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DIFFERENTIAL MODULATION BY SIGMA-1 RECEPTORS OF μ-OPIOID-INDUCED ANTINOCICEPTION AND SIDE EFFECTS: ROLE OF PERIPHERAL SIGMA-1 RECEPTORS

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A mis padres, por apoyarme en cada una de las decisiones que he tomado a lo largo de mi vida.

A Enrique

"La ciencia es el fundamento de todo progreso, que mejora la vida humana y alivia el sufrimiento"

"Science is at the base of all the progress that lightens the burden of life and lessens its suffering"

Irène Joliot-Curie

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RESUMEN

1. ANTECEDENTES, HIPÓTESIS Y OBJETIVOS

1.1. Antecedentes

Los fármacos opioides, especialmente los agonistas de receptores μ (tales como morfina, fentanilo, oxicodona, buprenorfina o tramadol), son muy utilizados en clínica para el tratamiento del dolor moderado a severo (Pergolizzi et al., 2008; Schäfer, 2010; Pasternak and Pan, 2011; Al-Hasani and Bruchas, 2011). Los receptores opioides se localizan en diferentes áreas del sistema nervioso central (tanto en diversos núcleos supraespinales como a nivel espinal) y periférico (ganglios de la raíz dorsal, DRG), implicadas en la modulación del dolor (Bigliardi-Qi et al., 2004; Khalefa et al., 2012). Se piensa que el efecto analgésico de los agonistas μ se debe principalmente a su interacción con receptores opioides localizados en el sistema nervioso central, particularmente a nivel supraespinal (p.ej. Christie et al., 2000; Khalefa et al., 2012). Sin embargo, en los últimos años el estudio del posible papel de los receptores opioides periféricos en la analgesia opioide ha cobrado un gran interés (p.ej. Stein et al., 2003; Sehgal et al., 2011).

Además de la analgesia, estos fármacos producen otros efectos derivados principalmente de sus acciones a nivel central, incluyendo nauseas, confusión mental y depresión respiratoria, entre otros efectos adversos de relevancia clínica (revisado en Waldhoer et al., 2004 y Al-Hasani and Bruchas, 2011). Los opioides también producen efectos periféricos, ya que disminuyen el tránsito intestinal principalmente por su efecto inhibitorio de la actividad del plexo mientérico (Holzer et al., 2009; Brock et al., 2012). Este efecto de los opioides se utiliza clínicamente para el tratamiento sintomático de la diarrea, concretamente mediante el uso de loperamida. Este fármaco es un agonista opioide que actúa exclusivamente a nivel periférico (Menéndez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012), y que, por lo tanto, es capaz de inhibir el tránsito intestinal aunque carece de efectos centrales (Gallelli et al., 2010; Layer et al., 2010), por lo que no se utiliza clínicamente como analgésico. Desafortunadamente, la inhibición del tránsito intestinal también es producida por los opioides analgésicos (con penetrabilidad central) y tiene un gran impacto en la calidad de vida del paciente, ya que el estreñimiento producido por estos fármacos es la causa principal de abandono voluntario de medicación opioide por los pacientes (Dhingra et al., 2013).

El receptor sigma (σ) fue identificado por Martin y colaboradores en 1976. Tras su descubrimiento fue erróneamente clasificado como un subtipo de receptor opioide, y más tarde se confundió con el sitio de unión de la fenciclidina en el receptor NMDA (*N*-metil-*D*-aspartato). Actualmente, los receptores σ se clasifican como una entidad farmacológica independiente (revisado por Cobos et al., 2008; Zamanillo et al., 2013). Estudios bioquímicos y farmacológicos evidencian la existencia de dos subtipos de receptores σ, denominados σ_1 y σ_2 (ver Cobos et al., 2008 para referencias). El subtipo σ_1 está caracterizado en mayor profundidad y es el objeto de estudio de esta Tesis Doctoral.

La farmacología del receptor σ_1 está actualmente bien descrita, y hoy en día existen fármacos selectivos para este receptor. Estos fármacos incluyen a los antagonistas selectivos BD-1063, BD-1047, NE-100 y S1RA, y a los agonistas selectivos PRE-084 y (+)-pentazocina (Cobos et al., 2008; Zamanillo et al., 2013). El receptor σ_1 ha sido clonado y no muestra homología con los receptores opioides ni con ninguna otra proteína de mamíferos (ver Hayashi y Su, 2003; Guitart et al., 2004 y Cobos et al., 2008, para referencias). Su clonaje ha permitido el desarrollo de ratones knockout σ_1 (KO-σ_1) (Langa et al., 2003), facilitando así el estudio de la función de estos receptores. El receptor σ_1 tiene un papel neuromodulador, atribuible a su acción chaperona sobre otros receptores y canales implicados en diversos procesos fisiopatológicos (Aydar et al., 2002; Kim et al., 2010; Navarro et al., 2010 y 2013; Su et al., 2010; Balasuriya et al., 2012; Kourrich et al., 2012 y 2013). Entre los receptores susceptibles de la modulación por el receptor σ_1 destacan los receptores opioides μ. Ambos receptores pueden interaccionar físicamente, y el antagonismo farmacológico del receptor σ_1 es capaz de incrementar la señalización opioide, medida como incremento de la fijación de $[^{35}S]GTP\gamma S$ en respuesta al agonista μ DAMGO (Kim et al., 2010).

El receptor σ_1 se localiza en áreas clave para el procesamiento del dolor e implicadas en la analgesia opioide. Estas áreas incluyen al asta dorsal de la médula espinal, la sustancia gris periaqueductal, la médula rostroventral, o los ganglios de la raíz dorsal (Alonso et al., 2000; Kitaichi et al., 2000; Ueda et al., 2001; Roh et al., 2008b). Sin embargo, todavía no se ha comparado cuantitativamente la expresión del receptor σ_1 en áreas del sistema nervioso central y periférico, por lo que se desconoce la/s localizaciones en las que es más abundante.

Aunque la inhibición genética o farmacológica del receptor σ_1 no altera el dolor nociceptivo inducido por la aplicación de un estímulo agudo térmico o mecánico puntiforme (p.ej. Chien y Pasternak, 1994; De la Puente et al., 2009; Entrena et al., 2009a y b; Marrazzo et al., 2011; Romero et al., 2012), es capaz de potenciar el efecto antinociceptivo de agonistas opioides (revisado por Zamanillo et al., 2013). Cabe destacar que esta potenciación de la antinocicepción opioide por la inhibición del receptor σ_1 se había examinado únicamente frente a estímulos de naturaleza térmica (p.ej. Chien and Pasternak, 1993 y 1994; Marrazzo et al., 2011). La ontogénesis y mecanismos neuroquímicos de la analgesia opioide frente a estímulos térmicos y mecánicos son diferentes (Kuraishi et al., 1985; Tseng et al., 1995; Wegert et al., 1997; Sato et al., 1999), por lo que la modulación de la antinocicepción opioide frente a estímulos térmicos por el receptor σ_1 no es necesariamente extrapolable a estímulos mecánicos.

En los estudios previos, el efecto de potenciación de la antinocicepción opioide por la inhibición del receptor σ_1 había sido atribuido a efectos centrales, concretamente supraespinales (King et al., 1997; Pan et al., 1998; Mei y Pasternak, 2002 y 2007; Marrazzo et al., 2006), y el posible papel del receptor σ_1 periférico en esta potenciación estaba totalmente inexplorado. Una herramienta útil para diseccionar el origen central o periférico de los efectos de los opioides es el uso de antagonistas opioides carentes de penetrabilidad central, como por ejemplo la naloxona metiodida (Menéndez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012). Sin embargo, no se conoce la sensibilidad al antagonismo de los receptores opioides periféricos de la potenciación de la antinocicepción opioide por la inhibición σ_1, o si la inhibición periférica de este receptor es suficiente como para potenciar la antinocicepción opioide.

Además, estudios previos mostraron que el agonismo farmacológico del receptor σ_1 no modula algunos efectos adversos de los opioides (inhibición del tránsito intestinal y letalidad inducida por morfina) (Chien y Pasternak, 1994). Sin embargo, se desconocía si el incremento de la antinocicepción opioide por la inhibición del receptor σ_1 podría ir acompañado por incrementos en efectos no analgésicos de los opioides, lo que limitaría su uso clínico potencial como adyuvante de los fármacos opioides.

1.2. Hipótesis y objetivos

Teniendo en cuenta los antecedentes anteriormente mencionados, la **hipótesis principal** de esta Tesis Doctoral fue que los receptores σ_1 podrían estar implicados en la modulación de la antinocicepción opioide periférica frente a un estímulo mecánico, y que la inhibición de los receptores σ_1 podría potenciar diferencialmente la antinocicepción opioide sin alterar otros efectos no analgésicos (adversos) de los fármacos opioides.

Para comprobar esta hipótesis, nuestro **primer objetivo** fue estudiar la influencia de la inhibición del receptor σ_1 en el dolor nociceptivo producido por un estímulo mecánico romo en presencia o ausencia de la administración sistémica (subcutánea, s.c.) de diversos analgésicos opioides de relevancia clínica. La inhibición del receptor σ_1 se efectuó mediante el uso de ratones KO-σ_1 o mediante su bloqueo farmacológico sistémico (con BD-1063, BD-1047, NE-100 o S1RA) en ratones salvajes. Los opioides evaluados incluyeron a los productos con penetrabilidad central morfina, fentanilo, oxicodona, buprenorfina y tramadol.

El **segundo objetivo** de esta Tesis Doctoral fue determinar el papel de los receptores opioides periféricos en la antinocicepción opioide frente a un estímulo mecánico romo, en presencia y ausencia de la modulación del efecto opioide por la inhibición del receptor σ_1. Para conseguir este objetivo se siguieron diferentes estrategias experimentales:

1) Comparar la inhibición por un antagonista opioide periférico (naloxona metiodida) de los efectos antinociceptivos producidos por la administración sistémica (s.c.) de los agonistas opioides anteriormente nombrados, en una situación control y durante la inhibición del receptor σ_1 (en ratones KO-σ_1 o en ratones salvajes tratados por vía sistémica con antagonistas del receptor σ_1).

2) Estudiar si la inhibición del receptor σ_1 (en ratones KO-σ_1 o mediante su bloqueo farmacológico sistémico en ratones salvajes) es capaz de poner de manifiesto una acción antinociceptiva del agonista opioide de acción periférica loperamida.

3) Estudiar si la inhibición farmacológica local del receptor σ_1 es capaz de incrementar la antinocicepción mecánica periférica inducida por la administración sistémica de los diferentes agonistas opioides evaluados. Para alcanzar este objetivo administramos los opioides por vía s.c. junto con BD-1063 o S1RA por vía intraplantar (i.pl.) y evaluamos la sensibilidad del efecto antinociceptivo al antagonista opioide periférico naloxona metiodida (como indicador de que el efecto antinociceptivo implica la activación de receptores opioides periféricos).

4) Estudiar si la inhibición del receptor σ_1 es capaz de permitir la expresión del efecto antinociceptivo de la administración local de morfina (usado como prototipo de agonista opioide). Para ello estudiamos los efectos de la administración i.pl. de morfina tanto en una situación control como en ratones KO-σ_1, así como durante la inhibición farmacológica local del receptor σ_1 (mediante la administración i.pl. de BD-1063, BD-1047, NE-100 y S1RA) en ratones salvajes.

Puesto que los datos experimentales del presente trabajo sugieren que existen receptores σ_1 periféricos capaces de modular los efectos antinociceptivos periféricos de los opioides, el **tercer objetivo** de esta Tesis Doctoral fue comparar la expresión del receptor σ_1 en diversas áreas del sistema nervioso central y periférico involucradas en la analgesia opioide (para poder vincular los efectos comportamentales de la inhibición del receptor σ_1 en la antinocicepción opioide periférica con su localización anatómica). Para alcanzar este objetivo realizamos experimentos de Western blot (con un anticuerpo específico del receptor σ_1) en muestras de tejido nervioso central (amígdala basolateral, médula rostroventral, sustancia gris periaqueductal, región dorsal de la médula espinal) y periférico (ganglios de la raíz dorsal de la médula espinal).

El **cuarto objetivo** de esta Tesis Doctoral fue demostrar que el incremento en el efecto antinociceptivo de los opioides que encontramos en los ratones KO-σ_1 no se debe a cambios adaptativos en los receptores opioides μ periféricos o centrales y que la modulación de la antinocicepción opioide causada por los antagonistas del receptor σ_1 no se debe a efectos cruzados de los opioides en los receptores σ_1 o de los antagonistas σ_1 en los receptores opioides μ. Para alcanzar este objetivo se realizaron estudios de saturación de la fijación de [^{3}H]DAMGO (un radioligando selectivo del receptor μ) a tejido de cerebro, médula espinal y piel de la pata y estudios de desplazamiento por los fármacos opioides y σ_1 de la unión de [^{3}H]DAMGO y [^{3}H](+)-pentazocina (un radioligando selectivo del receptor σ_1) a sus sitios de fijación específicos.

Finalmente, teniendo en cuenta la relevancia clínica de los efectos no analgésicos (principalmente adversos) de los opioides, **el quinto objetivo** de esta Tesis Doctoral fue estudiar la modulación por el receptor σ_1 de otros efectos opioides diferentes de la antinocicepción. Para ello, exploramos los efectos de la inhibición del receptor σ_1 en: a) la hiperlocomoción inducida por morfina, un efecto de origen central (supraespinal) observado en los ratones tras la administración de opioides (Hnasko et al., 2005), y b) la inhibición del tránsito intestinal inducida por analgésicos opioides (morfina y fentanilo) así como por el antidiarreico loperamida. Este segundo efecto no analgésico opioide es de particular interés para la posible aplicabilidad futura de los hallazgos de esta Tesis Doctoral, ya que como se ha comentado anteriormente es un efecto opioide con una gran relevancia clínica y de origen principalmente periférico, al igual que la antinocicepción opioide frente a estímulos mecánicos cuando está inhibida la función del receptor σ_1 (como demuestra esta Tesis Doctoral).

2. MÉTODOS

2.1. Animales de experimentación

Los experimentos fueron realizados en ratones hembra de la cepa CD-1 (Charles River, Barcelona, España) y en ratones KO-σ_1 (Esteve, Barcelona, España). Los ratones KO-σ_1 se generaron en un fondo genético CD-1 como se describió previamente (Entrena et al., 2009a). Los animales fueron manipulados de acuerdo con la Directiva del Consejo de Comunidades Europeas de 24 de Noviembre de 1986 (86/609/ECC). El protocolo experimental fue aprobado por el Comité de Ética de Experimentación Animal de la Universidad de Granada.

2.2. Fármacos y administración de fármacos

Los agonistas opioides utilizados fueron los opioides de acción periférica y central morfina, fentanilo, oxicodona, buprenorfina y tramadol, y el agonista opioide periférico loperamida. Los antagonistas opioides utilizados fueron naloxona y su derivado cuaternario de acción periférica naloxona metiodida. Los antagonistas selectivos σ_1 utilizados fueron BD-1063, BD-1047, NE-100 y S1RA. Como agonista selectivo σ_1, y para confirmar la selectividad de los efectos de los antagonistas σ_1, utilizamos al PRE-084. Todos los fármacos se disolvieron en salino, con la excepción de la loperamida, que fue disuelta en DMSO al 1% en salino. Las administraciones sistémicas de los fármacos se realizaron mediante inyecciones s.c. en la zona interescapular, usando un volumen de 5 ml/kg. Cuando se estudiaron los efectos de la asociación sistémica de varios fármacos, estos se administraron en diferentes zonas de la región interescapular para evitar posibles interacciones físico-químicas. Las administraciones locales de los fármacos se realizaron mediante administraciones i.pl. usando un volumen de 20 µl.

2.3. Ensayos de nocicepción frente a un estímulo mecánico romo (test de presión de la pata)

2.3.1. Descripción general del procedimiento de evaluación de la respuesta dolorosa

Los animales fueron habituados durante 1 hora en la habitación de experimentación. Tras ser convenientemente inmovilizados fueron evaluados aplicando un estímulo mecánico romo sobre las patas traseras tal y como se describió previamente (Menéndez et al., 2005), con pequeñas modificaciones. El aparato utilizado fue un analgesímetro de presión (Modelo 37215, Ugo-Basile, Varese, Italia). Se aplicó una estructura cónica con la punta redondeada, y a una intensidad constante, sobre la parte dorsal de las patas traseras del animal. Se evaluó el tiempo de latencia hasta la aparición de la respuesta de forcejeo del animal, utilizada como indicador de dolor. Cada ratón fue evaluado dos veces en cada pata de forma alterna y dejando un minuto entre medida y medida. Se estableció un tiempo de corte de 50 segundos para minimizar la posibilidad de ocasionar un daño tisular.

2.3.2. Comparación de la respuesta dolorosa en ratones salvajes y knockout σ_1

Con objeto de comparar la respuesta dolorosa entre ratones salvajes y desprovistos del receptor σ_1 (KO-σ_1) ante un estímulo mecánico como el que se acaba de describir, se aplicó sobre las patas traseras de los ratones una presión de una intensidad determinada comprendida en un amplio rango de presiones (100-600 g), y se registró el tiempo de latencia para cada una de ellas. A la hora de cuantificar la respuesta evaluada, se consideró la media de los tiempos de latencia de las dos medidas realizadas en cada pata. Se utilizó un grupo diferente de animales para cada una de las presiones evaluadas, con objeto de evitar una posible sensibilización de la pata debida a las estimulaciones repetidas.

2.3.3. Evaluación del efecto de la inhibición del receptor σ_1 en la antinocicepción opioide

Los efectos de los fármacos se evaluaron usando una intensidad de estimulación de 450 g. Esta intensidad de estimulación se escogió en base al experimento comentado en el apartado anterior, ya que el tiempo de latencia de respuesta ofrecía una amplia ventana para la detección de posibles incrementos inducidos por los tratamientos evaluados (ver sección 3.1. para una información más detallada).

Cuando los analgésicos opioides fentanilo, morfina, oxicodona y tramadol se administraron sistémicamente, estos se inyectaron vía s.c. 30 minutos antes de la evaluación comportamental. Sin embargo, buprenorfina fue administrada 1 hora antes ya que se ha descrito previamente que su máximo efecto antinociceptivo es más tardío que para otros opioides (Yassen et al., 2005). Para estudiar el efecto sistémico de los antagonistas σ_1, estos fueron administrados por vía s.c. 5 minutos antes de la administración sistémica del fármaco opioide. Cuando se estudió el efecto de la administración local de los fármacos, estos se administraron 5 minutos antes de la evaluación, para minimizar la probabilidad de la absorción sistémica de los mismos. Para evaluar los efectos del PRE-084, naloxona o naloxona metiodida, estos fármacos o su solvente fueron administrados 5 minutos antes de la solución de los agonistas μ. En los experimentos donde los fármacos se administraron exclusivamente por vía sistémica, se consideró la media de los tiempos de latencia de las dos medidas realizadas en cada pata. Sin embargo, cuando alguno de los tratamientos farmacológicos se administró por vía i.pl. con objeto de estudiar su efecto a nivel local, la media de las dos evaluaciones obtenidas en cada pata fueron analizadas de forma independiente.

2.4. Determinación de los efectos de la inhibición del receptor σ_1 en efectos no analgésicos de los opioides: hiperlocomoción e inhibición del tránsito intestinal

2.4.1. Evaluación de la hiperlocomoción inducida por morfina

Para evaluar el efecto de la morfina en la actividad locomotora horizontal de los animales, estos fueron monitorizados en cajas de evaluación provistas de detectores de infrarrojos (Med associated Inc., St Albans, VT, EE.UU.). Los animales fueron habituados en las cajas durante 90 minutos tras los cuales se administró la morfina o su solvente, y se evaluó la distancia recorrida por el ratón durante 30 minutos, comenzando el registro 30 minutos tras la administración del opioide.

2.4.2. Evaluación de la inhibición del tránsito intestinal inducida por opioides

Para estudiar el efecto de los opioides en el tránsito intestinal se utilizó el procedimiento previamente descrito (Chien y Pasternak, 1994), con pequeñas modificaciones. Tras 8 horas de ayuno (con acceso libre a agua), morfina, fentanilo, loperamida o sus solventes fueron administrados por vía s.c.; 30 minutos tras la inyección s.c. se administró vía oral 0.3 ml de carbón activado (0.5 g/ml). 30 minutos tras la administración de este último, es decir, 1 hora después de la administración del fármaco, los animales fueron sacrificados por dislocación cervical. El intestino delgado fue aislado y se midió la distancia recorrida por el carbón activado. En los experimentos en los que se asoció al tratamiento opioide un antagonista σ_1, este último se administró vía s.c. 5 minutos antes de la solución del opioide.

2.5. Estudio de la expresión del receptor σ_1 en diversas áreas del sistema nervioso mediante “western blotting”

Se realizaron determinaciones en muestras provenientes de diferentes áreas del sistema nervioso central y periférico implicadas en el dolor y la analgesia opioide. Concretamente estudiamos la expresión del receptor σ_1 en la amígdala basolateral (BLA), la médula rostroventral (RVM), la sustancia gris periacueductal (PAG), la zona dorsal del ensanchamiento lumbar medular (dSC), y los ganglios de la raíz dorsal (DRGs) de la médula espinal (a nivel de L4-L5, correspondientes a la inervación de la pata). Las muestras fueron homogeneizadas por sonicación en una solución tamponada, y la concentración de proteínas se midió mediante la técnica de Bradford. Las muestras fueron almacenadas a -80ºC hasta su uso. Se utilizaron muestras tanto de ratones salvajes como de ratones KO-σ_1 (usados como control de la especificidad del procedimiento).

Las proteínas de las muestras fueron separadas mediante electroforesis en geles de SDS-acrilamida (12 %) y transferidas a membranas de nitrocelulosa. Tras 1 hora de inmersión en una solución estándar de bloqueo, las membranas se incubaron durante toda la noche con un anticuerpo monoclonal de ratón frente al receptor σ_1 (1:1000, ref. sc-137075, Santa Cruz Biotechnology, Dallas, TX, USA) y con el anticuerpo de ratón frente a β-actina (1:2500, ref. sc-81178, Santa Cruz Biotechnology) utilizada como control de carga. Las membranas fueron lavadas (3x) para posteriormente ser incubadas con un anticuerpo secundario anti-IgG de ratón conjugado con peroxidasa (1:2500, ref. sc-2005, Santa Cruz Biotechnology). Las bandas se revelaron mediante una técnica de quimioluminiscencia (ECL Prime Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK). El análisis de las bandas se realizó con el programa Quantity One (Bio-Rad). Los datos se representaron como la razón de la intensidad de las bandas de σ_1 con respecto a las de β-actina.

2.6. Ensayos de fijación de radioligando

Los estudios de fijación se realizaron en la fracción sinaptosomal cruda (fracción P_2) obtenida de tejidos de cerebro, médula y piel plantar de la pata trasera según el protocolo descrito en trabajos previos de nuestro grupo de investigación (Cobos et al., 2005 y 2006; Entrena et al., 2009a y b). Las muestras de piel de pata fueron previamente congeladas en nitrógeno líquido para favorecer así su homogeneización, según se ha descrito con anterioridad (Baamonde et al., 2007).

Con el fin de descartar posibles cambios adaptativos en los receptores μ que pudieran ser responsables de los efectos observados en los ratones KO-σ_1, comparamos las propiedades de la fijación del radioligando μ [^{3}H]DAMGO (afinidad y/o número máximo de sitios de fijación reconocidos) en los tres tejidos objeto de estudio, tanto en ratones salvajes como KO-σ_1. Para descartar posibles efectos directos de los ligandos σ_1 sobre los receptores μ, estudiamos la afinidad de todos ligandos σ_1 utilizados en los experimentos comportamentales por los receptores μ marcados con [^{3}H]DAMGO. Estos últimos experimentos se realizaron únicamente en membranas cerebrales de ratones salvajes. Los ensayos de fijación de [^{3}H]DAMGO se realizaron según el protocolo previamente descrito (Narita et al., 2001).

De manera recíproca, se estudió la posible afinidad de los fármacos opioides (agonistas y antagonistas) por los receptores σ_1 marcados con el radioligando selectivo [^{3}H](+)-pentazocina. Estos ensayos se realizaron en membranas cerebrales de ratones salvajes siguiendo el protocolo descrito previamente por nuestro grupo (Entrena et al., 2009a y b). Todos los fármacos fríos (opioides o ligandos σ_1) fueron disueltos en agua ultrapura a excepción de loperamida, que fue disuelta en etanol. La concentración de etanol en el medio de incubación no superior al 0.1 % (vol/vol).

3. RESULTADOS Y DISCUSIÓN

Los resultados de esta Tesis Doctoral han sido publicados en Neuropharmacology y Journal of Pharmacology and Experimental Therapeutics (Sánchez-Fernández et al., 2013 y 2014), como se indica en el Capítulo “Published Papers”. Para la elaboración de este resumen hemos combinado ambos artículos, para facilitar la comprensión de nuestro trabajo.

3.1. Comparación de la respuesta dolorosa frente a un estímulo mecánico romo en ratones salvajes y knockout σ_1

Como esperábamos, la latencia de respuesta disminuyó conforme aumentó la intensidad del estímulo mecánico romo (desde 100 a 600 g). Los valores registrados en ratones salvajes y KO-σ_1 fueron muy similares, no mostrando diferencias significativas a ninguna de las intensidades de estimulación evaluadas. Estos datos concuerdan con la ausencia de diferencias entre ratones salvajes y KO-σ_1 en la respuesta comportamental frente a un estímulo mecánico de carácter puntiforme (p.ej. de la Puente et al., 2009; Entrena et al., 2009a y b; Nieto et al., 2012), y amplían la descripción del fenotipo sensorial de los ratones KO-σ_1 utilizando un tipo diferente de estimulación mecánica.

Cabe destacar que la latencia de respuesta usando una intensidad de 450 g fue de en torno a 1-2 segundos en ambos genotipos, por lo que se seleccionó para el resto de experimentos, ya que proporcionaba una ventana amplia para poder valorar el efecto antinociceptivo de los fármacos a evaluar.

3.2. Comparación del efecto de la administración sistémica de agonistas opioides μ de acción central en ratones salvajes y knockout σ_1

El efecto antinociceptivo de los opioides fue evaluado como el incremento en el tiempo de latencia de respuesta con respecto a los ratones tratados con sus solventes, al aplicar el estímulo doloroso de 450 g. La administración s.c. de los analgésicos opioides fentanilo (0.04-0.32 mg/kg), oxicodona (0,75-4 mg/kg) o morfina (0.5-16 mg/kg) indujo un efecto antinociceptivo dosis dependiente en ratones salvajes. Sin embargo, la administración de buprenorfina (0,06-0.48 mg/kg, s.c.) indujo un efecto antinociceptivo estadísticamente significativo solo a la dosis más alta en estos ratones, mientras que en el caso de tramadol (5-40 mg/kg, s.c.) no se observó efecto alguno. Estos resultados indican un alto grado de exigencia de este modelo comportamental para la detección de efectos antinociceptivos. Cuando las mismas dosis de estos fármacos opioides fueron evaluadas en ratones KO-σ_1 se observó un marcado aumento de sus efecto antinociceptivos, de modo que la dosis de fármaco necesaria para obtener un efecto significativo en ratones KO-σ_1 fue menor que en ratones salvajes, obteniéndose en todos los casos un desplazamiento hacia la izquierda de las curvas dosis-respuesta en los ratones KO-σ_1. Además, el efecto antinociceptivo conseguido con las dosis más elevadas de los fármacos estudiados fue marcadamente superior en los ratones KO-σ_1 en comparación con los valores obtenidos en ratones salvajes.

Por lo tanto, nuestros resultados demuestran que la inhibición genética del receptor σ_1 potencia la antinocicepción mecánica inducida por la administración sistémica de diversos agonistas µ usados en clínica como analgésicos, sin alterar la respuesta comportamental en ausencia de tratamiento con agonistas opioides. Este aumento del efecto antinociceptivo ante un estímulo mecánico registrado en los ratones KO-σ_1 contradice lo observado por otros autores, cuyos resultados mostraban la ausencia de modulación de la analgesia opioide en los ratones KO-σ_1 al aplicar un estímulo de naturaleza térmica (Vidal-Torres et al., 2013). Estas diferencias podrían atribuirse a la modulación diferencial de los mecanismos neuroquímicos que subyacen a la antinocicepción mecánica y térmica inducida por opioides en los ratones mutantes, ya que se han descrito diferencias en los mecanismos de la analgesia opioide según el tipo de estimulación sensorial (Kuraishi et al., 1995; Wegert et al., 1997).

3.3. Efecto de la administración de antagonistas selectivos σ_1, y de su asociación con la administración sistémica de agonistas opioides µ de acción central en el dolor nociceptivo producido por un estímulo mecánico en ratones salvajes

Con objeto de replicar farmacológicamente el incremento del efecto antinociceptivo observado en los ratones KO-σ_1, evaluamos los efectos de la administración sistémica (s.c.) de antagonistas σ_1 en la antinocicepción opioide.

Encontramos que el efecto de una dosis de morfina (4 mg/kg, s.c.) que produjo un efecto antinociceptivo mínimo en ratones salvajes fue incrementado de manera muy marcada por los antagonistas σ_1 BD-1063 (1-32 mg/kg, s.c.), BD-1047 (32 mg/kg, s.c.), NE-100 (4 mg/kg, s.c.) y S1RA (32 mg/kg, s.c.), confirmando que el incremento de la antinocicepción morfínica detectado en el ratón KO-σ_1 no es una peculiaridad de este ratón, sino que es extensible al bloqueo farmacológico del receptor σ_1. Además, el mínimo o nulo efecto antinociceptivo inducido por el tratamiento s.c. de dosis bajas de fentanilo (0,08 mg/kg), oxicodona (2 mg/kg), buprenorfina (0.24 mg/kg), o tramadol (40 mg/kg), también fue incrementado de manera marcada por el antagonista σ_1 BD-1063 (32 mg/kg, s.c.). El incremento por BD-1063 en la antinocicepción inducida por fentanilo (0,08 mg/kg, s.c.) fue replicado por el antagonista σ_1 S1RA (64 mg/kg, s.c.). Estos resultados muestran que el bloqueo farmacológico de los receptores σ_1 puede incrementar el efecto antinociceptivo de varios fármacos opioides usados en clínica como analgésicos. El mecanismo analgésico de los diversos opioides µ se solapa solo parcialmente (Ocaña et al., 1995; Sánchez-Blázquez et al., 2001; Pasternak, 2004; Smith, 2008; Pasternak y Pan, 2011; Raehal et al., 2011), por lo que la modulación por el bloqueo farmacológico de los receptores σ_1 de la antinocicepción inducida por una variedad de analgésicos opioides, es particularmente relevante con vistas a un posible uso clínico futuro de los antagonistas σ_1 como adyuvantes de los fármacos opioides.

La selectividad del antagonismo σ_1 en el incremento del efecto antinociceptivo inducido por los fármacos opioides evaluados fue confirmada mediante dos estrategias. En primer lugar, el incremento del efecto de los antagonistas σ_1 en la antinocicepción opioide (para todos los agonistas opioides evaluados) fue revertido completamente por la administración sistémica del agonista selectivo σ_1 PRE-084 (32 mg/kg, s.c.). En segundo lugar, el antagonismo farmacológico del receptor σ_1 (por BD-1063, BD-1047, NE-100 y S1RA) aumentó el efecto de la morfina (3 mg/kg) en los animales salvajes pero no en el caso de los ratones KO-σ_1. Esta estrategia ha sido utilizada anteriormente para demostrar la selectividad de los efectos farmacológicos (Petrus et al., 2007; Vidal-Torres et al., 2013; González-Cano et al., 2013), ya que si los fármacos carecen de efecto en animales desprovistos de su diana farmacológica, indica que no producen efectos adicionales en otras dianas que puedan explicar sus acciones.

Nuestros datos muestran sin lugar a dudas que el antagonismo farmacológico del receptor σ_1 es capaz de modular la antinocicepción opioide frente a estímulos mecánicos, extendiendo los datos previamente publicados sobre la potenciación opioide frente a estímulos térmicos inducida por la inhibición del receptor σ_1 (p.ej. Chien and Pasternak, 1993 y 1994; Marrazzo et al., 2011; Vidal-Torres et al., 2013).

A pesar de este marcado incremento en la antinocicepción opioide inducido por la administración sistémica de antagonistas σ_1, estos no alteraron las respuestas comportamentales de los animales frente al estímulo mecánico romo en ausencia del tratamiento con opioides. Esto concuerda con la ausencia de efecto de los antagonistas σ_1 frente al dolor nociceptivo inducido por estímulos mecánicos puntiformes descrito previamente (p.ej. Entrena et al., 2009a y b; Romero et al., 2012; Nieto et al., 2012), e indica, junto con los datos obtenidos en ratones KO-σ_1, la ausencia de modulación del dolor agudo nociceptivo mecánico por la inhibición del receptor σ_1 en ausencia de opioides.

3.4. Efectos de la inhibición del receptor σ_1 en la antinocicepción inducida por la loperamida

Además de los opioides analgésicos anteriormente nombrados, estudiamos el efecto del agonista opioide periférico loperamida en una situación control y de inhibición del receptor σ_1. En concordancia con estudios previos, la administración s.c. de loperamida (1-4 mg/kg) no indujo ningún efecto en la nocicepción aguda frente al estímulo mecánico en ratones salvajes (Menendez et al., 2005; Sevostianova et al., 2005). Sin embargo, los ratones KO-σ_1 mostraron un marcado incremento de la latencia de respuesta al ser tratados con este agonista opioide periférico. Este efecto antinociceptivo fue mayor incluso que el obtenido en ratones salvajes tratados con opioides mayores como fentanilo, morfina u oxicodona, lo que sugiere un marcado incremento de la acción opioide periférica en estos ratones mutantes.

El bloqueo farmacológico sistémico (s.c.) del receptor σ_1 por la administración de BD-1063 (32 mg/kg) o S1RA (64 mg/kg,) fue capaz de desenmascarar un marcado efecto antinociceptivo por la administración de loperamida (4 mg/kg, s.c.) en ratones salvajes. Este efecto de los antagonistas σ_1 fue mediado por la interacción con su diana farmacológica, ya que fue revertido completamente por la administración s.c. del agonista σ_1 PRE-084 (32 mg/kg).

Estos resultados sugieren que el receptor σ_1, además de modular a nivel central el efecto antinociceptivo de los opioides frente a estímulos térmicos, como se había descrito previamente (King et al., 1997; Pan et al., 1998; Mei y Pasternak, 2002 y 2007; Marrazzo et al., 2006), es capaz de modular el efecto antinociceptivo opioide a nivel periférico frente a estímulos mecánicos.

3.5. Contribución de los receptores opioides periféricos a la antinocicepción inducida por la administración sistémica (subcutánea) de agonistas opioides μ en condiciones normales y de inhibición del receptor σ_1

Dado el incremento por la inhibición del receptor σ_1 de la antinocicepción inducida por loperamida, los siguientes experimentos fueron dirigidos a evaluar la contribución de los receptores opioides periféricos en la antinocicepción inducida por los diversos agonistas μ evaluados en este estudio, tanto en una situación control como durante la inhibición del receptor σ_1. Para ello, realizamos experimentos de asociación de los distintos agonistas opioides con el antagonista opioide periférico naloxona metiodida.

La administración de naloxona metiodida (2–8 mg/kg, s.c.) a ratones salvajes tratados con dosis analgésicas de fentanilo (0.16 mg/kg, s.c.), morfina (16 mg/kg, s.c.) o buprenorfina (0.48 mg/kg, s.c.), no alteró el tiempo de latencia de respuesta de los animales. Estos datos concuerdan con la visión clásica de la producción de analgesia opioide a nivel central, en la que los receptores opioides periféricos juegan un papel mínimo (Greenwood-Van Meerveld y Standifer, 2008; Joshi et al., 2008; Thomas et al., 2008; Khalefa et al., 2012). Sin embargo, la antinocicepción inducida por oxicodona (4 mg/kg, s.c.) en ratones salvajes sí fue revertida por naloxona metiodida, aunque parcialmente, y únicamente a la dosis más alta evaluada de este antagonista opioide periférico (8 mg/kg, s.c.). Esta reversión parcial del efecto de la oxicodona es consistente con datos clínicos que sugieren que parte de los efectos analgésicos de la oxicodona pueden producirse a nivel periférico (Olesen et al., 2010).

En cambio, la asociación del antagonista opioide con penetrabilidad central naloxona (0.5 mg/kg, s.c.) revirtió completamente el efecto antinociceptivo inducido por fentanilo, morfina, buprenorfina u oxicodona.

Estos datos indican que en nuestras condiciones experimentales, el efecto antinociceptivo de los agonistas opioides en ratones salvajes es debido principalmente a acciones centrales, con la excepción de la oxicodona que mostró cierta sensibilidad al antagonismo de los receptores opioides periféricos.

Para estudiar la participación de los receptores opioides periféricos en el incremento de la antinocicepción opioide durante la inhibición del receptor σ_1, utilizamos dosis sistémicas de agonistas opioides que producen un marcado tiempo de latencia tanto en ratones KO-σ_1 como en ratones salvajes tratados con el antagonista σ_1 BD-1063 (32 mg/kg, s.c.), pero con un efecto mínimo o nulo en ratones control. El incremento antinociceptivo por la inhibición σ_1 (genética o farmacológica) observado en animales tratados s.c. con fentanilo (0.08 mg/kg), oxicodona (2 mg/kg), morfina (4 mg/kg), buprenorfina (0.24 mg/kg), tramadol (40 mg/kg) o loperamida (4 mg/kg) fue revertido completamente, y en todos los casos, por una dosis baja de naloxona metiodida (2 mg/kg). Los resultados obtenidos con BD-1063 asociado a fentanilo o loperamida fueron replicados por el tratamiento con otro antagonista σ_1, el S1RA (64 mg/kg, s.c.).

Esta sensibilidad al antagonismo opioide periférico durante la inhibición del receptor σ_1 indica que cuando el agonismo opioide y la inhibición σ_1 se realizan sistémicamente, el incremento en el efecto antinociceptivo ocurre principalmente a nivel periférico. Nuestros resultados no contradicen los datos que habían sido publicados hasta ahora, en los que se describe el papel modulador del receptor σ_1 de la analgesia opioide a nivel central (King et al., 1997; Pan et al., 1998; Mei y Pasternak 2002 y 2007; Marrazzo et al., 2006), aunque demuestran la gran importancia del receptor σ_1 en la modulación de la analgesia opioide periférica.

3.6. Efectos de la inhibición local (intraplantar) del receptor σ_1 en la antinocicepción mecánica inducida por la administración sistémica de agonistas opioides μ

El paso siguiente fue evaluar si la inhibición del receptor σ_1 a nivel local era suficiente para conseguir un aumento en la antinocicepción mecánica inducida por agonistas opioides μ administrados por vía sistémica. Para ello, administramos a ratones salvajes por vía sistémica (s.c.) las mismas dosis de agonistas opioides que producían escaso o nulo efecto antinociceptivo, descritas en el apartado anterior, e inyectamos i.pl. los antagonistas selectivos σ_1. La administración local de BD-1063 (50-200 μg) a animales tratados sistémicamente con fentanilo, incrementó de manera dosis-dependiente el tiempo de latencia en la pata inyectada con el antagonista σ_1, pero no en la pata contralateral, indicando que la potenciación del efecto antinociceptivo del opioide se estaba produciendo efectivamente a nivel local. Encontramos resultados similares asociando la administración local de BD-1063 (200 μg, i.pl.) con oxicodona, morfina, buprenorfina, tramadol o loperamida; en estos experimentos detectamos un incremento marcado en la latencia de respuesta al estimular al animal en la pata inyectada, pero no al evaluar al animal en la pata contralateral. Para asegurarnos de que este efecto no era debido a alguna peculiaridad del BD-1063, replicamos el experimento mediante la asociación de S1RA (200 μg, i.pl.) con la administración sistémica de fentanilo o loperamida, obteniendo resultados virtualmente idénticos a los obtenidos con BD-1063.

Este incremento del efecto antinociceptivo opioide producido por la administración local de antagonistas σ_1, fue revertido por la administración del agonista σ_1 PRE-084 (32 mg/kg, s.c.) en todos los casos, poniendo de manifiesto la especificidad del bloqueo local del receptor σ_1. Además, esta potenciación local de la antinocicepción opioide fue revertida por la administración de naloxona metiodida (2 mg/kg, s.c.), lo que confirma la participación de los receptores opioides periféricos en los efectos observados.

Por lo tanto, de estos experimentos se deduce que el bloqueo local del receptor σ_1 es suficiente como para incrementar marcadamente la función de los receptores opioides periféricos, resultando en una marcada antinocicepción opioide a nivel local tras el tratamiento con dosis bajas sistémicas de agonistas opioides.

3.7. Efecto de la inhibición del receptor σ_1 en los efectos antinociceptivos de la administración local (intraplantar) de morfina

Otra aproximación experimental utilizada fue comparar los efectos antinociceptivos frente al estímulo mecánico inducidos por la administración local de un opioide representativo, la morfina.

La administración local de morfina (50–200 µg, i.pl.) en ratones salvajes no indujo efecto antinociceptivo alguno. Estos resultados concuerdan con los estudios previos anteriormente mencionados en los que el efecto analgésico (en el dolor nociceptivo) de los opioides se atribuye a la activación de los receptores opioides a nivel central. Sin embargo, contrastan con resultados previos en los que la administración local de morfina es efectiva frente a estímulos térmicos (p.ej. Kolesnikov et al., 1996 y 2000), resaltando las diferencias entre los efectos opioides frente a estímulos mecánicos o térmicos.

Cuando administramos por vía i.pl. estas mismas dosis de morfina a ratones KO-$\sigma_{1,}$ observamos un incremento dosis dependiente muy marcado en el tiempo de latencia de la pata inyectada, alcanzando valores cercanos al tiempo de corte. El efecto de la administración i.pl. de morfina se produjo a nivel local, ya que en la pata contralateral de estos ratones no se observó ningún incremento detectable del tiempo de latencia de respuesta.

A la vista de estos resultados, nos planteamos si el antagonismo farmacológico local de los receptores σ_1 en ratones salvajes podría incrementar el efecto antinociceptivo mecánico de la morfina administrada localmente. Para ello, estudiamos las respuestas de animales salvajes coadministrados intraplantarmente con morfina (200 µg) y el antagonista σ_1 BD-1063 (12,5-200 µg). La asociación del antagonista σ_1 con la morfina indujo un aumento dosis dependiente del tiempo de latencia de respuesta en la pata inyectada, sin alterar los valores de la pata contralateral. Además, la administración i.pl. no sólo de BD-1063 (100 µg), sino también de otros antagonistas σ_1, tales como BD-1047 (50 µg), NE-100 (50 µg) o S1RA (100 µg), también incrementó marcadamente el tiempo de latencia de forcejeo de animales salvajes coadministrados con morfina (100 µg). Sin embargo, ninguno de estos antagonistas σ_1 fue capaz de incrementar la antinocicepción inducida por la coadministración local de morfina (100 µg) en ratones KO-σ_1. La reproducibilidad de los resultados con distintos antagonistas σ_1, junto con la ausencia de efecto de estos fármacos en ratones desprovistos de su diana farmacológica indica la especificidad de los efectos observados.

Estos resultados sugieren que el receptor σ_1 periférico inhibe tónicamente y de manera marcada la analgesia opioide periférica a estímulos mecánicos, y que bloqueando localmente al receptor σ_1 se puede conseguir un marcado efecto antinociceptivo incluso por la administración local de morfina.

3.8. Expresión del receptor σ_1 a nivel del sistema nervioso central y periférico

Los resultados obtenidos en nuestro trabajo ponen de manifiesto por primera vez la importancia de la modulación del receptor σ_1 en la antinocicepción opioide periférica. Con objeto de obtener una explicación anatómica para estos resultados, comparamos la expresión del receptor σ_1 en áreas del sistema nervioso implicadas en la analgesia opioide, a nivel supraespinal (BLA, RVM y PAG), espinal (dSC) y en el sistema nervioso periférico (DRG). En todas las muestras de ratones salvajes se pudo detectar una banda de inmunoreactividad en una posición ligeramente más elevada a la del marcador de peso molecular de 25 kDa, lo que concuerda con el peso molecular del receptor σ_1 de ratón (28 kDa) (Pan et al., 1998). A pesar de que esta banda fue detectable en todas las áreas exploradas, la expresión del receptor σ_1 fue notoriamente más elevada (8-10 veces superior) en el DRG que en cualquier área del sistema nervioso central analizada. No detectamos la banda inmunoreactiva de 28 kDa correspondiente al receptor σ_1 en muestras de las mismas áreas procedentes de ratones KO-σ_1, lo que indica la especificad del anticuerpo anti-σ_1 empleado.

Esta mayor expresión del receptor σ_1 en el DRG en comparación con la obtenida a nivel del sistema nervioso central aboga por una participación importante del receptor σ_1 periférico en la nocicepción.

3.9. Modulación de efectos adversos opioides (hiperlocomoción e inhibición del tránsito intestinal) por la inhibición del receptor σ_1

Dado el marcado incremento en la antinocicepción opioide por la inhibición del receptor σ_1, nos preguntamos si ocurriría igual con otros efectos derivados de la administración de opioides. Exploramos dos efectos opioides no analgésicos diferentes: la hiperlocomoción, un efecto central observado en los ratones tras la administración de opioides (Hnasko et al., 2005), y la inhibición del tránsito intestinal, de origen principalmente periférico (Al-Hasani and Bruchas, 2011).

La administración de morfina (4-16 mg/kg, s.c.) indujo un incremento dosis dependiente de la actividad locomotora tanto en animales salvajes como en KO-σ_1. Sin embargo, los ratones de ambos genotipos mostraron valores similares en la distancia recorrida cuando recibieron el mismo tratamiento, sin diferencias significativas entre ellos.

Por otra parte, la inhibición del receptor σ_1 en ratones KO-σ_1 o mediante la administración de BD-1063 (32 mg/kg, s.c.) a ratones salvajes, no alteró la distancia recorrida en el intestino delgado por un bolo de carbón activo (como indicador del tránsito intestinal) con respecto a ratones control, registrándose valores de en torno a 30 cm en todos los casos. En cambio, la administración s.c. de morfina (1–8 mg/kg), fentanilo (0.04–0.16 mg/kg) o loperamida (0.125-1 mg/kg) indujo una inhibición dosis dependiente del tránsito intestinal y su efecto fue similar en ratones salvajes y KO-σ_1. Además, el bloqueo farmacológico del receptor σ_1 con BD-1063 (32 mg/kg, s.c.) tampoco modificó la inhibición del tránsito inducida por fentanilo o loperamida. La ausencia de modulación por la inhibición del receptor σ_1 de las alteraciones en el tránsito intestinal inducidas por los opioides ha sido replicada recientemente por un estudio realizado en paralelo (Vidal-Torres et al., 2013).

Por lo tanto, pese a que la inhibición del receptor σ_1 incrementa marcadamente la antinocicepción opioide (como se describe en los apartados anteriores), no incrementa otros efectos no analgésicos de los opioides. Estos efectos diferenciales de la inhibición del receptor σ_1 en la función opioide sugieren que estos receptores podrían expresarse en tipos concretos de neuronas implicadas en la transmisión del dolor pero no en otros procesos, o bien que los mecanismos de potenciación del efecto opioide por la inhibición del receptor σ_1 en distintos tipos neuronales es diferente. Independientemente de las razones de esta modulación diferencial de los efectos beneficiosos y adversos de los opioides por la inhibición del receptor σ_1, nuestros resultados sugieren que el bloqueo del receptor σ_1 podría incrementar el rango terapéutico de los opioides, potenciando la analgesia sin alterar otros efectos perniciosos derivados de su uso.

3.10. Ensayos de saturación de [^{3}H]DAMGO en membranas de médula espinal, cerebro y piel plantar de la pata trasera en ratones salvajes y KO-σ_1

Para descartar posibles cambios adaptativos en los receptores μ que pudieran ocasionar los efectos observados en los ratones KO-σ_1 (usados ampliamente en este estudio), comparamos la fijación del radioligando μ [^{3}H]DAMGO en la fracción P_2 obtenida de cerebro, médula espinal y piel plantar de ratones salvajes y KO-σ_1.

[^{3}H]DAMGO se unió de una forma saturable a las membranas obtenidas de cerebro y médula espinal de ratones salvajes y KO-σ_1. No se encontraron diferencias estadísticamente significativas entre los valores de la constante de disociación en el equilibrio (K_D) ni en el número máximo de sitios de fijación (Bmax) de este radioligando entre las muestras de membranas de cerebro o médula espinal proveniente de ratones salvajes y KO-σ_1, por lo que la ausencia del receptor σ_1 no altera la afinidad de este radioligando por el receptor μ ni el número de receptores μ en cerebro o médula espinal. Debido a la cantidad tan limitada de P_2 obtenida de piel plantar no se pudo realizar la curva de saturación completa en dichas muestras. Por lo tanto, para obtener una estimación del número máximo de sitios de fijación ($B_{\max}$) de [^{3}H]DAMGO en membranas piel plantar de ratones salvajes y KO-σ_1, usamos una única concentración saturante (20 nM) de este radioligando y comparamos los resultados con los obtenidos en membranas de cerebro y médula espinal. Tanto en ratones salvajes como en KO-σ_1 encontramos valores de fijación más elevados en las muestras provenientes de sistema nervioso central que de piel plantar, aunque sin encontrar diferencias significativas para el mismo tipo de muestra entre ambos genotipos.

Por lo tanto, nuestros resultados comportamentales no pueden explicarse por cambios de densidad o afinidad de los receptores opioides μ en los ratones KO-σ_1.

3.11. Afinidad de los ligandos σ_1 por el sitio de unión de [^{3}H]DAMGO, y de los fármacos opioides por el sitio de unión de [^{3}H](+)-pentazocina

Para descartar posibles efectos directos entre los fármacos σ_1 y el receptor μ, y de los fármacos opioides con el receptor σ_1, se realizaron ensayos de competición utilizando [^{3}H]DAMGO y [^{3}H](+)-pentazocina para marcar selectivamente los receptores μ y σ_1, respectivamente.

Como control de ligandos fríos con afinidad demostrada por los receptores μ y σ_1 usamos morfina y BD-1063, respectivamente. Morfina inhibió de manera concentración dependiente la fijación de [^{3}H]DAMGO, mientras que BD-1063 inhibió la fijación de [^{3}H](+)-pentazocina, el efecto de ambos fármacos fue concentración-dependiente y se produjo en el rango nM, como había sido descrito previamente (p.ej. Entrena et al., 2009b; Nakamura et al., 2013). Esto indica que en nuestras condiciones experimentales somos capaces de detectar fármacos con afinidad tanto por el receptor μ como por el receptor σ_1.

Los ligandos σ_1 BD-1063, BD-1047, NE-100, S1RA o PRE-084 no consiguieron desplazar la fijación de [^{3}H]DAMGO. Estos resultados confirman lo que ya se había descrito para algunos de estos ligandos en especies y/o tejidos diferentes (Matsumoto et al., 1995; Kim et al., 2010; Romero et al., 2012). Además, ninguno de los fármacos opioides utilizados en los estudios comportamentales (fentanilo, morfina, oxicodona, buprenorfina, tramadol, loperamida, naloxona o naloxona metiodida) inhibió de forma significativa la fijación de [^{3}H](+)-pentazocina. Entre estos fármacos opioides, sólo se había comprobado anteriormente que morfina y naloxona carecían de afinidad por el receptor σ_1 (Walker et al., 1990). Nuestro estudio muestra que esta falta de afinidad por el receptor σ_1 es extensible a muchos más ligandos opioides.

Por lo tanto, podemos descartar que la potenciación de la antinocicepción opioide inducida por los antagonistas σ_1 pudiera deberse a la unión de éstos a los receptores opioides μ, o por la unión de los fármacos opioides al receptor σ_1.

4. CONCLUSIONES

4.1. Conclusiones específicas

1) La inhibición del receptor σ_1 (en ratones KO-σ_1 o mediante el antagonismo farmacológico a nivel sistémico o local en ratones salvajes) no altera la respuesta nociceptiva frente a un estímulo mecánico romo.

2) El efecto antinociceptivo ante un estímulo mecánico, derivado de la administración sistémica de agonistas opioides μ usados en clínica como analgésicos (fentanilo, morfina, oxicodona, buprenorfina y tramadol) es incrementado tanto en ratones KO-σ_1 como por el bloqueo farmacológico sistémico del receptor σ_1 en ratones salvajes.

3) La potenciación de la antinocicepción opioide en ratones KO-σ_1, así como en ratones salvajes tratados sistémicamente con antagonistas σ_1, es extensible al agonista opioide periférico loperamida.

4) El efecto antinociceptivo frente a un estímulo mecánico tras la administración sistémica de analgésicos opioides a ratones salvajes en condiciones normales (en ausencia de la inhibición del receptor σ_1) es eminentemente central. Sin embargo, la potenciación de la antinocicepción opioide en ratones KO-σ_1, así como en ratones salvajes tratados sistémicamente con antagonistas σ_1, depende completamente de la activación de receptores opioides periféricos, puesto que es abolida por la naloxona metiodida.

5) El bloqueo farmacológico del receptor σ_1 a nivel local es suficiente para potenciar el efecto antinociceptivo periférico producido por la administración sistémica de fármacos opioides, tanto de los usados en terapéutica como analgésicos como de la loperamida.

6) Pese a que la administración local de morfina no produce ningún efecto antinociceptivo frente a un estímulo mecánico, es capaz de inducir un efecto marcado en ratones KO-σ_1 o incluso en ratones tratados localmente con antagonistas σ_1.

7) El receptor σ_1 se expresa en mayor medida en el sistema nervioso periférico (ganglio de la raíz dorsal espinal) que en diversas áreas centrales implicadas en la analgesia opioide (tanto a nivel supraespinal como espinal), lo que apoya el papel del receptor σ_1 en la nocicepción a nivel periférico.

8) La inhibición del receptor σ_1 no interfiere en otros efectos opioides diferentes de la antinocicepción, incluyendo la hiperlocomoción inducida por morfina (de origen central) y la disminución del tránsito intestinal inducidos por morfina, fentanilo o loperamida (de origen periférico).

9) No se producen cambios adaptativos en los ratones KO-σ_1 en cuanto a densidad o afinidad de los receptores opioides μ en cerebro, médula espinal o piel plantar, que contribuyan a explicar la potenciación de la antinocicepción opioide en estos animales.

10) Los ligandos opioides μ utilizados *in vivo* (fentanilo, morfina, oxicodona, buprenorfina, tramadol, loperamida, naloxona y naloxona metiodida) carecen de afinidad por el receptor $\sigma_{1.}$ Asimismo, los ligandos σ_1 utilizados (BD-1063, BD-1047, NE-100, S1RA y PRE-084) carecen de afinidad por el receptor μ. Por lo tanto, nuestros resultados conductuales no pueden explicarse por la interacción directa de los ligandos σ_1 con los receptores opioides μ, o de los ligandos opioides con los receptores σ_1.

4.2. Conclusión general

La inhibición del receptor σ_1, tanto a nivel sistémico como local, incrementa la antinocicepción opioide a nivel periférico frente a un estímulo mecánico, de lo que se deduce que el receptor σ_1 es un freno biológico a la analgesia opioide periférica. Esta potenciación de la analgesia opioide no conlleva un incremento de efectos no analgésicos centrales o periféricos (hiperlocomoción e inhibición del tránsito intestinal, respectivamente) derivados de la administración de opioides. Por lo tanto, el antagonismo σ_1 podría tener relevancia clínica como adyuvante, a nivel sistémico o local, para incrementar la analgesia opioide sin incrementar los efectos adversos de los opioides.

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INTRODUCTION

1. THE SOMATOSENSORY SYSTEM

The somatosensory system requires a complex acquisition y processing of information directed to the adaptation of living organisms to the external environment. It compress sensory receptors, which are specialized structures that assume the function of sensing the changes of their environment and transform them into nerve signals that provide to the central nervous system information from numerous internal and external events.

Somatic sensibility has four major modalities: discriminative touch (required to recognize the size, shape, and texture of objects and their movement across the skin), proprioception (the sense of static position and movement of the limbs and body), temperature sense (warmth and cold), and nociception (the signaling of tissue damage or chemical irritation, typically perceived as pain or itch). Nociception is the neural process whereby damaging or potentially damaging stimulus provokes a response mechanism to avoid (as much as possible) tissue damage, and therefore plays a pivotal role for the survival of the organism (Gardner et al., 2000).

The processing of external stimuli is initiated by the activation of diverse populations of cutaneous and subcutaneous receptors. The information is transmitted from the body surface to the central nervous system for its interpretation and finally for action. When peripheral sensory neurons are activated, they carry these signals to the spinal cord, and sensory information is then transmitted to the brain by several different ascending pathways through the spinal cord to the brainstem and thalamus to reach the primary somatosensory cortex. There are also descending systems which modulate (facilitating or inhibiting) the transmission of ascending pain signals. All these processes will be described further on.

1.1. Primary afferent fibers

Central nervous system receives the information of the environment and organism state through highly specialized sensory fibres. These fibers in the skin detect sensory stimuli of a diverse nature, such as thermal, mechanical and chemical. Electrophysiological studies reveal the existence of fast conducting nerve fibers, generally activated by innocuous mechanical stimuli (Aβ-fibers), and slower conducting fibers typically activated by painful stimuli (Aδ- and C-fibers). The speed of action potential transmition is determined by fiber diameter and degree of myelination (Handwerker, 2006; Meyer et al., 2008), as it will be described in detail below.

1.1.1. Aβ-fibers

Most Aβ-fibers are low-threshold mechanoreceptors (LTM) which detect pressure, stretch or hair movement, playing therefore an essential role for discriminative touch and proprioception. Aβ-fibers have distinct specialized endings which detect specific types of non-noxious mechanical stimuli, including Merkel's disks, Meissner's corpuscles, Ruffini's endings and Pacini's corpuscles (Hall, 2011a). These specialized endings are in marked contrast with the bare endings of nociceptive fibers (Gardner et al., 2000) (see Fig. 1.1). The Aβ fibers are characterized by a large diameter (6-12 µm), high myelination and the consequent rapid speed conduction (30-70 m/s) (Hall, 2011a). In fact, Aβ-fibers exhibit the highest speed conduction among afferent fibers (as shown in Fig. 1.2).

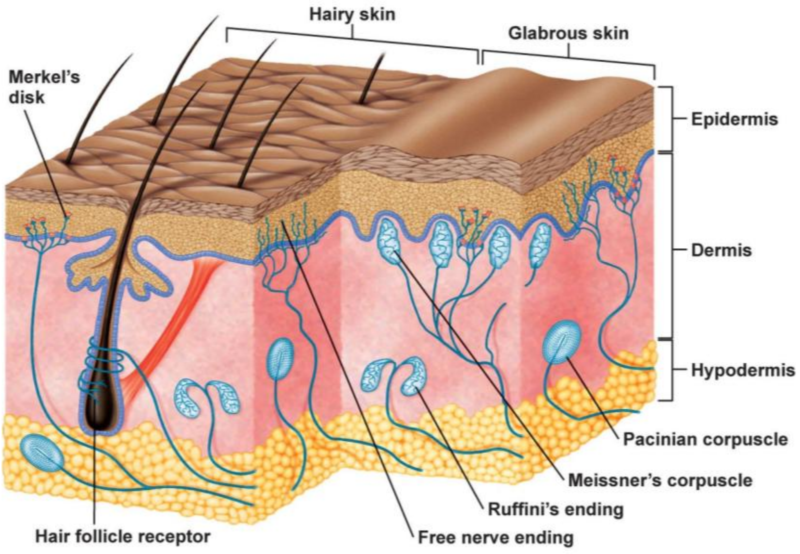

Hairy skin
Glabrous skin
Merkel's disk
Epidermis
Dermis
Hypodermis
Pacinian corpuscle
Meissner's corpuscle
Ruffini's ending
Free nerve ending
Hair follicle receptor

Fig. 1.1. Mechanoreceptors in hairy and glabrous skin of the human hand (taken from Stanfield and Germann, 2006).

