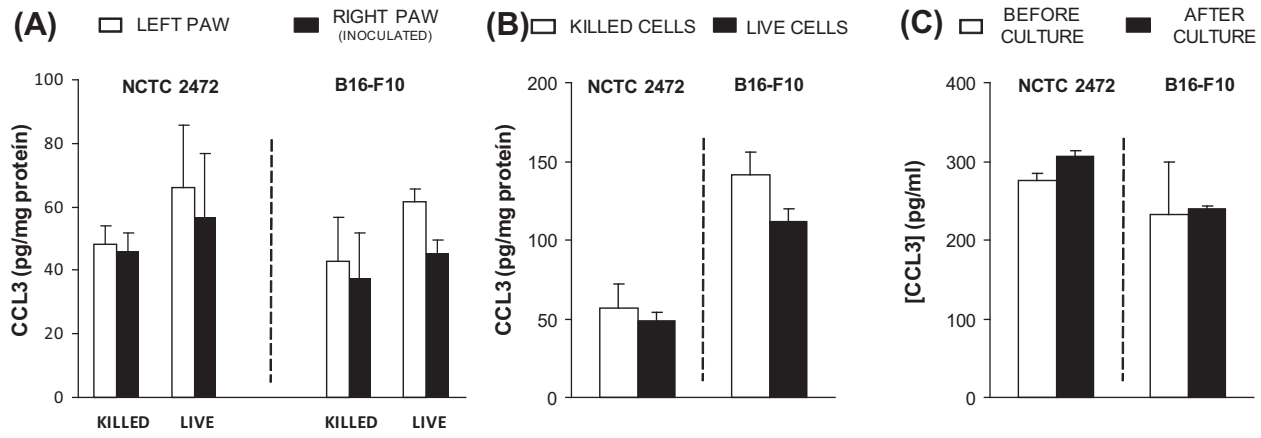

Fig. 4. The intratibial inoculation of either killed or live NCTC 2472 or B16-F10 cells did not induce significant changes in the concentration of CCL3 measured by ELISA in homogenates of the tibial region (A; $n = 9$) or the lumbar spinal cord (B; $n = 4$). No increase in CCL3 concentration was measured in NCTC 2472 and B16-F10 culture media in the absence of cells and after cell confluence (C; $n = 5$).

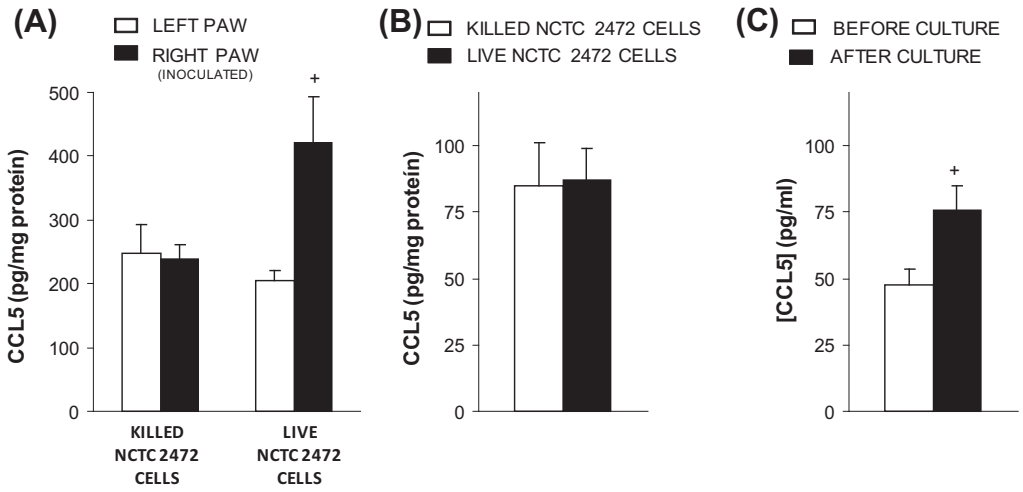

Fig. 5. The concentration of CCL5 measured by ELISA was increased in homogenates of the tibial region (A; $n = 4$), but not the lumbar spinal cord (B; $n = 4$) of mice inoculated with live NCTC 2472 cells. A significant increase of CCL5 concentration was also detected when comparing the values obtained in the NCTC 2472 culture medium in the absence of NCTC 2472 cells and after their confluence (C; $n = 5$). Means and their S.E.M. are represented. $^{+}P < 0.05$, compared with its corresponding, contralateral, left paw or control group, Student's t test.

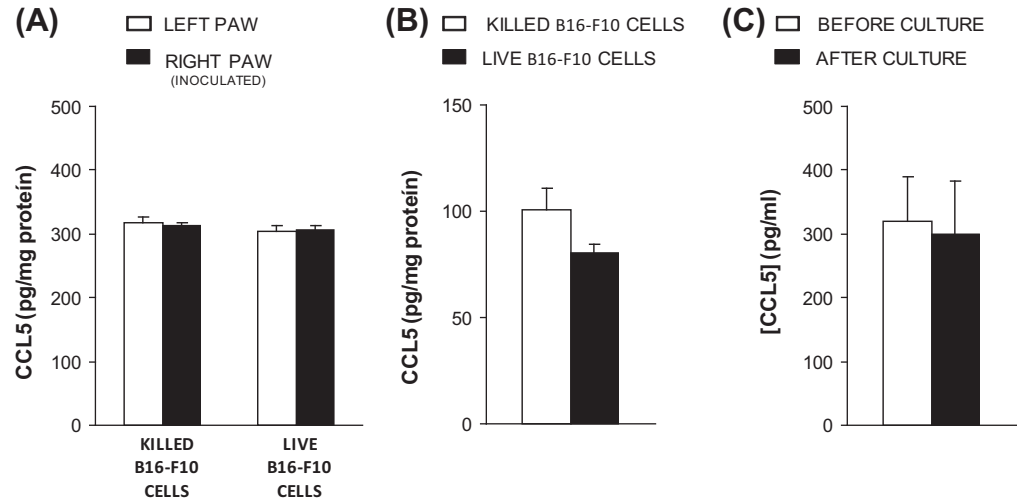

Fig. 6. The intratibial inoculation of B16-F10 cells did not induce significant changes in the concentrations of CCL5 measured by ELISA in homogenates of the tibial region (A; $n = 4$) or the lumbar spinal cord (B; $n = 4$). No increase in CCL5 concentration was detected in the culture media of B16-F10 cells when comparing the values obtained in the absence of cells and after cell confluence (C; $n = 5$).

showed the involvement of CCR1 and the endogenous chemokine CCL5. Thus, in order to complete this view, we have assayed the effect of the CCR5 antagonist, DAPTA, in osteosarcoma-evoked thermal hyperalgesia. As shown in Fig. 7C, the administration of 1 mg/kg of DAPTA did not modify NCTC 2472-evoked thermal hyperalgesia, suggesting that this type of tumoral hypernociception is independent on CCR5 stimulation.

