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DEPARTAMENTO DE FARMACOLOGÍA

ROLE OF SIGMA-1 RECEPTORS IN CAPSAICIN-INDUCED MECHANICAL HYPERSENSITIVITY: STUDIES WITH SIGMA-1 LIGANDS, KNOCKOUT MICE AND ANTISENSE OLIGODEOXYNUCLEOTIDES

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"El más bello sentimiento que uno puede experimentar es sentir misterio. Esta es la fuente de todo arte verdadero, de toda verdadera ciencia. Aquel que nunca ha conocido esta emoción, que no posee el don de maravillarse ni encantarse... sus ojos están cerrados..."

Albert Einstein

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RESUMEN

ANTECEDENTES, HIPÓTESIS Y OBJETIVOS

La existencia de los denominados receptores sigma (σ) fue propuesta por Martin y colaboradores en 1976. Inicialmente, fueron considerados un subtipo de receptor opiode y más tarde fueron confundidos con el sitio de unión de la fenciclidina en el receptor de NMDA (*N*-metil-*D*-aspartato). Hoy en día, los receptores σ son considerados como una entidad farmacológica independiente (revisado por Guitart et al., 2004; Monnet y Maurice, 2006), en la que se han caracterizado farmacológicamente dos subtipos, denominados σ_1 y σ_2 (revisado por Matsumoto et al., 2003; Cobos et al., 2008a). El receptor σ_1 ha sido clonado en humanos y roedores, incluyendo ratones (Hanner et al., 1996; Prasad et al., 1998; Pan et al., 1998), y no muestra homología con ninguna otra proteína de mamíferos (Guitart et al., 2004; Monnet y Maurice, 2006), mientras que el receptor σ_2 no ha sido clonado todavía.

Actualmente, la farmacología del receptor σ_1 está bien caracterizada, y hay disponibles agonistas selectivos, como (+)-pentazocina y PRE-084, y antagonistas selectivos, como BD-1063, BD-1047 y NE-100 (Guitart et al., 2004; Hayashi y Su, 2004; Cobos et al., 2008a). Algunos neuroesteroides, psicoestimulantes y antipsicóticos también se unen a los receptores σ_1 (Maurice et al., 2001; Monnet y Maurice, 2006). Entre los antipsicóticos, el haloperidol (HP) merece una consideración especial, porque aunque es conocido principalmente como un antagonista del receptor D_2, muestra una afinidad equiparable por los receptores σ_1 (Bowen et al., 1990; Matsumoto y Pouw, 2000), sobre los que se comporta como un antagonista (Maurice et al., 2001; Hayashi y Su, 2004). Sus metabolitos, haloperidol metabolito I (HP-Met-I) y II (HP-Met-II), son también antagonistas σ_1 (Cendán et al., 2005a), y muestran una afinidad preferencial por

los receptores σ_1 en relación con la que presentan por los receptores D_2 (Bowen et al., 1990; Matsumoto y Pouw, 2000).

Los receptores sigma-1 (σ_1) están implicados en la nocicepción, entre otros procesos. Se distribuyen en áreas del sistema nervioso central con gran importancia en el control del dolor, tales como las láminas superficiales del asta dorsal de la médula espinal, el locus coeruleus y la médula rostroventral (Alonso et al., 2000; Kitaichi et al., 2000). La potenciación de la antinocicepción opiode por los antagonistas del receptor σ_1 ha sido documentada ampliamente (Chien y Pasternak, 1993; Marrazzo et al., 2006; Mei y Pasternak, 2002 y 2007), aunque los receptores σ_1 también están implicados en la nocicepción en ausencia de opiodes. De hecho, estudios realizados por nosotros y otros grupos de investigación han demostrado que tanto la inactivación genética (en ratones "knockout" σ_1) como el antagonismo farmacológico de los receptores σ_1, producen efectos antinociceptivos en el test de la formalina (Cendán et al., 2005a y 2005b, Kim et al., 2006). Cabe destacar, que en la segunda fase del dolor inducido por formalina está implicado el fenómeno de sensibilización central, en el cual se produce una fosforilación del receptor de NMDA a nivel espinal, induciéndose así una potenciación de su actividad (Sawynok y Liu, 2004).

Este fenómeno de sensibilización central ocurre tanto durante el dolor inflamatorio, como en el dolor neuropático; es iniciado por la estimulación mantenida de los nociceptores periféricos, y resulta en una potenciación de la sensibilidad de las neuronas implicadas en la transmisión del dolor a nivel tanto del asta dorsal de la médula espinal como en el cerebro (Woolf y Salter, 2000, Ji y Woolf, 2001, Ji et al., 2003). El desarrollo y mantenimiento de la sensibilización central depende en gran medida de la activación del receptor de NMDA (Willis et al., 2001; Ji and Woolf,

2001). Los receptores σ_1 juegan un papel importante en la modulación de la actividad del receptor de NMDA (revisado por Debonnell y Montigny, 1996; Cobos et al., 2008a), así como en su fosforilación (Kim et al., 2006 y 2008); además, estudios recientes muestran que los receptores σ_1 pueden incluso modular el dolor agudo inducido por la administración intratecal de NMDA (Kim et al., 2008). Teniendo en cuenta estos antecedentes, la **hipótesis principal** de está Tesis Doctoral fue que los receptores σ_1 podrían estar implicados en la sensibilización central de las vías del dolor, y que los fármacos que actúan sobre estos receptores podrían inhibir este proceso, pudiendo ser útiles para el tratamiento de ciertos tipos de dolor que impliquen este fenómeno. En particular, nuestro estudio se centra en el papel del receptor σ_1 en uno de los síntomas más característicos de la sensibilización central y del dolor patológico: la sensibilización a un estímulo mecánico (alodinia mecánica).

La inyección de capsaicina induce un incremento en la sensibilidad al dolor en el área próxima al sitio de inyección de capsaicina (área de hipersensibilidad mecánica secundaria), que resulta del proceso de sensibilización central (Sang et al., 1996; Baron, 2000). Los cambios en la hipersensibilidad mecánica inducida por capsaicina han sido usados como modelo para estudiar las consecuencias comportamentales del tratamiento farmacológico de la sensibilización central (por ejemplo, Park et al., 1995; Baumgärtner et al., 2002; Gottrup et al., 2004; Bingham et al., 2005). Además, la hipersensibilidad mecánica inducida por capsaicina se considera un modelo predictor del dolor neuropático, ya que no sólo mimetiza los cambios sensoriales de esta enfermedad (Baumgärtner et al., 2002; Klein et al., 2005), sino que los fármacos antineuropáticos disminuyen la hipersensibilidad mecánica inducida tanto por capsaicina, como por una neuropatía (Gottrup et al., 2004; Joshi et al., 2006; Dworkin et al., 2007; Hagen et al.,

2008; Nieto et al., 2008). Sin embargo, no se ha descrito previamente el posible papel de los receptores σ_1 en la sensibilización a estímulos mecánicos inducida por capsaicina. Además, la implicación de estos receptores en el dolor inducido por una estimulación mecánica nociva en animales no sensibilizados tampoco ha sido estudiada con anterioridad.

Teniendo en cuenta estos antecedentes, el **objetivo principal** de esta Tesis Doctoral fue determinar si el receptor σ_1 podría ser considerado como una nueva diana farmacológica para el tratamiento de la alodinia mecánica o del dolor nociceptivo mecánico de carácter puntiforme. Para cumplir este objetivo, utilizamos tres herramientas experimentales: ratones deprovistos del receptor σ_1 (knockout σ_1), fármacos antagonistas y agonistas para el receptor σ_1, y oligodesoxinucleótidos antisentido (ASOs, abreviado del inglés "antisense oligodeoxynucleotides") frente al receptor σ_1.

La disponibilidad de ratones knockout σ_1 (desarrollados por Langa et al., 2003) ofrece grandes posibilidades para el estudio funcional de estos receptores, por lo que el **primer objetivo** de esta Tesis Doctoral fue comparar las características del dolor nociceptivo mecánico y de la alodinia mecánica inducida por capsaicina, en ratones salvajes y knockout para el receptor σ_1.

Como ha sido mencionado anteriormente, hay disponible en la actualidad una gran variedad de ligandos para el receptor σ_1. Por lo tanto, el **segundo objetivo** de esta Tesis Doctoral, fue evaluar si el bloqueo farmacológico del receptor σ_1, tanto por antagonistas selectivos (BD-1063, BD-1047 y NE-100), como por antagonistas no selectivos de este receptor (haloperidol y sus metabolitos), modula la alodinia mecánica y/o el dolor

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nociceptivo; así como estudiar si la coadministración con un agonista selectivo σ_1 (PRE-084) revierte los efectos de los antagonistas σ_1.

La sensibilización central en la médula espinal se acompaña de cambios sustanciales en el procesamiento de la información nociceptiva a nivel cerebral, produciéndose un fenómeno similar al de sensibilización central a nivel supraespinal, que contribuye al incremento de la sensibilidad al dolor (revisado por Porreca et al., 2002; Ji et al., 2003). Estos cambios en la actividad supraespinal relacionados con el dolor han sido documentados ampliamente en el modelo de estimulación mecánica en el área de hipersensibilidad mecánica secundaria inducida por capsaicina (Iadarola et al., 1998; Baron et al., 1999; Iannetti et al., 2005; Zambreanu et al., 2005). Sin embargo, el posible papel modulador de los receptor σ_1 supraespinales en este proceso no ha sido estudiado con anterioridad. Por lo tanto, el **tercer objetivo** de esta Tesis Doctoral fue evaluar los efectos en la alodinia mecánica inducida por capsaicina y en el dolor nociceptivo mecánico (en animales no sensibilizados con capsaicina) de la inhibición supraespinal de los receptores σ_1, inducida por la inyección intracerebroventricular de dos ASOs diferentes para el receptor σ_1.

MÉTODOS

Animales de experimentación

Todos los experimentos fueron realizados en ratones hembra de la cepa CD-1 (Charles River, Barcelona, España) con un peso de 25-30 g. Para obtener los ratones knockout σ_1 de la cepa CD-1, los animales previamente generados por Langa y colaboradores (2003) fueron cruzados durante 10 generaciones con ratones CD-1 puros, asegurando de esta manera una pureza de la cepa CD-1 de al menos un 99% (Wong et

al., 2002); los ratones portadores de la mutación fueron posteriormente cruzados hasta obtener individuos knockout homocigotos, los cuales fueron utilizados en el curso de esta Tesis Doctoral. Los ratones 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 en Investigación de la Universidad de Granada, España.

A. Experimentos *in vivo*

Evaluación de la respuesta comportamental frente a la estimulación mecánica. Aspectos generales

Los animales fueron colocados individualmente dentro de los compartimentos de evaluación (situados en una plataforma elevada con un suelo de maya metálica) durante dos horas, con el fin de habituarlos a las condiciones de experimentación. Tras este periodo de tiempo, los animales fueron inyectados intraplantarmente (i.pl.) con capsaicina (o su solvente, DMSO 1% en salino fisiológico) en la pata trasera derecha, e inmediatamente devueltos a sus compartimentos. Quince minutos después de la inyección de capsaicina (tiempo de máxima sensibilización) o su solvente, se aplicó el estímulo mecánico puntiforme a través de la maya metálica, utilizando un algesímetro electrónico (*Dynamic Plantar Aesthesiometer*, Ugo Basile, Varese, Italia). Este aparato utiliza un único filamento rígido (con un diametro de 0.5 mm), que fue dirigido electrónicamente hacia la parte ventral de la pata trasera derecha que previamente había sido inyectada. El filamento fue aplicado a una distancia aproximada de 5 mm desde el lugar de la inyección en dirección hacia los dedos. Cuando el animal retira la pata, el estímulo mecánico cesa automáticamente, quedando registrado el tiempo de latencia de

la respuesta. Este tipo de registro nos permite utilizar el tiempo de latencia de retirada de la pata como un indicador de la percepción del dolor en los ratones evaluados. Cada animal fue evaluado tres veces, dejando un intervalo de 0,5 min entre cada una de las estimulaciones, y con un tiempo máximo de estimulación de 50 s (tiempo de corte).

Para cuantificar la fuerza necesaria para inducir la retirada de la pata, evaluamos el efecto producido por la aplicación del filamento en un amplio rango de intensidades de estimulación (0,05 – 8 g de fuerza), tanto en animales previamente inyectados con capsaicina (1 μg) como en los inyectados con su solvente (DMSO 1%). Este método nos permitió estudiar la relación entre la intensidad de la estimulación y la respuesta producida en el animal (curva fuerza-respuesta), que permite cuantificar el grado de dolor nociceptivo mecánico para cada intensidad de estimulación (en ratones no sensibilizados), así como valorar, en animales sensibilizados, la hipersensibilidad mecánica inducida por una dosis constante (1 μg) de capsaicina. Además, este tipo de curvas hace posible la comparación entre ambas condiciones experimentales (dolor nociceptivo e hipersensibilidad mecánica) mediante el cálculo del parámetro Fuerza Eficaz 50 (FE_{50}), que se define como la intensidad de estimulación (en g de fuerza) necesaria para producir la mitad de la reducción máxima del tiempo de latencia de retirada de la pata.

Para estudiar el efecto de los distintos tratamientos utilizados en esta Tesis Doctoral, usamos dos tipos diferentes de estimulación mecánica. Para evaluar los efectos en la alodinia mecánica utilizamos una estimulación de 0,5 g de fuerza, ya que esta intensidad del estímulo no indujo retirada de la pata en animales tratados con el solvente de la capsaicina (es decir, fue un estímulo inocuo), mientras que redujo marcadamente el tiempo de latencia de retirada de la pata en ratones sensibilizados con

capsaicina. Para la evaluación de los efectos de los tratamientos en el dolor mecánico puntiforme, los animales inyectados con DMSO (no sensibilizados) fueron estimulados con el filamento a una intensidad de 4 g de fuerza. Esta intensidad de estimulación produjo una rápida retirada de la pata en animales no sensibilizados (estímulo nocivo), siendo el tiempo de latencia de esta respuesta similar a aquel obtenido en los animales sensibilizados con capsaicina y estimulados a una intensidad de 0,5 g de fuerza. Por consiguiente, tanto una estimulación con 4 g de fuerza en animales no sensibilizados como un estímulo de 0,5 g en animales sensibilizados con capsaicina indujeron respuestas comportamentales equivalentes, de modo que estas aproximaciones nos permitieron comparar los efectos de los distintos tratamientos estudiados en el dolor nociceptivo mecánico y en la alodinia mecánica inducida por capsaicina.

Comparación de la alodinia mecánica y del dolor nociceptivo mecánico puntiforme en ratones salvajes y knockout para el receptor σ_1

Para comparar las características del dolor nociceptivo mecánico y de la alodinia mecánica inducida por capsaicina, en ratones salvajes y knockout σ_1 **(primer objetivo)**, utilizamos dos aproximaciones experimentales diferentes. En primer lugar, realizamos una curva fuerza-respuesta (es decir, intensidad de los estímulos *vs* tiempo de latencia de retirada de la pata) en ratones salvajes y knockout σ_1, previamente inyectados con capsaicina (1 μg) o su solvente (DMSO 1%). Esta aproximación nos permitió comparar la respuesta comportamental de ratones salvajes y ratones knockout σ_1, frente al dolor nociceptivo mecánico y frente a la alodína mecánica. En segundo lugar, los animales fueron inyectados intraplantarmente con diferentes dosis de capsaicina (0,125 – 4 μg) o su solvente, para ser posteriormente estimulados con una intensidad de 0,5 g de fuerza

(estímulo no doloroso en condiciones normales). Con esta segunda aproximación pudimos obtener una relación entre la dosis de capsaicina administrada y el grado de alodinia mecánica inducido (disminución del tiempo de latencia de retirada de la pata), tanto en los ratones salvajes como en los ratones knockout σ_1.

Evaluación de los efectos de los fármacos en la alodínia mecánica y en el dolor nociceptivo mecánico

Para cumplir el **segundo objetivo** de esta Tesis Doctoral, evaluamos los efectos de varios ligandos σ_1, tanto en la hipersensibilidad mecánica inducida por capsaicina como en el dolor nociceptivo mecánico puntiforme, y comparamos sus efectos con aquellos producidos por varios fármacos control.

Para ello, usamos tanto antagonistas selectivos para el receptor σ_1 (BD-1063, BD-1047 y NE-100) como no selectivos (HP y sus metabolitos I, II y III) (Hayashi and Su, 2004; Guitart et al., 2004; Cobos et al., 2008a). Además, con el fin de controlar la influencia del antagonismo dopaminérgico en los efectos inducidos por el haloperidol y sus metabolitos, evaluamos el efecto del (-)-sulpiride, un antagonista de los receptores D_2/D_3 que carece de afinidad por los receptores σ_1 (Freedman et al., 1994; Matsumoto and Pouw, 2000). Igualmente, comparamos los efectos producidos por los ligandos σ_1 con los ejercidos por fármacos con actividad antialodínica previamente descrita (gabapentina, pregabalina y tetrodotoxina) (Dworkin et al., 2007; Hagen et al., 2008; Nieto et al., 2008). Como control de fármacos activos frente a la alodinia mecánica y el dolor nociceptivo, usamos mexiletina y clonidina (Khandwala et al., 1997; Paqueron et al., 2003). Como control negativo utilizamos rofecoxib, un fármaco antiinflamatorio (Moore et al., 2005), que carece tanto de actividad antinociceptiva como antialodínica

en animales sin inflamación (Bingham et al., 2005; Padi y Kulkarni, 2004). Con el objetivo de identificar la posible implicación de los receptores σ_1 en los efectos inducidos por los fármacos anteriormente mencionados, evaluamos los efectos del agonista selectivo σ_1 PRE-084 (Su et al., 1991) *per se* en la alodinia mecánica y en el dolor nociceptivo mecánico, así como si este fármaco podría revertir los efectos de los antagonistas σ_1 y de los fármacos antineuropáticos evaluados.

Para estudiar los efectos de estos fármacos, los animales fueron inyectados subcutáneamente (s.c.) con las soluciones de los fármacos (o su solvente) en la región interescapular en un volumen de 5 ml/kg; 45 minutos después (es decir, 15 minutos después de la administración de capsaicina o su solvente), evaluamos la alodinia mecánica o el dolor nociceptivo mecánico (como se describe anteriormente). Cuando utilizamos PRE-084 para revertir los efectos de los fármacos evaluados, la solución de PRE-084 fue inyectada (via s.c.) inmediatamente antes de la otra solución. Cada inyección fue realizada en áreas corporales diferentes para evitar la mezcla de las soluciones de los fármacos, evitando así cualquier interacción fisicoquímica entre ellas que pudiera interferir con los resultados.

Evaluación de los efectos de la inhibición supraespinal de los receptores σ_1 en la alodinia mecánica y en el dolor nociceptivo mecánico

Para evaluar los efectos en la alodinia mecánica inducida por capsaicina y en el dolor nociceptivo mecánico, de la inhibición de los receptores σ_1 localizados supraespinalmente **(tercer objetivo)**, los animales fueron tratados intracerebroventricularmente (i.c.v.), durante 4 dias consecutivos, con dos oligodesoxinucleótidos antisentido (ASOs) fosfotiolados diferentes, aunque ambos

dirigidos frente a la secuencia de ADNc del receptor σ_1 de ratón. Estos ASOs fueron diseñados para hibridar con áreas diferentes del ARNm diana. El ASO-A hibridaría con una secuencia posterior a la del codón de inicio (desde +77 a +97) (King et al., 1997; Pan et al., 1998); mientras que el ASO-B fue diseñado para hibridar con la secuencia que contiene al codón de inicio (desde -11 a +9) (Ueda et al., 2001).

Para comprobar la especificidad de los efectos inducidos por ambos ASOs, los animales fueron inyectados i.c.v. con dos oligodesoxinucleótidos fosfotiolados control (COs). Como control del ASO-A se intercambiaron tres pares de bases dentro de su secuencia, para generar el CO-A (King et al., 1997; Pan et al., 1998). Como control del ASO-B, su secuencia fue aleatorizada, para obtener el CO-B. Esta secuencia no mostró ninguna complementariedad con otra secuencia de ADNc registrada en la Base de Datos GenBank (NIH, Bethesda, MD, USA). La evaluación de la hipersensibilidad mecánica inducida por capsaicina y del dolor nociceptivo mecánico puntiforme fue realizada cinco y once días tras la primera administración de los ASOs σ_1, COs o su solvente (es decir, 24 horas y una semana después de la finalización de los tratamientos), utilizando el protocolo descrito en apartados anteriores.

B. Experimentos *in vitro*

Ensayos de fijación de [^{3}H](+)-pentazocina

Para estudiar los receptores σ_1 en fracción sinaptosomal cruda (fracción P_2) de cerebro completo de ratón (obtenida siguiendo el protocolo descrito por González y cols., 2001), realizamos ensayos de fijación de radioligando, marcando los receptores σ_1 con [^{3}H](+)-pentazocina (un radioligando selectivo del receptor σ_1). Para estudiar el número de receptores σ_1 en ratones salvajes y knockout σ_1, realizamos **ensayos de**

saturación de radioligando. Para estudiar la afinidad por los receptores σ_1 de cerebro de ratón de los fármacos evaluados en los ensayos *in vivo,* se realizaron **ensayos de competición** de radioligando. Estos ensayos de fijación fueron realizados usando los protocolos previamente descritos (Cobos et al., 2007), levemente modificados. Brevemente, las suspensiones de membranas cerebrales (en una concentración final de proteína de 1 mg/ml) fueron incubadas con [^{3}H](+)-pentazocina (a una concentración final de 5 nM en los ensayos de competición y de 0,1 – 37 nM en los experimentos de saturación) y con el fármaco frío o su solvente, a 30 ºC, pH 8, durante 240 minutos. La fijación no específica fue definida como la fijación retenida en presencia de 10 μM de HP. La reacción se detuvo con 5 ml de Tris 10 mM pH 7,4 enfriado en hielo, y el radioligando unido fue separado del libre mediante filtración (Brandel cell harvester; Brandel Instruments, SEMAT Technical Ltd., UK). Los filtrados de las muestras se realizaron a través de filtros de fibra de vidrio (Whatman GF/B), previamente humedecidos con una solución de polietilenimina al 5% durante al menos una hora. Posteriormente la radioactividad contenida en los filtros se midió en un contador de centelleo líquido (Beckman Coulter España S.A).

RESULTADOS Y DISCUSIÓN

Comparación de la alodinia mecánica y del dolor mecánico puntiforme en ratones salvajes y knockout para el receptor σ_1

Para comparar el grado de alodinia mecánica y de dolor mecánico puntiforme en ratones salvajes y knockout σ_1, los animales fueron tratados i.pl. con 1 μg de capsaicina o su solvente (DMSO 1%). Posteriormente, se les aplicó el filamento a diferentes intensidades de estimulación (que variaron desde estimulaciones inocuas a nocivas), con

objeto de construir una curva fuerza-respuesta (ver Métodos para más detalles). La mutación en los ratones knockout σ_1 fue confirmada mediante ensayos de fijación de [^{3}H](+)-pentazocina.

A medida que la fuerza del estímulo mecánico aplicado en la pata fue aumentando, el tiempo de latencia para la retirada de la pata fue disminuyendo, de modo similar en los ratones salvajes y en los knockout σ_1. Esto sugiere que la mutación en los ratones knockout para el receptor σ_1 no afectó a la percepción del estímulo mecánico. La inyección de capsaicina en los animales salvajes indujo una disminución de la FE_{50} de 9,4 veces, en comparación con los ratones salvajes tratados con el solvente de la capsaicina (DMSO 1%). En cambio, la curva fuerza-respuesta para los ratones knockout σ_1 tratados con 1 μg de capsaicina, fue equivalente a la de los animales knockout tratados con DMSO. Por lo tanto, la inyección de capsaicina indujo hipersensibilidad mecánica en los ratones salvajes, pero no en los knockout para el receptor σ_1.

También comparamos los efectos de diferentes dosis de capsaicina en los ratones salvajes y en los knockout σ_1, evaluando el tiempo de latencia de retirada de la pata tras la estimulación con el filamento a una intensidad inocua (0,5 g de fuerza). Cuando los ratones salvajes fueron inyectados con capsaicina (0,125 – 4 μg), el tiempo de latencia de retirada de la pata se redujo marcadamente y de manera dosis-dependiente (es decir, se indujo alodinia mecánica). Esta alodinia mecánica fue máxima tras la administración de 1 – 2 μg de capsaicina. Sin embargo, bajo las mismas condiciones experimentales, los ratones knockout σ_1 mostraron sólo una modesta reducción del tiempo de latencia de retirada de la pata inyectada con capsaicina.

En resumen, encontramos que los ratones knockout σ_1 no fueron capaces de ser sensibilizados por capsaicina. Sin embargo, la inactivación genética de los receptores σ_1

no afectó ni a la percepción del estímulo mecánico, ni a la respuesta motora necesaria para la retirada de la pata (ya que los animales salvajes y los knockout σ_1, no sensibilizados, mostraron respuestas equivalentes). Además, bajo nuestras condiciones experimentales, los ratones salvajes fueron sensibilizados al estímulo mecánico puntiforme tras la administración de capsaicina, como se había descrito previamente (Gilchrist et al., 1996; Joshi et al., 2006), por lo que nuestros resultados no pueden ser justificados por algún tipo de limitación metodológica.

Efectos de los fármacos en la alodinia mecánica inducida por capsaicina y en el dolor nociceptivo mecánico puntiforme, en ratones salvajes

La administración subcutánea tanto de los antagonistas selectivos σ_1 (BD-1063, BD-1047 y NE-100), como de los antagonistas no selectivos (HP, HP-Met-I y HP-Met-II), indujo un efecto antialodínico de manera dosis dependiente; es decir, los antagonistas σ_1 evaluados incrementaron el tiempo de latencia de retirada de la pata inyectada con capsaicina, cuando fue estimulada con el filamento a una intensidad de 0,5 g de fuerza. Los ensayos de competición con el radioligando mostraron que todos estos fármacos desplazaron a la [^{3}H](+)-pentazocina de su sitio específico de fijación en membranas de cerebro de ratón, lo que demuestra que tienen afinidad por el receptor σ_1. Cabe destacar que tanto (-)-sulpiride (usado como control de antagonista dopaminérgico), como un metabolito adicional del HP, el HP-Met-III, carecieron tanto de actividad antialodínica como de afinidad por los receptores σ_1, según mostraron los ensayos de fijación de radioligando realizados.

Cuando coadministramos los antagonistas del receptor σ_1 con el agonista selectivo σ_1 PRE-084, encontramos que este compuesto, a dosis que no modificaron el tiempo de

latencia de retirada de la pata en animales tratados con capsaicina, previniendo el efecto antialodínico inducido por todos los antagonistas σ_1 evaluados. Estos resultados indican que los antagonistas σ_1 evaluados (tanto los selectivos como los no selectivos), inhibieron la sensibilización inducida por capsaicina a través del bloqueo del receptor σ_1.

También estudiamos los efectos de los antagonistas σ_1 en el dolor nociceptivo mecánico de carácter puntiforme. Cuando administramos estos fármacos a las mismas dosis que produjeron un efecto antialodínico máximo, no se produjo incremento alguno en la latencia de retirada de la pata de los ratones no sensibilizados (tratados con DMSO 1%), y estimulados con un estímulo nocivo puntiforme (filamento con una intensidad de 4 g de fuerza); es decir, estos fármacos no ejercieron ningún efecto antinociceptivo. Es destacable que tanto, la actividad antialodínica de los antagonistas σ_1, como su ausencia de efecto contra el dolor nociceptivo, están en concordancia con los resultados obtenidos en los ratones knockout σ_1.