Merkel’s disks are non-encapsulated nerve terminals whose endings have expanded disk-like termini in contact with Merkel cells (Gardner et al., 2000). These mechanoreceptors are localized in the basement membrane that separates the epidermis from the dermis layer of glabrous and hairy skin (Fig. 1.1). Merkel’s disks can be grouped in touch spots (or Iggo dome), a very sensitive structure specialized to transmit the information necessary to detect the textures of an object (Gardner et al., 2000; Boulais and Misery, 2008; Hall, 2011a). They are slowly adapting receptors, producing nerve impulses for the duration of the stimulus. They are most responsive to extremely low vibration frequencies (5-15 Hz), being responsible for providing stable signals for determining continuous mechanical stimuli on the skin (Gardner et al., 2000; Hall, 2011a).

Hair follicle receptors are nerve endings which form a network around the hair. In the hair follicle Merkel cells are also localized in the outer root sheath. These mechanoreceptors are rapid adaptation sensing the moving of the hair (Gardner et al., 2000; Boulais and Misery, 2008).

Meissner´s corpuscles are loosely encapsulated sensory receptors located in the dermal papillae (interdigitations of dermis into the epidermis) of glabrous skin (Fig. 1.1). When the corpuscle is deformed by light pressure, these nerve encapsulated terminals are stimulated. They can adapt very fast after initial stimulation (less than a second after being stimulated). They are particularly sensitive to the movement of objects on the surface of the skin being able to sense light touch stimuli. Also they are responsive to midrange (20-50 Hz) frequency vibration (Gadner et al., 2000; Hall, 2011a).

Ruffini’s endings are located in dermis (Fig. 1.1), especially in the palm, in firgernails and joints. These receptors sense stretch of the skin or bending of the fingernails as these stimuli compresses the nerve endings. Mechanical information sensed by Ruffini´s endings, particularly in the palms contributes to our perception of the shape of grasped objects. These nerve terminals adapt very slowly and consequently they are important for communicating a continuous state of deformation in the tissue. Also, Ruffinni endings located at the joints participate in the detection of the extent of joint rotation (Gadner et al., 2000; Hall, 2011a).

Pacini’s corpuscles are located below the skin deeper in the tissues (Fig. 1.1). They are an onion-like structure, measuring 0.5 × 1.0 mm. They are one of the body’s largest sensory receptors, having a diameter of 8 to 13 μm and conducting at 50 to 80 metres per second. The Pacini corpuscle is a rapidly adapting receptor. When subjected to sustained pressure, it produces an impulse only at the beginning and the end of the stimulus. They are especially important to detect vibration, the Pacini’s corpuscles have the lowest thresholds for high frequencies (~250 Hz) and deep pressure (Boulais and Misery 2008; Hall, 2011a).

A small population of Aβ-fibers exhibit high thresholds for mechanical stimuli and are therefore nociceptive neurons (e.g. Wenk et al., 2005). However, they are not as well studied as the Aβ-LTM and there is not much information about their biology (Priestley, 2009).

It is worth pointing out that in spite of the variety of the types of mechanical stimuli detectable by Aβ-fibers, they are insensitive to thermal stimuli.

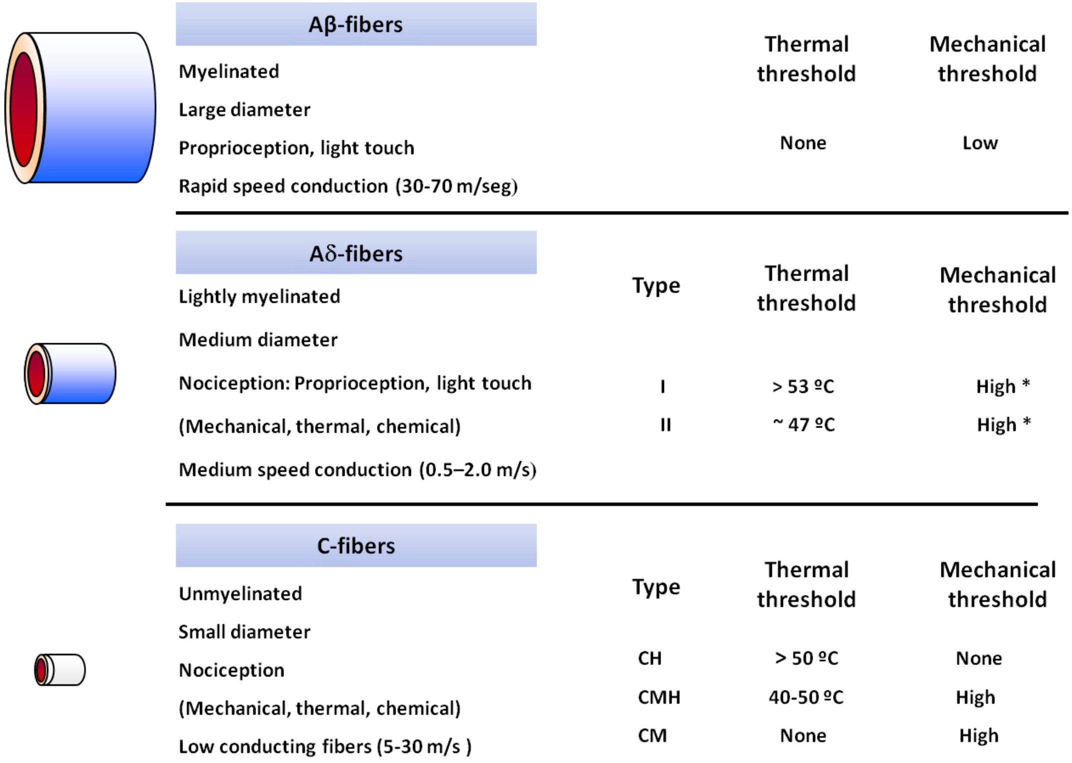
1.1.2. C- and Aδ-fibers

Noxious mechanical, chemical or thermal stimuli are detected by peripheral sensory neurons named nociceptors which, in contradistinction to the complexity of the nerve endings of Aβ-fibers, show bare nerve endings (see Fig. 1.1). The term nociceptor comes from the Latin *Nocere* (to harm or damage). It was introduced by Charles Sherrington (1897-1952) in a collection of ten lectures titled *The integrative Action of the Nervous System* (Sherrington, 1906), describing free afferent nerve endings able to be activated by painful stimuli. The neurophysiologist enunciated specific primary afferents with different properties to those of nerves devoted to the recognition of non-painful stimuli (reviewed by Handwerker, 2006).

Nociceptors are usually classified according to their anatomical features and physiological characteristics of their axons, such as diameter, myelination and conduction velocity (Fig. 1.2). C-fibers have small diameter (1µm), unmyelinated axons and are slowly conducting fibers with conduction velocities ranging between 0.5–2.0 m/s (depending on the specific C-subtype); Aδ-fibers are characterized by their thinly myelinated axons which support faster conduction velocities than C-fibers (typically between 5-30 m/s) (Fig. 1.2). It is thought that the fast onset of the first pain sensation is mediated by fast conducting Aδ-fibers, whereas the long latency of the second pain sensation is consistent with the activation of the slower conducting C-fibers (Schepers and Ringkamp, 2009). Receptive fields of nociceptors depend on the area covered by their branching into the skin, and this depends on nociceptor class, its location and the animal size (Handwerker, 2006). C-fiber branches are generally profuse, whereas the branches of Aδ-fibers cluster in separated small spots within a small area. These differences support that stimuli sensed by Aδ-fibers can be more precisely located than those detected by C-nociceptors (Farquhar-Smith, 2008; Dubin and Patapoutian, 2010).

1.1.2.1. Sensitivity of C- and Aδ-fibers to different types of sensory stimulation

Both C- and Aδ-nociceptors can be categorized attending to the kind of stimulus that activates them: mechanical (M), heat (H) and cold stimulus (Farquhar-Smith, 2008; Dubin and Patapoutian, 2010). However, they often do not respond exclusively to a single type of sensory stimulation, so overlapping neuronal populations of both C- and Aδ-nociceptors are responsible for temperature sensing and the transmission of noxious stimuli.



Aβ-fibers

Myelinated
Large diameter
Proprioception, light touch
Rapid speed conduction (30-70 m/seg)

Thermal threshold	Mechanical threshold
None	Low

Aδ-fibers

Lightly myelinated
Medium diameter
Nociception: Proprioception, light touch
(Mechanical, thermal, chemical)
Medium speed conduction (0.5–2.0 m/s)

Type	Thermal threshold	Mechanical threshold
I	> 53 ºC	High *
II	~ 47 ºC	High *

C-fibers

Unmyelinated
Small diameter
Nociception
(Mechanical, thermal, chemical)
Low conducting fibers (5-30 m/s)

Type	Thermal threshold	Mechanical threshold
CH	> 50 ºC	None
CMH	40-50 ºC	High
CM	None	High

Fig. 1.2. Classification and characteristics of peripheral afferent fibers (modified from Julius and Basbaum, 2001). *: if sensitive to mechanical stimuli.

According to their sensitivity to mechanical stimuli, they are termed as mechanical sensitive afferents (MSAs) and mechanically insensitive afferents (MIAs). In the initial classification, all MIAs were erroneously referred as silent nociceptors (Meyer et al., 2008) since their responsiveness to thermal stimuli was not initially taken into account for this classification. Further studies subclassified both MSAs and MIAs attending to their sensitivity to heat stimuli. MSAs can also respond to both mechanical and heat stimuli (mechano-heat sensitive nociceptors, MH) or exclusively to mechanical stimulations (M) (Dubin and Patapoutian, 2010). Usually, if a fibre responds to heat and mechanical stimuli, it will be activated by chemical stimuli as well. Thus C- and Aδ-nociceptors sensitive to mechanical and thermal stimuli may also be referred as *polymodal* nociceptors (Davis et al., 1993). MIAs, can be sensitive to heat (heat nociceptors), or might do not respond to either mechanical or heat. In this latter case, they are properly defined as silent nociceptors (mechanically insensitive and heat insensitive afferents, MiHi) (Dubin and Patapoutian, 2010, Meyer et al., 2008), although they might develop sensitivity to noxious mechanical or heat stimuli after tissue injury or inflammation, when they are sensitized by inflammatory mediators (Farquhar-Smith, 2008; Dubin and Patapoutian, 2010).

The sensitivity of primary afferents to cold stimulus has been much more recently explored than their sensitivity to mechanical and heat stimuli. It seems clear that noxious cold is also sensed by mixed populations of both C and Aδ-nociceptors. Noxious cold stimuli cause distinct sensations such as pricking, burning and aching (Davis and Pope, 2002; Harrison and Davis, 1999), which suggest that different fiber populations are activated by these stimuli. In fact, when Aδ-fibers are blocked (by ischemic-compression) and C-fibers are active, the cold pain is experienced as a burning sensation, and the pricking sensation is greatly reduced (Davis, 1998). This suggests that the sensory experience induced by noxious cold stimuli depends on the integration of the information provided by both thinly myelinated and unmyelinated fibers.

Although both C- and Aδ-nociceptors are able to detect mechanical and thermal stimuli, they strongly differ in the proportion of fibers responsive to these types of sensory stimuli, thermal and mechanical thresholds, conduction velocity and several other particular characteristics that clearly distinguish these two types of primary afferents.

1.1.2.1.1. Subtypes of C-fibers

The majority of C-fibers can be activated by heat (Priestley et al., 2009, Meyer et al., 2008). Heat thresholds for activating C-fibers in hairy skin range between 37 and 49 ºC, which includes temperatures from warm to painful heat (45 ºC and above). This indicates that C-fibers code for both warm sensations and heat pain (Schepers and Ringkamp, 2009).

Most fibers exclusively responsive to heat (CHs) express TRPV1 (Lawson et al., 2008). However, most of the heat responsive C-fibers are also able to respond to mechanical stimuli (CMHs) (Lawson et al., 2008) and are therefore included under the category of polymodal receptors (Handwerker, 2008; Lawson et al., 2008). Mechanosensitive C-afferents are responsive to several types of mechanical stimuli, including von Frey hair stimulation and blunt pressure (Handwerker, 2006; Beissner et al., 2010). Although both CHs and CMHs are able to respond to heat stimuli, their thermal threshold differ, since CH fibers usually respond at higher thresholds (~48 ºC and above) than CMHs, and are therefore more likely to code for painful heat rather than for non-noxious heat (Fig. 1.2). The response to heat stimuli increases with the temperature of stimulation, and therefore, they can code for stimulus intensity (Schepers and Ringkamp, 2009; Dubin and Patapoutian, 2010). Most of the C-fibers responsive to cold stimuli fall in the CMH category, and their threshold temperatures vary over a wide range with values above 20 ºC to values below 10 ºC, and there is a positive correlation between discharge frequency of these C-fiber afferents and the absolute value of cold temperature stimulus (Campero et al., 1996).

A smaller population of C-nociceptors (10-15%) responds only to mechanical nociceptive stimuli (CMs), being their mechanical threshold similar than for CMHs (Fig. 1.2) (Dubin and Patapoutian, 2010). Other types of mechanosensitive C-fibers have been described, and are not nociceptive fibers. These units are termed C-tactile afferents (CT). They respond to innocuous stroking stimulus of hairy skin, but do not respond to heat or chemical stimulation (Olausson et al., 2008; McGlone and Reilly, 2010). These tactile fibers are present in hairy skin of the forearm and in the thigh, and they have never been reported in glabrous skin (Nordin, 1990; Vallbo et al., 1993 and 1999; Edin, 2001). CT fibers have a conduction velocity around 0.6-1.2 m/s (Vallbo et al., 1993), small receptive fields (only a few millimetres in diameter), and respond to forces of 250 mg or lower (Wessberg et al., 2003). Therefore, tactile sensibility in hairy skin is mediated by not only by Aβ-fibers but also by slowly conducting unmyelinated low-threshold mechanoreceptive C-afferentes that respond preferentially to light touch.

1.1.2.1.2. Subtypes of Aδ-fibers

As for C-fibers, A-afferent nociceptors can be classified into afferents sensitive to heat (but not to mechanical) stimuli (AH), and MSAs fibers, which include mechano-heat nociceptors (AMH), and nociceptors sensitive only to mechanical stimuli (AM). In addition to this classification, the heat sensitive A-fibers (AHs and AMHs) can be classified according to their heat threshold into type I and type II A-nociceptors.

Type I have a relatively high-threshold to heat stimuli (median threshold >53 ºC). However, if a lower heat stimulus is maintained over time these fibers can be activated. Sensitization conditions, such as after burn injury, can also decrease the heat threshold of Type I A-fibers (Treede et al., 1995; Basbaum et al., 2009). Type II fibers have lower heat threshold (~47 ºC) than type I nociceptors (Fig. 1.2). The areas innervated by type I and II neurons also differ: whereas type I afferents are present in glabrous and hairy skin, type II fibers are exclusively present in hairy skin which may explain the lack of first heat pain from the palm of the hand (Treede et al., 1995; Basbaum et al., 2009; Schepers and Ringkamp, 2009).

Most heat responsive Aδ-fibers fall in the type I category (Dubin and Patapoutian, 2010). This population of A-fibers respond to stepped heat stimuli with short latency and vigorous burst activity. They have a fast conduction velocity (13.3 ± 0.8 m/s) and mediate the first acute pain response to noxious heat (Dubin and Patapoutian, 2010). However, MSA-nociceptors mediate the first pain induced by pinprick and other intense mechanical stimulus (Basbaum et al., 2009). The group of MSAs unresponsive to heat (AMs) are the most abundant (15-50%) of Aδ-fibers, but there is a significant population sensitive to both mechanical and heat stimuli (AMHs). Most AMHs show a type I thermal threshold (AMHI), although there are also some AMH type II nociceptors (AMHII). The conduction velocity of AMHI is faster than any other A-nociceptive subtype (Treede et al., 1995, Dubin and Patapoutian, 2010; Meyer et al., 2008). The mechanical threshold among the types of AMHs differs, and it is lower for AMHI than for AMHII. Therefore, although AMHs are sensitive to both mechanical and thermal stimuli, AMHIs show a preferential sensitivity to mechanical stimulus and

AMHIIs for thermal stimulation. As for C-fibers, most A-fibers responsive to cold are also MHs (polymodal). They can be activated by noxious cold being their response proportional to the stimulus intensity (Simone and Kajander, 1996 and 1997).

1.1.3. Dorsal root ganglia (DRGs): classification of DRG neurons by size and molecular markers

The dorsal root ganglia (DRGs) contain the cell bodies of primary afferent neurons and they are localized just lateral and along the spinal cord (outside the CNS). On the other hand, somatosensory information from cranial structures (the face, lips, oral cavity, conjunctiva, and dura mater) is transmitted by the trigeminal sensory neurons grouped in trigeminal ganglions. Although DRGs and trigeminal ganglions could be consider equivalent (Gardner et al., 2000), they exhibit differences (see Price and Flores, 2007; Hu et al., 2013) which will not be described in detail in this chapter. In this section it will be described the morphological features of the different types of DRG neurons, as well as the molecular markers which characterize them. These molecular markers are thought to be involved in the transduction of the different modalities of sensory information and can be used therefore to classify DRG neurons depending on their responsiveness.

The DRG neurons are pseudounipolar neurons. Both the peripheral terminal and the central axonal branch emanate from a common axonal stalk. The peripheral branch innervate the target (skin, organs, muscles and joints) and the central axonal branch synapse with second order neurons in the spinal cord to transmit the sensory information collected from the periphery to the central nervous system (Basbaum et al., 2009; Priestley, 2009). However, it is worth pointing out that peripheral terminals have not exclusively a mere receptive function, since they can release a variety of molecules able to influence local tissue environment under some circumstances. This can be exemplified by the vasodilatation and extravasation of plasma proteins induced by nociceptor activity (neurogenic inflammation) (Basbaum et al., 2009).

DRG neurons can be classified in three different populations by the size of their soma: large, intermediate and small. Electrophysiological studies showed that large DRG neurons are associated with Aβ-fibers, intermediate size neurons with Aδ-fibers, and the smaller neurons with C-fibers (reviewed by Willis and Coggeshall, 2004). The molecular properties of each of these neuronal populations are heterogenous and related to their sensitivity to the different sensory stimuli, since these molecular markers include channels and other molecules involved in sensory transduction (Basbaum et al., 2009; Gardner et al., 2000).

Among all molecular markers of DRG neurons, the better studied is probably the transient receptor potential (TRP) cation channels family, in particular TRPV1 receptors. A high percentage of the small diameter DRG neurons express TRPV1 (Caterina et al., 1997; Tominaga and Caterina, 2004; Priestley, 2009). This channel is sensitive to heat stimuli, and it is thought to be one the main heat sensors, being their thermal activation threshold of ~43°C. Capsaicin and related vanilloid compounds are also able to open these channels producing a burning sensation (Priestley, 2009).

The TRPV1 positive DRGs neurons are heterogeneous, and they express a variety of other molecular markers. Thus, three different populations can be distinguished within DRG neurons. About half of the TRPV1 positive DRG small neurons are peptidergic neurons since they express calcitonin-gene related peptide (CGRP) and might co-express other neuropeptides such as substance P (SP), somastotatin and galanin, among others (see Fig. 1.3). Among the other molecular markers present in this neuronal population, TRPA1 has received much attention in the last years. This receptor is expressed in small diameter neurons mostly in a subset of TRPV1-expressing cells (Tominaga and Caterina, 2004; Kobayashi et al., 2005). TRPA1 channels are sensitive to electrophilic chemical irritants, such as allyl-isothiocyanate (mustard oil) and other pungent chemicals including allicin (from garlic), cinnamaldehyde (cinnamon), methysalicylate (wintergreen), eugenol (cloves), and gingerol (ginger) (Kwan et al., 2006; Basbaum et al., 2009). It is thought that TRPA1 receptor acts as a cooling sensor, and its cold threshold for activation is 17 ºC (Kwan et al., 2006). Although it has been proposed to also play a role in mechanoperception, this issue remains currently unclear (Bautista et al., 2006; Petrus et al., 2007; Kwan et al., 2006 and 2009).

Another significant population of small DRG neurons are TRPV1 negative neurons. This population corresponds to nonpeptidergic neurons since they do not contain neuropeptides, but they can be identified typically by the *Griffonia simplicifolia* isolectin (IB4). Although in the mouse it is clear that there is almost no overlap between TRPV1 and IB4 expressing neurons (Zwick et al., 2002; Lawson et al., 2008), in the rat the differentiation between these populations is not that straightforward, since there is a significant overlap between these markers (e.g. Zwick et al., 2002).

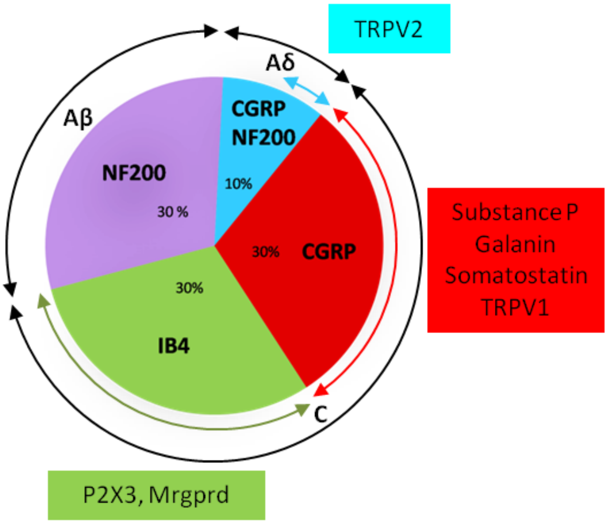


Fig. 1.3. Pie chart summarizing the main neurochemical populations of mouse DRG neurons (Taken from Priestley, 2009 with modifications). There is a varying percentage of C-nociceptors, not represented in the figure, that simultaneously express IB4 and CGRP/TRPV1 (see text for details).

IB4 positive neurons might express distinct sensory transducers, such as P2X3, Mrgprd (Mas-related G-protein-coupled receptor subtype d) and TRPM8 (Zwick et al., 2002; Kobayashi et al., 2005; Cavanaugh et al., 2009). 87% of these nonpeptidergic neurons are P2X3-positive in the mouse (Zwick et al., 2002). This receptor responds to purinergic compounds associated with injury, such as ATP (Chen et al., 1995). Mrgprd is also expressed in mice IB4 positive neurons, constituting 90% of all nonpeptidergic cutaneous C-fibers. It is thought that Mrgprd expressing cells play a major role in the transduction of mechanical stimuli (Cavanaugh et al., 2009). TRPM8 is expressed in only a small subset of TRPV1 negative small neurons. This receptor is another member of the TRP family, and as TRPA1, it has been proposed as a cold sensor. This channel is activated by cool temperatures in a subset of neurons also different to TRPA1-expressing cells. TRPM8 threshold is of 23-25ºC, which is higher than for TRPA1, and this temperature feels cool but not painfully cold (Bandell et al., 2004; Kwan et al., 2006). This point to that TRPM8 plays a role in the coding of non-noxious cold, but it is not essential for cold nociception (Bautista et al., 2007). This channel can also be activated by chemicals such as menthol, spearmint and icilin, which induce a cold sensation when are applied to the skin (Bandell et al., 2004; Kwan et al., 2006).

Other major population of DRG neurons is constituted by NF200-expressing neurons (Fig. 1.3). NF-200 is considered to be a marker for mechanosensory myelinated neurons. This marker is expressed in both large and medium size neurons but not in small diameter DRG neurons. Most medium size neurons (Aδ nociceptors) also express CGRP and another member of the TRP family, the TRPV2. This receptor (as TRPV1) can be activated by heat, but with a much higher threshold (53ºC). It has been suggested that TRPV2 is the heat transducer for type I A-nociceptors (Caterina et al., 1999; Leffler et al., 2007). Unlike TRPV1, this channel is not activated by capsaicin (Caterina et al 1999; Leffler et al., 2007). Some Aδ nociceptors also express molecular markers initially thought to be restricted to C-nociceptors, such as TRPV1, which is purportedly the heat sensor present in AMHII neurons (Dubin and Patapoutian, 2010; Mitchell et al 2010; Abraham et al., 2011), and also TRPM8 (Kobayashi et al., 2005; Bautista et al., 2007).

The population of large DRG neurons expresses other TRP receptor members, such as TRPC1, 3 and 6. These receptors are thought to play an important role sensing light touch (Garrison et al., 2011; Quick et al., 2012). In addition to these molecules, Piezo 2 (a stretch-activated ion channel) has been recently identified as a mechanotransducer in

NF-200 expressing DRG neurons, although it is unclear if they participate in sensing mechanical pain, touch or both (Coste et al., 2010; Nilius and Honoré, 2012).

The expression patterns of the molecules described above might change under some circumstances, contributing to the adaptive response of the somatosensory system to pathological pain conditions. Examples of these adaptive changes are the abnormal expression of SP (normally exclusively expressed by nociceptors) by Aβ-fibers during inflammation or nerve injury (Boulais and Misery, 2008), and the expression of TRPV1 receptors by IB4 positive neurons induced by inflammation (Breese et al., 2005). These adaptive changes in the molecular characteristics of primary sensory neurons will not be described in detail in this chapter.

1.2. Spinal Cord

A transverse section of the spinal cord shows white matter, composed of ascending and descending axons, most of which are myelinated, and gray matter, composed of neuron bodies and glial cells. The gray matter is located centrally, with two dorsal horns and two ventral horns (reviewed by Millan, 1999). The spinal cord dorsal horn has a very important role in the nociceptive transmission since it is an integrative area that receives inputs from primary afferents that innervate the skin and deeper tissues of the body. These primary fibers synapse in the dorsal horn with second-order neurons which send the sensory information to supraspinal structures through the ascending pathways, and also with the descending modulatory pathways originated from supraspinal sites (reviewed by Millan, 1999 and Todd, 2010). Interneurons are also present in the spinal cord, and they play a major role in the modulation of the ascending and descending sensory information. Their function will be described in the Section 1.5.1.

1.2.1. Laminae organization of the spinal cord and primary afferent fibers received in the dorsal horn

Rexed divided the grey matter of the cat dorsal horn into 10 distinct laminae based on variations in the size and packing density of neurons in 1952, and since that date this scheme has been applied to other species. Following the diagram made by Rexed, the dorsal horn was constituted by laminae I (marginal layer), laminae II (substantia gelatinosa), III and IV (nucleus propius) and V and VI (deep layers). Lamina VII corresponds to the intermediate grey matter, laminae VIII and IX comprise the medial and lateral ventral horn, respectively, and lamina X is the region surrounding the spinal canal (Todd, 2010).

Due to the importance of the dorsal horn in both normal sensory processing and in pathological conditions, many studies have been made to discern the functionality of the different laminae and the type of fibers that synapse with them. In general, myelinated low-threshold mechanoreceptive afferents (Aβ-fibers from hair follicles and skin) arborize in an area extending from the inner portion of lamina II (IIi) to lamina V. A branch of Aβ-fibers ascends without synapting with second order neurons in the spinal cord, to constitute the dorsal columns, as it will be described in the Section 1.3.

On the other hand the great majority of nociceptive primary afferent fibers terminate in the superficial dorsal horn to synapse with second order neurons. Aδ and C peptidergic afferents innervate lamina I and the outer part of lamina II (IIo), while the nonpeptidergic C-afferents terminate in the inner part of lamina II (IIi) (Fig. 1.4) (Liu et al., 2007; Cavanaugh et al., 2009; Basbaum et al., 2009; Priestley, 2009; Todd, 2010). This is schematically presented in Fig. 1.4. However, the real situation is more complicated to what is shown in the diagram, since not all C- and Aδ- fibers terminate in the superficial layers of the dorsal horn, as some Aδ fibers terminate in lamina V, and C-fibers of visceral origin also terminate in laminae V–VII and X (Willis and Coggeshall, 1991; Byers and Bonica, 2001). In addition, recent studies have identified a group of cooling-specific C-afferents that terminate in lamina I (Dhaka et al., 2008).

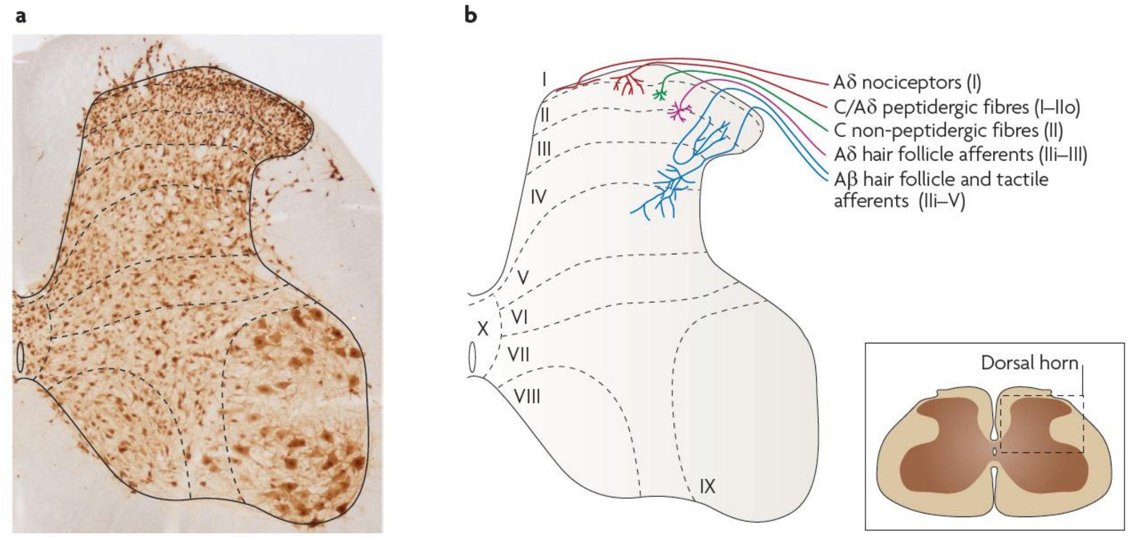

a
b
I
II
III
IV
V
VI
VII
VIII
IX
X
Aδ nociceptors (I)
C/Aδ peptidergic fibres (I–IIo)
C non-peptidergic fibres (II)
Aδ hair follicle afferents (IIi–III)
Aβ hair follicle and tactile afferents (IIi–V)
Dorsal horn

Fig. 1.4. Laminar organization of the spinal cord. (a) A transverse section of rat mid-lumbar spinal cord immunostained using an antibody (Anti-NeuN) that specifically labels neurons (b) Simplified scheme of the primary afferent inputs received in the dorsal horn (taken from Todd, 2010).

1.2.2. Second order neurons in the spinal cord

Primary afferents synapse with distinct populations of second order neurons in the spinal cord. Based on the response of second order neurons to nociceptive input, they can be classified into three subtypes: specific-nociceptive, wide-dynamic range and non-nociceptive neurones (reviewed by Schaible and Grubb, 1993; Calvino and Grilo, 2006).

Specific nociceptive neurons receive information from Aδ- and C- fibers, and are mostly concentrated in laminae I and II, but are also found in deeper layers such as in laminae V-VII. These neurons respond only to high intensity thermal or mechanical peripheral stimuli (Willis and Coggeshall, 1991; Craig, 1996; Calvino and Grilo, 2006).

Wide-dynamic range (WDR) neurons receive information from both non-nociceptive (Aβ) and nociceptive (Aδ and C) fibers. Their firing frequency increases with stimulus intensity, thereby they are able to code stimulus intensity (Schaible and Grubb, 1993; Calvino and Grilo, 2006). WDR neurons are found predominantly in lamina V, as well as in laminae IV and VI, although they are present in smaller numbers in laminae I and II, as well as in lamina X and the ventral horn (Calvino and Grilo, 2006).

Non-nociceptive neurons are found primarily in laminae II, III and IV, but a few may also be present in lamina I. They respond exclusively to low-intensity stimuli and therefore do not play a role in integrating nociceptive information (see Meyer et al., 2008; Calvino and Grilo, 2006).

1.3. Ascending sensory pathways

The sensitive information received by sensory afferents is transmitted from spinal cord to brain through the ascending pathways: the dorsal columns and the anterolateral system.

1.3.1. Ascending pathways through the dorsal columns

The dorsal columns of the spinal cord (Fig. 1.5) are mainly formed by collateral central branches of low threshold mechanoreceptor, in the ipsilateral (same side as the corresponding DRG) side of the spinal cord (Willis, 2007). However, a smaller number of fibers of the dorsal columns correspond to axons of second order neurons originated in the spinal cord (mainly in the nucleus propius, and laminae III, IV and X of the spinal gray matter), the so called postsynaptic dorsal column (PSDC) neurons (Willis and Coggeshall 2003). Fibers from these dorsal columns are distributed in two fasciculi, denominated gracile and cuneate. Fibers from sacral and lumbar afferents form the fasciculus gracile, and the fibers from thoracic and cervical afferents form the fasciculus cuneate. Fibers from these fasciculi synapse with neurons in the homonymous nuclei in the medulla oblongata. These neurons send then axons that cross to the contralateral side (opposite side of the input), forming the medial lemniscus (and therefore this pathway is called dorsal column-medial lemniscal -DCML- pathway) to synapse to the ventral nuclei of the thalamus. After synapsing in the thalamus, still on the contralateral side, the pathway continues to the primary somatic sensory cortex (Purves et al., 2006; Hall, 2011a).

Since this pathway transmits information mainly from LTM receptors, it is responsible for the perception of fine touch, pressure, vibration and conscious proprioception to the cerebral cortex. Although this pathway is mainly devoted to the transmission of nonnociceptive information, it has been recently shown that the PSDC cells located in lamina X transmit information about noxious stimulation received from afferent fibers innervating inflamed visceral organs (Al-Chaer et al. 1996b, 1997, Paleček and Willis, 2003; Paleček, 2004).

The proprioceptive and tactile information that comes from the face is transmitted to the thalamus by a different route, the somatic trigeminal sensory system (Purves et al., 2006), and it will not be described here.

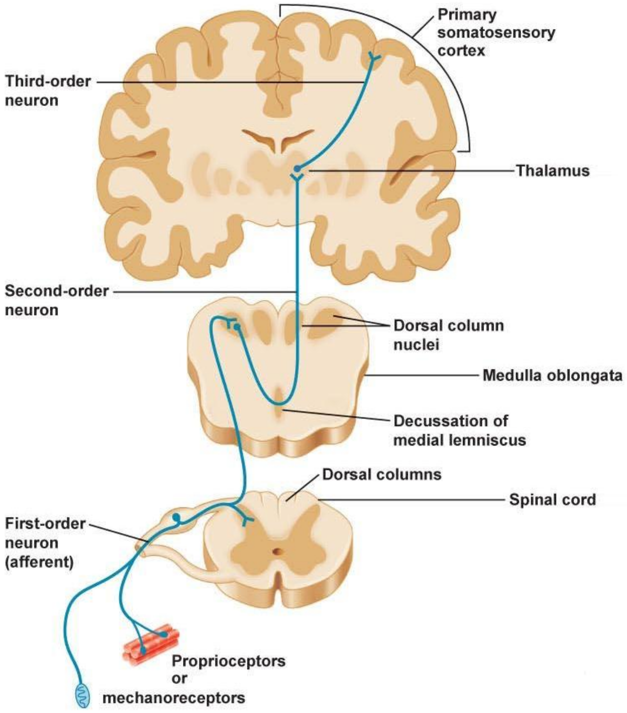

Primary somatosensory cortex
Third-order neuron
Thalamus
Second-order neuron
Dorsal column nuclei
Medulla oblongata
Decussation of medial lemniscus
Dorsal columns
Spinal cord
First-order neuron (afferent)
Proprioceptors or mechanoreceptors

Fig. 1.5. Dorsal column-medial lemniscal pathway (taken from Purves, 2006).

1.3.2. Anterolateral system

The anterolateral system (ALS) is a complex and multifaceted system which not only transmits the information of noxious (painful) and thermal stimuli to the brain, but also indicate the location, intensity and type of stimulus (thermal or mechanical), and is also involved in the affective-motivational component of the sensations, leading to the unpleasantness of the pain experience, and in the autonomous activation following a noxious stimulus. However, and in contradistinction to the DCML pathway, the ALS is thought to play a minor role on propioception and fine touch.

The ALS anatomically differs from the DCLM system in several aspects, but the most characteristics are probably the following two: 1) in contradistinction to the fibers in the dorsal columns, which are originated from collaterals of the primary afferents, ascending fibers in the ALS come from second order neurons located primarily in the dorsal horn (postsynaptic to primary afferent fibers); and 2) while the fibers of the DCML pathway decussate to the contralateral side in the medulla oblongata, the ALS crosses in the spinal cord (Basbaum and Jessel, 2000).

Ascending fibers of the ALS can be classified depending on if they directly or indirectly project to the thalamus. The tract which directly synapses with the thalamus is denominated neospinothalamic tract (nSTT), and constitutes the classical lateral spinothalamic tract. The spinothalamic tract projecting indirectly to the thalamus is termed paleospinothalamic tract (pSTT).

The painful stimulations to the face are conveyed by a distinct tract, called the spinal trigeminal tract which is described in detail elsewhere (Terman and Bonica, 2001; Craig, 2003; Purves et al., 2006; Dostrovsky and Craig, 2008)

1.3.2.1. Neospinothalamic tract (nSTT)

nSTT neurons reside mainly in lamina I, and are activated mainly by Aδ-nociceptors (Hall, 2011b). Projections of nSTT neurons decussate in the anterior white commissure, within one or two segments rostral to the cells of origin, to ascend in the white matter of the contralateral anterolateral system, to synapse with the thalamus (to the ventroposterolateral and ventroposteroinferior nuclei). The interruption (surgical or traumatic) of this tract results in the lack of sensation to noxious cutaneous stimuli applied to the contralateral side of the body at spinal segments below the level of the lesion (Hall, 2011b). However, at sacral and upper cervical levels, a significant number (~26%) of these axons ascend in the ipsilateral anterolateral system (Craig, 2003; Ness and Randich, 2010).

After synapsing in the thalamus, a third order neuron projects to the primary somatosensory cortex (which remains in the contralateral to the stimulated primary afferent). Since nSTT activates the primary somatosensory cortex, it is thought to be implicated in the localization and intensity coding of pain sensations (Ness and Randich, 2010) (Fig. 1.6). The anatomy and the physiological role of the primary somatosensory cortex will be more extensively described in Section 1.4.

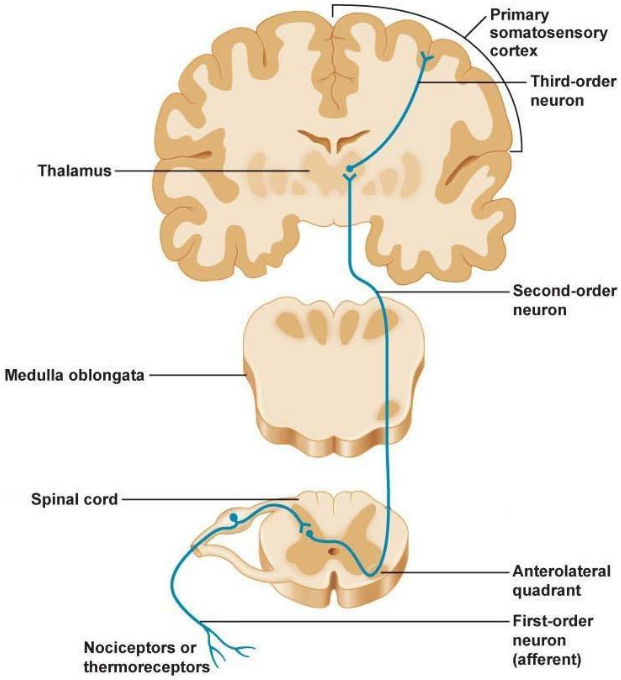

Primary somatosensory cortex
Third-order neuron
Thalamus
Second-order neuron
Medulla oblongata
Spinal cord
Anterolateral quadrant
First-order neuron (afferent)
Nociceptors or thermoreceptors

Fig. 1.6. Neospinothalamic tract (taken from Purves, 2006).

1.3.2.2. Paleospinothalamic tract (pSTT)

The pSTT is phylogenetically older than the nSTT, and it conveys information mainly from C-afferents (although also from Aδ-fibers) (Hall, 2011b). In contradistinction to the nSTT, a higher proportion of pSTT fibers ascend bilaterally and not only in the contralateral side to the primary afferent (Ness and Randich, 2010). The most clear difference between pSTT and nSTT is that only 10-25% of pSTT fibers reach directly the thalamus, and they first synapse with other brain areas such as the reticular formation (spinoreticular tract), mesencephalon (spinomesencephalic tract) or hypothalamus (spinohypothalamic tract), before sending projections to the thalamus (these three distinct tracts are detailed in the next sections) (Fig. 1.7). The thalamus then projects to the somatosensory cortex, but much more diffusedly than for the nSTT. This makes that pain transmitted by the pSTT is much more poorly localized than that transmitted by the nSTT (Hall, 2011b).

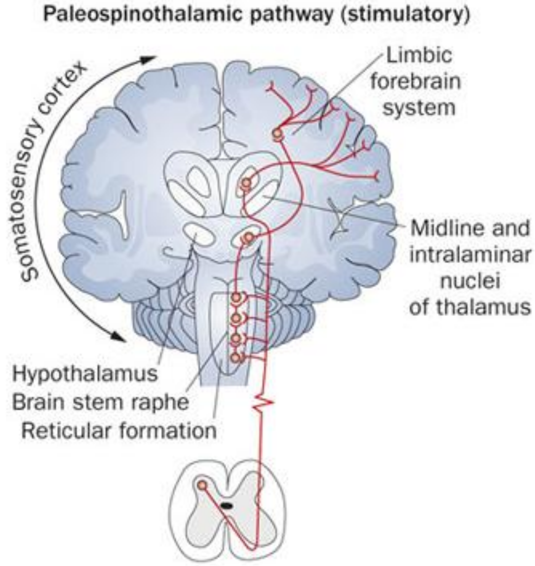

Paleospinothalamic pathway (stimulatory)
Somatosensory cortex
Limbic forebrain system
Midline and intralaminar nuclei of thalamus
Hypothalamus
Brain stem raphe
Reticular formation

Fig. 1.7. Paleospinothalamic tract (taken from Borenstein, 2010).

1.3.2.2.1. Spinoreticular tract

The spinoreticular tract transmit nociceptive, thermal (nonnociceptive), and nondiscriminatory (crude) touch signals from the spinal cord to the thalamus indirectly, by forming multiple synapses in the reticular formation prior to their thalamic projections. This tract is furnished with input from C-fibers that synapse on interneurons in laminae II and III, which in turn have polysynaptic influence on projection neurons in laminae V-VIII. Also, the reticular formation receives collaterals from lamina I STT axons. The third order neurons in the reticular formation project to intralaminar thalamic nuclei and the posterior thalamus (reticulothalamic fibers). The intralaminar nucleus then projects to the striatum and wide areas of cerebral cortex, whereas the posterior nucleus projects to the secondary somatosensory cortex and posterior insular cortex. These cortex areas are responsible to elicit the arousal and wakefulness after an injury, that will enable the organism to evade the painful stimuli (Terman and Bonica, 2001; reviewed by Craig, 2003; Dostrovsky and Craig, 2008; Willis, 2009).

Spinoreticular axons also project to nuclei in the rostroventral medulla (RVM) such as the nucleus gigantocellularis, paragigantocellularis and raphe magnus (Ness and Randich, 2010), which are key areas involved in the descending modulatory pathways, as it will be described in the Section 1.5.2.

1.3.2.2.2. Spinomesencephalic tract

The spinomesencephalic tract comprises axons of dorsal horn cells in laminae I and V. This pathway ascends mainly in the contralateral anterolateral tract, although some axons from lamina I ascend in the dorsolateral funiculus. The spinomesencephalic tract projects to the periaqueductal gray (PAG), which contains neurons that are part of descending pathway that regulates pain transmission (Calvino and Grilo, 2006). Part of the spinomesencephalic tract ends just above the PAG, in the superior colliculus and nucleus cuneiformis, located in the tectum of the midbrain. Therefore, this part of the spinomesencephalic tract is also called spinotectal tract. The superior colliculus and nucleus cuneiformis participate in coordination of motor avoidance behaviour evoked by painful stimuli. Another prominent target of the spinomesencephalic tract is the parabraquial nuclear complex nuclei. Neurons in the lateral parabranchial nucleus project to the amygdala through the spino-parabrachio-amygdalar pathway, and might therefore be involved in affective and emotional responses to pain (Calvino and Grilo, 2006; Ness and Randich, 2010).

1.3.2.2.3. Spinohypothalamic tract

This tract includes axons which projects to or through the hypothalamus. Spinohypothalamic fibers transmit somatosensory information, including nociceptive spinal input (somatic/visceral). These fibers arises from cells in laminae I, V, VII and X of the dorsal horn and ascend in the anterolateral system, where the axon or collateral branches of the spinohypothalamic neurons terminate in both sides of the hypothalamus, thalamus, superior colliculus, and reticular formations in the midbrain, pons, and medulla. As one of the main spinolimbic tracts, this pathway is associated with the autonomic and reflex responses (i.e. neuroendocrine and cardiovascular) to nociception, and to emotional changes caused by pain. (Basbaum and Jessel, 2000; Terman and Bonica, 2001; Willis, 2009).

1.4. The somatosensory cortex

The cortex is divided in different areas according to their functional and anatomical characteristics. There exist distinct cortical structures for motor, sensory, cognitive emotional or autonomic functions (Ness and Randich, 2010). Only the somatosensory cortex will be described here.

The somatosensory cortex (SSC) is involved in the processing of somatic sensory information. The SSC comprises two different regions, the primary somatic sensory cortex (S-I), and secondary somatic sensory cortex (S-II) (Purves et al., 2006; Ness and Randich, 2010). The S-I is located in the postcentral gyrus of the parietal lobe and consists of four functional areas: Brodmann′s areas 1, 2, 3a, and 3b (Fig. 1.8A). Results obtained in experiments in nonhuman primates indicated that neurons of each Brodmann´s area respond depending on the type of stimulus. The areas 3b and 1 are responsive to cutaneous stimuli, while the neurons in area 3a responds to stimulation of the propioceptors; finally the neurons in area 2 process both tactile and proprioceptive stimuli (Purves et al., 2006). S-I process the information from the contralateral part of the body, receiving projections from the ventral posterior nucleus. The S-I is precisely organized somatotopically. Thereby, in S-I certain areas of the body are better represented than others (the face, oral cavity or hands, etc.) (Fig. 1.8B), which is related to the receptors density in the body (Bonica, 1990; Martin and Jessel, 2000; Ness and Randich, 2010). Importantly, direct intracerebral electrical stimulation of S-I failed to elicit painful sensations (Lorenz and Hauck, 2010). Therefore, although S-I contribute to the discriminative analysis of painful stimuli, it needs to act in conjunction with other areas to perceive a stimulation as painful (Lorenz and Hauck, 2010).

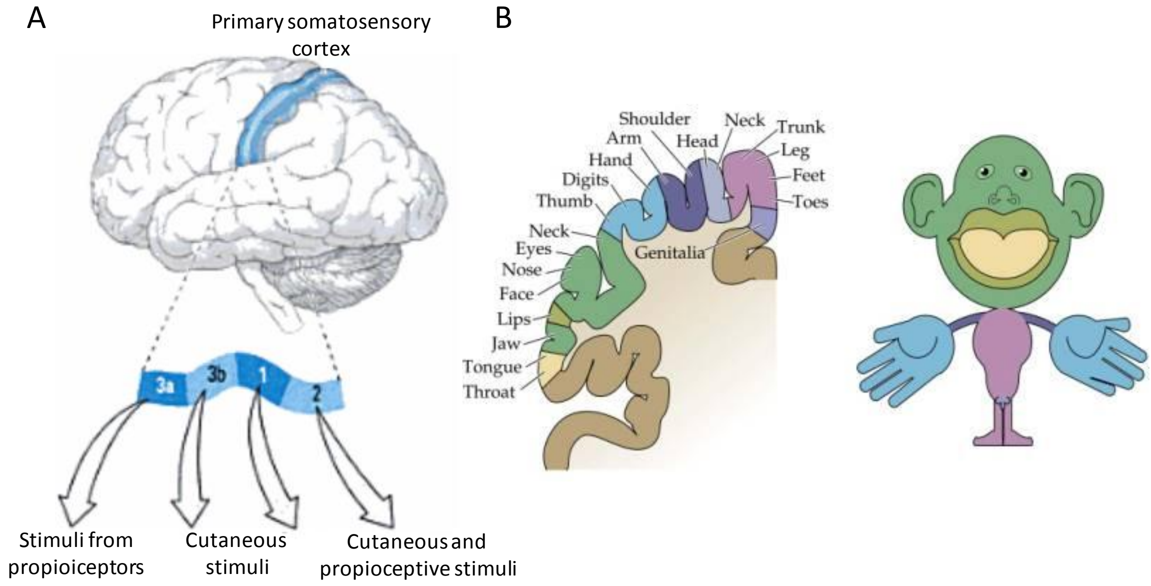

A
Primary somatosensory cortex
3a
3b
1
2
Stimuli from propioiceptors
Cutaneous stimuli
Cutaneous and propioceptive stimuli
B
Shoulder
Neck
Trunk
Arm
Head
Leg
Hand
Feet
Digits
Toes
Thumb
Neck
Eyes
Genitalia
Nose
Face
Lips
Jaw
Tongue
Throat

Fig. 1.8. (A) Schematic anatomical localization of primary somatosensory cortex (modified from Kolb and Whishan, 2003. (B) Somatotopic organization of human primary somatosensory cortex (taken from Purves et al., 2006).

The S-II is situated lateral and posterior to S-I and in contradistinction to S-I, it responds to sensory stimuli bilaterally (Purves et al., 2006; Ness and Randich 2010).

S-II receives convergent projections from the S-I and sends projections to limbic structures such as the amygdala and hippocampus and to the somatic sensory fields in the insular region (Martin and Jessel, 2000; Lorenz and Hauck, 2010). Therefore, S-II is thought to play a major role in pain-induced attention, learning and memory, as well as in the emotional aspects of pain experience (Chen et al., 2008).

1.5. Pain modulation: the gate control theory and the descending control systems

The sections above discussed the anatomical pathways involved in the transmission of nociceptive information from the periphery to the central nervous system. However, there is also a modulatory system within the central nervous system that influences pain transmission and processing: the spinal gate control and the modulatory descending pathways.

1.5.1. The gate control theory

It was in the early 1960s when neurophysiological studies provided evidence that modulation of dorsal horn cells could be affected by input coming from the periphery but also from supraespinal descending systems. This idea was introduced by Patrick Wall and Ronald Melzack as the *gate control theory* (Melzack and Wall, 1965). They found that the stimulation of low-threshold mechanoreceptors (LTM), corresponding to myelinated Aβ-fibers, decreases the response of dorsal horn neurons to unmyelinated nociceptors (C-fibers), whereas blockade of conduction in myelinated fibers enhances the response of dorsal horn neurons. Therefore, the activity of spinal cord neurons in response to nociceptor activation can be modulated by nonnociceptive fibers (reviewed by Bonica et al., 1990; Jessel and Kelly, 2000). The action of LTM was explained by the presence of inhibitory interneurons in the superficial laminae of the dorsal horn, which receive inputs from both nociceptive and nonnociceptive primary afferents, and synapse with the dorsal horn projection neurons. These interneurons are present in a very high density in the superficial laminae of dorsal horn, and make up approximately

25% of cells in laminae I-II and 40% of those in lamina III (Todd and Sullivan, 1990). Inhibitory interneurons act as a “gate” allowing or reducing pain transmission in the dorsal horn as a function of the LTM and nociceptor activity. LTM activation is able to increase inhibitory interneuron activity, thereby closing the gate and blocking the transmission of nociceptive signals to supraspinal structures, which leads to inhibition of pain. However, the activation of nociceptive fibers inhibits the activity of inhibitory interneurons opening the gate and facilitating the transmission of nociceptive signals to supraspinal structures (Fig. 1.9). This initial model suffered some modifications to include also facilitatory interneurons in the substantia gelatinosa, which can be activated by nociceptive fibers contributing to the stimulation of the dorsal horn neurons, whereas the inhibitory interneuron was activated by LTM to decrease the activity of the nociceptive dorsal horn neuron (Wall, 1978). Importantly, the “gate” was shown to be also controlled by descending supraspinal fibers, which synapse with the modulatory interneurons to modify the activity of dorsal horn projection neurons (Fig. 1.9). These descending modulatory influences on pain will be detailed below.

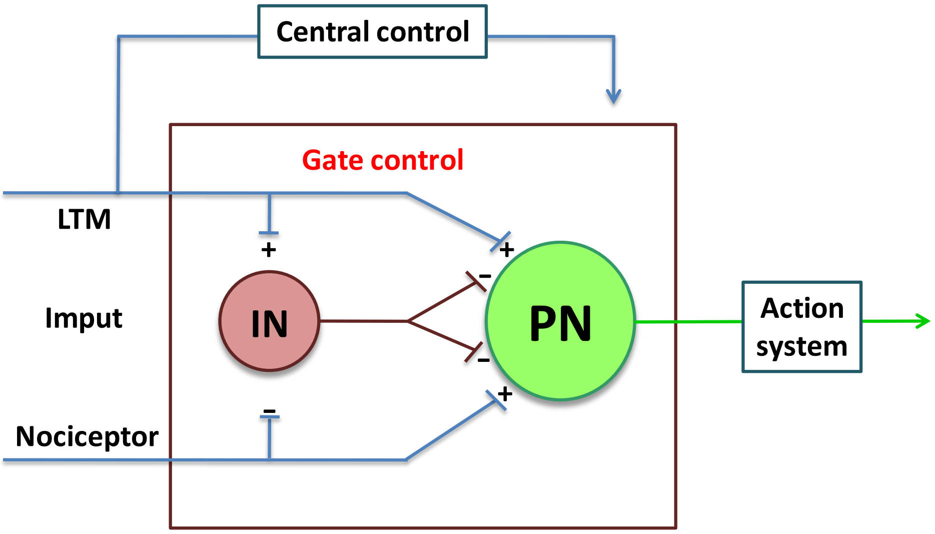


Fig. 1.9. Schematic representation of the gate control model in the spinal dorsal horn. LTM: low-threshold mechanoreceptors; IN: inhibitory interneurons; PN: projection neuron.

1.5.2. Descending modulatory influences from the brainstem.

Cerebral cortex projects to two key components of limbic system: the amygdala and the hypothalamus which in turn send descending projections to the brainstem (Bonica et al., 1990). There are three main brainstem areas involved in the descending modulation of pain: the PAG, the RVM and the locus coeruleus (LC). Their relations are summarized in Fig. 1.10, and detailed below.

As described in the preceding sections, the PAG receives afferent fibers from the ALS. Reynolds (1969) demonstrated that electrical stimulation of PAG induced analgesia, which was referred as "stimulation-produced analgesia". The effects of stimulation-produced analgesia may last from seconds to even hours.

Few neurons in the PAG project directly to the dorsal horn; instead they make excitatory connections with neurons of the RVM, in particular with serotonergic neurons in the nucleus raphe magnus (NRM) and nucleus reticularis gigantocellularis. These nuclei are considered a final common relay of descending serotonergic projections from supraspinal sites (Ossipov et al., 2010).

Activation of RVM neurons can generate either facilitation or inhibition of pain transmission. This dual control results from the activity of different neuronal subpopulations within the RVM. These populations are termed ON, OFF or neutral cells. ON cells increase their activity immediately before a pain response occurs, and are thought to exert a facilitating role for the nociceptive transmission. However, OFF cells show a tonic activity which is stopped immediately before the pain response, and are thought to be responsible for the descending inhibition of nociception. The net effect of RVM on pain modulation depends therefore on the balance between ON and OFF cells activity. Neutral cells were initially characterized by their absence of response to nociceptive stimuli, although they might respond to nociceptive stimuli applied to other body parts (Fields, 1992; Porreca et al., 2002; Calvino and Grilo, 2006).

Furthermore, both PAG and RVM project to the noradrenergic LC, which sends inputs to different nuclei of PAG and RVM and also directly to spinal cord. Fibers from LC are a major source of noradrenergic projections to the spinal cord to inhibit pain transmission (Calvino and Grilo, 2006; Ossipov et al., 2010; Ness and Randich, 2010).

Descending serotonergic and noradrenergic projections from the brainstem course through the dorsolateral and ventrolateral funiculi (DLF and VLF, respectively) to synapse with primary afferent terminals, ascending projection neurons and modulatory interneurons in the spinal cord, controlling therefore the “gate” of pain transmission (Ossipov et al., 2010). The roles of the fibers in the DLF and VLF are distinct. While DLF carries predominantly descending inhibitory signals, and consequently lesions in the DLF disrupt the supraspinal inhibition of nociceptive stimuli (Ness and Randich, 2010), VLF transmits predominantly facilitatory influences from supraspinal origin (Zhuo and Gebhart, 1992). Therefore, the balance between the inhibitory and facilitatory descending systems determines the overall level of excitability of the neurons in the dorsal horn.

There is another cerebral cortex descending modulatory system, the corticospinal tract that sends terminals from sensory cortex directly to dorsal horn spinal cord. Neurons in this pathway also project to several areas such as striatum, reticular ventrobasal posterior and intralaminar thalamic nuclei, and to mesencephalon and reticular formation. Although its function is not well known it is thought to enhance the inhibitory function of the mesencephalic and medullary structures explained above (Bonica et al., 1990).

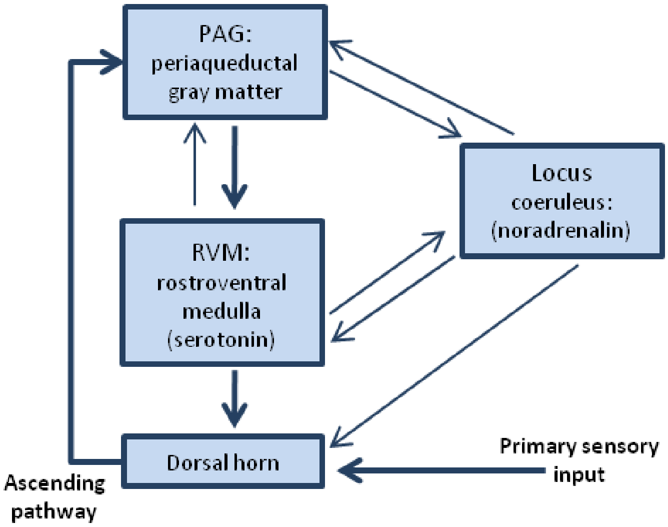

PAG:
periaqueductal
gray matter
Locus
coeruleus:
(noradrenalin)
RVM:
rostroventral
medulla
(serotonin)
Dorsal horn
Primary sensory
input
Ascending
pathway

Fig. 1.10. Spinal-medullary-spinal negative feedback loop underlying an endogenous analgesic system called into play by nociceptive stimuli (modified from Basbaum and Fields, 1984).