DISCUSSION

The present experiments were designed to investigate the involvement of the chemokine receptor CCR1 in the bone cancer-induced hypernociception in mice. We have initially assessed the effect of the CCR1 antagonist J113863 administered systemically, intrathecally or peritumorally, on the thermal hyperalgesia evoked in mice after the intratibial inoculation of either NCTC 2472 fibrosarcoma cells (Schwei et al., 1999; Menéndez et al., 2003) or B16-F10 melanoma cells (Curto-Reyes et al., 2008). Thermal hyperalgesia measured in C3H/He mice 4 weeks after the inoculation of NCTC 2472 cells was inhibited by the s.c. administration of J113863. This effect was reproduced after the injection of small doses of J113863 in the vicinity of the tumor, suggesting that CCR1 present at the tumoral environment can counteract neoplastic hyperalgesia. Furthermore, the administration of J113863 also inhibited mechanical hyperalgesia, but not mechanical allodynia, evoked by the inoculation of NCTC 2472 cells. In contrast with the antihyperalgesic

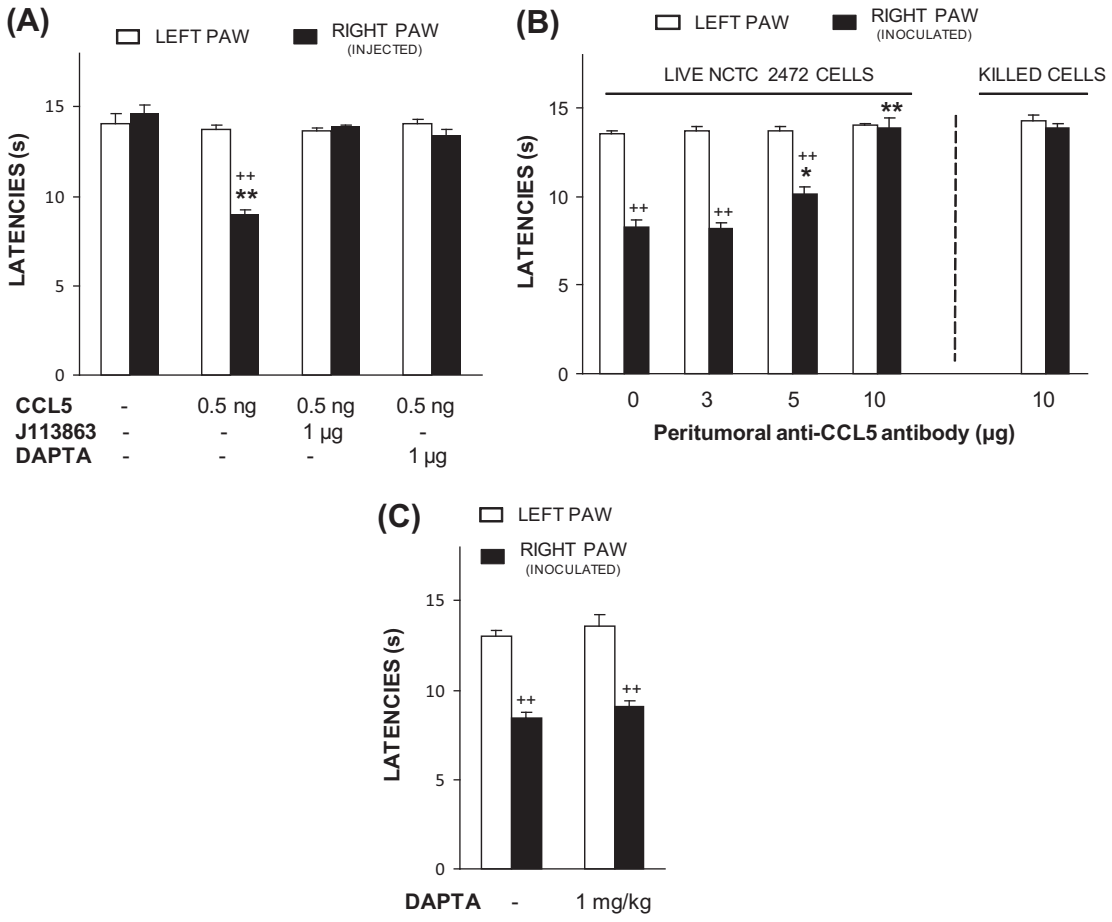

Fig. 7. (A) Thermal hyperalgesia evoked 30 min after the intraplantar injection of 0.5 ng of CCL5 into the right hind paw of naive C3H/He mice and inhibition by the coadministration of 1 µg of the CCR1 antagonist J1138633 or 1 µg of the CCR5 antagonist DAPTA ($n = 5$). Means and their S.E.M. are represented. $^{++}P < 0.01$, compared with its corresponding left paw, Student's t test. $^{**}P < 0.01$ compared with solvent-treated group, Newman–Keuls test. (B) Inhibition of thermal hyperalgesia measured in mice intratibially inoculated with NCTC 2472 cells after the peritumoral administration of an anti-CCL5 antibody (3–10 µg). The lack of effect on the withdrawal latencies of the maximal dose administered to mice inoculated with killed tumoral cells is shown at the right hand side of the graph. Means ($n = 5$) and their S.E.M. are represented. $^{++}P < 0.01$ compared with its corresponding left paw, Student's t test; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with solvent-treated group, Dunnett's t test. (C) Lack of effect of the s.c. administration of the CCR5 antagonist DAPTA (1 mg/kg) on thermal hyperalgesia measured in mice intratibially inoculated with NCTC 2472 cells ($n = 5$). $^{++}P < 0.01$, compared with its corresponding left paw, Student's t test.

effect evoked by the CCR1 antagonist in mice inoculated with NCTC 2472 cells, the lack of antihyperalgesic or antiallodynic effect of J113863 in mice inoculated with B16-F10 cells seems to indicate that the participation of CCR1 agonists is not a general rule for tumoral pain and depends on the type of tumor studied.

The analgesic effect derived from the inhibition of peripheral CCR1 has been previously demonstrated by the perineural administration of siRNA against CCR1 in mice with sciatic nerve injury (Kiguchi et al., 2010b). Besides, although the previous description of the role of spinal CCR1 in neuropathic pain (Kiguchi et al., 2010a) supported a possible spinal role of this receptor in neoplastic pain, the absence of antinociceptive effects following the i.t. administration of J113863 discards their participation in this type of tumoral hyperalgesia. It could perhaps be thought that the administration of higher doses into the spinal cord could evoke analgesic effects. However, the appearance of motor side-effects impeded to test this possibility.

Apart from thermal hyperalgesia, J113863 inhibited mechanical hyperalgesia measured by a paw pressure test, but not mechanical allodynia measured by the von Frey test in mice inoculated with NCTC 2472 cells. We have previously observed a similar profile with other drugs able to modulate tumoral hypernociception by acting peripherally. Thus, antihyperalgesic but not antiallodynic effects were produced in tumor-bearing mice by peripherally acting opiates (Menéndez et al., 2005; Curto-Reyes et al., 2008), the interleukin-1 receptor antagonist anakinra (Baamonde et al., 2007) or the CCR2 antagonist RS 504393 (Pevida et al., 2012). In accordance, drugs that inhibited both tumoral hyperalgesia and allodynia in bone cancer-bearing mice, such as cannabinoid type 2 receptor agonists, evoked their antiallodynic effect by exclusively acting at the spinal cord (Curto-Reyes et al., 2010). These results seems to suggest that, mechanical allodynia in tumor-bearing mice is probably more related to spinal sensitisation whereas tumoral hyperalgesia could be

particularly due to the peripheral sensitisation of nociceptors.

The inhibition of tumoral hyperalgesia after the peripheral blockade of CCR1 could be due to either the up-regulation of CCR1 or the increased presence of an endogenous ligand in response to tumor development. Although a previous report studying the involvement of CCR1 on neuropathic pain has demonstrated that the expression of CCR1 can be increased at injured nerves (Kiguchi et al., 2010b), we have focussed our study on the possibility that an augmented concentration of chemokines able to activate CCR1 could occur. We have initially studied the possible involvement of CCL3, one of the main endogenous CCR1 agonists (Sarau et al., 1997) that can evoke nociceptor sensitisation (Zhang et al., 2005) and participates in neuropathic hypernociception acting at peripheral level (Kiguchi et al., 2010b; Kim et al., 2011). However, ELISA measurements of CCL3 levels in spinal or tumor homogenates of mice inoculated with NCTC 2472 or B16-F10 cells as well as in their respective culture media reveal that CCL3 concentrations remain unaltered. These data suggest that CCR1-mediated hyperalgesia in mice inoculated with fibrosarcoma cells does not depend on CCL3 release.