Así mismo, se comparó la actividad de los antagonitas σ_1 evaluados con la de varios fármacos controles. En primer lugar, estudiamos la afinidad de estos fármacos control por los receptores σ_1, encontrando que todos estos fármacos control carecieron de afinidad por estos receptores. Los efectos antialodínicos y antinociceptivos de los fármacos controles fueron evaluados con la misma metodología que fue utilizada para evaluar los efectos de los antagonistas σ_1. Observamos que pregabalina, gabapentina y tetrodotoxina fueron capaces de inhibir la alodinia mecánica inducida por capsaicina, sin ejercer ningún efecto antinociceptivo frente al dolor mecánico puntiforme. Estos resultados eran esperables, ya que estudios previos describieron que estos fármacos inhibían la alodinia mecánica en modelos de dolor neuropático (Joshi et al., 2006; Han

et al., 2007; Nieto et al., 2008), a dosis que no ejercieron efectos antinociceptivos en los animales control (Fields et al., 1997; Joshi et al., 2006; Nieto et al., 2008). Por otro lado, mexiletina y clonidina mostraron efectos tanto antialodínicos como antinociceptivos. Estos datos también concuerdan con los resultados previamente descritos para estos dos fármacos (Khandwala et al., 1997; Honda et al., 2002; Paqueron et al., 2003). Por otra parte, el rofecoxib (utilizado como control negativo) careció tanto de actividad contra la alodinia mecánica como contra el dolor nociceptivo mecánico puntiforme, lo cual también es congruente con los datos previamente descritos acerca de la ausencia de efecto de este fármaco en la hipersensibilidad mecánica inducida por una neuropatía o por la administración de capsaicina (Padi y Kulkarni, 2004; Bingham et al., 2005). Estos resultados obtenidos con los fármacos control, indican que la metodología usada en esta Tesis Doctoral es adecuada y fiable para estudiar la alodinia mecánica y el dolor nociceptivo, y además ayudan a validar los resultados obtenidos con los antagonistas del receptor σ_1.

También cabe destacar que los efectos antialodínicos de pregabalina, mexiletina o tetrodotoxina no pudieron ser revertidos con la coadministración de PRE-084. Estos reultados evidencian que el bloqueo del receptor σ_1 podría ser una nueva diana farmacológica contra la hipersensibilidad mecánica, que no es compartida por otros fármacos antineuropáticos conocidos.

Efectos de la administración intracerebroventricular de oligodesoxinucleótidos antisentido del receptor σ_1 en la alodinia mecánica inducida por capsaicina y en el dolor nociceptivo en ratones salvajes

El tratamiento durante cuatro días consecutivos con ASO-A y ASO-B, inhibió de modo dosis-dependiente la alodinia mecánica inducida por capsaicina (es decir, incrementó de manera dosis-dependiente el tiempo de latencia de retirada de la pata inyectada con capsaicina, al ser estimulada con el filamento a una intensidad de 0,5 g de fuerza), cuando los animales fueron evaluados 24 h tras la última administración de los ASOs en estudio. Este efecto inducido por los ASOs desapareció completamente cuando evaluamos a los animales una semana después de la última inyección i.c.v. Por lo tanto, ninguno de estos ASOs indujo un cambio permanente de la hipersensibilidad mecánica inducida por capsaicina. Por otro lado, ni el ASO-A ni el ASO-B exhibieron ningún efecto antinociceptivo contra un estímulo mecánico nocivo puntiforme (4 g de fuerza).

Como era esperable, la administración repetida por vía i.c.v. de los CO-A y CO-B (usados como controles de ASO-A y ASO-B, respectivamente), o del salino (solvente de los oligodesoxinucleótidos), no ejerció ningún efecto en la alodinia mecánica o en el dolor nociceptivo, a ninguno de los tiempos evaluados. Estos datos refuerzan la especificidad de los efectos antialodínicos inducidos por la inhibición del receptor σ_1 por los ASOs, e indican que el procedimiento de administración i.c.v. no produjo ninguna alteración cerebral capaz de modificar la respuesta al estímulo mecánico en las diferentes condiciones experimentales.

Estos hallazgos sugieren que los receptores σ_1 participan en las modificaciones que ocurren en el procesamiento de la información dolorosa que ocurren a nivel

supraespinal tras la sensibilización con capsaicina, sin interferir en los mecanismos fisiológicos cerebrales implicados en el dolor nociceptivo mecánico.

CONCLUSIONES

Conclusiones específicas

1. La inactivación genética de los receptores σ_1 (en ratones knockout σ_1) inhibió completamente la hipersensibilidad mecánica inducida por la capsaicina en el ratón.
2. El bloqueo farmacológico de los receptores σ_1, mediante antagonistas tanto selectivos como no selectivos, indujo efectos antialodínicos de manera dosis-dependiente. Estos efectos fueron revertidos con el agonista selectivo σ_1 PRE-084.
3. La inhibición supraespinal de los receptores σ_1 inducida por oligodesoxinucleótidos antisentido frente al receptor σ_1 (inyectados i.c.v.), inhibió la hipersensibilidad mecánica inducida por capsaicina. Este efecto fue transitorio, desapareciendo completamente en el plazo de una semana tras la última administración.
4. La inactivación genética de los receptores σ_1, el antagonismo farmacológico, o la inhibición supraespinal de estos receptores por oligodesoxinucleótidos antisentido, no interfirió con la percepción del dolor nociceptivo mecánico.

Conclusión general

Los receptores σ_1 juegan un papel importante en los mecanismos implicados en la hipersensibilidad mecánica inducida por capsaicina (pero no en el dolor nociceptivo mecánico). Por consiguiente, el bloqueo de los receptores σ_1 podría ser una nueva diana farmacológica para el tratamiento de la alodinia mecánica.

SUMMARY

BACKGROUND, HYPOTHESIS AND GOALS

The existence of sigma (σ) receptors was first proposed by Martin and collaborators in 1976. Initially considered a subtype of opioid receptor and later confused with phencyclidine binding sites in NMDA (*N*-methyl-*D*-aspartate) receptors, σ receptors are now considered a distinct pharmacological entity (reviewed by Guitart et al., 2004; Monnet and Maurice, 2006). Two subtypes (σ_1 and σ_2) have been pharmacologically characterized (reviewed by Matsumoto et al., 2003; Cobos et al., 2008a). The σ_1 receptor has been cloned in humans and rodents, including mice (Hanner et al., 1996; Prasad et al., 1998; Pan et al., 1998), and it shows no homology with other mammalian proteins (Guitart et al., 2004; Monnet and Maurice, 2006). The σ_2 receptor has still not been cloned.

The pharmacology of σ_1 receptors is now well characterized, and selective agonists, such as (+)-pentazocine and PRE-084, and antagonists, such as BD-1063, BD-1047 and NE-100, are both available (Guitart et al., 2004; Hayashi and Su, 2004; Cobos et al., 2008a). Some neurosteroids, psychostimulants and antipsychotics also bind to σ_1 receptors (Maurice et al., 2001; Monnet and Maurice, 2006). Among the antipsychotics, haloperidol (HP) deserves special consideration because, although mainly known as a D_2 receptor antagonist, it shows the same affinity for D_2 as for σ_1 receptor (Bowen et al., 1990; Matsumoto and Pouw, 2000) and exhibits σ_1 receptor antagonistic activity (Maurice et al., 2001; Hayashi and Su, 2004). Its metabolites haloperidol metabolite I (HP-Met-I) and II (HP-Met-II) are also σ_1 antagonists (Cendán et al., 2005a) and display preferential activity at σ_1 receptors *versus* dopamine D_2 receptors (Bowen et al., 1990; Matsumoto and Pouw, 2000).

Sigma-1 (σ_1) receptors are involved in nociception, among other processes. They are distributed in the central nervous system at areas of major importance for pain control, such as the superficial layers of the spinal cord dorsal horn, the periaqueductal gray matter, the locus coeruleus and the rostroventral medulla (Alonso et al., 2000; Kitaichi et al., 2000). The enhancement of opioid-induced antinociception by σ_1 receptor antagonists has been extensively documented (Chien and Pasternak, 1993; Marrazzo et al., 2006; Mei and Pasternak, 2002 and 2007), but σ_1 receptors may also play a role in nociception in the absence of opioid drugs. In fact, studies performed by our group and other researchers demonstrated that both genetic inactivation and pharmacological antagonism of σ_1 receptors produce antinociceptive effects in the formalin test (Cendán et al., 2005a and b; Kim et al., 2006). Interestingly, the second phase of the formalin test involves a phenomenon of central sensitization, in which the enhancement of spinal NMDA receptor activity by its phosphorylation plays a facilitating role (Sawynok and Liu, 2004).

Central sensitization occurs in pathological pain (e.g. inflammatory and neuropathic pain); it is triggered by sustained peripheral nociceptor input and results in enhanced responsiveness of pain transmission neurons in spinal cord dorsal horn and brain (Woolf and Salter, 2000; Ji and Woolf, 2001; Ji et al., 2003). NMDA receptor activation plays a pivotal role in the development and maintenance of central sensitization (Willis et al., 2001; Ji and Woolf, 2001). Sigma-1 receptors play an important modulatory role in NMDA receptor activity (reviewed by Debonnell and Montigny, 1996; Cobos et al., 2008a), NMDA receptor phosphorylation (Kim et al., 2006 and 2008), and even in modulating acute pain induced by intrathecal administration of NMDA (Kim et al., 2008). Therefore, the **main hypothesis** of this

Doctoral Thesis was that σ_1 receptors may play a role in the central sensitization of pain pathways and that σ_1 receptor-acting drugs may inhibit central sensitization, which would be useful for the treatment of types of pain involving this phenomenon. In particular, we centred our study on the role of σ_1 receptors in sensitization to mechanical stimulus, one of the most characteristic signs of central sensitization and pathological pain.

Intradermal injection of capsaicin induces an increase in pain sensitivity in the area surrounding the capsaicin injection (area of secondary mechanical hypersensitivity), which results from central sensitization (Sang et al., 1996; Baron, 2000). Changes in capsaicin-induced mechanical hypersensitivity have been used to study the behavioural consequences of drug treatment for central sensitization (e.g. Park et al., 1995; Baumgärtner et al., 2002; Gottrup et al., 2004; Bingham et al., 2005). In addition, capsaicin-induced mechanical hypersensitivity is considered a surrogate model of neuropathic pain, since it mimics the sensory changes of this disease (Baumgärtner et al., 2002; Klein et al., 2005), and several antineuropathic drugs are reported to diminish the mechanical hypersensitivity induced by capsaicin or neuropathy (e.g. Gottrup et al., 2004; Joshi et al., 2006; Dworkin et al., 2007; Hagen et al., 2008; Nieto et al., 2008). However, the possible role of σ_1 receptors on sensitization to capsaicin-induced mechanical stimulus is unknown. Furthermore, no study has been published on the involvement of these receptors in pain induced by noxious mechanical stimulation in nonsensitized animals.

With this background, the **main goal** of this Doctoral Thesis was to determine whether the σ_1 receptor can be considered a new pharmacological target for the treatment of mechanical allodynia or punctate mechanical nociceptive pain. Three experimental tools were used for this purpose: σ_1 receptor knockout mice,

pharmacological antagonists and agonist for σ_1 receptor and injection of σ_1 receptor antisense oligodeoxynucleotides (ASOs).

The availability of σ_1 receptor knockout mice (developed by Langa et al., 2003) offers interesting possibilities for studying the functions of these receptors. Consequently, the **first specific goal** of this Doctoral Thesis was to compare the characteristics of mechanical nociceptive pain and capsaicin-induced mechanical allodynia between wild-type and σ_1 receptor knockout mice.

As mentioned above, a wide variety of σ_1 receptor-acting drugs are available. Therefore the **second specific goal** of this Doctoral thesis was to evaluate whether the pharmacological blockade of σ_1 receptor with σ_1 selective antagonists (BD-1063, BD-1047 and NE-100) and σ_1 non-selective antagonists (haloperidol and its metabolites) modulates mechanical allodynia and/or nociceptive pain, and whether coadministration with a σ_1 selective agonist (PRE-084) reverses the effects of these σ_1 receptor antagonists.

Central sensitization in spinal cord is accompanied by substantial changes in the brain processing of nociceptive information, producing a central sensitization-like phenomenon at supraspinal level, which contributes to the enhanced pain sensitivity (reviewed by Porreca et al., 2002; Ji et al., 2003). These changes in pain-related supraspinal activity have been extensively documented during mechanical stimulation of the area of secondary mechanical hypersensitivity induced by capsaicin (Iadarola et al., 1998; Baron et al., 1999; Iannetti et al., 2005; Zambreanu et al., 2005). However, the possible modulatory role of supraspinal σ_1 receptors on this process remains unknown. Therefore, the **third specific goal** of this Doctoral Thesis was to test the effects on capsaicin-induced mechanical allodynia and on mechanical nociceptive pain (in animals

not sensitized with capsaicin) of the supraspinal inhibition of σ_1 receptors induced by intracerebroventricular injection of two different σ_1 receptor ASOs.

METHODS

Experimental animals

Female CD-1 mice (Charles River, Barcelona, Spain) weighing 25 – 30 g were used throughout the experiments. To obtain CD-1 σ_1 receptor knockout mice, previously generated animals (Langa et al., 2003) were backcrossed for 10 generations onto the CD-1 background to ensure that less than 1% of the genetic material from the original background remained (Wong et al., 2002); mice harbouring the mutation were then bred to homozygosity and used in the experiments of this Doctoral Thesis. Mice were always handled in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC). The experimental protocol was approved by the Ethical Research Committee of the University of Granada, Spain.

A. In vivo assays

Evaluation of the behavioural response to mechanical stimulation. General aspects.

Animals were placed into individual test compartments (situated on an elevated mesh bottom platform) for two hours in order to habituate them to the test conditions. After this time period, the animals were injected intraplantarly with capsaicin (or its solvent, 1% DMSO in physiological saline) into the right hind paw and immediately returned to the compartment. Fifteen minutes after the administration of capsaicin (time to maximum sensitization) or its solvent, punctate mechanical stimulation was applied with a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) through the

mesh-bottomed platform. Briefly, a nonflexible filament (0.5 mm diameter) was electronically driven into the ventral side of the paw previously injected with capsaicin or solvent (i.e., the right hind paw), at least 5 mm away from the site of the injection. When a paw withdrawal response occurred, the stimulus was automatically terminated and the response latency time was automatically recorded. Therefore, we used paw withdrawal latency time as an indicator of pain perception in the evaluated mice. A cut-off time of 50 s was used. In each test session, each mouse was tested in three trials separated by 0.5-min intervals.

The force needed to induce a behavioural response in sensitized and non-sensitized mice was quantified by evaluating the effect of filament application at a wide range of intensities (0.05 – 8 g force) in animals previously injected with capsaicin (1 μg) or its solvent (DMSO 1%). This method allowed us to construct a force-response curve to quantify the degree of mechanical nociceptive pain at different intensities of mechanical stimulation in non-sensitized mice and to determine the mechanical hypersensitivity induced by a fixed dose (1 μg) of capsaicin.

When the effects of treatments were tested, two different types of mechanical stimulation were used. Mechanical allodynia in capsaicin-injected mice was evaluated by applying a mechanical stimulation of 0.5 g force, since this intensity of mechanical stimulus did not induce paw withdrawal (i.e., it was an innocuous stimulus) in capsaicin solvent-treated mice but markedly reduced paw withdrawal latency time in capsaicin-sensitized mice. Mechanical punctate pain was evaluated by stimulating non-sensitized animals with the filament at 4 g force. This intensity of stimulation induced a rapid paw withdrawal in non-sensitized mice (i.e. it was a noxious stimulus), resulting in a withdrawal latency time similar to the response obtained in the capsaicin-sensitized

animals when stimulated at 0.5 g force. Therefore, because equivalent behavioural responses were induced by 4-g force stimulation in non-sensitized animals and 0.5-g force stimulation in capsaicin-sensitized animals, this approach allowed us to compare the effects of treatments on mechanical nociceptive pain and mechanical allodynia.

Comparison of mechanical allodynia and punctate nociceptive pain between wild-type and σ_1 knockout mice

We used a double experimental approach to compare the characteristics of mechanical nociceptive pain and capsaicin-induced mechanical allodynia between wild-type and σ_1 receptor knockout mice **(first specific goal)**. Firstly, we constructed a force-response curve (intensity of stimuli vs. paw-withdrawal latency time) in wild-type and σ_1 knockout mice previously injected with capsaicin (1 μg) or its solvent (1% DMSO). This method allowed us to compare the behavioural response to mechanical nociceptive pain or mechanical hypersensitivity at different intensities of mechanical stimulation between wild-type and σ_1 knockout animals. Secondly, animals were intraplantarly administered with different doses of capsaicin (0.125-4 μg) or its solvent and stimulated with the filament at 0.5 g force (non-painful stimulus under normal conditions). The latter approach yielded a relationship between the dose of capsaicin injected and the degree of mechanical allodynia (decrease in paw-withdrawal latency time) in both wild-type and σ_1 receptor knockout mice.

Evaluation of drug effects on capsaicin-induced mechanical hypersensitivity and punctate mechanical nociceptive pain

To achieve the **second specific goal**, we evaluated the effects of several σ_1 receptor-acting drugs on capsaicin-induced mechanical hypersensitivity and on punctate

mechanical nociceptive pain, comparing these effects with those induced by various control drugs.

We used selective (BD-1063, BD-1047 and NE-100) and non-selective σ_1 antagonists (HP and its metabolites HP-Met-I, II and III) (Hayashi and Su, 2004; Guitart et al., 2004; Cobos et al., 2008a). The influence of dopaminergic antagonism on the effects induced by haloperidol and metabolites was controlled for by evaluating the effect of (-)-sulpiride, a D_2/D_3 receptor antagonist that is devoid of activity on σ_1 receptors (Freedman et al., 1994; Matsumoto and Pouw, 2000). In addition, we compared the effects induced by these σ_1 ligands with those induced by the known antiallodynic control drugs gabapentin, pregabalin and tetrodotoxin (Dworkin et al., 2007; Hagen et al., 2008; Nieto et al., 2008). We used mexiletine and clonidine as controls for drugs active against mechanical allodynia and nociceptive pain, (Khandwala et al., 1997; Paqueron et al., 2003). As a negative control, we used the anti-inflammatory drug rofecoxib (Moore et al., 2005), which is devoid of both antinociceptive and antiallodynic activity in animals without inflammation (Bingham et al., 2005; Padi and Kulkarni, 2004). In addition, possible involvement of σ_1 receptors in the effects of the evaluated drugs was determined by testing whether the effects of the selective σ_1 agonist PRE-084 (Su et al., 1991) might reverse the effects of the σ_1 antagonists and antineropathic drugs tested on capsaicin-induced mechanical allodynia and mechanical nociceptive pain.

The effects of these drugs were studied by subcutaneously (s.c.) injecting animals with 5 ml/kg drug solutions (or their solvent) in the interscapular region and evaluating mechanical allodynia or mechanical nociceptive pain at 45 min (15 min after capsaicin administration), as described in the above section. When PRE-084 was used to reverse

the effects of test drugs, the PRE-084 solution was injected s.c. immediately before the other drug solution. Each injection was performed in different areas to avoid mixtures of drug solutions that might interfere with the results through physicochemical interaction.

Evaluation of effects of supraspinal inhibition of σ_1 receptors on mechanical allodynia and mechanical nociceptive pain

Effects of supraspinal inhibition of σ_1 receptors in capsaicin-induced mechanical allodynia and mechanical nociceptive pain **(third specific goal)** were investigated by intracerebroventricularly (i.c.v.) treating the animals on four consecutive days with two phosphorothioate-modified antisense oligodeoxynucleotides (ASOs) against the mouse σ_1 receptor cDNA sequence. These ASOs were designed to target different areas of the mouse σ_1 mRNA. ASO-A targets a sequence downstream from the initiation codon (from +77 to +97) (King et al., 1997; Pan et al., 1998) and ASO-B an area spanning the initiation codon (from -11 to +9) (Ueda et al., 2001).

The specificity of the effects of the two σ_1 ASOs was tested by injecting mice i.c.v. with two additional phosphorothioate-modified control oligodeoxynucleotides (COs). As control for ASO-A, we used a previously designed mismatch oligodeoxynucleotide in which three pairs of bases were switched, (King et al., 1997; Pan et al., 1998). As control for ASO-B, we completely scrambled the respective sequence to obtain CO-B. This sequence shows no possible hybridization to any other known cDNA sequence registered in the GenBank Database (NIH, Bethesda, MD, USA). Capsaicin-induced mechanical hypersensitivity and mechanical punctate nociceptive pain were evaluated at days 5 and 11 after the first administration of σ_1

ASOs, COs or its solvent (i.e. 24 h and 1 week, respectively, after end of treatments), using the protocol described in previous sections.

B. In vitro assays

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

Radioligand binding assays were performed to study σ_1 receptors in crude synaptosomal (P_2) fraction from whole mice brain, obtained following the protocol described by González et al., 2001. We studied the number of σ_1 receptors in wild-type and σ_1 knockout mouse brain membranes by using *in vitro* ***saturation binding assays***, labelling σ_1 receptors with the selective σ_1 radioligand [^{3}H](+)-pentazocine. The affinity for wild-type mouse brain σ_1 receptors of all drugs tested in the *in vivo* assays was determined by using [^{3}H](+) pentazocine ***competition binding assays***. Binding assays were performed using previously described protocols (Cobos et al., 2007) with slight modifications. Briefly, resuspended membrane preparations (in a final protein concentration of 1 mg/ml) were incubated with [^{3}H](+) pentazocine (final concentration of 5 nM in competition assays and 0.1 – 37 nM in saturation experiments) and with the cold drug or its solvent at 30 °C (pH 8) for 240 min. Nonspecific binding was defined as the binding retained in the presence of 10 μM HP. The reaction was stopped with 5 ml 10 mM ice-cold Tris (pH 7.4). The bound and free radioligands were separated by rapid filtration under vacuum with a Brandel cell harvester (Brandel Instruments, SEMAT Technical Ltd., UK) on Whatman GF/B glass fibre filters (presoaked for ≥ 1 hr with 0.5% polyethylenimine). The radioactivity was then measured with a liquid scintillation spectrometer (Beckman Coulter España S.A).

RESULTS AND DISCUSSION

Comparison of mechanical allodynia and punctate pain in wild-type and σ_1 knockout mice

The degree of mechanical allodynia and mechanical nociceptive pain was compared between wild-type and σ_1 knockout mice by applying the filament at different intensities ranging from innocuous to noxious stimuli in mice treated i.pl. with 1 μg capsaicin or its solvent (1% DMSO) and constructing a force-response curve (see Methods for details). Mutation in σ_1 receptor knockout mice was confirmed by [^{3}H](+)-pentazocine binding assays.

When the force applied to the paw was increased, the paw-withdrawal latency time was similarly decreased in DMSO-treated σ_1 receptor knockout and wild-type mice, suggesting that the mutation in σ_1 receptor knockout mice does not affect mechanical perception. Capsaicin injection in wild-type animals induced a 9.4-fold shift to the left of the force-response curve in comparison to their control (DMSO-treated) wild-type animals. However, the force-response curve obtained in capsaicin-treated σ_1 knockout mice was equivalent to that found in DMSO-treated knockout animals. Therefore, capsaicin 1 μg induces mechanical hypersensitivity in wild-type animals but not in σ_1 knockout animals.

We also compared the effects of different doses of capsaicin on paw withdrawal latency time between wild-type and σ_1 receptor knockout mice stimulated with the filament at a normally innocuous intensity (0.5 g force). When capsaicin (0.125-4 μg) was administered i.pl. to wild-type mice, it induced a marked and dose-dependent reduction in paw withdrawal latency time (i.e. it induced mechanical allodynia), which

was maximal at 1-2 μg of capsaicin. However, under the same experimental conditions, σ_1 receptor knockout mice showed only a modest reduction in withdrawal latency time of the capsaicin-injected paw.

In summary, we found that the σ_1 receptor knockout mice could not be sensitized by capsaicin. However, the genetic inactivation of σ_1 receptors did not affect mechanical stimulus perception or the motor response necessary to produce paw withdrawal (since non-sensitized wild-type and σ_1 knockout mice showed equivalent responses). Methodological limitations did not account for these results, since wild-type animals were sensitized to the mechanical punctate stimulus by capsaicin under our experimental conditions, as previously reported (Gilchrist et al., 1996; Joshi et al., 2006).

Effects of drugs on punctate mechanical nociceptive pain and capsaicin-induced mechanical hypersensitivity

The subcutaneous administration of the selective σ_1 receptors antagonists BD-1063, BD-1047 and NE-100 and the non-selective σ_1 antagonists HP, HP-Met-I and HP-Met-II induced a dose-dependent antiallodynic effect; i.e. they increased the withdrawal latency time in the capsaicin-injected hind paw when stimulated with a filament at 0.5 g force. Competition binding assays showed that all of these drugs displaced [^{3}H](+)-pentazocine from its specific binding sites in mouse brain membranes. Interestingly, another metabolite of HP, HP-Met-III, and also (-)-sulpiride (used as control for dopaminergic antagonism) were devoid of antiallodynic activity and affinity for σ_1 receptors in binding studies.

When we coadministered the σ_1 receptor antagonists with the selective σ_1 receptor agonist PRE-084, we found that this compound, at doses that did not modify paw withdrawal latency in capsaicin-treated animals, prevented the antiallodynic effect induced by all of the σ_1 antagonists tested. These data indicate that both the selective and non-selective σ_1 receptor antagonists tested inhibited the sensitization induced by capsaicin through σ_1 receptor blockade.

We also studied the effects of these drugs on punctate mechanical pain. These drugs, administered at the doses that showed maximal antiallodynic effects, did not increase paw withdrawal latencies after stimulation with a noxious punctuate mechanical stimulus (filament at 4 g force) in the paw of nonsensitized (1% DMSO-treated) mice, i.e., these drugs exerted no antinociceptive effect on punctate mechanical nociceptive pain. The antiallodynic activity of σ_1 antagonists and their lack of effect against nociceptive pain agreed with the results obtained in σ_1 knockout mice.

We also compared the activity of the σ_1 antagonists tested with that of several control drugs. Firstly, we demonstrated that all control drugs used in this study were devoid of affinity for σ_1 receptors. Then, we evaluated their antiallodynic and antinociceptive effects with the same methods used to evaluate the effects of σ_1 receptor antagonists. Pregabalin, gabapentin and tetrodotoxin were able to inhibit capsaicin-induced mechanical allodynia but exerted no antinociceptive effect on punctuate mechanical pain. These effects were expected, since previous studies reported that these drugs inhibited mechanical allodynia in models of neuropathic pain (Joshi et al., 2006; Han et al., 2007; Nieto et al., 2008) at doses that did not exert antinociceptive effects in control animals (Field et al., 1997; Joshi et al., 2006; Nieto et al., 2008). On the other hand, mexiletine and clonidine showed both antiallodynic and antinociceptive effects against

mechanical stimuli. These data also agree with previous reports on these two drugs (Khandwala et al., 1997; Honda et al., 2002; Paqueron et al., 2003). Conversely, the negative control rofecoxib was devoid of activity against mechanical allodynia or punctuate mechanical pain, which is congruent with previous reports of the lack of effect of rofecoxib on mechanical hypersensitivity induced by neuropathy or by capsaicin administration (Padi and Kulkarni, 2004; Bingham et al., 2005). Data obtained with control drugs indicate that the methodology used in this Doctoral thesis is adequate and reliable to study both mechanical allodynia and nociceptive pain, and they validate the results obtained with σ_1 receptor antagonists.