2. μ-OPIOID DRUGS: MECHANISM OF ACTION, THERAPEUTIC USE AND SIDE EFFECTS

2.1. Opioid system: historical overview

As described in the previous chapter, pain is a fundamental characteristic of the physiological mechanisms of protection from tissue damage. However, when pain is persistent or too severe it loses its protective role, and pain management is needed. Therefore, trying to relieve pain has been one of the most constant human searches (Haigh and Blake, 2001).

The opium, product extracted from poppy (*Papaver somniferum*) is among the oldest medications known to treat a number of medical problems including pain but also cough and diarrhoea. The psychoactive and the rewarding properties of this extract are also well known since centuries ago (Kieffer, 1999, Pasternak and Pan, 2013). The active ingredients of opium are alkaloid compounds. The most active component of opium is morphine, which was isolated in 1805 by Friedrich Sertürner, a german pharmacy apprentice, who called it morphine after the god of dreams Morpheus (Kieffer, 1999; Gutstein and Akil, 2001). Subsequently, other alkaloids were isolated from opium such as codeine (metilmorphine), thebaine (dimetilmorphine), papaverine and noscapine. Afterwards semisynthetic opioids as heroine and also synthetic opioids such as fentanyl were synthesized (reviewed by Florez, 2008).

Pharmacological studies developed for years shows the existence of an opioid system expressed throughout the nociceptive neural circuitry and in regions of the central nervous system included in reward and emotion-related brain structures, among other areas (reviewed by Al-Hasani and Bruchas, 2011). The opioid system is composed of three major receptor types known as μ-, δ- and κ-opioid receptors (reviewed by Kieffer, 1999; Pasternak and Pan, 2013). Genes encoding these opioid receptors have been cloned and characterized (Akil et al., 1984, Satoh and Minami, 1995). Overall, μ receptor agonists display the best antinociceptive activity, and are the ones most frequently used in therapeutics (reviewed by Kieffer, 1999). Accordingly, this chapter focuses on μ-opioid receptors and their clinically relevant uses.

2.2. Endogenous opioid peptides

The existence of a specific receptor for morphine and other opioid drugs led to think that this receptor should play an important physiological role in pain control and in the existence of endogenous ligands able to activate opioid receptors.

It was observed that the electrical stimulation of different brain areas produced antinociception, which was reversed by the administration of the opioid antagonist naloxone (Akil et al., 1972 and 1976). These findings indicated that endogenous opioids were produced within the central nervous system, and led to subsequent experiments aimed to search for the responsible molecules. These endogenous opioid ligands were identified as peptides with opioid-like activity and affinity for opioid receptors (reviewed by Pasternak and Pan, 2013).

The first endogenous opioid ligands discovered were the pentapeptides enkephalins, Met- and Leu-enkephalin (Hughes et al., 1975). Soon after, other peptides were aisolated: β-endorphin, dynorphin A and B, α-neoendorphin, and the, endomorphines 1 and 2. Remarkably, all endogenous opioids peptides except endomorphines share a four amino acids sequence (Tyr-Gly-Gly-Phe) at the *N*-terminus, with differing extensions and sequence at the *C*-terminus, which can vary from just one additional aminoacid (as for Leu-enkephalin or Met-enkephalin) to as much as 27 extra aminoacids (as for dynorphin A) (see Table 2.1.) (Florez, 2008; Pasternak and Pan, 2013).

The endogenous opioid ligands show different selectivity for the opioid receptor subtypes. Enkephalins have higher affinity for δ-opioid receptors, Dynorphin A for κ_1, while β-endorphin binds with high affinity to μ receptors (and also to δ receptors, although with a lower affinity) (Pasternak and Pan, 2013).

Table 2.1. Mammalian endogenous opioids peptides.

[Leu^5]enkephalin	**Tyr-Gly-Gly-Phe**-Met
[Met^5]enkephalin	**Tyr-Gly-Gly-Phe**-Leu
Dynorphin A	**Tyr-Gly-Gly-Phe**-Met-Thr-Ser-Gly-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-lle-lle-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Dynorphin B	**Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-lle-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
α-Neoendorphin	**Tyr-Gly-Gly-Phe**-Leu-Arg-Lys-Tyr-Pro-Lys
Endomorphin-1	Tyr-Pro-Trp-Phe-NH$_2$
Endomorphin-2	Tyr-Pro-Phe-Phe-NH$_2$

2.3. Opioid drugs: classification by intrinsic efficacy

Opioid drugs usually show affinity for more than one opioid receptor subtype, although the opioid drugs used in the clinical practice mainly exert their effects through µ opioids receptors (Kieffer, 1999; Pasternak and Pan, 2011; Pasternak and Pan, 2013). These drugs can be classified according to their origin (natural, semisynthetic and synthetic), chemical structure or intrinsic activity (Florez, 2008). Among these classifications, the one based on the intrinsic activity of the compound is the widest used, since this parameter clearly influences the effects of the drug (both in the clinical practice and in preclinical research). According to their intrinsic activity the opioids drugs can be classified as:

- Pure agonists: drugs which display a maximum intrinsic activity and therefore the highest efficacy inducing signalling through its receptor. This group includes known µ agonists such as morphine, oxycodone, fentanyl (and its derivatives such as sufentanyl), heroin and methadone (Florez, 2008).
- Partial agonists: drugs that act with a lower intrinsic activity than a pure agonist. The most relevant example is the partial µ agonist buprenorphine (Vadivelu and Hines, 2007).
- Mixed agonist-antagonist: opioid drugs which act as agonists of a concrete subtype of opioid receptor while acting as antagonists of a different subtype.

One representative example is pentazocine, which is a κ agonist but also a μ antagonist (Hoskin and Hanks, 1991).

- Agonists with additional mechanisms: some drugs in addition to opioid agonism show additional mechanisms which contribute to their analgesic effects. These include drugs such as tapentadol and tramadol, which in addition to μ agonism inhibits the reuptake of neurotransmitters. Specifically, tapentadol inhibits the recaptation of noradrenalin (Hartrick and Rozek, 2011; Raffa et al., 2012), whereas tramadol inhibits the reuptake of both serotonin and noradrenalin (Mongin, 2007; Raffa et al., 2012).
- Antagonists: drugs that have affinity for opioid receptors but lack of intrinsic activity and therefore of efficacy. The most widely used opioid antagonist is the centrally-penetrant naloxone, which shows affinity for all three opioid receptor subtypes (although with some preference for μ-opioid receptors). This drug is used in preclinical research or in the clinical practice to reverse the effect of opioid agonists (Mueller-Lissner, 2010; Cui et al., 2014; Mauger et al., 2014). Another centrally-penetrant opioid antagonist with clinical use is naltrexone (Corder et al., 2013; Syed and Keating, 2013). In addition, there have been developed opioid antagonists with limited accessibility to the CNS, to reverse exclusively the peripheral effects induced by opioid agonists. These drugs include naloxone methiodide, which has been extensively used in preclinical research (González-Rodríguez et al, 2010; Baamonde et al., 2006), and methylnaltrexone which has been recently introduced in the clinical practice to reverse clinically relevant peripheral opioid side effects (which will be described later in the Section 2.6).

An additional classification of opioid drugs is done in base of their analgesic efficacy at their therapeutic doses. This classification will be described in the Section 2.5.1.2.

2.4. The μ-opioid receptor and its molecular mechanisms of signaling

The μ-opioid receptor is a member of the G-protein-coupled receptor (GPCR) superfamily (Quock, 1999; Costantino et al., 2012). It has a typical GPCR structure with seven transmembrane helices, and with the *N*- and *C*-terminus facing at the extracellular and the intracellular side, respectively (Connor and Christie, 1999; Knapman and Connor, 2014) (Fig. 2.1).

The G proteins coupled to μ-opioid receptors are heterotrimeric proteins composed by a Gα subunit (tipically of the inhibitory subtype) that binds guanosine diphosphate (GDP) and is coupled to the receptor, and the Gβγ dimer which is anchored to the plasma membrane and binds to Gα (Raehal et al, 2011). Once the opioid agonist binds to the opioid receptor, GTP is exchanged by GDP in the Gα subunit. Then the Gα dissociates from the Gβγ subunits, and is able to interact with other cytoplasmatic proteins. The activated Gα subunit, inhibits the activity of adenylate cyclase (AC) with the consequent decrease in cAMP production and thereby protein kinase A functioning (Al-Hashani and Bruchas, 2011) (Fig. 2.1). In addition, the Gβγ dimer, which is still anchored in the membrane, now is able to interact with several membrane targets. Specifically, the Gβγ subunit inhibits the activity of several plasmalemmal voltage-dependent Ca^{2+} channels (P/Q-type, N-type, and L-type), while opening G-protein-regulated inwardly rectifying potassium channels (GIRKs) (Mark and Herlitze, 2000; Ocaña et al., 2004; Al-Hasani and Bruchas, 2011) (Fig. 2.1). Importantly, the Gα subunit contributes to the modulation of the effect of the Gβγ dimer to GIRKs (e.g. Mark and Herlitze 2000). All these events contribute to the decrease of the activity of the neurons stimulated by μ agonists, by hyperpolarizing the cell (Al-Hasani and Bruchas, 2011; Raehal et al., 2011) (Fig. 2.1).

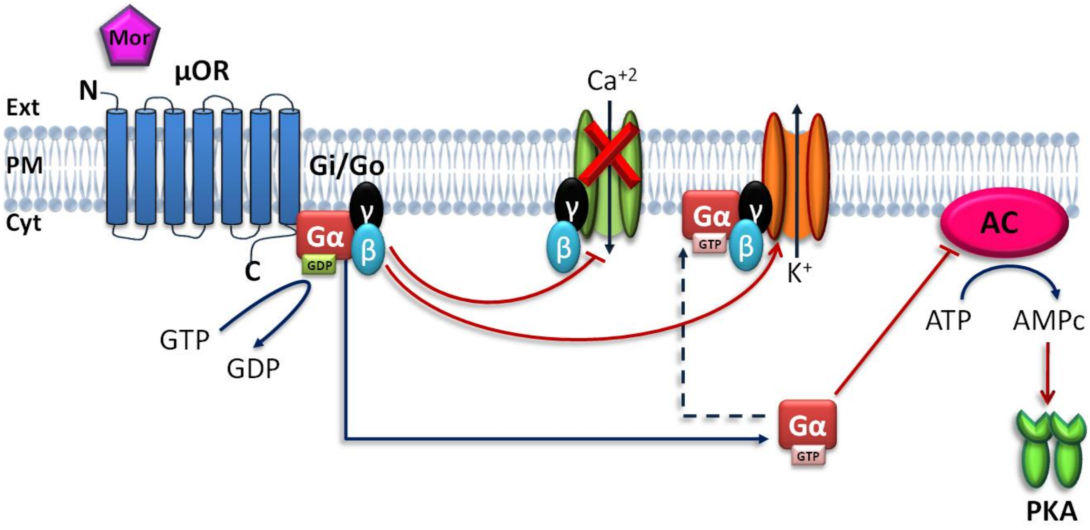


Fig. 2.1. Opioid signaling. The opioid agonists such as morphine (Mor) activate μ-opioid receptors (μOR). The activated Gα subunit inhibits adenylate cyclase (AC), and so the AMPc production thereby diminishing protein kinase A (PKA) activation. The βγ complex inhibits voltage dependent Ca^{+2} channels and opens G-protein-regulated inwardly rectifying K^+ channels (GIRKs). Activated Gα subunit also modulates the effect of βγ complex on K^+ channels.

2.4.1. μ-opioid receptor splice variants and the differences in the signaling induced by μ agonists

The gene encoding for the μ-opioid receptor (MOR-1) is comprised by a number of exons that can combine to form a wide range of splice variants (Fig. 2.2); MOR-1 itself contains 4 exons. The first 3 exons encode both the *N*-terminus and all 7 transmembrane domains, whereas exon 4 encodes only the last 12 amino acids in the *C*-terminus. The major series of the variants of μ-opioid receptors are constituted by receptors with seven transmembrane domains in which the exon 4 is replaced with a set of alternative exons (Fig. 2.2). This splicing pattern is relatively conserved in mice, rats or humans (Pasternak, 2010 and 2014). The binding pocket for all of these variants might be identical, as it is comprised by the 7 transmembrane domains encoded by exons 1, 2, and 3. However, the differences in the amino acid sequence at the *C*-terminus appear to be important, since the signaling (measured as the increase in $[^{35}S]GTP\gamma S$ binding) induced by several μ agonists differ between these spliced versions of the receptor, and not equally for all µ agonists tested, indicating that these spliced variants could exhibit a preferential activation by specific drugs (e.g. Pasternak et al., 2004; Bolan et al., 2004; Pan et al., 2005 and 2009).

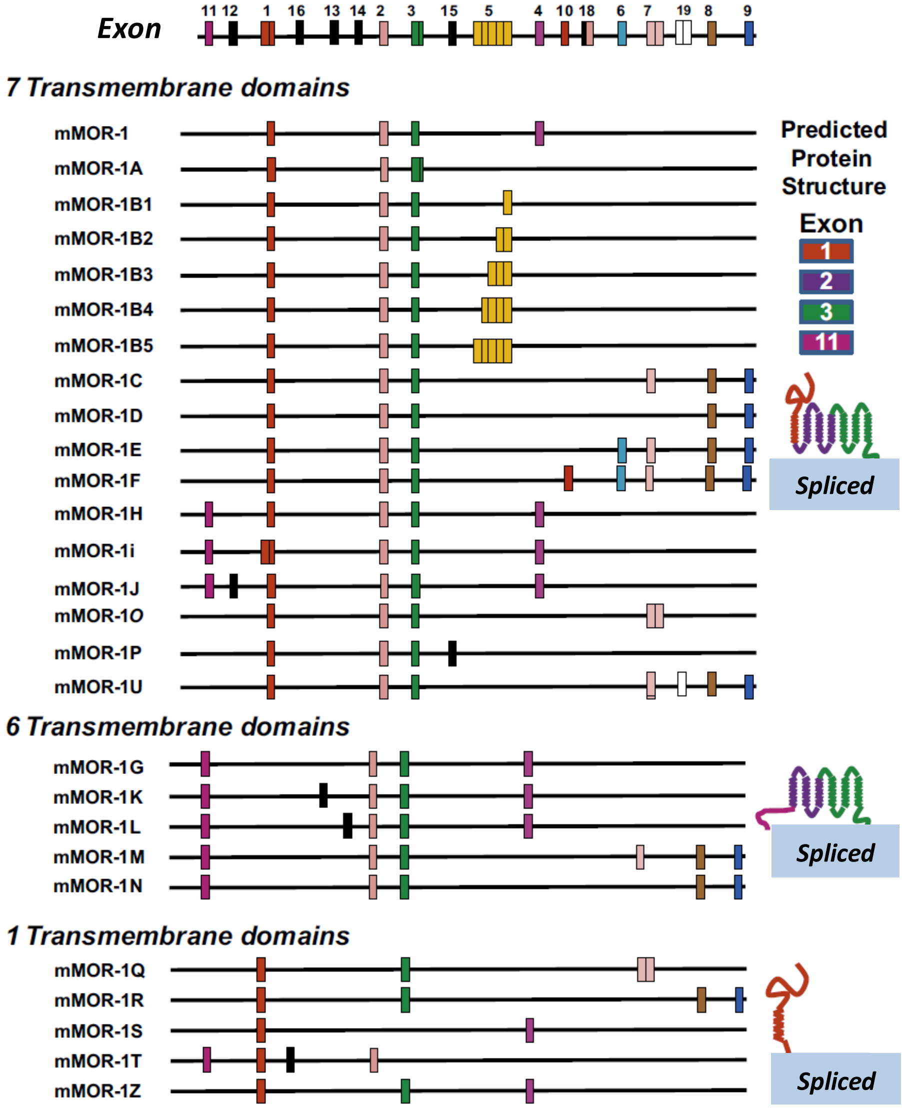

Exon
11 12 1 16 13 14 2 3 15 5 4 10 18 6 7 19 8 9
7 Transmembrane domains
mMOR-1
mMOR-1A
mMOR-1B1
mMOR-1B2
mMOR-1B3
mMOR-1B4
mMOR-1B5
mMOR-1C
mMOR-1D
mMOR-1E
mMOR-1F
mMOR-1H
mMOR-1i
mMOR-1J
mMOR-1O
mMOR-1P
mMOR-1U
Predicted Protein Structure
Exon
1
2
3
11
Spliced
6 Transmembrane domains
mMOR-1G
mMOR-1K
mMOR-1L
mMOR-1M
mMOR-1N
Spliced
1 Transmembrane domains
mMOR-1Q
mMOR-1R
mMOR-1S
mMOR-1T
mMOR-1Z
Spliced

Fig. 2.2. Schematic representation of the MOR-1 splice variants (Taken from Pasternak, 2014).

Complementary detailed studies showed that not all µ agonists activate the same pattern of Gα subunits *in vivo*. The array of Gα subunits known to be activated by µ opioids, include the inhibitory Gi1, Gi2, Gi3, Go1, Go2 and Gz, but also Gq and G11 (Raffa et al., 1994; Sánchez-Blázquez et al., 1995, 1999 and 2001; Garzón et al., 1996).

Morphine and DAMGO were shown to predominantly activate Gi2 and Gz (and additionally Gq for DAMGO). However, methadone and buprenorphine are able to activate different combinations of a broader spectrum of Gα subunits, and in particular heroin is able to activate all Gα subtypes mentioned above (Sánchez-Blázquez et al., 1995 and 2001) (Fig. 2.3). Therefore, the spliced variants of the receptor exhibit a preferential activation by specific drugs, and specific opioids induce distinct signaling patterns. Importanty, the effectors of different μ-opioid drugs are also only partially overlapping (see Urban et al., 2007 and Raehal et al., 2011 for reviews), which might be influenced by the distinct signaling of the different opioid drugs. One clear example is the recruitment of ATP-sensitive K^+ channels for the antinociceptive effect of only some μ agonists, which include morphine, buprenorphine or methadone, while other agonists such as fentanyl or levorphanol do not need of the activation of these channels for inducing antinociception (Ocaña et al., 1995).

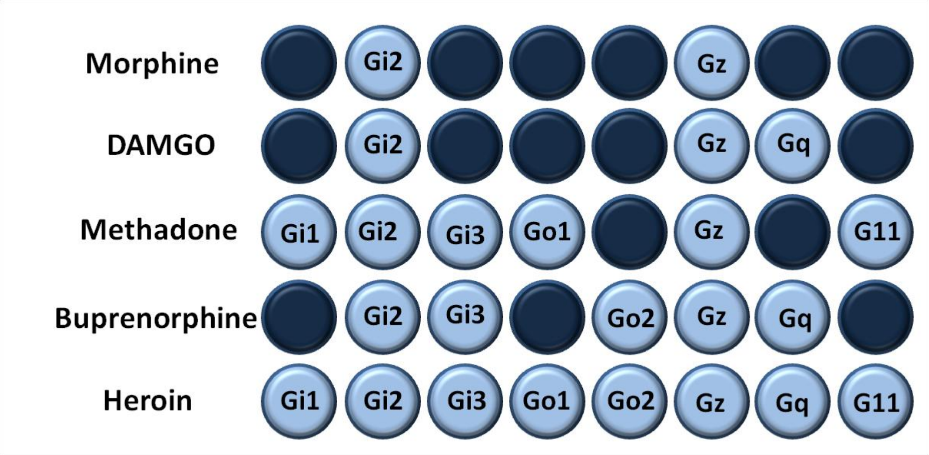


Fig. 2.3. Activation patterns of Gα subunits depend on the μ agonist tested (Taken from Sánchez-Blázquez et al., 2001, with slight modifications).

In addition, there are splice variants which code for opioid receptors of 6 or even only one transmembrane domain (reviewed by Pasternak, 2014) (Fig. 2.2), which by definition do not fit to the 7 transmembrane domain protein of a GPCR, and therefore are not coupled to G proteins. The 6 transmembrane variant of the receptor still retains binding to some known µ agonists, such as fentanyl, although do not bind other classic drugs such as morphine (Majumdar et al., 2011 and 2012). Importantly, activation of this truncated receptor retains antinociceptive activity while showing limited respiratory depression or constipation (Majumdar et al., 2011 and 2012), which are troublesome opioid side-effects, as it will be described in the Section 2.6. Although the role of the single transmembrane domain versions of the µ receptor are still largely unclear, it has been recently shown that they could exhibit a chaperone-like activity by minimizing the degradation of the full length versions of the receptor, and therefore increasing the protein levels (and therefore the actions) of the later (Xu et al., 2013).

2.5. Therapeutic use of µ-opioid drugs

Analgesia is the most extended therapeutic use of opioids, but they can be also prescribed as antitussive and antidiarrheal drugs, among other clinical uses.

2.5.1. Opioid analgesia

Analgesia is the most important therapeutic effect induced by opioids. Although the activation of all subtypes of opioid receptors leads to analgesia, the great majority of opioid analgesic drugs used in therapeutics are µ agonists (Marker et al., 2005; Al-Hasani and Bruchas, 2011; Pasternak, 2013). In this section we will summarize the modulation of the pain pathways by µ-opioid drugs as well as their clinical use as analgesics, highlighting their place in the World Health Organization (WHO) analgesic ladder and their rational use in therapeutics.

2.5.1.1. Modulation of pain pathways by µ-opioid drugs

µ-opioid receptors are located along the pain pathways, including several areas of the central nervous system (both in supraspinal nuclei or at spinal level) as well as in the periphery (dorsal root ganglia, DRG) (Bigliardi-Qi et al., 2004; Khalefa et al., 2012). It is thought that opioid drugs exert their analgesic effects mainly at central levels. Although they can elicit a robust analgesia when administered at the spinal cord (e.g.

Yaksh and Rudy, 1976; Wagemans et al., 1997; Mercadante, 1999), they are particularly active at supraspinal sites (e.g. Christie et al., 2000; Ossipov et al., 2010; Khalefa et al., 2012; Ringkamp and Raja, 2012), where µ-opioid receptors are expressed at their highest density (e.g. Arvidsson et al., 1995; Khalefa et al., 2012; Ringkamp and Raja, 2012).

Among the several supraspinal structures which participate in opioid analgesia, the most prominent ones are those constituting the modulatory descending pain pathways. As described in the Section 1.5.2, these areas include the PAG, LC and RVM. Administration of opioids into any of these three sites is enough to elicit analgesia (e.g. Millan, 2002; Porreca et al., 2002; Mei and Pasternak, 2007). These three areas are interconnected and participate coordinately in pain control (Fields, 1984). The relationship between their actions is such that the simultaneous administration of low (inactive) doses of morphine into the PAG and RVM is able to elicit strong antinociceptive effects that are far higher than additive (i.e. synergistic) (Rossi et al., 1993). RVM play a particular role on the descending modulation of pain, since it receives afferents from both PAG and LC and is thought to be the final common relay in both the descending inhibition and facilitation of pain (reviewed by Porreca et al., 2002; Millan, 2002). This dual pain control is the result from the activity of two distinct neuronal subpopulations within the RVM: the ON and OFF cells. ON cells are thought to exert a facilitating role for the nociceptive transmission, and increase their activity immediately before a pain response occurs; moreover, the activation of ON cells is able to inhibit the activity of OFF cells. These latter neurons show a tonic activity which is stopped immediately before the pain response, and they are thought to be responsible for the descending inhibition of nociception. Therefore, these two cell types act coordinately, and a nociceptive stimulation is able to induce simultaneously both an increase in the activity of ON cells and a decrease in the activity of OFF cells, resulting on pain (see Fig. 2.4 left panel) (Fields, 1992; Porreca et al,. 2002; Calvino and Grilo, 2006; Ossipov et al., 2010). Opioid effects are particularly well studied in the RVM. Opioid administration is able to decrease the activation of ON cells (and not that of OFF cells), resulting in the disinhibition of OFF cells and therefore eliciting analgesia (reviewed by Porreca et al., 2002; Ossipov et al., 2010) (see Fig. 2.4 right panel).

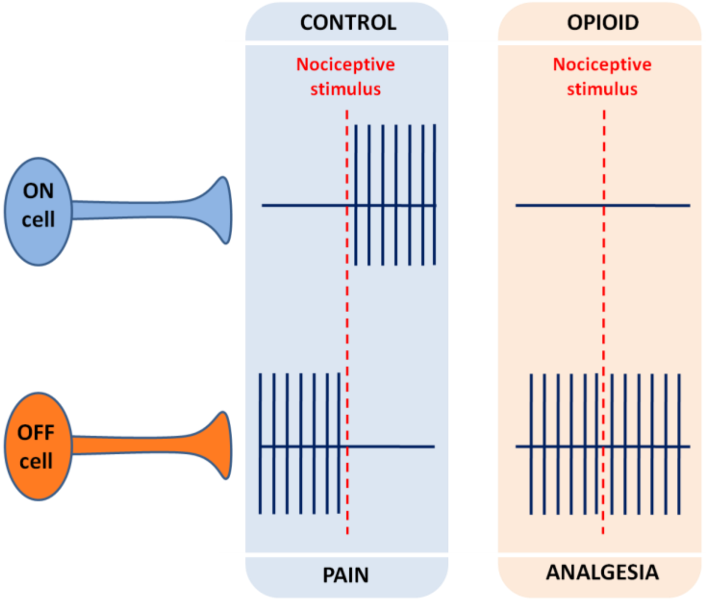


Fig. 2.4. ON and OFF cells activity in the rostroventral medulla (RVM) during application of a nociceptive stimulus and after opioid administration.

Although most of the analgesic effects of opioids are attributed to their actions at central levels, peripheral opioid receptor activation can also elicit analgesia in rodents (Craft et al., 1995; Kayser et al., 1995; Kolesnikov et al., 1996 and 2000; Shannon and Lutz, 2002) and humans (reviewed by Stein et al., 2003; Sehgal et al., 2011), but their effects are thought to be limited in comparison to their central actions (Greenwood-Van Meerveld and Standifer, 2008; Joshi et al., 2008; Thomas et al., 2008; Khalefa et al., 2012; Ringkamp and Raja, 2012).

2.5.1.2. Clinical use as analgesics

The WHO developed a 3-step “analgesic ladder” to guide pain management, which is based on the intensity of pain (see Fig. 2.5). In accordance with WHO recommendations, if pain intensity is mild a nonopioid analgesic, such as a nonsteroidal anti-inflammatory drug (NSAID) or acetaminophen should be used. If pain intensity is too high to be appropriately managed with this type of drugs alone (i.e. mild to moderate pain), weak opioids such as codeine or tramadol (the so called step II opioids) should be used. If pain is too intense (i.e. moderate to severe), other opioids with a higher analgesic efficacy are needed. These opioid drugs are called step III opioids and include drugs such as morphine, fentanyl, oxycodone or buprenorphine (Pergolizzi et al., 2008; Christo and Mazloomdoost, 2008; Leppert, 2011). Both step II and III opioids are generally associated to NSAIDs or acetaminophen to minimize the opioid dose administered (Christo and Mazloomdoost, 2008). It is worth pointing out that although analgesic efficacy increases as we climb the ladder, adverse events are also more pronounced and might not be well tolerated by the patient (Christo and Mazloomdoost, 2008; Whistler, 2012).

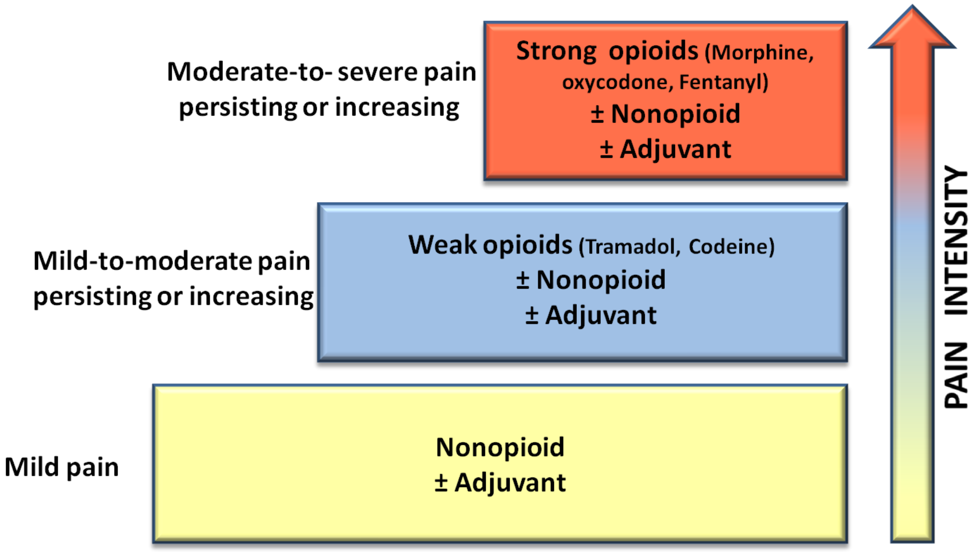

Moderate-to- severe pain persisting or increasing
Strong opioids (Morphine, oxycodone, Fentanyl)
± Nonopioid
± Adjuvant
Mild-to-moderate pain persisting or increasing
Weak opioids (Tramadol, Codeine)
± Nonopioid
± Adjuvant
Mild pain
Nonopioid
± Adjuvant
PAIN INTENSITY

Fig. 2.5. The WHO "analgesic ladder" for pain treatment.

Importantly, although all opioid analgesics used in clinics target the μ subtype, their pharmacological effects might vary unpredictably between patients, with some patients achieving good relief with a particular opioid, while another patient may be better managed with a different one. This strongly argues for the individualization of the opioid treatment for each patient (e.g. Ballantyne and Mao, 2003; Pasternak, 2010 and

2014). These clinical findings could be replicated in different strains of mice, which show striking differences in the analgesic sensitivity to opioid antinociception, and not to the same degree or direction for all opioids; for instance, morphine had a much lower antinociceptive effect in CXBK mice than in CD-1 mice, whereas heroin behaved in the opposite way (reviewed by Pasternak, 2004). These clinical and preclinical observations can hardly be explained by the existence of a common mechanism of action for all μ opioids, and support that μ-opioid drugs do not bind to a single receptor, but they might interact with a large number of receptor variants with different activation profiles elicited by the different drugs, as previously commented.

2.5.2. Antitussive properties of opioid drugs

Cough is considered a defensive reflex mechanism, which protects the airways by ejecting obstructive or potentially harmful substances. However, chronic cough is also a distressing characteristic symptom of many inflammatory airways' diseases (Belvisi and Hele, 2009). Both bronchopulmonary C-fibers and polymodal Aδ-fibers subsets (the so called "cough receptors") can participate in the cough process (Reynolds et al., 2004). After their activation, they stimulate the brainstem cough centre through vagal afferent pathways, eliciting the cough reflex (reviewed by Canning, 2011). It is thought that the antitussive properties of opioids are predominantly central (e.g. Reynolds et al., 2004), and a consequence of the decreased activity of the cough center induced by opioid receptor activation (although the signal transduction involved is not as well described as the one involved in analgesia) (Belvisi and Hele, 2009). Peripheral opioid actions in the airway cough sensors might also participate in the antitussive effects of opioids, however it is thought that their contribution is much limited in comparison to that from central opioid receptors (Reynolds et al., 2004).

Although all centrally acting opioids effectively decrease the cough reflex, codeine is often considered the 'gold standard' in antitussive therapy since it has a better side-effect profile than the stronger opioids (Reynolds et al., 2004; Chung 2008; Al-Hasani and Bruchas, 2011).

2.5.3. Antidiarrheal

Although gastrointestinal transit can be influenced by both central and peripheral opioid receptors, the central contribution to this effect is thought to be limited (Holzer et al., 2009), contrary to analgesia and the antitussive effects of opioid drugs described above. Opioid receptors are located in the gastrointestinal tract on the myenteric and submucosal plexuses (Brock et al., 2012; Khalefa et al., 2012), and enteric neurons at these locations synthesize and release opioid peptides, so the opioidergic system is thought to have a physiological role on gastrointestinal transit function (Greenwood-Van Meerveld et al., 2004; Holzer et al., 2009).

Opioid drugs can also interact with opioid receptors on the gastrointestinal tract (Holzer et al., 2009; Brock et al., 2012). Opioid agonism in this location induces a decrease of gut motility and fluid secretion as well as increase in sphinter contraction (DeHaven-Hudkins et al., 2008; Holzer et al., 2009; Brock et al., 2012).

Although the inhibition of gastrointestinal transit is considered an adverse side effect of opioid analgesics (see Section 2.6), this effect of opioid agonists is therapeutically used for the symptomatic treatment of acute and chronic diarrhea (Holzer, 2009; Gallelli et al., 2010). Not all opioid agonists have this clinical application, since their central effects limit their use. The opioid most frequently used for this therapeutic purpose is loperamide. This particular opioid agonist has little CNS effect due to its active efflux transport from the CNS into the blood by transporters including P-glycoprotein (Upton, 2007), but as its peripheral actions are prominent it has a marked effect on gastrointestinal transit (Layer et al., 2010; Gallelli et al., 2010).

2.6. Opioid side effects

Along with their therapeutic effects, opioids have the potential to produce important side effects. Management of opioid-induced adverse events is still today a major clinical challenge, since they occur frequently and reduce the quality of life of patients, being often the cause of treatment discontinuation (Cherny et al., 2001; Al-Hashani and Bruchas, 2011; Annemans, 2011; Brock et al., 2012). As for the therapeutically useful effects of opioids, their side events (or most of them) derive from the inhibitory actions of opioids on neuronal functioning. In most cases these side effects are produced by the simultaneous actions of opioids at both central and peripheral levels, although for each concrete effect the peripheral or central contribution varies, as it will be detailed below. The most relevant opioid-induced side effects and their most prominent origins (central or peripheral) are listed below (Table 2.2.).

Table 2.2. Opioid side effects distributed by their primary location of induction (central or peripheral).

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

The actions summarized in this table are observed for all clinically available opioid agonists. Although several of these effects are known to have both a central and a peripheral component, for clarity purposes only the predominant location of each side effect was considered for this table.

Among the peripheral side effects of opioid drugs, those related to the gastrointestinal tract are the most frequent, being the constipation the more prevalent symptom produced by prolonged opioid treatment and one of the main reasons for patients' voluntary withdrawal from opioid medication (Brock, 2012; Dhingra et al., 2013). Constipation is mainly caused by the inhibition of gastrointestinal motility, intestinal secretion and sphincter contraction induced by opioid receptors localized on the enteric nervous system, but might also present a central component (see Section 2.5.2 for details). Constipation is also accompanied by other symptoms such as incomplete evacuation, abdominal distention, abdominal discomfort and gastroesophageal reflux, which can lead to secondary complications such as anorexia, which can altogether profoundly impair the quality of life of patients (Holzer, 2010; Brock, 2012; Dhingra et al., 2013). Hence, several treatment strategies are available to alleviate these side effects. Traditionally, laxative therapy has been used to prevent constipation, however clinical studies have showed that it is clearly insufficient (see Greenwood-Van Meerveld and Standifer, 2008 for references). The recent introduction of the peripherally-restricted opioid receptor antagonist methylnaltrexone in the clinical practice (trade name Relistor) has opened up a new window for controlling opioid-induced constipation without compromising the central analgesic actions of opioids (Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008; Holzer, 2010; Gatti and Sabato, 2012; Mori et al., 2013).

On the other hand, nausea and vomiting, which is one of the most frequent and severe symptoms after acute treatment with opioids (Florez, 2008), are mainly produced centrally by the stimulation of the chemoreceptor vomiting center in the area postrema (Bastami, 2014). However, the inhibition of gastrointestinal transit is also thought to play a role on the induction of nausea and vomiting, and in fact treatment with methylnaltrexone is able to partially relieve these side effects of opioids (Rosow et al., 2007; Gatti and Sabato, 2012; Sharma and Jamal, 2013).

Sedation and confusion, are centrally-mediated opioid side effects which mainly occur with initiation of therapy or dose escalation (Florez, 2008), and both are among the most commonly reported adverse effects of opioids (Davies et al., 2009). Opioid-induced sedation occurs on a continuum ranging from full consciousness to complete loss of consciousness (coma), the later happening more frequently in cases of intoxication (Jarzyna et al., 2011; Mégarbane et al., 2007). During opioid intoxication, coma is often accompanied with other two centrally-mediated opioid side effects: severe respiratory depression and accused miosis (pinpoint pupils) (Sporer et al., 1996; Florez, 2008; Mégarbane et al., 2007). If this symptomatic triad (coma, respiratory depression and pinpoint pupils) can be reversed by naloxone, it is considered a pathognomonic sign of opioid intoxication (Sporer et al., 1996; Florez, 2008; Mégarbane et al., 2007).

Although respiratory depression induced by opioids is relatively uncommon, in some cases (of opioid intoxication) is life-threatening (Dahan et al., 2013). Its frequency increases when an underlying pulmonary disease, interactions with other drugs or renal failure exits, and during an overdose by opioid misuse (Florez, 2008; Pasternak and Pan, 2013; Dahan et al., 2013). Respiratory alterations caused by opioids are mainly originated at central level by depression of central respiratory centers, but they also have a peripheral component, as opioids can decrease the sensitivity of peripheral chemo-receptors to carbon dioxide (Pattinson, 2008; Florez, 2008; Radke et al., 2014). Another central effect of opioids that can aggravate the respiratory alterations induced by opioids is trunk rigidity which although it is an uncommon effect, increases its frequency when the opioid drug used is highly lipophilic, such as fentanyl or derivatives (Al-Hasani and Bruchas, 2011; Coruh et al., 2013). Miosis is a central effect occurring at the autonomic segment of the oculomotor nerve, and although it is present at analgesic doses of opioids it does not induce any relevant distress. It is used clinically as an indicator of possible opioid intoxication when is very pronounced, and experimentally as an indicator of the central effects of opioids, since it is sensitive to naloxone but not to methylnaltrexone (Rosow et al., 2007).

The psychotomimetic alterations induced by a chronic treatment with opioids are directly related to the concentration reached in the CNS, and therefore vary depending on the liposolubility of the specific opioid (Martín and Goicoecha, 2008).

In addition to these effects, opioids are well known to increase the tone of the trigone, decrease detrusor tone, decrease the urge to void, and inhibit the voiding reflex, all of them factors contributing to the urinary retention, which may lead to the overdistension of the bladder producing pain and bladder injury (Kuipers et al., 2004; Rosow et al., 2007; Harris, 2008; Zand et al., 2014). Although this adverse event of opioids is clinically significant, in particular in elder men (Florez, 2008), its incidence has proven surprisingly difficult to estimate (Rosow et al., 2007). Intrathecal administration (i.t.) of opioids is well known to decrease urinary function, and therefore activation of peripheral opioid receptors is not needed for the occurrence of this effect (e.g. Kuipers et al., 2004; Zand et al., 2014). However, the association of methylnaltrexone with systemic opioids was shown to decrease at some extent urinary retention, which might be due to the reversion of the actions of opioids in parasympathetic nerves innervating the bladder (Rosow et al., 2007; Reichle and Conzen, 2008).

Pruritus, a subjective unpleasant and irritating sensation that provokes an urge to scratch, is also very often reported in patients with opioid treatment (Davies et al., 2009). The symptoms typically start at the trunk, nose, around the eyes and in other facial areas (Szarvas et al., 2003; Kumar and Indu-Sing, 2013). This opioid effect is thought to be mainly central in origin as it happens often after epidural or intrathecal administration (Szarvas et al., 2003; Kumar and Indu-Sing, 2013), but peripheral opioid receptors might play a role as well, since topical treatment with opioid antagonists can relieve some types of pruritus (Bigliardi et al., 2007).

The cardiovascular complications during a treatment with morphine are rare in normotensive patients. However, opioids may affect cardiovascular function by acting in the kidneys, blood vessels and even in the heart, decreasing blood pressure and heart rate. The frequency of cardiovascular complications increases at high doses of opioids or in patients with a preexistent heart disease (Florez, 2008; Martín and Goicoechea, 2008; Harris, 2008).

Interestingly, it is known that the chronic use of opioids, reduces the immune response and promotes the development of infections. These actions of opioids in immune system can be direct (by stimulation of opioid receptors expressed immune cells), or indirect (by neuro-immune interactions) (Florez, 2008; Martín and Goicoechea, 2008; Shen et al., 2014).

A major limitation to the long-term use of opioids is the development of tolerance and dependence (Cherny et al., 2001; Harris, 2008), which will be detailed in the next section.

2.7. Tolerance and dependence

Tolerance is a pharmacologic phenomenon that develops with the repeated used of some drugs, including opioids. The development of tolerance reduces the effects of opioids and therefore increased doses of opioids are needed to maintain the same level of analgesia (Ballantyne and Mao, 2003; Raehal et al., 2011). Importantly, although tolerance to the analgesic effect of opioids is often produced, tolerance is not (or it is minimally) developed for other nonanalgesic effects of these drugs, such as constipation (Brock et al., 2012; Pan and Pasternak, 2013). This is particularly worrisome, since the dose escalation needed for the maintenance of analgesia increase in parallel the severity of some opioid side effects that are not attenuated over time (Martín and Goicoecha, 2008; Schmittner et al., 2009).

The activation of opioid receptors by an agonist induce the phosphorylation of the opioid receptor by G protein-coupled receptor kinases (GRKs), inhibiting the association of the receptor to the G proteins. This process leads to the desensitization of the receptor to further agonist stimulations. β-arrestins (of the subtype 2) can then bind to the phosphorylated receptor promoting its internalization by endocytosis. Following endocytosis receptors can be dephosphorylated and recycled to the cell surface in a fully active state, thereby resensitizing cells to agonist. However, if agonist exposure is persistent, the endocytosis process will end up in the degradation of the opioid receptor. This will decrease the number of opioid receptors available in the plasma membrane (downregulation), and consequently decreasing the effects of the opioids (reviewed by Kohout and Lefkowitz, 2003; Waldhoer et al., 2004; Garzón et al., 2008; Pasternak and

Pan, 2013). Although this is thought to be a general mechanism for the development of desensitization/tolerance, it is not applicable equally to all μ agonists. For instance morphine is known to induce a minimal internalization of the receptor, while DAMGO is shown to robustly induce this process (reviewed by Waldhoer et al., 2004; Garzón et al., 2008; Pasternak and Pan, 2013).

Changes during tolerance not only involve changes in the opioid receptor, but has also changes in second messenger systems (as adenyl cyclase superactivation), as well as in glutamatergic neurotransmission (through the NMDA-nitric oxide cascade) (reviewed by Waldhoer et al., 2004; Garzón et al., 2008; Pasternak and Pan, 2013). In addition, prolonged treatment with opioids enhances the release of excitatory neurotransmitters including glutamate, CGRP, and Substance P from nociceptive primary afferent fibers within the spinal cord, which contribute to the analgesic tolerance (reviewed by King et al., 2005; Waldhoer et al., 2004).

Interestingly, patients highly tolerant to the analgesic effect of one specific μ-opioid drug may regain (at least partially) their sensitivity when switched to a different μ-opioid. This observation led to the clinical practice of opioid rotation. When the dose escalation with one opioid fails (the balance between analgesia and side effects is decompensated), the incomplete cross-tolerance between opioid drugs may permit a lower (relative) dose of a new opioid to be used (reviewed by Pasternak, 2010; Pasternak and Pan, 2013). This clinical observation has been replicated in the preclinical setting in which it was clearly shown that several μ opioid drugs show incomplete cross-tolerance (reviewed by Pasternak, 2004). This does not reconcile with the existence of a unique μ opioid receptor or with signalling pathways and mechanisms for tolerance common for all μ opioid agonists, but might be influenced by the diversity of μ opioid receptors and the known differences in the signalling and effectors between the different μ opioid drugs (see Section 2.4.1), and of course by the differences in the mechanisms of the desensitization/resensitization of the receptor by different μ agonists (as previously commented).

In addition to tolerance, physical dependence, which results in the necessity for continued administration of the drug to prevent the development of symptoms of opioid withdrawal, can ensue in patients. The withdrawal syndrome can be precipitated by the abrupt withdrawal of the opioid treatment or by the administration of opioid drugs with lower intrinsic efficacy (including of course opioid antagonists) (Walsh and Eissenberg, 2003; Waldhoer et al., 2004; Paronis and Bergman, 2010). Tolerance and dependence may share partially overlapping mechanisms (reviewed by Waldhoer et al., 2004); as an example, both tolerance and dependence can be blocked by NMDA receptor antagonists (Trujillo and Akil, 1991; Kolesnikov et al., 1993; Gónzalez et al., 1997). However, the overlapping between the mechanisms of tolerance and dependence are not complete. For instance it is known that β-arrestin-2 is needed for the development of opioid tolerance, but does not influence dependence (Bohn et al., 2000).

From a clinical standpoint, opioid withdrawal is one of the most powerful factors driving addictive behaviors. Addiction is a complex phenomenon, distinct to dependence, which is characterized by intense drug-seeking behavior and the compulsive use of the drug (Williams et al., 2001; Florez, 2008). Opioids are able to modulate neural brain circuits associated with reward (positive reinforcement), so opioid administration can motivate the repeated use of the drug simply for pleasure (Waldhoer et al., 2004). It is generally thought that opioid drugs induce addiction when they activate the rewarding areas in the absence of significant pain (i.e. during recreational use), and that when they are used for treating severe pain the tendency to abuse is exceptional (Williams et al., 2001; Florez, 2008). However, in the last few years it has been detected a high increase in the abuse of opioid analgesics, which is thought to be due to the recent increased number of their prescriptions (Højsted and Sjøgren, 2007).

3. SIGMA-1 (σ_1) RECEPTORS AND PAIN: FROM CENTER TO PERIPHERY

Despite the progress made in the understanding of pain mechanisms, pain management is still a major clinical need (Cobos and Portillo-Salido, 2013; Finnerrup et al., 2013; Drageset et al., 2014; Pergolizzi et al., 2014). Therefore, it is important to search for new pharmacological targets to obtain new therapies or to improve existing treatments. Here we will summarize the discovery of σ_1 receptors, their pharmacology, anatomical and subcellular distribution, as well as their modulatory role on neurotransmission. Finally, we will extensively describe the experimental evidences which leaded to propose σ_1 receptors as a potential new pharmacological target for pain treatment from two different perspectives: the modulation of opioid effects on acute pain, and the modulation of pain hypersensitivity on pathological pain. In this latter section we will place into an adequate frame the results obtained during this Doctoral Thesis, highlighting our findings as the first evidences of a role of peripheral σ_1 receptors on pain modulation and the impact of these findings on the field.

3.1. Discovery of σ receptors

Initial studies misclassified σ receptors as a subtype of opioid receptors, and later with the binding site for phencyclidine (PCP) within the *N*-methyl-*D*-aspartate (NMDA) glutamate receptor (Martin et al., 1976; Zukin et al., 1984). These early confusions were due to the complex pharmacology of the first σ ligand deeply characterized, (±)-SKF-10,047. Whereas (–)-SKF-10,047 binds to opioid receptors, and therefore its effects are reversible by naloxone, the (+)-isomer lacks affinity for opioid receptors but binds to two different naloxone insensitive sites: the PCP binding site and to an additional benzomorphan binding site (Walker et al., 1990; Cobos et al., 2008; Robson et al., 2012). This latter binding site was termed σ receptor (Quirion et al., 1992) and still retains the same designation (Cobos et al., 2008; Zamanillo et al., 2013 for references).

Early studies demonstrated the existence of two subtypes of σ receptors, which differed on their molecular mass (29 kDa and 18-21.5 kDa for σ_1 and σ_2, respectively) (Hellewell and Bowen, 1990), and also in their pharmacological profile. While σ_1 binding sites display stereospecificity towards dextrorotatory isomers of benzomorphanes, σ_2 binding sites display certain selectivity to levorotatory isomers (Quirion et al., 1992). The cloning of the σ_1 receptor strongly helped to establish σ_1 receptors as a unique entity (as it will be described in the next section). Only the characteristics and mechanisms of σ_1 receptors will be discussed in the present chapter.

3.2. Cloning and structural characteristics of σ_1 receptor

A significant progress in the knowledge of the molecular structure of σ_1 receptors was possible after its cloning. σ_1 receptor was firstly cloned from guinea pig liver (Hanner et al., 1996), and later from other tissues and species, as mouse kidney, a JAR human choriocarcinoma cell line, and in the rat and mouse brain (see Cobos et al., 2008 for references). The cloning of σ_1 receptors allowed to unveil both its structure and function. This receptor is a single polypeptide, composed by 223 amino acids with high sequence homology between several species (Seth et al., 2001). Interestingly, this receptor shows no homology with any other mammalian protein, but shares approximately 30% identity with the yeast gene that encodes the C7–C8 sterol isomerase (Moebius et al., 2001), which might explain at some extent its affinity for neurosteroids (Maurice et al., 2001 and 2006), that as it will be described in the next section are putative σ_1 endogenous ligands. Although initial studies proposed a structure of σ_1 receptors with a single transmembrane region (Hanner et al., 1996; Seth et al., 1997; Dussossoy et al., 1999), further studies made clear that σ_1 receptors have two transmembrane segments (Aydar et al., 2002). This later structure has been refined in subsequent studies, reporting the existence of two additional hydrophobic segments (one of them partially overlapping the second transmembrane domain), corresponding to steroid binding domain-like sites and purportedly responsible for ligand binding (Chen et al., 2007; Pal et al., 2007 and 2008). More recently, it was described the existence of a chaperone domain (Hayashi and Su, 2007) within residues 112-223 of the σ_1 receptor (Ortega-Roldan et al, 2013) (see Fig. 3.1). This domain confers to this receptor the ability to modify the function of a variety of target proteins, and therefore explaining the modulatory actions of σ_1 receptors that will be detailed in subsequent sections. As it will be described in the Section 3.4, σ_1 receptors can be localized in plasma and ER membranes. In both cases the loop connecting the two transmembrane domains is cytoplasmic, whereas the *N*- and *C*- termini are both facing to the ER lumen (when located in the ER) or to the extracellular side (when located in the plasma membrane) (Pabba, 2013).

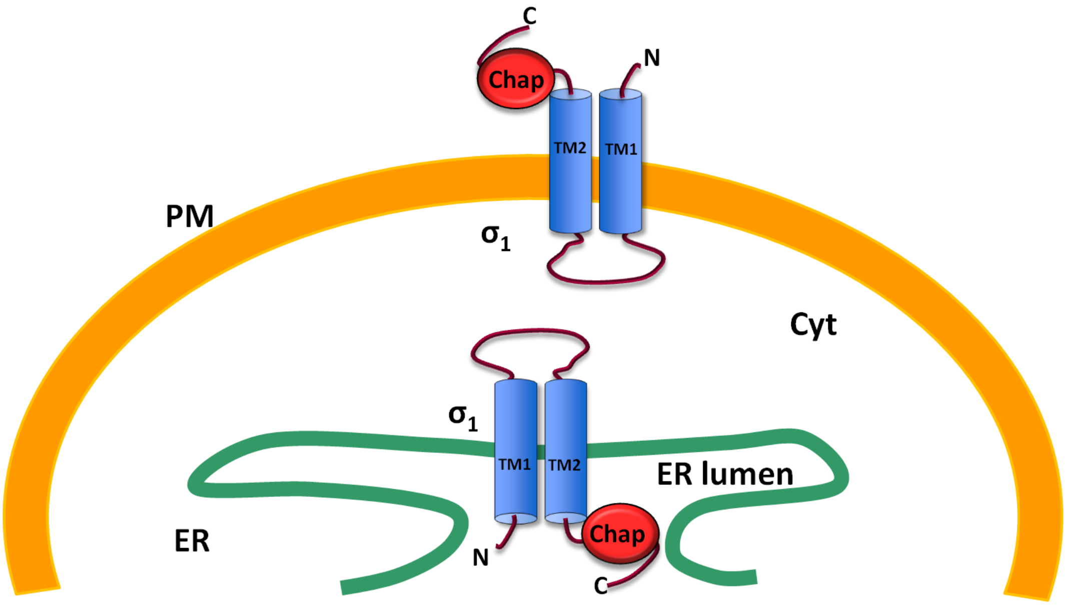


Fig. 3.1. Structure of σ1 receptors. These receptors exhibit two transmembrane domains (TM1 and TM2 in the figure) and a chaperone domain (Chap) close to C-terminus. The loop connecting the two transmembrane domains is located in the cytoplasm (Cyt), whereas the N- and C- termini are both facing to the endoplasmic reticulum (ER) lumen, when located in the ER, or to the extracellular side, when located in the plasma membrane (PM) (modified from Pabba, 2013).

Although the σ_1 receptor described above is the better understood, the existence of a splice variant of σ_1 receptors has been recently discovered. This short form contains less than half aminoacids in comparison to the large form of σ_1 receptors (Shioda et al., 2012). Although the short σ_1 receptor has a similar location than the large form, it likely lacks chaperone activity and ligand binding ability, as the coding region of the chaperone and ligand binding domains is truncated (Shioda et al., 2012).

Importantly, the cloning of σ_1 receptor allowed the design of specific antisense oligodeoxynucleotides (e.g. King et al., 1997; Pan et al., 1998) and the development of σ_1-receptor knockout mice (Langa et al., 2003). These tools have been extensively used for studying the role of these receptors on pain (as it will be described in the Section 3.6).

3.3. Pharmacological profile of σ_1 receptors

σ_1 receptor binds bezomorphans displaying stereospecificity for their dextrorotatory isomers, and also bind to a broad catalogue of compounds with high to moderate affinity, including: antipsychotics (e.g. haloperidol), antidepressants (e.g. fluvoxamine), antitussives (e.g. carbetapentane) and drugs of abuse (e.g. cocaine) (see Hayashi and Su, 2004; Cobos et al., 2008; Su et al., 2010 for references). Moreover, allosteric modulators of σ_1 receptors have also been described, such as the anticonvulsant drug phenytoin (Musacchio et al., 1989; Cobos et al., 2005 and 2006).

Neurosteroids (pregnenolone, dehydroepiandrosterone, progesterone, allopregnanolone and their sulfate esters) are considered the most likely naturally occurring σ_1 endogenous ligands (Maurice et al., 2001; Hayashi and Su, 2004; Cobos et al., 2008). In recent years it has been reported the existence of another putative endogenous ligand, *N,N*-dimethyltryptamine (DMT), which is a natural hallucinogen (Fontanilla, 2009). However, the affinities of most neurosteroids or DMT for σ_1 receptors fall in the µM range (Schwarz et al., 1989; Cobos et al., 2008; Fontanilla, 2009), and do not appear to be high enough for endogenous ligands. Therefore, the endogenous σ_1 ligands (if any) of σ_1 receptors have not yet been unequivocally defined.

Although the endogenous σ_1 ligands are still unclear, there have been developed some selective and high affinity σ_1 drugs for studying σ_1 receptor function. These include the prototypical σ_1 agonists (+)-pentazocine and PRE-084, and the σ_1 antagonists BD-1047, NE-100, BD-1063 (reviewed by Hayashi and Su, 2004; Cobos et al., 2008), and more recently the σ_1 antagonist S1RA, which has been shown to exhibit an exquisite selectivity for σ_1 receptors lacking affinity for 170 additional targets (Romero et al., 2012). This latter drug is currently in Phase II clinical trials for pain treatment, after completing a Phase I study showing excellent kinetics and tolerability (Abadías et al., 2013).

3.4. Anatomical and subcellular distribution of σ_1 receptors

The distribution of σ_1 receptor has been studied at anatomical and subcellular levels, and the knowledge of its location has provided information for the understanding of its possible functions.

At anatomical level, σ_1 receptors are widely localized in the central nervous system (CNS), including important areas for pain control, such as superficial layers of the dorsal horn, the periaqueductal gray matter, the locus coeruleus and rostroventral medulla (Roh et al., 2008b; Zamanillo et al., 2013; Sánchez-Fernández et al., 2014). Recently, it was also reported that σ_1 receptor is also present in the peripheral nervous system in both neuronal bodies of the dorsal root ganglion (DRG) (Bangaru et al., 2013) and in Schwann cells, specifically in their cytoplasm and in the paranodal region of Ranvier nodes (Palacios et al., 2004). Interestingly, although most functional pain studies focused on the role of central σ_1 receptors on pain processing, we recently found (as a part of this Doctoral Thesis) that σ_1 receptors are present in the DRGs at much higher density than in pain-related CNS areas (Sánchez-Fernández et al., 2014).

Besides to be found in the nervous system, σ_1 receptor is also expressed in a variety of nonnervous organs, including endocrine organs, gastrointestinal tract, liver, kidney, spleen and heart, among others, although its function in nonnervous tissue is much less investigated (e.g. Bowen, 2000; Stone et al., 2006; Bhuiyan and Fukunaga, 2011).

At subcellular level, the σ_1 receptor is localized as highly clustered globular structures enriched in cholesterol and neutral lipids in biological membranes, including microsomal, mitochondrial, nuclear and plasma membranes (Alonso et al., 2000; Hayashi and Su, 2004). Recent studies showed that σ_1 receptors are found to be particularly enriched in mitochondrion-associated endoplasmic reticulum (ER) membrane (MAM) (Hayashi and Su, 2007; Hayashi et al., 2011), and its function there will be detailed below.

3.5. σ_1 receptor as a calcium-sensing and ligand-operated chaperone

The MAM plays an important role in the transfer of Ca^{2+} from the endoplasmic reticulum to mitochondria, stimulating oxidative metabolism and regulating Ca^{2+} homeostasis (e.g. Pinton et al., 2008). σ_1 receptors constitutively interact through its chaperone domain with BiP (immunoglobulin heavy chain-binding protein) (Hayashi and Su, 2007; Su and Hayashi, 2010; Ortega-Roldan et al., 2013). This latter protein is another chaperone involved in the folding and assembly of proteins in the ER. In stress situations, when Ca^{2+} decreases in the ER or when σ_1 receptors bind to an agonist, the complex σ_1 receptor-BiP is dissociated and its chaperone activity increases. Then, σ_1 receptor is translocated to the ER reticular network to bind unstable IP_3 (inositol 1,4,5-trisphosphate) receptors (Fig. 3.2A). On the other hand, σ_1 antagonism is able to completely prevent the effect of σ_1 agonists by blocking the dissociation of σ_1 receptors from BiP (Fig. 3.2B) (Hayashi and Su, 2007; Ortega-Roldan et al., 2013).

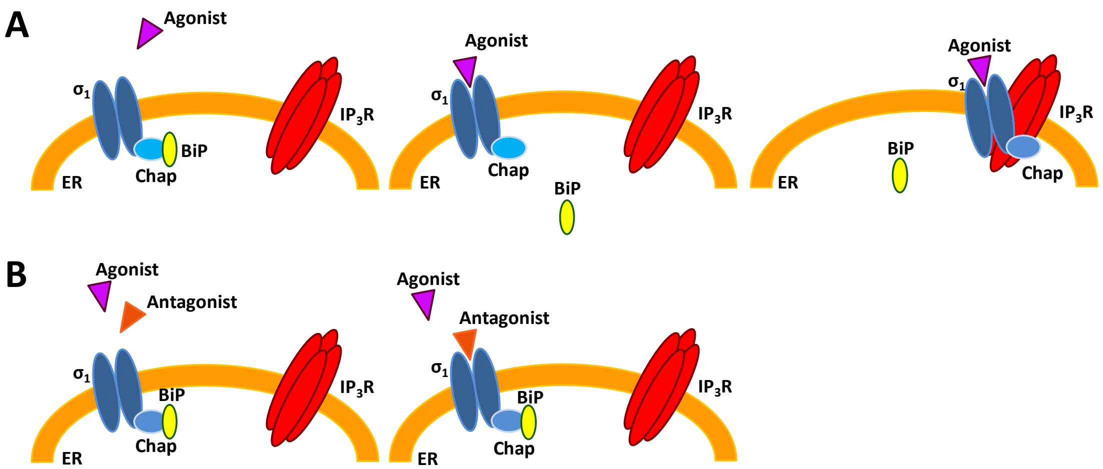


Fig. 3.2. Proposed mechanism for the modulation of IP_3 (inositol 1,4,5-trisphosphate) receptors at the endoplasmic reticulum (ER) by σ_1 receptors. (A) In the resting state, σ_1 receptors interact to BiP (immunoglobulin heavy chain-binding protein) through its chaperone (Chap) domain. The complex σ_1 receptor-BiP can be dissociated in response to a σ_1 agonist, with the consequent binding of σ_1 receptors to IP_3 receptors. (B) Blockade of the action of σ_1 agonists by σ_1 antagonists.