CCL5 was our next candidate to be the endogenous chemokine acting at CCR1 to induce thermal hyperalgesia in mice inoculated with NCTC 2472 cells. This chemokine binds CCR1 with high affinity (Sarau et al., 1997) and its release from several types of tumors has been previously described (Cambien et al., 2011; Soria et al., 2012). Furthermore, the involvement of CCL5 in some types of neuropathic hypernociception has been previously reported (Liou et al., 2012, 2013). In the particular field of bone cancer-induced pain, a recent report describes the involvement of spinal CCL5 in the development of mechanical hyperalgesia in rats intratibially inoculated with Walker 256 cells (Hang et al., 2013). The fact that the levels of CCL5 detected in our ELISA experiments performed in homogenates of tumor-bearing paws are significantly higher than the values obtained in the contralateral ones or in homogenates prepared from paws coming from mice inoculated with NCTC 2472 killed cells, supports the participation of CCL5 in the hyperalgesia evoked by NCTC 2472 cells. Interestingly, in accordance with the lack of effect of J113863 in mice inoculated with B16-F10 or when i.t. administered to mice inoculated with NCTC 2472 cells, CCL5 levels were unaltered in the paws or the spinal cord of mice inoculated with B16-F10 cells and in the spinal cord of mice inoculated with NCTC 2472 cells. Further supporting that CCL5 could be the CCR1 agonist responsible for thermal hyperalgesia in mice inoculated with NCTC 2472 cells, we have observed that these cells, but not B16-F10, release CCL5 "*in vitro*". Thus, besides a possible up-regulation of CCR1 that was not explored in the present study, the increased levels of CCL5 at tumoral level offer an explanation for the antihyperalgesic effects induced through the blockade of CCR1 in tumor–bearing mice.

A necessary premise to consider CCL5 as a possible mediator of tumoral hyperalgesia should be the demonstration that this molecule can evoke peripheral hyperalgesic responses in mice. In accordance with a previous report describing the instauration of mechanical allodynia after its intradermal injection into the hind paw of rats (Oh et al., 2001), we have observed that the administration of an i.pl. acute dose of CCL5 to naïve C3H/He mice reduces thermal withdrawal latencies. Considering that CCL5 could act as a CCR1 ligand, we initially tested the ability of the CCR1 antagonist J113863 to prevent this CCL5-evoked hyperalgesia and we observed that the coadministration of 1 μg of J113863 together with CCL5 supressed the hyperalgesic effect evoked by CCL5. In any case, we have also considered that CCL5 could also produce hyperalgesia through the activation of other receptors. CCR5 shows several properties compatible with its involvement in CCL5-evoked hyperalgesia: it is stimulated by CCL5, it is expressed in DRG (Oh et al., 2001) and its activation can lead to hyperalgesic consequences, as demonstrated in nerve-injured mice (Kiguchi et al., 2010b). The fact that CCL5-mediated thermal hyperalgesia was completely suppressed by the coadministration of the CCR5 antagonist DAPTA indicates that exogenously administered CCL5 can evoke thermal hyperalgesia in mice, not only through the stimulation of CCR1 but also by activating peripheral CCR5. Based on the above-mentioned data, it could be proposed that the hyperalgesic effect induced by CCL5 could be mediated through the activation of CCR1 and/or CCR5 located on nociceptors. However, we cannot discard the possibility that the binding of CCL5 to receptors expressed in other cells, such as keratinocytes (Spiekstra et al., 2007) or bone cells (Wintges et al., 2013) or even the CCL5-evoked recruitment of immune cells (Liou et al., 2012, 2013) could also participate in the establishment of hyperalgesia evoked by exogenously administered CCL5.

Once established that CCL5 can evoke thermal hyperalgesia in mice through the activation of CCR1 or CCR5, our next aim was to elucidate whether this chemokine could play a hyperalgesic role in tumor-bearing mice. In order to check the involvement of CCL5 in the hyperalgesia measured in mice inoculated with NCTC 2472 cells, we assayed the effect of a specific anti-CCL5 antibody. The fact that the administration of this antibody did not modify thermal latencies in mice inoculated with killed NCTC 2472 cells, but completely inhibited tumoral hyperalgesia, strongly suggests that the increased levels of CCL5 could mediate the hyperalgesic responses measured in mice inoculated with NCTC 2472 cells. In addition, the lack of effect of the CCR5 antagonist DAPTA to inhibit tumoral hyperalgesia when administered at a dose 100 times higher than that previously reported as effective in rats (Rosi et al., 2005), seems to indicate that, although CCL5 can evoke CCR5-mediated hyperalgesia in naïve mice, thermal hyperalgesia measured in mice inoculated

with NCTC 2472 cells is unrelated to CCR5 stimulation whereas, as shown in the experiments performed with J113863, probably depends on the stimulation of CCR1.

The expression of CCR1 in different peripheral cell lines raises the question of which might be the possible location where the blockade of CCR1 leads to the inhibition of tumoral hyperalgesia. Initially, the most immediate explanation could be the direct action of J113863 on CCR1 located in nociceptors, since these receptors are present in small- to medium-diameter neurons of DRG, where they are coexpressed with transient receptor potential vanilloid 1 (TRPV1) in >85% of small-diameter neurons (Zhang et al., 2005). Moreover, it has been described that CCR1 can contribute to TRPV1-mediated nociceptor sensitisation through a protein kinase C (PKC)-dependent pathway (Zhang et al., 2005). In this sense, it seems noteworthy of the previous demonstration that thermal hyperalgesia evoked by NCTC 2472 cells can be inhibited by blocking TRPV1 (Menéndez et al., 2006). In any case, although the inhibition of a putative CCR1-mediated sensitisation of TRPV1 could explain the effect of J113863 on tumoral hyperalgesia, the possibility that the blockade of CCR1 expressed in immune cells such as activated T cells, monocytes, neutrophils and eosinophils (Zhang et al., 2005) or even in osteoclast precursors (Yu et al., 2004) could contribute to the antihyperalgesic effect evoked by J113863 cannot be discarded.

The present results related to CCL5, together with those referred to the role played by CCL2 in mice inoculated with NCTC 2472 cells (Pevida et al., 2012), suggest that the release of a particular chemokine from a tumoral cell line in culture could help to predict the efficacy of a chemokine receptor antagonist to counteract tumoral hyperalgesia. In accordance with this proposition, the blockade of CCR1 and CCR2 (Pevida et al., 2012) evokes antihyperalgesic properties in mice inoculated with NCTC 2472 cells, their corresponding agonists, CCL5 and CCL2, are released by this tumoral cell line in culture and increased concentrations of both chemokines are found at the tumors induced by them. Furthermore, B16-F10 cells do not release any of these chemokines, their levels are not enhanced in tumoral homogenates and CCR1 or CCR2 receptor antagonists are ineffective to inhibit the corresponding tumoral hyperalgesia as shown in the present study for CCL5 and previously for CCL2 (Pevida et al., 2012).