Interestingly, the antiallodynic effects of pregabalin, mexiletine and tetrodotoxin could not be reversed by the coadministration of PRE-084. Therefore, σ_1 receptor blockade may be a novel pharmacological target against mechanical hypersensitivity that is not shared by other known antineuropathic drugs.

Effects of intracerebroventricular administration of σ_1 receptor antisense oligodeoxynucleotides on nociceptive pain and capsaicin-induced mechanical allodynia

Antisense treatment with ASO-A or ASO-B for four consecutive days dose-dependently inhibited capsaicin-induced mechanical allodynia (i.e. they dose-dependently increased the withdrawal latency time in the capsaicin-injected hind paw stimulated with a filament at 0.5 g force) when animals were evaluated at 24 h after the last ASO-administration. These effects were completely recovered at 1 week after the last i.c.v. injection. Therefore, neither ASO-A nor ASO-B induced any permanent change in capsaicin-induced mechanical hypersensitivity. Furthermore, neither ASO-A

nor ASO-B exhibited any antinociceptive effect against a noxious punctate mechanical stimulus (4 g force).

As expected, the repeated i.c.v. administration of CO-A or CO-B (used as controls for ASO-A and ASO-B, respectively) or saline (oligodeoxynucleotides solvent) exerted no effect on mechanical allodynia or nociceptive pain at any time. These data argue strongly for the specificity of the antiallodynic effects of σ_1 receptor inhibition induced by the σ_1 ASOs and indicate that the i.c.v. injection procedure produced no brain alteration able to modify the response to the mechanical stimulus under the different experimental conditions.

These findings suggest that σ_1 receptors participate in the modifications of processing at supraspinal level after sensitization with capsaicin but are not involved in the physiological brain mechanisms implicated in mechanical nociceptive pain.

CONCLUSIONS

Specific conclusions

1. Genetic inactivation of σ_1 receptors (in σ_1 knockout mice) completely inhibits capsaicin-induced mechanical hypersensitivity in mice.

2. Pharmacological antagonism of σ_1 receptors with selective or non-selective drugs produces dose-dependent antiallodynic effects. These effects are reversed with the selective σ_1 agonist PRE-084.

3. Supraspinal inhibition of σ_1 receptors induced by selective antisense oligodeoxinucleotides (injected i.c.v.) dose-dependently inhibits capsaicin-induced mechanical hypersensitivity. This effect completely disappears at one week after antisense administration.

4. Sigma$_1$ receptor genetic inactivation, pharmacological antagonism and supraspinal inhibition of σ_1 receptors with antisense oligodeoxinucleotides do not interfere with the perception of mechanical nociceptive pain.

General conclusion

Sigma$_1$ receptors play a pivotal role in the mechanisms underlying capsaicin-induced mechanical hypersensitivity but not in mechanical nociceptive pain. Therefore, σ_1 receptors blockade may be a novel pharmacological target for the treatment of mechanical allodynia.

INTRODUCTION

1. NOCICEPTIVE TRANSMISSION PATHWAYS

1.1. Nociceptors and primary afferent fibers

The term nociceptor comes from the Latin *nocere* which means to damage and was introduced by the famous Oxford physiologist Charles Sherrington who won the Nobel Prize for medicine in 1932. In a monograph published in the first years of the last century (Sherrington, 1906) he defined the term nociceptor as 'receptor' that respond selectively to stimuli that cause damage or threaten to cause damage. Currently, nociceptors unit are defined as peripheral afferent nerve units that connect with a central nervous system area devoted to the processing of pain and nocifensive reflexes. Nociceptor units usually show extensive terminal arborisation and therefore have more than one peripheral nociceptor terminal (Fig. 1.1.). Generally, the size of the peripheral receptive fields depends not only on the size of the animal and on the location, but also on the nociceptor class. Nociceptor terminals comprise several membrane receptors and channels involved in the transduction of nociceptive signals.

Nociceptors usually are classified taking into account two criteria: the kind of stimulus that activated them and the characteristics of their afferent nerve fibers. The responsiveness of nociceptors to different kinds of stimuli has been studied for decades and several subtypes have been established (Handwerker, 2006). In most early studies, only heat and mechanical stimuli were used to studying the nociceptors. Currently, these nociceptors can be distinguished, on the basis of their response to squeezing (mechanical) stimuli, between mechanically sensitive afferents (MSAs) and mechanically insensitive afferents (MIAs), the latter of which by definition have high mechanical thresholds or are unresponsive to mechanical stimuli (Meyer et al., 2007). If

a nociceptor responds to heat and mechanical stimuli, it will in most cases respond to chemical stimuli as well and may also be referred to as polymodal nociceptor (Davis et al., 1993). Primary afferent fibers can be categorized on the basis of diameter and presence or absence of myelin into three groups: large myelinated (Aβ), fine myelinated (Aδ) and unmyelinated (C) fibers. In general, Aβ fibers respond to innocuous mechanical stimuli, and most of those innervating the skin are tactile or hair-follicle afferents. However, some Aβ afferents function as nociceptors. In contrast, the majority of fine afferents (C and Aδ fibers) are nociceptors (Lawson, 2002). Another useful criterion to classify nociceptors was established by the work of Erlanger and Gasser (Erlanger and Gasser, 1924; Gasser and Erlanger, 1929) who distinguished between peripheral nerve fibers according to their conduction velocity. On the basis of this concept, most nociceptors belong to the group of nerve fibers with slow conduction velocity, i.e. slowly conducting myelinated fibers (type Aδ) and in particular the unmyelinated fibers (type C).

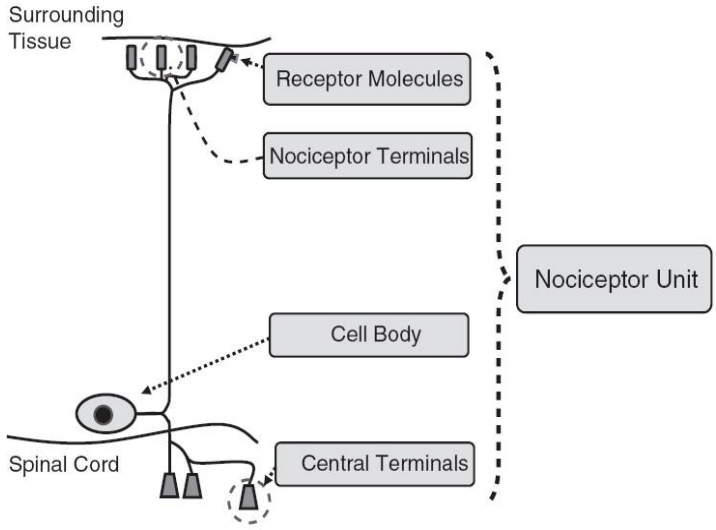

Figure 1.1. Schematic diagram of the different parts a nociceptor unit (Figure taken from Handwerker, 2006).

1.1.1. C-fiber nociceptors

Morphologically, these fibers are 1 μm wide or less, are unmyelinated and its axonal conduction velocity is about 1 m/s. They can be MSA or MIAs. The C-fiber MSA nociceptors can be classified as C mechano-heat (CMH) nociceptors and C mechano (CM) nociceptors. The former respond to mechanical and heat stimuli, and most also respond to chemical stimuli, and can therefore be considered C-polymodal nociceptors. However, C mechano (CM) nociceptors respond to high intensity (noxious) mechanical but not heat stimuli. However, some low-threshold C-fiber mechanoreceptors (LTMs) probalby contribute to visceral pain (Handwerder, 2006).The C-fiber MIA can be classified in several groups depending on their response to heat, cold or irritant stimuli.

1.1.1.1. C-fiber MSA (mechanically sensitive afferent)

A) Polymodal nociceptors (CMH nociceptors)

C-polymodal nociceptors comprise the major class of C-fiber afferents from mammalian skin, which have been extensively characterized in animals (Bessou and Perl, 1969) and humans (Torebjork, 1974). They respond to firm pressure, heat, and irritant chemicals. These units have been described in humans, monkeys, and many subprimate species, and are also found in hairy and glabrous skin (see Lynn and Perl, 1977 for references). Their quantitative properties vary markedly from unit to unit and between species and skin areas. Typically, conduction velocities average 0.8 - 0.9 m/s in larger mammals, punctate pressure thresholds are reported to be 0.5 - 5 g, and heat thresholds 40-55 ºC (Lynn and Carpenter, 1982). Receptive fields for CMH nociceptors increase with body size, but usually consist of a single zone with clear borders. The

receptive field size in humans is closed to 100 mm^2 (Schmidt et al., 1997). A subgroup of polymodal nociceptors has been reported to discharge in response to extreme cold (e.g., Georgopoulos, 1976). Responses have also been reported to a range of irritants including dilute acid, histamine, bradykinin and capsaicin (see Lynn et al, 1996 for references). Importantly, the TRPV-1 receptor (transient receptor potential vanilloid type 1), a receptor molecule in the terminal membrane of C-polymodal nociceptors, is activated by capsaicin and heat (> 43 ºC) (Caterina et al., 1997).

B) C-mechanical nociceptor (CM nociceptor)

A small number of C-fibers have pressure thresholds in the nociceptor range, but do not respond to heat (Bessou and Perl, 1969). The mechanical thresholds of CMH and CM nociceptors are similar. The receptive field size appears to scale with the size of the animal, and in humans is closed to 100 mm^2 (Schmidt et al., 1995 and 1997).

C) Low-threshold C-fiber mechanoreceptors

Low-threshold C-fiber mechanoreceptors are strongly activated by innocuous mechanical stimuli moved slowly across of their receptive field, and they also respond to pinprick stimuli, but do not respond to heat (Bessou and Perl. 1969). Signaling in these fibers activates the insular cortex. Since this system is poor at encoding discriminative aspects of touch but well-suited to encoding slow, gentle touch, LTM fibers in hairy skin may be part of a system for processing pleasant and socially relevant aspects of touch (Olausson et al., 2008). However, the LTMs probably contribute to visceral pain under physiological conditions and not only under pathological conditions (Handwerker, 2006).

1.1.1.2. C-fiber MIA (mechanically insensitive afferent)

This group of fibers consists of several classes of C-fiber MIAs. Heat nociceptors (CH) are C-afferent units firing preferentially to noxious heating, but only weakly or not at all to pressure. They have been reported in small numbers in several species (see Lynn and Perl, 1996 for references).

Cold nociceptors that only fire to severe cooling, although reported, are rare (Baumann et al., 1991). However, a significant population of nociceptors with responses to both strong pressure and to cooling, termed mechanical-cold nociceptors, has been described in the skin of the guinea pig and rabbit (Lynn and Perl, 1996).

Other studies have detected the existence of C-afferent MIA fibers that in undamaged skin, vigorously respond only to irritant chemical stimuli, such as mustard oil or capsaicin (Schmidt et al., 1995 and 2000).

1.1.2. A-fiber nociceptors

These are myelinated fibers, this characteristic permit a higher conduction velocity than C-fiber, <30 to 70 m/s depending on their wide of diameter. A-fiber nociceptors are thought to evoke pricking pain, sharpness. They respond with higher discharge frequencies than C-fiber, therefore the discriminable information supplied to the central nervous system is greater (Slugg et al 2000). Two types of A-fiber nociceptor have been identificated, type I and type II (Treede et al., 1998).

1.1.2.1. Type I

These nociceptors are typically responsive to heat, mechanical and chemical stimuli, and may therefore be referred to as A-polymodal nociceptors or AMHs. Type I

has a higher heat threshold and a lower mechanical threshold than type II. The heat thresholds depend on the duration of the stimulation, and are high (typically >53°C) in response to short (1 s) heat stimuli, but lower (40–50 ºC) in response to long (30 s) stimuli (Treede et al., 1998). Heat sensitivity in type I fibers is most likely mediated by vanilloid receptor-like protein 1, called the TRPV-2 receptor (previously known as VRL-1), because it has a similar high threshold for activation by heat and is expressed in neurons with small myelinated axons (Caterina et al 1999). These fibers also sensitize after a burn or chemical injury, and probably play a role in the development of hyperalgesia. Type I AMHs are seen in hairy and glabrous skin (Campbell et al 1979). Their receptive field size is similar to that of CMHs, but the presence of hot spots (more sensitive regions within the receptive field) to mechanical stimuli is much more obvious (Meyer et al. 2007). They have been suggested to mediate the first pain to noxious mechanical stimuli (Treede et al., 1998). The mean conduction velocity for type I AMHs is 25 m/s and can be as high as 70 m/s depending on the diameter of the fiber. Thus, based on conduction velocity criteria, type I AMHs fall into a category between that of Aδ and Aβ fibers, with two-thirds belonging to type I Aδ and one third Aβ (Djouhri and Lawson, 2004).

1.1.2.2. Type II

Their thresholds to mechanical stimuli place the majority of these fibers in the MIA category, and many have no demonstrable response to mechanical stimuli. The response of type II fibers to heat and chemical stimuli resemble those in CMHs, and they may also be mediated by the vanilloid receptor TRPV-1. Type II fibers have a lower heat threshold to short stimuli (median 46 ºC) with a prompt, short-lasting heat

response (Treede et al., 1998). Therefore, they are thought to signal the first pain sensation from heat, and may also contribute to the pain due to the application of capsaicin to the skin (Ringkamp et al., 2001). Their mean conduction velocity, < 30 m/s, is lower than that of type I AMHs. They are only found in hairy skin, and all have Aδ fibers (Treede et al 1998).

1.2. Dorsal root ganglion

The dorsal root ganglion (DRG) contains the cell bodies of primary afferent neurons (Fig. 1.1.), which are pseudounipolar neurons. The sensory receptors, which are located in the skin, muscles or joints and detect mechanical, thermal or noxious stimuli, are the peripheral terminals of DRG neurons. Their central terminals are localized in the dorsal horn of the spinal cord, and release neurotransmitters and neuromodulators in response to peripheral stimuli (reviewed by Zhang and Bao, 2006).

DRG neurons can be classificated in three different populations: Large, associated with Aβ fibers, intermediate, related with Aδ fibers, and small with C fibers (reviewed by Willis and Coggeshall, 2004). Two major subsets of small DRG neurons have been identified. One subset is nerve growth factor (NGF)-sensitive small DRG neurons, which express the neuropeptides calcitonin gene-related peptide (CGRP) and substance P. The other subset is composed of non peptidergic neurons, which are sensitive to glial cell line-derived neurotrophic factor (GDNF). Both types of neurons release glutamate and ATP, among other neurotransmitters and respond similarly to acute noxious stimulation, but the peptidergic afferents are more likely to play a role in inflammatory pain, whereas non-peptidergic afferents may be more characteristically involved in neuropathic pain (Willcockson and Valtschanoff, 2008). These neurons give rise to a

single axon that bifurcates into peripheral and central processes. The central process enters in the dorsal horn of spinal cord and synapse directly with the projection neurons in laminas (second-order neurons) or with interneurons that then synapse with a projection neuron (Zhang and Bao, 2006).

1.3. Spinal cord

The spinal cord is a key component of the nociceptive transmission pathways. It is a integrative area that receives inputs from peripheral nociceptors, through the primary afferent fibers that connect with second order neurons, and from supraspinal locations, via descending modulatory pathways (see section 1.4).

1.3.1. Structure of the 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. Rexed (1952) described 10 laminas within the gray matter (Fig. 1.2.), six in the dorsal horn, three in the ventral horn, and one around the central canal. Of these, laminae I (marginal layer), II (substantia gelatinosa), III and IV (nucleus propius), and V and VI (dorsal nucleus) comprise the dorsal horn. Lamina VII corresponds to the intermediate zone (intermediolateral nucleus). Laminas VIII and IX comprise the medial and lateral ventral horn respectively (motor neurons of the anterior horn), and lamina X is the region surrounding the canal. The two most superficial laminas, I and II_0 (the outer part of lamina II), together with the deeper laminas V and VI, and lamina X constitute the regions predominantly involved in the

reception, processing and rostral transmission of nociceptive information (reviewed by Millan, 1999).

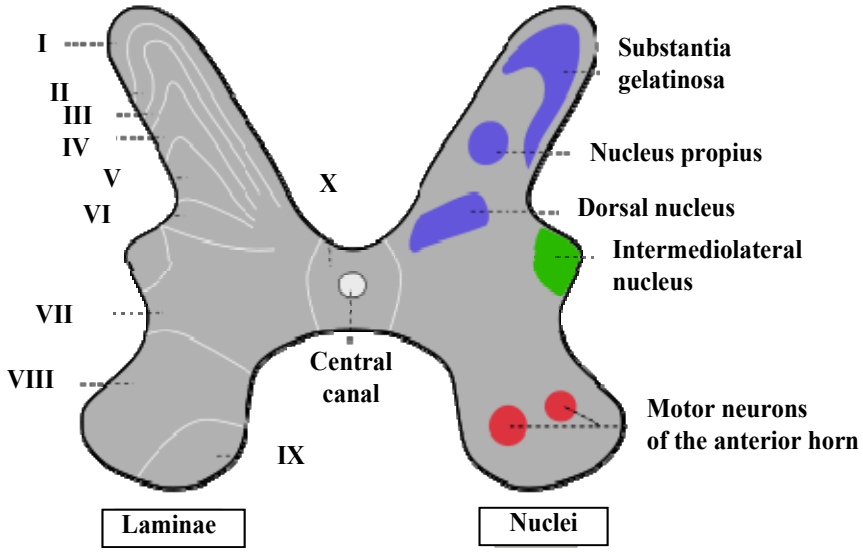

Figure 1.2. Organization of laminas and nuclei of the dorsal horn (Figure taken from Sławomirski and Głuszak , 1986)

1.3.2. Spinal projections of nociceptive primary afferents: Second-order neurons in the dorsal horn

The great majority of nociceptive primary afferent fibers terminate in the superficial dorsal horn (laminae I and IIo), although some Aδ fibers terminate in lamina V, and C-fibers of visceral origin also terminate in laminas V–VII and X, sometimes bilaterally (Willis and Coggeshall, 1991; Byers and Bonica, 2001).

Within the dorsal horn, three basic types of second-order neurones can be identified based on the nature of their response to nociceptive input: nociceptive-specific, wide-dynamic range and non-nociceptive neurones (reviewed by Schaible and

Grubb, 1993). The typically-silent, nociceptive-specific neurons are most concentrated in laminas I and II_0, although they are also found in deeper laminas (V and VI). Their main afferents are Aδ and C fibers; therefore these nociceptive-specific neurons respond to noxious mechanical or noxious thermal stimuli or both, but not to innocuous stimulation, i.e., they produce fire action potentials when a painful stimulus is detected at the periphery. The ability of nociceptive-specific neurones to encode stimulus intensity is limited (Willis and Coggeshall, 1991; Craig, 1996).

Multireceptorial or wide-dynamic range neurons manifest considerable convergence from cutaneous, muscle and visceral input (Schaible and Grubb, 1993). They are found predominantly in lamina V, as well as in laminas IV and VI, although they are also encountered in laminas I and II_0, as well as in lamina X and the ventral horn. "Wide-dynamic range" refers to the fact that they respond to both low- and high-intensity peripheral stimuli, and their firing frequency increases linearly or exponentially with stimulus intensity, thereby coding stimulus intensity. Wide-dynamic range neurons receive Aβ-, Aδ- and C-fiber inputs, and respond to a large range of mechanical stimuli ranging from innocuous to strongly nociceptive stimuli. They also respond to a variety of other stimuli (innocuous or noxious thermal and chemical stimuli), and show viscerosomatic convergence (Calvino and Grilo, 2006). Wide-dynamic range neurons in deeper laminas are most clearly involved in C fiber-mediated processes of sensitization and amplification contributing to prolonged pain.

Non-nociceptive neurons are found primarily in laminas II, III and IV, but a few may also occur in lamina I. They respond only to low-intensity peripheral stimuli and play no role in integrating nociceptive information (see Meyer et al., 2007; Calvino and Grilo, 2006).

1.4. Ascending nociceptive pathways

Nociceptive sensory signals are transmitted mainly by the anterolateral ascending system, which carries information chiefly about pain and temperature and also relays some tactile information. The three major pathways of the anterolateral system are: direct projections to the thalamus (i.e. the spinothalamic tract, STT), direct projections to numerous nuclei in the brainstem (i.e. the spinobulbar or spinoreticular tract), and projections to several structures of the mesencephalon (the spinomesencephalic tract). Other ascending pathways are the spinohypothalamic tract and spinopontine-amygdaloid pathway. The dorsal column postsynaptic system (DCPS) and spinocervical tract are alternatives that also carry nociceptive signals, although classical accounts do not consider them to be involved in this function (Byers and Bonica, 2001).

1.4.1. Spinothalamic tract

The STT is not a monolithic pathway; but originates in three distinct regions of the spinal grey matter: the most superficial layer of the dorsal horn (lamina I), the deep dorsal horn, (IV-V), and the intermediate zone and medioventral horn (VII-VIII). Almost half of the STT cells are located in lamina I, about one quarter is found in laminas IV-V and the remaining quarter are in laminas VII-VIII. Each of the three main populations of STT neurons is dominated by afferent inputs from a different constellation of primary afferent fibers. Accordingly, these populations of STT cells display different patterns of functional activity (Meyer et al., 2007).

The axons of STT cells generally cross in the dorsal and ventral spinal commissures to reach the white matter of the contralateral spinal cord within one or two segments rostral to the cells of origin. Ascending STT axons are concentrated in two

locations: the middle of the lateral funiculus (the classical neospinothalamic tract), which transmits mainly information about pain and temperature, and the middle of the anterior funiculus (the classical paleospinothalamic tract) which transmits mainly information about crude touch (Byers and Bonica, 2001; reviewed by Craig et al., 2003).

1.4.1.1. Spinothalamic tract projection sites

The STT terminates in different regions of the thalamus, as detailed below (Craig, 2003; Meyer et al., 2007, for a review).

The neospinothalamic tract has few synapses and projects to the posterior portion of the ventromedial (VMpo) and the ventroposterior lateral (VPL) nuclei of the thalamus. The VMpo serves as a thalamocortical relay nucleus for lamina I STT cells. This nucleus projects topographically to the dorsal margin of the posterior insular cortex buried within the lateral sulcus, and constitutes an interoceptive sensory representation of the physiological condition of the body. Embedded within this projection are distinct, highly resolved representations of several sensations from the body, including pain, temperature, itch, muscle and visceral sensations, and (C-fiber) touch, i.e. they are important for processing signals relevant for homeostasis, as most of the population of neurons are nociceptive-specific neurones. The STT terminations in the VPL originate primarily from cells in laminas IV-V, which in turn receive many monosynaptic input from nociceptive Aδ-fibers and polysynaptic inputs from C-fibers from the skin, muscle or viscera. They respond to both low- and high-threshold stimuli. Therefore most laminas IV-V STT cells are WDR. A long-held view is that STT input to VPL plays a

role in pain. The VPL projects to the region of the retroinsular (vestibular) and primary somatosensory cortex in the lateral sulcus.

The paleospinothalamic tract is originated from lamina II, III and V cells. The nerve cells that furnish the paleospinothalamic tract are wide dynamic range neurons. There is a moderately dense STT projection to the ventrocaudal part of the mediodorsal nucleus. This nucleus project to area 24c in the cortex at the fundus of the anterior cingulate sulcus (limbic motor cortex), rather than to the orbitofrontal and prefrontal cortex where the remainder of mediodorsal nucleus projects. This STT component is important for the affective and motivational aspect of pain. This tract makes a synaptic connection in different locations, originating spinoreticular, spinomesencephalic and spinohypothalamic tracts (see sections 1.5.2, 1.5.3 and 1.5.4). The innervation of these three tracts is bilateral because some of the ascending fibers do not cross to the opposite side of the cord.

1.4.2. Spinoreticular tract

The neurons of the spinoreticular are originated primarily in laminas I, V, VII and VIII of the spinal cord and they terminate in many sites throughout the brainstem. Spinal projections to the brainstem are important for the integration of nociceptive activity with processes that subserve homeostasis and behavioral states. There are also pathways that indirectly convey nociceptive activity to the forebrain following integration in the brainstem. In addition, spinal input to the brainstem influences the modulation of both spinal and forebrain activity, which can affect the experience of pain (Meyer et al., 2007).

Anatomical evidence indicates that ascending spinoreticular projections terminate mainly in four major areas of the brainstem (Wiberg et al., 1987; Craig, 2003), described below.

The regions of catecholamine cell groups (A1-A7). Lamina I projections to the A5, A6 and A7 groups in the dorsolateral pons influence noradrenergic and encephalinergic bulbospinal cells that modulate nociceptive and autonomic spinal activity.

The parabrachial nucleus. Spinal input to the parabrachial nucleus originates primarily from lamina I neurons, with a weak contribution from lamina IV-VI cells. Lamina I input to the parabrachial nucleus thus provides a substrate for the integration of nociceptive activity with general visceral (homeostatic) afferent activity.

The periaqueductal grey (PAG). Moderately dense spinal input occurs in the lateral and ventrolateral (caudal) portions of the PAG and adjacent tegmentum. Stimulation of different portions of the PAG can elicit aversive behaviors, cardiovascular changes and (opioidergic or nonopioidergic) antinociceptive modulation. Notably, portions of the PAG that receive spinal input also have ascending projections to the hypothalamus and medial thalamus.

The brainstem reticular formation. A portion of the dorsomedial reticular formation of the medulla (subnucleus reticularis dorsalis) receives spinal input from lamina I and V cells, and contains neurons with nociceptive responses from large receptive fields. However, these cells project back to the dorsal horn or rostrally to the ventromedial thalamus and thence to the entire frontal cortex. This is so-called 'spinoreticulothalamic' pathway. A modulatory role for these cells has been proposed in pain behaviour, that could serve the motivational and arousal aspects of pain.

1.4.3. Spinomesencephalic tract

Spinomesencephalic tract neurons terminate in several structures of the mesencephalon, especially the PAG, the nucleus cuneiformis, and the superior colliculus. These connections produce affective and aversive behaviors such as fear, associated with pain. The spinomesencephalic tract input to the PAG activates the system for descending pain modulation, which produces endogenous analgesia (Byers and Bonica, 2001).

1.4.4. Spinohypothalamic tract

The spinohypothalamic tract is originated bilaterally from cells in laminas I, V, VII and X over the entire length of the cord (Dado et al., 1994). The contralateral hypothalamus receives axon endings, either directly from the spinohypothalamic tract or indirectly from the spinoparabrachial-hypothalamic tract. The hypothalamus is involved in controlling autonomous nervous system responses to pain, and in releasing hormones that contribute to stress control (Calvino and Grilo, 2006).

1.4.5. Other ascending pathways

The spinopontine-amygdaloid pathway is originated from layer I of the dorsal horn and connects to the amygdaloid complex after relaying in the lateral parabrachial nucleus. This pathway may be involved in affective and emotional responses to pain (Calvino and Grilo, 2006).

The spinocervical tract is originated chiefly in laminas I, III, IV and V, ascends in the ipsilateral dorsolateral funiculus, and terminate in the lateral cervical nucleus. From here, third-order neurons constitute the cervicothalamic tract, which sends their axons to

the contralateral ventral funiculus, where they run rostrally to reach the brainstem. At this level they join the medial lemniscus and ascend to terminate in the midbrain and VPL. The importance of the spinocervical tract varies among species, it is an important nociceptive pathway in the cat, but is much reduced in the lower primates and is considered absent or vestigial in human (Byer and Bonica, 2001).