It has been very recently reported that the binding of σ_1 receptor to either agonists or antagonists leads to distinct conformational changes in the receptor, so that σ_1 agonists increase the separation between the *N*- and *C*-termini, while σ_1 antagonists produce the opposite effect (Gómez-Soler et al., 2014). These conformational changes in response to ligands might be related to the intracellular dynamics of σ_1 receptor, modulating its ability to form complexes with other proteins such as IP_3 receptors. The association between σ_1 and IP_3 receptors leads to the decrease of the degradation of the later, with the consequent enhancement of Ca^{2+} signalling from the ER into the mitochondria (Fig. 3.3). All of the described above correspond to the functions of the large isoform of σ_1 receptors. However, the short variant of σ_1 receptors appears to have opposing effects, holding a regulatory role on the functions of the large isoform by counteracting its actions on Ca^{+2} influx (Shioda et al., 2013).

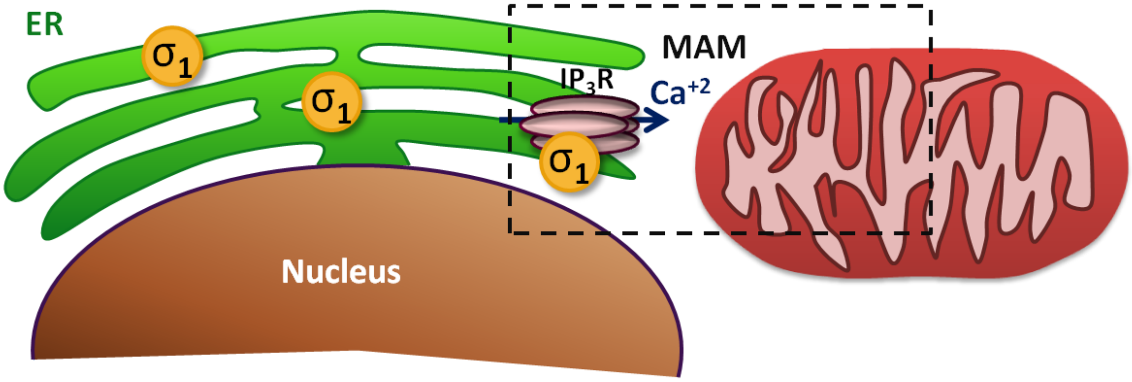


Fig. 3.3. Potentiation of Ca^{2+} influx from the endoplasmic reticulum (ER) to mitochondria by σ_1 receptors located at the mitochondrion-associated ER membrane (MAM). Taken from Crottès et al., 2013 with modifications.

3.5.1. σ_1 receptors as modulators of the activity of receptors and channels through protein-protein interaction: modulation of opioid and NMDA receptors

Although σ_1 receptor in resting conditions mainly reside at the MAM, under prolonged cellular stress conditions, σ_1 receptors translocate to other areas of the cells, such as the plasmalemmal area within the extended ER reticular network, or to the plasma membrane itself (Hayashi and Su, 2007; Su et al., 2010; Crottès et al., 2013). Once σ_1 receptors translocate, they can physically interact in their new location with different membrane targets, acting as a regulatory subunit of a wide variety of receptors and channels (Su et al., 2010; Kourrich et al., 2012).

These membrane targets include voltage dependent K^+ channels (Kv1.2, Kv1.4 and Kv1.5) (Aydar et al., 2002; Kourrich et al., 2013), L-type voltage dependent Ca^{2+} channels (VDCC) (but not other types of VDCC) (Tchedre et al., 2008; Gonzalez et al., 2012), acid-sensing ion channels of the subtype 1a (ASIC1a) (Herrera et al., 2008; Carnally et al., 2010), $GABA_A$ receptors (Kourrick et al., 2013) and G-protein coupled receptors (GPCR) such as dopamine D_1 and D_2 receptors (Navarro et al., 2010 and 2013). Although all these targets have been extensively documented to participate on pain neurotransmission (Ocaña et al., 2004; Lingueglia et al., 2006; Wemmie et al., 2006; Jaggi and Sing, 2011; Pradhan et al., 2012; Youn et al., 2013; Liu et al., 2014), two additional receptors targeted by the chaperoning activity of σ_1 receptors have been strongly linked to the effects of σ_1 receptors on animal models of pain: opioid receptors (Kim et al., 2010) and NMDA receptors (Balasuriya et al., 2014; Sánchez-Blázquez et al., 2014) (Fig. 3.4). As it will be extensively described in the next sections, σ_1 receptors have been proposed to impact on acute pain when opioid drugs are administered, and on tonic/chronic pain through the modulation of NMDA receptors.

The direct interaction between σ_1 and μ-opioid receptors has been reported recently (Kim et al., 2010). These receptors can be co-immunoprecipitated and it has been shown that σ_1 receptors negatively modulate μ-opioid signalling, since σ_1 antagonism is able to increase GTPγS in response to the μ-opioid agonist DAMGO (Kim et al., 2010). Although a direct coupling between σ_1 and other opioid receptor subtypes has not been demonstrated yet, it is likely that this occurs since σ_1 inhibition is widely reported to modulate κ- and δ-opioid mediated effects or signalling (e.g. Chien and Pasternak, 1994; King et al., 1997; Pan et al., 1998; Mei and Pasternak, 2002; Kim et al., 2010). It is worth pointing out that in addition to opioid receptors, several protein targets of σ_1 receptors are known to participate in opioid effects. For instance, L-type Ca^{2+} channels are among the downstream effectors of opioid signalling (Al-Hasani and Bruchas, 2011), and opioid antinociception is known to be modulated by NMDA receptors (reviewed by Pasternak and Pan, 2013). Therefore, the behavioral impact on the modulation of opioid effects by σ_1 receptors, which will be described in the next section, might be the result from simultaneous complex interactions between several membrane targets of the σ_1 receptors and not exclusively from the direct modulation of opioid receptors.

The modulation of NMDA receptors appears to be more complex. It has been reported that σ_1 receptors can modulate small conductance calcium-activated K^+ (SK) channels, which can be inhibited by σ_1 agonism (Martina et al., 2007). The activity of SK channels is known to inhibit NMDA receptors, and therefore σ_1 receptor activation can enhance the activity of NMDA receptors through this indirect mechanism (Martina et al., 2007) (Fig. 3.4). In addition to the indirect modulation of NMDA receptors through SK channels, it has been recently described that σ_1 receptors can directly modulate NMDA receptors through protein-protein interaction with the NR1 subunit (Balasuriya et al., 2013; Sánchez-Blázquez et al., 2014). These recent results successfully explained at the biochemical level the widely reported modulation of NMDA responses by σ_1 ligands in early electrophysiological experiments (reviewed by Bermack and Debonnel, 2005 and Monnet et al., 2006).

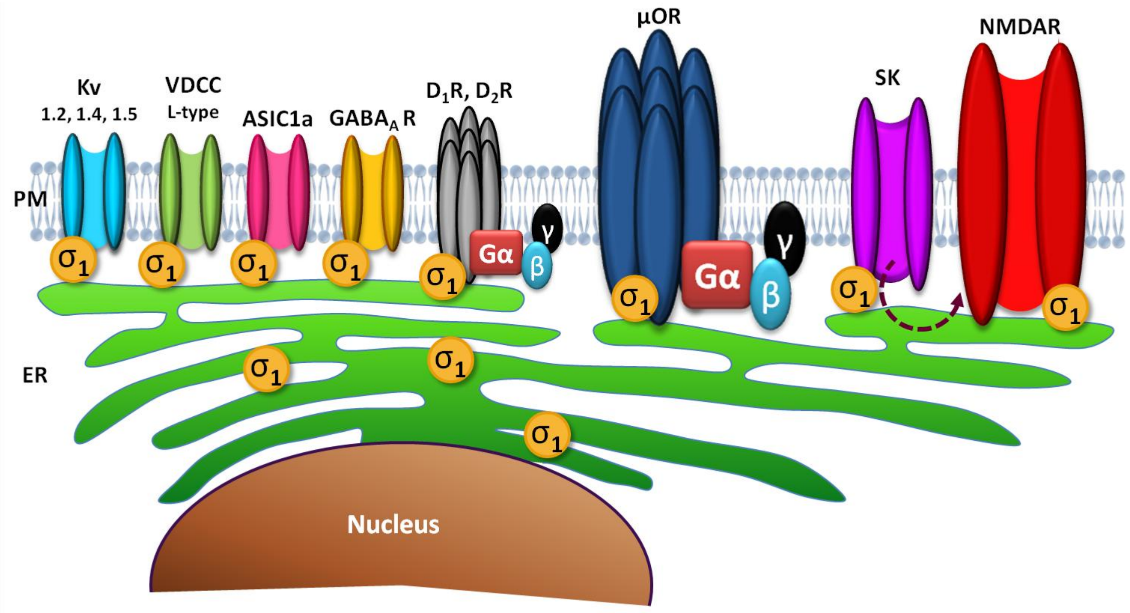


Fig. 3.4. Main protein targets of σ_1 receptors located at the plasma membrane (PM). σ_1 receptors located in the endoplasmic reticulum (ER) reticular network can interact through protein-protein interactions with several receptors and channels located in the PM. These protein targets include: voltage-gated K^+ channels (Kv1.2, 1.4, 1.5), L-type voltage dependent calcium channels (VDCC), acid-sensing ion channels of the subtype 1a (ASIC1a), $GABA_A$ receptors ($GABA_AR$), small conductance calcium-activated K^+ channels (SK), *N*-methyl-*D*-aspartate receptor (NMDAR) and G-protein coupled receptors (CGRP) such as dopamine (D_1R and D_2R) receptors and μ-opioid receptor (μOR). Until now the best studied σ_1 interactions have been those that implied μOR, NMDAR and SK channels, which have been represented at a larger size for illustrative purposes.

Interestingly, the heteromers formed by σ_1 and NMDA receptors are not limited to these proteins, since it has been recently reported that these complexes can harbor the cannabinoid receptor 1 (CB1) (Sánchez-Blázquez et al., 2014), expanding the modulatory role of σ_1 receptors on NMDA function to other membrane targets. At this time it is unknown if these complexes can be formed with other proteins important for pain processing. The discovery of these complexes, together with the variety of receptors and channels involved in neurotransmission which are targeted by σ_1 receptors, point to that the precise mechanisms by which σ_1 receptors modulate pain transmission are still at this time only very partially unveiled.

Rencently it has been reported that σ_1 receptors besides forming heteromers such as those described above, these receptors are also able to form homodimers and/or homomultimers. However, their physiological significance is not clear at the moment (Chu et al., 2013).

3.6. Evidence for the role of σ_1 receptors on animal models of pain

The involvement of σ_1 receptors on pain has been studied from several perspectives. Initially, and probably because of their early confusion with opioid receptors, the interaction between opioid antinociception and the effects of σ_1 drugs was explored in models of acute pain. The spectrum of pain models in which the role of σ_1 receptors was explored has been more recently greatly expanded to cover several types of pain from different aetiologies. In this section we will summarize the most important findings on the modulatory role of σ_1 receptors on preclinical pain models either in combination with opioid agonists or alone.

3.6.1. Modulation of opioid effects by σ_1 receptors

In contradistinction to opioid drugs, which can relieve acute pain from either thermal or mechanical stimuli (e.g. Ocaña et al., 2004; Pasternak and Pan, 2013), this is not the case of σ_1 drugs (e.g. Chien and Pasternak, 1994; Cendán et al., 2005b; Entrena et al., 2009; Sánchez-Fernández et al., 2013; Tejada et al., 2014). However, σ_1 receptors have been thoroughly shown to modulate opioid-induced antinociception to acute thermal stimuli (tail-flick test), particularly at central levels. We recently expanded these results by testing the modulation of opioid antinociception in peripheral tissue and to mechanical stimuli (paw pressure test), as part of this Doctoral Thesis. In addition to opioid antinociception, the possible role on nonanalgesic effects of opioids has been also explored (partially reviewed by Zamanillo et al., 2013). These findings will be summarized in the present section.

3.6.1.1. Modulation by σ_1 receptors of opioid-induced thermal antinociception

Although most experiments on the modulation of opioid antinociception by the σ_1 system have been performed using μ agonists (mainly morphine), there is also a broad body of evidence showing modulation of κ- and δ- opioid antinociception by σ_1 receptors. These experiments and their most relevant conclusions are summarized below.

3.6.1.1.1. Modulation by σ_1 receptors of μ-opioid-induced thermal antinociception

Pasternak's research group was the first to report the modulation of opioid antinociception by σ_1 drugs, much earlier than the recent discovery of the physical association between σ_1 and opioid receptors. They reported that the systemic administration of the selective σ_1 agonist (+)-pentazocine and the non-selective σ_1 drug 1,3-di(2-tolyl)guanidine (DTG) diminished the antinociceptive effect induced by the systemic administration of the prototypical μ-opioid agonist morphine (Chien and Pasternak, 1993 and 1994). On the other hand, haloperidol, a non-selective σ_1 antagonist, greatly enhanced morphine antinociception (Chien and Pasternak, 1993, 1994 and 1995a), which was abolished by the selective μ receptor antagonist β-funaltrexamine indicating that the activation of μ-opioid receptors was participating in the enhanced antinociception (Chien and Pasternak, 1994).

Although it is now widely assumed that haloperidol acts as a potent σ_1 antagonist (Cobos et al., 2008; Hayashi and Su, 2004), this drug binds to several other receptors such as dopamine receptors (mainly D_2 receptors) (Jaen et al., 1993), σ_2 receptors (e.g. Bowen et al., 1990; Moison et al., 2003) and NMDA receptors (e.g. Shim et al., 1999).

Among all pharmacological targets of haloperidol, the main targets of this neuroleptic are classically thought to be dopamine receptors, and in fact this drug is used in therapeutics as an antipsychotic (Donnelly et al., 2013). Interestingly, (-)-sulpiride, a known D_2 antagonist devoid of affinity for σ_1 sites (Bowen et al., 1990; Cobos et al., 2007), had no effect on morphine antinociception (Chien and Pasternak, 1993 and 1994), and both (+)-pentazocine and haloperidol were still able to modulate the antinociception induced by morphine in D_2 knockout mice (King et al., 2001), conclusively discarding the possible involvement of dopaminergic antagonism on the enhanced morphine antinociception induced by haloperidol. These experiments greatly contributed to the establishment of haloperidol as a prototypical σ_1 antagonist which although it is far from the desired selectivity on σ_1 receptors is still widely used in basic research on σ_1 receptors (Cendán et al., 2005b; Entrena et al., 2009b; Caplan, 2012, Delaunois et al., 2013). In addition, were the first evidences of the presence for a tonically active anti-opioid σ system, and led to subsequent deeper studies on the modulatory role of σ_1 receptors on opioid antinociception.

More recently, experiments using the systemic administration of other more selective σ_1 antagonists replicated the increase induced by haloperidol on the thermal antinociception produced by systemic morphine. Morphine thermal antinociception was enhanced by a spipethiane derivative (named as compound 9 in the original description of its effects, in Piergentili et al., 2010), and very recently the highly selective σ_1 antagonist S1RA (Vidal-Torres et al., 2014). Importantly, morphine is not the only μ agonist used in clinics shown to be modulated by σ_1 antagonism, since S1RA also increases the antinociceptive effect to thermal stimuli induced by the systemic administration of fentanyl, oxycodone, codeine, buprenorphine and tramadol (Vidal-Torres et al., 2013). Therefore, the modulation of morphine thermal antinoception is not restricted to this μ-opioid drug but is extensive to other relevant μ agonists.

The results of the studies testing the combination of systemic treatments with μ-opioids and σ_1 drugs on acute heat nociception (tail-flick test) are summarized in table 3.1.

Table 3.1. Summary of the effects on acute heat nociception (tail-flick test) of the combination of systemic treatments with μ-opioids and σ_1 drugs in WT mice.

Opioid receptor subtype	**Opioid drug**	**σ_1 receptor agonist/antagonis**	**σ_1 drug**	**Effect on opioid antinociception**	**References**
μ	Morphine	Agonist	(+)-Pentazocine	Inhibition	Chien and Pasternak, 1993 and 1994
			DTG		Chien and Pasternak, 1994
		Antagonist	Haloperidol	Enhancement	Chien and Pasternak, 1993, 1994 and 1995a
			Unnamed spipethiane derivative		Piergentili et al., 2010
			S1RA		Vidal-Torres et al., 2013
	Fentanyl	Antagonist	S1RA	Enhancement	Vidal-Torres et al., 2013
	Oxycodone				
	Codeíne				
	Buprenorphine				
	Tramadol				

Systemic treatments were performed via intraperitoneal (i.p.) administration in the study by Vidal-Torres et al., 2013, and subcutaneously in the rest of studies.

In agreement with the lack of effect of σ_1 drugs on acute thermal nociception (in the absence of opioid drugs), σ_1 receptor knockout (σ_1-KO) mice do not exhibit any deficit in the responses to thermal stimuli (De la Puente et al., 2009; Vidal-Torres et al., 2013; Tejada et al., 2014). However, in contradistinction to the enhancement of μ-opioid induced thermal antinociception by the pharmacological inhibition of σ_1 receptors, σ_1-KO mice do not exhibit any alteration in the effect induced by the systemic administration of morphine, sufentanyl, fentanyl or buprenorphine on thermal nociception (Vidal-Torres et al., 2013) (Table 3.2.). Although these results are apparently contradictory, might be explained by the development of compensatory mechanisms in the mutant mice (Vidal-Torres et al., 2013). Conflicting results between the pharmacological and genetic inhibition of σ_1 receptors has been reported in other pain models (as it will be described in the Section 3.6.2, and also in previous pain studies of targets other than σ_1 receptors (e.g., Petrus et al., 2007; Bonin et al., 2011), or in fields different than pain research (e.g., Guscott et al., 2005; Voss et al., 2010).

Therefore, the development of compensatory mechanisms is not a peculiarity of σ_1-KO mice or pain research with mutant animals but extensive to other targets and to other fields of study. Regardless of the nature of these compensatory mechanisms, σ_1-KO mice can be used to test the specificity of drugs, since their pharmacological target is absent and it would be expected that a drug targeting the absent receptor will be devoid of effect. In this context, the σ_1 antagonist S1RA was unable to increase μ-opioid induced thermal antinociception in σ_1-KO mice (Vidal-Torres et al., 2013), supporting on-target mechanisms for the effect of this drug.

The results of the studies testing the effects of μ opioids on σ_1–KO mice on acute heat nociception are summarized in table 3.2.

Further studies were conducted to determine the anatomical location of the modulation of μ-opioid thermal antinociception by σ_1 receptors. The antinociception induced by the intracerebroventricular (i.c.v.) administration of morphine was decreased by the σ_1 agonist (+)-pentazocine either systemically (Chien and Pasternak, 1994) or i.c.v. administered (Mei and Pasternak, 2002). Conversely, the antinociceptive effect induced by systemic morphine could be decreased by i.c.v. treatment with (+)-pentazocine (Mei and Pasternak, 2002). These results indicate that the inhibitory effects on morphine antinociception induced by σ_1 agonism could be produced at supraspinal levels. The presence of the tonically active anti-opioid σ_1 system at supraspinal locations was determined by several approaches. The antinociceptive effect elicited by the supraspinal (i.c.v.) administration of DAMGO was enhanced by the systemic administration of the σ_1 antagonist (+)-MR200 (Marrazzo et al., 2006), and the antinociceptive effect of supraspinal morphine was enhanced by the central administration of specific antisense oligodeoxynucleotides (ASOs) to inhibit the expression of σ_1 receptors (Pan et al., 1998; Mei and Pasternak, 2002). Interestingly, the antinociceptive effect induced by spinal morphine was not affected by either i.c.v. or i.t. treatment with the σ_1 agonist (Mei and Pasternak, 2002), although it could be decreased by systemic (+)-pentazocine (Chien and Pasternak, 1994), which might indicate that the activation of both spinal and supraspinal σ_1 sites might be necessary to functionally impact on spinal morphine antinociception (Mei and Pasternak, 2002), and that although σ_1 receptors are expressed at either supraspinal and spinal sites (Alonso et al., 2000; Mei and Pasternak, 2002; Sánchez-Fernández et al., 2014), the modulation of opioid antinociception by spinal σ_1 receptors is less promiment than that produced by supraspinal σ_1 receptors.

Table 3.2. Summary of the effects on acute heat (tail-flick test) and mechanical (paw pressure) nociception of μ opioids in σ_1-KO mice.

		Effect of σ_1 receptor knockout on opioid antinociception		
Opioid drug	**Route**	**Thermal**		**Mechanical**
		without S1RA	**with S1RA (s.c.)**	
Morphine	s.c.	No effects	abolishment of the potentiation of μ-opioid antinociception induced by S1RA	Enhancement
	i.pl	n.t.	n.t.	
Fentanyl	s.c.	No effects	abolishment of the potentiation of μ-opioid antinociception induced by S1RA	
Oxycodone				
Buprenorphine				
Tramadol		n.t.	n.t.	
Loperamide				

Abbreviations: s.c., subcutaneous; i.pl., intraplantar; n.t., not tested.
Codeine and sufentanyl were evaluated in the tail-flick test σ_1-KO mice, with similar effects than the other opioids tested, but these results have been omitted in this table for clarity. Results on thermal nociception can be found in Vidal-Torres et al., 2013, and results on mechanical nociception can be found in Sánchez-Fernández et al., 2013 and 2014.

The specific supraspinal sites involved in the modulation of morphine antinociception by σ_1 receptors were determined by subsequent experiments. The rostroventral medulla (RVM), the periaqueductal gray (PAG) and the locus coeruleus (LC) constitute important sites for the circuit responsible of the descending modulation of pain (Fields, 2000), and μ-opioids strongly act in these nuclei to elicit analgesia (Porreca et al., 2002). Although σ_1 receptor is expressed in these three areas (Alonso et al., 2000; Sánchez-Fernández et al., 2014), the modulation of morphine antinociception differed depending on the injection site (Mei and Pasternak, 2007). The microinjection of morphine into any of these three regions elicited antinociception, which was decreased by the co-administration of (+)-pentazocine in the same region (although the PAG was much less sensitive than the other two sites). Blockade of the σ_1 receptors with haloperidol in the RVM markedly enhanced the antinociceptive actions of coadministered morphine, implying a strong tonic activity of the σ_1 system in this site. This effect was mimicked by down-regulation of RVM σ_1 receptors using specific σ_1-ASOs. However, no tonic σ_1 activity was observed in either the LC or the PAG (evidenced by the lack of response to haloperidol coadministered with morphine in these regions). Highlighting the marked role of σ_1 receptors in the RVM, the pharmacological agonism of σ_1 receptors in this site was able to modulate the antinociception elicited from morphine microinjected into the PAG, whereas haloperidol in the RVM enhanced PAG morphine analgesia (Mei and Pasternak, 2007). These results illustrate the importance on morphine antinociception of brainstem σ_1 receptors, and in particular of those located in the RVM.

The results of the studies testing the effects on acute heat nociception of the combination of μ-opioids and σ_1 drugs or σ_1-ASOs, administered at specific central nervous system sites, are summarized in table 3.3.

Table 3.3. Summary of the effects on acute heat nociception (tail-flick test) of the combination of μ-opioids and σ_1 drugs or antisense oligodeoxynucleotides (ASOs), administered at specific central nervous system sites.

Opioid receptor subtype	**Opioid drug**	**Route**	**σ_1 receptor agonist/antagonist**	**σ_1 drug or σ_1-ASO**	**Route**	**Effect on opioid antinociception**	**References**
μ	Morphine	s.c.	Agonist	(+)-Pentazocine	i.c.v	Inhibition	Mei and Pasternak, 2002
		i.c.v.	Agonist	(+)-Pentazocine	s.c.	Inhibition	Chien and Pasternak, 1994
					i.c.v		Mei and Pasternak, 2002
			-	ASO	i.c.v	Enhancement	Pan et al., 1998; Mei and Pasternak, 2002
		PAG	Agonist	(+)-Pentazocine	PAG	Inhibition	Mei and Pasternak, 2007
					RVM		
		LC			LC		
		RVM			RVM		
		RVM	Antagonist	Haloperidol	RVM	Enhancement	Mei and Pasternak, 2007
		PAG			PAG	No effect	
					RVM	Enhancement	
		LC			LC	No effect	
		RVM	-	ASO	RVM	Enhancement	
		PAG					
		i.t.	Agonist	(+)-Pentazocine	s.c.	Inhibition	Chien and Pasternak, 1994
					i.c.v .	No effect	Mei and Pasternak, 2002
					i.t.		Mei and Pasternak, 2002
	DAMGO	i.c.v	Antagonist	(+)-MR200	s.c.	Enhancement	Marrazzo et al., 2006

Abbreviations: s.c., subcutaneous; i.c.v., intracerebroventricular; i.t., intrathecal; RVM, rostroventral medulla; PAG, periaqueductal gray matter; LC, locus coeruleus; ASO, antisense oligodeoxynucleotide.

3.6.1.1.2. Modulation by σ_1 receptors of κ-and δ-opioid-induced thermal antinociception

In addition to the modulation of μ-opioid antinociception by σ_1 receptors, the effects of the systemic administration of σ_1 drugs on the thermal antinociception induced by the systemic administration of agonists of other opioid receptors was tested. Similar to the results found with the modulation of μ-opioid antinociception by σ_1 drugs, systemic σ_1 agonism (by the prototypic σ_1 agonist (+)-pentazocine and the purported σ_1 agonist (±)-PPCC) inhibited the antinociception induced by the systemic administration of the κ_1-opioid agonist U50,488H (Chien and Pasternak, 1994; Ronsisvalle et al., 2001; Prezzavento et al., 2008). On the other hand, systemic administration of the σ_1 antagonists haloperidol, MR200 and (-)-MRV3 were able to enhance U50,488H-induced antinociception (Chien and Pasternak, 1994 and 1995a; Ronsisvalle et al., 2001; Marrazzo et al., 2011). However, the σ_1 antagonist BD-1047 unexpectedly induced a decrease of the antinociception induced by U50,488H (Prezzavento et al., 2008). Although BD-1047 is widely used as a σ_1 antagonist (e.g. Hayashi and Su, 2004; Cobos et al., 2008), its selectivity has been tested in a panel of only 10 receptors (Matsumoto et al., 1995). Therefore, a possible explanation of these discordant effects induced by BD-1047 might be that this drug could be interacting to unknown target/s interfering with opioid antinociception.

In addition, the σ_1 antagonist haloperidol was also able to increase the antinociception induced by the κ_3-opioid agonist naloxone benzoylhydrazone (NalBzoH) (Chien and Pasternak, 1994 and 1995a) and that induced by the nonselective κ-opioid analgesic (-)-pentazocine (Chien and Pasternak, 1995b). (-)-Pentazocine is a peculiar drug because it has high affinity for several other receptors including σ_1 receptors (although with a considerably lower affinity than its dextrogyre isomer) behaving as a σ_1 agonist (Chien and Pasternak., 1995b). Due to this agonistic activity over σ_1 receptors, high doses of (-)-pentazocine are thought to be able to inhibit its own opioid-induced antinociception, which results in animals in a biphasic dose-response curve (Chien and Pasternak, 1995b). The interest of this observation is that pentazocine (as a racemic mixture) is used in humans patients as an opioid analgesic (Adeniji and Atanda, 2013;

King et al., 2013), and both (+)-pentazocine and (-)-pentazocine through σ_1 agonism could be interfering in the opioid analgesia induced by (-)-pentazocine. However, the relevance in the clinical practice of the interaction of pentazocine with σ_1 receptors is still unknown.

The results of the studies testing the combination of systemic treatments with κ opioids and σ_1 drugs on acute heat nociception (tail-flick test) are summarized in Table 3.4.

Table 3.4. Summary of the effects on acute heat nociception (tail-flick test) of the combination of systemic treatments with κ opioids and σ_1 drugs.

Opioid receptor subtype	Opioid drug	σ_1 receptor agonist/antagonist	σ_1 drug	Effect on opioid antinociception	References
κ	U50,488H	Agonist	(+)-Pentazocine	Inhibition	Chien and Pasternak, 1994; Ronsisvalle et al., 2001 and Prezzavento et al., 2008
			(±)-PPCC		Prezzavento et al., 2008
		Antagonist	Haloperidol	Enhancement	Chien and Pasternak, 1994 and 1995a
			MR200		Ronsisvalle et al., 2001
			(-)-MRV3		Marrazzo et al., 2011
			BD-1047	Inhibition	Prezzavento et al., 2008
	NalBzoH	Antagonist	Haloperidol	Enhancement	Chien and Pasternak, 1994 and 1995a
	(-)-Pentazocine	Antagonist	Haloperidol	Enhancement	Chien and Pasternak, 1995b

All systemic treatments were performed by the subcutaneous administration of the σ_1 drugs.

As for the modulation of µ-opioid antinociception by σ_1 receptors, it has been reported that supraspinal σ_1 receptors play a pivotal role on the antinociceptive effects induced not only by the κ agonists U50,488H and NalBzoH, but also by the δ agonist DPDPE. It was shown that the supraspinal administration of (+)-pentazocine inhibited the antinociception induced by U50,488H and NalBzoH systemically administered, and that induced by the i.c.v. administration of DPDPE (Mei and Pasternak, 2002), while σ_1 antisense treatments increased the antinociceptive effects of all opioid drugs tested

(King et al., 1997; Pan et al., 1998; Mei and Pasternak, 2002), demonstrating the presence of the tonic activation of the σ_1 system inhibiting κ antinociception. In agreement with the tonic supraspinal inhibitory control of κ and δ antinociception by σ_1 receptors, it was found that the systemic administration of the σ_1 antagonist (+)-MR200 was able to enhance the antinociception induced by the supraspinal administration of either U50,488H or DPDPE (Marrazzo et al., 2006). Interestingly, the systemic administration of haloperidol was able to enhance the antinociceptive effect induced by spinal DPDPE (Chien and Pasternak, 1994), although it is unclear if the modulatory effect of haloperidol was produced at spinal or supraspinal levels (or both).

The results of the studies testing the effects on acute heat nociception of the combination of κ or δ opioids and σ_1 drugs or σ_1-ASOs, administered at specific central nervous system sites, are summarized in table 3.5.

Taking into account all these studies together, it seems clear that in addition to the modulation by central σ_1 receptors (particularly supraspinally) of the thermal antinociception by μ agonists, they also strongly modulate κ- and δ-mediated antinociception.

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Table 3.5. Summary of the effects on acute heat nociception (tail-flick test) of the combination of κ- or δ- opioids and σ_1 drugs or antisense oligodeoxynucleotides (ASOs), administered at specific central nervous system sites.

Opioid receptor subtype	**Opioid drug**	Route	**σ_1 receptor agonist/antagonist**	**σ_1 drug or σ_1-ASO**	**Route**	**Effect on opioid antinociception**	**References**
κ	U50,488H	s.c.	Agonist	(+)-Pentazocine	i.c.v.	Inhibition	Mei and Pasternak, 2002
			-	ASO	i.c.v.	Enhancement	King et al., 1997; Pan et al., 1998
		i.c.v.	Antagonist	(+)-MR200	s.c.	Enhancement	Marrazzo et al., 2006
	NalBzoH	s.c.	Agonist	(+)-Pentazocine	i.c.v	Inhibition	Mei and Pasternak, 2002
			-	ASO	i.c.v	Enhancement	Mei and Pasternak, 2002; Pan et al., 1998; King et al., 1997
δ	DPDPE	i.c.v.	Agonist	(+)-Pentazocine	i.c.v	Inhibition	Mei and Pasternak, 2002
			-	ASO		Enhancement	Mei and Pasternak, 2002; Pan et al.,1998
		i.c.v.	Antagonist	(+)-MR200	s.c.	Enhancement	Marrazzo et al., 2006
		i.t.		Haloperidol			Chien and Pasternak, 1994

Abbreviations: s.c., subcutaneous; i.c.v., intracerebroventricular; i.t., intrathecal; ASO, antisense oligodeoxynucleotide; NalBzoH, Naloxone benzoylhydrazone; DPDPE, [D-Pen^2, D-Pen^5]enkephalin.

3.6.1.2. Modulation by σ_1 receptors of μ-opioid-induced mechanical antinociception

Bearing in mind that the neurochemical mechanisms underlying opioid-induced mechanical and thermal antinociception differ (Kuraishi et al., 1985; Sato et al., 1999; Tseng et al., 1995; Wegert et al., 1997), the previously described modulation of opioid-induced thermal antinociception by σ_1 receptors was not necessarily expected to be applicable to mechanical stimulation. In addition, although the role of central σ_1 receptors on opioid antinociception has been explored in the past (as thoroughly described in the sections above), the possible role of peripheral σ_1 receptors on opioid antinociception was absolutely unknown. Therefore, the main goal of this Doctoral Thesis was to explore the role of σ_1 receptors on opioid mechanical antinociception, and specifically focusing on the role of peripheral σ_1 receptors in the modulation of the antinociception. Since the most clinically relevant opioid drugs are agonists of the μ subtype (Kieffer, 1999; Pasternak and Pan, 2011; Pasternak and Pan, 2013), we tested a variety of μ opioids in clinical use

We found that σ_1–KO mice showed an enhanced mechanical antinociception induced by morphine, fentanyl, oxycodone, buprenorphine and tramadol (Sánchez-Fernández et al., 2013 and 2014). These data are in contrast with the absence of modulation of opioid thermal antinociception in these mutant mice purportedly by compensatory mechanisms (Vidal-Torres et al., 2013), as described in the Section 3.6.1.1.1 and in Table 3.2., and may be attributable to the known differences in the neurochemical mechanisms of thermal and mechanical opioid antinociception mentioned above, which may be affected differentially by possible compensatory mechanisms in σ_1-KO mice. Systemic σ_1 pharmacological σ_1 blockade replicated the increase in μ-opioid antinociception seen in the σ_1-KO for all opioids tested (Sánchez-Fernández et al., 2013 and 2014). Interestingly, this σ_1 inhibition (by either σ_1 knockout or systemic σ_1 pharmacological antagonism) was sufficient to unmask strong antinociceptive effects by loperamide (Sánchez-Fernández et al., 2014), a peripherally restricted μ-opioid agonist used in therapeutics as an antidiarrheal drug (Layer et al., 2010; Gallelli et al., 2010), and the intraplantar (i.pl.) administration of σ_1 antagonists was able to increase the antinociception to mechanical stimuli induced by the systemic administration of all μ opioids tested (Sánchez-Fernández et al., 2014). Reinforcing the peripheral mediation of the effects observed, the enhanced antinociception by σ_1 inhibition was very sensitive to peripheral opioidantagonism (i.e. to the peripherally restricted opioid antagonist naloxone methiodide) (Sánchez-Fernández et al., 2014). In addition, and adding evidence to the peripheral antinociceptive synergism between σ_1 inhibition and opioid agonism, we found that the intraplantar administration of morphine showed a marked enhanced antinociception in σ_1-KO mice and in wild-type mice coadministered with several σ_1 antagonists in the morphine-injected paw (Sánchez-Fernández et al., 2013). These results clearly show that the modulation of μ-opioid antinociception by σ_1 receptors is not restricted to either thermal stimuli or to central sites, and constituted the first reported evidence of a role of peripheral σ_1 receptors on pain.

The results obtained on the modulatory role of σ_1 receptors of μ-opioid induced mechanical antinociception are summarized in Tables 3.2. and 3.6. (for the experiments performed in σ_1-KO mice and using σ_1 drugs, respectively), and will be exhaustively presented in the Chapter "Published Papers" of this Doctoral Thesis.

Further research will be needed to test whether the modulation of peripheral opioid antinociception by σ_1 receptors is restricted to μ agonists or if it is extensive to other types of opioids, and to test whether this peripheral modulation of opioid mechanical antinociception is extensive to pain induced by thermal stimuli. Although there are undoubtedly still some gaps on this field of research, the discovery of the modulatory role of peripheral σ_1 receptors on the effects of opioids on acute pain planted the seed for subsequent studies on the modulation of other types of pain by peripheral σ_1 receptors, described in the section below.

Table 3.6. Summary of the effects on acute mechanical nociception (paw pressure test) of the combination of opioids and σ_1 drugs

Opioid drug	Route	σ_1 receptor agonist/antagonist	σ_1 drug	Route	Effect on opioid antinociception	References
Morphine	s.c.	Antagonist	BD-1063	s.c.	Enhancement	Sánchez-Fernández et al., 2013 and 2014
				i.pl.	Enhancement	Sánchez-Fernández et al., 2014
			BD-1047	s.c.	Enhancement	Sánchez-Fernández et al., 2013
			NE-100			
			S1RA	s.c.	Enhancement	Sánchez-Fernández et al., 2013 and 2014
		Agonist	PRE-084	s.c.	No effect	Sánchez-Fernández et al., 2013
	i.pl.	Antagonist	BD-1063	i.pl.	Enhancement	Sánchez-Fernández et al., 2013
			BD-1047			
			NE-100			
			S1RA			
Fentanyl	s.c.	Antagonist	BD-1063	s.c.	Enhancement	Sánchez-Fernández et al., 2014
				i.pl.		
			S1RA	s.c.		
				i.pl.		
Oxycodone	s.c.	Antagonist	BD-1063	s.c.	Enhancement	Sánchez-Fernández et al., 2014
				i.pl.		
Buprenorphine	s.c.	Antagonist	BD-1063	s.c.	Enhancement	Sánchez-Fernández et al., 2014
				i.pl.		
Tramadol	s.c.	Antagonist	BD-1063	s.c.	Enhancement	Sánchez-Fernández et al., 2014
				i.pl.		
Loperamide	s.c.	Antagonist	BD-1063	s.c.	Enhancement	Sánchez-Fernández et al., 2014
				i.pl.		
			S1RA	s.c.		
				i.pl.		

Abbreviations: s.c., subcutaneous; i.pl., intraplantar

3.6.1.3. Modulation by σ_1 receptors of non-analgesic (adverse) effects of opioids

As it has been described in the Chapter “µ-Opioid drugs: mechanism of action, therapeutic use and side effects” although opioid drugs are widely used in therapeutics for pain management, their well-known side effects strongly limit their use (Benyamin et al., 2008; Al-Hasani and Bruchas, 2011; Ringkamp and Raja, 2012).

Before the experiments presented in this Doctoral Thesis were conducted very few studies existed about the role of σ_1 receptor in non-analgesic opioid effects. The only previously published study was performed by Chien and Pasternak, who reported that unlike the decrease on the antinociceptive effects of opioids induced by σ_1 agonism, the inhibition of gastrointestinal transit or lethality induced by morphine were not affected by the administration of (+)-pentazocine (Chien and Pasternak, 1994). However, the effect of σ_1 inhibition in non-analgesic opioid effects remained untested. The importance of the study of the modulation by σ_1 antagonists of non-analgesic effects of opioids is that if they would equally increase analgesic and non-analgesic opioid effects, it would decrease the interest for the possible future clinical use of σ_1 antagonists as opioid adjuvants to enhance opioid-induced analgesia.

At present, the possible modulation by σ_1 inhibition on a wide variety of non-analgesic opioid effects has been explored in rodents. These include hyperlocomotion, mydriasis, withdrawal, tolerance, dependence and gastrointestinal transit inhibition. Changes in pupil size are a well-known centrally-induced non-analgesic effect of opioids. Although in humans opioid drugs induce myosis (Al-Hasani and Bruchas, 2011), in mice they induce mydriasis (Stav et al., 1992; Vidal-Torres et al., 2013). S1RA administration did not modify the pupillary diameter when administered alone, and did not modify morphine-induced mydriasis in rodents (Vidal-Torres et al., 2013). Other paradoxically and centrally-induced non-analgesic opioid effect is the sedation which is often produced in humans (Al-Hasani and Bruchas, 2011) while increasing the locomotor activity in mice (Hnasko et al., 2005). We found that morphine-induced hyperlocomotion was not modified either by σ_1 inhibition (σ_1-KO) mice (Sánchez-Fernández et al., 2013; see Chapter "Published Papers” for a detailed description of these findings). Furthermore, σ_1 pharmacological antagonism (by S1RA) did not change the severity of somatic manifestations of naloxone-induced morphine withdrawal nor the development of morphine tolerance in mice (Vidal-Torres et al., 2013), which are very relevant opioid side effects in humans (Al-Hasani and Bruchas, 2011). σ_1 antagonism was also effective in restoring morphine antinociception in morphine-tolerant mice, and interestingly, the rewarding effects of morphine (evaluated by place conditioning) were antagonized (Vidal-Torres et al., 2013). This later result although in opposite direction to the widely reported enhanced opioid antinociception by σ_1 antagonism, agree with additional preclinical studies which proposed the use of σ_1 antagonists as promising tools for the treatment of addiction and dependence induced by other drugs of abuse (e.g. Matsumoto, 2009).

As a part of this Doctoral Thesis, we reported that gastrointestinal transit inhibition induced by morphine, fentanyl and loperamide, was unaltered by σ_1 receptor inhibition (Sánchez-Fernández et al., 2013 and 2014) (see Chapter "Published Papers"). The lack of involvement of σ_1 receptor in the inhibition of gastrointestinal transit has been confirmed in a parallel study (Vidal-Torres et al., 2013). This opioid non-analgesic effect has a high clinical relevance, since is one of the main reasons for patients' voluntary withdrawal from opioid medication (Dhingra et al., 2013), and in contradistinction to the other opioid side effects, it is produced mainly peripherally (Al-Hasani and Bruchas, 2011; Ringkamp and Raja, 2012).

Therefore, σ_1 receptors do not appear to modulate either centrally- or peripherally-induced opioid non-analgesic effects. Although the mechanisms responsible of the differential modulation of opiod antinociception and their non-analgesic effects by σ_1 receptors are unclear, it can be concluded that σ_1 antagonism is a promising pharmacological tool for differentially enhancing the analgesic effects of opioids while minimizing their side effects.

3.6.2. Pain modulation by σ_1 receptors in the absence of opioid drugs

In addition to the described role of σ_1 receptors on the modulation of opioid effects on acute nociceptive pain induced by either thermal or mechanical stimuli, it has been widely reported that σ_1 inhibition might play a role in the absence of opioids to ameliorate other types of pain, in particular those produced by chemical irritation or sensitization of pain pathways.

3.6.2.1. σ_1 receptors and pain induced by chemical irritants: formalin and capsaicin

Formalin is one of the chemical irritants most widely used in pain research (Le Bars et al., 2001). Formalin injection induces a biphasic pain response in rodents: an initial acute pain response (first phase) due to the direct activation of nociceptors, followed by a prolonged tonic response (second phase) characterized by spontaneous activity of primary afferent neurons together with functional changes in the spinal cord (central sensitization) accompanied by an edematous process (Le Bars et al., 2001; Sawynok and Liu, 2004). Although in pain studies formalin is classically administered in the paw, it can be also injected into the trigeminal area (e.g. Chavelou et al., 1995) to study orofacial pain, which is a distinct type of pain known to be resistant to analgesic treatment (see Sarlani et al., 2005).

The first reported evidences on the role of σ_1 receptors on a chemically-induced pain model were performed in the formalin test. σ_1-KO mice showed a significant decrease of both nociceptive phases after intraplantar formalin (Cendán et al., 2005a). Moreover, the systemic pharmacological antagonism of σ_1 receptors, using either non-selective (such as haloperidol) and selective drugs (such as S1RA), also decreased both phases of formalin-induced pain when the chemical irritant was administered into the paw (Cendán et al., 2005b; Romero et al., 2012; Díaz et al., 2013; Lan et al., 2014; Gómez-Soler et al., 2014; Vidal-Torres et al., 2014) or into the trigeminal area (Roh and Yoon, 2014).

Since the involvement of central sensitization in the second phase of formalin-induced pain is prominent, further studies focused on the role of spinal σ_1 receptors on this pain model. It was found that the i.t. administration of σ_1 antagonists reduced formalin induced pain (Kim et al., 2006; Vidal-Torres et al., 2014), and this was accompanied by a reduction of the phosphorylation (activation) of the NR1 subunit of the NMDA at either protein kinase C (PKC) and protein kinase A (PKA) sites (Kim et al., 2006), which plays a pivotal role in central sensitization (Latremoliere and Woolf, 2009). Although it is unknown whether σ_1 receptors can directly modulate the activity of intracellular kinases, taking into account that σ_1 receptors can directly bind to the NR1 subunit of NMDA receptors (Balasuriya et al., 2013; Sánchez-Blázquez et al., 2014), one tempting speculation to explain (at least partially) the decrease in pNR1 by σ_1 antagonism could be that the accessibility of the phosphorylation sites of the NR1 would depend on the activation state of σ_1 receptors, and that σ_1 inhibition would lead to a decrease in the accessibility to these sites by the intracellular kinases.

Recently it has been explored the role of σ_1 receptors at other anatomical locations in formalin-induced pain. It was found that supraspinal σ_1 antagonism increased noradrenaline levels in the spinal cord, suggesting the involvement of descending inhibitory pathways in the mechanism of action of σ_1 inhibition to decrease pain (Vidal-Torres et al., 2014). Interestingly, it was recently reported that σ_1 antagonism in the place of the administration of formalin was also able to reduce pain behavior, indicating that peripheral σ_1 receptors also participate in the decrease of formalin-induced pain by σ_1 inhibition (Vidal-Torres et al., 2014). These results indicate that σ_1 receptors facilitate formalin-induced pain at central sites (both spinally and supraspinally) and also peripherally.

Further experiments were performed using capsaicin as a chemical irritant. The intradermal injection of capsaicin induces a decrease in the mechanical threshold of the area surrounding the injection even if it was not stimulated by capsaicin (area of secondary hypersensitivity), which is produced by central sensitization (Sang et al, 1996; Baron, 2000). The interest of exploring the effects of σ_1 inhibition on capsaicin-induced secondary mechanical hypersensitivity was that this model is widely used in clinical research to test the effects of drugs on mechanical allodynia (e.g. Eisenach et al., 2002; Gottrup et al., 2004) and it is considered to be a surrogate model of neuropathic pain, since anti-neuropathic drugs show antiallodynic activity in this test in both humans and rodents (Gottrup et al., 2004; Joshi et al., 2006). It has been shown that σ_1 knockout mice did not sensitize to mechanical stimuli in response to capsaicin

(Entrena et al., 2009a), and this phenotype was mimicked by σ_1 antagonists, such as the non-selective drug haloperidol or selective σ_1 antagonists including BD-1063 or S1RA (Entrena et al., 2009a and b; Romero et al., 2012; Díaz et al., 2013).

Importantly, the effects of σ_1 inhibition on either capsaicin-induced secondary mechanical hypersensitivity or formalin-induced pain were not reversed by the opioid antagonist naloxone (Cendán et al., 2005b and Entrena et al., 2009b, respectively), definitively indicating that these effects are independent to the modulation of the opioidergic system, and that σ_1 receptors can act through other mechanisms to decrease pain transmission.

Both formalin and capsaicin are known C-fiber activators, although through distinct receptors. While formalin activates TRPA1, capsaicin stimulates TRPV1 (Kerstein et al., 2009; Dubin and Patapoutian, 2010). The repetitive activation of C-fibers is known to increase the excitability of spinal cord neurons, a phenomenon named wind-up which can contribute to the establishment of central sensitization (Herrero et al., 2000; Latremoliere and Woolf, 2009). This process is known to be inhibited in σ_1–KO mice (de la Puente et al., 2009) or by S1RA in WT animals (Romero et al., 2012), which support the role of σ_1 receptors on the hyperexcitability of spinal neurons that contribute to pain hypersensitivity after the injection of formalin or capsaicin.

The utilities of capsaicin administration as a pain model are not restricted to somatic pain, since this chemical algogen can be also administered in the gut to produce visceral pain. Visceral pain induced by capsaicin shows two distinct components: an intense (acute) pain and refereed mechanical hyperalgesia, and these two components have been reported in both humans (Drewes et al., 2003; Schmidt et al., 2004) and rodents (Laird et al., 2001). The pathophysiological mechanisms and response to drug treatments are different in somatic and visceral pain (Cerveró and Laird, 1999). In fact, in contradistinction to somatic afferents, visceral afferents are known to be unable to trigger wind-up in the spinal cord (reviewed by Herrero et al., 2000), hence the interest of testing the effects of σ_1 receptor inhibition on a visceral pain model. Systemic administration of a variety of selective σ_1 antagonists (BD-1063, S1RA and NE-100) attenuated both acute pain-related behaviours and refereed mechanical hyperalgesia induced by intracolonic capsaicin (González-Cano et al., 2013). Interestingly, σ_1 knockout mimicked the effects of σ_1 antagonists in the decrease of acute pain-like behaviours induced by intracolonic capsaicin but did not show any amelioration in refereed hyperalgesia. However, σ_1 antagonists were devoid of effect in σ_1-KO mice, indicating that the effects of these drugs are specifically mediated by σ_1 receptors, and that the absence of phenotype in refereed hyperalgesia showed by σ_1-KO mice was due to the development of compensatory mechanisms (González-Cano et al., 2013).

There have not been yet performed studies to dissect the anatomical location of pain modulation by σ_1 receptors on nociceptive behaviors or sensory hypersensitivity induced by capsaicin (either administered in somatic or visceral tissues), although taking into account the accumulated evidence of the role of σ_1 receptors on central sensitization it is likely that central σ_1 receptors play a role in the effects observed. However, taking into account the mixed contribution of peripheral and central σ_1 receptors to pain induced by other chemical irritants (formalin), the participation of peripheral σ_1 receptors to capsaicin-induced pain or sensitization cannot be discarded, and further studies are needed to clarify this issue.

3.6.2.2. σ_1 receptors and neuropathic pain

Neuropathic pain is one of the most challenging types of chronic pain conditions to treat, and new therapeutic tools are strongly needed (Zhou et al., 2011; Attal, 2012). The early results of the amelioration of formalin-induced pain and capsaicin-induced secondary mechanical hypersensitivity by σ_1 inhibition led to further research on the role of σ_1 receptors on neuropathic pain, since central sensitization is a key feature of this important pathological state (Latremoliere and Woolf, 2009).

In experimental animals (and humans), peripheral neuropathic pain can be produced by nerve trauma and by the administration of several agents with neurotoxic properties, such as antineoplastics (reviewed by Campbell and Meyer, 2006; Cobos and Portillo-Salido, 2013). It was reported that σ_1-KO mice do not develop signs of either cold or mechanical allodynia after traumatic nerve injury, although they showed a normal heat hyperalgesia (De la Puente et al., 2009). However, systemic repeated administrations of the selective σ_1 antagonist S1RA, starting before the neuropathy was established (immediately after the injury), avoided the development of not only neuropathic cold and mechanical allodynia but also heat hyperalgesia (Romero et al., 2012). These effects of σ_1 antagonists disappeared after discontinuation of the treatment and their repeated administration did not induce tolerance to the antihypersensitivity effects (Romero et al., 2012). The efficacy in preventing neuropathic heat hyperalgesia by S1RA and the normal development of this sensory alteration in injured σ_1-KO mice suggests again that compensatory mechanisms in pain pathways involved in heat sensitivity might be developed in σ_1-KO mice.

The ameliorative effects of S1RA on neuropathic hypersensitivity were not limited to its preemptive administration, since systemic administration of this σ_1 antagonist was able to fully reverse sensory hypersensitivity once the neuropathy was fully established (several days after the trauma) (Romero et al., 2012; Díaz et al., 2012; Bura et al., 2012). Importantly, neuropathic rodents were shown to freely administer themselves S1RA once neuropathy was established, to reverse not only mechanical and cold allodynia and heat hyperalgesia, but also neuropathic anhedonia (measured as a decreased preference to sweet solution) as an indicator of the emotional negative state induced by the pain condition, which reflects both the self-assessment of the successful efficacy of the treatment and the deep positive effects on the emotional state of the rodents (Bura et al., 2012).

Similar to the results found after traumatic nerve injury, σ_1-KO mice did not develop sensory hypersensitivity (mechanical and cold) after the administration of the antineoplastic drug paclitaxel (Nieto et al., 2012 and 2014), a first-line chemotherapeutic agent for the treatment of several types of cancer which frequently produces painful peripheral neuropathies as one of its major side-effects (Argyriou et al., 2008). In addition, systemic treatment with σ_1 antagonists (BD-1063 and S1RA) not only abolished mechanical and cold allodynia once neuropathy was fully developed

(Nieto et al., 2012), but were able to fully prevent the hypersensitivity associated to the neuropathy (which did not manifest again even after the discontinuation of the treatment with the σ_1 antagonists), suggesting that σ_1 inhibition had a protective role for the neuronal toxicity induced by the taxane (Nieto et al., 2012 and 2014).

Further studies were conducted to examine the role of spinal σ_1 receptors on peripheral neuropathic pain. It was shown that σ_1 receptor protein was transiently up-regulated in the dorsal spinal cord in the early days after the nerve injury (Roh et al., 2008b), and that repeated i.t. administration of the σ_1 antagonist BD-1047 prevented the full development of neuropathic mechanical hypersensitivity (Roh et al., 2008b; Choi et al., 2013), pointing to the importance of spinal σ_1 receptors in the development of neuropathic hypersensitivity. In fact, in neuropathic animals σ_1 inhibition decreased the phosphorylation of the NR1subunit of NMDA receptors in the spinal cord (Roh et al., 2008b), the phosphorylation of different nitrogen-activated protein kinases, such as extracellular signal-regulated kinase (ERK) 1/2 (De la Puente et al., 2009; Nieto et al., 2012) and p38 (Moon et al., 2013), and the activity of NADPH oxidase 2 (Nox2) with the subsequent reduction in the production of reactive oxygen species in neuropathic animals (Choi et al., 2013). All these molecules are known to play a major role on central sensitization (Latremoliere and Woolf, 2009). Conversely, intrathecal σ_1 agonism (by the selective drugs PRE-084 or (+)-pentazocine) induces a neuropathic-like phenotype in mice, resulting in sensory hypersensitivity (Roh et al., 2008a, 2010 and 2011; Ohsawa et al., 2011; Choi et al., 2013) and triggering similar mechanisms to those ameliorated by σ_1 inhibition in neuropathic animals (Roh et al., 2008a, 2010 and 2011; Ohsawa et al., 2011; Choi et al., 2013). Although the known modulation of NMDA receptors by σ_1 receptors could undoubtedly play a role in the effects induced by σ_1 drugs, it is hard to believe that all the above mentioned biochemical events fully derive from this interaction. Bearing in mind the mechanism of action of σ_1 receptor as a chaperone able to modulate numerous of targets by protein-protein interaction, including several ion channels and GPCRs involved in neurotransmission (see 3.5.1.), it would not be surprising that in the next future it will be demonstrated the functional relevance of these and other protein-protein interactions in an appropriate pain context.

Although there is no doubt for the pronociceptive role of σ_1 receptors in the spinal cord, it is worth pointing out that the sensory hypersensitivity induced by σ_1 agonists when administered i.t. (Roh et al., 2008a, 2010 and 2011; Choi et al., 2013) is not found when the agonists are administered systemically (Prezzavento et al., 2008; Entrena et al., 2009a and b; Tejada et al., 2014), supraspinally (e.g. Mei and Pasternak, 2002) or peripherally (Tejada et al., 2014), at doses able to reverse the effect of σ_1 antagonists; which indicates that the drugs were interacting with σ_1 receptors and producing effects. Although it is hard to explain these apparently contradictory findings, it might indicate that the concentrations of σ_1 agonists obtained after i.t. administration to induce hypersensitivity are higher in the spinal cord than those obtained by systemic treatments, or that alternatively, the procedure for the spinal administration of the drugs might be priming the nociceptive system making it more susceptible to the modulation by σ_1 receptor activation. These hypotheses need to be tested.

Interestingly, although the role of spinal σ_1 receptors in the development of neuropathic mechanical allodynia is clear, i.t. administration of the σ_1 antagonist BD-1047 in the induction phase of the neuropathy failed to alter thermal hyperalgesia (Roh et al., 2008b), and when the σ_1 antagonist was i.t. applied after the neuropathy was fully established it was devoid of effect in both mechanical and thermal hypersensitivity (Roh et al., 2008b). These results are in marked contrast with the previously commented high efficacy of systemically administered σ_1 antagonists on both mechanical and thermal hypersensitivity either before the neuropathy was established or when it was fully developed (Romero et al., 2012; Díaz et al., 2012; Bura et al., 2012; Nieto et al., 2012 and 2014), and point to that in addition to the participation of σ_1 receptors at the spinal level, these receptors at other locations might also contribute to the amelioration of the neuropathic pain phenotype produced by systemically administered σ_1 antagonists. There are not studies yet that show the effects of σ_1 antagonism at supraspinal levels in neuropathic pain models, which would be clarifying for expanding the central effects of σ_1 receptors on neuropathic pain. However, it has been recently reported that σ_1 receptors are down-regulated in DRG neurons after traumatic nerve injury (Bangaru et al., 2013), and that σ_1 knockout or systemic treatment with the σ_1 antagonist BD-1063 were able to prevent mitochondria abnormalities (as a sign of the toxicity) in myelinated A-fibers of the saphenous nerve in paclitaxel-treated mice (Nieto et al., 2014), which are thought to play a major role to the neuropathic pain induced by this antineoplastic (e.g. Flatters et al., 2006). These results are the first indicating the involvement of peripheral σ_1 receptors in the pathophysiological changes occurring during peripheral painful neuropathy, although they need to be expanded to clarify the role of peripheral σ_1 receptors on neuropathic pain processing.

In summary, σ_1 receptors clearly modulate central sensitization during neuropathic pain. Although most studies on neuropathic pain and σ_1 receptors have been focused on their role of at central levels, the evidence indicates that the spinal effects of σ_1 antagonists do not fully account for the effects seen in mice systemically treated with σ_1 antagonists, and that σ_1 receptors at other locations (such as in the peripheral nervous system) might be contributing to the ameliorative effects of the systemic administration of σ_1 antagonists on neuropathic pain. Further studies are warranted to clarify the role of peripheral σ_1 receptors in the aberrant sensory gain occurring during neuropathic pain.

3.6.2.3. σ_1 receptors and inflammatory pain

Inflammatory pain is a major type of clinical pain (e.g., Scholz and Woolf, 2002; Woolf, 2004). In contrast to neuropathic pain, inflammatory pain is characterized by a more pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the milieu of inflammatory mediators released at the inflammation site (see Scholz and Woolf, 2002; Ji, 2004; Patapoutian et al., 2009; Latremoliere and Woolf, 2009, for reviews). In contradistinction to the large amount of studies focused on σ_1 receptors and neuropathic pain, the role of these receptors on inflammatory pain has been only very recently described.