In any case, it seems likely that tumoral cells are not the only source of chemokines at tumor environment. Bone cells themselves can release several chemokines and, in fact, the ability of different cell types involved in bone remodeling to secrete either CCL5 (Yu et al., 2004) or CCL2 (Wu et al., 2013) has been previously demonstrated. Although the release of these two chemokines from bone cells present in tumors induced by the presence of NCTC 2472 cells has not been studied, the increased presence of IL-1β (Baamonde et al., 2007), a factor able to evoke their release from bone cells (Yu et al., 2004), seems to favor this possibility. Since CCL5 (Yu et al., 2004) or CCL2 (Sul et al., 2012) can exacerbate osteolytic mechanisms and the activation of CCR1 favors osteoclastogenesis in a bone neoplastic process such as multiple myeloma (Vallet et al., 2007), it could be speculated that the different concentrations of chemokines, such as CCL2 or CCL5, present in the tumors evoked by NCTC 2472 or B16-F10 cells could even contribute to the development of the different bone pathological features induced by these cells.

Our data add new information relative to the involvement of CCL5 in pathological experimental pain. A very recent report described the involvement of spinal CCL5 in a model of bone cancer-induced pain in rats (Hang et al., 2013) and previous reports, focussed on neuropathic settings, have described the up-regulation of CCL5 in injured nerves (Oh et al., 2001) and the reduction of behavioral hypersensitivity when the biological activity of CCL5 is inhibited (Liou et al., 2012, 2013). In the context of bone cancer-induced pain, and based on the involvement of CCR1 in osteoclast maturation (Yu et al., 2004), it should be a particularly interesting theoretical possibility that the antihyperalgesic effect evoked by the blockade of CCR1 could be accompanied by the prevention of bone injury itself. Supporting this possibility, it has been shown that the chronic administration of CCR1 antagonists reduces tumor burden and osteolytic damage in a murine model of multiple myeloma (Dairaghi et al., 2012). The recent availability of CCR1 antagonists that are being tested in clinical trials for the management of several diseases bearing an inflammatory component such as rheumatoid arthritis, multiple sclerosis or chronic obstructive pulmonary disease (Gladue et al., 2010), opens the opportunity that these new drugs could perhaps be also considered for bone cancer-induced pain.

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4. Resumen de resultados y discusión general

En los experimentos incluidos en este trabajo se estudió la posible participación de los receptores CCR2 y CCR1 y de diferentes quimiocinas endógenas capaces de activarlos, en la hipernocicepción de origen tumoral así como en la hiperalgesia producida por el antineoplásico paclitaxel en ratones. Los experimentos en animales con tumor se realizaron en dos modelos experimentales que han sido utilizados en nuestro laboratorio durante los últimos años en los que se inoculan por vía intratibial diferentes tipos de células tumorales (Menéndez et al., 2003; Curto-Reyes et al., 2008). Las líneas celulares fueron la NCTC 2472 derivada de fibrosarcoma (Schwei et al., 1999) y la B16-F10, derivada de melanoma (Sabino et al., 2003), ambas capaces de provocar el crecimiento de tumores óseos cuyo desarrollo conlleva la producción de hiperalgesia y alodinia. Puesto que las células NCTC 2472 provocan el desarrollo de un tumor fundamentalmente osteolítico (Menéndez et al., 2003) mientras que las B16-F10 originan un patrón mixto donde las lesiones osteolíticas conviven con procesos osteoblásticos de neoformación ósea (Curto-Reyes et al., 2008), el trabajo con ambos tipos de tumores permite valorar nuestras hipótesis en dos situaciones que reproducen situaciones patológicas diferenciadas. Este abordaje parece interesante puesto que cabe pensar que diferentes tipos de lesiones que aparecen en distintas neoplasias óseas puedan propiciar la mayor o menor participación de mediadores hipernociceptivos. Junto a estos experimentos realizados con tumores óseos y, puesto que, además del dolor producido por los propios procesos tumorales, los fármacos utilizados en el tratamiento del cáncer pueden, a su vez, generar síntomas dolorosos, uno de los estudios incluidos en la presente tesis trata de evaluar la posible implicación de algunas quimiocinas en la hipernocicepción producida por un antineoplásico de amplio uso como es el paclitaxel.

Inicialmente, de acuerdo con publicaciones que describían la implicación de los receptores CCR2 y CCR1 en la hiperalgesia y alodinia de origen neuropático (White et al., 2005; Jeon et al., 2011; Kiguchi et al., 2010a; Kiguchi et al., 2010b; Knerlich-Lukoschus et al., 2011) se estudió el efecto producido por antagonistas selectivos para dichos receptores en los dos modelos mencionados de tumores tibiales. A partir de los datos obtenidos en estos ensayos de comportamiento, se diseñaron experimentos con la intención de complementar la información en diferentes aspectos. Así, se realizaron ensayos de western blot para medir la expresión de receptores de quimiocinas o de RT-PCR cuantitativa para conocer el nivel de expresión del mRNA correspondiente, se cuantificaron mediante ELISA los niveles de algunas quimiocinas en diferentes localizaciones o se diseñaron experimentos inmunohistoquimicos para tratar de conocer con más detalle la expresión de las quimiocinas a nivel espinal o la posible participación de la activación glial en sus efectos. La mayor parte de nuestros experimentos corresponden a estudios sobre los receptores CCR2 y su agonista endógeno

CCL2. Ello se debe a que observamos que el sistema CCR2/CCL2 participaba tanto a nivel periférico como espinal en el dolor tumoral, lo que acabó recogiéndose en dos publicaciones separadas (publicaciones 1 y 2). Además, en el estudio realizado con paclitaxel, se pudo observar cómo estos receptores participan también en la hiperalgesia inducida por este antineoplásico y estos resultados se recogieron en otro artículo (publicación 3). En contraste con ello, puesto que los receptores CCR1 sólo participan en la hipernocicepción de origen tumoral a nivel periférico pero no espinal y, a su vez, no están implicados en los efectos del paclitaxel, los resultados relativos a ellos se pudieron agrupar en un único artículo (publicación 4). A continuación se comentan y se discuten los resultados obtenidos organizados en las publicaciones mencionadas.

En los experimentos recogidos en la **publicación 1** (Pevida et al., Naunyn-Schmiedeberg's Arch Pharmacol 385:1053-1061, 2012) se estudió el posible efecto antihiperalgésico y antialodínico producido por la administración del antagonista de CCR2, RS504393, en ratones inoculados con células NCTC 2472 o B16-F10, así como la posible correlación de los efectos antinociceptivos observados con la presencia aumentada de la quimiocina CCL2, que es su principal agonista endógeno.

La administración, sistémica de RS504393 inhibió la hiperalgesia térmica y mecánica, pero no la alodinia mecánica, observada en ratones inoculados con células NCTC 2472 mientras que este antagonista de CCR2 fue prácticamente ineficaz en ratones inoculados con células B16-F10. El efecto antihiperalgésico producido por el RS504393 parece debido a su acción local ya que se reprodujo tras la administración del fármaco sobre el tumor (peritumoral) pero no cuando el fármaco se inyectó en la pata contralateral. Asimismo, tanto la demostración del bloqueo de la hiperalgesia térmica tras la administración peritumoral de un anticuerpo anti-CCL2 como el hallazgo de niveles aumentados de CCL2 en la pata afectada por el tumor observado en ensayos de ELISA apoyan la idea de que la activación de CCR2 pueda deberse al aumento de CCL2 a nivel tumoral. De hecho, el aumento en los niveles de CCL2 en ratones tratados con células NCTC 2472 había sido detectado previamente tras la inoculación en el calcáneo (Khasabova et al., 2007). Sin embargo, aunque estos autores postularon que este mecanismo podría provocar hiperalgesia, ningún trabajo previo había explorado esta posibilidad en ensayos de comportamiento.