The dorsal column pathway is composed of branches of primary afferent fibers and of the axons of postsynaptic dorsal column (PSDC) neurons (Willis and Coggeshall, 2004). The PSDC neurons are located in the nucleus proprius and in the vicinity of the central canal in the spinal gray matter, and project to the gracile and cuneate nuclei. The PSDC fibers ascend to higher levels in the medial lemniscus and terminate principally in the VPL nucleus of the thalamus (Palecec, 2004). Traditionally, the dorsal column has been thought to carry only fibers activated by innocuous stimuli such as touch and propioception, but a role for the PSDC neurons in the transmission of visceral pain has been suggested (Willis et al., 1999).

1.5. Supraspinal structures and descending modulatory pathways

As early as 1911, Head and Holmes explicitly postulated modulatory influences on pain. They proposed that the thalamus was the center for pain perception, and that the neocortex, the discriminative perception center, continuously modulated the responses of the thalamus to noxious stimuli. According to their hypothesis, pain modulation was a necessary part of the ongoing process of discriminative sensation. Clear-cut examples of descending modulation of sensory transmission were subsequently described. Hagbarth and Kerr (1954) provided the first direct evidence that supraspinal sites control ascending (presumably sensory) pathways, and Carpenter and

colleagues demonstrated descending control of sensory input to ascending pathways (Carpenter et al., 1965). However, the existence of a specific pain modulatory system was first clearly articulated in 1965 by Melzack and Wall in their gate control theory of pain (Melzack and Wall, 1965). Supraspinal influences on the gate were proposed, although there was limited evidence at that time for descending control of nociception. Shortly thereafter, Wall (1967) demonstrated that structures in the brainstem tonically inhibit nociresponsive neurons in the spinal cord.

The supraspinal structures most important in the descending modulation of pain are the PAG and the rostroventral medulla (RVM). These structures receive inputs from several supraspinal and spinal locations, and project to the spinal cord through three major descending pathways (corticospinal, reticulospinal and raphespinal tracts), evoking the stimulation of the inhibitory interneurons in the spinal cord to produce analgesia (Byers and Bonica, 2001).

1.5.1. Supraspinal structures

1.5.1.1. Periaqueductal grey

The discovery of the pain-modulating role of the PAG was a critical advance in understanding the mechanisms of pain modulation. Subsequent research demonstrated that the PAG is part of a central nervous system circuit that controls nociceptive transmission at the level of the spinal cord (Fields et al., 1991). In animals, PAG stimulation inhibits simple noxious stimulus-evoked reflexes such as the tail-flick or paw withdrawal. Furthermore, nociceptive neurons in the spinal cord dorsal horn are selectively inhibited by PAG stimulation, the PAG projects to the locus coeruleus and

from there to the spinal cord dorsal horn but only minimally since the majority of projections are originated from RVM (Calvino and Grilo, 2006; Meyer et al., 2007).

The PAG integrates inputs from the limbic forebrain and diencephalon with ascending nociceptive input from the dorsal horn (Bandler and Keay, 1996). There are direct projections to the PAG from a number of medial prefrontal areas including the anterior cingulated, and from the insular cortex.

The amygdala, which receives massive input from both the hippocampus and the neocortex, is another major source of afferents to the PAG (Aggleton, 1992). Cortical afferents to the amygdala largely target its basolateral component. The basolateral amygdala then projects to the central nucleus, which in turn projects densely to the PAG (Rizvi et al., 1991). The central nucleus of the amygdala also receives nociceptive input, both directly from the spinal cord (Burstein and Potrebic, 1993; Gauriau and Bernard, 2004) and indirectly via a large projection from dorsal horn lamina I to the parabrachial nucleus (Gauriau and Bernard, 2002).

The nucleus accumbens, located in the ventromedial striatum, has also been implicated in pain modulation (Gear et al., 1999). The nucleus accumbens receives a major projection from the basolateral amygdala, and although the accumbens does not have a major direct projection to the PAG, it projects to the lateral hypothalamus and to the amygdala, which in turn project to the PAG (Zahm et al., 1999).

Major brainstem inputs to the PAG arise from the adjacent nucleus cuneiformis, the pontomedullary reticular formation, the locus coeruleus, and other brainstem catecholaminergic nuclei (Herbert and Saper, 1992). The PAG and adjacent nucleus cuneiformis receive a significant projection from the dorsal horn, including spinal lamina I nociceptive neurons. The PAG also projects rostrally to the medial thalamus

and orbital frontal cortex, raising the possibility of ascending control of nociception (Meyer et al., 2007, for references). The PAG is reciprocally connected with the rostroventral medulla.

1.5.1.2. Rostroventral medulla

The rostroventral medulla (RVM) includes the midline nucleus raphe magnus and the adjacent reticular formation that lies ventral to the nucleus reticularis gigantocellularis. Activation of the RVM neurons can generate either facilitation or inhibition of pain transmission under different conditions. This dual control results from the activity of two neuronal subpopulations in the RVM. The two cell classes, which are also present in the PAG and dorsolateral pons, exhibit phasic reciprocal changes in firing that precede nociceptor-elicited withdrawal reflexes. One class, termed "off cells", is characterized by a pause in firing that begins before the withdrawal reflex. The other class, called "on cells" is characterized by a burst of activity that begins before the reflex. Consistent with their role in pain modulation, RVM on- and off-cell axons project directly and selectively to dorsal horn laminas along the dorsolateral funiculus that relays nociceptive signals (Fields et al, 1995).

The RVM receives input from serotonin-containing neurons of the dorsal raphe and neurotensinergic neurons of the PAG. The RVM also receives a substantial direct projection from the medial preoptic region. Cortical afferents arise from the limbic and prelimbic cortex, including the anterior insula (Meyer et al., 2007).

Direct spinal projections to the RVM are sparse, but the RVM receives spinal information, possibly via the PAG and nucleus cuneiformis or through the nucleus reticularis gigantocellularis, which receives a large direct projection from nociceptive

spinoreticular neurons and projects massively to the RVM. The RVM also receives noradrenergic and encephalinergic input from the A5, A6 and A7 cell groups in the dorsolateral pons (Sagen and Proudfit, 1981). Of note is the finding that the antinociceptive effect of cholinergic stimulation in the RVM is substantially attenuated by inactivation of the A7 region (Nuseir et al., 1999).

1.5.2. Descending pathways

1.5.2.1. Corticospinal tracts

The corticospinal tracts are constituted by axons of corticospinal neurons from the somatosensorial cortex which pass ipsilaterally in the brainstem, where fibers are given off to the trigeminal spinal nucleus. Most of the remaining fibers cross at the pyramidal decussation to the opposite side, where they descend in the dorsolateral funiculus (lateral corticospinal tract), and a smaller portion descends ipsilaterally as the anterior corticospinal tract. Corticospinal afferents from the somatosensory cortex terminate in laminas I-VII. Corticospinal fibers in laminas I and II exert a direct postsynaptic control on dorsal horn neurons (Byers and Bonica, 2001).

1.5.2.2. Reticulospinal and raphespinal tracts

The PAG has descending connections to the RVM that play a critical role in pain modulation, particularly in its ventral part, comprising the medullary reticular nuclei and the nucleus raphe magnus. These sites in the rostral medulla are a mayor source of axons projecting principally via the raphespinal and reticulospinal tracts, which descend in the dorsolateral funiculus and terminate in several laminas in the dorsal horn of the spinal cord. The reticulospinal tract is in turn divided into anterior and medial

reticulospinal tracts, which originate in the nucleus gigantocellularis and send terminals to laminas VII and VIII. The lateral reticulospinal tract originates in the nucleus magnocellularis and sends terminals to laminas I, IIo and V-VIII. In contrast, the raphespinal tract originates in the nucleus raphe magnus and projects to all laminas except III, IV and VIII (see Byers and Bonica, 2001 for references).

1.5.3. Inhibitory interneurons

Represent a major subset of neurons in the superficial laminas of the dorsal horn that are excited by descending pathways originating from supraspinal structures. These neurons release the inhibitory amino acids GABA, glycine or opioids, which contribute to descending controls (Meyer et al., 2007).

GABAergic cells make up approximately 25 - 30% of the neurons in laminas I and II, and around 40% of those in lamina III (Todd and Sullivan, 1990). Immunocytochemical studies have suggested that most of the glycinergic neurons in laminas I-III are also GABAergic, although deeper laminas contain neurons that are glycinergic but not GABAergic (Todd and Sullivan, 1990). Not surprisingly, GABA and glycine are colocalized in many axon terminals in the dorsal horn and some of the synapses formed by these terminals have both $GABA_A$ and glycine receptors (Todd et al., 1996). Furthermore, glycine is released into the spinal cord dorsal horn by stimulation in the RVM, which inhibits the firing of nociceptive neurons (Sorkin et al., 1993). In contrast, the vast majority of opioid terminals in the dorsal horn derive from local opioidergic interneurons and enkephalinergic terminals and cells present in superficial dorsal horn, which show dense concentrations of μ-opioid receptors.

2. CAPSAICIN AND PAIN SENSITIZATION

2.1. Capsaicin

Capsaicin, first isolated by Thresh (1846), is the main active, pungent constituent in peppers of the genus *Capsicum*. This genus comprises approximately 20 species of *Capsicum* (family *Solanaceae*), which are widely distributed throughout the world. The five most frequently cultivated species are *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinese*, *Capsicum pendulum* and *Capsicum pubescens* (reviewed by Szallasi and Blumberg, 1999). *Capsicum* species are often known as chili peppers, and are used in culinary preparations and in folk medicine. They have been cultivated in South America for over 7000 years, and in the rest of the world since the 16th century. Traditional medicinal uses of capsicum peppers include appetite stimulation, treatment of gastric ulcers and rheumatism, and relief of toothaches (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). The pharmaceutical application of capsaicinoids is attributed to its antioxidant, anticancer, antiarthritic, and analgesic properties (see Prasad et al., 2006 for references).

Despite their early discovery, it was not until 1919 when Nelson reported that capsaicin is a condensation product of vanillylamine and an isomer of decylenic acid (fatty acid). Once the double bond in the isomer was found, Nelson and Dawson were able to firmly establish the chemical structure of capsaicin, known as 8-methyl-6-nonenoyl vanillylamide, in 1923 (reviewed by Govindarajan, 1986) (Fig. 2.1.).

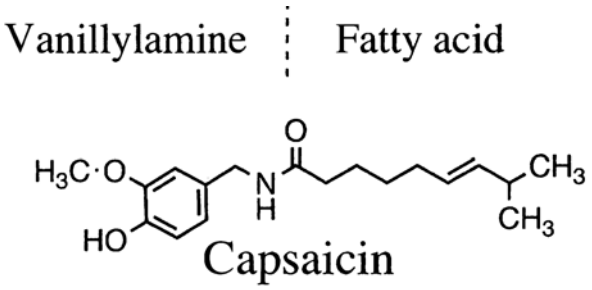

Figure 2.1. Capsaicin chemical structure

2.2. Transient receptor potential vanilloid-1 (TRPV-1)

Electrophysiological (Bevan and Szolcsanyi, 1990) and biochemical (Wood et al., 1988) studies showed that capsaicin excited nociceptors by increasing the permeability of the plasma membrane to cations, but the molecular mechanism underlying this phenomenon was unclear. Previous studies by Szolcsanyi and Jancso-Gabor (1975 and 1976) found that capsaicin derivatives show structure–function relationships and evoke responses in a dose-dependent manner, therefore the most likely mechanism of action was thought to be via a receptor site, later called vanilloid receptor-1 (VR-1). This model was supported by patch clamp studies with capsazepine, a competitive capsaicin antagonist (Bevan et al., 1992), and by the discovery of resiniferatoxin, an extremely potent capsaicin analogue, that mimics the cellular actions of capsaicin (Szallasi and Blumberg, 1989). These and many other studies formed the groundwork that led to the cloning of a capsaicin or vanilloid-gated ion channel by David Julius' group, who showed that this receptor belongs to the transient receptor potential (TRP) family (Caterina et al., 1997); therefore it was named TRPV-1 in later studies.

2.2.1. Cloning and molecular structure of TRPV-1

The TRPV-1 receptor was first isolated from a rat cDNA library, based on the channel's sensitivity to capsaicin and the ability of capsaicin to activate calcium influx in dorsal root ganglion (DRG) neurons. The TRPV-1 cDNA contains a 2541-nucleotide open reading frame that encodes a protein of 838 amino acids with a predicted relative molecular mass of 95 kDa (Caterina et al., 1997). Its shows with clear sequence homology to several other proteins, including all members of the *Drosophila melanogaster* TRP family, and the OSM-9 protein of *Caenorhabditis elegans*, a protein involved in olfaction, mechanosensation and olfactory adaptation in this species (Harteneck et al., 2000).

TRPV-1 is an ionotropic receptor that is Ca^{+2}–permeable and acts as a nonselective cation channel. Structurally, the functional TRPV-1 channel is a tetrameric membrane protein with four identical subunits assembled around a central aqueous pore. Each subunit shows a topology of six transmembrane segments with a pore region between the fifth and sixth segment, and cytoplasmic N and C termini (Fig. 2.2.). These channels contain three or four ankyrin domains in the N terminal that interact with cytosolic proteins. Amino acid Tyr511 at the N-end of the third transmembrane domain has been identified as a critical structural determinant of the capsaicin binding site. This receptor also exhibits a cytosolic C terminal which contains phosphoinositide- and calmodulin-binding domains, as well as consensus sites for protein kinase A (PKA) and C (PKC) and Ca^{+2} – calmodulin-dependent kinase II (CAMKII) phosphorylation (reviewed by Ferrer-Montiel et al., 2004) (Fig. 2.2.). Functional studies have attributed to the C terminus an important role on inflammation-induced TRPV-1 potentiation, as well as the receptor thermal sensitivity (see Garcia-Sanz et al., 2004 for references). In

addition, the C terminal of TRPV-1 contains a TRP box that shows similarity to the highly conserved TRP domain of the TRP protein family, referred to as the TRP-like domain. This domain in TRPV-1 acts as an association domain involved in the tetramerization of receptor subunits into functional channels, suggesting that the homologous TRP domain in the TRP protein family may function as a general, evolutionary conserved association domain involved in subunits multimerization (Garcia-Sanz et al., 2004).

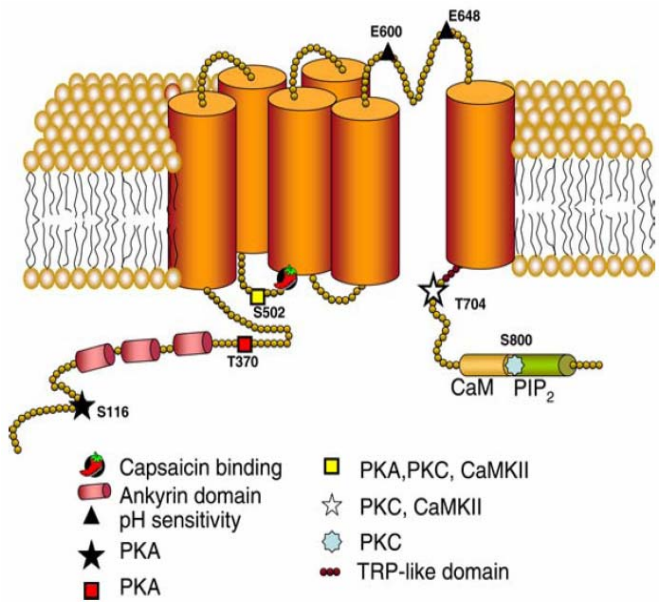

Figure 2.2. Topological model of a vanilloid receptor (TRPV-1) subunit exhibiting six putative transmembrane domains, an intracellular N-terminal, and a C-end domain. Amino acid residues specific for capsaicin activation, as well as residues important for phosphorylation by protein kinases A and C (PKA, PKC) and Ca^{+2}-calmodulin-dependent kinase II (CaMKII), are highlighted in both intra-cytoplasmic regions. The TRP-like, calmodulin (CAM)- and phosphatidylinositol-4,5-bisphosphate (PIP_2)-binding domains are also depicted. A functional TRPV-1 receptor contains four of these subunits assembled around a central pore formed from the fifth and sixth segments of the four subunits. (Figure taken from Planell-Cases et al., 2005)

2.2.2. Localization of TRPV-1 receptors

Capsaicin sensitivity is considered a principal pharmacological trait of a major subpopulation of nociceptive sensory neurons in dorsal horn, trigeminal and nodose sensory ganglia. Most capsaicin-sensitive nociceptors are C-fibers, but another, less numerous population consists of Aδ fibers (reviewed by Caterina and Julius, 2001). Indeed, expression of TRPV-1 protein was observed in the terminal of afferent fibers projecting to the superficial layers of the spinal cord dorsal horn, specifically in laminas I and II in the spinal cord, to which unmyelinated C-fibers project (Caterina and Julius, 2001). Furthermore, TRPV-1 has also been found in various brain areas, including dopaminergic neurons, hypothalamic neurons, the locus coeruleus in the brainstem, and in various layers of the cortex (Mezey et al., 2000). This receptor has also been observed in the nucleus of the solitary tract and area postrema, which receive vagal projections from visceral organs through the nodose ganglia. These observations, together with the fact that TRPV-1 protein is detected in nerve endings of the bladder, indicate that TRPV-1 is expressed in both central and peripheral terminals of small-diameter sensory neurons (Tominaga and Julius, 2000).

In addition, TRPV-1 receptor has been identified in non-neuronal cells in physiological and pathological processes. In the skin, for example, TRPV1-positive cells have been associated with Meissner corpuscles, and have been located in keratinocytes. More recently, its expression was reported in mast cells, where activation of TRPV-1 may release mast cell mediators that bind to histamine and proteinase-activated receptors on sensory terminals. TRPV-1 is also expressed in visceral tissue in the gastrointestinal tract within the myenteric and submucous plexus, and in some enteric intrinsic neurons. Under pathological conditions TRPV-1 has been implicated in

gastrointestinal tract hypermotility, abdominal pain associated with functional bowel disorders, and in the neurogenic component of pancreatitis (reviewed by Planell-Cases et al., 2005).

2.2.3. Pharmacological profile of the TRPV-1 receptor

To date, the known activators of TRPV-1 include several vanilloids. The most widely used are capsaicin and resiniferatoxin (isolated from the cactus-like plant *Euphorbia resinifera*), which functions as an ultrapotent capsaicin analogue. Others vanilloids known to activate TRPV-1 receptors are piperine, the active ingredient in black pepper, and zingerone, responsible for the piquancy of ginger (Sterner and Szallasi., 1999). Endogenous ligands known as endovanilloids can also activate TRPV-1 receptors. These endovanilloids are derived from the metabolism of arachidonic acid, e.g., the endocannabinoid anandamide, some lipoxygenase products of arachidonic acid, and N-arachidonoyl dopamine (reviewed by van der Stelt and Di Marzo, 2004). In addition, some antagonists have been described, such as the synthetic competitive vanilloid receptor antagonist capsazepine (Walpole et al., 1994). TRPV-1 can be also activated by protons (pH) and by high temperatures in the noxious range, suggesting that it functions as a transducer of painful thermal stimuli *in vivo* (reviewed by Tominaga and Julius, 2000).

2.3 Capsaicin-induced pain sensitization: general aspects

In early studies of pain sensitization, summarized by Lewis (Lewis, 1942) and by Hardy and coworkers (Hardy et al., 1967), the skin of human volunteers was damaged by a mild burn, by mechanical injury or by electric shocks. More recently, a number of

investigators have used intradermal injections of capsaicin to produce these pain states (LaMotte et al., 1991 and 1992; Ali et al., 1996; Liu et al., 1998).

The main behavioral manifestations of pain sensitization are hyperalgesia and allodynia. The International Association for the Study of Pain defines hyperalgesia as "an increased response to a stimulus which is normally painful", and allodynia as "pain due to a stimulus which does not normally provoke pain" (www.iasp-pain.org). Hyperalgesia and allodynia in an area that has been injured are referred to as primary hyperalgesia and allodynia, whereas hyperalgesia and allodynia in an adjacent, undamaged part of the body are known as secondary hyperalgesia and allodynia. Primary hyperalgesia and allodynia are characterized by enhanced pain to both heat and mechanical stimuli, whereas secondary hyperalgesia and allodynia are characterized by enhanced pain to mechanical but not heat stimuli (LaMotte et al., 1992; Ali et al., 1996). The mechanism of primary hyperalgesia may involve a contribution of processes integrated in the central nervous system, but can predominantly be explained by events that happen in peripheral nociceptors and result in their sensitization (LaMotte et al., 1992). However, primary afferent fibers that supply the area that develops secondary mechanical hyperalgesia and allodynia maintain a normal level of excitability; i.e., they are not sensitized (Baumann et al., 1991). Thus, secondary mechanical hyperalgesia and allodynia must result from an enhanced responsiveness of central nociceptive neurons or 'central sensitization' rather than from sensitization of primary afferents (Baron, 2000; Willis, 1991).

An intradermal injection of capsaicin activates TRPV-1 receptors located at the internal surface of the membrane on subpopulations of primary afferent neurons, C- and some Aδ fiber nociceptors. The result is immediate burning pain and peripheral

sensitization that follows neurogenic inflammation (Li et al., 2008), as well as enhanced pain sensitivity in the area surrounding capsaicin injection due to central sensitization (Baron, 2000; Willis, 2001). Capsaicin-induced burning pain is maximal initially, but decreases progressively over about 15–20 min. Moreover, at the injection site there is a zone of hyperesthesia or primary hyperalgesia (Sang et al., 1996) that can be attributed to the excitotoxic effect of capsaicin on capsaicin-sensitive primary afferent fibers (Holzer, 1991). Primary mechanical and heat hyperalgesia develop rapidly in the vicinity of the injection. In addition, the intradermal injection of capsaicin produces secondary allodynia or hyperalgesia, in response to innocuous or noxious stimuli respectively applied to adjacent unaltered skin (Torebjörk et al., 1992). Secondary pain sensation develops over a period of about 15 min in a progressively enlarging area of skin surrounding the injection site, and then slowly decrease. Depending on the dose of capsaicin that is injected, secondary mechanical allodynia may last several hours and secondary mechanical hyperalgesia about a day (Ali et al., 1996). Taking into account that the secondary mechanical hypersensitivity induced by capsaicin is widely considered a feature of central sensitization (Park et al., 1995; Gottrup et al., 2004), which greatly contributes to neuropathic pain, capsaicin-induced mechanical hypersensitivity is considered a surrogate model of neuropathic pain (Baumgartner et al., 2002; Klein et al., 2005).

2.4. Capsaicin-induced peripheral sensitization

Sensitization of primary afferent nociceptive terminals (peripheral sensitization) arising from activation of TRPV-1 receptors is presumed to be an initial step in the process by which neurogenic inflammation occurs, and this sensitization contributes to

mechanical and heat primary hyperalgesia (see Ren et al., 2004 for references). Peripheral sensitization, i.e., a reduction in the pain threshold and an increase in responsiveness of the peripheral endings of primary afferent nociceptive fibers, can be produced by the intradermal injection of capsaicin (Jancsó et al. 1967 and 1968). It is generally accepted that the mechanisms underlying in the peripheral sensitization include a direct action of capsaicin on TRPV-1 receptors, whose activation causes an influx of Ca^{2+} and together voltage-gated calcium channels activated induce an increase of intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$). This increase of $[Ca^{2+}]i$ induced by capsaicin into nerve endings causes the exocytosis of inflammatory mediators and their release into the periphery, producing both neurogenic inflammation (i.e., inflammatory symptoms resulting from the activation of primary sensory neurons) and sensitization of primary afferent nociceptors (reviewed by Szallasi and Blumberg, 1999).

Several studies support the notion that pro-inflammatory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are major initiators of neurogenic inflammation (reviewed by Meyerson and Vasko, 2002). Other neuropeptides such as neuropeptide Y are also secreted by sensory neurons in response to irritant stimuli, and seem to play an important role in neurogenic inflammation. Moreover, pro-algesic mediators such as nerve growth factor (NGF), protons, histamine, cytokines, prostaglandins, neurokinins, chemokines, glutamate and ATP also contribute to this inflammatory process (see Planells-Cases et al., 2005 for references). All these molecules sensitize nociceptors, either by directly modulating the sensitivity of membrane receptors or by upregulating intracellular signaling cascades (Fig. 2.3). For example, it has been demonstrated that activation of a PKA-dependent pathway by inflammatory mediators such as prostaglandins influences capsaicin- or heat- mediated

actions in sensory neurons, probably by acting on TRPV-1. These results suggest that PKA plays a pivotal role in the development of hyperalgesia and inflammation by inflammatory mediators. On the other hand, PKC-dependent phosphorylation of TRPV-1 occurs downstream from activation of G_q-coupled receptors by several inflammatory mediators including ATP, bradykinin, and prostaglandins. While the phosphorylation of TRPV-1 by CaMKII is activated by the increased of $[Ca^{2+}]i$ (reviewed by Tominaga and Tominaga, 2005) (Fig. 2.3.). These intracellular pathways of activation of PKC, PKA and CaMKII will be described in detail below (section 2.5.2.1.). Consequently the excitability of the peripheral terminal membrane increases, facilitating peripheral sensitization.

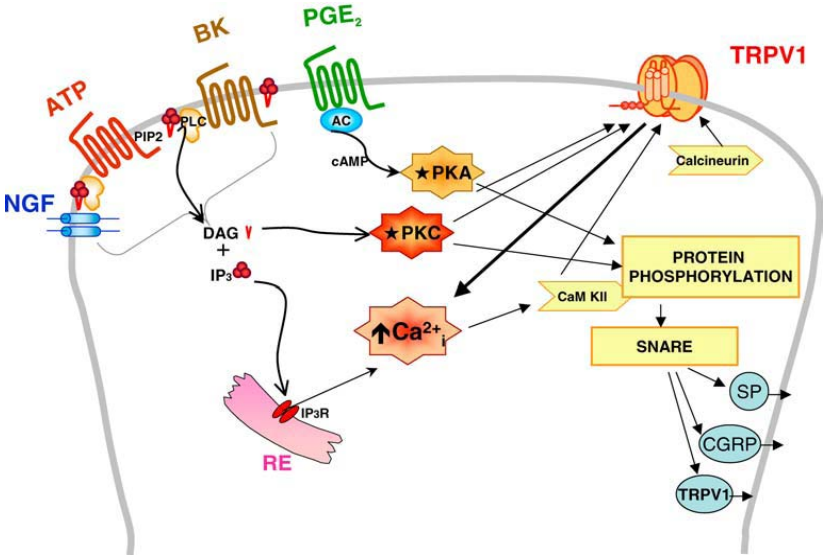

Figure 2.3. Intracellular pathways involved in TRPV-1 activation and neuropeptide release under inflammatory conditions. Nerve growth factor (NGF) and bradikinin (BK) stimulate G-protein-coupled receptors which, in turn, signal through phospholipase C (PLC), PKA, PKC and CaMKII pathways to increase TRPV-1 channel activity and release Ca^{2+} to the cytosol. Increased $[Ca^{2+}]_i$ activates PKC and CaMKII, which in addition to modulating TRPV-1, increase substance P (SP) and calcitonin-gene-related peptide (CGRP) release (taken from Planell-Cases et al., 2005).

Moreover, an increase in translation and transport of TRPV-1 to the peripheral nociceptor terminal from the DRG has been reported (Ji et al., 2002) (see section 2.4.2.), and other research has found increased transcription or altered trafficking of sodium channels (Scholz and Woolf, 2002). As a result of these processes, the activation threshold will be lower for pain sensations, strong sensory signalling is conveyed to the spinal cord and subsequently to specific brain regions, leading to an enhanced pain sensation.