Inflammatory pain can be induced in rodents by the administration of proinflammatory agents. While the administration of carrageenan is used for studying acute inflammation, other agents such as complete Freund’s adjuvant (CFA) induce a longer lasting inflammatory pain hypersensitivity (Sandkühler, 2009; Cobos and Portillo-Salido, 2013). It was reported that the systemic administration of several σ_1 antagonists, including the selective σ_1 drugs S1RA and BD-1063 and the well reported σ_1 antagonist (+)-MR200, were able to ameliorate acute inflammatory pain hypersensitivity (Parenti et al., 2014a and b; Tejada et al., 2014; Gris et al., 2014). In addition, systemically administered S1RA was also shown to decrease the sensory gain during chronic inflammation (Gris et al., 2014). As for other aspects of the pain phenotype described in the preceding sections σ_1-KO mice only partially replicated the ameliorative effects induced by σ_1 antagonists. While systemic σ_1 pharmacological antagonism was able to abolish inflammatory mechanical and heat hyperalgesia, and mechanical allodynia (Parenti et al., 2014a and b; Tejada et al., 2014; Gris et al., 2014), σ_1-KO mice showed an abolishment of inflammatory mechanical hyperalgesia (Tejada et al., 2014) but a normal thermal hypersensitivity and mechanical allodynia in response to inflammation (Tejada et al., 2014; Gris et al., 2014). However, σ_1 pharmacological antagonism did not have any effect on σ_1–KO mice (Tejada et al., 2014; Gris et al., 2014), indicating that the presence of σ_1 receptors is necessary for the ameliorative effects of the drugs.

Importantly, we showed that peripheral σ_1 pharmacological antagonism (by BD-1063 and S1RA) in the inflamed site was enough to fully abolish inflammatory hyperalgesia (both to heat and mechanical stimuli) (Tejada et al., 2014), indicating that the activity of σ_1 receptors during inflammation is needed for the sensory alterations induced by a painful inflammation. However, we lack for a satisfactory mechanistic explanation of these findings. It could be hypothesized that σ_1 receptors might be chaperoning proteins involved in the transduction of mechanical or thermal stimuli during inflammation, or that σ_1 receptors modulate the signaling of algesic mediators released in the inflamed site. In fact, σ_1 agonism is known to enhances bradykinin-induced Ca^{+2} signaling in neuronal-like cell cultures (Hayashi et al., 2000; Hong et al., 2004), and this compound is well known to be released during inflammation and to contribute to peripheral sensitization (Chuang et al. 2001; Wang et al., 2006; Patapoutian et al., 2009). However, this hypothesis needs to be tested in DRG neurons, and the specific pain mediators susceptible to this possible modulation remain to be elucidated.

The role of central σ_1 receptors on inflammatory hypersensitivity remains completely unexplored up to date, but taking into account the extensively described role of σ_1 receptors on central sensitization (as described in the preceding sections), and that this process is also prominent on inflammatory pain (Latremoliere and Woolf, 2009), it could be expected that central σ_1 receptors could also be participating on inflammatory pain.

In summary, these recent studies expand the therapeutic possibilities of σ_1 receptors beyond neuropathic pain states, showing that peripheral σ_1 receptors are a promising target for inflammatory pain treatment.

3.7. Conclusions and final remarks

We have reviewed the available evidence showing the prominent role of σ_1 receptors on different types of pain. At present time they are a very promising pharmacological target for neuropathic and inflammatory pain treatment as well as for the amelioration of nociceptive pain at either in an acute pain situation (lowering the doses of opioids necessary to reach therapeutic effects). In addition, in spite of the disparities between the results obtained using σ_1 antagonists and σ_1-KO mice by purported compensatory mechanisms, these mutant mice has been successfully used as a tool for testing the specificity of drug effects by exploring the expected lack of activity of σ_1 drugs in the absence of σ_1 receptors. Experiments using σ_1-KO mice also indicates that caution should be used in drawing conclusions when a mutant mouse strain is the only tool available to study a putative therapeutic target (not only σ_1 receptors). Finally, although σ_1 receptors have been considered classically a neuromodulatory protein acting at central levels, the recently reported activity of peripheral σ_1 receptors as a biological brake for opioid antinociception, pathophysiological processes in the peripheral nerve during neuropathy, and their role as pain facilitators during inflammation, open both new possible therapeutic utilities for σ_1 antagonists and a broad new field of study on the peripheral mechanisms of pain.

RATIONALE, HYPOTHESIS AND GOALS

1.1. Rationale

Opioid drugs, particularly µ agonists (such as morphine, fentanyl, oxycodone, buprenorphine or tramadol), are widely used in therapeutics for treatment of moderate to severe pain (Al-Hasani and Bruchas, 2011; Pasternak and Pan, 2011; Pergolizzi et al., 2008; Schäfer, 2010). µ-opioid receptors are located along the pain pathways, including several areas of the central nervous system (both in supraspinal nuclei or at spinal level) as well as in the periphery (e.g. dorsal root ganglia, DRG) (Bigliardi-Qi et al., 2004; Khalefa et al., 2012). It is thought that opioid drugs exert their analgesic effects mainly at central levels, particularly supraspinally (e.g. Christie et al., 2000; Khalefa et al., 2012). However, the study of the possible role of peripheral opioid receptors to opioid analgesia has gained considerable interest in recent years (e.g. Stein et al., 2003; Sehgal et al., 2011).

In addition to analgesia, opioid drugs produce other effects resulting from their actions at central level, including nausea, mental confusion and respiratory depression, among other clinically relevant side effects (reviewed in Waldhoer et al., 2004 and Al-Hasani and Bruchas, 2011). Opioids also produce effects at peripheral levels, since these drugs act on the myenteric plexus to decrease gastrointestinal transit (Holzer et al., 2009; Brock et al., 2012). This latter opioid effect is used in therapeutics for the symptomatic treatment of diarrhea, specifically by the use of loperamide. This µ-opioid agonist acts exclusively at peripheral level (Menendez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012) and is able to inhibit gastrointestinal transit without inducing central effects (Gallelli et al., 2010; Layer et al., 2010); therefore it is not clinically used as an analgesic. Unfortunately, inhibition of gastrointestinal transit is also produced by centrally penetrant opioid analgesics and has a marked impact on the quality of life of patients. In fact, constipation induced by opioid drugs is the main cause of voluntary withdrawal of opioid medication by patients (Dhingra et al., 2013).

The sigma (σ) receptor was identified by Martin et al., in 1976. After its discovery, it was misclassified as a subtype of opioid receptors, and later confused with the phencyclidine (PCP) binding site within the *N*-methyl-*D*-aspartate (NMDA) receptor, but σ receptors are currently classified as a distinct entity (reviewed by Cobos et al., 2008; Zamanillo et al., 2013). Biochemical and pharmacological studies show the existence of two subtypes of σ receptors, termed σ_1 y σ_2 (see Cobos et al., 2008 for references). The σ_1 subtype is more deeply characterized and is the topic of study of this Doctoral Thesis.

The pharmacology of σ_1 receptors is currently well described, and there are available several selective σ_1 drugs. These drugs include the selective antagonists BD-1063, BD-1047, NE-100 and S1RA, and the selective agonists PRE-084 and (+)-pentazocine (Cobos et al., 2008; Zamanillo et al., 2013.). The σ_1 receptor has been cloned and shows no homology with opioid receptors or any other mammalian protein (see Guitart et al., 2004; Hayashi and Su, 2003 and Cobos et al., 2008, for references.). Its cloning allowed the development of σ_1 knockout mice (σ_1-KO) (Langa et al., 2003), highly facilitating the study of the function of these receptors. σ_1 receptors have a neuromodulatory role attributable to its chaperone action on other receptors and channels involved in several pathophysiological processes (Aydar et al., 2002; Kim et al., 2010, Navarro et al., 2010 and 2013; Su et al., 2010; Balasurilla et al., 2012; Kourrich et al., 2012 and 2013). μ-opioid receptors are among the target proteins susceptible to the σ_1 modulatory activity. Both receptors physically interact, and the pharmacological antagonism of σ_1 receptors is able to increase opioid signaling, measured as the increase in $[^{35}S]GTP\gamma S$ binding in response to the μ agonist DAMGO (Kim et al., 2010).

The σ_1 receptor is widely localized in important areas for pain processing and opioid analgesia. These areas include the spinal cord dorsal horn, the periaqueductal gray matter, rostroventral medulla or the DRGs (Alonso et al., 2000; Kitaichi et al., 2000; Roh et al., 2008b; Ueda et al., 2001). However, the expression of the σ_1 receptor has not yet been quantitatively compared between areas of the central and peripheral nervous system. Therefore, the locations where σ_1 is more abundant are unknown.

Although the genetic or pharmacological inhibition of σ_1 receptors do not alter nociceptive pain induced by the application of acute thermal or punctate mechanical stimuli (e.g. Chien and Pasternak, 1994; De La Puente et al., 2009; Entrena et al., 2009a and b; Marrazzo et al., 2011; Romero et al., 2012), it is able to potentiate the antinociceptive effect of opioid agonists (reviewed by Zamanillo et al., 2013). Importantly, the potentiation of opioid antinociception by σ_1 receptor inhibition described in previous studies was examined exclusively using thermal stimuli (e.g. Chien and Pasternak, 1993 and 1994; Marrazzo et al., 2011). The ontogenesis and neurochemical mechanisms of opioid analgesia to thermal and mechanical stimuli are different (Kuraishi et al., 1985; Tseng et al., 1995; Wegert et al., 1997; Sato et al., 1999). Therefore, the previously described modulation of morphine thermal antinociception by σ_1 receptors is not necessarily expected to be applicable to mechanical stimulation, which remains unexplored.

In previous studies, the enhancement of opioid antinociception by the inhibition of σ_1 receptors was attributed to central effects, particularly at supraspinal levels (King et al., 1997; Pan et al., 1998; Mei and Pasternak, 2002 and 2007; Marrazzo et al., 2006). However the possible role of peripheral σ_1 receptor in the potentiation of opioid antinociceptive effects was completely unexplored. A useful procedure for clarifying the central or peripheral origin of opioid effects is to test their sensitivity to opioid antagonists lacking of central penetrability, such as naloxone methiodide (Menendez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012). However, the effect of peripheral opioid antagonism on the potentiation of opioid antinociception by σ_1 inhibition is unknown, as well as whether the inhibition of peripheral σ_1 receptors is enough to enhance opioid antinociception.

Furthermore, previous studies showed that pharmacological σ_1 receptor agonism does not modulate some adverse effects of opioids (inhibition of intestinal transit and morphine-induced lethality) (Chien and Pasternak, 1994). However, it was unknown whether the increase of opioid antinociception by σ_1 receptor inhibition could be accompanied by an increase in non-analgesic effects of opioids, limiting its potential clinical use as an opioid adjuvant.

1.2. Hypothesis and goals

Taking into account these antecedents, the **main hypothesis** of this Doctoral Thesis was that σ_1 receptors might be involved in the modulation of peripheral opioid antinociception to a mechanical stimulus, and that the inhibition of σ_1 receptors may differentially potentiate opioid antinociception without altering other opioid non-analgesic (adverse) effects.

To test this hypothesis, our **first goal** was to study the influence of σ_1 receptor inhibition on nociceptive pain induced by a blunt mechanical stimulus in the presence or absence of the systemic (s.c.) administration of several clinically relevant opioid analgesics. To fulfill this goal we used σ_1-KO mice and systemically administered several σ_1 antagonists (BD-1063, BD-1047, NE-100 or S1RA) to wild-type mice. The opioid analgesics evaluated included the centrally penetrant drugs morphine, fentanyl, oxycodone, buprenorphine and tramadol.

The **second goal** of this Doctoral Thesis was to determine the role of peripheral opioid receptors in opioid antinociception to a blunt mechanical stimulus, in the presence or absence of the inhibition of σ_1 receptors. We used several experimental approaches to achieve this goal:

1) To compare the effect of the inhibition by a peripheral opioid antagonist (naloxone methiodide) on the antinociception induced by the systemic administration (s.c.) of the above mentioned opioid agonists, in control conditions and when σ_1 receptors are inhibited (in σ_1-KO mice or in wild-type mice treated systemically with σ_1 receptor antagonists).

2) To study whether σ_1 receptor inhibition (in σ_1-KO mice or by the systemic σ_1 pharmacological blockade in wild-type mice) is able to unmask possible antinociceptive actions induced by the peripheral opioid agonist loperamide.

3) To study whether the local pharmacological inhibition of σ_1 receptors is able to increase the peripheral mechanical antinociception induced by the systemic administration of the opioid agonists tested. To do that, we administered the opioids via s.c. and BD-1063 or S1RA via i.pl.; moreover, as an indicator of the involvement of peripheral opioid receptors in the resulting antinociceptive effect, we evaluated its sensitivity to the peripherally-restricted opioid antagonist naloxone methiodide.

4) To study whether the inhibition of σ_1 receptors is able to allow the expression of the antinociceptive effect induced by the local administration of morphine (used as a prototype of opioid agonist). We studied the effects of i.pl. morphine both in wild-type mice and in σ_1-KO mice, as well as during the local pharmacological blockade of σ_1 receptors (by the i.pl. administration of BD-1063, BD-1047, NE-100 or S1RA) in wild-type mice.

Since the experimental data of this study suggest that σ_1 receptors are able to modulate the peripheral antinociceptive effects of opioids, the **third goal** of this Doctoral Thesis was to compare the expression of σ_1 receptors in several areas of the central and peripheral nervous system involved in opioid analgesia, to provide an anatomical support for the behavioral effects of σ_1 receptor inhibition on peripheral opioid antinociception. To achieve this goal we performed Western blot experiments (using a σ_1 receptor specific antibody) in samples from central nervous system (basolateral amygdala, rostroventral medulla, periaqueductal gray matter and dorsal spinal cord) and from peripheral nervous tissue (DRG).

The **fourth goal** of this Doctoral Thesis was to demonstrate that the increase in the antinociceptive effect of opioids found in σ_1-KO mice is not due to adaptive changes in the peripheral or central expression of μ-opioid receptors, as well as to demonstrate that the modulation of opioid antinociception induced by σ_1 receptor antagonists was not due to crossed effects between opioid drugs and σ_1 receptors or between σ_1 ligands and μ opioid receptors. To achieve this goal, we performed saturation [^{3}H]DAMGO (μ receptor selective radioligand) binding assays in brain, spinal cord and hind-paw plantar skin samples from both genotypes. In addition, we carried out competition binding assays of [^{3}H]DAMGO and [^{3}H](+)-pentazocine (a selective σ_1 receptor radioligand) using cold σ_1 and opioid ligands, respectively.

Finally, taking into account the clinical relevance of the non-analgesic (adverse) effects of opioids, the **fifth goal** of this Doctoral Thesis was to study the modulation by σ_1 receptors of opioid effects different from their antinociceptive actions. To fulfill this goal, we studied the effects of σ_1 receptor inhibition in: a) morphine-induced hyperlocomotion, which is a centrally mediated opioid effect in rodents (Hnasko et al., 2005), and b) gastrointestinal transit inhibition induced by opioid analgesics (morphine and fentanyl) and by the antidiarrheal drug loperamide. This second non-analgesic opioid effect is of particular relevance for the possible future applicability of the findings of this Doctoral Thesis, since as mentioned above, gastrointestinal transit inhibition is an opioid side effect with a high clinical relevance and it is induced peripherally, as well as the potentiation of opioid antinociception to mechanical stimuli by σ_1 receptor inhibition (as it is shown in this Doctoral Thesis).

PUBLISHED PAPERS

1. Potentiation of morphine-induced mechanical antinociception by σ_1 receptor inhibition: role of peripheral σ_1 receptors

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1.1. ABSTRACT

We studied the modulation of morphine-induced mechanical antinociception and side effects by σ_1 receptor inhibition. Both wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice showed similar responses to paw pressure (100-600 g), while systemically (subcutaneously) or locally (intraplantarly) administered σ_1 antagonists (BD-1063, BD-1047, NE-100 and S1RA) were also devoid of antinociceptive effects. However, σ_1-KO mice exhibited an enhanced mechanical antinociception in response to systemic morphine (1-16 mg/kg). Similarly, systemic treatment of WT mice with σ_1 antagonists markedly potentiated morphine-induced antinociception, and their effects were reversed by the selective σ_1 agonist PRE-084. Although the local administration of morphine (50-200 μg) was devoid of antinociceptive effects in WT mice, it induced dose-dependent antinociception in σ_1-KO mice. This effect was limited to the injected paw. Enhancement of peripheral morphine antinociception was replicated in WT mice locally co-administered with σ_1 antagonists and the opioid. None of the σ_1 antagonists tested enhanced morphine-antinociception in σ_1-KO mice, confirming a σ_1-mediated action. Morphine-induced side-effects (hyperlocomotion and inhibition of gastrointestinal transit) were unaltered in σ_1-KO mice. These results cannot be explained by direct interaction of σ_1 ligands with μ-opioid receptors or adaptive changes of μ-receptors in σ_1-KO mice, given that [^{3}H]DAMGO binding in forebrain, spinal cord, and hind-paw skin membranes was unaltered in mutant mice, and none of the σ_1 drugs tested bound to μ-opioid receptors. These results show that σ_1 receptor inhibition potentiates morphine-induced mechanical analgesia but not its acute side effects, and that this enhanced analgesia can be induced at peripheral level.

1.2. INTRODUCTION

Although sigma (σ) receptors were initially confused with opioid receptors, they are now considered a distinct entity. In contradistinction to the seven transmembrane domains of opioid receptors, typical of the G-protein coupled receptor family, the sigma-1 (σ_1) receptor only has two transmembrane domains. Furthermore, its sequence does not resemble any other known mammalian protein, underscoring its uniqueness (see Cobos et al., 2008; Guitart et al., 2004; Hayashi and Su, 2004, for reviews). The σ_1 receptor has been pharmacologically characterized and is known to exert neuromodulatory actions (Cobos et al., 2008), which may be attributable, at least in part, to its physical association with other receptors and channels (Aydar et al., 2002; Kim et al., 2010). σ_1 receptors are widely distributed in both the central and peripheral nervous system, including important pain control areas such as the spinal cord dorsal horn, periaqueductal gray matter, and dorsal root ganglia (Alonso et al., 2000; Kitaichi et al., 2000; Roh et al., 2008b; Ueda et al., 2001).

σ_1 receptor inhibition does not influence acute pain induced by thermal or punctate mechanical stimuli (e.g. Chien and Pasternak, 1994; De la Puente et al., 2009; Entrena et al., 2009a; Marrazzo et al., 2011; Romero et al., 2012). However, it induces a marked decrease in pain responses in experimental models of tonic/chronic pain involving central sensitization, such as formalin-induced pain (Cendán et al., 2005a and b; Kim et al., 2006; Romero et al., 2012), capsaicin-induced mechanical hypersensitivity (Entrena et al., 2009a and b; Romero et al., 2012), and neuropathic pain (De la Puente et al., 2009; Nieto et al., 2012; Roh et al., 2008b; Romero et al., 2012). Although σ_1 inhibition appears to have no effect on acute pain, it has been widely reported that σ_1 antagonism or downregulation (by antisense oligodeoxynucleotides) in the central nervous system greatly increases opioid-induced thermal antinociception (King et al., 1997; Marrazzo et al., 2006; Mei and Pasternak 2002 and 2007; Pan et al., 1998). These results indicate that the σ_1 system at central levels modulates both pain hypersensitivity and the effects of opioids in acute thermal nociception.

Most of the antinociceptive effects of morphine and other opioids have been attributed to their action on the central nervous system (e.g. Christie et al., 2000; Khalefa et al., 2012), but they can also have local antinociceptive effects under some circumstances (Sehgal et al., 2011; Stein et al., 2003). However, the possible modulatory role of σ_1 receptors on peripherally-mediated opioid antinociception is not known. Moreover, the ontogenesis and neurochemical mechanisms underlying morphine-induced mechanical and thermal antinociception differ (Kuraishi et al., 1985; Sato et al., 1999; Tseng et al., 1995; Wegert et al., 1997). Therefore, the previously described modulation of morphine thermal antinociception by σ_1 receptors is not necessarily expected to be applicable to mechanical stimulation, which remains unexplored. Consequently, the main aim of this study was to evaluate the role of σ_1 receptors in the modulation of morphine-induced mechanical antinociception, particularly in the periphery. To this end, we studied the effects of σ_1 receptor inactivation (σ_1-knockout) and of the systemic (subcutaneous) and local (intraplantar) administration of the known σ_1 receptor antagonists BD-1063, BD-1047, NE-100, and S1RA (Cobos et al., 2008; Díaz et al., 2012; Hayashi and Su 2004; Romero et al., 2012) on the mechanical antinociception induced by the systemic and local administration of morphine. We also assessed the specificity of the effects induced by the σ_1 antagonists by attempting to reverse them with the selective σ_1 agonist PRE-084 (Cobos et al., 2008; Su et al., 1991) and by testing their expected inactivity in σ_1-knockout (σ_1-KO) mice.

Morphine is widely used to treat moderate-to-severe pain, despite its clinically relevant side effects, which are mediated at both central (e.g. nausea, respiratory depression) and peripheral (e.g. constipation) levels (Al-Hasani and Bruchas, 2011; Waldhoer et al., 2004). An additional goal of the present study was to determine the possible influence of σ_1 receptor inhibition on some adverse effects of morphine. For this purpose, we compared the responses of wild-type (WT) and σ_1-KO mice to morphine-induced hyperlocomotion, an acute centrally-mediated side effect of morphine in rodents (Hnasko et al., 2005), and their responses to morphine-induced gastrointestinal transit inhibition, a major peripherally-mediated adverse effect of this opioid (Al-Hasani and Bruchas, 2011).

Finally, in order to rule out any direct effects of σ_1 ligands on μ-opioid receptors or any adaptive changes in μ-receptors that could account for effects observed in σ_1-KO animals, we compared [^{3}H]DAMGO binding properties between WT and σ_1-KO mice and studied the affinity of all σ_1 ligands used in the behavioral experiments for μ-opioid receptors in WT mice.

1.3. MATERIAL AND METHODS

1.3.1. Experimental animals

Experiments were performed in female wild-type (Charles River, Barcelona, Spain) and σ_1-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25-30 g. The knockout mice were generated on a CD-1 background as previously described (Entrena et al., 2009a). Animals were housed under a 12/12 h day/night cycle in temperature-controlled rooms (22 ± 2ºC) and were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water *ad libitum*. After arrival at our vivarium, animals were allowed to acclimatize for at least 4 days before experimental handling. Testing was performed during the light phase (from 9.00 h to 15.00 h) randomly throughout the estrous cycle. Animal care was carried out in accordance with institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council directive 86/609).

1.3.2. Radioligand, drugs, and drug administration

The σ_1 receptor antagonists used were: BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride), BD-1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide), NE-100 (*N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride), and the new selective σ_1 antagonist S1RA (4-[2-[[5-methyl-1-(2-naphtalenyl)1H-pyraol-3-yl]oxy]ethyl] morpholine hydrochloride) (Cobos et al., 2008; Díaz et al. 2012; Hayashi and Su 2004; Romero et al., 2012). BD-1047 and BD-1063 were purchased from Tocris

Cookson Ltd. (Bristol, United Kingdom), NE-100 was synthesized as previously described (Nakazato et al., 1999), and S1RA was synthesized and kindly supplied by Laboratorios Esteve. As σ_1 receptor agonist, we used PRE-084 ([2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride]) provided by Tocris Cookson Ltd. The μ-opioid receptor agonist morphine hydrochloride was obtained from the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health (Madrid, Spain).

For *in vivo* studies, all drugs were dissolved in sterile physiological saline (0.9% NaCl). To evaluate the effects of systemic treatments, 5 ml/kg of the drugs or their solvent were administered by subcutaneous injection (s.c.) into the interscapular zone. When the systemic effect of the association of two drugs was assessed, each injection was performed in different areas of the interscapular zone. The local effect of treatments was evaluated through the intraplantar (i.pl.) injection of 20 μL of the drug into the right hind-paw using a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a $30^{1/2}$-gauge needle. The control group received the same volume of sterile saline in the same manner. When morphine and a σ_1 receptor antagonist were associated, they were dissolved in the same solution and injected together to avoid paw lesions from multiple injections. In one experiment, morphine was injected in the right hind-paw and BD-1063 in the left hind-paw of the same animals.

For binding assays, the radioligand used to label μ-opioid receptors was $[^3H]$DAMGO (D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin), with a specific activity of 50.0 Ci/mmol (PerkinElmer Life Sciences, Boston, MA, USA). Naloxone HCl (Sigma-Aldrich, Madrid, Spain) was used to measure the non-specific binding. Dilutions from the stock $[^3H]$DAMGO solution were prepared with incubation buffer (50 mM HCl-Tris buffer, pH 7.44 at 25 ºC). Naloxone, morphine, BD-1063, BD-1047, NE-100, S1RA, and PRE-084 were dissolved in ultrapure water to make up a 1 mM stock solution, from which further dilutions were prepared with incubation buffer.

1.3.3. Evaluation of the behavioral response to paw pressure

Animals were placed in the experimental room for a 1-h acclimation period before starting the experiments. Then, after gently restraining the animals, blunt mechanical stimulation was applied to the hind-paws with an Analgesimeter (Model 37215, Ugo-Basile, Varese, Italy), as previously described by Menéndez et al. (2005) with slight modifications. Briefly, a cone-shaped paw-presser with a rounded tip was applied carefully (to avoid alarming the animal) to the dorsal surface of the hind-paw. The analgesimeter allowed a constant pressure (of varied intensity depending on the type of experiment, see below) to be applied to the paw until the animal showed a struggle reaction. A chronometer was manually activated at the start of the pressure application and stopped at the onset of the struggle reaction, and the response latency was recorded in seconds. The test was performed twice alternately in each hind-paw, with a 1-min interval between each stimulation. A 50-sec cut-off was established for each measurement to prevent tissue damage. In the systemic drug treatment experiments, animals showed a similar struggle response latency during the stimulation of each hind-paw, and the mean value of the two averaged measurements for each hind-paw was considered in the analyses. In the local (i.pl.) treatment experiments, the average of the two values was independently considered for each paw (injected and non-injected).

To test whether the lack of σ_1 receptor alters the response to paw pressure stimulation, we compared the responses of naïve WT and σ_1-KO mice, applying a wide range of pressures (100 - 600 g) to the hind-paws and recording the struggle response latency at each pressure, as described above. Each pressure was tested in a different group of animals to avoid paw sensitization from repeated stimulation. Based on these data, a pressure-response curve (stimulus pressure *vs.* latency time) was constructed for each genotype and was used to determine the optimal pressure for the subsequent experiments. A pressure of 450 g was always used as nociceptive stimulus to test the effect of the drugs because the response latency was markedly reduced at this pressure, offering a wide window to observe an increase in the latency up to the cut-off time (see

Fig. 1). Furthermore, this pressure was used in previous research on the analgesic effects of opioids in mice (Menéndez et al., 2005).

In experiments on the effects of the systemic administration of morphine or its solvent, these were s.c injected at 30 min before application of the mechanical stimulus to the hind-paws; in those on the effects of systemic administration of σ_1 drugs, these were s.c. injected at 5 min before injection of the opioid or solvent. When PRE-084 was used to reverse the effects of σ_1 receptor antagonists, it was s.c. injected immediately before the σ_1 antagonist solution. In the study of the local antinociceptive effects of morphine, σ_1 antagonist, or their combination, these were i.pl. injected at 5 min before application of the mechanical stimulus to the hind-paw to minimize their systemic absorption.

1.3.4. Assessment of morphine-induced hyperlocomotion

Ambulatory locomotion was monitored by using an infrared detector (Med associated Inc., St Albans, VT, USA) equipped with 48 infrared photocell emitters and detectors (12 photo sensors on front and back walls and 12 on each side wall). Mice were habituated to the evaluation chambers (27.5 cm wide x 27.5 cm long x 20 cm high) for 90 min before the s.c. administration of morphine or its solvent. After the injection, we immediately returned the animal to its evaluation cage and recorded the distance it travelled between 30 and 60 min post-injection. To avoid distracting factors that could interfere with the locomotor activity, the evaluation chambers were always in a sound-isolated testing room, and no experimenters were present at any time during the habituation or evaluation period except for the time needed to inject the drug.

1.3.5. Assessment of morphine-induced inhibition of gastrointestinal transit

Evaluation of gastrointestinal transit was performed following a previously published protocol (Chien and Pasternak, 1994) with modifications. Briefly, mice were fasted for 8 h with water available *ad libitum* before evaluation of the morphine effects. At 30 min after the s.c. administration of morphine or its solvent (saline), 0.3 ml of 0.5% (w/v) activated charcoal (2-12 μm powder, Sigma-Aldrich) suspended in distilled water was intragastrically administered. At 30 min after ingestion of the activated charcoal, mice were killed by cervical dislocation, and the small intestine from the pyloric sphincter to the ileocecal junction was isolated. The distance travelled by the leading edge of the charcoal meal was measured with a ruler for calculation of the gastrointestinal transit.

1.3.6. Membrane preparations for binding assays

Experiments were performed in crude synaptosomal membranes (P_2 fraction) obtained as previously described (Cobos et al., 2005, 2006) with slight modifications. Mice were killed by cervical dislocation, and the forebrain, spinal cord, and hind-paw plantar skin were rapidly removed. Forebrains and spinal cords were homogenized in 15 volumes (w/v) of 0.32 M sucrose-10 mM Tris-HCl, pH 7.4, with a Polytron homogenizer (model PT10-35, Kinematica AG, Basel, Switzerland). The same procedure was followed with the paw plantar skin except that it was frozen with liquid nitrogen before homogenization, as previously described (Baamonde et al., 2007). All homogenates were centrifuged (Avanti 30, Beckman Coulter España S.A., Madrid, Spain) at 1,000×g for 13 min, discarding the resulting pellets (P_1 pellets) and then centrifuging the supernatants at 21,000×g for 15 min to obtain the P_2 pellets. Each P_2 pellet (obtained from 2 forebrains, 5 spinal cords or the plantar skin of 20 hind-paws) was washed by resuspension in 15 mL of 10 mM Tris-HCl, pH 7.4, and centrifuged again at 21,000×g for 15 min. The entire process was performed at 4°C. Finally, each pellet was resuspended in 1 ml of 10 mM Tris-HCl, pH 7.4 and frozen in aliquots at −80°C. The binding characteristics of the tissue were stable for at least 1 month when stored at −80°C.

1.3.7. [^{3}H]DAMGO binding assays

Binding assays were performed as previously described (Narita et al., 2001) with slight modifications. Membrane aliquots, obtained as reported in the previous section, were slowly thawed and resuspended in fresh incubation buffer to obtain a final protein concentration of 400-500 µg/ml. Protein concentration was measured by the method of Lowry et al. (1951) with some modifications, using bovine serum albumin as the standard. Membrane solutions were incubated with 20 µl of [^{3}H]DAMGO solution and 20 µl of the cold ligand solution or its solvent at 25°C for 120 min, at a final volume of 500 µl. The final concentration of [^{3}H]DAMGO was 5 nM in competition assays and 0.25-45 nM in saturation assays. Because the amount of plantar skin sample was limited, a single saturating concentration (20 nM) of [^{3}H]DAMGO was used to estimate the maximum number of binding sites (B_{max}) labeled by the radioligand, as reported in other binding assays (Barturen and Garcia-Sevilla, 1992; Cobos et al., 2007). The same procedure was also used with forebrain and spinal cord samples to permit comparison of the results. In all experiments, non-specific binding was defined as the binding retained on the filter and membranes in the presence of 10 µM naloxone.

To stop the [^{3}H]DAMGO binding, 5 ml ice-cold filtration buffer (10 mM Tris pH 7.4) was added to the membrane solution. The bound and free radioligand were separated by rapid filtration under a vacuum using a Brandel cell harvester (Model M-12 T Brandel Instruments; SEMAT Technical Ltd., St Albans, Hertfordshire, UK) on Whatman GF/B glass fiber filters (SEMAT Technical Ltd.), which were pre-soaked with 0.5% polyethylenimine (Sigma-Aldrich) in 10 mM Tris pH 7.4, for at least 1 h prior to their utilization to reduce non-specific binding. The filters were washed twice with 5 ml volumes of the ice-cold filtration buffer and transferred to scintillation counting vials; then, a 4 ml liquid scintillation cocktail (Opti-phase Hisafe II; PerkinElmer Wallac, Loughborough, UK) was added and the mixture was equilibrated for at least 20 h. The radioactivity retained in the filter was measured by using a liquid scintillation spectrometer (Beckman Coulter España S.A.) with an efficiency of 52%. Each assay was conducted in triplicate.

1.3.8. Data analysis

Data were analyzed with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA). In behavioral assays, the dose-response curves of the drugs and the pressure-response curves were estimated by using the equation for a sigmoid plot. In the binding experiments, the equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}) from saturation assays were calculated by non-linear regression analysis of the results fitted to a rectangular hyperbola equation. The IC_{50} value (concentration of morphine that inhibited 50% of [^{3}H]DAMGO-specific binding) was calculated from competition assays using non-linear regression analysis, assuming one-site competition. The K_i value of morphine in forebrain membranes (indicating the affinity of the inhibitor for the receptor) was calculated with the Cheng-Prussoff equation: $K_i = IC_{50}/(1 + [L]/K_D)$, where $[L]$ is the concentration of radioligand used, and K_D is the value obtained by nonlinear regression analysis from the saturation experiment. Parameters obtained from non-linear regressions of binding assays were compared with Snedecor's F test to check the goodness-of-fit of different models that shared one or more parameters. When several means were compared, the statistical analysis was carried out using a one-way or two-way analysis of variance (ANOVA), depending on the experiment, followed by a Bonferroni *post-hoc* test. $P < 0.05$ was considered significant in all tests.

1.4. RESULTS

1.4.1. Comparison of mechanical sensitivity in wild-type and σ_1 knockout mice

The cone-shaped paw-presser was applied at different intensities (100 to 600 g) on the dorsal hind-paw of the animals, and response latency values were compared between WT and σ_1-KO mice. The struggle response latency decreased as the mechanical pressure on the dorsal hind-paw increased in both WT and σ_1-KO mice, which did not significantly differ in response latency at any pressure applied (Fig. 1); i.e., the WT and σ_1-KO mice showed equivalent responses to noxious paw pressure.

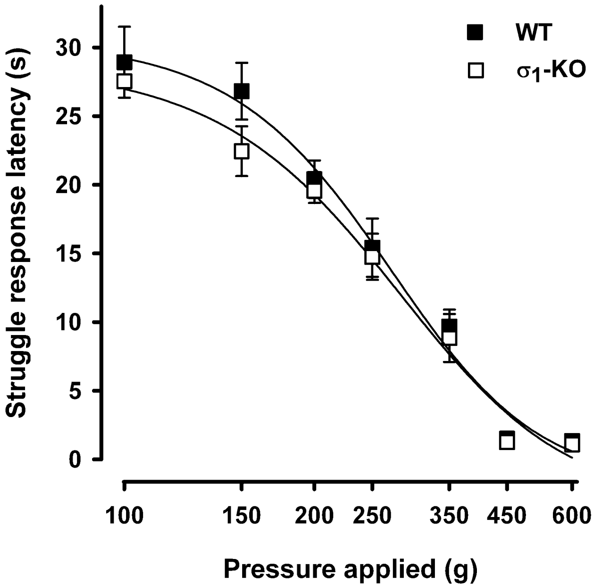

Fig. 1. Latency to struggle response evoked by blunt mechanical stimulation (100-600 g pressure) of the hind-paws of wild-type (WT) and σ_1 knockout (σ_1-KO) mice. Each point and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Each group was stimulated with only one pressure. There were no statistically significant differences between the values obtained in WT and σ_1-KO mice at any pressure applied (two-way ANOVA followed by Bonferroni test).

1.4.2. Effects of systemic (subcutaneous) morphine on mechanical nociception in wild-type and σ_1 knockout mice

The effects of s.c. morphine on mechanical nociception were evaluated as the increased response latency with respect to solvent-treated mice when noxious pressure (450 g) was applied to the hind-paw. The mechanical stimulus produced a fast and similar response in both WT (1.50 ± 0.14 s) and σ_1-KO (1.25 ± 0.25 s) mice treated with saline (Fig. 2, dose 0). Morphine administration (0.5-16 mg/kg, s.c.) induced a dose-dependent increase in response latency in WT mice, i.e., exerted mechanical antinociception, and this effect was significant at doses of 4 mg/kg ($P < 0.05$) or higher (Fig. 2, closed circles). We were not able to accurately test the effects on nociception of doses higher than 16 mg/kg of morphine, because the mice exhibited behavioral abnormalities (nervousness and stiffness) that hampered the behavioral evaluation. Morphine also induced a dose-dependent antinociceptive effect in σ_1-KO mice; however, in contrast to WT mice, they showed a strong and highly significant ($P < 0.001$) antinociception from a dose of 2 mg/kg, and latency values close to the cut-off time were obtained at doses of 4 mg/kg and higher (Fig. 2, open circles). Hence, the genetic inactivation of σ_1 receptors induced a clear and marked potentiation of systemic morphine-induced mechanical antinociception.

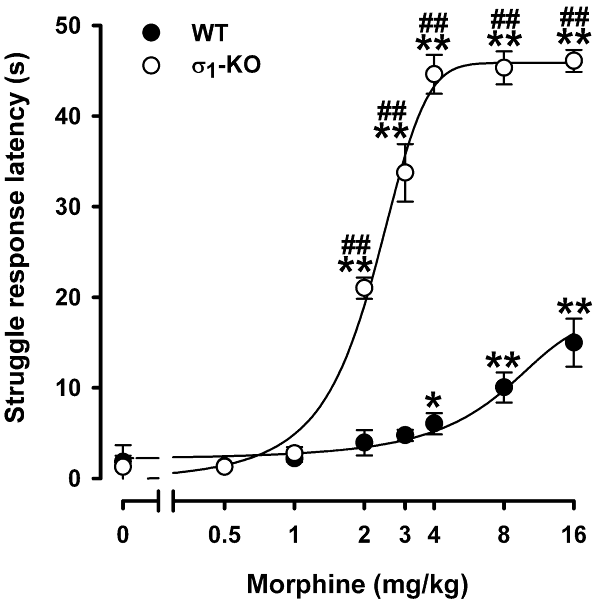

Fig. 2. Effects of the systemic (subcutaneous) administration of morphine on mechanical nociceptive pain in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with several doses of morphine (0.5-16 mg/kg) or its solvent (saline, dose 0). Each point and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in saline- and morphine-treated groups: $^{*}P < 0.05$; $^{**}P < 0.01$, and between the values obtained in WT and σ_1-KO mice at the same dose of morphine: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test).

1.4.3. Effects of systemic (subcutaneous) administration of selective σ_1 drugs on mechanical antinociception induced by systemic morphine

As reported above, 4 mg/kg (s.c.) of morphine elicited a slight but statistically significant increase in struggle response latency in WT mice submitted to noxious pressure (450 g) on the paw (see Fig. 2). When this morphine dose was associated with systemic administration of the selective σ_1 receptor antagonist BD-1063 in WT mice, a dose-dependent potentiation of the morphine-induced increase in response latency was observed (Fig. 3). WT mice treated with systemic morphine (4 mg/kg, s.c.) and with the highest tested dose of BD-1063 (32 mg/kg, s.c.) showed similar response latency values (42.87 ± 2.71 s, Fig. 3) to those observed in σ_1-KO mice treated with the same dose of morphine (44.6 ± 2.15 s, Fig. 2).

We evaluated the specificity of the effects of systemic BD-1063 on morphine-induced mechanical antinociception by testing whether the selective σ_1 agonist PRE-084 was able to reverse the effect of the σ_1 antagonist. In contrast to the effects induced by BD-1063, treatment with the selective σ_1 agonist PRE-084 (16 mg/kg, s.c.) did not significantly modify the antinociceptive effect of morphine, either at 4 mg/kg (Fig. 4) or at 16 mg/kg (data not shown). However, when PRE-084 (4-16 mg/kg, s.c.) was co-administered with BD-1063 (32 mg/kg, s.c.), it completely reversed the potentiation of morphine-induced mechanical antinociception by the σ_1 antagonist in a dose-dependent manner (Fig. 4).

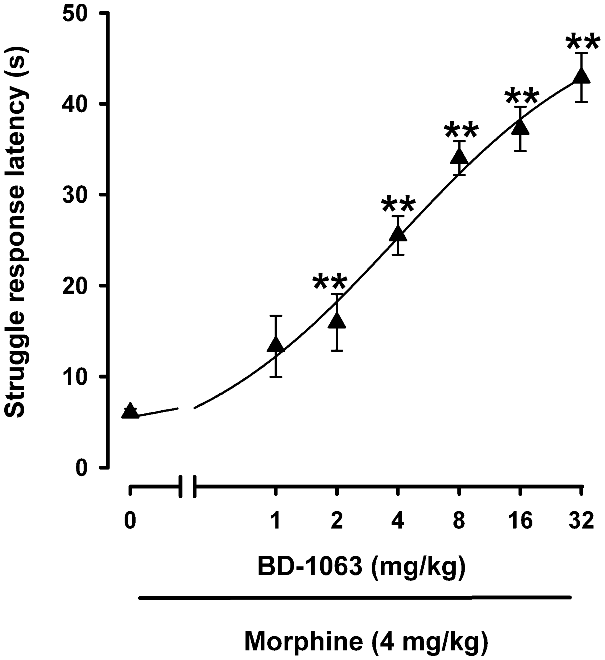

Fig. 3. Effects of systemic (subcutaneous) administration of BD-1063 on mechanical antinociception induced by systemic morphine in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with several doses of BD-1063 (1-32 mg/kg) or its solvent (dose 0) associated with morphine (4 mg/kg). Each point and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in the groups treated with BD-1063 and its solvent (dose 0): $**P < 0.01$ (one way ANOVA followed by Bonferroni test).

We confirmed the effects of the pharmacological antagonism of σ_1 receptors on morphine-induced antinociception by using a panel of selective σ_1 antagonists: BD-1047 (32 mg/kg, s.c.), NE-100 (4 mg/kg, s.c.) and SR1A (32 mg/kg, s.c.). All of these drugs mimicked the effects of BD-1063 on morphine-induced mechanical antinociception, increasing the response latency of morphine-treated WT mice, and their effects were dose-dependently reversed by PRE-084 (Fig. 4).

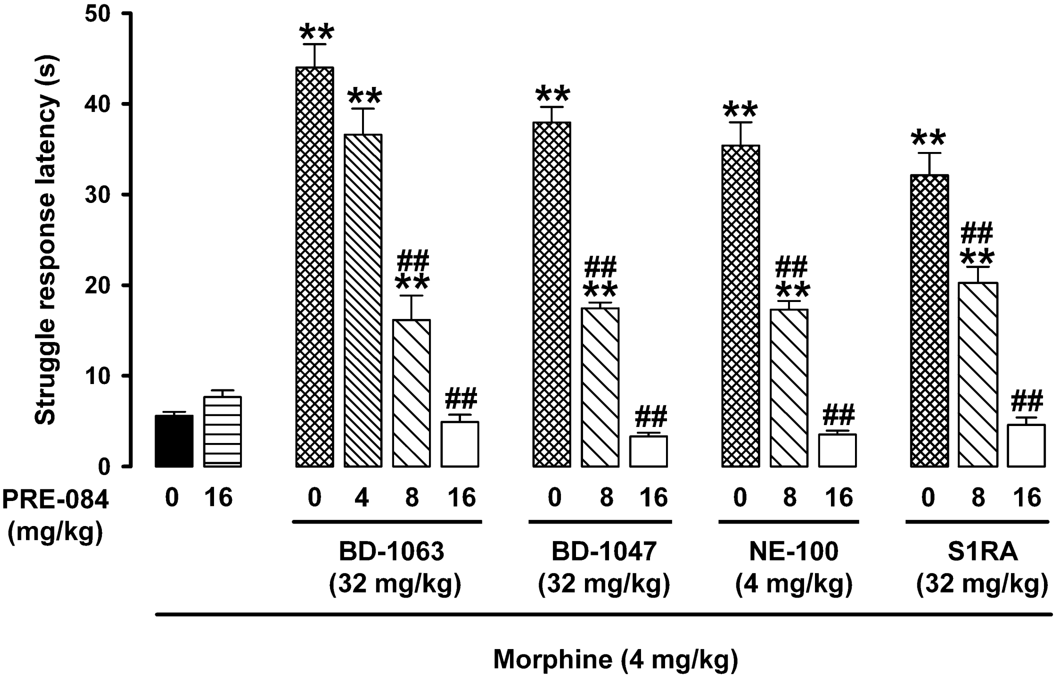

Fig. 4. Effect of the systemic (subcutaneous) administration of the σ_1 agonist PRE-084 on the mechanical antinociception induced by the systemic administration of morphine associated with several σ_1 antagonists in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with PRE-084 (4-16 mg/kg) or its solvent and the σ_1 antagonists BD-1063 (32 mg/kg), BD-1047 (32 mg/kg), NE-100 (4 mg/kg), or S1RA (32 mg/kg) or their solvent, associated with morphine (4 mg/kg). Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in the groups treated with morphine alone and associated with the σ_1 drugs: $^{**}P < 0.01$, and between the values obtained in mice given each σ_1 antagonist associated with PRE-084, with respect to each σ_1 antagonist associated with PRE-084 solvent (dose 0): $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

We further evaluated the selectivity of the effects induced by the σ_1 antagonists by testing their action on morphine-induced mechanical antinociception in σ_1-KO mice. A slightly lower morphine dose (3 mg/kg, s.c.) was used in this experiment in order to facilitate the detection of possible non-specific increases in struggle response latency induced by the σ_1 antagonists. Response latencies were significantly higher in WT mice co-administered s.c. with 3 mg/kg morphine and σ_1 antagonist than in those treated with morphine alone (Fig. 5, middle panel). However, none of the σ$_1$ antagonists further increased the morphine-induced mechanical antinociception in σ$_1$-KO mice (Fig. 5, right panel), suggesting that off-target effects do not significantly contribute to the potentiation of morphine-induced antinociception by these drugs in our experimental conditions. Response latency values did not significantly differ between those of WT mice treated by any of the σ$_1$ antagonists tested in combination with morphine and those of σ$_1$-KO mice treated with morphine alone (Fig. 5, middle and right panel), indicating that similar levels of enhanced morphine analgesia were induced by the systemic pharmacological blockade of σ$_1$ receptors and by their genetic inactivation.

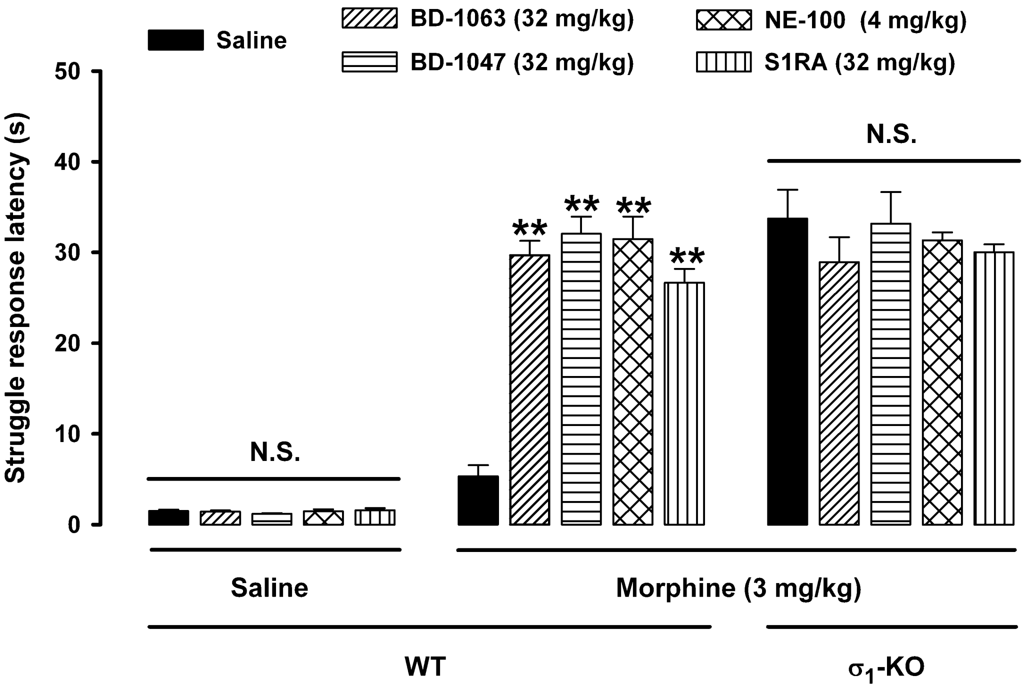

Fig. 5. Effects of the systemic (subcutaneous) administration of several σ$_1$ antagonists on the mechanical antinociceptive effect induced by systemic morphine in wild-type (WT) and σ$_1$ knockout (σ$_1$-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with the σ$_1$ antagonists BD-1063 (32 mg/kg), BD-1047 (32 mg/kg), NE-100 (4 mg/kg), or S1RA (32 mg/kg) or their solvent (saline), associated to morphine (3 mg/kg) or its solvent (saline). Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained in the groups treated with each σ$_1$ antagonist and their solvent in morphine-treated WT mice: $^{**}P < 0.01$. N.S.: no statistically significant differences between the values (two-way ANOVA followed by Bonferroni test).

None of the σ_1 antagonists administered alone was able to modify the struggle response latency in WT mice, i.e., they did not affect the responses to noxious pressure in the absence of morphine (Fig. 5, left panel). Therefore, the antinociceptive effects observed in WT mice treated with the association of morphine and σ_1 antagonist were synergistic rather than merely additive.

1.4.4. Local antinociceptive effects induced by intraplantar administration of morphine in wild-type and σ_1 knockout mice

We tested whether σ_1 receptors are able to modulate the antinociceptive effects of morphine in the periphery by the i.pl. administration of this opioid in WT and σ_1-KO mice. The i.pl. administration of morphine (50-200 µg) did not significantly modify the struggle response latency in WT mice in either the injected or non-injected paw (Fig. 6), indicating that these doses were unable to locally induce a significant mechanical antinociceptive effect in this genotype. In contrast, σ_1-KO mice i.pl injected with the same doses of morphine showed a marked dose-dependent increase in response latency in the injected paw, reaching values close to the cut-off time at the highest dose (200 µg) (Fig. 6). Latency values in the contralateral non-injected paw of σ_1-KO mice remained unchanged and undistinguishable from control values in mice treated with the solvent of morphine (Fig. 6), indicating that the antinociceptive effect of morphine in σ_1-KO mice was locally produced. Hence, the local effect of morphine against noxious pressure was potentiated in mice globally lacking functional σ_1 receptors.

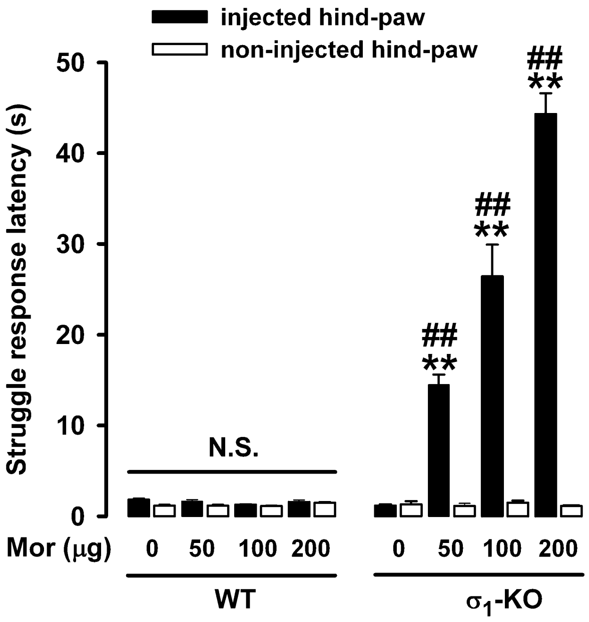

Fig. 6. Effect of the local (intraplantar) injection of morphine (Mor) on mechanical nociceptive pain in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the mice hind-paw injected with morphine (50-200 μg) or its solvent (dose 0), in comparison to that obtained during stimulation of the non-injected hind-paw. Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences between the values obtained stimulating the morphine-injected hind-paw versus those obtained stimulating the solvent-treated hind-paw (dose 0) in σ_1-KO mice: $^{**}P < 0.01$, and between the values obtained in the injected and non-injected hind-paws in σ_1-KO mice: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test). No statistically significant differences (N.S.) were found in the values from WT groups between the injected and non-injected hind-paws or between any dose of morphine with respect to its solvent (two-way ANOVA followed by Bonferroni test).

1.4.5. Potentiation of the local antinociceptive effect of morphine by pharmacological blockade of σ_1 receptors

We investigated whether local pharmacological antagonism of σ_1 receptors could also potentiate morphine-induced peripheral mechanical antinociception in WT mice by testing the effects of the i.pl. co-administration of the selective σ_1 receptor antagonist BD-1063 (12.5-200 μg) with 200 μg morphine in WT mice.

The intraplantar injection of BD-1063 alone did not produce antinociception (Fig. 7). The co-administration of BD-1063 with morphine in WT mice produced a dose-dependent increase in struggle response latency in the injected paw but not in the non-injected paw (Fig. 7). In addition, mice i.pl. administered with BD-1063 in the contralateral hind-paw to the morphine injection (200 µg each) showed no increase in struggle response latency (data not shown). These results rule out any possible systemic effect of the i.pl. administration of BD-1063 and demonstrate that BD-1063 is able to locally potentiate morphine-induced mechanical antinociception in the periphery.

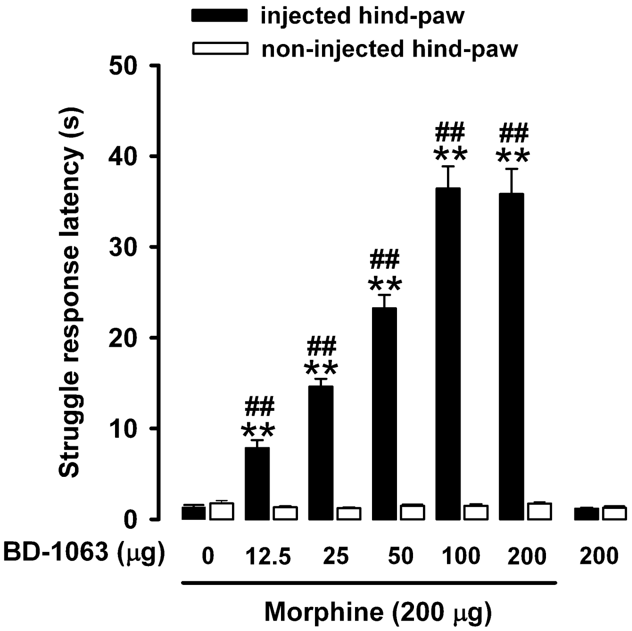

Fig. 7. Effect of the local (intraplantar) administration of BD-1063 associated with morphine on mechanical nociceptive pain in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure of the mice hind-paw injected with morphine (200 µg) or its solvent associated with BD-1063 (12.5-200 µg) or its solvent in comparison to values obtained during stimulation of the non-injected hind-paw. Statistically significant differences between the values obtained stimulating hind-paws injected with morphine and BD-1063 with respect to those treated with morphine alone (BD-1063 dose 0): $^{**}P < 0.01$, and between the values obtained from the injected and non-injected hind-paws: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test). No statistically significant differences were found among the values in the non-injected hind-paws with any treatment or between the values obtained in the injected and non-injected paw in the group treated with BD-1063 alone (two-way ANOVA).

In order to establish the specificity of these effects, we tested the effect of BD-1063 in σ_1-KO mice. For this experiment, we used a lower dose of morphine (100 µg) to facilitate detection of any non-specific increases in morphine-antinociception attributable to the i.pl. injection of the σ_1 antagonist. BD-1063 (100 µg) enhanced the effects of morphine in WT mice but did not significantly alter the response of morphine-treated σ_1-KO mice (Fig. 8, middle and right panel).

Likewise, the i.pl. administration of BD-1047 (50 µg), NE-100 (50 µg), or S1RA (100 µg) had no effects on the behavioral response in the absence of morphine (Fig. 8, left panel). However, these drugs increased the response latency in the injected paw of morphine-treated (100 µg, i.pl.) WT mice (Fig. 8, middle panel), although not in their non-injected paw (data not shown). In contrast, the co-administration of these σ_1 antagonists with morphine did not potentiate the effect of the opioid in σ_1-KO mice (Fig. 8, right panel). These results support that the enhancement of locally-induced morphine antinociception produced by the σ_1 antagonists is mediated by their interaction with σ_1 receptors, and that no additional effects of these drugs are participating in the effects observed. In addition, the response latency of WT mice locally co-administered with σ_1 antagonist and morphine did not significantly differ from that obtained in σ_1-KO mice treated with morphine alone (Fig. 8, middle and right panel). This finding indicates that a similar potentiation of local morphine antinociceptive effects was produced by local σ_1 pharmacological blockade and by genetic inactivation.

According to our results, the local pharmacological blockade of σ_1 receptors potentiates the mechanical antinociception induced locally by morphine but does not alter nociceptive responses in the absence of the opioid.

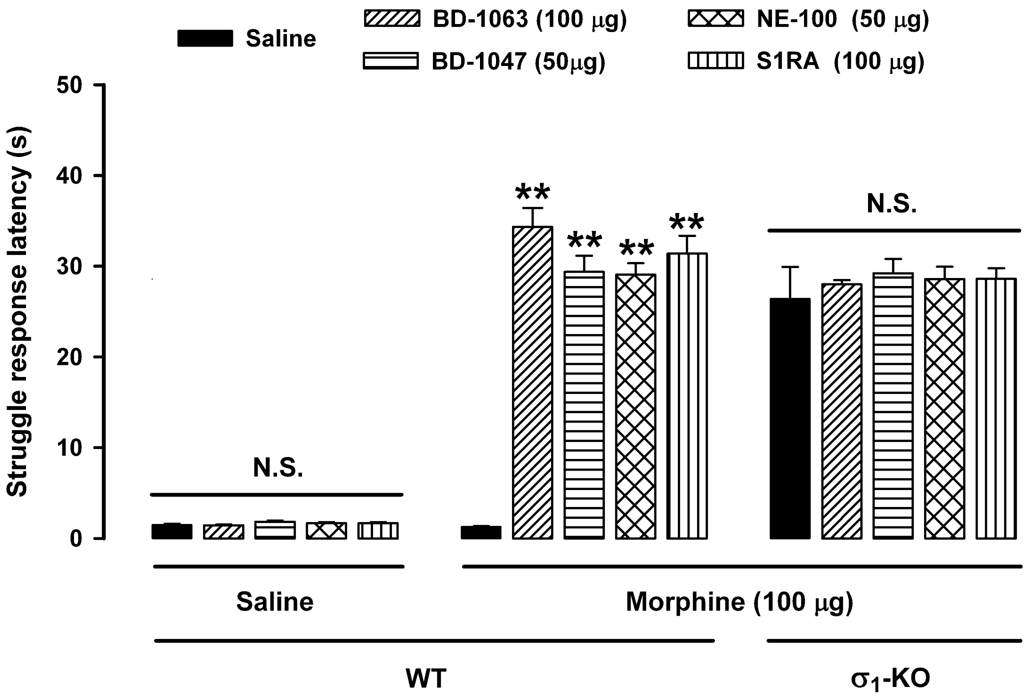

Fig. 8. Effects of the local (intraplantar) administration of several σ_1 antagonists on the mechanical antinociceptive effect induced by intraplantar morphine in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with the σ_1 antagonists BD-1063 (100 µg), BD-1047 (50 µg), NE-100 (50 µg), or S1RA (100 µg) or their solvent (saline) associated with morphine (100 µg) or its solvent (saline). Each bar and vertical line represents the mean ± SEM of values obtained in 8-10 animals. Statistically significant differences in values between morphine-injected WT mice treated with each σ_1 antagonist and those treated with its solvent: $^{**}P < 0.01$. N.S.: no statistically significant differences between the values (two-way ANOVA followed by Bonferroni test). None of the treatments produced any effect in the non-injected paw (data not represented in order to simplify the figure)

1.4.6. Morphine-induced side effects (hyperlocomotion and inhibition of gastrointestinal transit) in wild-type and σ_1 knockout mice

To determine whether the increased morphine-induced antinociception observed in σ_1-KO mice was accompanied by an increase in the non-analgesic effects of morphine, we tested morphine-induced hyperlocomotion and gastrointestinal transit inhibition. WT and σ_1-KO mice showed a similar ambulatory locomotion when injected with saline (Fig. 9A). Systemic administration of morphine (4-16 mg/kg, s.c.) induced a marked and dose-dependent increase in the distance travelled by both WT mice and morphine-injected σ1-KO mice, with no statistically significant differences between them (Fig. 9A).