El aumento observado en los niveles de CCL2 a nivel tumoral podría proceder de la liberación de esta quimiocina desde diferentes tipos celulares como las propias células NCTC 2472 (Schiller et al., 2009; Khasabova et al., 2007), las células óseas (Schiller et al., 2009), células inmunes presentes en la masa tumoral (Yoshimura et al., 2013; Stec et al., 2012) e

incluso también de los propios nociceptores (Dansereau et al., 2008; Tanaka et al., 2004). La capacidad de las células NCTC 2472 para liberar CCL2 se ha demostrado tanto en este trabajo como en otros previos (Schiller et al., 2009; Khasabova et al., 2007) mediante la medida de la concentración de CCL2 en el medio de cultivo de estas células tras alcanzar la confluencia. En contraste, las medidas realizadas en los DRG correspondientes a las aferencias de la zona del tumor (L3-L5) mostraron que las concentraciones de CCL2 son similares en animales inoculados con células vivas o muertas, sugiriendo que los nociceptores no contribuyen al aumento de CCL2. Ello supone una diferencia notable respecto a lo que parece ocurrir tras lesiones neuropáticas o inflamatorias, situaciones en las que los niveles de CCL2 en neuronas periféricas sí están aumentadas (White et al., 2005; Jeon et al., 2008; Jeon et al., 2011).

En cualquier caso, la aparición de hiperalgesia en respuesta al aumento de la concentración de CCL2 estaría de acuerdo con trabajos previos que muestran la capacidad de CCL2 para evocar hiperalgesia térmica (Qin et al., 2005; Jung et al., 2008), probablemente por sensibilizar nociceptores mediante la activación de mecanismos como la liberación de calcio intracelular en respuesta a la estimulación de la proteína cinasa C (Qin et al., 2005), la transactivación de los TRPV1 (Jung et al., 2008) o el aumento de la excitabilidad de los canales Nav1.8 (Belkouch et al., 2011). Previamente se había demostrado cómo la quimiocina CCL2 liberada en ratones inoculados con células NCTC 2472 en el calcáneo favorece cambios fenotípicos como la expresión de las subunidades α2δ1 de los canales de calcio en los nociceptores (Khasabova et al., 2007), lo que podría asimismo explicar la aparición de hiperalgesia. Resulta llamativo que, aunque el bloqueo de receptores CCR2 fue capaz de inhibir la hiperalgesia tumoral en ratones inoculados con células NCTC 2472, la administración de RS503493 no inhibió la alodinia presente en dichos animales. Una posible razón que puede explicar esta discrepancia podría estar relacionada con la mayor implicación de la sensibilización central en la producción de alodinia (Kim et al., 2012). No parece esperable que este proceso medular pueda ser inhibido por fármacos que actúan a nivel periférico, como ocurre con el RS504393 cuando se administra por vía peritumoral. De hecho, en nuestro laboratorio se ha observado previamente que la estimulación exclusivamente periférica de receptores periféricos opioides o cannabinoides en ratones con tumores óseos produce asimismo efecto antihiperalgésico pero no antialodínico (Baamonde et al., 2006; Curto-Reyes et al., 2008; Curto-Reyes et al., 2010).

Por último, resulta destacable el hecho de que el efecto analgésico del bloqueo de receptores CCR2 en ratones con tumor no parece un hecho generalizable a todos los tumores óseos sino que puede depender estrechamente del tipo de tumor, como se desprende de la ineficacia del RS503493 en ratones inoculados con la línea celular de melanoma B16-F10. La

constatación de que los niveles de CCL2 no están aumentados en estos ratones muestra cómo las características del proceso tumoral son determinantes para delimitar cuáles son los mediadores implicados en la amplificación de los procesos nociceptivos. La publicación 4 incide también en la participación selectiva en las respuestas hipernociceptivas observadas en ratones con estos diferentes tipos de tumor de otra quimiocina, la CCL5. En ese caso, vuelve a observarse que dicha quimiocina juega un papel hiperalgésico sólo tras la inoculación de células NCTC 2472 pero no B16-F10.

Para resumir los resultados obtenidos en este primer trabajo, se puede indicar que algunos tumores óseos, como el provocado tras la inoculación de células NCTC 2472, inducen hiperalgesia térmica o mecánica a través del aumento en los niveles de CCL2 que ocurre en respuesta al desarrollo del tumor. Como consecuencia, el bloqueo de receptores CCR2 podría constituir una herramienta útil para contrarrestar algunos síntomas dolorosos en aquellos tumores en donde se dé esta circunstancia. Un dato interesante en este sentido es la detección de niveles aumentados de CCL2 en diferentes tipos de tumores humanos que se asocian a la presencia de síntomas dolorosos, como es el pancreático (Chehl et al. 2009) o las metástasis tumorales derivadas de cáncer de próstata (Shirotake et al. 2012) o de mama (Soria et al. 2011).

Continuando con el estudio de la implicación de la quimiocina CCL2 y su receptor CCR2 en la hiperalgesia producida por la inoculación de células NCTC 2472 en ratones, el objetivo principal de la **publicación 2** (Pevida et al., Cellular and Molecular Neurobiology; Oct 10, epub ahead of print, 2013) fue estudiar si este sistema CCL2/CCR2 puede jugar también un papel hipernociceptivo actuando a nivel de la médula espinal.

La implicación de los receptores CCR2 espinales y de la CCL2 liberada a nivel medular en la hiperalgesia provocada por la inoculación intratibial de células NCTC 2472 se apoya en la inhibición dependiente de la dosis de la hiperalgesia térmica obtenida tras la administración i.t. del antagonista de CCR2, RS504393, o de un anticuerpo anti-CCL2. Nuestros resultados están de acuerdo con datos previos que muestran la implicación de CCL2 a nivel espinal en modelos de dolor neuropático (Serrano et al., 2010; Gao et al., 2009), daño quirúrgico (Peters y Eisenach, 2010) y, más recientemente, dolor neoplásico (Hu et al., 2012). Estos últimos datos relativos a dolor canceroso fueron obtenidos en ratas inoculadas con células de cáncer de mama Walker 256 y apuntan en el mismo sentido que nuestros resultados ya que, aunque en el trabajo mencionado no se estudia la participación funcional de receptores CCR2 en ensayos de comportamiento, sí se muestra el efecto antialodínico derivado de la administración espinal de un anticuerpo anti-CCL2 (Hu et al., 2012).

Con el fin de conocer si el efecto antihiperalgésico deriva de un incremento en el número de receptores CCR2 espinales, estos se cuantificaron mediante western blot y, contrariamente a lo descrito por otros autores (Vit et al., 2006; Hu et al., 2013), no se observó una regulación al alza de los mismos en la médula espinal. Por ello, se centró el estudio en la posibilidad de que el efecto del antagonista de receptores CCR2 y del anticuerpo anti-CCL2 pudiera ser debido a un aumento en la expresión espinal de CCL2 provocado por el desarrollo tumoral. Inicialmente, los niveles de CCL2 presentes en la médula espinal se midieron mediante ELISA pero, dado que en estos experimentos no se obtuvieron diferencias en la concentración de CCL2 presente en ratones con o sin tumor, empleamos técnicas inmunohistoquímicas para poder observar con mayor detalle posibles cambios localizados en la expresión de dicha quimiocina. En estos ensayos, se observó un incremento significativo en la expresión de CCL2 en las láminas más superficiales (I-II) de la médula espinal de ratones inoculados con células NCTC 2472. Posiblemente, la circunscripción de este aumento a un área tan limitada pueda ser la causa de que estos cambios no se detectasen por ELISA, ya que en esos experimentos se utilizaron homogeneizados completos de los segmentos L2-L6 de médula lo que probablemente dificulta la detección de cambios limitados a una pequeña porción de tejido. Tanto en animales con una lesión neuropática (Zhang y De Koninck, 2006; Van Steenwinckel et al., 2011; Zhang et al., 2012b) como en el estudio mencionado relacionado con dolor neoplásico experimental (Hu et al., 2012) se han descrito niveles aumentados de CCL2 y todo ello parece sugerir que la regulación al alza de CCL2 puede ser una característica común en la hiperalgesia de origen espinal asociada a diferentes tipos de dolor crónico.