2.4.1. Phosphorylation/dephosphorylation of the TRPV-1 receptor

A large body of evidence has now accumulated to suggest that TRPV-1 can act as a substrate for PKA, PKC and CaMKII (see Bhave and Gereau, 2004 for references). These kinases phosphorylate the receptor at specific intracellular sites, provoking the modulation of both vanilloid affinity for capsaicin and receptor sensitization or desensitization (Fig. 2.2.). The interaction of vanilloids with TRPV-1 receptor is dependent on the phosphorylation state of Ser502 and Thr704 (Jung et al., 2004). Ser502 is a consensus phosphorylation site for PKA, PKC and CAMKII, found between the second and third transmembrane domains, whereas Thr704 is located in a consensus sequence for PKC and CAMKII in the C-terminal (Jung et al., 2004) (Fig. 2.2.). Specifically, phosphorylation at Ser502 controls the activation and sensitization of TRPV-1 receptor by vanilloids (Jung et al., 2004). For example, once Ser502 is phosphorylated, TRPV-1 receptor is ready to be activated and sensitized if other key sites are phosphorylated by PKA or PKC. When only Thr704 is phosphorylated, TRPV-1 receptor is activated, but sensitization will not occur until Ser502 is phosphorylated by either PKC or PKA (Jung et al., 2004). The phosphorylation/dephosphorylation state of

Ser502 thus modulates vanilloid affinity and sensitization of the TRPV-1 receptor (Jung et al., 2004; Bhave et al., 2003). Moreover, the PKC phosphorylation site Ser800 is located in the center of the phosphatidylinositol biphosphate (PIP_2) binding site, suggesting that PKC activation enhances the capsaicin-activated current in nociceptive neurons due at least in part to disruption of the PIP_2–TRPV-1 interaction. An increase in intracellular Ca^{2+} after the TRPV-1 receptor has been opened by capsaicin would lead to dephosphorylation by phosphatase calcineurin, and therefore to TRPV-1 receptor desensitization (Jung et al., 2004). Interestingly, capsaicin-induced receptor desensitization can be inhibited by PKA-mediated phosphorylation of the N-terminal amino acids Thr-370 and S116 (Bhave et al., 2002).

2.4.2. Increased TRPV-1 receptor in nociceptive terminals

Peripheral neurogenic inflammation results in changes in neuropeptides, ion channels, and receptor levels in the cell bodies in the DRG (see Ji et al., 2002 for references). NGF is upregulated in inflammatory tissues (Woolf et al., 1994) and plays an important role in inflammatory pain by driving peripheral sensitization. Interestingly, the increase of translation and transport of TRPV-1 receptors to the peripheral terminal is initiated by retrograde transport of NGF released from inflamed tissue to soma. NGF phosphorylates p38 (phospho-p38), producing the increase of TRPV-1 receptor expression in the cell body, which is anterogradely transported to the peripheral terminals of the C-fibers (Fig.2.4.). These findings imply directed trafficking of the receptor to the peripheral axon after inflammation, which could actively contribute to the enhancement of the heat sensitivity (Ji et al., 2002) (Fig.2.4.).

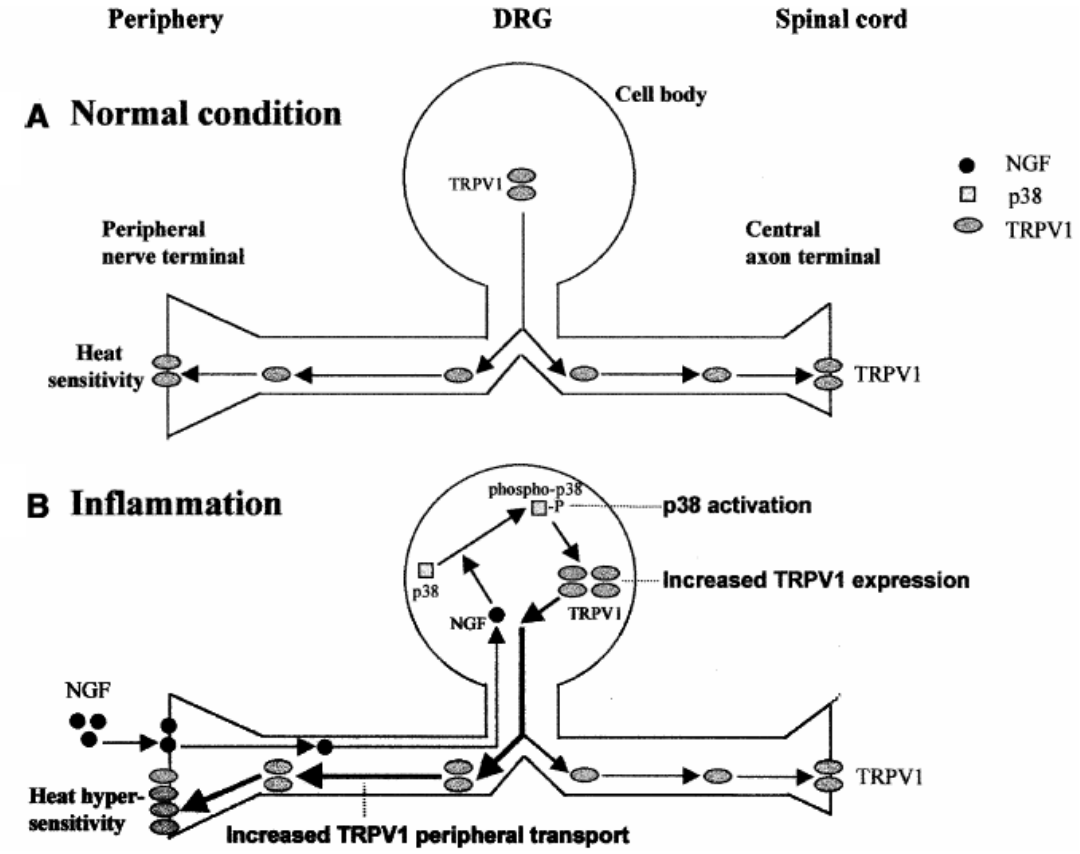

Figure 2.4. In the absence of peripheral inflammation, TRPV-1 is synthesized in the cell bodies of primary sensory neurons in the DRG and transported both to peripheral and central terminals, contributing to heat sensitivity in the periphery. After inflammation, NGF is produced in inflamed tissue, taken up by peripheral nerve terminals, and retrogradely transported to the cell body in the DRG. NGF in the cell body activates p38, which in turn increases TRPV-1 translation. Increased TRPV-1 is anterogradely transported to peripheral terminals, increasing heat sensitivity (taken from Ji et al., 2002).

2.5. Capsaicin-induced central sensitization in the spinal cord

Secondary allodynia and hyperalgesia following capsaicin injection are likely to be produced by sensitization of central nociceptive neurons, because capsaicin does not alter the sensitivity of sense organs that supply the skin in the secondary area. It has been shown that the release of several neurotransmitters and the activation of glutamate

and substance P receptors in the spinal cord play an important role in the central sensitization following capsaicin injection (Willis, 2001).

2.5.1. Neurotransmitters involved in triggering central sensitization in the spinal cord

The nociceptive primary afferent terminals (δ- and C-fibers) in the dorsal horn and the interneurons that they excite release excitatory amino acids, including glutamate and aspartate (Sorkin and McAdoo, 1993), as well as peptides such as substance P, in dorsal horn synapses (Khasabov et al., 2002). These amino acids, released from capsaicin-sensitive primary afferent terminals, increase significantly in the spinal dorsal horn after the intradermal injection of capsaicin, and are thought to contribute to central sensitization. As a consequence of these synaptic events, a number of signal transduction pathways involving PKA, PKC and CaMKII are activated, and lead to phosphorylation of synaptic receptors and intracellular proteins in dorsal horn neurons, which increases the spinal neuronal response to afferent inputs. The consequence of this modulation is the phenomenon called central sensitization (Dougherty and Willis, 1992; Willis, 2001).

2.5.1.1. Role of glutamate in central sensitization

Ueda and coworkers (1993 and 1994), using a fluorometric online monitoring system, demonstrated that capsaicin induced the release of glutamate from primary afferent terminals. Other evidence of the ability of capsaicin to evoke glutamate release from primary afferent neurons, particularly Aδ- and C-fiber afferents, was the distribution of released glutamate in laminas I, II, V and X in the spinal dorsal horn, the regions where these fibers terminate (Tohda and Kuraishi, 1996). The capsaicin-induced increase in glutamate is ipsilateral, and maximum increase in the dorsal horn was found

after 0 to 20 min; thereafter the concentration of glutamate returns to basal values in 40 to 60 min (Sorkin and McAdoo, 1993).

The glutamate released from terminal nociceptive afferent to the spinal dorsal horn activated ionotropic receptors such as *N*-methyl-*D*-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and kainate receptors, and also metabotropic receptors (mGluR) of the dorsal horn spinothalamic tract neurons. These receptors contribute to capsaicin-induced thermal and mechanical hypersensitivity (Sang., 1998; Soliman, 2005) (see section 2.5.2).

2.5.1.2. Role of aspartate in central sensitization

Although glutamate has been long favored as a transmitter of the primary afferent fibers, anatomical and physiological evidence suggests a role for aspartate in the capsaicin-induced effects of neurotransmission. The capsaicin-induced activation of nociceptive primary afferent Aδ- and C- fibers induce glutamate and aspartate release in the spinal cord (Kangrga and Randic, 1991). The capsaicin-induced increase in aspartate in the dorsal horn was ipsilateral, and the duration of this effect was similar to that of glutamate release (Sorkin and McAdoo, 1993). Whereas glutamate activates both NMDA and non-NMDA receptors, aspartate is thought to act preferentially on NMDA receptors (Kangrga and Randic, 1991).

2.5.1.3. Role of substance P in central sensitization

It has been shown that capsaicin evokes the release of substance P from primary sensory neurons (Theriault, 1979). Substance P is located in small-caliber afferent, Aδ- and C- fibers (McCarthy and Lawson, 1989), which is consistent with the observation that substance P is concentrated in regions of the dorsal horn where small-caliber

sensory fibers terminate (Ruda et al., 1986). Substance P and glutamate have been found to coexist in these primary afferents (DeBiasi and Rustioni, 1988), and it is generally recognized that glutamate interacts with substance P-containing sensory afferents to modulate nociceptive transmission from the periphery to the spinal dorsal horn (Cuesta et al., 1999). Substance P contributes to the development of central sensitization by activating neurokinin 1 receptors (NK-1) present in neurons of the superficial dorsal horn, most of which belong to the spinothalamic and spinoparabrachial tracts, which are involved in the ascending transmission of nociceptive information (Khasabov et al., 2002).

2.5.1.4. Other molecules involved in central sensitization

Nerve growth factor (NGF), when released at the site of inflammation, alters the sensitivity of peripheral terminals and is retrogradely transported from the periphery to the cell body (see section 2.4.2), activating specific signal transduction pathways (Hendry et al., 1974) that may, via the cAMP (cyclic adenosine monophosphate) response element binding protein (Greene and Kaplan, 1995), contribute to increased BDNF in the DRG. Specifically, the central actions of BDNF on membrane excitability may be mediated by tyrosine phosphorylation of the NMDA receptor, either directly via the TrkB receptor or indirectly via other tyrosine kinases, contributing to stimulus-induced hypersensitivity after inflammation. (Mannion et al., 1999).

It has also been reported that nitric oxide (NO), a diffusible molecule, plays an important role in nociceptive transmission. In this connection it has been shown that NO is released within the spinal cord dorsal horn following the intradermal injection of capsaicin, and this effect supports the idea that NO release in the spinal cord contributes

to the mechanical allodynia induced by capsaicin injection (Wu et al., 2000). Nitric oxide acts by activating soluble guanylate cyclase, which in turn catalyzes the production of cGMP (cyclic guanosine monophosphate). The NO-cGMP cascade plays a role in the process of central sensitization involved in mechanical allodynia (Lin et al., 1997).

2.5.2. Receptors involved in triggering central sensitization in the spinal cord

Among the receptors involved in the processes of central sensitization, this section focuses on the receptors for glutamate and substance P. The receptor for the latter neurotransmitter is the metabotropic neurokinin 1 (NK-1) receptor. Receptors for glutamate include non-NMDA glutamate receptors (Na^+/K^+-channels linked), NMDA glutamate receptors (Ca^{2+}-channel linked) and metabotropic glutamate receptors (G-protein coupled). In this section we describe the main structural and functional characteristics of these receptors.

2.5.2.1. Metabotropic receptors: Neurokinin-1 and glutamate receptors

Among the receptors coupled to G-protein, NK-1 and mGluR group I receptors (mGluR1 and 5) are coupled to the G_q/G_{11} α subunit, which mediates phospholipase C (PLC) activation (reviewed by Kew and Kemp, 2005). Stimulation of these receptors by substance P and glutamate leads to the activation of PLC and the subsequent generation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol from phosphatidylinositol 4,5 biphosphate (PIP_2) hydrolysis (Taylor et al., 1986). Activation of NK-1 and mGluR group I receptors by their ligands increases intracellular Ca^{2+}, which is produced by two distinct pathways: one pathway is the result of Ca^{2+} release from intracellular stores (Ca^{2+} mobilization from the endoplasmic reticulum) evoked primarily by IP_3; the other

pathway is the influx of extracellular Ca^{2+} through channels on the plasma membrane (Mochizuki-Oda et al., 1994). This increase in intracellular Ca^{2+} activates PKC, which in turn potentiates the effectiveness of the NMDA channel by phosphorylation (see below in section B.4.). In addition, activation of PKC inhibits GABA receptors. These changes are all believed to contribute to the enhanced sensitivity of dorsal horn neuronal responses to excitatory inputs (Lin et al., 1996).

Glutamate also activates mGluR group II (mGluR2 and 3) and group III receptors (mGluR4-8), which are G_i/G_o-protein-linked receptors associated with the cAMP transduction cascade. Activation of these receptors results in activation of adenylate cyclase, which converts ATP to cAMP, leading to an increase in cAMP formation in the cell. This increase activates PKA, resulting in the phosphorylation of proteins such as the NMDA receptors (see section B.4.) and AMPA receptors (see section A). Therefore, activation of cAMP via mGluR groups II and III can result in increased neuronal excitability, and may also contribute to central sensitization (Sluka, 1997).

2.5.2.2. Ionotropic glutamate receptors

A) AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and kainate receptors

AMPA receptors are hetero-oligomeric proteins formed by different combinations of GluR1, GluR2, GluR3 and GluR4 subunits, whereas kainate receptors are composed of two related subunit families, GluR5–7 and KA-1 and -2. The latter two (KA-1 and –-2) combine in heteromeric assemblies with members of the GluR5–7 subfamily to form functional receptors resembling native kainate receptors (reviewed by Kew and Kemp, 2005). Although the ion channels formed by these receptors are permeable to sodium

ions, their permeability to calcium is usually very low but varies according to the subunits involved, as shown by the finding that AMPA receptors lacking GluR2 are more permeable to calcium (Engelman et al., 1997; Sorkin et al., 1999). The activation of calcium-permeable AMPA receptors produces desensitization of colocalized NMDA receptors (Sorkin et al., 1999). Both AMPA and kainate receptors are synaptically localized in dorsal horn neurons in culture, and are expressed in lamina I and outer lamina II, areas associated with nociceptive processing (Engelman et al., 1997; reviewed by Kew and Kemp, 2005). In laminas IV and V GluR3 is more strongly expressed than the other subtypes, indicating that these AMPA receptors may also be postsynaptic to Aβ fibers, and participate specifically in touch-evoked pain (Furuyama et al., 1993; Sorkin et al., 1999).

In central sensitization induced by intradermal injection of capsaicin, PKA, PKC and calcium-calmodulin kinase II (CaMKII) are involved in the regulation of AMPA receptor GluR1 subunit phosphorylation (Fang et al., 2002 and 2003). Consequently these phosphorylated GluR1 subunits are recruited from the cytosol to the synaptosomal membrane, which increases the ratio of receptors at synapses (Galan et al., 2004), whereas GluR2/3 subunits are constitutively cycled in and out of the plasma membrane to maintain normal transmission (reviewed by Malinow and Malenka, 2002). On the other hand, the modulation of kainate receptors in capsaicin-induced central sensitization is unclear.

B) *N*-methyl-*D*-aspartate receptor

B.1. *Molecular organization and operation of N*-methyl-*D*-aspartate *receptors*

N-methyl-*D*-aspartate (NMDA) receptors are heteromeric, integral membrane proteins. Seven genes encode the NMDA receptor subunits NR1, NR2A–NR2D and NR3A–NR3B. The NR1 subunit undergoes extensive splicing to yield eight variants. Functional NMDA receptors are formed from the co-assembly of what is termed the obligatory NR1 glycine-binding subunit with NR2 (glutamate binding) and/or NR3 subunits (Stephenson, 2006). The subunits probably assemble as tetramers with stoichiometry $(NR1)_2(NR2)_2$, although this has not been unequivocally proved and accepted. *N*-methyl-*D*-aspartate receptor diversity is generated by the association of the obligatory NR1 subunit with various NR2 subunits to yield four major subtypes, NR1/NR2A, NR1/NR2B, NR1/NR2C and NR1/NR2D. In minor subpopulations, two types of NR2 subunit are shown to co-associate within the same receptor, e.g., $(NR1)_2$/NR2A/NR2B (Stephenson, 2006). The role of the NR3 subunit is unclear. One report showed that NR1/NR3A or NR1/NR3B subunits formed a novel excitatory glycine-gated receptor in oocytes, but the existence of such a receptor in vivo has yet to be proved (Chatterton et al., 2002). However, when coexpressed with NR1 and NR2, NR3A and NR3B act as modulators of ion flux, reducing single-channel conductance and permeability to Ca^{2+} (Sasaki et al., 2002).

B.2. *Functional domains in N-methyl-D-aspartate receptor subunits*

N-methyl-*D*-aspartate subunits all share a common membrane topology (Fig. 2.5.) characterized by a large extracellular *N*-terminal, a membrane region comprising three transmembrane segments (TM1, 3 and 4) plus a re-entrant pore loop (M2), an

extracellular loop between TM3 and TM4, and a cytoplasmic C-terminal, which varies in size depending on the subunit, and provides multiple sites of interaction with numerous intracellular proteins. The extracellular region of NMDA subunits (like that of other eukaryotic iGluR subunits) is organized as a tandem of two domains (Fig.2.5.), which share structural and functional homologies with two families of bacterial periplasmic proteins. The *N*-terminal domain shows sequence homology with the bacterial protein leucine/isoleucine/valine-binding protein, this domain plays an important role in subunit assembly (reviewed by Paoletti and Neyton, 2007).

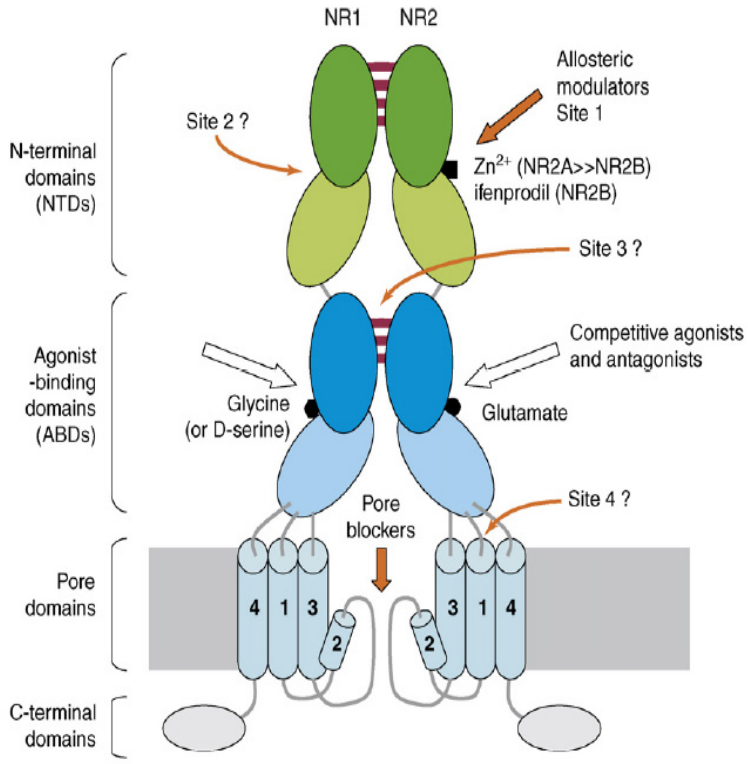

Figure 2.5. Potential sites for ligand binding at NMDA receptors. Most NMDA receptors are believed to assemble as tetramers in which two NR1 and two NR2 subunits are associated in a "dimer of dimers" quaternary architecture. For clarity, only one of the two NR1/NR2 heterodimers is shown (taken from Paoletti and Neyton, 2007).

Activation of NMDA receptor requires the simultaneous binding of two co-agonists: glutamate and glycine (or D-serine). The agonist binding domain binds glycine in NR1 and NR3, whereas NR2 binds glutamate (Yao and Mayer, 2006). The Ca^{2+}-permeable pore is formed by the re-entrant membrane domains M2 belonging to each of the four NR subunits that constitute the receptor. The sequences of the regions lining the pore are highly conserved in NR2 subunits, so permeation properties (i.e., single channel conductance, ionic selectivity), as well as affinity for the pore blocker Mg^{2+}, vary little among the different NR1/NR2 receptor subtypes. In contrast, incorporating the NR3 subunit markedly decreases single-channel conductance, Ca^{2+} permeability and Mg^{2+} blockade (Sasaki et al., 2002; reviewed by Paoletti and Neyton, 2007).

B.3. *N*-methyl-*D*-aspartate *receptor localization*

The NMDA receptor is widespread in postsynaptic densities of synapses in most brain structures such as the hippocampus, cerebral cortex and cerebellar cortex (Moriyoshi et al., 1991), as well as in the cervical spinal cord, dorsal root and vestibula ganglia, and in pineal and pituitary glands (Shigemoto et al., 1992). It is present in lower numbers in the olfactory bulb, neocortex, striatum, some thalamic and hypothalamic nuclei, and many reticular, sensory and motor neurons of the brainstem and spinal cord (Petralia et al., 1994). In addition, the NMDA receptor is located on glutamatergic primary afferent terminals in the spinal cord, particularly in inner lamina II, where it may facilitate the transmission of inputs to the spinal cord by increasing the release of neurotransmitter from the primary afferent terminal (Liu et al., 1994). Studies by Zou and coworkers in dorsal horn neurons demonstrated the presence of NR1

subunits of the NMDA receptor in the soma and dendrites of spinothalamic tract neurons, as well as in synaptic terminals in the spinal cord (Zou et al., 2000).

B.4. *Phosphorylation of NMDA receptor after intradermal capsaicin*

Early experiments showed that NMDA receptor-mediated currents can be potentiated by the activity of intracellular kinases (Gerber et al., 1989; Cerne et al., 1993; Ugolini et al., 1997). The enhancement of NMDA receptor activity by the action of intracellular kinases can also be produced *in vivo* after intradermal capsaicin injection.

As described above (section 2.5.1.), large increases in intracellular Ca^{2+} concentration are induced by the activation of NK-1, mGluRs, AMPA and NMDA receptors in spinal cord. These and other processes activate various signal transduction cascades, including those involving protein kinases such as CAMKII, PKC, PKG and PKA (Willis, 2001; Ji and Woolf, 2001). The most widely studied effects of post-capsaicin protein kinase activation are those derived from the activity of PKC and PKA. These intracellular kinases are able to phosphorylate the NR1 subunit of the NMDA receptor, which potentiates NMDA receptor activity (reviewed by Wyneken et al., 2004). They phosphorylate different sites on the NMDA receptor: PKA phosphorylates NR1 in Ser897, whereas PKC specifically phosphorylates the subunit NR1 in Ser890 and Ser896 (Tingley et al., 1997; Zou et al., 2002; Brenner et al., 2004), resulting in a reduction of the Mg^{2+} blockade of NMDA receptors (Chen and Huang, 1992; Moriguchi et al., 2006). It has also been shown that the NR1 subunit can be phosphorylated by Ser-897 alone or by Ser-897 and Ser-896 together but not by Ser-896 alone, i.e., the NR1

subunit can be phosphorylated by both PKs simultaneously or by PKA alone but not by PKC alone (Gao et al., 2007).

The enhanced phosphorylation of NR1 after capsaicin administration can be detected in spinal cord dorsal horn neurons, especially in spinothalamic tract cells (Zou et al., 2000), and it is thought to play a pivotal role in capsaicin-induced central sensitization (Willis, 2000). Anatomically, there are some differences between the phosphorylation induced by PKA and PKC. Enhancement of PKA-mediated phosphorylation of NR1 has been detected in the deep and superficial laminae of the spinal cord dorsal horn, whereas PKC phosphorylates NR1 in the deep laminae of the spinal cord but not in its superficial laminae. Both kinase activities play a crucial role in the mechanical hypersensitivity induced by capsaicin (Zou et al., 2002).

Importantly, enhancement of NR1 phosphorylation in spinal cord is also present in pathological pain (neuropathic and inflammatory pain) models, and it is considered to play a pivotal role in pain hypersensitivity in these states (Ultenius et al., 2006; Zhao et al., 2006).

In summary, the activity of several protein kinases (e.g., PKA and PKC) is enhanced in the spinal cord not only after intradermal capsaicin injection but also in pathological pain states. These protein kinases phosphorylate NMDA receptors at specific sites, enhancing NMDA receptor activity and thereby contributing to the central sensitization of pain.

2.5.3. Gene expression implicated in central sensitization induced by capsaicin in spinal cord.

Stimulation of the NMDA receptor, voltage-gated calcium channels and metabotropic receptors evokes activation of the Ras/MAPK (mitogen-activated protein kinase) signalling pathway, which involves the action of several protein kinases, including PKC, PKA, Raf and MEK. This leads to the phosphorylation of ERK (extracellular signal-regulated kinase), which is a member of the MAPK family (reviewed by Kolch, 2000; Ji and Woolf, 2001) (Fig. 2.6.). It has been demonstrated that the intradermal injection of capsaicin induces ERK activation in dorsal horn neurons, which can be detected as early as 1 min after the capsaicin injection (Ji et al., 1999). Phosphorylated ERK (pERK) is found in the medial superficial dorsal horn of the spinal cord on the ipsilateral (stimulated) side after capsaicin administration, where primary nociceptive afferents from the hind paw terminate (reviewed by Ji and Woolf, 2001; Walker et al., 2007). Phosphorylation of ERK also occurs in the spinal cord in neuropathic and inflammatory pain states (Zhuang et al., 2005; Choi et al., 2006) and after acute thermal (heat and cold) or mechanical (prick) noxious stimulation but not after innocuous mechanical stimulation (Ji and Woolf, 2001). Therefore, the activation of ERK appears to be important in the pain signalling system.

pERK is likely to cause posttranslational modifications *via* phosphorylation of key substrates such as receptors, ion channels and kinases, thereby modifying membrane excitability and synaptic plasticity (Ji and Woolf, 2001). In addition, a fraction of pERK translocates to the nucleus of nociceptive neurons, and this MAPK is implicated in the activation by phosphorylation of transcription factors CREB (cAMP response element binding) and Elk-1 (Ets-like transcription factor) (see Yu and Yezierski, 2005 for

references). Phosphorylated CREB binds to the CRE sites on the promoter region of the DNA and initiates the transcription of genes, including c-fos, dynorphin, and BDNF. On the other hand, phosphorylated Elk-1 binds to the SRE (serum response element) sites and consequently initiates the transcription of c-fos gene (Fig. 2.6.) (reviewed by Ji and Woolf, 2001; Kawasaki et al., 2004).