Gastrointestinal transit values did not significantly differ between saline-treated WT and σ1-KO mice, with the charcoal meal travelling about 30 cm of the small intestine in both genotypes (Fig. 9B). Morphine (1-8 mg/kg, s.c.)-treated mice of both genotypes showed a dose-dependent decrease in gastrointestinal transit of a similar magnitude (Fig. 9B).

Therefore, two different non-analgesic effects of morphine were unaltered by the genetic inactivation of σ1 receptors.

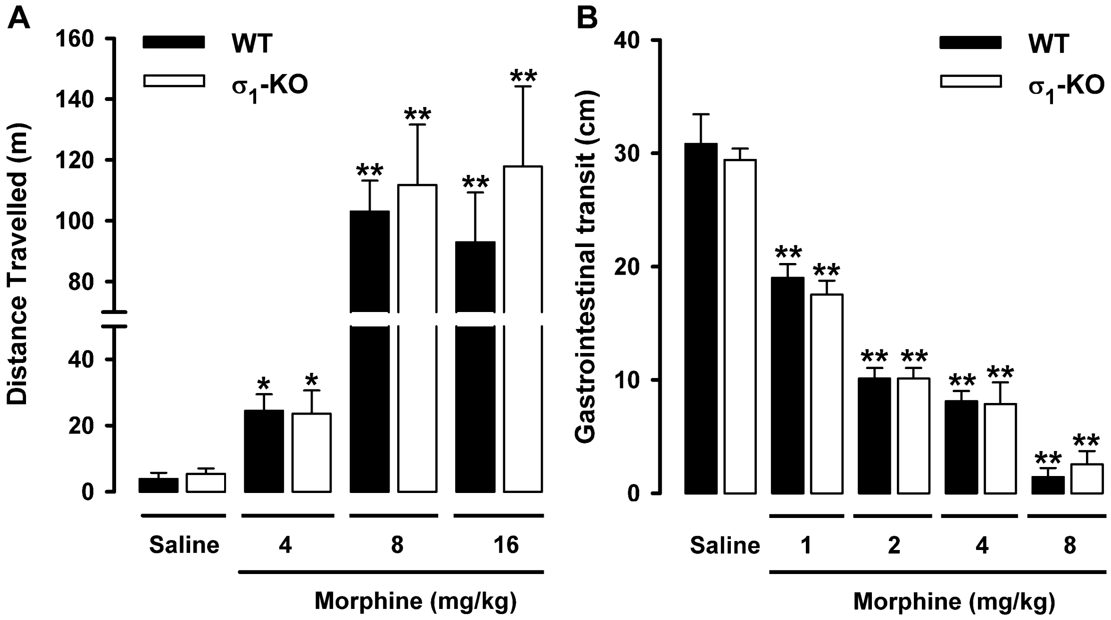

Fig. 9. Effects of the systemic (subcutaneous) administration of morphine or saline on (A) locomotor activity and (B) gastrointestinal transit of wild-type (WT) and σ_1 knockout (σ_1-KO) mice. (A) Mice were injected with morphine or saline, and the distance that they travelled between 30 and 60 min post-injection was recorded. Each bar and vertical line represents the mean ± SEM of values obtained in 8-13 mice. (B) At 30 min after the injection of morphine or saline, mice were intragastrically administered with 0.5 % charcoal suspension. Transit of the charcoal was measured at 30 min after its ingestion. Each bar and vertical line represents the mean ± SEM of values obtained in 6 mice. Statistically significant differences between the values obtained in saline- and morphine-treated groups: $^{**}P$ <0.01. No statistically significant differences were found between genotypes under the same treatment (two-way ANOVA followed by Bonferroni test).

1.4.7. [^{3}H]DAMGO saturation binding assays in spinal cord, forebrain, and hind-paw skin membranes from wild-type and σ_1 knockout mice

Saturation assays showed that the selective μ-opioid receptor radioligand [^{3}H]DAMGO bound in a saturable manner to forebrain and spinal cord membranes (P_2 fraction) from both WT and σ_1-KO mice (Fig. 10A). Replicates were fitted by nonlinear regression analysis to hyperbolic equations. In the case of the forebrain membranes, no significant differences were found between WT and σ_1-KO mice in [^{3}H]DAMGO equilibrium dissociation constant (K_D) values (1.530 ± 0.212 nM *vs.* 1.172 ± 0.166 nM, respectively; $P > 0.05$) or in the maximal number of receptors (B_{max}) (0.137 ± 0.004 *vs.* 0.133 + 0.004 pmol/mg of protein, respectively; $P > 0.05$). Likewise, in the spinal cord membranes, no significant differences between WT and σ_1-KO mice were found in K_D (2.028 ± 0.269 *vs.* 1.861 ± 0.226 nM, respectively; $P > 0.05$) or B_{max} (0.179± 0.005 *vs.* 0.180 ± 0.004 pmol/mg of protein, respectively; $P > 0.05$) values.

The [^{3}H]DAMGO B_{max} in paw skin membranes was estimated by using a single saturating concentration (20 nM) of this radioligand. For a better comparison, we used the same concentration of the radioligand to determine the estimated B_{max} in both spinal cord and forebrain membranes. Using this approach, we obtained very similar [^{3}H]DAMGO-specific binding values to those obtained with the full saturation assay in both WT (0.180 ± 0.012 and 0.1266 ± 0.003 pmol/mg of protein for spinal cord and forebrain membranes, respectively) and σ_1-KO (0.183 ± 0.013 and 0.122 ± 0.07 pmol/mg of protein for spinal cord and forebrain membranes, respectively) mice (Fig. 10B). [^{3}H]DAMGO binding sites were much less abundant in the paw skin membranes than in the tissues from the central nervous system, with no significant differences between the genotypes (0.030 ± 0.002 and 0.030 ± 0.001 pmol/mg of protein for WT and σ_1-KO mice, respectively) (Fig. 10B).

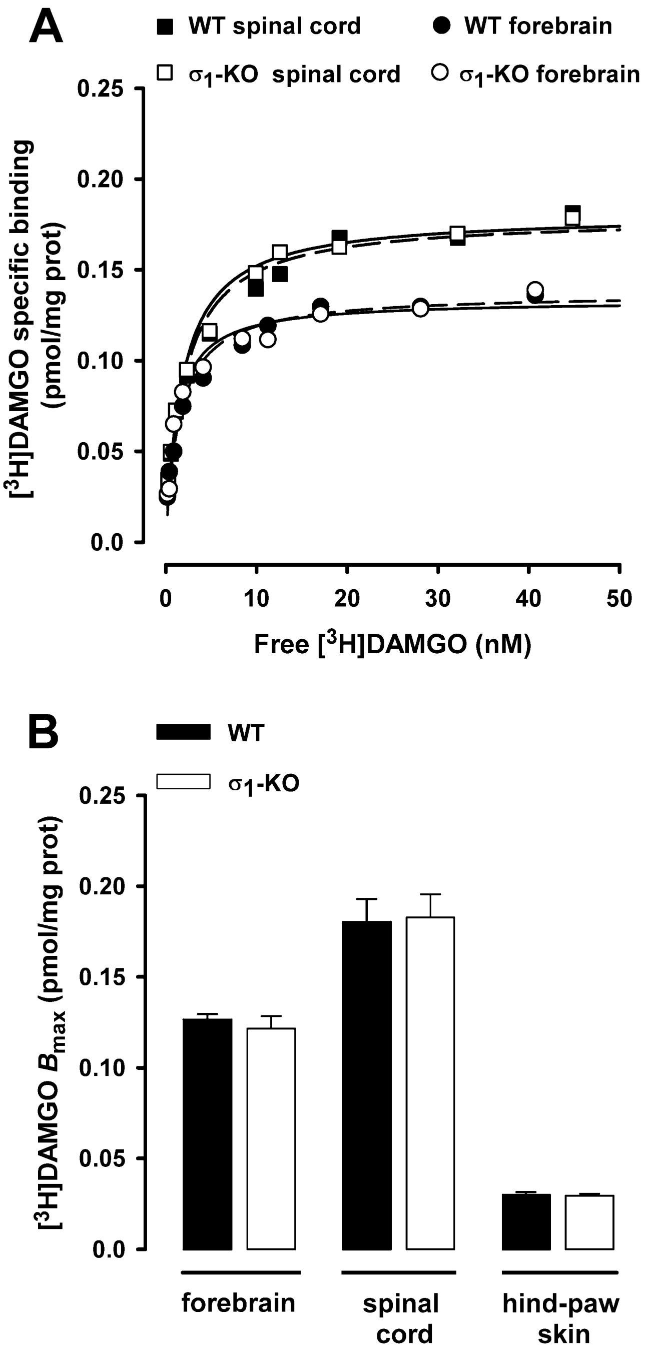

Fig. 10. [^{3}H]DAMGO binding assays in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. (A) [^{3}H]DAMGO saturation assays in forebrain and spinal cord membranes (P_2 fraction) from WT (closed symbols) or σ_1-KO (open symbols) mice. Experiments were performed by incubating the membranes for 120 min at 25ºC with several concentrations of [^{3}H]DAMGO (0.25-45 nM). (B) [^{3}H]DAMGO B_{max} values in forebrain, spinal cord, and hind-paw skin membranes. B_{max} values were estimated by incubating the membranes with a single saturating concentration of [^{3}H]DAMGO (20 nM) under the experimental conditions described above. All experiments were carried out in the presence of 10 µM naloxone (to define non-specific binding) or its solvent. The data shown represent three experiments carried out in triplicate.

1.4.8. Affinity of selective σ_1 ligands and morphine for [^{3}H]DAMGO binding sites in forebrain membranes from wild-type mice

We used competition binding assays to test the binding of the studied drugs to [^{3}H]DAMGO-labeled µ-receptors in forebrain membranes from WT mice. As expected, the [^{3}H]-DAMGO-specific binding was concentration-dependently inhibited by morphine, which showed an affinity (K_i) value of 3.746 ± 0.319 nM. However, the specific binding of [^{3}H]DAMGO was not inhibited by any of the selective σ_1-ligands tested (BD-1063, BD-1047, NE-100, S1RA, or PRE-084), therefore demonstrating negligible affinity of these drugs for [^{3}H]DAMGO binding sites (Fig. 11).

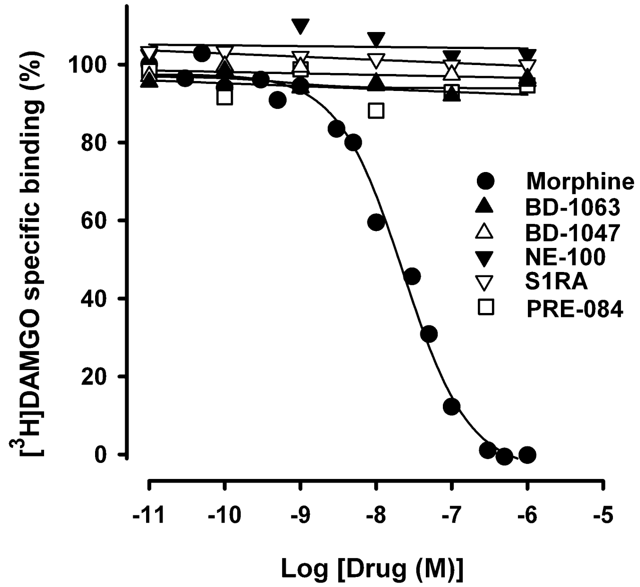

Fig. 11. Inhibition by unlabeled drugs of [^{3}H]DAMGO binding to forebrain membranes (P_2 fraction) in wild-type mice. Membranes were incubated for 120 min at 25ºC with 5 nM [^{3}H]DAMGO and increasing concentrations of morphine or the σ_1 ligands BD-1063, BD-1047, NE-100, S1RA, or PRE-084. Naloxone (10 µM) was used to define the non-specific binding. Data are the average of three experiments carried out in triplicate.

1.5. DISCUSSION

In this study, pharmacological antagonism or genetic inactivation of σ_1 receptors induced a strong functional synergism with the mechanical antinociceptive effect of morphine, without altering its non-analgesic effects (hyperlocomotion and inhibition of gastrointestinal transit). Furthermore, this synergistic interaction occurred at the peripheral level. None of these findings have been previously reported.

We found that the pain-like responses evoked by a blunt mechanical stimulus were virtually identical between σ_1-KO and WT mice over a wide range of pressure intensities. Neither were they altered by the local or systemic administration of σ_1 antagonists. These findings expand the results found in previous studies reporting that σ_1-KO mice or WT mice treated with σ_1 antagonists showed unaltered responses against different sensory modalities of acute nociceptive pain, including mechanical (punctate) and thermal (cold and heat) stimulation (e.g. Chien and Pasternak, 1994; De la Puente et al., 2009; Entrena et al., 2009a; Marrazzo et al., 2011; Nieto et al., 2012; Romero et al., 2012). These results suggest that the basic mechanisms for perceiving these stimuli and the motor response for producing the pain-like responses are intact in σ_1-KO mice and are not altered by the pharmacological blockade of σ_1 receptors in WT mice. However, despite the apparent absence of σ_1 receptor involvement in acute nociception, we found that the mechanical antinociceptive effect of morphine was markedly enhanced in σ_1-KO mice and that this effect was mimicked by the systemic administration of several prototypic σ_1 antagonists in WT mice. Our results extend previous reports on the potentiation of opioid-induced thermal antinociception by σ_1 inhibition (Chien and Pasternak, 1993 and 1994; Marrazzo et al., 2011 and 2006; Mei and Pasternak, 2002 and 2007; Pan et al., 1998; Ronsisvalle et al., 2001). Taken together, these findings suggest that a tonically active antiopioid σ_1 system modulates both the mechanical and thermal antinociception induced by morphine.

We found that the i.pl. administration of morphine had no analgesic effect against mechanical stimuli in naïve mice over a wide range of doses. This finding is in contrast to previous reports showing that local morphine is effective against thermal stimuli (e.g. Kolesnikov et al., 1996, 2000), highlighting the differences between the effects of this opioid against thermal and mechanical stimuli. The lack of effect of local morphine demonstrated here is in agreement with clinical reports showing that the local application of opioid agonists (including morphine) to uninjured tissue does not reliably produce analgesic effects (reviewed by Stein et al., 2003), and it is consistent with the preferentially central action of opioids to induce analgesia in either humans or rodents (e.g. Christie et al., 2000; Thomas et al., 2008; Khalefa et al., 2012). Because of this preferentially central localization of opioid-induced analgesia, previous studies focused on the role of σ_1 receptors at central levels in modulating this opioid-mediated effect, demonstrating that the central administration of either σ_1 antagonists or antisense oligodeoxynucleotides enhances morphine-induced thermal antinociception (Mei and Pasternak, 2002 and 2007; Pan et al., 1998). In the present study, we show that σ_1-KO mice locally treated with morphine and WT mice locally co-administered with this opioid and σ_1 receptor antagonist exhibit a strong synergistic mechanical antinociceptive effect at the site of the administration of the combined drug solution but not at a site distant from its injection (contralateral paw) which suggests that the interaction is produced locally. This view is further supported by the fact that WT mice treated with morphine (200 µg) in one paw and BD-1063 (200 µg) in the contralateral one evidenced no antinociception in either paw (data not shown). Hence, our data reveal for the first time that the tonic inhibition of morphine analgesia by σ_1 receptors is also present at the periphery and is strongly involved in mechanical nociceptive pain.

We found similar levels of ([^{3}H]DAMGO-labeled) µ-opioid receptors in σ_1-KO and WT mice in all tissues examined, and the affinity of this radioligand for its binding site was not altered in σ_1-KO mice. In addition, all σ_1 ligands tested showed a negligible affinity for µ-opioid receptors from mouse forebrain membranes, as previously reported for some of these σ_1-ligands in other tissues or species (Kim et al., 2010; Matsumoto et al., 1995; Romero et al., 2012). Hence, our results cannot be explained by abnormal µ-opioid receptors in the σ_1-KO mice or by a direct interaction of the σ_1 drugs used in this study with µ-opioid receptors.

Several of our findings indicate that a selective σ_1 receptor action is involved in the modulation of morphine-induced mechanical antinociception. Firstly, the enhancement of morphine-induced mechanical antinociception in σ_1-KO mice was replicated, at a similar magnitude, by all of the selective σ_1 antagonists tested in WT mice. Secondly, the selective σ_1 agonist PRE-084, which had no effect on morphine-induced antinociception, was able to reverse the effects of the systemic σ_1 antagonists. Finally, none of the σ_1 antagonists tested (administered either systemically or locally) further enhanced morphine-induced antinociception in σ_1-KO mice, indicating that off-target effects do not account for the effects observed. The similarities in the antinociceptive effects of morphine between σ_1-KO mice and σ_1 antagonist-treated WT mice, together with the clear σ_1 pharmacology of these effects, strongly suggest that the effects observed are mediated by σ_1 receptor inhibition. A recent study indicates a possible mechanism for these effects. Thus, the prototypic σ_1 receptor antagonist BD-1047 was found to increase DAMGO-induced G-protein couple receptor signaling (measured as the increase in $[^{35}S]GTP\gamma S$ binding) without altering opioid receptor binding, and σ_1 receptors and μ-opioid receptor were shown to physically interact (Kim et al., 2010). Therefore, basal σ_1 receptor activity may tonically reduce μ-opioid receptor signaling, explaining the increase in morphine analgesia by σ_1 receptor inhibition.

Previous research on opioid-induced thermal antinociception showed that the selective σ_1 agonist (+)-pentazocine could decrease morphine-induced thermal antinociception, indicating that tonic inhibition of this analgesic effect of morphine can be enhanced by further σ_1 activation (Chien and Pasternak, 1993 and 1994; Mei and Pasternak, 2002 and 2007). However, under our conditions, PRE-084 did not influence the effect of morphine on mechanical stimuli, suggesting that the tonic inhibition of morphine-induced mechanical antinociception by σ_1 receptors was already maximal and could not be further increased by exogenous σ_1 activation. The role of σ_1 receptors in animal models of tonic/chronic pain has been thoroughly studied over the past decade (Cendán et al., 2005a and b; De la Puente et al., 2009; Entrena et al., 2009a and b; Kim et al., 2006; Nieto et al., 2012; Roh et al., 2008b; Romero et al., 2012). Modulation of these pain behaviors by σ_1 receptors is thought to be located at central levels, because σ_1 receptor activation in the spinal cord triggers central sensitization to induce mechanical allodynia (Kim et al., 2008; Roh et al., 2010 and 2011; Ohsawa et al., 2011), likely contributing through this mechanism to the modulation of activity-induced spinal sensitization (De la Puente et al., 2009; Romero et al., 2012). We previously demonstrated that the effect of σ_1 antagonists on some of those behavioral outcomes (formalin-induced pain and capsaicin-induced mechanical hypersensitivity) was insensitive to naloxone treatment (Cendán et al., 2005a; Entrena et al., 2009b).

Therefore, these effects of σ_1 antagonists do not appear to depend on modulation of the opioid system.

Opioids, and particularly morphine, are considered to be the “gold standard” for pain management (Christie et al., 2000; Waldhoer et al., 2004). However, several serious side effects associated with acutely administered morphine, including constipation, sedation, respiratory depression, and nausea, represent substantial drawbacks to its use (reviewed in Al-Hasani and Bruchas, 2011 and Waldhoer et al., 2004). Two strategies have been proposed to minimize the adverse effects of opioids. One of these approaches is to administer an adjuvant drug with synergistic analgesic effects in order to minimize the dose of the opioid (and hence its side effects) while maintaining acceptable levels of analgesia. One example is the combination of non-steroidal anti-inflammatory drugs or anticonvulsants with opioids (Christie et al., 2000; Mao et al., 2011). In the present study, we show that, despite the strong potentiation of the analgesic effects of morphine observed in σ_1-KO mice, σ_1 receptors do not appear to influence two morphine-induced side effects, i.e., hyperlocomotion and gastrointestinal transit inhibition. Hyperlocomotion is a common side effect of opioids in rodents (e.g. Cobos et al., 2012; Elhabazi et al., 2012) and reflects an increase in supraspinal dopamine release (Hnasko et al., 2005), whereas the inhibition of gastrointestinal transit mainly results from opioid action at peripheral level (Al-Hasani and Bruchas., 2011). In agreement with our data, a previous study found that the selective σ_1 agonist (+)-pentazocine did not modify the morphine-induced decrease in gastrointestinal transit or lethality (Chien and Pasternak, 1994). The differential impact of σ_1 receptors on the modulation of opioid analgesia and side effects may suggest that σ_1 receptors are present in a specific subset of opioid receptor-expressing neurons that are more involved in pain pathways or, alternatively, that further mechanisms besides the direct modulation of opioid signaling may participate in the analgesia enhancement.

A different approach to reducing the side effects of opioids is to target peripheral opioid antinociception in order to minimize undesirable centrally mediated effects (reviewed by Sehgal et al., 2011; Stein et al., 2003). Here we show that σ_1 inhibition is able to potentiate local morphine analgesia, producing an even greater antinociceptive effect than is induced by systemic morphine at the highest dose administered in this study (16 mg/kg). Consequently, the potentiation of peripherally mediated morphine analgesia by σ_1 receptor antagonists may offer safer and improved therapeutic outcomes in pain management.

1.6. CONCLUSIONS

In summary, we found that systemic σ_1 receptor inhibition synergistically enhances morphine-induced mechanical antinociception without modifying a centrally-induced (hyperlocomotion) or peripherally-induced (constipation) side effect of morphine. Enhancement of morphine analgesia by σ_1 inhibition can be produced locally, indicating that σ_1 receptors tonically inhibit peripheral opioid functioning. The local combination of morphine with σ_1 receptor blockade may represent a strategy to minimize the adverse effects of morphine by differentially potentiating its therapeutic analgesic actions.

1.7. ACKNOWLEDGEMENTS

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2. Modulation of peripheral μ-opioid analgesia by σ_1 receptors

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2.1. ABSTRACT

We evaluated the effects of σ_1 receptor inhibition on µ-opioid-induced mechanical antinociception and constipation. σ_1-knockout mice exhibited marked mechanical antinociception in response to several µ-opioid analgesics (fentanyl, oxycodone, morphine, buprenorphine and tramadol) at systemic (subcutaneous, s.c.) doses that were inactive in wild-type mice, and even unmasked the antinociceptive effects of the peripheral µ-opioid agonist loperamide. Similarly, systemic (s.c.) or local (intraplantar) treatment of wild-type mice with the selective σ_1 antagonists BD-1063 or S1RA potentiated µ-opioid antinociception; these effects were fully reversed by the σ_1 agonist PRE-084, showing the selectivity of the pharmacological approach. The µ-opioid antinociception potentiated by σ_1 inhibition (by σ_1 receptor knockout or σ_1 pharmacological antagonism) was more sensitive to the peripherally-restricted opioid antagonist naloxone methiodide than opioid antinociception under normal conditions, indicating a key role for peripheral opioid receptors in the enhanced antinociception. Direct interaction between the opioid drugs and σ_1 receptor cannot account for our results, since the former lacked affinity for σ_1 receptors (labeled with $[^3H](+)$-pentazocine). A peripheral role for σ_1 receptors was also supported by their higher density (western blot results) in peripheral nervous tissue (dorsal root ganglia) than in several central areas involved in opioid antinociception (dorsal spinal cord, basolateral amygdala, periaqueductal gray and rostroventral medulla). In contrast to its effects on nociception, σ_1 receptor inhibition did not alter fentanyl- or loperamide-induced constipation, a peripherally-mediated nonanalgesic opioid effect. Therefore, σ_1-receptor inhibition may be used as a systemic or local adjuvant to enhance peripheral µ-opioid analgesia without affecting opioid-induced constipation.

2.2. INTRODUCTION

Opioid drugs, particularly agonists of the µ-receptor subtype, are widely used to treat moderate-to-severe pain (Pasternak and Pan, 2011; Al-Hasani and Bruchas, 2011). Opioid receptors are located at different sites along the pain-processing pathway, including both the central (spinal cord and different supraspinal nuclei) and peripheral (dorsal root ganglion [DRG] and peripheral nerve terminals) nervous system (Khalefa et al., 2012, Bigliardi-Qi et al., 2004). The antinociceptive effect of systemic opioids is thought to be produced mainly at the central (particularly supraspinal) level (Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008; Joshi et al., 2008; Khalefa et al., 2012), although peripheral opioid receptors might also participate (Kayser et al., 1995; Craft et al., 1995; Shannon and Lutz, 2002).

The sigma-1 (σ_1) receptor has been cloned and its sequence does not show homology with opioid receptors or any other known mammalian protein; it is therefore currently considered a unique entity (Cobos et al., 2008; Zamanillo et al., 2013). Inhibition of σ_1 receptor function either by the systemic administration of σ_1 antagonists or by σ_1 receptor knockdown does not influence acute nociception per se (Cendán et al., 2005b; Entrena et al., 2009b; De la Puente et al., 2009; Nieto et al., 2012; Romero et al., 2012; Sánchez-Fernández et al., 2013). However, σ_1 inhibition is able to enhance opioid signaling (Kim et al., 2010) and to potentiate the antinociceptive effect of systemic opioids (e.g. Chien and Pasternak, 1993 and 1994; Marrazzo et al., 2011; Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). Opioid antinociception can be potentiated by central σ_1 inhibition (King et al., 1997; Pan et al., 1998; Mei and Pasternak 2002 and 2007; Marrazzo et al., 2006), and we recently reported that the local peripheral coadministration of σ_1 antagonists and morphine also resulted in markedly enhanced antinociception (Sánchez-Fernández et al., 2013). However, it is unknown whether peripheral mechanisms are involved in the antinociception induced by systemic opioids when σ_1 receptors are inhibited. We hypothesize that the contribution of peripheral opioid receptors to overall antinociception induced by the combination of systemic opioids and σ_1 inhibition might be more relevant than the contribution of these receptors to the effect of systemic opioids under normal conditions. The main goal of this study was to test this hypothesis.

To do so, we compared the effect of the peripherally restricted opioid antagonist naloxone methiodide (Menéndez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012) on the mechanical antinociception induced by systemic µ-opioids in the presence or absence of σ_1 receptor inhibition. We used several clinically relevant µ-opioids with different intrinsic activities and blood–brain barrier permeabilities. These opioid drugs include the centrally active analgesics fentanyl, morphine, oxycodone, tramadol and buprenorphine (Pergolizzi et al., 2008; Schäfer 2010), and the peripherally restricted µ-agonist loperamide, used clinically as an antidiarrheal drug (Layer et al., 2010; Gallelli et al., 2010). To inhibit σ_1 receptors we used σ_1-knockout (σ_1-KO) mice and systemic and local treatments of wild-type (WT) mice with the selective σ_1 antagonists BD-1063 and S1RA. Moreover, because the data from the present study support the importance of peripheral σ_1 receptors as modulators of opioid antinociception, we compared the expression of σ_1 receptors in peripheral (DRG) and central areas (dorsal spinal cord, basolateral amygdala, periaqueductal gray and rostroventral medulla) known to be involved in opioid antinociception (Millan, 2002). Finally, to rule out possible direct interactions between the opioid drugs tested here and σ_1 receptors, we determined their affinity for σ_1 receptors.

Opioid-induced constipation is the most clinically relevant peripheral side effect of µ-opioids (Benyamin et al 2008; Al-Hasani and Bruchas, 2011; Ringkamp and Raja, 2012), and it is one of the main reasons for patients’ voluntary withdrawal from opioid medication (Dhingra et al., 2012). We recently showed that although morphine-induced antinociception was potentiated in σ_1-KO mice, morphine-induced constipation remained unaltered (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). However, it is unknown whether these differential effects of σ_1 inhibition also occur with other µ-opioids. Therefore, an additional goal of this study was to test the effects of σ_1 inhibition on constipation induced by two very different µ-opioids: fentanyl, a centrally penetrant drug, and loperamide, a peripherally restricted drug.

2.3. MATERIAL AND METHODS

2.3.1. Experimental animals

Most experiments were performed in female WT (Charles River, Barcelona, Spain) and σ_1-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25-30 g. The knockout mice were backcrossed for 10 generations to a CD-1 genetic background as described previously (Entrena et al., 2009a). Some experiments were also performed in male WT mice to ensure that sex differences did not affect our results. All animals were kept in our animal facilities for a minimum of 7 days before the experiments. Animals were housed under controlled environmental conditions: 12/12 h day/night cycle, constant temperature (22 ± 2 °C) with free access to water and food (standard laboratory diet, Harlan Teklad Research Diet, Madison, WI, USA). Behavioral testing was done during the light phase (from 9.00 h to 15.00 h) and randomly throughout the estrous cycle. Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council directive 86/609), and with the guidelines for the investigation of experimental pain in conscious animals (Zimmermann 1983).

2.3.2. Radioligand, drugs and drug administration

The opioid drugs and their suppliers were: the µ-opioid agonists morphine hydrochloride (from the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), fentanyl citrate, oxycodone hydrochloride, buprenorphine hydrochloride and loperamide (all from Sigma-Aldrich Química SA, Madrid, Spain), tramadol (supplied by Laboratorios Esteve, Barcelona, Spain) and the opioid antagonists naloxone hydrochloride and naloxone methiodide (Sigma-Aldrich Química SA). BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) (Tocris Cookson Ltd., Bristol, UK), and S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride) (synthesized and kindly supplied by Laboratorios Esteve, Barcelona, Spain) were used as selective σ_1 antagonists (Cobos et al., 2008; Romero et al., 2012). PRE-084 ([2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride]) (Tocris Cookson Ltd.) was used as a selective σ_1 agonist (Hayashi and Su, 2004; Cobos et al., 2008).

All drugs used for the in vivo studies were dissolved in sterile physiological saline (NaCl 0.9%) except loperamide, which was dissolved in 1% dimethylsulfoxide (DMSO, Merck KGaA, Darmstadt, Germany) in ultrapure water. The solutions of the σ_1 ligands BD-1063 and PRE-084 were appropriately alkalinized with NaOH. To evaluate the effects of systemic treatments, drugs or their solvents were injected subcutaneously (s.c.) into the interscapular zone in a volume of 5 mL/kg. When the effect of the association of several drugs was tested, each drug was injected into a different area of the interscapular zone. To study the local effects of BD-1063 or S1RA, these drugs were injected intraplantarly (i.pl.) into the right hindpaw at a volume of 20 μL with a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a 301/2–gauge needle. Each control group received the same volume of drug vehicle.

For binding assays, the radioligand was the selective σ_1 agonist [^{3}H](+)-pentazocine (Cobos et al., 2006), with a specific activity of 34.8 Ci/mmol (PerkinElmer Life Sciences, Boston, MA, USA). Dilutions from the stock [^{3}H](+)-pentazocine solution were prepared with ice-cold incubation buffer (50 mM Tris–HCl buffer, pH 8 at 30 °C). The cold drugs tested were the opioids: fentanyl, oxycodone, morphine, buprenorphine, loperamide, tramadol, naloxone and naloxone methiodide. BD-1063 was used as a control with known high-affinity for σ_1 receptors (Cobos et al., 2007; Entrena et al., 2009a). All drugs were dissolved at a concentration of 1 mM in ultrapure water with the exception of loperamide, which was dissolved in absolute ethanol. Further dilutions were prepared with incubation buffer. The final maximal concentration of ethanol in the incubation medium was 0.1% (vol/vol), which did not affect [^{3}H](+)-pentazocine binding (Cobos et al., 2005 and 2006).

2.3.3. Evaluation of mechanical nociception (paw pressure)

The effects of the drugs on mechanical nociception were evaluated with an Analgesimeter (Model 37215, Ugo-Basile, Varese, Italy) according to methods described previously (Menéndez et al., 2005; Sánchez-Fernández et al., 2013). Briefly, the hindpaw of the mice was stimulated with a constant pressure of 450 g using a cone-shaped paw-presser with a rounded tip until the animal showed a struggle reaction. Immediately thereafter, the stimulus was stopped and the response latency (in seconds) was recorded. The test was done twice alternately to each hindpaw at intervals of 1 min between each stimulation, with a 50-s cutoff for each determination. The antinociceptive effects of μ-agonists were evaluated 30 min after s.c. administration except for buprenorphine, which was administered s.c. 1 h before the evaluation, since the onset of its antinociceptive effect is known to be much slower than for other opioids (Yassen et al., 2005). To study the effects of the systemic administration of BD-1063 or S1RA on μ-opioid antinociception, these drugs (or its solvent) were administered s.c. 5 min before the μ-agonists. When drugs were administered systemically the struggle response latency was calculated as the mean of the two averaged times in each hindpaw, since no differences between sides were seen in the response of each hindpaw. To study the local effects of σ_1 inhibition on μ-opioid antinociception, BD-1063, S1RA or their solvent were administered i.pl. and the animals were evaluated 5 min after the injection to minimize possible systemic effects induced by the drug. In experiments to determine the local effects of the σ_1 antagonists, we calculated the average of the two values from the injected and noninjected hindpaws independently. To test the effects of PRE-084, naloxone or naloxone methiodide on μ-opioid antinociception in the presence or absence of σ_1 inhibition, these drugs or their solvent were administered s.c. 5 min before the μ-agonist solution, when appropriate.

The experimenters who evaluated the behavioral responses were blinded to the treatment group and genotype of each experimental animal.

2.3.4. Evaluation of opioid-induced inhibition of gastrointestinal transit

Gastrointestinal transit was estimated as previously described (Sánchez-Fernández et al., 2013). Briefly, 8 h before the experiment, food was withheld and water was available ad libitum. BD-1063 or its solvent was injected s.c., and 5 min later fentanyl, loperamide or their solvent was injected s.c. Thirty minutes after the opioid was given, the mice received 0.3 mL of a 0.5% (wt/vol) activated charcoal suspension (2–12 µm powder, Sigma-Aldrich Química SA) in distilled water. The mice were killed by cervical dislocation 30 min after the activated charcoal was administered (i.e. 60 min after administration of the opioid); then the small intestine was removed from the pyloric sphincter to the ileocecal junction and straightened to measure the distance travelled by the leading edge of the charcoal meal.

2.3.5. Western blotting

The basolateral amygdala (BLA), rostroventral medulla (RVM), periaqueductal grey matter (PAG), dorsal spinal cord of the lumbar enlargement (dSC) and lumbar (L4-L5) root ganglia (DRG) were carefully removed from naïve WT and σ_1-KO mice. The tissue was homogenized by sonication in a buffer solution (50 mM Tris, 150 nM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 1% NP-40, 1mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 25 mM NaF, 0.5% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail, all from Sigma-Aldrich Quımica SA). Protein concentration in the tissue homogenate was measured with the Bradford assay. The samples were stored at −80 °C until use.

Twenty-five micrograms of protein was loaded on 12% (wt/vol) SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Madrid, Spain). The membranes were blocked at room temperature for 1 h with blocking buffer containing 5% dry skim milk in T-TBS (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5). Then the membranes were incubated overnight at 4 °C with a mouse monoclonal antibody that recognized σ_1 receptor (1:1,000, sc-137075, Santa Cruz Biotechnology, Dallas, TX, USA). Mouse monoclonal anti-β-actin antibody (1:2,500, sc-81178, Santa Cruz Biotechnology) was used as a loading control. Both primary antibodies were diluted in T-TBS containing 0.5% dry skim milk. The membranes were washed (3 × 10 min) with T-TBS and incubated for 1 h at room temperature with horseradish peroxidase-linked goat anti-mouse IgG (sc-2005, Santa Cruz

Biotechnology), diluted to 1:2,500 in T-TBS containing 0.5% dry skim milk. The membranes were washed (6 × 10 min) with T-TBS, and the bands were visualized with an enhanced chemiluminescence method (ECL Prime Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions (5 min incubation at room temperature). Densitometric analysis of immunoreactive bands was done with Quantity One software (Bio-Rad). The data are presented as the ratio of the intensity of the σ_1 receptor bands to the β-actin bands. To estimate the molecular weight of the resulting immunoreactive bands, when the samples were loaded we included in the same gel a mixture of 10 blue-stained recombinant proteins of different molecular weights (Precision Plus Protein All Blue Standards, Bio-Rad).

2.3.6. [^{3}H](+)-Pentazocine competition binding assays

Binding assays were done in WT mouse brain membranes (P_2 fraction) as previously described (Entrena et al., 2009a and b; Sánchez-Fernández et al., 2013). Briefly, membrane solutions (460 µL) were incubated at a final protein concentration of 0.8 mg/mL with 20 µL of several concentrations of the cold drug or its solvent and 20 µL [^{3}H](+)-pentazocine (final concentration of 5 nM) for 240 min at 30 °C, pH 8. Five milliliters of ice-cold filtration buffer (Tris 10 mM, pH 7.4) was added to the tubes, and the solutions were rapidly filtered (Brandel cell harvester Model M-12 T, Brandel Instruments, SEMAT Technical, St. Albans, Hertfordshire, UK) over Whatman GF/B glass fiber filters (SEMAT Technical) pre-soaked with 0.5% polyethylenimine in Tris 10 mM, pH 7.4. The filters were washed twice with 5 mL of ice-cold filtration buffer. Then 4 mL of liquid scintillation cocktail (CytoScint scintillation counting solution, MP Biomedicals, Irvine, CA, USA) was added to each filter. On the next day, their radioactivity level was measured by liquid scintillation spectrometry (Beckman Coulter España SA, Madrid, Spain). Nonspecific binding was defined as the binding retained in the presence of a high concentration of BD-1063 (1 µM), and was always less than 20% of total binding.

2.3.7. Data analysis

The data were analyzed with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA). In behavioral assays, the dose-response curves of the drugs were estimated with equations for a sigmoid plot. For binding assays, the concentration of unlabeled BD-1063 that inhibited 50% of $[^3H]$(+)-pentazocine specific binding (IC_{50}) and its standard error were calculated from the inhibition curve with nonlinear regression analysis from the equation for a sigmoid plot, assuming one-site competition. To compare different mean values from in vivo studies, one-way or two-way analysis of variance (ANOVA) was used depending on the experiment; a Bonferroni post hoc test was done in both cases. $P < 0.05$ was considered significant in all tests.

2.4. RESULTS

2.4.1. Effects of systemic (subcutaneous) μ-opioid receptor agonists on mechanical nociception in wild-type and σ_1 knockout mice

Mechanical antinociception induced by the s.c. administration of several opioid drugs was measured as the increase in struggle response latency in drug-treated mice with respect to control (solvent-treated) mice when constant pressure (450 g) was applied to the hindpaw. The struggle response latency was similarly short in WT and σ_1-KO solvent-treated mice (Fig. 1A and B, dose 0).

As expected, the s.c. administration of the opioids fentanyl (0.04 – 0.32 mg/kg), oxycodone (0.75 – 4 mg/kg) or morphine (0.5 – 16 mg/kg) induced a dose-dependent mechanical antinociceptive effect in WT mice. The highest doses tested of fentanyl, oxycodone and morphine increased the latency values 20-fold, 13-fold and 10-fold, respectively, compared to solvent-treated WT mice (Fig. 1A, closed symbols). Buprenorphine (0.06 – 0.48 mg/kg, s.c.) evoked a significant mechanical antinociceptive effect only at the highest dose tested, increasing the latency 6-fold compared to solvent-treated WT mice. However, tramadol (5 – 40 mg/kg, s.c.) and the peripheral μ-agonist loperamide (1 – 4 mg/kg, s.c.) did not induce a significant increase in struggle response latency in WT mice at the doses used here (Fig. 1B, closed symbols).

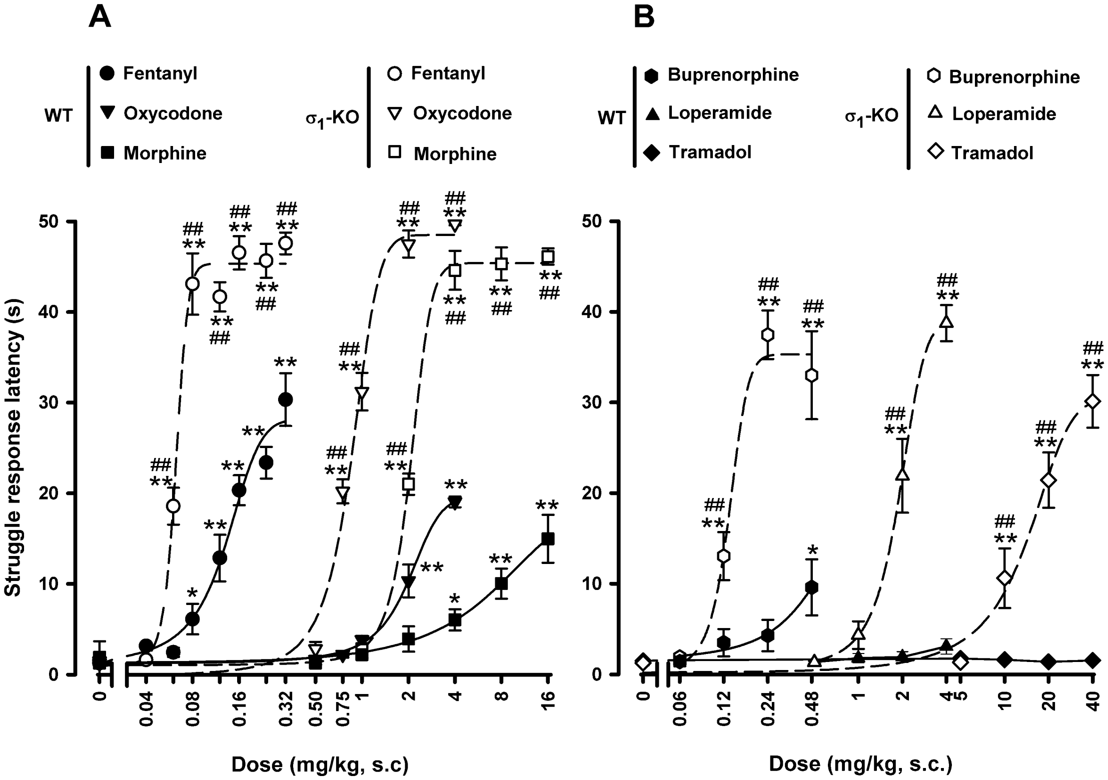

Fig. 1. Mechanical antinociception induced by the systemic administration of several µ-opioid receptor agonists in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaws of mice treated subcutaneously with several doses of: (A) fentanyl, oxycodone, or morphine; and (B) buprenorphine, loperamide or tramadol, or their solvents (doses 0). Each point and vertical line represents the mean ± SEM of values obtained in 8–10 animals. The morphine dose-response curve for comparison with the other opioids is from Sánchez-Fernández et al., 2013. Statistically significant differences between the values obtained in solvent- and opioid-treated groups: $^{*}P < 0.05$; $^{**}P < 0.01$, and between the values obtained in WT and σ_1-KO mice at the same dose of a given opioid: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test).

In σ_1-KO mice the antinociceptive effect in response to fentanyl, oxycodone, morphine or buprenorphine was greater than in WT mice. The doses of these drugs needed to induce significant mechanical antinociception in σ_1-KO mice were much lower than in WT mice, resulting in a displacement to the left of the dose-response curves (Fig. 1A and B). In addition, tramadol or loperamide treatments, which had no effect in WT mice under our experimental conditions, evoked marked antinociception in σ_1-KO mice (Fig.

1B, open symbols). The largest antinociceptive effects in σ_1-KO mice were recorded after treatment with fentanyl, oxycodone or morphine, and struggle response latency reached values close to the cutoff time (50 s) (Fig. 1A, open symbols). Interestingly, σ_1-KO animals treated with the highest doses of buprenorphine, tramadol or even the peripheral µ-agonist loperamide showed latencies higher than those in WT mice treated with fentanyl, oxycodone or morphine (Fig. 1B, open symbols). Therefore, the genetic inactivation of σ_1 receptors clearly potentiated the mechanical antinociception induced by the systemic administration of µ-opioid agonists, including antinociception induced by the peripherally restricted opioid loperamide.

2.4.2. Contribution of peripheral opioid receptors to antinociception induced by the systemic (subcutaneous) administration of µ-opioid analgesics in wild-type mice

To shed light on the role of peripheral opioid receptors in the mechanical antinociception induced by µ-opioid analgesics in WT mice, we tested the sensitivity of this antinociception to the peripherally restricted opioid antagonist naloxone methiodide.

In WT mice treated s.c. with fentanyl (0.16 mg/kg), oxycodone (4 mg/kg), morphine (16 mg/kg) or buprenorphine (0.48 mg/kg) and pretreated with the naloxone methiodide solvent, struggle response latency increased markedly in comparison to untreated mice (Fig. 2). Tramadol and loperamide were not evaluated in this experiment because they did not induce any antinociceptive effect at any dose tested in WT mice (Fig. 1B). Pretreatment with naloxone methiodide (2 – 8 mg/kg) did not alter the increase in struggle response latency induced by fentanyl, morphine or buprenorphine (Fig. 2). However, the antinociception induced by oxycodone was partially reversed by the highest dose of this peripheral opioid antagonist (Fig. 2). As expected, the centrally-penetrant opioid antagonist naloxone (0.5 mg/kg, s.c.) was able to completely reverse the antinociceptive effect induced by all µ-agonists tested: latencies were undistinguishable from those in untreated mice (Fig. 2).

Therefore, among the opioid analgesics tested in WT mice, only oxycodone showed partial sensitivity to peripheral opioid antagonism.

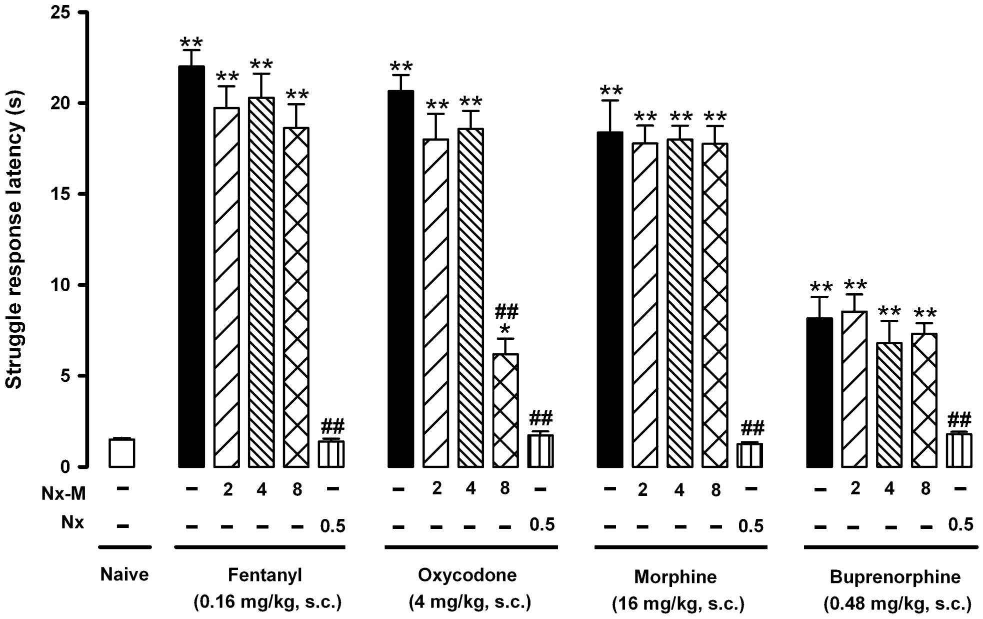

Fig. 2. Contribution of peripheral opioid receptors to the antinociception induced by the systemic administration of several µ-agonists in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of naïve mice, and animals treated subcutaneously with fentanyl (0.16 mg/kg), oxycodone (4 mg/kg), morphine (16 mg/kg) or buprenorphine (0.48 mg/kg), associated with naloxone (Nx, 0.5 mg/kg), naloxone methiodide (Nx-M, 2-8 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in naïve mice and animals treated with the µ-opioid analgesics: **P* < 0.05, ***P* < 0.01; and between the groups treated with the different µ-opioid agonists alone and associated with Nx or Nx-M: ## *P* < 0.01 (one-way ANOVA followed by Bonferroni test).

2.4.3. Contribution of peripheral opioid receptors to antinociception induced by the systemic (subcutaneous) administration of µ-agonists in σ_1 knockout mice

To explore the contribution of peripheral opioid receptors (sensitive to naloxone methiodide) to the enhanced mechanical antinociception seen in σ_1-KO mice, we used doses of µ-agonists that induced little or no antinociception in WT mice but induced maximal antinociception in σ_1-KO mice.

In σ_1-KO mice treated with fentanyl (0.08 mg/kg, s.c.), latencies approached the cutoff time (44.97 ± 1.32 s). This effect was reversed by naloxone methiodide in a dose-dependent manner (0.5 – 2 mg/kg, s.c), and a dose as low as 2 mg/kg completely reversed the potentiation of fentanyl antinociception seen in σ_1-KO mice (Fig. 3A). This dose of naloxone methiodide was chosen to test the involvement of peripheral opioid receptors in the mechanical antinociception induced by several other μ-agonists in σ_1-KO mice. The mechanical antinociception induced by the s.c. administration of oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg) in σ_1-KO mice was completely abolished by naloxone methiodide (Fig. 3B).

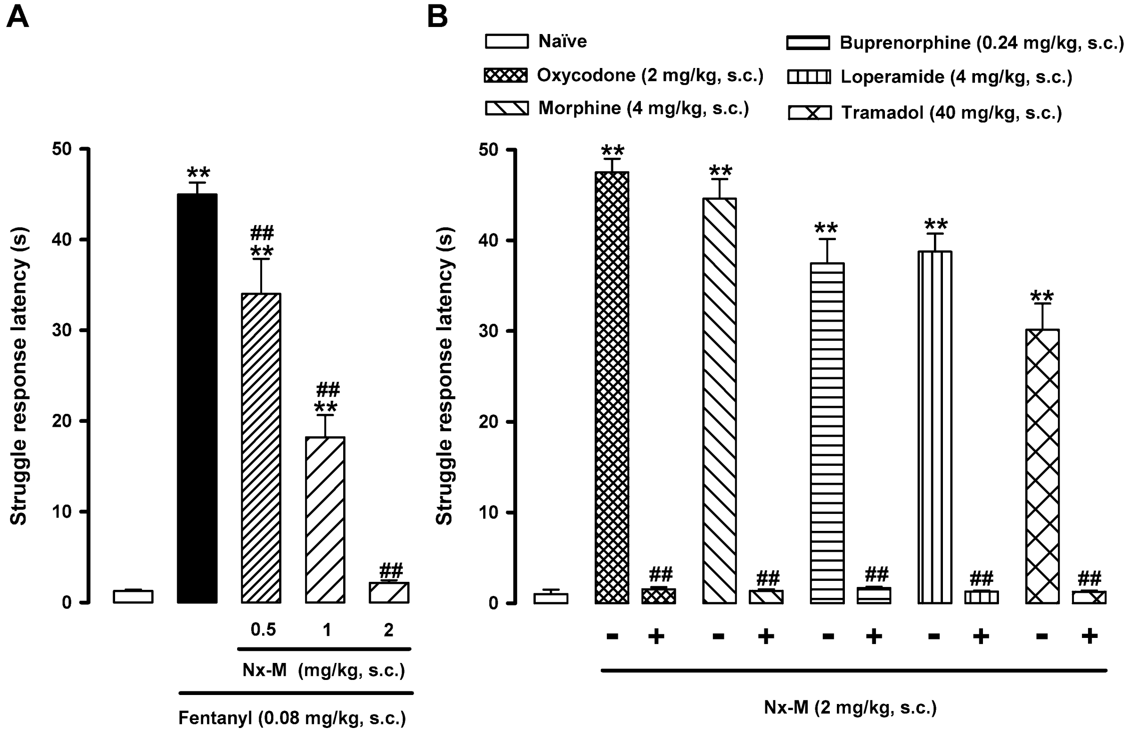

Fig. 3. Contribution of peripheral opioid receptors to the antinociception induced by the systemic administration of low doses of several μ-agonists in σ_1 knockout mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaws of (A) mice treated subcutaneously with the combination of fentanyl (0.08 mg/kg), and naloxone methiodide (Nx-M, 0.5-2 mg/kg) or its solvent, and mice treated with the solvent of both drugs (white bar), or (B) naïve mice and mice treated subcutaneously with oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg), associated with Nx-M (2 mg/kg) or its solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in mice treated and not treated with the μ-opioid analgesics: $^{**}P < 0.01$; and between the groups treated with different μ-opioid agonists associated with Nx-M or its solvent: $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

Therefore, the peripheral opioid antagonist naloxone methiodide was able to fully reverse the enhanced mechanical antinociception induced by several μ-agonists in σ_1-KO mice.

2.4.4. Effects of systemic (subcutaneous) administration of the selective σ_1 antagonist BD-1063 on mechanical antinociception induced by μ-agonists in WT mice: involvement of σ_{1-} and peripheral opioid receptors

We tested whether the pharmacological blockade of σ_1 receptors in WT mice would replicate the phenotype seen in σ_1-KO mice, potentiating μ-opioid analgesia.

BD-1063 (8 – 32 mg/kg) increased the antinociception induced by a low dose of fentanyl (0.08 mg/kg, s.c.) in a dose-dependent manner (Fig. 4A). To confirm the selectivity of this effect, we tested its sensitivity to treatment with PRE-084, a σ_1 agonist. PRE-084 (8 – 32 mg/kg) completely reversed, in a dose-dependent manner, the potentiation of fentanyl antinociception induced by BD-1063 (Fig. 4B).

To test the involvement of peripheral opioid receptors in the potentiation of fentanyl-induced antinociception by systemic treatment with BD-1063, we assayed the sensitivity of this effect to naloxone methiodide. A dose of 2 mg/kg (s.c.) of this peripheral opioid antagonist completely reversed the antinociception induced by the association of BD-1063 with fentanyl (Fig. 4B). BD-1063, PRE-084 and naloxone methiodide did not modify struggle response latency per se (data not shown).

Our results were not affected by sex differences, since as described above for female mice, the fentanyl-induced antinociception in response to the systemic administration of BD-1063 (32 mg/kg, s.c.) was also greatly increased in male mice. This enhanced antinociception was also abolished by PRE-084 (32 mg/kg, s.c.) or Nx-M (2 mg/kg, s.c.) in male mice (data not shown).

We also studied the effects of the association of BD-1063 (32 mg/kg, s.c.) with other μ-opioid receptor agonists administered at doses that induce little or no significant antinociceptive activity in WT mice.

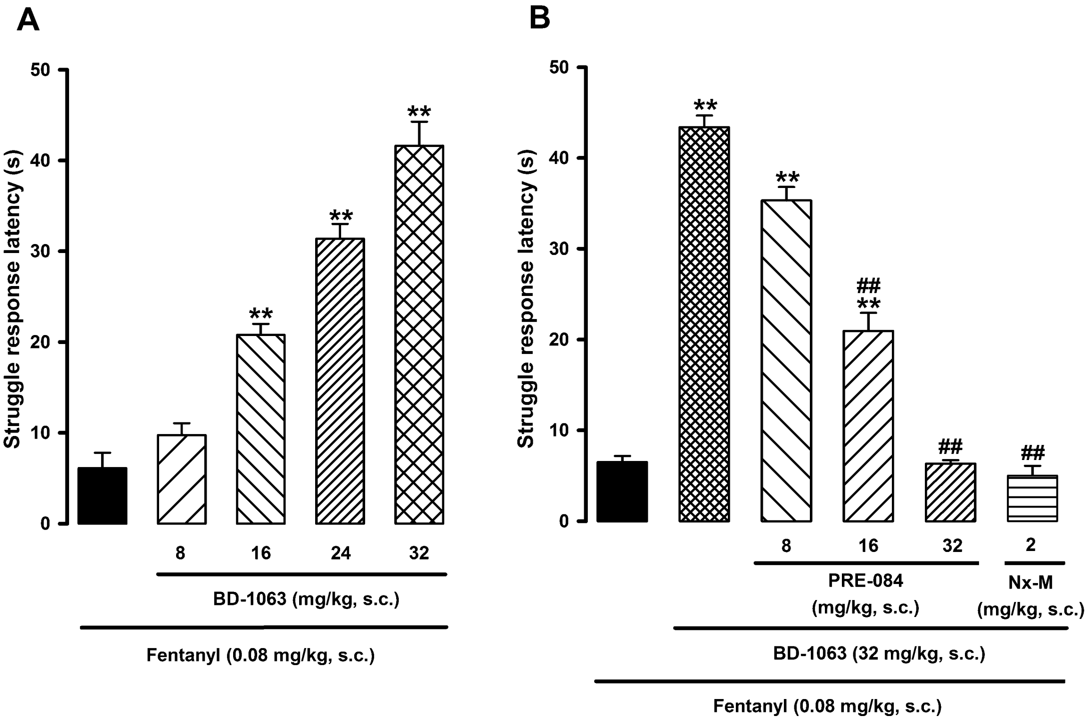

Fig. 4. Contribution of σ_1 and peripheral opioid receptors to the antinociception induced by the association of fentanyl (0.08 mg/kg) and BD-1063, both administered systemically to wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaws of (A) mice treated subcutaneously (s.c.) with fentanyl and BD-1063 (8-32 mg/kg) or its solvent, and (B) mice s.c. given fentanyl (0.08 mg/kg) or fentanyl (0.08 mg/kg) + BD-1063 (32 mg/kg) associated to PRE-084 (8-32 mg/kg, s.c.), naloxone methiodide (Nx-M, 2 mg/kg, s.c.) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with fentanyl associated with BD-1063 or its solvent: $^{**}P < 0.01$; and between the values obtained in mice treated with the combination of fentanyl and BD-1063, and its association with PRE-084 or Nx-M: $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

The association of BD-1063 with oxycodone (2 mg/kg, s.c.), morphine (4 mg/kg, s.c.), buprenorphine (0.24 mg/kg, s.c.), tramadol (40 mg/kg, s.c.) or even loperamide (4 mg/kg, s.c.) induced a statistically significant increase in struggle response latency compared to animals treated with the opioid drugs alone (Fig. 5). This increase was completely reversed by PRE-084 (32 mg/kg, s.c.) (Fig. 5). Naloxone methiodide (2 mg/kg, s.c.) decreased the effect of the association of BD-1063 and the µ-agonists to the same extent as the effect of µ-opioids administered alone, with the exception of the combination of BD-1063 and oxycodone: the effect of this combination was decreased more markedly than the effect of oxycodone alone (Fig. 5).