Teniendo en cuenta la información recogida en trabajos previos relacionados con dolor neuropático, la liberación de esta quimiocina a nivel espinal podría ocurrir fundamentalmente desde los nociceptores (Thacker et al., 2009; Zhang y De Koninck, 2006; Van Steenwinckel et al., 2011) o desde las células gliales (Gao et al., 2009, 2010). El hecho de que no se obtuviera ninguna modificación ni de los niveles de mRNA de CCL2 medidos por RT-PCR cuantitativa, ni de la concentración de CCL2 medida por ELISA en los DRG correspondientes a L3-L5 de los animales inoculados con células NCTC 2472 vivas, sugería que el aumento de CCL2 a nivel espinal no era debido a su liberación desde los nociceptores. Por lo tanto, se exploró la posibilidad de que las células gliales fuesen la fuente desde la que se libera CCL2. Para ello, se estudió en primer lugar si el desarrollo tumoral provocaba la activación de astroglía y microglía y si este fenómeno podría estar implicado en la hiperalgesia térmica que presentan estos ratones. De acuerdo con datos previos (Honoré et al., 2000b; Hald et al., 2009a; Vit et al., 2006), pudimos observar mediante ensayos inmunohistoquímicos con marcadores de astroglía (GFAP) y microglía (Iba-1) que ambos tipos de células gliales se activaban en respuesta al

desarrollo del tumor. Asimismo, se comprobó mediante ensayos de comportamiento que la administración intratecal de inhibidores de la activación de astroglía (aminoadipato) y microglía (minociclina) disminuye la hiperalgesia inducida por el desarrollo del tumor. A continuación, se realizaron ensayos inmunohistoquímicos de doble tinción en la que la colocalización de los anticuerpos frente a Iba-1 y GFAP con el anticuerpo anti-CCL2 demostró que la quimiocina CCL2 puede ser expresada tanto por células de astroglía como de microglía en ratones con tumores óseos. Este resultado es compatible con la posibilidad de que ambos tipos de células gliales liberen esta quimiocina en la médula espinal de los ratones inoculados con células NCTC 2472 y concuerda con datos previos que muestran la liberación de CCL2 por astroglía tras un daño neuropático (Gao et al., 2009, 2010) o el incremento de CCL2 microglial en el hipocampo tras daño neuronal (Babcock et al., 2003). Por lo tanto, aunque nuestros datos no excluyen que una parte de esta CCL2 pudiera ser liberada desde neuronas espinales, sí permiten formular la hipótesis de que dicha quimiocina implicada en la hiperalgesia tumoral pueda ser liberada en las láminas I-II de la médula espinal por las células gliales activadas en respuesta al desarrollo del tumor.

Por último se exploró la posibilidad de que, además de ser fuente de CCL2 en animales portadores de tumor, la astroglía y microglía pudieran ser, a su vez, activadas por esta quimiocina. Esta posibilidad parecía factible ya que datos previos de nuestro laboratorio demostraban que la administración espinal de la quimiocina CCL2 provoca hiperalgesia térmica mediada, al menos en parte, a través de la activación de células gliales (Baamonde et al., 2011). Para estudiar si la activación de receptores CCR2 podría ser causante de la activación de células gliales espinales, se analizó si la administración intratecal repetida del antagonista de receptores CCR2, RS504393, podría modificar el incremento en la expresión de GFAP o de Iba-1 inducida por el tumor. Se observó que la activación astroglial, pero no microglial, depende en parte de la estimulación de CCR2, ya que el bloqueo de receptores CCR2 provoca una reducción significativa de la inmunoreactividad de GFAP en las láminas más superficiales de la médula. Resulta interesante señalar que la reducción de inmunoreactividad para GFAP correspondía precisamente a las láminas I-II que son aquellas donde habíamos demostrado la presencia aumentada de CCL2 en ratones inoculados con células NCTC 2472.

Globalmente, en este trabajo se demostró la participación de CCL2 y de los receptores CCR2 espinales en la hiperalgesia térmica inducida tras la inoculación de células tumorales NCTC 2472 en ratones y asimismo se comprobó que la función hiperalgésica espinal de esta quimiocina está estrechamente ligada a la activación de células gliales. En conjunto, los resultados recogidos en las publicaciones 1 y 2 mostraron la participación del sistema

CCR2/CCL2 en la hipernocicepción tumoral sugiriendo la posibilidad de que la modulación farmacológica de este sistema pueda resultar interesante para el control del dolor canceroso.

En la **publicación 3** (Pevida et al., Brain Res. Bull. 95:21-27, 2013) se estudió un nuevo aspecto relacionado con la posible participación de la quimiocina CCL2 y los receptores CCR2 en el contexto del dolor experimental relacionado con el cáncer. En lugar de estudiar su implicación en dolor neoplásico como se hizo en las dos publicaciones anteriores, se planteó en este caso un estudio sobre su participación en la hiperalgesia al frío producida por la administración del antineoplásico paclitaxel en ratones.

La aparición de síntomas dolorosos, especialmente alodinia al frío, en pacientes tratados con paclitaxel es un problema que, aunque muy variable en cuanto a su intensidad (Ghoreishi et al., 2012), puede limitar su utilidad clínica (Argyriou et al., 2008; Wolf et al., 2008). A nivel experimental, se ha descrito la aparición de diversas manifestaciones de hipernocicepción en respuesta al tratamiento con paclitaxel. Así, en animales sometidos a tratamientos con este antineoplásico se pudo medir hiperalgesia frente al calor (Polomano et al., 2001; Pascual et al., 2005; Matsumoto et al., 2006; Burgos et al., 2012), al frío (Peters et al., 2007) o a estímulos mecánicos (Authier et al., 2000; Polomano et al., 2001; Hidaka et al., 2009) y alodinia frente a estímulos mecánicos (Smith et al., 2004; Pascual et al., 2005; Liu et al., 2010; Naguib et al., 2012) o al frío (Polomano et al., 2001; Nieto et al., 2008). En nuestro caso, seleccionamos una pauta descrita en un trabajo previo en el que se administra una dosis única de 10 mg/kg de paclitaxel (Hidaka et al., 2009) y medimos las latencias de retirada de la pata sometida a un estímulo de 0 ºC por contacto con una placa fría. Se trabajó de un modo similar al habitualmente utilizado en la placa caliente unilateral (Menéndez et al, 2002) y el único cambio significativo fue la realización de una medida única en cada pata debido a la dificultad de reproducir las latencias de retirada tras la realización de medidas repetidas en el mismo ratón. En estos ensayos, se observó que las latencias de retirada de la pata de la placa fría se reducían significativamente en ratones tratados con paclitaxel durante los primeros 7 días tras la administración del fármaco, indicando la presencia de hiperalgesia a estímulos nociceptivos fríos.