In summary, the activation of ERK by its phosphorylation may contribute to the regulation of gene transcription after C-fibre activation by capsaicin and may therefore contribute to the pain hypersensitivity after capsaicin administration.

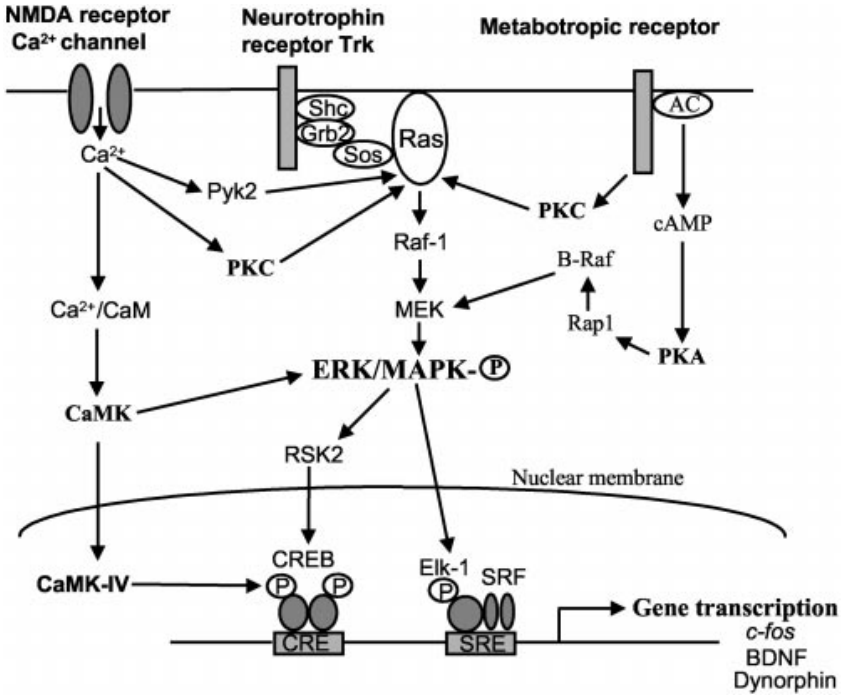

Figure 2.6. ERK/MAPK activation pathways (taken from Ji and Woolf, 2001).

2.6. Supraspinal changes implicated in the capsaicin-induced mechanical hypersensitivity

The use of PET (positron emission tomography) and fMRI (functional magnetic resonance imaging) has enabled the identification of the cerebral network that is active during nonpainful mechanical stimulation and capsaicin-induced mechanical hypersensitivity. The application of a nonpainful mechanical stimulus, under control conditions, activated various cortical areas (primary somatosensory cortex, parietal association cortex, bilateral secondary somatosensory cortex), contralateral inferior parietal lobule and contralateral insula (Iadarola et al., 1998; Baron et al., 1999; Maihöfner et al., 2004). Interestingly, during capsaicin-induced secondary mechanical hyperalgesia, the brain responses were partially overlapped with those induced by nonpainful mechanical stimulation in the nonsensitized area, but several additional areas were also activated. The contralateral inferior frontal cortex, the ipsilateral insula and bilateral thalamus were activated during capsaicin-induced mechanical hypersensitivity (Iadarola et al., 1998; Maihöfner et al., 2004; Zambreanu et al., 2005); all of these areas are implicated in the sensory-perceptual network (Iadarola et al., 1998). Other areas activated during capsaicin-induced mechanical hypersensitivity were the contralateral prefrontal cortex and posterior parietal cortex, which are linked to hypervigilance and/or increased attention towards pain, as seen in several clinical pain disorders (Baron et al., 1999; Zambreanu et al., 2005). It was also reported that capsaicin-induced secondary mechanical hyperalgesia evoked activation of the cerebellum (Zambreanu et al., 2005). Increased cerebellar activity has often been attributed to the intention of motor response or withdrawal (Saab and Willis, 2003). However, studies on psychological aspects of

pain found activity changes in the cerebellum (Smith et al., 2002), suggesting a higher cognitive function for the cerebellum in pain processing (Zambreanu et al., 2005). Interestingly, these changes in brain activity during capsaicin-induced secondary mechanical hyperalgesia are similar to those produced in neuropathic pain (Moisset and Bouhassira, 2007).

Furthermore, it has been shown that the activity of nucleus cuneiformis and periaqueductal gray matter are increased during capsaicin-induced secondary mechanical hyperalgesia (Zambreanu et al., 2005). As described in section 1.6.1.1., these brainstem structures have a long-recognized capacity for descending modulation of pain, since the periaqueductal gray and the nucleus cuneiformis are the main sources of input to the RVM. Therefore, descending modulatory pathways may also be altered during capsaicin-induced mechanical hypersensitivity, as reported for mechanical sensitization in models of inflammatory or neuropathic pain (see Urban and Gebhart, 1999 for references).

3. SIGMA-1 RECEPTORS

3.1. Historical overview: Discovery of sigma receptors and sigma receptor subtypes

Sigma (σ) receptors were first described as a subclass of opioid receptors (Martin et al., 1976) to account for the psychotomimetic actions of (±)-SKF-10,047 (*N*-allylnormetazocine) and other racemic benzomorphans. This early confusion was due to the complex pharmacology of this racemic compound; later studies showed that (–)-SKF-10,047 binds to μ and κ opioids, whereas the (+)-isomer lacks affinity for opioid receptors but binds to PCP (phencyclidine) binding sites with low affinity, and to a different site with high affinity, which currently retains the designation of σ (Matsumoto et al., 2003; Walker

et al., 1990; amongst others).

Two different σ sites were distinguished based on their different drug selectivity pattern and molecular mass; these two σ sites are now known as σ_1 and σ_2 receptors (Hellewell and Bowen, 1990). It was reported that σ_1 binding sites display stereospecificity towards dextrorotatory isomers of benzomorphans, whereas σ_2 binding sites display reverse selectivity, i.e., levorotatory isomers display higher affinity than dextrorotatory isomers of σ ligands (Hellewell and Bowen, 1990; Quirion et al., 1992). The molecular weight was found to differ between the two σ receptors subtypes: the σ_1 receptor is a 29-kDa single polypeptide first cloned in 1996 (Hanner et al., 1996), whereas σ_2 receptors have not yet been cloned and have an apparent molecular weight of 18-21.5 kDa according to photolabeling studies (Hellewell et al., 1994; Pal et al., 2007). In spite of intensive efforts in research on the σ_2 subtype in recent years (partially reviewed in Bowen, 2000; Nuwayhid and Werling, 2006; Matsumoto et al., 2007; Monassier et al., 2007), the σ_1 subtype is much better characterized, and is the focus of this Doctoral thesis.

$Sigma_1$ receptors have been thoroughly studied in an attempt to elucidate their possible neuropharmacological applications, mainly in learning and memory processes, depression and anxiety, schizophrenia, analgesia and some effects of certain drugs of abuse. In this Doctoral thesis we describe some aspects of the general biology of σ_1 receptors, but focus on σ_1 ligand neuropharmacology and the role of σ_1 receptors in behavioral animal studies, which have contributed greatly to the understanding of the possible neuropharmacological properties of σ_1 receptors. Non-neuropharmacological effects of σ_1 ligands such as cardiovascular effects or their effects on cancer and immunity, and their antitussive effects, are not covered in this Doctoral thesis.

3.2. Molecular characteristics, distribution and pharmacological profile of sigma$_1$ receptors

3.2.1. Cloning and structure of σ_1 receptors

Significant progress in our knowledge of σ receptors was made when the σ_1 receptor was cloned. The σ_1 receptor is a 29-kDa single polypeptide which was first cloned in guinea-pig liver (Hanner et al., 1996), and later in mouse kidney, a JAR human choriocarcinoma cell line, and in the rat and mouse brain (reviewed in Guitart et al., 2004). The protein is composed by 223 amino acids and shows the typical σ_1 binding profile (Kekuda et al., 1996; Seth et al., 1998; Hanner et al., 2004). The amino acid sequence of the σ_1 receptor cloned from the human cell line is highly homologous to the σ_1 receptor cloned from the other species (Seth et al., 2001), and shows no homology with other mammalian proteins, but shares approximately 30% identity with the yeast gene that encodes the C7–C8 sterol isomerase (Moebius et al., 1997), and contains an endoplasmic reticulum retention signal at the NH_2 terminus (Hanner et al., 1996; Seth et al., 1997). Cloning of the σ_1 receptor has contributed greatly to research in this field, making it possible to design specific antisense oligodeoxynucleotides to study σ_1 receptor function (as will be described below) and develop σ_1-receptor knockout mice (Langa et al., 2003).

Several structures have been proposed for σ_1 receptors. Initial studies proposed a single transmembrane domain structure (Hanner et al., 1996; Dussossoy et al., 1999). More recently, Aydar and coworkers presented evidence that the σ_1 receptor in the plasma membrane has two transmembrane segments (when expressed in *Xenopus laevis* oocytes) with the NH_2 and COOH termini on the cytoplasmic side of the membrane (Aydar et al., 2002). Recent studies proposed that in addition to the hydrophobic regions that constitute

the putative transmembrane domains, there are two additional hydrophobic segments (one of them partially overlapping the second transmembrane domain), which were proposed to be steroid binding domain-like sites (Chen et al., 2007), and suggesting the existence of two different domains for ligand binding in the σ_1 receptor (Pal et al., 2007), as previously proposed in earlier experiments (Bowen et al., 1989). This proposed model is illustrated in Figure 3.1. The pharmacological characterization of these putative domains merits further study.

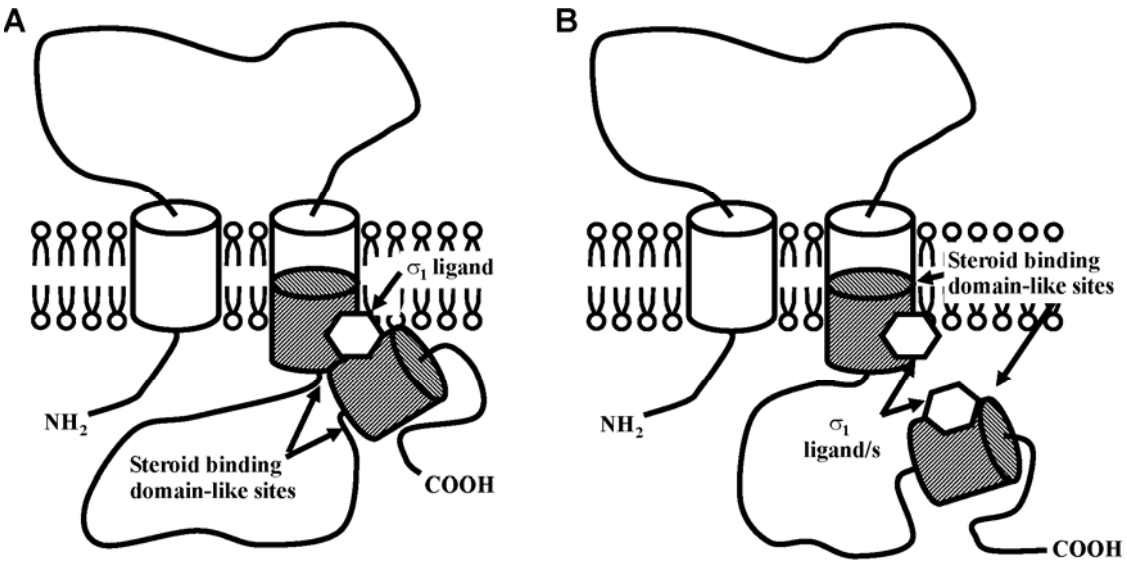

Figure 3.1. Putative model for σ_1 receptors proposed by Pal and coworkers (Pal et al., 2007). Open cylinders represent the two putative transmembrane domains. Closed cylinders represent the steroid binding domain-like sites and the open hexagon represents a putative σ_1 ligand. A, Possible spatial arrangement of the ligand binding site involving both steroid binding domain-like sites. B, Alternative model for ligand interaction with the σ_1 receptor.

3.2.2. Anatomical and subcellular distribution of σ_1 receptors

3.2.2.1. Anatomical distribution of σ_1 receptors

At the anatomical level σ_1 receptors are widely distributed in peripheral organs (Stone

et al., 2006) and different areas of the central nervous system, where they have been thoroughly studied. They are widely distributed in the brain, but concentrated in specific areas involved in memory, emotion and sensory and motor functions (reviewed in Bermack and Debonnel, 2007; Guitart et al., 2004; Monnet and Maurice, 2006). In these studies high to moderate levels of σ_1 receptors were associated with the hippocampus, especially in the dentate gyrus, hypothalamus, olfactory bulb, several cortical layers, pons, the septum, the central gray, locus ceruleus, dorsal raphe, the substantia nigra pars compacta, the red nucleus and various motor cranial nerve nuclei. The cerebellum is not particularly enriched in σ_1 receptors, although some of its areas, such as the Purkinje cell layer, have been reported to show considerable densities of σ_1 receptors. In addition to the brain, σ_1 receptors are also numerous in the spinal cord, mainly in the superficial layers of the dorsal horn (Alonso et al., 2000).

3.2.2.2. Subcellular distribution of σ_1 receptors

The subcellular distribution of σ_1 receptors was firstly studied with radioligand binding in subcellular fractions, and more recently with immunochemical methods. Binding experiments with the σ_1 radioligands [^{3}H](+)-SKF-10,047, [^{3}H](+)-3-PPP and [^{3}H](+)-pentazocine showed that σ_1 receptors are located in several types of mouse, rat and guinea pig brain membrane. These binding sites are more abundant in microsomal membranes, which are consistent with the endoplasmic reticulum retention signal of the cloned σ_1 receptor (Hanner et al., 1996; Seth et al., 1997), but they are also present in nuclear, mitochondrial and synaptic membranes (Itzhak et al., 1991; Cagnotto et al., 1994; DeHaven-Hudkins et al., 1994; Cobos et al., 2007). Immunohistochemical studies further confirmed the existence of σ_1 receptors in the endoplasmic reticulum not only in neurons

(Alonso et al., 2000), but also in many other cell types such as oligodendrocytes (Palacios et al., 2003), lymphocytes (Dussossoy et al., 1999), retinal cells (Jiang et al., 2006) and certain cancer cells (Hayashi and Su, 2004). Detailed studies by Hayashi and Su in NG108 cells showed that σ_1 receptors are located as highly clustered globular structures enriched in cholesterol and neutral lipids in the nuclear envelope and endoplasmic reticulum (reviewed in Hayashi and Su, 2004). In neurons from the rat hypothalamus and hippocampus, electron microscopy studies showed that σ_1 receptor immunostaining was mostly associated with neuronal perikarya, the membrane of mitochondria, some cisternae of the endoplasmic reticulum and dendrites, where it was localized in the limiting plasma membrane including the postsynaptic thickening (Alonso et al., 2000).

3.2.3. Pharmacological profile of σ_1 receptors: xenobiotics and endogenous ligands

3.2.3.1. Exogenous ligands for σ_1 receptors

As it has been described above (see section 3.1), one characteristic that distinguishes σ_1 binding sites from σ_2 receptors is that the σ_1 receptor displays stereospecificity towards dextrorotatory isomers of benzomorphans (such as SKF-10,047 or pentazocine) (Hellewell and Bowen, 1990; Quirion et al., 1992). An interesting aspect of σ_1 receptor pharmacology is that these receptors can bind, with high to moderate affinity, a wide spectrum of known compounds of very different structural classes and with different therapeutic and pharmacological applications, such as neuroleptics (e.g. haloperidol, nemopramide), antidepressants (e.g. fluvoxamine, clorgyline), antitussives (carbetapentane, dextromethorphan, dimemorfan), drugs for the treatment of neurodegenerative disorders such as Parkinson's disease (amantadine) or Alzheimer's disease (memantine, donepezil), and drugs of abuse (cocaine, methamphetamine) (Table 1). As for many other receptors,

some allosteric modulators have been described for σ_1 receptors, including the anticonvulsant drugs phenytoin (DPH) and ropizine (Table 1). The modulation of σ_1 radioligand binding by DPH has been conventionally assumed to be a characteristic difference between σ_1 and σ_2 binding sites (see Quirion et al., 1992; Maurice et al., 2001c for reviews). However, we recently reported that DPH also discriminates between different σ_1 ligands depending on their activities on σ_1 receptors (Cobos et al., 2005 and 2006).

Haloperidol deserves special consideration among the σ ligands, because it is the most widely used σ_1 antagonist in research on σ_1 receptors, and its affinity is high enough to bind σ_1 receptors in humans after a single oral dose (Stone et al., 2006). In fact, haloperidol binds with similarly high affinity to dopamine D_2 receptors and σ receptors, but its metabolites display preferential activity at σ receptors compared to dopamine D_2 receptors (Bowen et al., 1990). Particularly interesting is the reduced metabolite of haloperidol (haloperidol metabolite II), which has high affinity for σ_1 and σ_2 receptors but shows much lower affinity for D_2 receptors than the original compound (Bowen et al., 1990; Matsumoto and Pown, 2000). This compound was recently shown to be an irreversible σ_1 ligand (Cobos et al., 2007).

Some selective and high affinity σ_1 drugs have been developed and are considered prototypical σ_1 ligands. Examples are the σ_1 agonists (+)-pentazocine, PRE 084, JO-1784 and SA4503, and the σ_1 antagonists BD 1063 and NE-100. Table 1 summarizes the pharmacological activities on σ_1 receptors, σ subtype selectivity and other known pharmacological activities of some σ ligands used in research (and also in therapeutics). Currently the number of σ ligands is increasing rapidly with the development of new compounds (Collina et al., 2007; Marrazzo et al., 2002; Matsumoto et al., 2001a; Ronsisvalle et al., 2000, among others).

3.2.3.2. Putative endogenous σ_1 ligands: neurosteroids

Although the endogenous ligands for σ_1 receptors have not yet been defined unequivocally, currently the neurosteroids are considered the most probable endogenous σ_1 ligands. This term, first used by Baulieu, identifies steroids that are synthesized in the central and peripheral nervous systems, and includes pregnenolone, dehydroepiandrosterone (DHEA), their sulfate esters, progesterone, and allopregnenolone (reviewed in Baulieu, 1998). The physiological actions of neurosteroids include genomic actions and nongenomic neuromodulatory actions, the latter of which are presumably related with σ_1 receptors (see Monnet and Maurice, 2006 for a detailed review). The interaction between neurosteroids and σ_1 receptors was first suggested in 1988 (Su et al., 1988) from *in vitro* experiments in guinea pig brain and spleen. Of the steroids tested, progesterone was the most (Schwarz et al., 1989) potent inhibitor of σ_1-specific radioligand binding; however, whether neurosteroids are the endogenous ligands of the σ_1 receptor remains controversial because the affinity of progesterone for σ_1 receptors does not appear to be high enough for an endogenous ligand. In addition, other steroids such as DHEAS (DHEA sulfate), pregnenolone sulfate, testosterone and deoxycorticosterone exhibited even lower affinities for σ_1 receptors than progesterone (Hayashi and Su, 2004). However, some reports support that neurosteroids are the σ_1 receptor endogenous ligands. In many experimental paradigms, progesterone behaved like other known σ_1 antagonists, and DHEA and pregnenolone sulfate act as other known σ_1 agonists (see Maurice et al., 2006; Monnet and Maurice, 2006, for an extensive review). The exogenous administration of neurosteroids led to a dose-dependent inhibition of *in vivo* σ_1 radioligand binding (Maurice et al., 1996; Waterhouse et al., 2007), and modifications in endogenous levels of neurosteroids (e.g., after adrenalectomy, castration, ovariectomy or during pregnancy)

affected σ_1 responses (Bergeron et al., 1996 and 1999; Urani et al., 2001). The cloned σ_1 protein presents homologies with the steroid binding site of several steroidogenic enzymes, which supports the specific interaction of σ_1 receptors with neurosteroids (Maurice et al., 2006; Chen et al., 2007; Pal et al., 2007). We have therefore included them in Table 1 as putative σ_1 endogenous ligands.

Table 1. Pharmacology of some usual σ_1 receptor ligands.

Compound	Subtype selectivity	Affinity for σ_1 site*	Function on σ_1 site	Other activities
Benzomorphans				
(+)-Pentazocine	σ_1 [a]	+++ [a]	Agonist [a]	-
(–)-Pentazocine	σ_1/σ_2 [b]	++ [b]	Agonist [c]	κ_1 agonist, μ_1, μ_2, ligand, low affinity δ, and κ_3 opioid ligand [c]
(+)-SKF-10,047	σ_1 [a]	+++ [a]	Agonist [a]	NMDA receptor ligand [a]
Antipsychotics				
Chlorpromazine	σ_1/σ_2 [d]	++ [a]	? [a]	Dopamine D_2 antagonist [a]
Haloperidol	σ_1/σ_2 [a]	+++ [a]	Antagonist [a]	Dopamine D_2 and D_3 antagonist [t]; σ_2 agonist [i]
Nemonapride	σ_1/σ_2? [a]	+++ [a]	? [a]	Dopamine D_2 antagonist [a]
Antidepressants				
Clorgyline	σ_1 [e]	+++ [e]	Agonist? [f]	Irreversible monoamine oxidase A inhibitor [e]
Fluoxetine	σ_1 [g]	+ [g]	Agonist [a]	Selective 5-HT reuptake inhibitor [g, a]
Fluvoxamine	σ_1 [g]	+++ [g]	Agonist [a]	Selective 5-HT reuptake inhibitor [g, a]
Imipramine	σ_1 [g]	++ [g]	Agonist [a]	Monoamine reuptake inhibitor [a]
Sertraline	σ_1 [g]	++ [g]	Agonist [f]	Selective 5-HT reuptake inhibitor [g]
Antitussives				
Carbetapentane	σ_1/σ_2 [h]	+++ [h]	Agonist [i]	Muscarinic antagonist [h]
Dextromethorphan	σ_1 [j]	++ [j]	Agonist [i]	NMDA receptor allosteric antagonist [u]
Dimemorfan	σ_1/σ_2 [j]	++ [j]	Agonist [j, k]	?
Parkinson's and/or Alzheimer's disease				
Amantadine	?	+ [l]	Agonist? [l]	NMDA antagonist, antiviral properties [v]
Donepezil	σ_1/σ_2? [m]	+++? [m]	Agonist [ad, ae, af]	Cholinesterase inhibitor [m]
Memantine	?	+ [l]	Agonist? [l]	NMDA antagonist, antiviral properties [v]
Drugs of abuse				
Cocaine	σ_1/σ_2 [n]	+ [a, n]	Agonist [a]	Monoamine transporters inhibitor, amongst other actions [w]
MDMA	σ_1/σ_2 [o]	+ [o]	?	Preferential SERT inhibitor, among other actions [y]
Metamphetamine	σ_1/σ_2 [p]	+ [p]	?	Preferential DAT inhibitor, amongst other actions [z]

Putative endogenous ligands (neurosteroids)				
DHEAS	σ_1 [a]	+ [a]	Agonist [a]	$GABA_A$ negative modulator [i]
Pregnenolone sulfate	σ_1 [a]	+ [a]	Agonist [a]	NMDA positive/$GABA_A$ negative modulator [i]
Progesterone	σ_1 [a]	+ [q, r, s]	Antagonist [a]	NMDA negative/$GABA_A$ positive modulator [i]
Anticonvulsants				
Phenytoin (DPH)	σ_1 [b]	Not applicable	Allosteric Modulator [q, r, b]	Delayed rectifier K^+ channel blocker [aa]; T-type Ca^{2+} current inhibitor [ab]; Na^+ current inhibitor [ac]
Ropizine	σ_1 [b]	Not applicable	Allosteric modulator [b]	?
Other σ drugs				
BD 737	σ_1/σ_2 [ag]	+++ [ah]	Agonist [ah]	-
BD 1008	σ_1/σ_2 [a]	+++ [a]	Antagonist [a]	σ_2 agonist? [az]
BD 1047	σ_1 [ai]	+++ [ai]	Antagonist [ai]	β adrenoceptor ligand [ai]
BD 1063	σ_1 [ai]	+++ [ai]	Antagonist [ai]	-
BMY 14802	σ_1/σ_2 [d]	++ [d]	Antagonist [ah]	5-HT_{1A} agonist [aw]
DTG	σ_1/σ_2 [a]	+++ [a]	? [a]	σ_2 agonist [i]
Dup 734	σ_1 [a]	+++ [a]	Antagonist [ah]	5-HT_2 antagonist [ay]
Eliprodil (SL-82.0715)	σ_1/σ_2 [aj]	++ [aj]	? [a]	NMDA antagonist, σ_1 adrenoceptor ligand [aj]
E-5842	σ_1 [ak]	+++ [ak]	Antagonist [ah]	Low to moderate affinity for dopamine, 5-HT and glutamate receptors [ak]
Haloperidol Metabolite I	σ_1 [d]	++ [al, d]	Antagonist [am]	-
Haloperidol Metabolite II	σ_1/σ_2 [d]	+++ [al, d]	Irreversible antagonist [al]	Dopamine D_2 and D_3 ligand [t]
4-IBP	σ_1/σ_2 [an]	+++ [an]	Agonist [f]	Dopamine D_2 ligand [an]
JO-1784 (Igmesine)	σ_1 [a]	+++ [a]	Agonist [a]	-
Metaphit	σ_1/σ_2 [ao]	++ [al]	Irreversible antagonist [ao]	Acylator of PCP and σ_2 binding sites [ao]
(+)-MR 200	σ_1/σ_2 [ap]	+++ [ap]	Antagonist [aq]	-
MS-377	σ_1 [a]	+++ [a]	Antagonist [a]	-
NE-100	σ_1 [a]	+++ [a]	Antagonist [a]	-
OPC-14523	σ_1/σ_2 [a]	+++ [a]	Agonist [ah]	Agonist of pre- and post-synaptic 5-HT_{1A} receptors [ar]; SERT inhibitor [as]
Panamesine (EMD 57445)	σ_1/σ_2? [a]	+++? [a]	Antagonist [ah]	One of its metabolites is a dopaminergic antagonist [a]
(+)-3-PPP	σ_1/σ_2 [at]	++ [q, r]	Agonist [a]	σ_2 agonist [i]; NMDA receptor ligand [au]; dopaminergic agonist [a]
PRE 084	σ_1 [a]	+++ [a]	Agonist [a]	-
Rimcazole (BW-234U)	σ_1/σ_2 [av]	+ [a]	Antagonist [a]	DAT inhibitor [av]
SA4503	σ_1 [a]	+++ [a]	Agonist [a]	-
SR 31742A	?	+++ [a]	?	High affinity for C8-C7 sterol isomerase [a]

[*] K_i or K_D values: +++ <50 nM; ++ <500 nM; + <10 μM

?: Not studied or unclear at the moment

-: No other pharmacological target has been described

[a] Hayashi and Su, 2004a, [b] Walker et al., 1990, [c] Chien and Pasternak, 1995b, [d] Matsumoto and Pouw, 2000, [e] Itzhak et al., 1991, [f] Bermack and Debonnel, 2005a, [g] Narita et al., 1996, [h] Calderon et al, 1994, [i] Maurice et al., 2001c, [j] Shin et al., 2005, [k] Wang et al., 2003, [l] Peeters et al., 2004, [m] Kato et al., 1999, [n] Matsumoto et al., 2002, [o] Brammer et al., 2006, [p] Nguyen et al., 2005, [q] Cobos et al., 2005, [r] Cobos et al., 2006, [s] Hong et al., 2004, [t] Jaen et al., 1993, [u] LePage et al., 2005, [v] Chen and Lipton, 2006, [w] Rothman and Baumann, 2003, [y] Green et al., 2003, [z] Fleckenstein et al., 2007, [aa] Nobile and Lagostena, 1998, [ab] Todorovic and Lingle, 1998, [ac] Rush and Elliott, 1997, [ad] Maurice et al., 2006, [ae] Meunier et al., 2006a, [af] Meunier et al., 2006b, [ag] Hellewell et al., 1990, [ah] Guitart et al., 2004, [ai] Matsumoto et al., 1995, [aj] Hashimoto and London, 1995, [ak] Guitart et al., 1998, [al] Cobos et al., 2007, [am] Cendan et al., 2005, [an] John et al., 1994, [ao] Bluth et al., 1989, [ap] Ronsisvalle et al., 2001, [aq] Marrazzo et al., 2006, [ar] Bermack and Debonnel, 2007, [as] Tottori et al., 2001, [at] Hellewell and Bowen, 1990, [au] Matsumoto et al., 2001a, [aw] Matos et al., 1996, [ay] Tam et al., 1992, [az] Maurice et al., 1999.