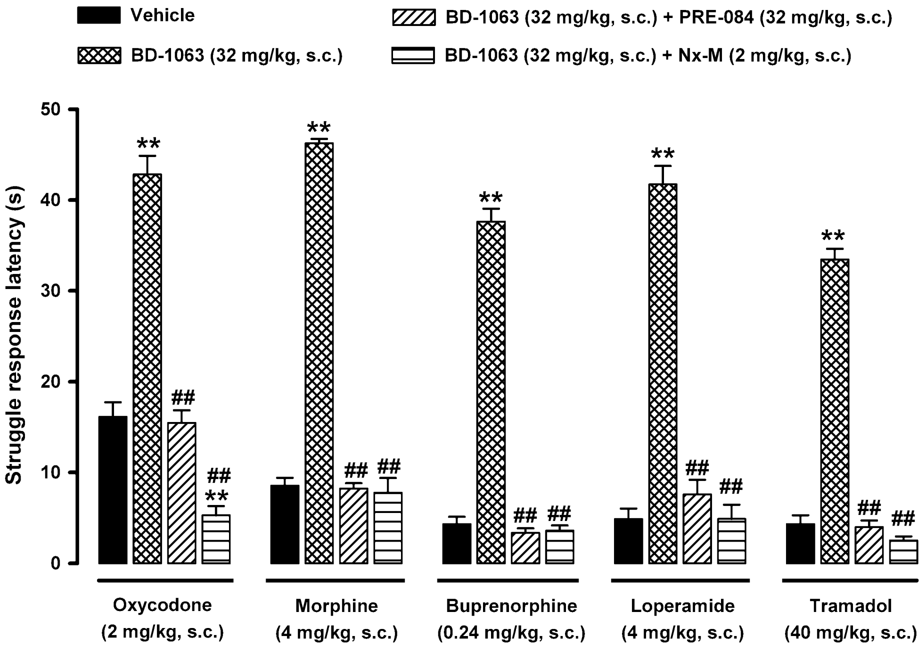

Fig. 5. Contribution of σ_1 and peripheral opioid receptors to the antinociception induced by the association of several μ-agonists and BD-1063, both administered systemically to wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of mice treated subcutaneously (s.c.) with BD-1063 (32 mg/kg) or its solvent in combination with oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg), and its association with the s.c. administration of PRE-084 (32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with different μ-opioid agonists associated with BD-1063 or its solvent: $^{**}P < 0.01$; and between the values obtained in mice treated with the combination of a given μ-agonist with BD-1063, and its association with PRE-084 or Nx-M: $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

Therefore, systemic treatment with the σ_1 antagonist BD-1063 synergistically enhanced the antinociception induced by the systemic administration of all μ-agonists tested. This effect was sensitive to both the σ_1 receptor agonist PRE-084 and the peripheral opioid antagonist naloxone methiodide.

2.4.5. Effects of local (intraplantar) administration of the selective σ_1 antagonist BD-1063 on mechanical antinociception induced by µ-agonists in wild-type mice: involvement of σ_1 and peripheral opioid receptors

We also tested whether the local administration of BD-1063 into the hindpaw was sufficient to enhance µ-opioid-induced mechanical antinociception. As in the experiments described in the preceding section, low doses of systemic µ-agonists were injected s.c. in WT mice, but BD-1063 was injected i.pl. to test for locally-induced effects.

In WT mice, fentanyl injected s.c. (0.08 mg/kg) and BD-1063 solvent (saline) injected i.pl. led to similarly short struggle response latencies in the treated paw and the contralateral (untreated) paw (Fig. 6A). However, when this dose of fentanyl was associated to BD-1063 (50-200 µg, i.pl.), a dose-dependent increase in latency was seen only in the treated hindpaw (black bars in Fig. 6A).

To test the σ_1 specificity of the effects induced by local treatment with BD-1063, we used the σ_1 agonist PRE-084, and also evaluated the effects of naloxone methiodide to determine whether peripheral opioid receptors were involved in the enhanced antinociception induced by the combination of fentanyl and local σ_1 pharmacological blockade. PRE-084 (8-32 mg, s.c.) completely reversed, and in a dose-dependent manner, the mechanical antinociceptive effect induced by the combination of fentanyl (0.08 mg/kg, s.c.) and BD-1063 (200 µg, i.pl.) (Fig. 6B). Treatment with naloxone methiodide (2 mg/kg, s.c.) was also able to completely abolish the local potentiation of fentanyl-induced antinociception by BD-1063 (200 µg, i.pl.) (Fig. 6B). Neither PRE-084 nor naloxone methiodide induced any change in latencies of the noninjected paw in animals treated with systemic fentanyl (white bars in Fig. 6B).

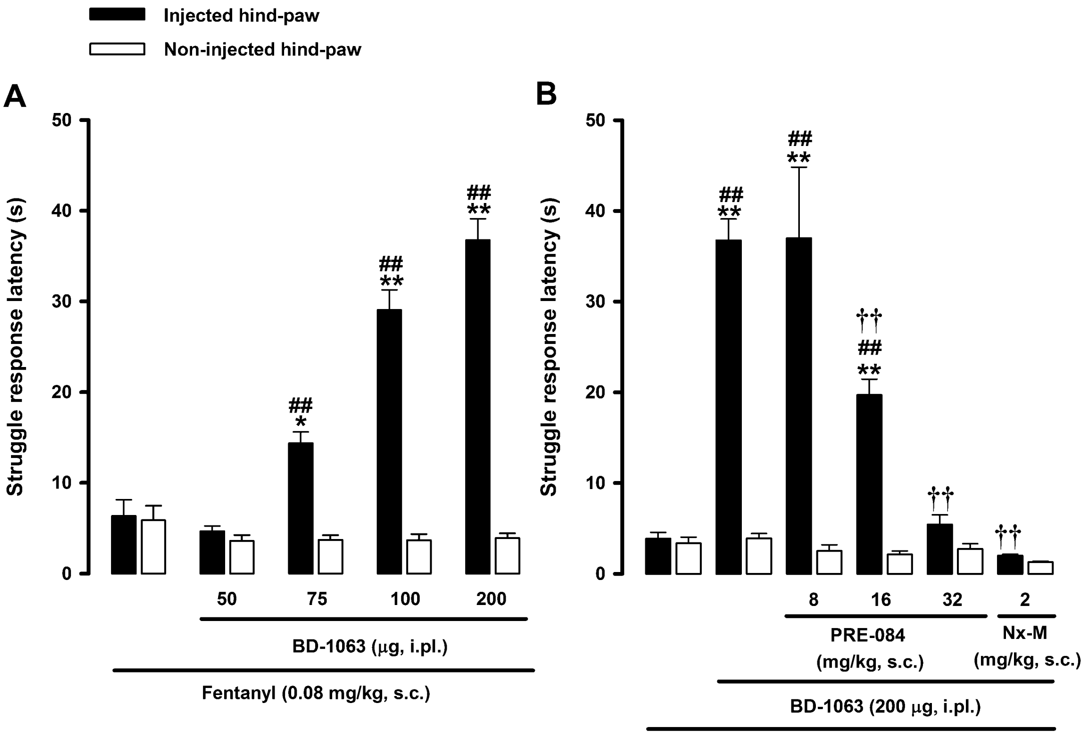

Fig. 6. Contribution of σ_1 and peripheral opioid receptors to the effects of the local administration of BD-1063 on the antinociception induced by systemically administered fentanyl in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of mice treated subcutaneously (s.c.) with: (A) fentanyl (0.08 mg/kg) + intraplantar (i.pl.) BD-1063 (50-200 µg) or its solvent, and (B) fentanyl (0.08 mg/kg, s.c.) + BD-1063 (200 µg, i.pl.) in animals pretreated s.c. with PRE-084 (8-32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animal. (A and B) Statistically significant differences between the values obtained upon stimulation after the injection of BD-1063 or its solvent in the hindpaw: $*P < 0.05$, $**P < 0.01$; and between the values obtained from the injected and noninjected hindpaws: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test). (B) Statistically significant differences between the values obtained in mice treated with the combination of fentanyl with BD-1063, and its association with PRE-084 or Nx-M: †† $P < 0.01$ (two-way ANOVA followed by Bonferroni test).

We also tested the association of local treatment of BD-1063 (200 µg, i.pl.) with low doses of the other µ-agonists. Local treatment with BD-1063 was also able to increase struggle response latencies in animals that were given (s.c.) oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg) (Fig. 7). This effect was only seen in the treated hindpaw, whereas latencies in the untreated hindpaw remained unaltered (data not shown). This increased latency in the hindpaw treated with BD-1063 in mice that were given µ-agonists was abolished by treatment with either PRE-084 (32 mg/kg, s.c.) or naloxone methiodide (2 mg/kg, s.c.) (Fig. 7). As in the experiments described in the preceding section, PRE-084 reversed the effects of the local administration of BD-1063, but not beyond the level of the effects of the µ-agonists alone (Fig. 7). Naloxone methiodide decreased latencies in animals treated with BD-1063 together with µ-agonists to the same extent as the effect of the opioids administered alone, with the exception of the combination of BD-1063 and oxycodone: the effect of this combination was decreased more markedly than the effect of oxycodone alone (Fig. 7).

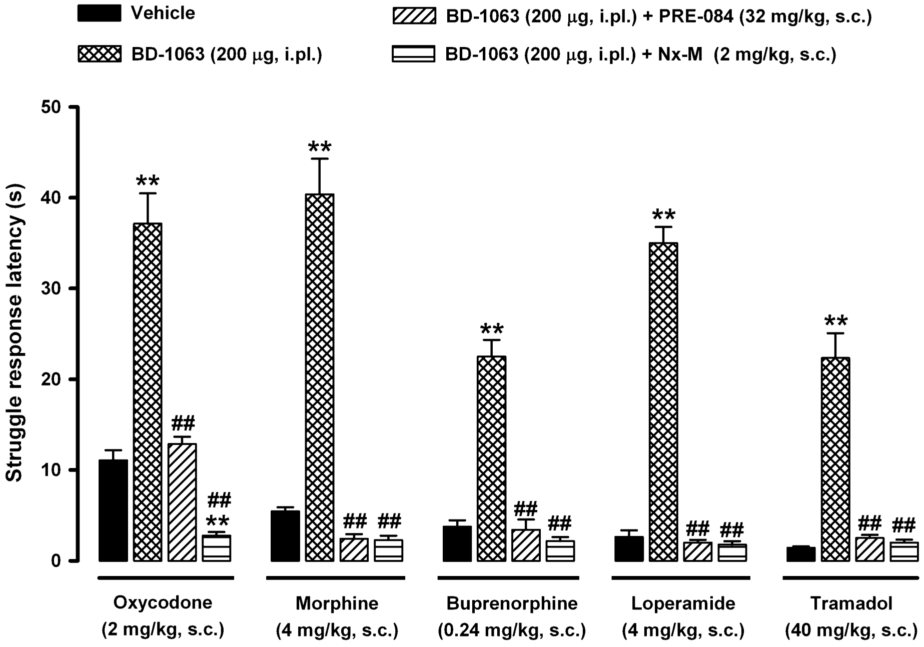

Fig. 7. Contribution of σ_1 and peripheral opioid receptors to the effects of the local administration of BD-1063 on the antinociception induced by several µ-agonists administered systemically to wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the injected hindpaw in mice treated intraplantarly with BD-1063 (200 µg) or its solvent in combination with the subcutaneous (s.c.) administration of oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg), and its association with the s.c. administration of PRE-084 (32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with the different µ-opioid agonists alone and associated with BD-1063: $^{**}P < 0.01$; and between the values obtained in mice treated with the combination of a given µ-agonist with BD-1063, and its association with PRE-084 or Nx-M: $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

Therefore, local treatment with the σ_1 antagonist BD-1063 synergistically enhanced the antinociception induced by all μ-agonists tested when they were given systemically. This effect was observed only in the treated paw and was sensitive to both σ_1 agonism and peripheral opioid antagonism.

2.4.6. Effects of systemic (subcutaneous) and local (intraplantar) administration of the selective σ_1 antagonist S1RA on mechanical antinociception induced by fentanyl and loperamide in wild-type mice: involvement of σ_1- and peripheral opioid receptors

To test whether the effects induced by BD-1063 were replicable by a different σ_1 antagonist, we evaluated the effects of S1RA on the antinociceptive effects induced by two different μ-opioids: the centrally active fentanyl and the peripherally restricted loperamide.

The systemic administration of S1RA (64 mg/kg, s.c.) increased the struggle response latencies in animals treated s.c. with either fentanyl (0.08 mg/kg) or loperamide (4 mg/kg) (Fig. 8A). The i.pl. administration of S1RA (200 μg) was also able to increase the response latency in mice treated with these μ-opioid agonists (Fig. 8B), and this enhanced antinociception was detectable in the injected but not in the contralateral paw (data not shown). As described in the preceding sections regarding the effects of BD-1063, treatment with either the σ_1 agonist PRE-084 (32 mg/kg, s.c.) or the peripheral opioid antagonist naloxone methiodide (2 mg/kg, s.c.) abolished the effects on opioid antinociception of S1RA administered either systemically or locally (Fig. 8A and B, respectively).

Therefore, systemic or local treatment with the σ_1 antagonist S1RA synergistically enhanced the antinociception induced by the systemic administration of fentanyl or loperamide, and these effects were fully reversed by either σ_1 agonism or peripheral opioid antagonism.

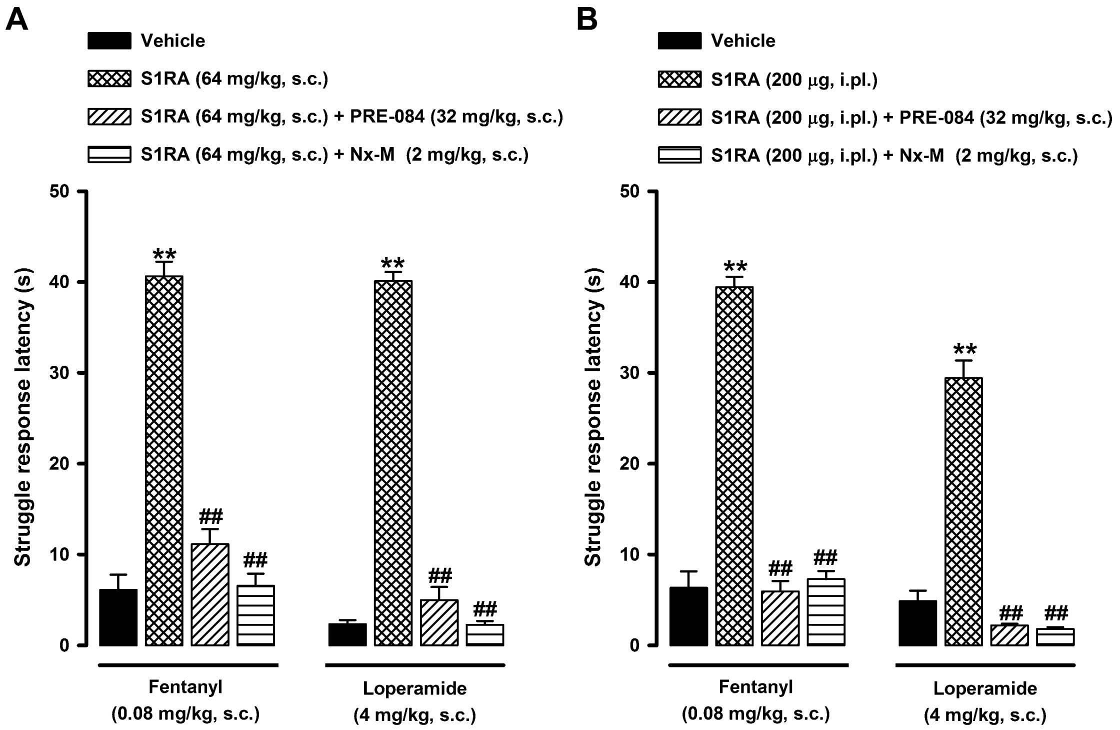

Fig. 8. Contribution of σ_1 and peripheral opioid receptors to the effects of the systemic and local administration of S1RA on the antinociception induced by systemically administered fentanyl and loperamide in wild-type mice. Animals were treated (A) subcutaneously (s.c.) or (B) intraplantarly (i.pl.) with S1RA (64 mg/kg and 200 µg, respectively) in combination with fentanyl (0.08 mg/kg, s.c.) or loperamide (4 mg/kg, s.c.), and its association with the s.c. administration of PRE-084 (32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. The results represent the struggle response latency in mice during stimulation with 450 g pressure on the hindpaws (A) or on the injected hindpaw (B). Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with the µ-opioid agonists associated with S1RA or its solvent: $^{**}P < 0.01$; and between the values obtained in mice treated with the combination of a given µ-agonist with S1RA, and its association with PRE-084 or Nx-M: $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

2.4.7. Sigma-1 receptor expression in the central and peripheral nervous system

To obtain anatomical support for the marked behavioral effects of local σ_1 receptor blockade on opioid antinociception, we compared the expression of σ_1 receptors in different areas of the nervous system involved in opioid analgesia, including supraspinal (BLA, RVM and PAG), spinal (dSC) and peripheral nervous locations (DRG). All samples from WT mice yielded immunoreactive bands at a molecular weight slightly higher than 25 kDa (Fig. 9A), which is consistent with the molecular weight of the cloned σ_1 receptor from the mouse (~28 kDa) (Pan et al., 1998).

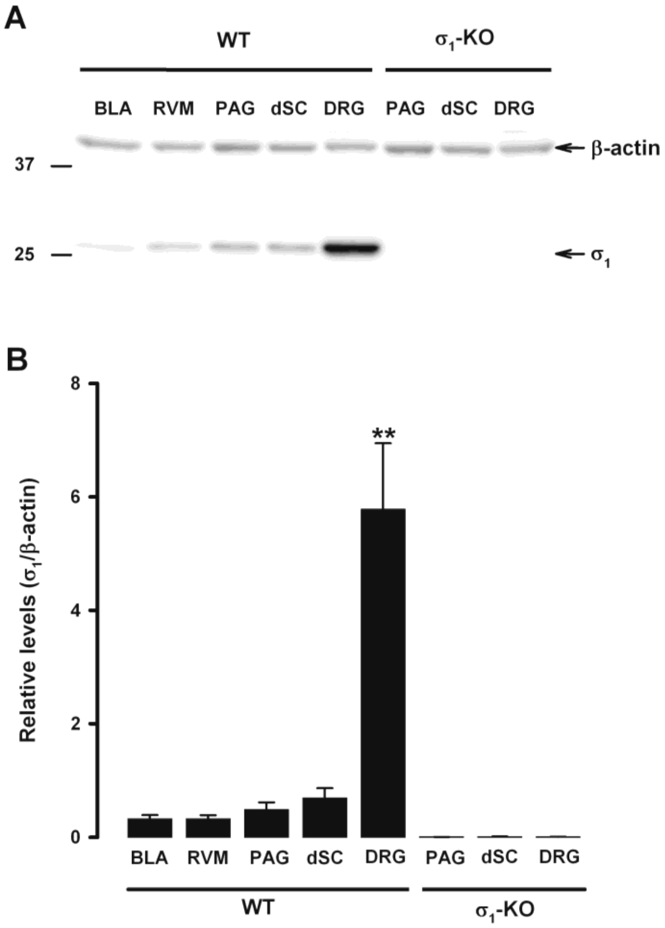

Fig. 9. Expression of sigma-1 (σ_1) receptors in the basolateral amygdala (BLA), rostroventral medulla (RVM), periaqueductal grey matter (PAG), dorsal spinal cord from the lumbar enlargement (dSC), and lumbar dorsal root ganglia (DRGs) in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. (A) Representative immunoblots for σ_1 receptors in WT and σ_1-KO mice. β-actin was used as the loading control. The migration positions of molecular weight standards (in kDa) are shown to the left of the gel. (B) Quantification of immunoblotting for the σ_1 receptor in WT and σ_1-KO mice. Each bar and vertical line represents the mean ± SEM of the densitometric values obtained in 8 animals. The σ_1 receptor band intensities were relativized to those of their corresponding β-actin loading control bands. Statistically significant differences between the values obtained in samples of central nervous system regions (BLA, RVM, PAG, dSC) and the lumbar dorsal root ganglia (DRGs) from WT mice $^{**}P < 0.01$. No σ_1 receptor expression was found in samples from σ_1-KO mice.

We found no significant differences in σ_1 receptor band intensities among all central areas in WT mice (Fig. 9B); however, σ_1 receptor density was much higher in DRG samples than in any of the central areas examined (Fig. 9A and B). To verify the specificity of the anti-σ_1 receptor antibody, we tested its immunoreactivity in samples from σ_1-KO mice. We found no immunoreactive σ_1 receptor bands in PAG, dSC or DRG samples from σ_1-KO animals (Fig. 9A and B), or in samples of BLA or RVM from these mice (data not shown). The absence of these bands in σ_1-KO samples argues for the specificity of the σ_1 antibody used. In addition, we were unable to detect immunoreactive bands in WT samples when tested in the absence of anti-σ_1 antibody (data not shown), which further confirms the specificity of the procedure.

The high level of σ_1 receptor expression in peripheral nervous tissue argues for a possible major role of peripheral σ_1 receptors in nociception.

2.4.8. Effects of fentanyl and loperamide on gastrointestinal transit in wild-type mice, wild-type mice treated with BD-1063, and σ_1 knockout mice

Gastrointestinal transit distances did not differ significantly between WT mice, WT mice treated with BD-1063 (32 mg/kg, s.c.) and σ_1-KO mice that had been given the fentanyl (Fig. 10A) or loperamide solvents (Fig. 10B). In all cases the charcoal meal traversed about 30 cm of the small intestine. Both fentanyl (0.04 – 0.16 mg/kg, s.c.) and loperamide (0.125 – 1 mg/kg, s.c.) induced a dose-dependent decrease in gastrointestinal transit in WT mice (white bars in Fig. 10A and 10B). For each dose of fentanyl (Fig. 10A) or loperamide (Fig. 10B), this decrease was similar in WT mice treated with BD-1063 and in σ_1-KO mice. Therefore, σ_1 inhibition alone did not alter gastrointestinal transit and did not modify the effects of fentanyl or loperamide on this outcome.

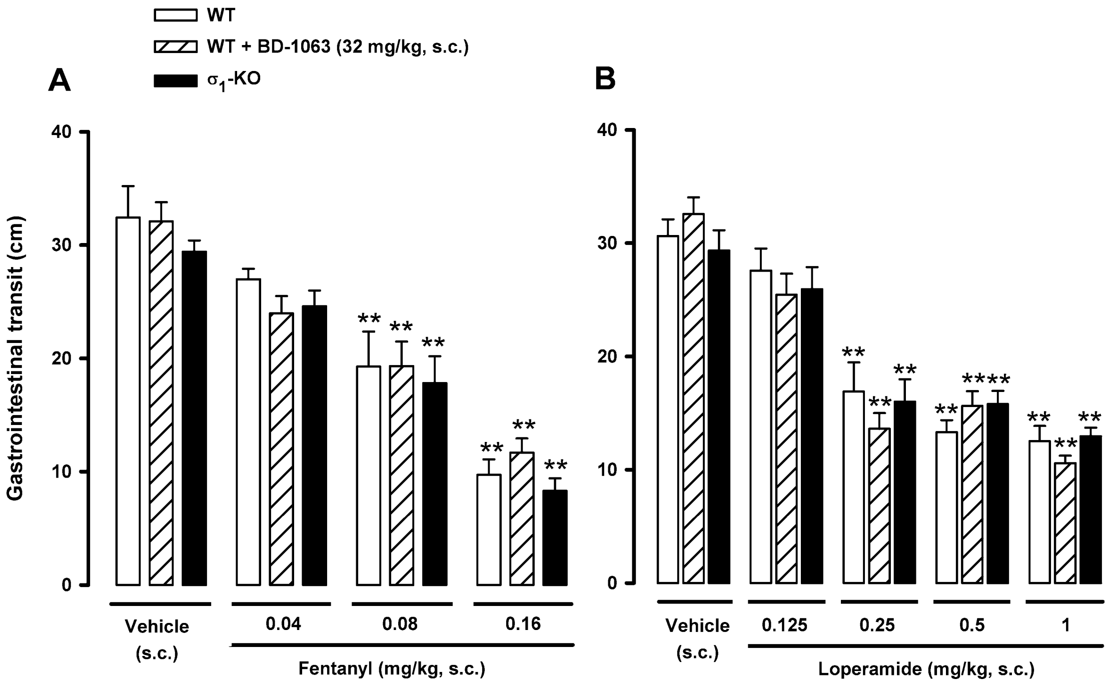

Fig. 10. Effects of the systemic (subcutaneous, s.c.) administration of several doses of fentanyl (A) and loperamide (B) on gastrointestinal transit of wild-type mice (WT), WT mice treated with BD-1063 (32 mg/kg, s.c.), and σ_1 knockout mice (σ_1-KO). BD-1063 or its solvent was injected, and 5 min later the animals were treated with fentanyl, loperamide or their solvents. 30 minutes after the administration of the opioid drug, the mice were given a 0.5% charcoal suspension intragastrically. Transit of the charcoal was measured 30 min after administration. Each bar and vertical line represents the mean ± SEM of values obtained in 8-12 mice. Statistically significant differences between the values obtained in saline- and opioid-treated groups: $^{**}P < 0.01$. No statistically significant differences were found between genotypes with the same treatment, or between animals treated and not treated with BD-1063 (two-way ANOVA followed by Bonferroni test).

2.4.9. Affinity of μ-opioid drugs for [^{3}H](+)-pentazocine binding sites

We used competition binding assays to test the affinity of the opioid drugs assayed in vivo for [^{3}H](+)-pentazocine-labeled σ_1 receptors in brain membranes from WT mice. As expected, the known σ_1 antagonist BD-1063 inhibited [^{3}H](+)-pentazocine specific binding in a concentration-dependent manner, with an IC_{50} value of 40.21 ± 3.24 nM. This value was similar to that found in previous studies (Entrena 2009a; Cobos et al., 2005, 2006 and 2007). However, none of the opioid drugs (fentanyl, oxycodone, morphine, buprenorphine, tramadol, loperamide, naloxone or naloxone methiodide) significantly inhibited [^{3}H](+)-pentazocine binding at any concentration tested (ranging from 10-10 to 10-6 M), and therefore their affinity for $[^3H]$(+)-pentazocine binding sites was considered negligible (Fig. 11).

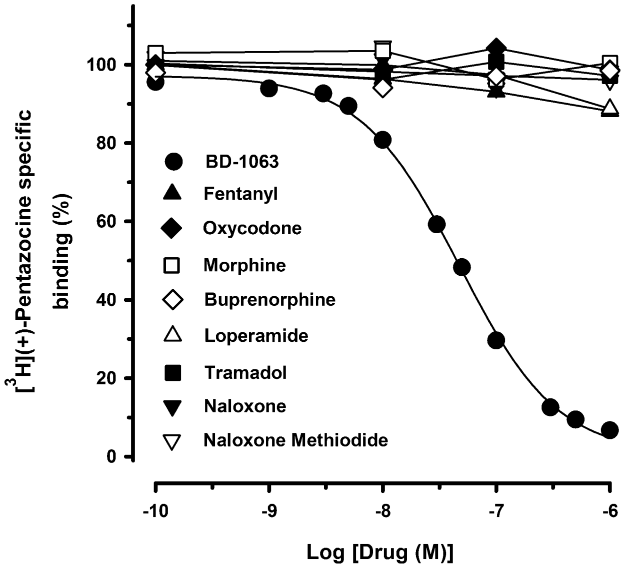

Fig. 11. Inhibition by unlabeled drugs of $[^3H]$(+)-pentazocine binding to brain membranes (P_2 fraction) in wild-type mice. $[^3H]$(+)-pentazocine (5 nM) was incubated with 0.8–1 mg/mL brain membrane protein at 30 °C, pH 8, for 240 min and increasing concentrations of BD-1063, fentanyl, oxycodone, morphine, buprenorphine, loperamide, tramadol, naloxone or naloxone methiodide. The data shown are the averages of two experiments carried out in triplicate.

2.5. DISCUSSION

We found that several centrally penetrant µ-opioid analgesics (fentanyl, oxycodone, morphine, buprenorphine and tramadol) and the peripheral µ-agonist loperamide, at systemic doses which induce little or no antinociception in control animals, have a marked antinociceptive effect when boosted by σ_1 receptor inhibition (σ_1–KO mice or σ_1 pharmacological blockade). We show that this enhanced opioid antinociception is mediated peripherally, since it can be achieved by local σ_1 pharmacological blockade, and is sensitive to peripheral opioid antagonism. However, the increase in peripheral opioid antinociception by σ_1 receptor inhibition is not accompanied by increased inhibition of opioid-induced gastrointestinal transit, a known peripherally–mediated side effect of opioids.

It has been proposed that the analgesic mechanisms of different µ-opioids used in clinical settings overlap only partially (Ocaña et al., 1995; Pasternak, 2004; Pasternak and Pan 2011; Smith, 2008; Raehal et al., 2011). Hence the importance of testing whether the widely reported enhancement of morphine antinociception by σ_1 inhibition (e.g. Chien and Pasternak 1993; Mei and Pasternak 2007; Díaz et al., 2009; Sánchez-

Fernández et al., 2013) also occurs with other clinically relevant µ-opioids. It was recently reported that the systemic (subcutaneous) administration of S1RA enhanced antinociception against a thermal stimulus induced by morphine and other systemically administered centrally-penetrant µ-opioid analgesics (fentanyl, oxycodone, buprenorphine and tramadol) (Vidal-Torres et al., 2013). We now extend those results by showing that this σ_1 antagonist and also BD-1063 enhance opioid antinociception against a different type of nociceptive (mechanical) stimulus, and more importantly, that the enhanced antinociception is mediated peripherally (see below). Moreover, we show that opioid-induced mechanical antinociception is clearly potentiated in σ_1-KO mice, which contrasts with the previously reported absence of modulation of opioid thermal antinociception in σ_1-KO mice (Díaz et al., 2009; Vidal-Torres et al., 2013). These apparently contradictory results seem to be related to the type of sensory stimulation used, and may be attributable to the known differences in the neurochemical mechanisms of thermal and mechanical opioid antinociception (Kuraishi et al., 1995; Wegert et al., 1997). These mechanisms may be affected differentially by possible compensatory mechanisms in σ_1-KO mice.

In addition to centrally-acting µ-opioid analgesics, we also tested the effects of the peripheral µ-opioid agonist loperamide under normal conditions and during σ_1 inhibition. In agreement with previous reports, loperamide had no antinociceptive effects in WT mice (Menendez et al., 2005; Sevostianova et al., 2005). However, in σ_1–KO mice or WT mice treated systemically with BD-1063 or S1RA, we observed a profound antinociceptive effect in response to loperamide. Therefore, σ_1 inhibition is sufficient to unmask the strong antinociceptive effects of this peripherally acting µ-opioid. This finding evidences that peripheral µ-opioid analgesia is enhanced by σ_1 inhibition. In fact, the local (intraplantar) administration of σ_1 antagonists was able to enhance µ-antinociception in response to all µ-agonists tested. This enhanced antinociception occurred only in the paw injected with the σ_1 antagonist, but not in the contralateral paw, clearly indicating that the effects of these σ_1 antagonists occurred locally. The selective σ_1 agonist PRE-084 reversed the effects of BD-1063 or S1RA (administered either systemically or locally), arguing for a σ_1-mediated action.

Interestingly, PRE-084 was unable to reverse the enhanced antinociception seen in σ_1-KO mice treated with fentanyl, supporting on-target mechanisms in the effects induced by this σ_1 agonist (data not shown).

To investigate the role of peripheral opioid receptors in the antinociceptive effect of systemically administered μ-agonists under normal conditions and during σ_1 inhibition, we tested the sensitivity of antinociception to peripheral opioid antagonism in both situations. The antinociceptive effects of the opioids fentanyl, morphine and buprenorphine in WT mice (in the absence of σ_1 blockade) were sensitive to the centrally penetrant opioid antagonist naloxone. However, they were resistant to the peripherally restricted antagonist naloxone methiodide. These findings support a preferential location of the antinociceptive effects of μ-opioids at central levels under our experimental conditions, and are consistent with previous studies (Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008; Joshi et al., 2008; Khalefa et al., 2012; Ringkamp and Raja, 2012). Among the opioids tested in WT mice (in the absence of σ_1 blockade), only oxycodone had an antinociceptive effect that was partially reversible by naloxone methiodide, and this only occurs at the highest dose (8 mg/kg) of the antagonist. Our findings are consistent with clinical data suggesting that part of the analgesic effects of oxycodone may be mediated peripherally (Olesen et al., 2010). The sensitivity to peripheral opioid antagonism is diametrically different when opioid antinociception is enhanced by σ_1 inhibition. A dose as low as 2 mg/kg of naloxone methiodide was enough to completely abolish the opioid antinociception induced by all μ-agonists tested, not only in WT mice treated locally with a σ_1 antagonist (in which its effects are clearly peripherally mediated), but also in σ_1–KO mice or WT mice treated systemically with the σ_1 antagonists. This does not argue against the widely reported potentiation of opioid antinociception by central σ_1 receptor inhibition (King et al., 1997; Pan et al., 1998; Mei and Pasternak 2002 and 2007; Marrazzo et al., 2006), but indicates that when both opioid agonism and σ_1 inhibition are induced systemically, the increase in antinociception occurs mainly at the peripheral level. In support of the peripheral location of these modulatory effects of σ_1 receptors, we found much higher levels of these receptors in the DRG than in several areas of the central nervous system that play a key role in opioid antinociception.

Among the opioid drugs tested in the present study, only morphine and naloxone had previously been shown to lack affinity for σ_1 receptors (Walker et al., 1990). Here we show that this lack of σ_1 affinity is shared by other opioid drugs (fentanyl, oxycodone, buprenorphine, tramadol, loperamide and naloxone methiodide). In addition, it is known that the σ_1 drugs tested here (BD-1063, S1RA and PRE-084) do not bind to μ-opioid receptors (Matsumoto et al., 1995; Sánchez-Fernández et al., 2013). Therefore, our results support a functional link between peripheral σ_1 receptors and the μ-opioid system rather than interactions of σ_1 ligands with μ opioid receptors or opioid drugs with σ_1 receptors.

It was recently reported that σ_1 antagonism potentiates μ-opioid signaling (measured as the increase in DAMGO-induced [^{35}S]GTPγS binding), providing a mechanistic explanation for the enhanced opioid antinociception by σ_1 inhibition (Kim et al. 2010). Previous studies of thermal nociception show that σ_1 inhibition potentiates μ, κ and δ opioid antinociception at central levels (King et al., 1997; Mei and Pasternak, 2002). Therefore, the modulation of peripheral μ opioid antinociception by σ_1 inhibition that we report here might occur with other subtypes of opioid receptors, although this hypothesis remains to be tested.

Clinically, a dose-limiting factor in obtaining maximal analgesia with systemic opioids is the risk of adverse side effects. Some of these effects are mediated through peripheral opioid receptors (constipation), whereas others are mediated at supraspinal sites (addiction, dependency, nausea, somnolence, respiratory depression) (Benyamin et al 2008; Greenwood-Van Meerveld and Standifer, 2008; Al-Hasani and Bruchas, 2011; Ringkamp and Raja, 2012). Targeting peripheral opioid receptors has been proposed as a strategy to minimize opioid-induced side effects that are produced centrally (Sehgal et al., 2011; Ringkamp and Raja, 2012). However, since most of the analgesia from systemic opioids is produced normally at central sites, it seems difficult to dissociate antinociceptive effects from centrally-induced side effects. In this regard, we previously reported that despite the potentiation of antinociception by σ_1 inhibition, the central side effects of morphine (such as hyperlocomotion, physical dependence or mydriasis) were not altered (Sánchez-Fernández et al., 2013; Vidal-Torres et al, 2013). Here we show that the enhancement of opioid antinociception by systemic σ_1 inhibition occurs primarily at peripheral levels, which might explain the lack of potentiation of morphine-induced central side effects. Interestingly, morphine-induced constipation, which is the most clinically relevant peripheral side effect of μ-opioids, was also not modulated by σ_1 receptors (Chien and Pasternak, 1994; Sánchez-Fernández et al., 2013; Vidal-Torres et al, 2013); however, it is unknown whether this characteristic is shared by other opioids. To find out, we tested two additional μ-opioids of very different characteristics: the central analgesic fentanyl and the peripherally-acting loperamide. Importantly, the gastrointestinal transit inhibition induced by these opioids was unaffected in either σ_1–KO mice or WT mice treated with BD-1063. Regardless of the exact mechanistic nature of the differential modulation of opioid antinociception and adverse events by σ_1 inhibition, our findings point to a potentially beneficial avenue of research aimed at improving the safety profile of opioid drugs.

In summary, we found that σ_1 receptor inhibition enhanced the peripheral opioid antinociception induced by clinically relevant μ-agonists, but did not increase opioid-induced constipation. These data support the conclusion that peripheral σ_1 receptors are a biological brake to μ-opioid antinociception, and that either systemic or local σ_1 receptor inhibition is potentially useful as an adjuvant to enhance peripheral μ-opioid analgesia. Combinations of σ_1 antagonists and μ-opioid agonists may be of therapeutic interest in terms of both efficacy and safety, and merit clinical studies.

2.6. ACKNOWLEDGEMENTS

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CONCLUSIONS

SPECIFIC CONCLUSIONS

1) Inhibition of σ_1 receptor function (in σ_1-KO mice or by the systemic or local σ_1 pharmacological antagonism in wild-type mice) does not alter the nociceptive response to a blunt mechanical stimulation.
2) The mechanical antinociceptive effect induced by the systemic administration of µ-opioid agonists clinically used as analgesics (fentanyl, morphine, oxycodone, buprenorphine and tramadol) is increased in σ_1-KO mice and in wild-type mice treated systemically with σ_1 antagonists.
3) Inhibition of σ_1 receptor function, in σ_1-KO mice or by the systemic σ_1 pharmacological antagonism in wild-type mice, is able to unmask antinociceptive effects induced by the peripherally restricted opioid agonist loperamide.
4) Mechanical antinociception induced by the systemic administration of opioid analgesics to wild-type mice under normal conditions (in the absence of σ_1 receptor inhibition) is mainly produced at central levels. However, the potentiation of opioid antinociception in σ_1-KO mice and in wild-type mice treated systemically with σ_1 antagonists completely depends on the activation of peripheral opioid receptors, because it is abolished by naloxone methiodide.
5) Local σ_1-pharmacological blockade is enough to enhance the peripheral antinociceptive effects induced by the systemic administration of opioid drugs clinically used as analgesics as well as to unmask an antinociceptive effect of loperamide.
6) Although the local administration of morphine does not produce any antinociceptive effect against a mechanical stimulus, it induces a marked effect in σ_1-KO or in wild-type mice locally treated with σ_1 antagonists.
7) The expression of σ_1 receptors is higher in the peripheral nervous system (DRG) than in several central areas involved in opioid analgesia (at either supraspinal or spinal levels), which supports the role of σ_1 receptors in nociception at the peripheral level.

8) Inhibition of σ_1 receptor function does not interfere with other non-analgesic opioid effects, including morphine-induced hyperlocomotion (a centrally mediated effect) and the decrease of gastrointestinal transit (a peripherally mediated effect) induced by morphine, fentanyl and loperamide.

9) σ_1-KO mice do not present adaptive changes in density or affinity of μ-opioid receptors in the brain, spinal cord or hind-paw plantar skin which could account for their enhanced opioid antinociception.

10) The μ-opioid ligands used (fentanyl, morphine, oxycodone, buprenorphine, tramadol, loperamide, naloxone and naloxone methiodide) lack affinity for σ_1 receptors. Also, the σ_1 ligands used (BD-1063, BD 1047, NE-100, S1RA and PRE-084) are devoid of affinity for μ opioid receptors. Therefore, our behavioral results cannot be explained by direct interaction of opioid drugs with σ_1 receptors, or σ_1 ligands with μ-opioid receptors.

GENERAL CONCLUSION

Systemic or local inhibition of σ_1 receptor function increases peripheral opioid antinociception to mechanical stimuli, suggesting that the σ_1 receptor is a biological brake to peripheral opioid analgesia. This potentiation of opioid analgesia is not accompanied by an increase of non-analgesic central or peripheral (hyperlocomotion and inhibition of gastrointestinal transit, respectively) opioid effects. Therefore, σ_1 antagonism might be clinically useful as a systemic or local adjuvant to increase opioid analgesia without increasing opioid side effects.

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LIST OF ABBREVIATIONS

AC: adenylate cyclase

AH: heat-sensitive A-fibers

ALS: anterolateral system

AM: mechanosensitive A-fibers

AMH: mechano-heat sensitive A-fibers

AMHI: heat-mechano-sensitive A-fibers type I

AMHII: heat-mechano-sensitive A-fibers type II

ANOVA: analysis of variance

ASIC1a: acid-sensing ion channels of the subtype 1a

ASOs: antisense oligodeoxynucleotides

ATP: adenosine triphosphate

BD-1063: 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride

BD-1047: *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide

BiP: immunoglobulin heavy chain-binding protein

BLA: basolateral amygdala

B_{max}: maximum number of binding sites

ºC: celsius degrees

cAMP: 3´-5´cyclic adenosine monophosphate

CGRP: calcitonin-gene related peptide

Chap: chaperone domain

CM: mechanosensitive C-fibers

CMHs: mechano-heat sensitive C-fibers

CNS: central nervous system

CT: C-tactile afferents

Cyt: cytoplasm

DAMGO: $[^3H]$[D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin

DCML: dorsal column-medial lemniscal

DLF: dorsolateral funiculi.

DMT: *N*,*N*-dimethyltryptamine

DMSO: dimethylsulfoxide

DPDPE: [D-Pen^2,DPen^5] enkephalin

DRG: dorsal root ganglia

dSC: dorsal spinal cord;

DTG: 1,3-di(2-tolyl)guanidine

D_1R: dopamine 1 receptor

D_2R: dopamine 2 receptor

GABA: gamma aminobutyric acid

$GABA_AR$: gamma aminobutyric acid receptor type A

GTPγS: guanosine 5'-O-(γ-thio)triphosphate

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

Fig.: Figure

GDP: guanosine diphosphate

GIRKs: G-protein-regulated inwardly rectifying potassium channels

H: heat sensitive nociceptors

IB4: isolectin B4

IC_{50}: concentration of unlabeled drug that inhibited 50% of radioligand-specific binding

i.c.v.: intracerebroventricular

IN: interneuron

i.pl.: intraplantar

IP_3: inositol 1,4,5-trisphosphate

i.t.: intrathecal

K_D: equilibrium dissociation constant

K_i: inhibition constant

KO: knockout

Kv: voltage-gated K^+ channels

kDa: kilodalton

LC: locus coeruleus

LTM: low-threshold mechanoreceptors

L4-L5: lumbar vertebrae 4 and 5

M: mechanosensitive nociceptors

MAM: mitochondrion-associated endoplasmic reticulum membrane

mg: milligrams

MH: mechano-heat sensitive nociceptors

MIA: mechanically insensitive afferent

MiHi: mechanically insensitive and heat insensitive afferents

mM: millimolar

µM: micromolar

µm: micrometer

m/s: meters per second

Mor: morphine

MOR-1: gene encoding for the µ-opioid receptor

µOR: µ-opioid receptor

Mrgprd: mas-related G-protein-coupled receptor subtype d

MSA: mechanical sensitive afferent

NADPH: nicotinamide adenine dinucleotide phosphate

NalBzoH: naloxone benzoylhydrazone

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

NF-200: neurofilament 200

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

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

Nox2: NADPH oxidase 2

NRM: nucleus raphe magnus

NSAID: nonsteroidal anti-inflammatory drug

nSTT: neospinothalamic tract

n.t.: not tested

Nx: naloxone

Nx-M: naloxone methiodide

PAG: periaqueductal gray

PCP: phencyclidine

PM: plasma membrane

PN: projection neuron

pNR1: phosphorylated NMDA receptor NR1 subunit

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

PSDC: postsynaptic dorsal column

pSTT: paleospinothalamic tract

P_1: crude nuclear fraction

P_2: crude synaptosomal fraction

RVM: rostroventral medulla

s.c.: subcutaneous

STT: neospinothalamic tract

SSC: somatosensory cortex

SK: small conductance calcium-activated K^+ channels,

SP: substance P

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

S-I: primary somatic sensory cortex

S-II: secondary somatic sensory cortex

σ receptor: sigma receptor

σ_1 receptor: sigma-1 receptor

σ_2 receptor: sigma-2 receptor

TM1: transmembrane domain 1

TM2: transmembrane domain 2

TRP: transient receptor potential

TRPA1: transient receptor potential type A1

TRPC1: transient receptor potential canonical 1

TRPC3: transient receptor potential canonical 3

TRPC6: transient receptor potential canonical 6

TRPM8: transient receptor potential type M8

TRPV1: transient receptor potential vanilloid type 1

TRPV2: transientreceptor potential vanilloid type 2

T-TBS: tween 20 in Tris-*buffered* saline

U50,488H: trans-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide

VDCC: L-type voltage dependent calcium channels

VLF: ventrolateral funiculi

WDR: wide-dynamic range

WHO: World Health Organization

WT: wild-type

IIi: inner part of lamina II

IIo: outer of lamina II

(+)-MR200: (+)-methyl (1*R*,2*S*)-2-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate

(±)-PPCC: 1R,2*S*/1*S*,2R)-2-[4-hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl

REFERENCES

Abadias M, Escriche M, Vaqué A, Sust M, Encina G (2013) Safety, tolerability and pharmacokinetics of single and multiple doses of a novel sigma-1 receptor antagonist in three randomized phase I studies. *Br J Clin Pharmacol*. 75:103-17.

Abraham TS, Chen ML, Ma SX (2011) TRPV1 expression in acupuncture points: response to electroacupuncture stimulation. *J Chem Neuroanat.* 41:129-36.

Adeniji AO and Atanda OO (2013) Randomized comparison of effectiveness of unimodal opioid analgesia with multimodal analgesia in post-cesarean section pain management. *J Pain Res*. 6:419-24.

Argyriou AA, Koltzenburg M, Polychronopoulos P, Papapetropoulos S, Kalofonos HP (2008) Peripheral nerve damage associated with administration of taxanes in patients with cancer. *Crit Rev Oncol Hematol.* 66:218-28.

Akil H, Mayer DJ, Liebeskind JC (1972) Monoaminergic mechanisms involved in analgesia caused by stimulation of the brain stem in rats *J Physiol (Paris)*. 65:185A.

Akil H, Mayer DJ, Liebeskind JC (1976) Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science.* 191:961-2.

Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, Walker JM (1984) Endogenous opioids: biology and function. *Annu Rev Neurosci*. 7:223-55.

Al-Chaer ED, Lawand NB, Westlund KN, Willis WD (1996) Pelvic visceral input into the nucleus gracilis is largely mediated by the postsynaptic dorsal column pathway. *J Neurophysiol.* 76:2675-90.

Al-Hasani R and Bruchas MR (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology.* 115:1363-1381.

Alonso G, Phan V, Guillemain I, Saunier M, Legrand A, Anoal M, Maurice T (2000) Immunocytochemical localization of the sigma$_1$ receptor in the adult rat central nervous system. *Neuroscience.* 97: 155-170.

Annemans L (2011) Pharmacoeconomic impact of adverse events of long-term opioid treatment for the management of persistent pain. *Clin Drug Investig*. 31:73-86.

Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R (1995) Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. *J Neurosci*. 15:3328-3341.

Attal N (2012) Neuropathic pain: mechanisms, therapeutic approach, and interpretation of clinical trials. *Continuum (Minneap Minn).* 18:161-75.

Aydar E, Palmer CP, Klyachko VA, Jackson MB (2002) The sigma receptor as a ligand-regulated auxiliary potassium channel subunit. *Neuron.* 34:399-410.

Baamonde A, Lastra A, Juárez L, García-Suárez O, Meana A, Hidalgo A, Menéndez L (2006) Endogenous beta-endorphin induces thermal analgesia at the initial stages of a murine osteosarcoma. *Peptides.* 27:2778-85.

Baamonde A, Curto-Reyes V, Juárez L, Meana A, Hidalgo A, Menéndez L (2007) Antihyperalgesic effects induced by the IL-1 receptor antagonist anakinra and increased IL-1beta levels in inflamed and osteosarcoma-bearing mice. *Life Sci*. 81:673-682.

Balasuriya D, Stewart AP, Edwardson JM (2013) The σ-1 receptor interacts directly with GluN1 but not GluN2A in the GluN1/GluN2A NMDA receptor. *J Neurosci.* 33:18219-24.

Ballantyne JC and Mao J (2003) Opioid therapy for chronic pain. *N Engl J Med*. 349:1943-53.

Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ, Patapoutian A (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron.* 41:849-57.

Bangaru ML, Weihrauch D, Tang QB, Zoga V, Hogan Q, Wu HE (2013) Sigma-1 receptor expression in sensory neurons and the effect of painful peripheral nerve injury. *Mol Pain.* 9:47.

Baron R (2000) Capsaicin and nociception: from basic mechanisms to novel drugs. *Lancet.* 356:785-7.

Barturen F and Garcia-Sevilla JA (1992) Long term treatment with desipramine increases the turnover of α_2-adrenoceptors in the rat brain. *Mol Pharmacol.* 42:846-855.

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

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. *Cell.* 139:267-84.

Bastami S, Gupta A, Zackrisson AL, Ahlner J, Osman A, Uppugunduri S (2014) Influence of UGT2B7, OPRM1 and ABCB1 Gene Polymorphisms on Morphine Use. *Basic Clin Pharmacol Toxicol*. [Epub ahead of print]

Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D (2006) TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell.* 124:1269-82.

Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature*. 448:204-8.

Beissner F, Brandau A, Henke C, Felden L, Baumgärtner U, Treede RD, Oertel BG, Lötsch J (2010) Quick discrimination of A_{delta} and C fiber mediated pain based on three verbal descriptors. *PLoS One*. 5:e12944.

Belvisi MG and Hele DJ (2009) Cough sensors. III. Opioid and cannabinoid receptors on vagal sensory nerves. *Handb Exp Pharmacol.* 187:63-76.

Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R (2008) Opioid complications and side effects. *Pain Physician.* 11:105-120.

Bigliardi PL, Stammer H, Jost G, Rufli T, Büchner S, Bigliardi-Qi M (2007) Treatment of pruritus with topically applied opiate receptor antagonist. *J Am Acad Dermatol*. 56:979-88.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG (2000) Mu-opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature.* 408:720-3.

Bolan EA, Pan YX, Pasternak GW (2004) Functional analysis of MOR-1 splice variants of the mouse mu opioid receptor gene Oprm. *Synapse.* 51:11-8.

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

Bonin RP, Labrakakis C, Eng DG, Whissell PD, De KoninckY, Orser BA (2011) Pharmacological enhancement of δ-subunit-containing $GABA_A$ receptors that generate a tonic inhibitory conductance in spinal neurons attenuates acute nociception inmice. *Pain.* 152:1317-1326

Borenstein D (2010) The role of the rheumatologist in managing pain therapy. *Nat Rev Rheumatol.* 6:227-31.

Boulais N and Misery L (2008) The epidermis: a sensory tissue. *Eur J Dermatol.* 18:119-27.

Bowen WD, Moses EL, Tolentino PJ, Walker JM (1990) Metabolites of haloperidol display preferential activity at sigma receptors compared to dopamine D-2 receptors. *Eur J Pharmacol.* 177:111-8.

Bowen WD (2000) Sigma receptors: recent advances and new clinical potentials. *Pharm Acta Helv*. 74:211-8.

Bowen WD, Moses EL, Tolentino PJ, Walker JM (1990) Metabolites of haloperidol display preferential activity at sigma receptors compared to dopamine D-2 receptors. *Eur J Pharmacol.* 177:111-8.

Breese NM, George AC, Pauers LE, Stucky CL (2005) Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. *Pain*. 115:37-49.

Brock C, Olesen SS, Olesen AE, Frøkjaer JB, Andresen T, Drewes AM (2012) Opioid-induced bowel dysfunction: pathophysiology and management. *Drugs.* 72:1847-65.

Bura AS, Guegan T, Zamanillo D, Vela JM, Maldonado R (2012) Operant self-administration of a sigma ligand improves nociceptive and emotional manifestations of neuropathic pain. *Eur J Pain.* 17:832-43.

Byers MR and Bonica JJ (2001). Peripheral pain mechanisms and nociceptor plasticity. In: Bonica’s Management of Pain. 4th ed. Fissheman S, Ballantyne and Rathmell JP eds. Lippincott Williams & Wilkins. Philadelphia.

Calvino B and Grilo RM (2006) Central pain control. *Joint Bone Spine*. 73:10-6.

Campbell JN and Meyer RA (2006) Mechanisms of neuropathic pain. *Neuron.* 52:77-92.

Campero M, Serra J, Ochoa JL (1996). C-polymodal nociceptors activated by noxious low temperature in human skin. *J Physiol*. 497:565-72.

Caplan JP (2012) Delirium, sigma-1 receptors, dopamine, and glutamate: how does haloperidol keep the genie in the bottle?. *Crit Care Med*. 40:982-3.

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*. 398:436-41.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* 389:816-24.

Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc Natl Acad Sci*. 106:9075-80.

Cendán CM, Pujalte JM, Portillo-Salido E, Montoliu L, Baeyens JM (2005a) Formalin-induced pain is reduced in σ_1 receptor knockout mice. *Eur J Pharmacol.* 511:73-74.

Cendán CM, Pujalte JM, Portillo-Salido E, Baeyens JM (2005b) Antinociceptive effects of haloperidol and its metabolites in the formalin test in mice. *Psychopharmacology (Berl).* 182:485-493.

Cervero F and Laird JM (1999) Visceral pain. *Lancet.* 353:2145-8.

Chao MV and Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns (4,5) P2-mediated inhibition. *Nature* 411:957-962

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. *Nature.* 377:428-31.

Chen Y, Hajipour AR, Sievert MK, Arbabian M, Ruoho AE (2007) Characterization of the cocaine binding site on the sigma-1 receptor. *Biochemistry.* 46:3532-42.

Cherny N, Ripamonti C, Pereira J, Davis C, Fallon M, McQuay H, Mercadante S, Pasternak G, Ventafridda V (2001) Strategies to manage the adverse effects of oral morphine: an evidence-based report. *J Clin Oncol*. 19:2542-54.

Chien CC and Pasternak GW (1993) Functional antagonism of morphine analgesia by (+)-pentazocine: evidence for an anti-opioid sigma-1 system. *Eur J Pharmacol.* 250:R7-R8.

Chien CC and Pasternak GW (1994) Selective antagonism of opioid analgesia by a sigma system. *J Pharmacol Exp Ther*. 271: 1583-1590.

Chien CC, and Pasternak GW (1995a) Sigma antagonists potentiate opioid analgesia in rats. *Neurosci Lett*. 190:137-9.

Chien CC and Pasternak GW (1995b) (-)-Pentazocine analgesia in mice: interactions with a sigma receptor system. *Eur J Pharmacol*. 294:303-8.

Choi SR, Roh DH, Yoon SY, Kang SY, Moon JY, Kwon SG, Choi HS, Han HJ, Beitz AJ, Oh SB, Lee JH (2013) Spinal sigma-1 receptors activate NADPH oxidase 2 leading to the induction of pain hypersensitivity in mice and mechanical allodynia in neuropathic rats. *Pharmacol Res*. 74:56-67.

Christie MJ, Connor M, Vaughan CW, Ingram SL, Bagley EE (2000) Cellular actions of opioids and other analgesics: implications for synergism in pain relief. *Clin Exp Pharmacol Physiol.* 27:520-523.

Christo PJ and Mazloomdoost D (2008) Cancer pain and analgesia. *Ann N Y Acad Sci*. 1138:278-98.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Wang H, Ehnerta C, Brenner GJ, Woolf C (2006) Bradykinin and peripheral sensitization. *Biol Chem.* 387:11–14

Chung KF (2008) Currently available cough suppressants for chronic cough. *Lung.* 186:S82-7.

Clavelou P, Dallel R, Orliaguet T, Woda A, Raboisson P (1995) The orofacial formalin test in rats: effects of different formalin concentrations. *Pain*. 62:295-301

Cobos EJ, Baeyens JM, Del Pozo E (2005) Phenytoin differentially modulates the affinity of agonist and antagonist ligands for σ_1 receptors of guinea pig brain. *Synapse*. 55: 192-195.

Cobos EJ, Lucena G, Baeyens JM, Del Pozo E (2006) Differences in the allosteric modulation by phenytoin of the binding properties of the σ_1 ligands [^{3}H](+)-pentazocine and [^{3}H]NE-100. *Synapse.* 59:152-161.

Cobos EJ, Del Pozo E, Baeyens JM (2007) Irreversible blockade of sigma-1 receptors by haloperidol and its metabolites in guinea pig brain and SH-SY5Y human neuroblastoma cells. *J Neurochem*. 102: 812-825.

Cobos EJ, Entrena JM, Nieto FR, Cendán CM, Del Pozo E (2008) Pharmacology and therapeutic potential of sigma$_1$ receptor ligands. *Curr Neuropharmacol.* 6:344-366.

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

Cobos EJ and Portillo-Salido E (2013) "Bedside-to-Bench" Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. *Curr Neuropharmacol*. 11:560-91.

Connor M and Christie MD (1999) Opioid receptor signalling mechanisms. *Clin Exp Pharmacol Physiol.* 26:493-9.

Corder G, Doolen S, Donahue RR, Winter MK, Jutras BL, He Y, Hu X, Wieskopf JS, Mogil JS, Storm DR, Wang ZJ, McCarson KE, Taylor BK (2013) Constitutive μ-opioid receptor activity leads to long-term endogenous analgesia and dependence. *Science*. 341:1394-9.

Coruh B, Tonelli MR, Park DR (2013) Fentanyl-induced chest wall rigidity. *Chest.* 143:1145-6.

Costantino CM, Gomes I, Stockton SD, Lim MP, Devi LA (2012) Opioid receptor heteromers in analgesia. *Expert Rev Mol Med.* 14:e9.

Coste B, Mathur J, Schmidt M, Earley TJ, Ranade S, Petrus MJ, Dubin AE, Patapoutian A (2010) Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science.* 330:55-60.

Craft RM, Henley SR, Haaseth RC, Hruby VJ, Porreca F (1995) Opioid antinociception in a rat model of visceral pain: systemic versus local drug administration. *J Pharmacol Exp Ther*. 275:1535-42.

Craig AD (2003) Pain mechanisms: labeled lines versus convergence in central processing. *Annu Rev Neurosci*. 26:1-30.

Cui Y, Ostlund SB, James AS, Park CS, Ge W, Roberts KW, Mittal N, Murphy NP, Cepeda C, Kieffer BL, Levine MS, Jentsch JD, Walwyn WM, Sun YE, Evans CJ, Maidment NT, Yang XW (2014) Targeted expression of μ-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nat Neurosci.* 17:254-61.

Dahan A, Overdyk F, Smith T, Aarts L, Niesters M (2013) Pharmacovigilance: a review of opioid-induced respiratory depression in chronic pain patients. *Pain Physician.* 16:E85-94.