La administración sistémica del antagonista de receptores CCR2 RS504393, pero no del antagonista de receptores de CCR1 J113863, inhibió la hiperalgesia al frio inducida por paclitaxel en un rango de dosis similar al utilizado previamente para inhibir otros tipos de dolor neuropático (Zhang et al., 2012b). Puesto que la implicación de la CCL2 a nivel espinal había sido demostrada en otros modelos de dolor neuropático (Thacker et al., 2009; van Steenwinckel et al., 2011) se estudiaron los efectos de la administración i.t. tanto del

antagonista CCR2 como de un anticuerpo anti-CCL2 en ratones tratados con paclitaxel. Ambos tratamientos provocaron la inhibición de la hiperalgesia evocada por el antineoplásico. Por otra parte, en ensayos de ELISA se pudo observar el aumento de los niveles de CCL2 en la médula espinal en respuesta al tratamiento con paclitaxel. Estos datos sugieren que la activación del sistema CCR2/CCL2 en la médula espinal participa en la hiperalgesia producida por el paclitaxel. Puesto que este antineoplásico apenas puede atravesar la barrera hematoencefálica (Glantz et al., 1995; Wang et al., 2012a) parece probable que, como ocurre en respuesta a otras lesiones de tipo neuropático, los daños producidos por el paclitaxel en los nervios periféricos pueda ser la causante de la liberación de CCL2 en médula espinal.

A partir de este resultado, se trató de dilucidar si la activación de células gliales espinales podría estar implicada en el efecto hipernociceptivo inducido por el sistema CCL2/CCR2 en los ratones tratados con paclitaxel. Aunque este aspecto no había sido estudiado previamente en animales tratados con antineoplásicos, varios trabajos describen la participación de las células gliales en el efecto hipernociceptivo provocado por CCL2 en diferentes modelos de dolor neuropático (Calvo y Bennett, 2012; Schomberg y Olson, 2012), tal y como se comentó en relación con los resultados obtenidos en la publicación 2. El estudio inmunohistoquímico con marcadores de microglia (Iba-1) y de astroglia (GFAP) en la médula espinal mostró la activación microglial, pero no astroglial, en las láminas superficiales del asta dorsal de la médula espinal de los ratones tratados con paclitaxel. Si bien un resultado similar había sido descrito previamente en ratas tratadas con 18 mg/kg de paclitaxel (Peters et al., 2007), existen trabajos en los que se describe la activación de microglía y astroglía (Burgos et al 2012; Naguib et al, 2012) o la activación de astroglía exclusivamente (Zhang et al, 2012a). Una posible explicación para estas variaciones puede ser el empleo de diferentes pautas de administración del antineoplásico.

La activación microglial observada en ratones tratados con paclitaxel parece participar en la hiperalgesia al frío presente en estos ratones, ya que ésta se redujo tras la administración i.t. del inhibidor microglial, minociclina, a dosis similares a las eficaces en otros procesos neuropáticos (Narita et al, 2006). De acuerdo con ello, la eficacia de la minociclina para inhibir síntomas nociceptivos tanto a nivel periférico (Cata et al, 2008; Liu et al, 2010) como central (Burgos et al, 2012) había sido demostrada en ratas tratadas con paclitaxel. La activación microglial detectada en la zona superficial del asta dorsal de la médula de los ratones tratados con paclitaxel parece depender del aumento local de CCL2 ya que la administración i.t. de un anticuerpo anti-CCL2 durante 3 días la redujo significativamente. Por lo tanto, puesto que la neutralización de CCL2 espinal previene la activación de microglía y la inhibición de la microglía evocada por minociclina bloquea la hiperalgesia al frío inducida por paclitaxel, puede

proponerse que la hiperalgesia producida por este antineoplásico se debe a la liberación espinal de CCL2 y la consecuente activación de microglía.

En conjunto, nuestros resultados sugieren que la administración de paclitaxel en ratones produce la liberación espinal de CCL2 que, al actuar sobre los CCR2 provoca la activación de microglía, siendo este proceso fundamental en el establecimiento de la hiperalgesia al frío producida por el antineoplásico. Puesto que nuestro trabajo fue realizado en ratones tratados con una dosis única de paclitaxel, los resultados obtenidos parecen particularmente relacionados con la observación clínica de que este antineoplásico puede producir síntomas sensitivos a las 24-72 h de su primera administración (Dougherty et al., 2004; Argyriou et al., 2008). Ello abriría la puerta a la consideración de que la modulación del sistema CCL2/CCR2 podría ser útil para inhibir los síntomas neuropáticos asociados al tratamiento con paclitaxel.

Finalmente, el trabajo recogido en la **publicación 4** (Pevida et al., Neuroscience 259C:113-125, 2013) se relaciona con la inhibición del dolor neoplásico experimental a través del bloqueo de receptores CCR1, un aspecto que no había sido abordado en ninguna publicación previa. Tras observar inicialmente el efecto antinociceptivo producido tras la administración del antagonista de CCR1 J113863 en ratones inoculados con células NCTC 2472, se estudió qué quimiocinas podrían ser responsables de estimular este receptor en animales con tumor. La administración sistémica o peritumoral del antagonista de CCR1 J113863, inhibió de forma dependiente de la dosis la hiperalgesia térmica y mecánica pero no la alodinia observada en ratones inoculados con células NCTC 2472. El hecho de que la administración espinal de J113863 no modificase la hiperalgesia térmica en esos ratones sugería que el efecto deriva de una acción periférica debida al bloqueo de receptores CCR1 presentes en la zona tumoral. De hecho, la producción de efectos antinociceptivos derivados del antagonismo de CCR1 periféricos en ratones inoculados con células NCTC 2472 concuerda con la demostración previa de su participación en dolor neuropático (Kiguchi et al., 2010b). En cambio, nuestros datos parecen indicar que los efectos analgésicos derivados del bloqueo de receptores CCR1 espinales en modelos experimentales de dolor neuropático no serían extrapolables a situaciones de dolor neoplásico (Kiguchi et al., 2010a). Las dosis de J113863 ensayadas a nivel espinal no pudieron aumentarse debido a la aparición de alteraciones motoras. Por otra parte, tal y como se propuso en el caso de la inhibición de la hiperalgesia tumoral tras la administración de un antagonista de CCR2 actuando a nivel periférico (publicación 1), la ausencia de efectos antialodínicos en respuesta al bloqueo de receptores CCR1 periféricos podría estar relacionada con la participación de la sensibilización central en la producción de alodinia (Kim et al., 2012)

Un resultado similar al obtenido con el antagonista CCR2 ensayado en la publicación 1 fue la ausencia de implicación de los receptores CCR1 en la hipernocicepción provocada por la inoculación de células B16-F10. Así, la administración del antagonista CCR1 J113863, no mostró ninguna eficacia antinociceptiva tras su administración sistémica, peritumoral o espinal en este modelo, lo cual parece indicar que, de la misma manera que se describió en dicha publicación con el sistema CCL2/CCR2, la participación de determinadas quimiocinas en el dolor neoplásico no es una regla general sino que depende del tipo de tumor.