3.3. Modulation of cellular effects by sigma-1 receptors

One of the earliest questions about the cellular effects of σ_1 receptors concerned their possible coupling to G-proteins. This issue has been studied with different experimental approaches, and the results reported to date are as profuse as they are contradictory (reviewed in Bermack and Debonnel, 2005b; Guitart et al., 2004). Even some selective σ_1 agonists seemed to act through G-proteins (JO-1784), whereas others ((+)-pentazocine) did not under the same experimental conditions (Monnet et al., 1994). Now that the σ_1 receptor has been cloned (Hanner et al., 1996), it seems clear that the cloned receptor does not have the typical structure of a G-protein-coupled receptor with seven transmembrane domains; however, the existence of a metabotropic σ_1 receptor subtype different of the cloned type cannot be ruled out yet (e.g. Maruo et al., 2000). Although the coupling of σ_1 receptors to G-proteins remains controversial, the modulatory role of σ_1 receptors in the activity of some ion channels, different kinds of neurotransmission (mainly glutamatergic) and in second messenger systems, particularly the phospholipase C/protein kinase C/inositol 1,4,5-

trisphosphate (PLC/PKC/IP_3) system, has been extensively reported.

3.3.1. Modulation of plasmalemmal ion channels

3.3.1.1. Potassium channels

Potassium channels have been shown to constitute an important target for σ drugs. It has been shown that σ ligands inhibited K^+ currents in several experimental preparations (Soriani et al., 1999a and 1999b; Wilke et al., 1999; Lupardus et al., 2000; Zhang and Cuevas, 2005; Martina et al., 2007). In some of these studies, known σ_1 agonists and antagonists produced the same effects (Wilke et al., 1999; Zhang and Cuevas, 2005). These results might reflect the participation of σ_2 activity, since it was recently reported that σ_2 ligands can also modulate K^+ currents (Monassier et al., 2007). However, other recent studies showed that the selective σ_1 agonists (+)-pentazocine and JO-1784 reduced several K^+ currents in frog melanotropic cells (Soriani et al., 1999a and 1999b), and prevented the activation of small-conductance calcium-activated K^+ channels (SK channels) in rat hippocampal slices (Martina et al., 2007). These effects were reversed by known σ_1 antagonists (NE-100 or haloperidol) (Soriani et al., 1999; Martina et al., 2007). Regarding the molecular mechanism of the effects of σ_1 receptors in K^+ currents, it was proposed that σ_1 receptors and K^+ channels must be in close proximity for any functional interaction to occur (Lupardus et al., 2000; Mavlyutov and Ruoho, 2007), and in fact the heterologous expression of σ_1 receptors with voltage-gated K^+ channels Kv 1.4 and 1.5, in *Xenopus* oocytes, resulted in modulation of the channel function in the absence of any σ ligand, and greater modulation in the presence of SKF-10,047 (Aydar et al., 2002). Moreover, Kv 1.4 channel not only colocalized (Mavlyutov and Ruoho, 2007) but also co-immunoprecipitated with σ_1 receptor proteins, indicating that σ_1 receptors are directly associated with these K^+

channels (Aydar et al., 2002). In addition, σ_1 photolabeling with radioiodinated probes identified high-molecular-mass protein complexes, suggesting that σ_1 receptors may exist as oligomers or interact with protein partners either constitutively or through ligand binding (Pal et al., 2007). The formation of these complexes might help explain the wide variety of actions produced by σ_1 ligands in the central nervous system.

3.3.1.2. Calcium channels

Sigma$_1$ ligands have also been reported to modulate plasmalemmal voltage-dependent calcium channels. Interaction between σ receptors and Ca^{2+} channels was suggested from studies in which the increase in $[Ca^{2+}]_i$ mediated by depolarization was diminished by σ ligands in neuronal cultures or forebrain synaptosomes (reviewed in Bermack and Debonnel, 2005a, Guitart et al., 1998; Monnet et al., 2003). However, in some of these experiments σ_1 agonists and σ_1 antagonists produced the same effects, which might be also due to the participation of σ_2 receptors (reviewed in Monnet, 2005). In addition, the selective σ_1 agonists (+)-pentazocine and PRE 084 induced opposite effects on the increase in $[Ca^{2+}]_i$ induced by depolarization with KCl in NG108 cells, but both effects were reverted by σ_1 receptor antisense oligodeoxynucleotide (Hayashi and Maurice, 2000), suggesting that they were both mediated by the cloned σ_1 receptor. It therefore seems clear that more studies are necessary to clarify the role of these receptors in plasmalemmal voltage-dependent calcium channels.

3.3.2. Neurotransmitter systems and σ_1 receptors: modulation of N-methyl-D-aspartate (NMDA) neurotransmission

Many studies have shown that σ_1 receptors are able to modulate several neurotransmitter systems. It has been reported that σ_1 receptors can potentiate glutamatergic neurotransmission (partially reviewed in Bermack and Debonnel, 2005b and Monnet and Maurice, 2006), enhance cholinergic neurotransmission (partially reviewed in Maurice and Lockhart, 1997; Romieu et al., 2006), enhance serotonergic neurotransmission (reviewed in Bermack and Debonnel, 2005b), negatively modulate the GABAergic system (Garrone et al., 2000; Mtchedlishvili and Kapur, 2003), diminish noradrenaline release (Campana et al., 2002), and modulate dopaminergic neurotransmission (reviewed in Maurice et al., 2002). The direction of modulation of the dopaminergic system has been especially controversial because the contradictory results reported thus far make it difficult to reach solid conclusions. The conflicting results probably reflect the use of drugs with different degrees of selectivity for σ_1 receptors, and different routes of administration (reviewed extensively in Maurice et al., 2002).

Among the modulatory effects on different neurotransmitter systems by σ_1 receptors, the modulation of glutamatergic neurotransmission has been described in greater detail than others. It has been reported that σ_1 receptors agonists can enhance spontaneous glutamate release in the hippocampus (Dong et al., 2007; Meyer et al., 2007), potentiate glutamate release induced by brain-derived neurotrophic factor (Yagasaki et al., 2006), potentiate the increase in $[Ca^{2+}]_i$ induced by glutamate in pyramidal neurons (Monnet et al., 2003), and facilitate long-term potentiation in the rat hippocampus (Chen et al., 2006; Li et al., 2006; Martina et al., 2007). Of the three subtypes of glutamate-gated ion channels (NMDA, kainate and AMPA receptors), the connection between σ_1 and NMDA receptors has been

widely explored, mainly in studies of the NMDA-induced firing activity in the dorsal hippocampus. In this model σ_1 agonists such as the selective agonists JO-1784 and (+)-pentazocine, the putative agonist DHEA, and the antidepressants clorgyline and sertraline (but not paroxetine or tranylcypromine, which showed much lower affinities for σ_1 receptors) were able to modulate NMDA-induced firing. The effect of these ligands was reversed by known σ_1 antagonists such as haloperidol, NE-100 or BMY-14802, and also by the putative endogenous ligand progesterone [reviewed in Bermack and Debonnel, 2005b; Monnet and Maurice, 2006). Interestingly, in these studies σ_1 agonists showed a bell-shaped dose-response curve characterized by low-dose stimulation and high-dose inhibition. This type of dose-response curve indicates hormesis (Calabrese and Baldwin, 2003), and is well documented for σ_1 receptor activation not only in the modulation of NMDA-induced firing, but also in many other experimental approaches, as will be described below.

Particularly interesting are the studies that related steroidal tonus under physiological conditions with the σ-mediated potentiation of glutamatergic neurotransmission in the hippocampus. This effect was strongly affected by increased levels of progesterone in pregnancy (Bergeron et al., 1999), and by decreased levels of this neurosteroid after ovariectomy (Bergeron et al., 1996). A molecular mechanism was recently proposed by which σ_1 receptor activation increases the NMDA receptor response. Ca^{2+} entering the cells through the NMDA receptors activates a Ca^{2+}-activated K^+ current, underlain by SK channels, which in turn shunts the NMDA receptor responses. Selective σ_1 agonists prevented SK channel opening, and consequently increased the NMDA receptor response, emphasizing the importance of the σ_1 receptor as a postsynaptic regulator of synaptic transmission (Martina et al., 2007). Importantly, the modulation of several neurotransmitter systems mentioned above may be a consequence, at least partially, of the modulation of

NMDA receptors. It has been reported in this connection that σ_1 ligands can modulate dopaminergic (reviewed in Maurice et al., 2002), cholinergic (reviewed in Monnet and Maurice, 2006), serotonergic (Takahashi et al., 2001) or noradrenergic systems (reviewed in Bermack and Debonnel, 2005b) through NMDA receptors.

In summary, σ_1 receptors modulate several neurotransmitter systems, and it seems that the modulation of NMDA responses by σ_1 receptors plays a pivotal role in the modulation of neurotransmission by σ_1 ligands.

3.3.3. Sigma$_1$ receptors as modulators of intracellular messenger systems

The modulation of metabotropic responses by σ_1 receptors, particularly the increase in $[Ca^{2+}]_i$ after stimulation of IP_3 receptors at the endoplasmic reticulum, has been described in detail. The mechanism of modulation of the PLC/PKC/IP_3 system by σ_1 receptors appears to be a complex one involving the translocation of σ_1 receptors to the plasma membrane and the nucleus; this was proposed as a mechanism by which an intracellular receptor modulates metabotropic responses (Morin-Surun et al., 1999; Hayashi et al., 2000). Sigma$_1$ receptors are localized in highly clustered globular structures associated with the endoplasmic reticulum, which contain moderate amounts of free cholesterol and neutral lipids, forming lipid droplets (Hayashi et al., 2005), in which σ_1 receptor, ankyrin (specifically the ANK220 isomer) and the IP_3 receptors form a complex (Hayashi and Su, 2001). Sigma$_1$ receptor activation by agonists induces the dissociation of the σ_1 receptor-ANK220 complex from the IP_3 receptors (Hayashi and Su, 2001), potentiating the calcium efflux induced by receptors that activates the PLC system (such as receptors for bradykinin and brain-derived neurotrophic factor) (Hayashi et al., 2000; Hayashi and Su, 2001; Hong et al., 2004; Peeters et al., 2004; Yagasaki et al., 2006) (Fig. 3.2).

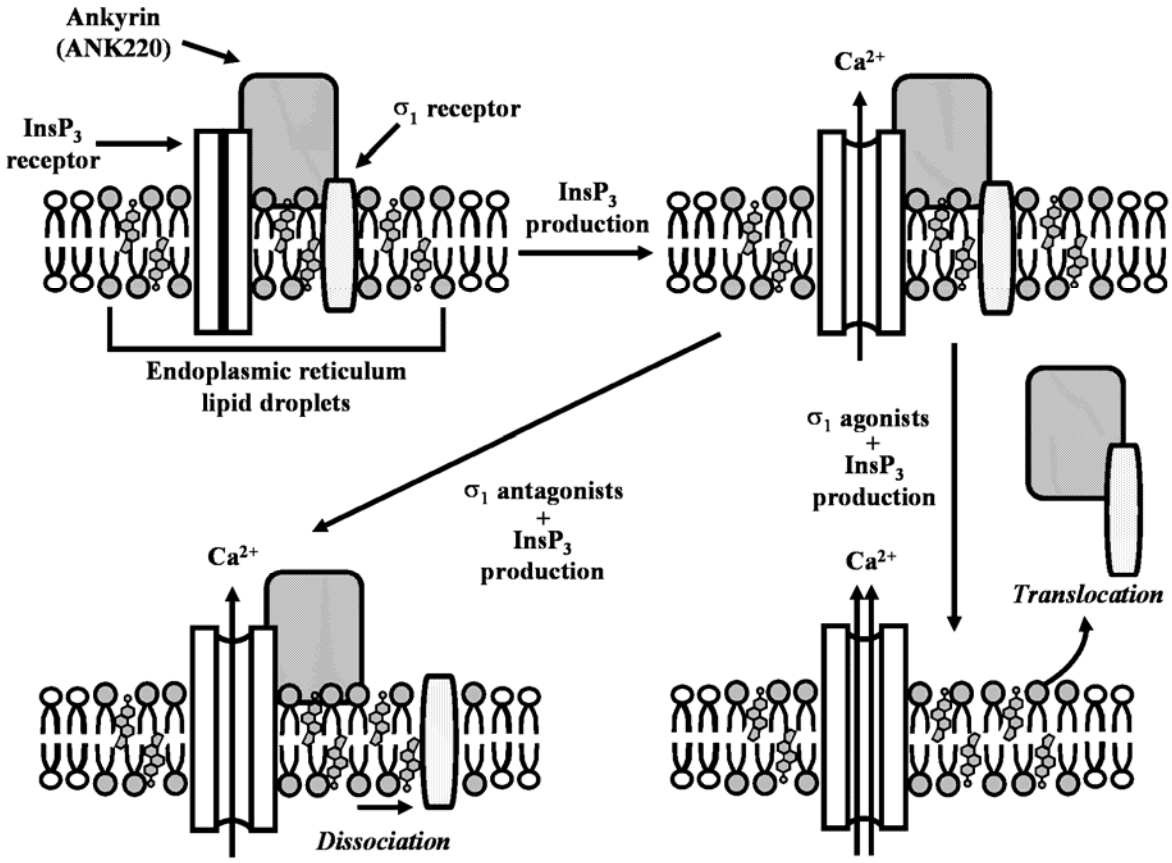

Figure 3.2. Model of modulation by σ_1 receptors of IP_3-mediated calcium efflux, proposed by Hayashi and Su (Hayashi and Su, 2001 and 2005a). IP_3 receptors, ANK220 and σ_1 receptor form a complex in lipid droplets on the endoplasmic reticulum, which contain moderate amounts of free cholesterol and neutral lipids. In the presence of a σ_1 agonist, the σ_1 receptor-ANK220 complex is dissociated from IP_3 receptors and translocated. As a result IP_3 binding to its receptor increases and Ca^{2+} efflux is enhanced. In the presence of a σ_1 antagonist, ANK220 remains coupled to IP_3 receptor, but σ_1 receptor is dissociated from the complex, impeding the potentiation of calcium efflux by σ_1 agonists.

The enhancement of calcium efflux, which followed a bell-shaped curve (Hayashi et al., 2000), has been reported not only with known selective σ_1 agonists such as PRE 084 or (+)-pentazocine (Hayashi et al., 2000; Hayashi and Su, 2001; Hong et al., 2004), but also with other compounds such as the neurosteroids pregnenolone, pregnenolone sulfate and DHEA (Hayashi et al., 2000; Hong et al., 2004), amantadine and memantine (Peeters et al.,

2004). In the presence of a σ_1 antagonist, σ_1 receptors are dissociated from ankyrin and IP_3 receptors, which remain on the endoplasmic reticulum (Hayashi and Su, 2001) where they impede the potentiation by σ_1 agonists of bradykinin-induced Ca^{2+} efflux (Hayashi et al., 2000; Hong et al., 2004; Peeters et al., 2004). This latter effect was also prevented by the putative σ_1 antagonist progesterone (Hayashi et al., 2000; Hong et al., 2004), and by specific σ_1 receptor antisense oligodeoxynucleotides (Hayashi et al., 2000). Under basal conditions σ_1 ligands did not affect $[Ca^{2+}]_i$ (Hayashi et al., 2000; Hong and Werling, 2002), and the cells needed to be stimulated to make appropriate levels of IP_3 available for the modulation of $[Ca^{2+}]_i$ by σ_1 receptor agonists. Additionally, the silencing of IP_3 receptors resulted in a decrease in σ_1 receptor mARN levels (Novakova et al., 2007), underscoring the relationship between this second messenger system and σ_1 receptors. This proposed model of modulation by σ_1 receptors of IP_3-mediated calcium efflux is illustrated in Figure 3.2.

An additional mechanism by which σ_1 receptors can modulate other receptors located in the plasma membrane was recently proposed. It was reported that σ_1 receptors can affect the levels of plasma membrane lipid rafts by changing the lipid components therein (Takebayashi et al., 2004). This membrane reconstitution could in turn affect the function of the proteins it contains, such as neurotransmitter receptors or tropic factor receptors. In fact, σ_1 receptors play an important role in neurite sprouting (see Hayashi and Su, 2005b for a more complete review).

In summary, σ_1 receptors translocate from lipid droplets on the endoplasmic reticulum when stimulated by agonists, modulating intracellular Ca^{2+} mobilizations at the endoplasmic reticulum after activation of the PLC/PKC/IP_3 system, and enhancing the cellular effects of different receptors.

3.4. Therapeutic potential of sigma$_1$ receptors

Given the widespread distribution of σ_1 receptors in the central nervous system and their modulatory role at cellular, biochemical and neurotransmission levels (see above), σ_1 ligands appear to be useful in different therapeutic fields such as depression and anxiety, amnesic and cognitive deficits, psychosis, analgesia and treatment for drugs of abuse. These potential therapeutic applications are reviewed briefly below.

3.4.1. Role of σ_1 receptors in learning and memory

The central cholinergic and glutamatergic neurotransmission systems play a crucial role in learning and memory functions. Cholinergic function is disturbed in some memory pathologies such as Alzheimer's disease and pathological ageing, in which deficits in cortical cholinergic activity have been observed (Bartus, 2000). In addition, NMDA receptors are involved in the induction of different forms of synaptic plasticity (such as long-term potentiation) which are thought to be the synaptic substrate for learning and memory processes (Riedel et al., 2003). As described in the section 3.3.2., σ_1 agonists facilitate long-term potentiation in the rat hippocampus. However, the administration of large doses of σ_1 agonists or antagonists (+)-SKF-10,047, (+)-pentazocine, PRE 084, JO-1784, SA4503, DTG, BMY 14802, haloperidol, BD 1047 or NE-100, or even the downregulation of σ_1 receptor expression by antisense oligodeoxynucleotides, failed to affect learning in control animals. This finding suggests that σ_1 receptors are not involved in normal memory functions (Maurice and Lockhart, 1997; Maurice et al., 1999; Maurice et al., 2001a; Maurice et al., 2006; Monnet and Maurice, 2006 for reviews). Bearing in mind the typical modulatory role of σ_1 receptors, it is not surprising that they have been found to modulate memory and learning processes when a state of pharmacological or pathological

imbalance is induced.

3.4.1.1. Role of σ_1 receptors in memory and learning impairment induced by drugs, chemicals or brain lesions affecting cholinergic or glutamatergic neurotransmission

The learning impairment induced by the cholinergic muscarinic antagonist scopolamine, the nicotinic antagonist mecamylamine, or by cortical cholinergic dysfunction induced by ibotenic acid injection in the basal forebrain were attenuated or reversed by several σ_1 agonists, including the selective σ_1 agonists (+)-pentazocine, JO-1874 and SA4503 (reviewed in Maurice and Lockhart, 1997; Maurice et al., 2001a; Monnet and Maurice, 2006). In addition, the memory impairments induced by the serotonin (5-HT) depleter *p*-chloroamphetamine (PCA), which also involves cholinergic dysfunction (Matsuno et al., 1993), were attenuated in a bell-shaped manner by the administration of (±)-pentazocine, (+)-3-PPP, DTG, and (+)-SKF-10,047 (Matsuno et al., 1993 and 1994). The effects of σ_1 agonists in scopolamine-induced amnesia were reversed by known σ_1 antagonists including haloperidol and NE-100, and by the downregulation of σ_1 receptor expression by specific antisense oligodeoxynucleotides (reviewed in Maurice and Lockhart, 1997; Maurice et al., 2001b; Monnet and Maurice, 2006). Interestingly, the putative σ_1 agonists pregnenolone sulfate and DHEAS were also effective in scopolamine-induced amnesia model, and their effects were reversed by NE-100 and progesterone (Maurice et al., 1999; Maurice et al., 2001c; Monnet and Maurice, 2006).

As noted above, NMDA receptors also play an important role in learning and memory processes. The σ ligands (+)-SKF-10,047, (+)-pentazocine, JO-1784, DTG, PRE 084 and SA4503, and also the putative endogenous σ_1 agonists DHEAS and pregnenolone sulfate attenuated the learning deficits induced by dizocilpine (MK-801), a noncompetitive NMDA

receptor antagonist, in rats and mice presented with different amnesic tasks. The anti-amnesic effect of σ_1 agonists was reverted by several known σ_1 antagonists such as haloperidol, BMY 14802, NE-100 and BD 1047, by the putative endogenous σ_1 antagonists progesterone (partially reviewed in Maurice et al., 1999 and 2001; Maurice et al., 2006; Monnet and Maurice, 2006), and by the administration of antisense oligodeoxynucleotides against σ_1 receptors (Maurice et al., 2001a and 2001b and 2006). Cholinesterase inhibitors such as rivastigmine, tacrine and donepezil also attenuated dizocilpine-induced learning impairments (Maurice et al., 2006); however, only the effect of donepezil (which is also a potent σ_1 ligand, see Table 1) was blocked by BD 1047 or antisense treatment (Maurice et al., 2006).

Repeated exposure to carbon monoxide gas induced long-lasting but delayed amnesia which was measurable about one week after exposure. Like models of ischemia, this model involves the neurotoxicity of excitatory amino acids, and the hippocampal cholinergic system appears markedly affected by hypoxic toxicity (reviewed in Maurice and Lockhart, 1997). $Sigma_1$ ligands have been shown to have neuroprotective properties in models of ischemia (partially reviewed in Maurice and Lockhart, 1997; Bucolo et al., 2006; Katnik et al., 2006). Consistent with this neuroprotective action is the observation that the σ ligands (+)-SKF-10,047, PRE 084, JO-1784 and DTG reversed carbon monoxide-induced amnesia, and their effects were prevented by NE-100, BMY 14802 and BD 1047 (partially reviewed in Maurice et al., 1999; Meunier et al., 2006b). Donepezil and some other cholinesterase inhibitors have also been tested in this behavioral model of amnesia, and it was found that all drugs showed anti-amnesic properties, but the pre-administration of BD 1047 blocked only the effect of donepezil (Meunier et al., 2006b). Interestingly, in this model of amnesia the σ_1 antagonists BD 1008 and haloperidol also showed anti-amnesic effects that were not

reversed by NE-100, so it was suggested that these drugs might produce their effects through their σ_2 agonistic activity (Maurice et al., 1999). The role of σ_1 receptors in these experimental models is summarized in Table 2.

3.4.1.2. Role of σ_1 receptors in cognitive impairments in ageing: Alzheimer disease

In models related with the memory deficits of ageing, σ_1 agonists were also effective in attenuating the learning deficits in aged mice, aged rats and in senescence-accelerated mice (reviewed in Maurice and Lockhart, 1997; Monnet and Maurice, 2006). Moreover, in the model of Alzheimer's disease-type amnesia induced by β_{25-35}-amyloid related peptide, which involves both cholinergic and glutamatergic neurotransmission through NMDA receptors (Maurice et al., 2001a), the σ_1 receptor agonists (+)-pentazocine, PRE 084, SA4503, (+)-SKF-10,047, the antitussive drug dimemorfan and the putative σ_1 agonists DHEAS and pregnenolone sulfate attenuated amnesia in a bell-shaped manner. The effects of σ_1 agonists were reverted by haloperidol, BD 1047 and the putative σ_1 antagonist progesterone (Maurice et al., 1998; Meunier et al., 2006b; Wang et al., 2003). Donepezil and other cholinesterase inhibitors were also tested in this behavioral model, but only the effects of donepezil were partially reversed by BD 1047, suggesting that the anti-amnesic effects of this drug involve both its cholinergic and σ_1 agonistic properties (Meunier et al., 2006b). These findings are consistent with the neuroprotective action of the σ_1 agonist PRE 084, which attenuated cell death in cultured cortical neurons co-incubated with β_{25-35}-amyloid related peptide, and this effect was reversed by the selective σ_1 antagonist NE-100 (Marrazzo et al., 2005). The effects of σ_1 ligands in ageing-related cognitive impairment are summarized in Table 2.

3.4.1.3. Other ameliorative effects of σ_1 agonists on learning and memory

Stress during pregnancy directly affects the neurophysiological development of the fetus with deleterious consequences observable throughout the individual's lifetime (Kofman, 2002), and can result in impairments in learning and memory processes (Meunier et al., 2004). The σ_1 agonist JO-1784 reversed the learning deficits induced by prenatal stress, in a BD 1063-sensitive manner (Meunier et al., 2004). In addition, it is known that repeated cocaine treatment *in utero* can induce learning and memory impairment in the offspring. It was recently found that this process can be reverted by the σ_1 agonist JO-1784 or DHEA, in a BD 1063-sensitive manner (Meunier and Maurice, 2004). On the other hand, cocaine administered at very low doses (much lower doses than those which induce learning and memory impairments) can enhance memory storage in mice (Introini-Collison and McGaugh, 1989). The ameliorating effects of cocaine on memory can be enhanced by the σ_1 agonist JO-1784 and also by the putative σ_1 agonist DHEA, and masked by the σ_1 antagonist BD 1047 and also by the putative σ_1 antagonist progesterone (Romieu et al., 2006). The hyperlocomotion, toxic effects, and reward properties induced by this psychostimulant are observed at much higher doses, and the effects of σ_1 ligands on these effects will be described later in section 3.4.5 on cocaine and σ_1 receptors. These effects of σ_1 agonists on cognitive impairment due to alterations during pregnancy, as well as their role in the ameliorative effects of low doses of cocaine, are summarized in Table 2.

In summary, σ_1 agonists appear to be promising pharmacological tools against memory and learning disorders resulting from pharmacological or pathological alterations (see Table 2). Among the memory and learning disorders, Alzheimer's disease (the most common form of late-life dementia) is characterized by a cognitive decline, and effective treatment remains elusive. Sigma$_1$ agonists could thus provide an alternative treatment

against the cognitive deficits of this disease.

Table 2. Summary of the effects of σ_1 receptors in experimental models of learning and memory (see references and text for detailed information).