Davies EC, Green CF, Taylor S, Williamson PR, Mottram DR, Pirmohamed M (2009) Adverse drug reactions in hospital in-patients: a prospective analysis of 3695 patient-episodes. *PLoS One*. 4:e4439.

Davis KD, Meyer RA, Campbell JN (1993) Chemosensitivity and sensitization of nociceptive afferents that innervate the hairy skin of monkey. *J Neurophysiol*. 69:1071-81.

Davis KD (1998) Cold-induced pain and prickle in the glabrous and hairy skin. *Pain*. 75:47-57.

Davis KD and Pope GE (2002) Noxious cold evokes multiple sensations with distinct time courses. *Pain*. 98:179-85.

De la Puente B, Nadal X, Portillo-Salido E, Sanchez-Arroyos R, Ovalle S, Palacios G, Muro A, Romero L, Entrena JM, Baeyens JM, Lopez-Garcia JA, Maldonado R, Zamanillo D, Vela JM (2009) Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. *Pain.* 145: 294-303.

DeHaven-Hudkins DL, DeHaven RN, Little PJ, Techner LM (2008) The involvement of the mu-opioid receptor in gastrointestinal pathophysiology: therapeutic opportunities for antagonism at this receptor. *Pharmacol Ther.* 117:162-87.

Delaunois A, De Ron P, Detrait E, Guyaux M (2013) Inhibitory effects of sigma-1 ligands on handling-induced tachycardia in conscious tethered rats. *Fundam Clin Pharmacol*. 27:354-63.

Dhaka A, Earley TJ, Watson J, Patapoutian A (2008) Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. *J Neurosci*. 28:566-75.

Dhingra L, Shuk E, Grossman B, Strada A, Wald E, Portenoy A, Knotkova H, Portenoy R (2013) A qualitative study to explore psychological distress and illness burden associated with opioid-induced constipation in cancer patients with advanced disease. *Palliat Med.* 27: 447-56

Díaz JL, Zamanillo D, Corbera J, Baeyens JM, Maldonado R, Pericàs MA, Vela JM, Torrens A (2009) Selective sigma$_1$ receptor antagonists: emerging target for the treatment of neuropathic pain. *Cent Nerv Syst Agents Med Chem.* 9:172-83.

Díaz JL, Cuberes R, Berrocal J, Contijoch M, Christmann U, Fernández A, Port A, Holenz J, Buschmann H, Laggner C, Serafini MT, Burgueño J, Zamanillo D, Merlos M, Vela JM, Almansa C (2012) Synthesis and biological evaluation of the 1-arylpyrazole class of σ(1) receptor antagonists: identification of 4-{2-[5-methyl-1-(naphthalen-2-yl)-1H-pyrazol-3-yloxy]ethyl}morpholine (S1RA, E-52862). *J Med Chem.* 55:8211-24.

Díaz JL, Christmann U, Fernández A, Luengo M, Bordas M, Enrech R, Carro M, Pascual R, Burgueño J, Merlos M, Benet-Buchholz J, Cerón-Bertran J, Ramírez J, Reinoso RF, Fernández de Henestrosa AR, Vela JM, Almansa C (2013) Synthesis and biological evaluation of a new series of hexahydro-2H-pyrano[3,2-c]quinolines as novel selective σ$_1$ receptor ligands. *J Med Chem*. 56:3656-65.

Donnelly L, Rathbone J, Adams CE (2013) Haloperidol dose for the acute phase of schizophrenia. *Cochrane Database Syst Rev*. 28;8:CD001951.

Dostrovsky JO and Craig AD (2008) Ascending projection systems. In: Wall and Melzack's Textbook of Pain. 5th ed. Saunders ed. Churchill Livingstone Elsevier. London.

Drageset J, Corbett A, Selbaek G, Husebo BS (2013) Cancer-Related Pain and Symptoms Among Nursing Home Residents: A Systematic Review. *J Pain Symptom Manage*. [Epub ahead of print]

Drewes AM, Schipper KP, Dimcevski G, Petersen P, Gregersen H, Funch-Jensen P, Arendt-Nielsen L (2003) Gut pain and hyperalgesia induced by capsaicin: A human experimental model. *Pain.* 104:333-41

Dubin AE and Patapoutian A (2010) Nociceptors: the sensors of the pain pathway. *J Clin Invest.* 120:3760-72.

Dussossoy D, Carayon P, Belugou S, Feraut D, Bord A, Goubet C, Roque C, Vidal H, Combes T, Loison G, Casellas P (1999) Colocalization of sterol isomerase and sigma1 receptor at endoplasmic reticulum and nuclear envelope level. *Eur J Biochem*. 263:377-86.

Edin B (2001) Cutaneous aferents provide information about knee joint movements in humans. *J Physiol*. 531:289-297.

Eisenach JC, Hood DD, Curry R (2002) Preliminary efficacy assessment of intrathecal injection of an American formulation of adenosine in humans. *Anesthesiology*. 96:29-34.

Elhabazi K, Trigo JM, Mollereau C, Moulédous L, Zajac JM, Bihel F, Schmitt M, Bourguignon JJ, Meziane H, Petit-demoulière B, Bockel F, Maldonado R, Simonin F (2012) Involvement of neuropeptide FF receptors in neuroadaptive responses to acute and chronic opiate treatments. *Br J Pharmacol*. 165: 424-435.

Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Gris G, Del PE, Zamanillo D, Baeyens JM (2009a) Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice. *Pain*. 143: 252-261.

Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Baeyens JM, Del Pozo E (2009b) Antagonism by haloperidol and its metabolites of mechanical hypersensitivity induced by intraplantar capsaicin in mice: role of sigma-1 receptors. *Psychopharmacology (Berl).* 205:21-33.

Farquhar-Smith WP (2008) Anatomy, physiology and pharmacology of pain. *Pain*. 9:3-7.

Fields HL (1984) Neurophysiology of pain and pain modulation. *Am J Med*. 77:2-8.

Fields HL (1992) Is there a Facilitating Component to Central Pain Modulation?. *APS Journal.* 1:139-141.

Finnerup NB, Scholz J, Attal N, Baron R, Haanpää M, Hansson P, Raja SN, Rice AS, Rief W, Rowbotham MC, Simpson DM, Treede RD (2013) Neuropathic pain needs systematic classification. *Eur J Pain.* 17:953-6.

Flatters SJ and Bennett GJ (2006) Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: evidence for mitochondrial dysfunction. *Pain*. 122:245-57.

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

Fontanilla D, Johannessen M, Hajipour AR, Cozzi NV, Jackson MB, Ruoho AE (2009) The hallucinogen *N*,*N*-dimethyltryptamine (DMT) is an endogenous sigma-1 receptor regulator. *Science*. 323:934-7.

Gallelli L, Colosimo M, Tolotta GA, Falcone D, Luberto L, Curto LS, Rende P, Mazzei F, Marigliano NM, De Sarro G, Cucchiara S (2010) Prospective randomized double-blind trial of racecadotril compared with loperamide in elderly people with gastroenteritis living in nursing homes. *Eur J Clin Pharmacol.* 66:37-144.

Gardner EP, Martin JH, Jessell TM (2000) The Bodily Senses. In: Principles of Neural Science. 3th ed. Kandel ER, Schwartz JH, Jessell TM eds. McGraw-Hill. New York.

Garrison SR, Dietrich A, Stucky CL (2012) TRPC1 contributes to light-touch sensation and mechanical responses in low-threshold cutaneous sensory neurons. *J Neurophysiol*. 107:913-22.

Garzón J, García-España A, Sánchez-Blázquez P (1997) Opioids binding mu and delta receptors exhibit diverse efficacy in the activation of Gi2 and G(x/z) transducer proteins in mouse periaqueductal gray matter. *J Pharmacol Exp Ther*. 281:549-57.

Garzón J, Rodríguez-Muñoz M, Sánchez-Blázquez P (2008) Do pharmacological approaches that prevent opioid tolerance target different elements in the same regulatory machinery?. *Curr Drug Abuse Rev.* 1:222-38.

Gatti A and Sabato AF (2012) Management of opioid-induced constipation in cancer patients: focus on methylnaltrexone. *Clin Drug Investig*. 32:293-301.

Gómez-Soler M, Fernández-Dueñas V, Portillo-Salido E, Pérez P, Zamanillo D, Vela JM, Burgueño J, Ciruela F (2014) Predicting the antinociceptive efficacy of σ_1 receptor ligands by a novel receptor fluorescence resonance energy transfer (FRET) based biosensor. *J Med Chem*. 57:238-42.

González P, Cabello P, Germany A, Norris B, Contreras E (1997) Decrease of tolerance to, and physical dependence on morphine by, glutamate receptor antagonists. *Eur J Pharmacol*. 332:257-62.

González-Rodríguez S, Hidalgo A, Baamonde A, Menéndez L (2010) Spinal and peripheral mechanisms involved in the enhancement of morphine analgesia in acutely inflamed mice. *Cell Mol Neurobiol*. 30:113-21.

González-Cano R, Merlos M, Baeyens JM, Cendán CM (2013) σ_1 receptors are involved in the visceral pain induced by intracolonic administration of capsaicin in mice. *Anesthesiology.* 118:691-700.

Gottrup H, Juhl G, Kristensen AD, Lai R, Chizh BA, Brown J, Bach FW, Jensen TS (2004) Chronic oral gabapentin reduces elements of central sensitization in human experimental hyperalgesia. *Anesthesiology*. 101:1400-8.

Greenwood-Van Meerveld B, Gardner CJ, Little PJ, Hicks GA, Dehaven-Hudkins DL (2004) Preclinical studies of opioids and opioid antagonists on gastrointestinal function. *Neurogastroenterol Motil.* 2:46-53.

Greenwood-Van Meerveld B, Standifer KM (2008) Methylnaltrexone in the treatment of opioid-induced constipation. *Clin Exp Gastroenterol.* 1:49-58.

Gris G, Merlos M, Vela JM, Zamanillo D, Portillo-Salido E (2014) S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in the carrageenan and complete Freund's adjuvant models in mice. *Behav Pharmacol*. 25:226-35.

Guitart X, Codony X, Monroy X (2004) Sigma receptors: biology and therapeutic potential. *Psychopharmacology (Berl).* 174: 301-319.

GuscottM, BristowLJ, HadinghamK, RosahlTW, BeerMS, Stanton JA, Bromidge F, Owens AP, Huscroft I, Myers J, Rupniak NM, Patel S, Whiting PJ, Hutson PH, Fone KC, Biello SM, Kulagowski JJ, McAllister G (2005) Genetic knockout and pharmacological blockade studies of the 5-HT7 receptor suggest therapeutic potential in depression. *Neuropharmacology* 48:492–502

Gutstein HB and Akil H (2001) Opioid Analgesics. In: The Pharmacological Basis of Therapeutics. 10th ed. Hardman JG, Limbird LE, Gilman AG, Goodman and Gilman's eds. McGraw-Hill. New York.

Haigh RC and Blake DR (2001) Understanding pain. Clin Med. 1:44-8. *Drugs*. 41:326-44.

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

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

Handwerker HO (2006) Chapter 3 Nociceptors: neurogenic inflammation. *Handb Clin Neurol*. 81:23-33.

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E, Glossmann H (1996) Purification, molecular cloning, and expression of the mammalian sigma1-binding site. *Proc Natl Acad Sci*. 93:8072-7.

Harris JD (2008) Management of expected and unexpected opioid-related side effects. *Clin J Pain.* Suppl 10:S8-S13.

Harrison JL and Davis KD (1999). Cold-evoked pain varies with skin type and cooling rate: a psychophysical study in humans. *Pain.* 83:123-135.

Hartrick CT and Rozek RJ (2011) Tapentadol in pain management: a μ-opioid receptor agonist and noradrenaline reuptake inhibitor. *CNS Drugs*. 25:359-70.

Hayashi T, Maurice T, Su TP (2000) Ca^{2+} signaling via σ_1-Receptors: novel regulatory mechanism affecting intracellular Ca^{2+} concentration. *J Pharmacol Exp Ther.* 293:788-98.

Hayashi T and Su TP (2003) Sigma-1 receptors (σ_1 binding sites) form raft-like microdomains and target lipid droplets on the endoplasmic reticulum: roles in endoplasmic reticulum lipid compartmentalization and export. *J Pharmacol Exp Ther*. 306:718-25.

Hayashi T and Su TP (2004) Sigma-1 receptor ligands: potential in the treatment of neuropsychiatric disorders. *CNS Drugs*. 18:269-284.

Hayashi T and Su TP (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca^{2+} signaling and cell survival. *Cell.* 131:596-610.

Hellewell SB and Bowen WD (1990) A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Res.* 527:244-53.

Herrero JF, Laird JM, López-García JA (2000) Wind-up of spinal cord neurones and pain sensation: much ado about something?. *Prog Neurobiol*. 61:169-203.

Hnasko TS, Sotak BN, Palmiter RD (2005) Morphine reward in dopamine-deficient mice. *Nature.* 438:854-857.

Højsted J and Sjøgren P (2007) Addiction to opioids in chronic pain patients: a literature review. *Eur J Pain.* 11:490-518.

Holzer P (2009) Opioid receptors in the gastrointestinal tract. *Regul Pept.* 155:11-7.

Holzer P (2010) Opioid antagonists for prevention and treatment of opioid-induced gastrointestinal effects. *Curr Opin Anaesthesiol*. 23:616-22.

Hong W, Nuwayhid SJ, Werling LL (2004) Modulation of bradykinin-induced calcium changes in SH-SY5Y cells by neurosteroids and sigma receptor ligands via a shared mechanism. *Synapse*. 54:102-10.

Hoskin PJ and Hanks GW (1991) Opioid agonist-antagonist drugs in acute and chronic pain states. *Drugs.* 41:326-44.

Hu ZL, Zhang X, Shi M, Tian ZW, Huang Y, Chen JY, Ding YQ (2013) Delayed but not loss of gliogenesis in Rbpj-deficient trigeminal ganglion. *Int J Clin Exp Pathol* 6:1261-71.

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature.* 258:577-80.

Jarzyna D, Jungquist CR, Pasero C, Willens JS, Nisbet A, Oakes L, Dempsey SJ, Santangelo D, Poloma (2011) American Society for Pain Management Nursing guidelines on monitoring for opioid-induced sedation and respiratory depression. *Pain Manag Nurs*. 12:118-145.

Jessell TM and Kelly DD (2000) Pain and Analgesia. In: Principles of Neural Science 4th ed. Kandel ER, Schwartz JH and Jessell TM eds. McGraw-Hill Interamericana. Madrid.

Ji RR (2004) Peripheral and central mechanisms of inflammatory pain, with emphasis on MAP kinases. *Curr Drug Targets Inflamm Allergy*. 3:299-303.

Joshi SK, Hernandez G, Mikusa JP, Zhu CZ, Zhong C, Salyers A, Wismer CT, Chandran P, Decker MW, Honore P. (2006) Comparison of antinociceptive actions of standard analgesics in attenuating capsaicin and nerve-injury-induced mechanical hypersensitivity. *Neuroscience* 143:587–96.

Joshi SK, Mikusa JP, Weaver B, Honore P (2008) Morphine and ABT-594 (a nicotinic acetylcholine agonist) exert centrally mediated antinociception in the rat cyclophosphamide cystitis model of visceral pain. *J Pain.* 9:146-56.

Kayser V, Lee SH, Guilbaud G (1995) Evidence for a peripheral component in the enhanced antinociceptive effect of a low dose of systemic morphine in rats with peripheral mononeuropathy. *Neuroscience.* 64:537-45.

Kerste*i*n PC, del Camino D, Moran MM, Stucky CL (2009) Pharmacological blockade of TRPA1 inhibits mechanical firing in nociceptors. *Mol Pain*. 5:19.

Khalefa BI, Shaqura M, Al-Khrasani M, Fürst S, Mousa SA, Schäfer M (2012) Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. *Eur J Pain.* 16:690-705.

Kieffer BL (1999) Opioids: first lessons from knockout mice. *Trends Pharmacol Sci.* 20:19-26.

Kim FJ, Kovalyshyn I, Burgman M, Neilan C, Chien CC, Pasternak GW (2010) Sigma 1 receptor modulation of G-protein-coupled receptor signaling: potentiation of opioid transduction independent from receptor binding. *Mol Pharmacol.* 77:695-703.

Kim HW, Kwon YB, Roh DH, Yoon SY, Han HJ, Kim KW, Beitz AJ, Lee JH (2006) Intrathecal treatment with σ_1 receptor antagonists reduces formalin-induced phosphorylation of NMDA receptor subunit 1 and the second phase of formalin test in mice. *Br J Pharmacol.* 148:490-498.

Kim HW, Roh DH, Yoon SY, Seo HS, Kwon YB, Han HJ, Kim KW, Beitz AJ, Lee JH, (2008) Activation of the spinal sigma-1 receptor enhances NMDA-induced pain via PKC- and PKA-dependent phosphorylation of the NR1 subunit in mice. *B J Pharmacol.* 154:1125-1134.

King MA, Bradshaw S, Chang AH, Pintar JE, Pasternak GW (2001) Potentiation of opioid analgesia in dopamine$_2$ receptor knock-out mice: evidence for a tonically active anti-opioid system. *J Neurosci*. 21:7788-92.

King CD, Goodin B, Glover TL, Riley JL, Hou W, Staud R, Fillingim RB (2013) Is the pain-reducing effect of opioid medication reliable? A psychophysical study of morphine and pentazocine analgesia. *Pain.* 154:476-83.

King M, Pan YX, Mei J, Chang A, Xu J, Pasternak GW (1997) Enhanced kappa-opioid receptor-mediated analgesia by antisense targeting the sigma1 receptor. *Eur J Pharmacol.* 331:R5-R6.

King T, Ossipov MH, Vanderah TW, Porreca F, Lai J (2005) Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance?. *Neurosignals.* 14:194-205.

Kitaichi K, Chabot JG, Moebius FF, Flandorfer A, Glossmann H, Quirion R (2000) Expression of the purported sigma$_1$ (σ_1) receptor in the mammalian brain and its possible relevance in deficits induced by antagonism of the NMDA receptor complex as revealed using an antisense strategy. *J Chem Neuroanat.* 20:375-387.

Knapman A and Connor M (2014) Cellular Signaling of Non-synonymous Single Nucleotide Polymorphisms of the Human μ-Opioid Receptor (OPRM1). *Br J Pharmacol*. [Epub ahead of print]

Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, Noguchi K (2005) Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with Aδ/C-fibers and colocalization with trk receptors. *J Comp Neurol*. 493:596-606.

Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol.* 63:9-18.

Kolesnikov YA, Chereshnev I, Pasternak GW (2000) Analgesic synergy between topical lidocaine and topical opioids. *J Pharmacol Exp. Ther*. 295:546-51.

Kolesnikov YA, Ferkany J, Pasternak GW (1993). Blockade of mu and kappa 1 opioid analgesic tolerance by NPC17742, a novel NMDA antagonist. *Life Sci.* 53:1489-94.

Kolesnikov YA, Jain S, Wilson R, Pasternak GW (1996) Peripheral morphine analgesia: synergy with central sites and a target of morphine tolerance. J *Pharmacol Exp Ther. 2*79: 502-506.

Kourrich S, Su TP, Fujimoto M, Bonci A (2012) The sigma-1 receptor: roles in neuronal plasticity and disease. *Trends Neurosci*. 35:762-71.

Kourrich S, Hayashi T, Chuang JY, Tsai SY, Su TP, Bonci A (2013) Dynamic interaction between sigma-1 receptor and Kv1.2 shapes neuronal and behavioral responses to cocaine. *Cell.* 152:236-47.

Kuipers PW, Kamphuis ET, van Venrooij GE, van Roy JP, Ionescu TI, Knape JT, Kalkman CJ (2004) Intrathecal opioids and lower urinary tract function: a urodynamic evaluation. *Anesthesiology*. 100:1497-503.

Kumar K and Singh SI (2013) Neuraxial opioid-induced pruritus: An update. J *Anaesthesiol Clin Pharmacol*. 29:303-307.

Kuraishi Y, Hirota N, Satoh M, Takagi H (1985) Antinociceptive effects of intrathecal opioids, noradrenaline and serotonin in rats: mechanical and thermal algesic tests. *Brain Res.* 326:168-71.

Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP (2006) TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*. 50:277-89.

Kwan KY, Glazer JM, Corey DP, Rice FL, Stucky CL (2009) TRPA1 modulates mechanotransduction in cutaneous sensory neurons. *J Neurosci*. 29:4808-19.

Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F (2001) A new model of visceral pain and referred hyperalgesia in the mouse. *Pain.* 92:335-42.

Lan Y, Chen Y, Xu X, Qiu Y, Liu S, Liu X, Liu BF, Zhang G (2014) Synthesis and biological evaluation of a novel sigma-1 receptor antagonist based on 3,4-dihydro-2(1H)-quinolinone scaffold as a potential analgesic. *Eur J Med Chem*. 79:216-30.

Langa F, Codony X, Tovar V, Lavado A, Giménez E, Cozar P, Cantero M, Dordal A, Hernández E, Pérez R, Monroy X, Zamanillo D, Guitart X, Montoliu L (2003) Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. *Eur J Neurosci.* 18:2188-96.

Latremoliere A and Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain*. 10:895-926.

Lawson JJ, McIlwrath SL, Woodbury CJ, Davis BM, Koerber HR (2008) TRPV1 unlike TRPV2 is restricted to a subset of mechanically insensitive cutaneous nociceptors responding to heat. *J Pain*. 9:298-308.

Layer P and Andresen V (2010) Review article: rifaximin, a minimally absorbed oral antibacterial, for the treatment of travellers' diarrhoea. *Aliment Pharmacol Ther* 31:1155-1164.

Le Bars D, Gozariu M, Cadden SW (2001) Animal models of nociception. *Pharmacol Rev*. 53:597-652.

Leffler A, Linte RM, Nau C, Reeh P, Babes A (2007) A high-threshold heat-activated channel in cultured rat dorsal root ganglion neurons resembles TRPV2 and is blocked by gadolinium. *Eur J Neurosci*. 26:12-22

Liu Q, Vrontou S, Rice FL, Zylka MJ, Dong X, Anderson DJ (2007) Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. *Nat Neurosci*. 10:946-8.

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

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the folin phenol reagent. *J Biol Chem.* 193:265-271.

Majumdar S, Grinnell S, Le Rouzic V, Burgman M, Polikar L, Ansonoff M, Pintar J, Pan YX, Pasternak GW (2011) Truncated G protein-coupled mu opioid receptor MOR-1 splice variants are targets for highly potent opioid analgesics lacking side effects. *Proc Natl Acad Sci.* 108:19778-83.

Majumdar S, Subrath J, Le Rouzic V, Polikar L, Burgman M, Nagakura K, Ocampo J, Haselton N, Pasternak AR, Grinnell S, Pan YX, Pasternak GW (2012) Synthesis and evaluation of aryl-naloxamide opiate analgesics targeting truncated exon 11-associated μ opioid receptor (MOR-1) splice variants. *J Med Chem.* 55:6352-62.

Mao J, Gold MS, Backonja MM (2011) Combination drug therapy for chronic pain: a call for more clinical studies. *J Pain.* 12:157-166.

Mark MD and Herlitze S (2000) G-protein mediated gating of inward-rectifier K^+ channels. *Eur J Biochem.* 267:5830-6.

Marker CL, Luján R, Loh HH, Wickman K (2005) Spinal G-protein-gated potassium channels contribute in a dose-dependent manner to the analgesic effect of mu- and delta- but not kappa-opioids. *J Neurosci*. 25:3551-9.

Marrazzo A, Parenti C, Scavo V, Ronsisvalle S, Scoto GM, Ronsisvalle G (2006) In vivo evaluation of (+)-MR200 as a new selective sigma ligand modulating MOP, DOP and KOP supraspinal analgesia. *Life Sci.* 78:449-53.

Marrazzo A, Cobos EJ, Parenti C, Arico G, Marrazzo G, Ronsisvalle S, Pasquinucci L, Prezzavento O, Colabufo NA, Contino M, Gonzalez LG, Scoto GM, Ronsisvalle G (2011) Novel potent and selective sigma ligands: evaluation of their agonist and antagonist properties. *J Med Chem.* 54:3669-3673.

Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE (1976) The effects of morphine- and nalorphine- like drugs in the nondependent and morphinedependent chronic spinal dog. *J Pharmacol Exp Ther.* 197:517-532.

Martín MI and Goicoecha G (2008) Fármacos analgésicos opioides. In Farmacología Básica y Clínica. 18th ed. Lorenzo P, Moreno A, Lizasoain I, Leza JC, Moro MA and Portolés A eds. Panamericana. Madrid

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

Matsumoto RR, Bowen WD, Tom MA, Vo VN, Truong DD, and De Costa BR (1995) Characterization of two novel sigma receptor ligands: antidystonic effects in rats suggest sigma receptor antagonism. *Eu J Pharmacol.* 280:301-310.

Matsumoto RR (2009) Targeting sigma receptors: novel medication development for drug abuse and addiction. *Expert Rev Clin Pharmacol*. 2:351-8.

Mauger S, Fraser R, Gill K (2014) Utilizing buprenorphine-naloxone to treat illicit and prescription-opioid dependence. *Neuropsychiatr Dis Treat*. 10:587-598.

Maurice T, Urani A, Phan VL, Romieu P (2001) The interaction between neuroactive steroids and the sigma1 receptor function: behavioral consequences and therapeutic opportunities. *Brain Res Brain Res Rev*. 37:116-32.

Maurice T, Grégoire C, Espallergues J (2006) Neuro(active)steroids actions at the neuromodulatory sigma1 (σ_1) receptor: biochemical and physiological evidences, consequences in neuroprotection. *Pharmacol Biochem Behav.* 84:581-97.

McGlone F and Reilly D (2010) The cutaneous sensory system. *Neurosci Biobehav Rev.* 34:148-59.

Mei J and Pasternak GW (2002) σ_1 receptor modulation of opioid analgesia in the mouse. *J. Pharmacol Exp Ther.* 300:1070-1074.

Mei J and Pasternak GW (2007) Modulation of brainstem opiate analgesia in the rat by σ_1 receptors: a microinjection study. *J Pharmacol Exp Ther.* 322:1278-1285.

Melzack R and Wall PD (1965) Pain mechanisms: a new theory. *Science*. 150:971-9.

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

Mercadante S (1999) Problems of long-term spinal opioid treatment in advanced cancer patients. *Pain.* 79:1-13.

Mégarbane B, Declèves X, Bloch V, Bardin C, Chast F, Baud FJ (2007) Case report: quantification of methadone-induced respiratory depression using toxicokinetic/toxicodynamic relationships. *Crit Care*. 11:R5.

Meyer RA, Ringkamp M, Campbell JN, Raja SN (2008) Peripheral mechanisms of cutaneous nociception. In: Wall and Melzack's Textbook of Pain. 5th ed. Saunders ed. Churchill Livingstone Elsevier. London.

Millan MJ (1999) The induction of pain: an integrative review. *Prog Neurobiol*. 57:1-164.

Millan MJ (2002) Descending control of pain *Prog. Neurobiol.* 66:355-474.

Mitchell K, Bates BD, Keller JM, Lopez M, Scholl L, Navarro J, Madian N, Haspel G, Nemenov MI, Iadarola MJ (2010) Ablation of rat TRPV1-expressing Adelta/C-fibers with resiniferatoxin: analysis of withdrawal behaviors, recovery of function and molecular correlates. *Mol Pain*. 6:94.

Moison D, De Deurwaerdère P, Cagnotto A, Marrazzo A, Prezzavento O, Ronsisvalle G, Mennini T, Spampinato U (2003) Intrastriatal administration of sigma ligands inhibits basal dopamine release in vivo. *Neuropharmacology.* 45:945-53.

Mongin G (2007) Tramadol extended-release formulations in the management of pain due to osteoarthritis. *Expert Rev Neurother*. 7:1775-84.

Moon JY, Roh DH, Yoon SY, Kang SY, Choi SR, Kwon SG, Choi HS, Han HJ, Beitz AJ, Lee JH (2013) Sigma-1 receptor-mediated increase in spinal p38 MAPK phosphorylation leads to the induction of mechanical allodynia in mice and neuropathic rats. *Exp Neurol*. 247:383-91.

Mori T, Shibasaki Y, Matsumoto K, Shibasaki M, Hasegawa M, Wang E, Masukawa D, Yoshizawa K, Horie S, Suzuki T (2013) Mechanisms that underlie μ-opioid receptor agonist-induced constipation: differential involvement of μ-opioid receptor sites and responsible regions. *J Pharmacol Exp Ther.* 347:91-9.

Moebius FF, Reiter RJ, Hanner M, Glossmann H (1997) High affinity of sigma$_1$-binding sites for sterol isomerization inhibitors: evidence for a pharmacological relationship with the yeast sterol C_8-C_7 isomerase. *Br J Pharmacol.* 121:1-6.

Mueller-Lissner S (2010) Fixed combination of oxycodone with naloxone: a new way to prevent and treat opioid-induced constipation. *Adv Ther.* 27:581-90.

Musacchio JM, Klein M, Paturzo JJ (1989) Effects of dextromethorphan site ligands and allosteric modifiers on the binding of (+)-[^{3}H]3-(-3-hydroxyphenyl)-*N*-(1-propyl)piperidine. *Mol Pharmacol*. 35:1-5.

Nakamura A, Hasegawa M, Minami K, Kanbara T, Tomii T, Nishiyori A, Narita M, Suzuki T, Kato A (2013) Differential activation of the μ-opioid receptor by oxycodone and morphine in pain-related brain regions in a bone cancer pain model. *Br J Pharmacol* 168:375-88.

Nakazato A, Kumagai T, Ohta K, Chaki S, Okuyama S, Tomisawa K (1999) Synthesis and SAR of 1-alkyl-2-phenylethylamine derivatives designed from *N*,*N*-dipropyl-4-methoxy-3-(2-phenylethoxy)phenylethylamine to discover σ_1 ligands. *J Med Chem.* 42:3965-3970.

Narita M, Ozaki S, Ioka M, Mizoguchi H., Nagase H, Tseng L, Suzuki T (2001) Different motivational effects induced by the endogenous μ-opioid receptor ligands endomorphin-1 and -2 in the mouse. *Neuroscience*. 105:213-218.

Navarro G, Moreno E, Aymerich M, Marcellino D, McCormick PJ, Mallol J, Cortés A, Casadó V, Canela EI, Ortiz J, Fuxe K, Lluís C, Ferré S, Franco R (2010) Direct involvement of σ-1 receptors in the dopamine D_1 receptor-mediated effects of cocaine. *Proc Natl Acad Sci.* U S A. 107:18676-81.

Navarro G, Moreno E, Bonaventura J, Brugarolas M, Farré D, Aguinaga D, Mallol J, Cortés A, Casadó V, Lluís C, Ferre S, Franco R, Canela E, McCormick PJ (2013) Cocaine inhibits dopamine D_2 receptor signaling via sigma-1-D_2 receptor heteromers. *PLoS One*. 8:e61245.

Ness T and Randich A (2010) Substrates of Spinal Cord Nociceptive Processing. In Bonica's Management of Pain. 4th ed. Fissheman S, Ballantyne and Rathmell JP eds. Lippincott Williams & Wilkins. Philadelphia.

Nieto FR, Cendán CM, Sánchez-Fernández C, Cobos EJ, Entrena JM, Tejada MA, Zamanillo D, Vela JM, Baeyens JM (2012) Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. *J Pain.* 13:1107-1121.

Nieto FR, Cendán CM, Cañizares FJ, Cubero MA, Vela JM, Fernández-Segura E, Baeyens JM (2014) Genetic inactivation and pharmacological blockade of sigma-1 receptors prevent paclitaxel-induced sensory-nerve mitochondrial abnormalities and neuropathic pain in mice. *Mol Pain.* 10:11.

Nilius B and Honoré E (2012) Sensing pressure with ion channels. *Trends Neurosci*. 35:477-86

Nordin M (1990) Low-threshold mechanoreceptive and nociceptive units with unmyelinated (C) fibres in the human supraorbital nerve. *J Physiol (Lond.).* 426:229-240.

Ocaña M, Del Pozo E, Barrios M, Baeyens JM (1995) Subgroups among mu-opioid receptor agonists distinguished by ATP-sensitive K^+ channel-acting drugs. *Br J Pharmacol.* 114:1296-302.

Ocaña M, Cendán CM, Cobos EJ, Entrena JM, Baeyens JM (2004) Potassium channels and pain: present realities and future opportunities. *Eur J Pharmacol*. 500:203-19.

Ohsawa M, Carlsson A, Asato M, Koizumi T, Nakanishi Y, Fransson R, Sandström A, Hallberg M, Nyberg F, Kamei J (2011) The dipeptide Phe-Phe amide attenuates signs of hyperalgesia, allodynia and nociception in diabetic mice using a mechanism involving the sigma receptor system. *Mol Pain.* 31:7-11.

Olausson H, Cole J, Rylander K, McGlone F, Lamarre Y, Wallin BG, Krämer H, Wessberg J, Elam M, Bushnell MC, Vallbo A (2008) Functional role of unmyelinated tactile afferents in human hairy skin: sympathetic response and perceptual localization. *Exp Brain Res*. 184:135-40.

Olesen AE, Upton R, Foster DJ, Staahl C, Christrup LL, Arendt-Nielsen L, Drewes AM (2010) A pharmacokinetic and pharmacodynamic study of oral oxycodone in a human experimental pain model of hyperalgesia. *Clin Pharmacokinet.* 49:817-827.

Ortega-Roldan JL, Ossa F, Schnell JR (2013) Characterization of the human sigma-1 receptor chaperone domain structure and binding immunoglobulin protein (BiP) interactions. *J Biol Chem*. 288:21448-57.

Ossipov MH, Dussor GO, Porreca F (2010) Central modulation of pain. *J Clin Invest.* 120:3779-87.

Pabba M (2013) The essential roles of protein-protein interaction in sigma-1 receptor functions. *Front Cell Neurosci.* 7:50.

Pal A, Hajipour AR, Fontanilla D, Ramachandran S, Chu UB, Mavlyutov T, Ruoho AE (2007) Identification of regions of the sigma-1 receptor ligand binding site using a novel photoprobe. *Mol Pharmacol*. 72:921-33.

Pal A, Chu UB, Ramachandran S, Grawoig D, Guo LW, Hajipour AR, Ruoho AE (2008) Juxtaposition of the steroid binding domain-like I and II regions constitutes a ligand binding site in the sigma-1 receptor. *J Biol Chem*. 283:19646-56.

Palacios G, Muro A, Verdú E, Pumarola M, Vela JM (2004) Immunohistochemical localization of the sigma1 receptor in Schwann cells of rat sciatic nerve. *Brain Res.* 1007:65-70.

Paleček J and Willis WD (2003) The dorsal column pathway facilitates visceromotor responses to colorectal distention after colon inflammation in rats. *Pain.* 104:501-7.

Paleček J (2004) The role of dorsal columns pathway in visceral pain. *Physiol Res.* 1:S125-30.

Pan YX, Mei J, Xu J, Wan BL, Zuckerman A, Pasternak GW (1998) Cloning and characterization of a mouse sigma1 receptor. *J Neurochem.* 70:2279-2285.

Pan YX; Xu JY; Xu MM; Yu R; Bolan E; Gilbert AK; Pasternak GW (2009). Isolation and expression of three new splice variants, mMOR-1A, mMOR-1O and mMOR-1P, from mouse mu opioid receptor gene. *Synapse*. 68:144-52.

Pan L, Xu J, Yu R, Xu MM, Pan YX, Pasternak GW (2005) Identification and characterization of six new alternatively spliced variants of the human mu opioid receptor gene. *Oprm. Neurosci*. 133, 209e220

Parenti C, Turnaturi R, Arico G, Marrazzo A, Prezzavento O, Ronsisvalle S, Scoto GM, Ronsisvalle G, Pasquinucci L (2012) Antinociceptive profile of LP1, a non-peptide multitarget opioid ligand. *Life Sci.* 90:957-961.

Parenti C, Marrazzo A, Aricò G, Cantarella G, Prezzavento O, Ronsisvalle S, Scoto GM, Ronsisvalle G (2014) Effects of a selective sigma 1 antagonist compound on inflammatory pain. *Inflammation*. 37:261-6

Paronis CA and Bergman J (2011) Buprenorphine and opioid antagonism, tolerance, and naltrexone-precipitated withdrawal. *J Pharmacol Exp Ther.* 336:488-95.

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

Pasternak GW (2004) Multiple opiate receptors: deja vu all over again. *Neuropharmacology*. 47:312-323.

Pasternak GW (2010) Molecular insights into mu opioid pharmacology: From the clinic to the bench. *Clin J Pain*. Suppl. 10:S3-9.

Pasternak GW and Pan YX (2011) Mu opioid receptors in pain management. *Acta Anaesthesiol Taiwan.* 49:21-25.

Pasternak GW and Pan YX (2013) Mu opioids and their receptors: evolution of a concept. *Pharmacol Rev*. 65:1257-317.

Pasternak GW (2014) Opioids and their receptors: Are we there yet? *Neuropharmacology*. 76:198-203.

Pattinson KT (2008) Opioids and the control of respiration. *Br J Anaesth*. 100:747-58.

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

Pergolizzi JV, Raffa RB, Taylor R J (2014) Treating Acute Pain in Light of the Chronification of Pain. *Pain Manag Nurs*. 15:380-390.

Petrus M, Peier AM, Bandell M, Hwang SW, Huynh T, Olney N, Jegla T, Patapoutian A (2007) A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain*.17;3:40.

Piergentili A, Amantini C, Del Bello F, Giannella M, Mattioli L, Palmery M, Perfumi M, Pigini M, Santoni G, Tucci P, Zotti M, Quaglia W (2010) Novel highly potent and selective sigma 1 receptor antagonists related to spipethiane. *J Med Chem*. 53:1261-9.

Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. *Trends Neurosc.* 25:319-25.

Price TJ and Flores CM (2007) Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *J Pain*. 8:263-72

Priestley JV (2009) Neuropeptides: Sensory Systems. In Synaptic Plasticity in Pain textbook. Malcangio Marzia ed. Springer. UK.

Prezzavento O, Parenti C, Marrazzo A, Ronsisvalle S, Vittorio F, Aricò G, Scoto GM, Ronsisvalle G (2008) A new sigma ligand, (+/-)-PPCC, antagonizes kappa opioid receptor-mediated antinociceptive effect. *Life Sci.* 82:549-53.

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, Lamantia AS, Mcnamara JO, Williams SM (2006) Sistema Somatosensitivo. In Tratado de Neurociencia. 3th Ed. Purves D eds. Panamericana. Madrid.

Quick K, Zhao J, Eijkelkamp N, Linley JE, Rugiero F, Cox JJ, Raouf R, Gringhuis M, Sexton JE, Abramowitz J, Taylor R, Forge A, Ashmore J, Kirkwood N, Kros CJ, Richardson GP, Freichel M, Flockerzi V, Birnbaumer L, Wood JN. (2012) TRPC3 and TRPC6 are essential for normal mechanotransduction in subsets of sensory neurons and cochlear hair cells. *Open Biol*. 2:120068.

Quirion R, Bowen WD, Itzhak Y, Junien JL, Musacchio JM, Rothman RB, Su TP, Tam SW, Taylor DP (1992) A proposal for the classification of sigma binding sites. *Trends Pharmacol Sci*. 13:85-6.

Quock RM, Burkey TH, Varga E, Hosohata Y, Hosohata K, Cowell SM, Slate CA, Ehlert FJ, Roeske WR, Yamamura HI (1999) The delta-opioid receptor: molecular pharmacology, signal transduction, and the determination of drug efficacy. *Pharmacol Rev*. 51:503-32.

Radke JB, Owen KP, Sutter ME, Ford JB, Albertson TE (2014) The effects of opioids on the lung. *Clin Rev Allergy Immunol*. 46:54-64

Raehal KM, Schmid CL, Groer CE, Bohn LM (2011) Functional selectivity at the μ-opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol Rev*. 63: 1001-1019.

Raffa RB, Martinez RP, Connelly CD (1994) G-protein antisense oligodeoxyribonucleotides and mu-opioid supraspinal antinociception. *Eur J Pharmacol.* 258:R5-7.

Raffa RB, Buschmann H, Christoph T, Eichenbaum G, Englberger W, Flores CM, Hertrampf T, Kögel B, Schiene K, Straßburger W, Terlinden R, Tzschentke TM (2012) Mechanistic and functional differentiation of tapentadol and tramadol. *Expert Opin Pharmacother*. 13:1437-49.

Reichle FM and Conzen PF (2008) Methylnaltrexone, a new peripheral mu-receptor antagonist for the prevention and treatment of opioid-induced extracerebral side effects. *Curr Opin Investig Drugs*. 9:90-100.

Reynolds DV (1969) Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science.* 164:444-5.

Reynolds SM, Mackenzie AJ, Spina D, Page CP (2004) The pharmacology of cough. *Trends Pharmacol Sci*. 25:569-76.

Ringkamp M and Raja SN (2012) Dissecting the relative contribution of central versus peripheral opioid analgesia: are the analgesic and adverse effects of opioids inseparable?. *Eur J Pain*. 16:621-3.

Roh DH, Choi SR, Yoon SY, Kang SY, Moon JY, Kwon SG, Han HJ, Beitz AJ, Lee JH (2011) Spinal neuronal NOS activation mediates sigma-1 receptor-induced mechanical and thermal hypersensitivity in mice: involvement of PKC-dependent GluN1 phosphorylation. *Br J Pharmacol*. 163: 1707-1720.

Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Kim KW, Han HJ, Beitz AJ, Lee JH (2008a) Intrathecal administration of sigma-1 receptor agonists facilitates nociception: involvement of a protein kinase C-dependent pathway (2008) *J Neurosci Res.* 86:3644-54.

Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Kim KW, Han HJ, Beitz AJ, Na HS, Lee JH (2008b) Intrathecal injection of the σ_1 receptor antagonist BD1047 blocks both mechanical allodynia and increases in spinal NR1 expression during the induction phase of rodent neuropathic pain. *Anesthesiology.* 109:879-889.

Roh DH, Yoon SY, Seo, HS, Kang SY, Moon JY, Song S, Beitz AJ, Lee JH (2010) Sigma-1 receptor-induced increase in murine spinal NR1 phosphorylation is mediated by the PKCα and ε, but not the PKCξ, isoforms. *Neurosci Lett.* 477:95-99.

Roh DH and Yoon SY (2014) Sigma-1 receptor antagonist, BD1047 reduces nociceptive responses and phosphorylation of p38 MAPK in mice orofacial formalin model. *Biol Pharm Bull*. 37:145-51.

Romero L, Zamanillo D, Nadal X, Sanchez-Arroyos R, Rivera-Arconada I, Dordal A, Montero A, Muro A, Bura A, Segales C, Laloya M, Hernandez E, Portillo-Salido E, Escriche M, Codony X, Encina G, Burgueño J, Merlos M, Baeyens JM, Giraldo J, Lopez-Garcia JA, Maldonado R, Plata-Salaman CR, Vela JM (2012) Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization. *Br J Pharmacol*. 166:2289-2306.

Ronsisvalle G, Marrazzo A, Prezzavento O, Cagnotto A., Mennini T, Parenti C, Scoto GM (2001) Opioid and sigma receptor studies. New developments in the design of selective sigma ligands. *Pure Appl Chem*. 73:1499-1509.

Robson MJ, Noorbakhsh B, Seminerio MJ, Matsumoto RR (2012) Sigma-1 receptors: potential targets for the treatment of substance abuse. *Curr Pharm Des*. 18:902-19.

Rosow CE, Gomery P, Chen TY, Stefanovich P, Stambler N, Israel R (2007) Reversal of opioid-induced bladder dysfunction by intravenous naloxone and methylnaltrexone. *Clin Pharmacol Ther*. 82:48-53.

Rossi GC, Pasternak GW, Bodnar RJ (1993) Synergistic brainstem interactions for morphine analgesia. *Brain Res*. 624:171-80.

Sánchez-Blázquez P, García-España A, Garzón J (1995) In vivo injection of antisense oligodeoxynucleotides to G alpha subunits and supraspinal analgesia evoked by mu and delta opioid agonists. *J Pharmacol Exp Ther*. 275:1590-6.

Sánchez-Blázquez P, Rodríguez-Díaz M, DeAntonio I, Garzón J (1999) Endomorphin-1 and endomorphin-2 show differences in their activation of mu opioid receptor-regulated G proteins in supraspinal antinociception in mice. *J Pharmacol Exp Ther.* 291:12-8.

Sánchez-Blázquez P, Gómez-Serranillos P, Garzón J (2001) Agonists determine the pattern of G-protein activation in mu-opioid receptor-mediated supraspinal analgesia. *Brain Res Bull.* 54:229-35.

Sánchez-Blázquez P, Rodríguez-Muñoz M, Herrero-Labrador R, Burgueño J, Zamanillo D, Garzón J (2014) The calcium-sensitive Sigma-1 receptor prevents cannabinoids from provoking glutamate NMDA receptor hypofunction: implications in antinociception and psychotic diseases. *In J Neuropsychopharmacol.* 31:1-13.

Sánchez-Fernández C, Nieto FR, González-Cano R, Artacho-Cordón A, Romero L, Montilla-García A, Zamanillo D, Baeyens JM, Entrena JM, Cobos EJ (2013) Potentiation of morphine-induced mechanical antinociception by σ_1 receptor inhibition: Role of peripheral σ_1 receptors. *Neuropharmacology*. 70:348-58.

Sánchez-Fernández C, Montilla-García Á, González-Cano R, Nieto FR, Romero L, Artacho-Cordón A, Montes R, Fernández-Pastor B, Merlos M, Baeyens JM, Entrena JM, Cobos EJ (2014) Modulation of peripheral μ-opioid analgesia by σ1 receptors. *J Pharmacol Exp Ther*. 348:32-45.

Sandkühler J (2009) Models and mechanisms of hyperalgesia and allodynia. Physiol Rev. 89:707-58.

Sang CN, Gracely RH, Max MB, Bennett GJ (1996) Capsaicin-evoked mechanical allodynia and hyperalgesia cross nerve territories. Evidence for a central mechanism. *Anesthesiology*. 85:491-6.

Sarlani E, Balciunas BA, Grace EG (2005) Orofacial pain--Part I: Assessment and management of musculoskeletal and neuropathic causes. *AACN Clin Issues*. 16:333-46.

Satoh M and Minami M (1995) Molecular pharmacology of the opioid receptors. *Pharmacol Ther.* 68:343-64.

Sato T, Sakurada S, Takahashi N, Sakurada T, Tan-No K., Wako K, Kisara K (1999) Contribution of spinal μ_1-opioid receptors to morphine-induced antinociception. *Eur J Pharmacol.* 369:183-187.

Sawynok J and Liu XJ (2003) The Formalin Test: Characteristics and Usefulness of the Model. *In Reviews in Analgesia*. 7:145-163.

Schäfer M (2009) Opioids in Pain Medicine. In Guide to Pain Management in Low-Resources Setting. Kopf A and Patel NB eds. IASP Press. Seattle.

Schaible HG and Grubb BD (1993) Afferent and spinal mechanisms of joint pain. *Pain.* 55:5-54.

Schepers RJ and Ringkamp M (2009) Thermoreceptors and thermosensitive afferents. *Neurosci Biobehav Rev*. 33:205-12.

Schmittner J, Schroeder JR, Epstein DH, Krantz MJ, Eid NC, Preston KL (2009) Electrocardiographic effects of lofexidine and methadone coadministration: secondary findings from a safety study. *Pharmacotherapy*. 29:495-502.

Scholz J and Woolf CJ (2002) Can we conquer pain?. *Nat Neurosci*. Suppl 5:1062-7.

Schwarz S, Pohl P, Zhou GZ (1989) Steroid binding at sigma-"opioid" receptors. *Science.* 246:1635-8.

Sehgal N, Smith HS, Manchikanti L (2011) Peripherally acting opioids and clinical implications for pain control. *Pain Physician* 14:249-258.

Seth P, Leibach FH, Ganapathy V (1997) Cloning and structural analysis of the cDNA and the gene encoding the murine type 1 sigma receptor. *Biochem Biophys Res Commun*. 241:535-40.

Seth P, Ganapathy ME, Conway SJ, Bridges CD, Smith SB, Casellas P, Ganapathy V (2001) Expression pattern of the type 1 sigma receptor in the brain and identity of critical anionic amino acid residues in the ligand-binding domain of the receptor. *Biochim Biophys Acta*. 1540:59-67.

Sevostianova N, Danysz W, Bespalov AY (2005) Analgesic effects of morphine and loperamide in the rat formalin test: interactions with NMDA receptor antagonists. *Eur J Pharmacol* 525:83-90.

Shannon HE and Lutz EA (2002) Comparison of the peripheral and central effects of the opioid agonists loperamide and morphine in the formalin test in rats. *Neuropharmacology.* 42:253-61.

Sharma A and Jamal MM (2013) Opioid induced bowel disease: a twenty-first century physicians' dilemma. Considering pathophysiology and treatment strategies. *Curr Gastroenterol Rep*. 15:334.

Shen JC, Sun HL, Zhang MQ, Liu XY, Wang ZY, Yang JJ (2014) Flurbiprofen improves dysfunction of T-lymphocyte subsets and natural killer cells in cancer patients receiving post-operative morphine analgesia. *Int J Clin Pharmacol Ther*. [Epub ahead of print]

Shim SS, Grant ER, Singh S, Gallagher MJ, Lynch DR (1999) Actions of butyrophenones and other antipsychotic agents at NMDA receptors: relationship with clinical effects and structural considerations. *Neurochem Int*. 34:167-75.

Shioda N, Ishikawa K, Tagashira H, Ishizuka T, Yawo H, Fukunaga K (2012) Expression of a truncated form of the endoplasmic reticulum chaperone protein, σ_1 receptor, promotes mitochondrial energy depletion and apoptosis. *J Biol Chem*. 287:23318-31.

Simone DA and Kajander KC (1996) Excitation of rat cutaneous nociceptors by noxious cold. *Neurosci Lett.* 213:53-6.

Simone DA and Kajander KC (1997) Responses of cutaneous A-fiber nociceptors to noxious cold. *J Neurophysiol.* 77:2049-60.

Smith MT (2008) Differences between and combinations of opioids re-visited. *Curr Opin Anaesthesiol*. 21:596-601.

Schmidt B, Hammer J, Holzer P, Hammer HF (2004) Chemical nociception in the jejunum induced by capsaicin. *Gut.* 53:1109-16

Sporer KA, Firestone J, Isaacs SM (1996) Out-of-hospital treatment of opioid overdoses in an urban setting. *Acad Emerg Med*. 3:660-7.

Stein C, Schäfer M, Machelska H (2003) Attacking pain at its source: new perspectives on opioids. *Nat Med.* 9:1003-1008.

Stav A, Rabinowitz R, Korczyn AD (1992) Action of opioid agonist-antagonist drugs on the pupil and nociceptive responses in mice. *J Anesth*. 6:439-45.

Stanfield CL and Germann WJ (2006) Principles of Human Physiology. 3th ed. Etanfield C ed. Benjamin-Cummings Publishing Company. Massachusetts.

Szarvas S, Harmon D, Murphy D (2003) Neuraxial opioid-induced pruritus: a review. *J Clin Anesth.* 15:234-9.

Su TP, Wu XZ, Cone EJ, Shukla K, Gund TM, Dodge AL, Parish DW (1991) Sigma compounds derived from phencyclidine: identification of PRE-084, a new, selective sigma ligand. *J Pharmacol Exp Ther.* 259:543-550.

Su TP, Hayashi T, Maurice T, Buch S, Ruoho AE (2010) The sigma-1 receptor chaperone as an inter-organelle signaling modulator. *Trends Pharmacol Sci*. 31:557-66.

Syed YY and Keating GM (2013) Extended-release intramuscular naltrexone (VIVITROL®): a review of its use in the prevention of relapse to opioid dependence in detoxified patients. *CNS Drugs.* 27:851-61.

Tejada MA, Montilla-García A, Sánchez-Fernández C, Entrena JM, Perazzoli G, Baeyens JM, Cobos EJ (2014) Sigma-1 receptor inhibition reverses acute inflammatory hyperalgesia in mice: role of peripheral sigma-1 receptors. *Psychopharmacology (Berl).* [Epub ahead of print]

Terman GW and Bonica JJ (2001). Spinal mechanism and its modulation. In: *Bonica's Management of pain.* Loeser JD, Butler SH, Chapman CR. Turk D, eds. 3th ed. Lippincott Williams & Wilkins. Philadelphia.

Thomas J, Karver S, Cooney GA, Chamberlain BH, Watt CK, Slatkin NE, Stambler N, Kremer AB, Israel RJ (2008) Methylnaltrexone for opioid-induced constipation in advanced illness. *N Engl J Med*. 358:2332-43.

Todd AJ and Sullivan AC (1990) Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *J Comp Neurol.* 296:496-505.

Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci.* 11:823-36.

Tominaga M and Caterina MJ (2004) Thermosensation and pain. *J Neurobiol*. 61:3-12.

Treede RD, Meyer RA, Raja SN, Campbell JN (1995) Evidence for two different heat transduction mechanisms in nociceptive primary afferents innervating monkey skin. *J Physiol.* 483:747-58.

Trujillo KA and Akil H (1991) Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science*. 251:85-7.

Tseng LF, Collins KA, Wang Q (1995) Differential ontogenesis of thermal and mechanical antinociception induced by morphine and β-endorphin. *Eur J Pharmacol.* 277: 71-76.

Ueda H, Inoue M, Yoshida A, Mizuno K, Yamamoto H, Maruo J, Matsuno K, Mita S (2001) Metabotropic neurosteroid/σ-receptor involved in stimulation of nociceptor endings of mice. *J Pharmacol Exp Ther*. 298:703-710.

Upton RN (2007) Cerebral uptake of drugs in humans. *Clin Exp Pharmacol Physiol.* 34:695-701.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther*. 320:1-13.

Vadivelu N and Hines RL (2007) Buprenorphine: a unique opioid with broad clinical applications. *J Opioid Manag*. 3:49-58.

Vallbo ÅB, Olausson H, Wessberg J, Norrsell U (1993) A system of unmyelinated afferents for innocuous mechanoreception in the human skin. *Brain Res*. 628:301-304.

Vallbo ÅB, Olausson H, Wessberg J (1999) Unmyelinated afferents constitute a second system coding tactile stimuli of the human hairy skin. *J Neurophysiol*. 81:2753–2763.

Vidal-Torres A, de la Puente B, Rocasalbas M, Touriño C, Andreea Bura S, Fernández-Pastor B, Romero L, Codony X, Zamanillo D, Buschmann H, Merlos M, Baeyens JM, Maldonado R, Vela JM (2013) Sigma-1 receptor antagonism as opioid adjuvant strategy: Enhancement of opioid antinociception without increasing adverse effects. *Eur J Pharmacol*. 711: 63-72.

Vidal-Torres A, Fernández-Pastor B, Carceller A, Vela JM, Merlos M, Zamanillo D (2014) Effects of the selective sigma-1 receptor antagonist S1RA on formalin-induced pain behavior and neurotransmitter release in the spinal cord in rats. *J Neurochem.* 129:484-94.

Voss LJ, Melin S, Jacobson G, Sleigh JW (2010) GABAergic compensation in connexin36 knock-out mice evident during lowmagnesium seizure-like event activity. *Brain Res* 1360:49–55

Wagemans MF, Zuurmond WW, de Lange JJ (1997) Long-Term Spinal Opioid Therapy in Terminally Ill Cancer Pain Patients. *Oncologist.* 2:70-75.

Waldhoer M, Bartlett SE, Whistle JL (2004) Opioid receptors. *Annu Rev Biochem*. 73:953-990.

Walker JM, Bowen WD, Walker FO, Matsumoto RR, De Costa B, Rice KC (1990) Sigma receptors: biology and function. *Pharmacol Rev*. 42:355-402.

Wall PD (1978) The gate control theory of pain mechanisms. A re-examination and re-statement. *Brain.* 101:1-18.

Walsh SL and Eissenberg T (2003) The clinical pharmacology of buprenorphine: extrapolating from the laboratory to the clinic. *Drug Alcohol Depend*. 70 (Suppl 2):S13-27.

Wegert S, Ossipov MH, Nichols ML, Bian D, Vanderah TW, Malan TP Jr, Porreca F (1997) Differential activities of intrathecal MK-801 or morphine to alter responses to thermal and mechanical stimuli in normal or nerve-injured rats. *Pain.* 71:57-64.

Wenk HN, Brederson JD, Honda CN (2005) Morphine directly inhibits nociceptors in inflamed skin. *J Neurophysiol*. 95:2083-97.

Wessberg J, Olausson H, Fernström KW, Vallbo AB (2003) Receptive field properties of unmyelinated tactile afferents in the human skin. *J Neurophysiol*. 89:1567-1575.

Whistler JL (2012) Examining the role of mu opioid receptor endocytosis in the beneficial and side-effects of prolonged opioid use: from a symposium on new concepts in mu-opioid pharmacology. *Drug Alcohol Depend*. 121:189-204.

Williams J, Christie MJ, Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev*. 81:299-343.

Willis WD Jr (2007) The somatosensory system, with emphasis on structures important for pain. *Brain Res Rev*. 55:297-313.

Willis WD Jr (2009) Spinal cord pain systems. In: Encyclopedia of Neuroscience online. Squire LR, ed. online. Elsevier. http://www.sciencedirect.com/science/referenceworks/9780080450469.

Willis WD and Coggeshall RE (2004) Primary Afferente Neurons and the Spinal Dorsal Horn. In: Sensory mechanisms of the Spinal Cord. 3th ed. Kluwer Academic/Plenum Publishers. New York.

Woolf CJ (2004) American College of Physicians; American Physiological Society. Pain: moving from symptom control toward mechanism-specific pharmacologic management. *Ann Intern Med*. 140:441-51.

Xu J, Xu M, Brown T, Rossi GC, Hurd YL, Inturrisi CE, Pasternak GW, Pan YX (2013) Stabilization of the μ-opioid receptor by truncated single transmembrane splice variants through a chaperone-like action. *J Biol Chem*. 288:21211-27.

Yaksh TL, and Rudy TA (1976) Analgesia mediated by a direct spinal action of narcotics. *Science*. 192:1357–8.

Yassen A, Olofsen E, Dahan A, Danhof M (2005) Pharmacokinetic-pharmacodynamic modeling of the antinociceptive effect of buprenorphine and fentanyl in rats: role of receptor equilibration kinetics. *J Pharmacol Exp Ther.* 313:1136-1149.

Zamanillo D, Romero L, Merlos M, Vela JM (2013) Sigma 1 receptor: A new therapeutic target for pain. *Eur J Pharmacol.*16:78-93.

Zand F, Amini A, Asadi S, Farbood A (2014) The Effect of Methylnaltrexone on the Side Effects of Intrathecal Morphine after Orthopedic Surgery under Spinal Anesthesia. *Pain Pract*. [Epub ahead of print]

Zhou HY, Chen SR, Pan HL (2011) Targeting N-methyl-D-aspartate receptors for treatment of neuropathic pain. *Expert Rev Clin Pharmacol*. 4:379-88.

Zhuo M, Gebhart GF (1992) Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. *J Neurophysiol*. 67:1599-614.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain.* 16: 109-110.

Zukin SR, Brady KT, Slifer BL, Balster RL (1984) Behavioral and biochemical stereoselectivity of sigma opiate/PCP receptors. *Brain Res*. 294:174-7.

Zwick M, Davis BM, Woodbury CJ, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002) Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. *J Neurosci*. 22:4057-65.

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