La inhibición de la hiperalgesia provocada por las células NCTC 2472 por el bloqueo de receptores CCR1 periféricos podría ser debida a un incremento en la expresión de dicho receptor, tal y como describen Kiguchi y cols. (2010b) en un modelo de dolor neuropático, o bien a la regulación al alza de algún ligando endógeno capaz de estimularlos. Nuestro estudio se centró en la determinación de una posible presencia aumentada de dos quimiocinas, CCL3 y CCL5, capaces de producir respuestas funcionales en los DRG (Zhang et al., 2005b; Oh et al., 2001; Bolin et al., 1998; Bhangoo et al., 2007) y que además se han relacionado previamente con la nocicepción mediada a través del estímulo de receptores CCR1 (Zhang et al., 2005b; Eijkelkamp et al., 2010; Kiguchi et al., 2010b; Oh et al., 2001). La medida mediante ELISA de los niveles de CCL3 en homogeneizados de médula lumbar o de tumor así como en los medios de cultivo obtenidos tras la confluencia de las células NCTC 2472 o B16-F10 no mostró variaciones significativas en ningún caso, sugiriendo que la CCL3 no participa en los efectos hiperalgésicos medidos en ratones inoculados con células NCTC 2472. En consecuencia, se realizaron experimentos similares con la quimiocina CCL5. En este caso, se detectó un aumento en los niveles de CCL5 en los homogeneizados de patas de ratón inoculadas con células NCTC 2472 pero no en los procedentes de la médula espinal de estos ratones, lo que inicialmente concuerda con el hallazgo de efectos antinociceptivos periféricos pero no centrales del antagonista de CCR1. Además, también se observó que las células NCTC 2472 cultivadas “in vitro” son capaces de liberar CCL5. El siguiente paso fue intentar comprobar si, efectivamente, la CCL5 podría ser la quimiocina responsable de la hiperalgesia tumoral. Para que ello fuese verosímil deberían cumplirse las premisas de que la administración de CCL5 produjera hiperalgesia térmica en ratones y que ésta se produjera actuando sobre los receptores CCR1, ya que esta quimiocina puede unirse también a los CCR5 para producir hiperalgesia (Oh et al., 2001; Kiguchi et al., 2010b). Comprobamos cómo, de acuerdo con la descripción previa de que la administración intraplantar de CCL5 en ratas produce alodinia mecánica (Oh et al., 2001), la inyección en la pata de pequeñas dosis de CCL5 produce hiperalgesia térmica en ratones sanos. Esta hiperalgesia fue antagonizada tanto por el antagonista de receptores CCR1, J113863, como por el antagonista de CCR5 DAPTA lo

que, en principio, hacía posible que, en caso de participar en dolor tumoral, la CCL5 pudiera hacerlo a través de cualquiera de estos dos receptores. Inicialmente, se trató de comprobar si la CCL5 podría estar involucrada en la hiperalgesia producida por la inoculación de células NCTC 2472 estudiando el efecto de un anticuerpo anti-CCL5. La inhibición de la hiperalgesia tumoral tras la administración del anticuerpo anti-CCL5 junto con la incapacidad del antagonista CCR5, DAPTA, para inhibirla sugiere fuertemente que este efecto mediado por CCL5 deriva de la activación de receptores CCR1, y no CCR5. Estos datos sobre la participación del sistema CCL5/CCR1 a nivel periférico, junto con los publicados por otros autores durante la redacción de esta tesis describiendo la implicación de la CCL5 espinal en la hiperalgesia mecánica medida en un modelo de dolor neoplásico en ratas (Hang et al., 2013), constituyen las primeras descripciones de la implicación de esta quimiocina en el dolor neoplásico experimental.

Al igual que ocurrió en los trabajos que exploraban la implicación de CCL2/CCR2 (publicaciones 1 y 2) volvimos a observar que la activación de receptores CCR1 no participa en la hiperalgesia debida a la inoculación de células B16-F10. De acuerdo con ello, no se detectó aumento de la concentración de CCL5 ni en el medio de cultivo de estas células ni en homogeneizados de tumores o de médula espinal de animales inoculados con células B16-F10. Considerando estos datos junto con los obtenidos en trabajos anteriores, parece reseñable el posible papel predictivo de la capacidad de liberar una quimiocina en cultivo por parte de las células tumorales sobre la presencia a nivel tumoral de dicha quimiocina y su posible papel en la hiperalgesia generada por dicho tumor. Aun así, debe matizarse que las células tumorales no son la única fuente de quimiocinas en el entorno tumoral y tanto las células implicadas en la remodelación ósea (Yu et al., 2004) como las células inmunes (Katayama et al., 2002; Imai et al., 2013) presentes en el proceso también podrían participar en la liberación de mediadores, como la CCL5.

En resumen, en este cuarto trabajo se demuestra que la liberación de CCL5 a nivel tumoral participa en el desarrollo de hiperalgesia térmica y mecánica, pero no en la alodinia mecánica inducida por la inoculación intratibial de células NCTC 2472 en ratones. Por tanto, el antagonismo de receptores CCR1 periféricos podría ser eficaz para reducir la hiperalgesia tumoral en los tipos de tumores donde la quimiocina CCL5 ejerza un papel hiperalgésico.

Como comentario final, podemos decir que, aunque nuestro trabajo se desarrolló a nivel experimental utilizando dianas farmacológicas que no se utilizan hoy día en terapéutica, la disponibilidad reciente de anticuerpos monoclonales anti-CCL2 ensayados actualmente como antitumorales (Sandhu et al, 2013) o antagonistas CCR2, ensayados como analgésicos en dolor neuropático (Kalliomäki et al., 2013), así como de antagonistas CCR1 que están en fase de ensayo clínico para el tratamiento de diferentes afecciones con componente inflamatorio

como la artritis reumatoide (Gladue et al., 2010), abre la posibilidad de que estos nuevos fármacos pudieran considerarse en un futuro para el tratamiento del dolor por cáncer.

5. Conclusiones

1. La activación de receptores CCR2 por su ligando endógeno CCL2 tanto a nivel del tumor como en la médula espinal participa en la hiperalgesia tumoral evocada por la inoculación intratibial de células NCTC 2472 en ratones. Sin embargo, el sistema CCL2/CCR2 no está implicado en la alodinia provocada por el desarrollo de este tumor ni en los síntomas hipernociceptivos secundarios a la inoculación de células de melanoma B16-F10. Estos resultados sugieren que el bloqueo de CCR2 o la neutralización de CCL2 podrían ser estrategias útiles para inhibir algunos síntomas dolorosos presentes en determinados tipos de tumores aunque no parece que puedan ser eficaces en la mayoría de situaciones de dolor óseo de origen tumoral.

2. La implicación del sistema CCL2/CCR2 espinal en la hiperalgesia evocada por las células NCTC 2472 parece relacionada con la activación de microglía y astroglía. Nuestros resultados muestran que la CCL2 puede liberarse desde estas células gliales activadas como consecuencia del desarrollo del tumor y contribuir, a su vez, a la activación de astrocitos en las láminas superficiales de la médula espinal.

3. Los receptores CCR1 participan en la hiperalgesia evocada por células NCTC 2472 al ser activados por la quimiocina CCL5 liberada en el entorno tumoral, pero no en la médula espinal. Ello sugiere la posible utilidad de los antagonistas CCR1 o de los anticuerpos contra CCL5 en el control de algunos síntomas de dolor tumoral. Sin embargo, como ocurría en el caso de la CCL2, esta proposición no parece aplicable a nivel general y probablemente deba limitarse a aquellos tumores capaces de secretar CCL5.

4. El análisis de la capacidad de las dos líneas de células tumorales utilizadas para secretar las quimiocinas CCL2, CCL3 o CCL5 al medio de cultivo, las concentraciones de las mismas presentes en los correspondientes tipos de tumores y los efectos antihiperalgésicos provocados por fármacos que bloquean su acción, parece sugerir que la secreción “in vitro” de una quimiocina por parte de una línea tumoral puede ser un buen predictor tanto del aumento de sus niveles en el tumor como de la eficacia analgésica de los fármacos capaces de evitar sus efectos.

5. El desarrollo de hiperalgesia al frío tras la administración del antineoplásico paclitaxel es independiente de la activación de receptores CCR1 y está relacionado con el estímulo de receptores espinales de tipo CCR2 y la consiguiente activación microglial como consecuencia de la liberación de CCL2 en la médula espinal. Por lo tanto, podría proponerse la administración de antagonistas CCR2 o de anticuerpos contra CCL2 como posibles estrategias para controlar la hiperalgesia por paclitaxel.

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