<table>
<tr><th colspan="5">Involvement of σ_1 receptors in learning and memory</th></tr>
<tr><th colspan="5">Behavioral assays</th></tr>
<tr><th colspan="3"></th><th>Effect of σ_1 agonists</th><th>Effect of σ_1 antagonism</th></tr>
<tr><td rowspan="9">Cognitive impairment induced by</td><td rowspan="5">Drugs, chemicals or brain lessions</td><td>Scopolamine [a, b]</td><td>Improvement</td><td>Reversion of the effects of σ_1 agonists</td></tr>
<tr><td>Mecamylamine [a]</td><td rowspan="3">Improvement</td><td rowspan="3">Not tested</td></tr>
<tr><td>Basal forebrain lesion [a, b]</td></tr>
<tr><td>PCA [c, d]</td></tr>
<tr><td>Dizocilpine [a, b, e, f, g, h, i]</td><td rowspan="2">Improvement</td><td rowspan="2">Reversion of the effects of σ_1 agonists</td></tr>
<tr><td>CO [e, j, k, l, m]</td></tr>
<tr><td rowspan="3">Ageing-related diseases</td><td>Aged animals [b]</td><td>Improvement</td><td>Not tested</td></tr>
<tr><td>Senescence-accelerated mice [l, d]</td><td rowspan="2">Improvement</td><td rowspan="2">Reversion of the effects of σ_1 agonists</td></tr>
<tr><td>β_{25-35}-amyloid-related peptide (Alzheimer disease-type amnesia) [n, o, p]</td></tr>
<tr><td rowspan="2">Alterations during pregnancy</td><td>Stress [q]</td><td rowspan="2">Improvement</td><td rowspan="2">Reversion of the effects of σ_1 agonists</td></tr>
<tr><td>Cocaine administration [r]</td></tr>
<tr><td>Cognitive amelioration induced by</td><td colspan="2">Low doses of cocaine [s]</td><td>Enhancement</td><td>Inhibition</td></tr>
<tr><th colspan="5">Effects on mechanisms involved in memory and learning impairment or potentiation</th></tr>
<tr><td>Impairment</td><td colspan="2">Neuronal injury induced by ischemia [j, k, l] or β_{25-35}-amyloid-related peptide [t]</td><td>Neuroprotective effects</td><td>Reversion of the effects of σ_1 agonists</td></tr>
<tr><td>Potentiation</td><td colspan="2">Long-term potentiation [u, v, w]</td><td>Enhancement</td><td>Reversion of the effects of σ_1 agonists</td></tr>
</table>

* Some nonselective σ_1 antagonists exert neuroprotective effects (reviewed in Maurice and Lockhart, 1997), which may be due to a non-σ_1-mediated mechanism.

[a] Maurice et al., 2001a, [b] Monnet and Maurice, 2006, [c] Matsuno et al., 1993, [d] Matsuno et al., 1994, [e] Maurice et al., 1999, [f] Maurice et al., 2001b, [g] Maurice et al., 2001c, [h] Maurice et al., 2006b, [i] Maurice et al., 2006a, [j] Bucolo et al., 2006, [k] Katnik et al., 2006, [l] Maurice and Lockhart, 1997, [m] Meunier et al., 2006, [n] Maurice et al., 1998, [o] Meunier et al., 2006b, [p] Wang et al., 2003, [q] Meunier al., 2004, [r] Meunier and Maurice, 2004, [s] Romieu et al., 2006, [t] Marrazzo et al., 2005, [u] Chen et al., 2006, [v] Li et al., 2006, [w] Martina et al., 2007.

3.4.2. Role of σ_1 receptors in depression and anxiety

Several neurotransmitter systems are important in the pathophysiology of depression and anxiety. Depression likely involves dysfunction in brain areas that are modulated by monoaminergic systems such as the frontal cortex and the hippocampus (reviewed in Delgado and Moreno, 2000). Given that σ_1 receptors play a modulatory role in several neurotransmitter systems, and that they can bind several known antidepressants (Table 1), they have been studied as possible pharmacological tools against these mood disorders.

3.4.2.1. Depression and σ_1 receptors

The effects of σ_1 ligands were tested in behavioral studies used to predict the antidepressant activity of drugs. The selective σ_1 agonists SA4503 and (+)-pentazocine decreased immobility time in the tail suspension test, and this effect was antagonized by NE-100 (Ukai et al., 1998). Many σ_1 agonists have been tested in the forced swimming test, for example SA4503, (+)-pentazocine, JO-1784, DHEAS, pregnenolone sulfate, donepezil, and some novel σ selective compounds such as UMB23, among others. The decrease in immobility in the forced swimming test induced by the σ_1 agonists was blocked by known σ_1 antagonists (partially reviewed in Maurice et al., 1999; Urani et al., 2001; Skuza and Rogoz, 2002; Urani et al., 2002a; Maurice et al., 2006; Wang et al., 2007). Interestingly, the extracts of the flowering plant *Hypericum perforatum* (St. John's wort), which are used as

antidepressants, appear to exert their therapeutic actions through σ_1 receptors (reviewed in Mennini and Gobbi, 2004). Additional experiments have related endogenous neurosteroidal levels with σ_1 receptor function. In adrenalectomized and castrated mice, the effect of JO-1784 and PRE 084 in the forced swimming test was enhanced compared to control animals, and these effects were blocked by the selective σ_1 antagonist BD 1047 (Urani et al., 2001). In addition, the antidepressant efficacy of the selective agonist JO-1784 was enhanced in 12-month-old senescence-accelerated (SAM) mice, which showed decreased levels of progesterone (Phan et al., 2005). Moreover, in animals acutely treated with β_{25-35}-amyloid related peptide, which does not modify their immobility time, the effects of the selective σ_1 agonists JO-1784 and PRE 084 were facilitated, presumably because of a decrease in progesterone levels in the hippocampus (Urani et al., 2002b).

An important consideration is that σ_1 agonists were able to potentiate the firing of serotonergic neurons of the dorsal raphe nucleus, as early as after 2 days of treatment, whereas SSRI (selective serotonin reuptake inhibitor) - and monoamine oxidase inhibitor-induced changes took several weeks to emerge. The rapid effect of σ_1 agonists has been proposed to predict a more rapid onset of antidepressant efficacy compared to existing medications (Bermack and Debonnel, 2001). Because of the typically modulatory role of σ_1 receptors, OPC-14523, a compound with high affinity for σ_1 receptors, 5-HT_{1A} receptors, and serotonin transporter (SERT) (Table 1) was developed, and was found to produce a marked antidepressant-like effect in the forced swimming test after a single oral administration. This effect was reversed by both σ_1 and 5-HT_{1A} antagonists (Tottori et al., 2001). Moreover, and also in keeping with the modulatory role of σ_1 receptors, the combined administration of the selective σ_1 receptor agonist (+)-pentazocine and venlafaxine (Dhir and Kulkarni, 2007), or the co-administration of pramipexole and

sertraline (Rogoz and Skuza, 2006a), at subeffective doses, showed a synergistic antidepressant-like effect, as did the co-administration of SA4503 and memantine or amantadine (Skuza and Rogoz, 2006a). Importantly, the antidepressant-like effects of these drugs were reversed by known selective σ_1 antagonists, and also by progesterone (Rogoz and Skuza, 2006; Skuza and Rogoz, 2006a; Dhir and Kulkarni, 2007).

It is known that NMDA receptor subunit 1 is decreased in the prefrontal cortex or hippocampus of depressive patients (Law and Deakin, 2001; Nudmamud-Thanoi and Reynolds,2004). In the olfactory bulbectomized rat model of depression, animals show a decrease in NMDA receptor subunit 1 in these areas, and exhibit behavioral deficits which resemble the psychomotor agitation, loss of interest, and cognitive dysfunction of depression. Repeated treatment with SA4503 ameliorated the behavioral deficits, and also reversed the decrease in the protein expression of NMDA receptor subunit 1. These effects of SA4503 were blocked by the co-administration of NE-100 and by acute treatment with the NMDA receptor antagonist dizocilpine (Wang et al., 2007). These findings document the strong relationship between depression, NMDA receptors and σ_1 receptors.

In addition to the modulatory role of σ_1 receptors in NMDA- and 5-HT-mediated responses related with depression, a complementary mechanism of action of σ_1 ligands in this disorder has been reported to involve their effects in neuroplasticity processes. The mechanism of action of some antidepressants may involve neurotrophic actions (Nestler et al., 2002), and it was reported that treatment with (+)-pentazocine or the antidepressants imipramine and fluvoxamine (which exhibit affinity for σ_1 receptors, see Table 1) enhanced growth factor-induced neurite sprouting in PC12 cells, and also upregulated σ_1 receptors (Takebayashi et al., 2002). The enhancement of growth factor-induced neurite sprouting by these drugs was mimicked by the overexpression of σ_1 receptors (Takebayashi et al., 2004).

Table 3. Summary of the involvement of σ_1 receptors in depression (see references and text for additional information).

Involvement of σ_1 receptors in depression			
		Effect of σ_1 agonists	**Effect of σ_1 antagonists**
Behavioral experimental models	Tail suspension test [a]	Improvement	Reversion of the effects of σ_1 agonists
	Forced swimming test [b, c, d, e, f, g]		
	Olfactory bulbectomy [h]		
Mechanisms associated with antidepressant activity	Firing of serotonergic neurons [i, j]	Potentiation	Reversion of the effects of σ_1 agonists
	Neurotrophic actions [k]	Potentiation of growth factor-induced neurite sprouting	
Mechanisms associated with depression	Decrease of NMDA receptor subunit 1 [h]	Reversion	Reversion of the effects of σ_1 agonists

[a] Ukai et al., 1998, [b] Maurice et al., 2001a, [c] Maurice et al., 2006b, [d] Szuka and Rogoz, 1999, [e] Urani et al., 2001, [f] Urani et al., 2002a, [g] Wang et al., 2007a, [h] Wang et al., 2007b, [i] Bermack and Debonnel, 2001, [j] Tottori et al., 2001, [k] Takebayashi et al., 2002

3.4.2.2. Anxiety and σ_1 receptors

Evidence of anxiolytic activity of σ_1 ligands was reported in the conditioned fear stress model, in which (+)-SKF-10,047, JO-1784, the neurosteroids pregnenolone sulfate and DHEAS, and also the antitussive dextromethorphan attenuated the motor suppression induced by previous electric footshock (Kamei et al., 1996; Kamei et al., 1997; Noda et al., 2000; Urani et al., 2004), in a bell-shaped manner (Urani et al., 2004). In addition, the effects of σ_1 agonists on motor suppression were reversed by the known σ_1 antagonist NE-100 and progesterone (Noda et al., 2000; Urani et al., 2004). In contrast, (+)-

pentazocine lacked any effect in this model (Kamei et al., 1996; Kamei et al., 1997). Interestingly, the concentration of σ_1 active steroids was altered in the plasma and brain of stressed mice, and it was therefore hypothesized that endogenous levels of neurosteroids might be involved in the expression of conditioned fear stress responses via σ_1 receptors (Noda et al., 2000; Urani et al., 2004). In agreement with these results, animals treated chronically with $\beta_{1\text{-}40}$-amyloid related peptide, in which progesterone levels in the hippocampus and cortex were decreased, exhibited facilitation of the effect of the σ_1 agonists JO-1784, (+)-SKF-10,047 and DHEAS (Urani et al., 2004).

The effects of σ_1 ligands have also been assayed in other behavioral tests such as sexual dysfunction induced by stress, marble-burying behavior and colonic motor disturbances induced by fear. It was reported recently that DHEA attenuated stress-induced sexual dysfunction in rats in a NE-100 dependent manner (Mizuno et al., 2006). In the marble-burying behavior test, considered a potential model of obsessive–compulsive disorder on the basis of behavioral similarity, the effect of fluvoxamine was antagonized by BD 1063 and BD 1047, but not by the σ_2 antagonist SM-21, suggesting again that the interaction of fluvoxamine with σ_1 receptors contributes to its antidepressant effects. In addition, the σ_1 agonists (+)-SKF-10,047 and PRE 084 slightly inhibited marble-burying behavior (Egashira et al., 2007). Gue and coworkers (Gue et al., 1992) showed that JO-1784 suppressed stress-induced colonic motor disturbances induced by fear stress in rats, in a model that mimicked the gastrointestinal tract disorders frequently present in anxiety, and this effect was reversed by BMY 14802 (Gue et al., 1992). Subsequently, JO-1784 showed good results in clinical trials in a phase-1-model of functional diarrhea (Volz and Stoll, 2004). The results described above (summarized in Table 4) suggest that σ_1 receptors play an important role in the modulation of anxiety.

Table 4. Summary of the involvement of σ_1 receptors in anxiety (see references cited in the text for detailed information).

Involvement of σ_1 receptors on anxiety			
		Effect of σ_1 agonists	**Effect of σ_1 antagonists**
Behavioral experimental models	Conditioned fear stress [a, b, c, d]	Improvement	Reversion of the effects of σ_1 agonists
	Sexual dysfunction induced by stress [e]		
	Marble-burying behavior test [f]		
	Colonic motor disturbances induced by fear [g]		
Clinical trials (phase-1)	Functional diarrhea [h]	Improvement	Not tested

[a] Kamei et al., 1996, [b] Kamei et al., 1997, [c] Noda et al., 2000, [d] Urani et al., 2004, [e] Mizuno et al., 2006, [f] Egashira et al., 2007, [g] Gue et al., 1992, [h] Volz and Stoll, 2004

3.4.3. Schizophrenia and σ_1 receptors

The dopamine hypothesis of schizophrenia, which involves enhanced mesolimbic dopamine function, remains the dominant hypothesis for the pathophysiology of this disorder, particularly regarding the appearance of positive symptoms (Depatie and Lal, 2001). In addition, it is important to consider the glutamatergic system. In fact, the blockade of NMDA receptors by PCP induces schizophrenia-like psychosis in humans (Chavez-Noriega et al., 2005; van Berckel, 2003). Because several antipsychotics possess high to moderate affinities for σ_1 receptors (Table 1), researchers were inspired to test σ_1 receptor ligands in several animal models of schizophrenia.

3.4.3.1. Role of σ_1 receptors in behavioral models of schizophrenia in which dopaminergic function is prominently enhanced

In behavioral animal models in which the dopaminergic function is affected, such as apomorphine-induced climbing, amphetamine-induced locomotor activity, and behavioral sensitization by the repeated administration of psychostimulants, promising results have been reported using σ_1 antagonists (summarized in Table 5). The nonselective σ_1 antagonist BMY 14802, panamesine, E-5842 and MS-377 inhibit apomorphine-induced climbing (Taylor et al., 1993; Guitart et al., 1998; Takahashi et al., 1999; Sluka and Rogoz, 2006). In addition, DTG, SR 31742A, panamesine, rimcazole and E-5842 inhibit amphetamine-induced locomotor activity (Poncelet et al., 1993; Rückert et al., 1993; Guitart et al., 1998; Sluka and Rogoz, 2006). However, rimcazole and BD 1047 had little effect on apomorphine-induced climbing, and in addition, this latter compound had little effect on acute amphetamine-induced hyperlocomotion (Sluka and Rogoz, 2006). In models of behavioral sensitization with the repeated administration of psychostimulants —a pharmacological model of schizophrenia (Castner et al., 2004)— σ_1 antagonism inhibited sensitization to methamphetamine (Ujike et al., 1992; Akiyama et al., 1994; Takahashi et al., 2000) and cocaine (Witkin et al., 1993; Ujike et al., 1996). It was therefore suggested that σ_1 antagonists may be suitable for maintenance therapy in persons with stable schizophrenia rather than for the treatment of acute psychotic features.

3.4.3.2. Role of σ_1 receptors in behavioral models of schizophrenia in which glutamatergic function is prominently disturbed

As said before, in addition to dopaminergic dysfunction, alterations in glutamatergic neurotransmission are also involved in schizophrenia. $Sigma_1$ ligands modified animal

behavior in some glutamatergic models of this disease (summarized in Table 5). PCP-induced head weaving, which is insensitive to selective D_2 antagonists, was attenuated by NE-100, haloperidol, BMY 14802, Dup 734 and MS-377 (Takahashi et al., 1999; Hayashi and Su, 2005b). Recent reports also showed that BD 1047, rimcazole and panamesine attenuated PCP-induced head twitching (Skuza and Rogoz, 2006b). In addition, selective σ_1 receptor agonists such as (+)-pentazocine, and also 3-(+)-PPP and (+)-SKF-10,047, enhanced the psychotomimetic effect (hyperlocomotion) of dizocilpine in monoamine-depleted mice, and this enhancement was blocked by NE-100 (Okuyama et al., 1996), suggesting that σ_1 receptor blockade may be effective for negative symptoms of schizophrenia, which are hypothesized to be mediated, at least in part, by glutamatergic neurotransmission. Among the negative symptoms of schizophrenia, cognitive deficits are core features of the illness and predict vocational and social disabilities for patients (Kurtz, 2005). It has been extensively reported that σ_1 agonists play an important role in memory processes (as described in the section 3.4.1.). In fact, SA4503, DHEAS, and fluvoxamine (a SSRI with high affinity for σ_1 receptors), but not paroxetine (an SSRI without affinity for σ_1 receptors) improved the PCP-induced cognitive deficits in the novel object recognition test, and these effects were antagonized by the co-administration of NE-100 (Hashimoto et al., 2006). In addition, the antipsychotic (and also σ_1 antagonist) drug haloperidol was ineffective in this behavioral model (Hashimoto et al., 2005). These results suggest that σ_1 agonists are potentially useful for the cognitive deficits of schizophrenia.

3.4.3.3. Sigma$_1$ receptors and extrapyramidal side effects

The extrapyramidal effects of neuroleptics are considered one of the most problematic side effects of these drugs. It has been suggested that σ receptors mediate the

undesirable motor side effects of antipsychotic drugs (reviewed in Walker et al., 1990; Guitart et al., 2004), an effect classically attributed to the σ_2 subtype (e.g., Walker et al., 1993). Although it was found that the affinities of several neuroleptics for σ receptors (both σ_1 and σ_2) correlated well with their risk of producing acute dystonic reactions (Matsumoto and Pouw, 2000), it is known that the blockade of σ_1 receptors with other more selective antagonists such as NE-100 (Okuyama and Nakazato, 1996), MS-377 (Takahashi et al., 1999), E-5842 (Guitart et al., 1998) or BMY 14802 (Gewirtz et al., 1994) (at effective doses for the test used) does not induce motor side effect. These findings suggest that the blockade of σ_1 receptors is not enough in itself to induce extrapyramidal side effects, so additional mechanisms are probably be involved.

3.4.3.4. Clinical trials with σ_1 ligands in schizophrenia

Some clinical trials have been done with rimcazole, BMY 14802, eliprodil (SL-82.0715) and panamesine. The trials with rimcazole and BMY 14802 yielded inconclusive results (reviewed in Hayashi and Su, 2004); however, eliprodil reduced scores for negative but not positive symptoms, whereas panamesine reduced both positive and negative symptoms. However, a metabolite of panamesine has potent antidopaminergic properties which might explain its effect against the positive symptoms, so further research is needed to determine whether these effects are wholly or partly mediated by σ_1 receptors (reviewed in Hayashi and Su, 2004).

Table 5. Summary of the involvement of σ_1 receptors in schizophrenia (see references and text for detailed information).

Involvement of σ_1 receptors on schizophrenia				
			Effect of σ_1 agonists	**Effect of σ_1 antagonists**
Behavioral experimental models	Dopaminergic function prominently enhanced	Apomorphine-induced climbing [a, b, c, d]	Not tested	Inhibition
		Amphetamine-induced locomotor activity [a, b, e, f]		
		Behavioral sensitization induced by repeated administration of psychostimulants [g, h, i, j, k]		
	Glutamergic function prominently disturbed	PCP-induced stereotyped behaviors [b, c, l]	Not tested	Inhibition
		Dizocilpine-induced hyperlocomotion in monoamine depleted mice [m]	Enhancement	Reversion of the effects of σ_1 agonists
		PCP-induced cognitive deficits [n]	Improvement	Reversion of the effects of σ_1 agonists
Clinical trials	Only with BMY 14802, eliprodil and panamesine [o]		Not tested	Inconclusive results

[a] Guitart et al., 1998, [b] Skuza and Rogoz, 2006a, [c] Takahashi et al., 1999, [d] Taylor et al., 1993, [e] Poncelet et al., 1993, [f] Rückert and Schmidt, 1993, [g] Akiyama et al., 1994, [h] Takahashi et al., 2000, [i] Ujike et al., 1992, [j] Ujike et al., 1996, [k] Witkin et al., 1993, [l] Hayashi and Su, 2005b, [m] Okuyama et al., 1996, [n] Hashimoto et al., 2006, [o] Hayashi and Su, 2004

3.4.4. Sigma$_1$ receptors and analgesia

Sigma$_1$ receptors are distributed in the central nervous system in areas of great importance in pain control, such as the superficial layers of the spinal cord dorsal horn, the periaqueductal gray matter, the locus ceruleus and rostroventral medulla (Alonso et al., 2000; Kitaichi et al., 2000). As will be described below, they may be involved in the modulation of opioid analgesia, and may also play an important role in nociception in the

absence of opioid drugs.

3.4.4.1. Modulation of opioid analgesia by σ_1 receptors

Chien and Pasternak were the first to report the involvement of σ_1 receptors in analgesia (Chien and Pasternak, 1993): they clearly demonstrated that σ_1 receptors play an important role in the modulation of opioid analgesia in the tail-flick test. The systemic administration of σ_1 agonists, including the selective σ_1 agonist (+)-pentazocine, antagonized the antinociception induced by morphine in the tail-flick test (Chien and Pasternak, 1993, 1994 and 1995a; Mei and Pasternak, 2002). Further experiments with other opioids confirmed the role of σ_1 receptors in opioid analgesia. (+)-Pentazocine also diminished δ-, κ_1-, and κ_3-opioid antinociception (Chien and Pasternak, 1993; Mei and Pasternak, 2002; Ronsisvalle et al., 2001). In addition, σ_1 antagonists such as haloperidol and (+)-MR 200 not only reversed the effects of agonists, but also increased opioid-induced antinociception, indicating the presence of a tonically active anti-opioid σ_1 system (Chien and Pasternak, 1993, 1994 and 1995a; Ronsisvalle et al., 2001; Marrazzo et al., 2006).

The anatomical location of the modulation of opioid analgesia by σ_1 receptors has been determined with different routes of administration of opioids, σ_1 receptor ligands and antisense oligodeoxynucleotides. The intrathecal (i.t.) administration of (+)-pentazocine did not reverse the spinal (i.t.) analgesic effect of morphine in the tail-flick test, suggesting that the modulation of opioid analgesia by σ_1 receptors in this test does not occur at the spinal level (Mei and Pasternak, 2001). Interestingly, the supraspinal (intracerebroventricular, i.c.v.) administration of (+)-pentazocine decreased the analgesic effect of agonists for the κ and μ opioid receptors nalorphine and nalbuphine (Mei and Pasternak, 2001); in addition, the analgesia induced by the supraspinal (i.c.v.) administration of the selective μ-opioid

agonist DAMGO was enhanced by the σ_1 antagonist (+)-MR 200 administered subcutaneously (Marrazzo et al., 2006). Further experiments based on the selective blockade of σ_1 receptor synthesis by the i.c.v. administration of specific antisense oligodeoxynucleotides confirmed the supraspinal location of the modulation of opioid analgesia (King et al., 1997; Pan et al., 1998; Mei and Pasternak, 2002). Finally, a more detailed approach was tested recently by Mei and Pasternak (Mei and Pasternak, 2007), who used microinjections of morphine in conjunction with (+)-pentazocine, haloperidol, or both in three brainstem nuclei: the periaqueductal gray, rostroventral medulla and locus ceruleus. The activity of σ_1 receptors was found to differ depending on the area. Whereas both the locus ceruleus and rostroventral medulla were sensitive to (+)-pentazocine, the periaqueductal gray was not. The rostroventral medulla was particularly interesting, because it was the only region with evidence for tonic σ_1 activity (morphine-induced analgesia enhanced by haloperidol), and it was also able to modulate the analgesia from morphine administered to the periaqueductal grey.

In contradistinction to results in the tail-flick test, it was found that the systemic administration of (+)-SKF-10,047 or NE-100 was unable to modulate κ_1 opioid analgesia in the acetic acid-induced writhing test (Hiramatsu et al., 2002). Although the doses used in this study might have been too low to prevent the participation of σ_1 receptors in the modulation of κ_1 opioid analgesia in the acetic acid-induced writhing, i.c.v. treatment with σ_1 antisense oligodeoxynucleotides also failed to affect this response (Hiramatsu et al., 2004), suggesting that the supraspinal inhibition of σ_1 receptors does not affect κ opioid analgesia in this behavioral test. This findings may indicate that the supraspinal σ system modulates only some opioid analgesic effects, probably depending on the type of pain evaluated (i.e., depending on the behavioral model used). Further research with different

models is needed to characterize the role of the supraspinal σ system in opioid analgesia. The role of σ_1 receptors on opioid analgesia in behavioral experimental models is summarized in Table 6.

In addition, some recent reports showed that haloperidol and chlorpromazine, two neuroleptics that bind to σ sites (Table 1), inhibit the antianalgesia induced by nalbuphine in men (Gear et al., 2006). Although the authors did not attribute this inhibition to σ receptors, this possibility cannot be fully ruled out, and would suggest that interaction between the σ and opioid systems is important in clinical terms. However, this issue also needs to be addressed in further clinical studies.

3.4.4.2. Analgesic effect of σ_1 receptor ligands

The role of σ_1 ligands in the absence of opioid drug has also been investigated. Several σ_1 ligands or antisense treatments have been proved to be inactive in the tail-flick test (Cendán et al., 2005a; Chien and Pasternak, 1993, 1994 and 1995a; Pan et al., 1998; Mei and Pasternak, 2002; Marrazzo et al., 2006), as well as in the acetic acid-induced writhing test (Hiramatsu et al., 2002; Hiramatsu and Hoshino, 2004) (although higher doses of σ_1 ligands should be tested to ensure their lack of involvement in acetic acid-induced writhing). However, other reports showed that σ_1 receptors are able to modulate nociception in other behavioral tests in the absence of an opioid drug. Ueda and coworkers (Ueda et al., 2001) showed that the σ_1 agonists (+)-pentazocine and SA4503, (+)-3-PPP, and also the putative σ_1 agonists DHEAS and pregnenolone sulfate (administered intraplantarly) can induce nociception even when used alone in the nociceptive flexor response test. The effect of the σ_1 agonists was reverted by the known σ_1 antagonists NE-100, BD 1063 or the putative σ_1 endogenous antagonist progesterone (Ueda et al., 2001). Other studies in our

laboratory with the formalin test showed that formalin-induced nociception was attenuated not only by the systemic administration of haloperidol, haloperidol metabolite II and haloperidol metabolite I (with an order of potency which correlated with their affinity for σ_1 receptors) (Cendán et al., 2005a), but also in σ_1 receptor knockout mice (Cendán et al., 2005b). Recent experiments with the same behavioral test found that in contradistinction to the supraspinal action of σ_1 antagonists on the modulation of opioid analgesia, the i.t. administration of the σ_1 receptor antagonists BD 1047 and BMY 14802 dose-dependently reduced formalin-induced pain behaviors in the second phase but not in the first phase of the formalin test (Kim et al., 2006). This underscored the importance of spinal σ_1 receptors in the second phase of formalin-induced pain. These results were consistent with previous findings which showed that haloperidol, haloperidol metabolite I and haloperidol metabolite II were more effective in the second than in the first phase of formalin-induced pain (Cendan et al., 2005a). In agreement with these behavioral studies, it was also reported that antagonism of spinal σ_1 receptors suppressed phosphorylation of the NR1 subunit of spinal NMDA receptors (Kim et al., 2006), which are important for maintaining spinal sensitization associated with the second phase of the formalin test (South et al., 2003). Interestently, recent studies show that spinally administration of σ_1 agonists potentiate NMDA-induced acute pain behavior (Kim et al., 2008). From these results it was proposed that σ_1 receptors may be important in models in which spinal sensitization occurs (without ruling out other analgesic effects in other models), and in fact, the putative σ_1 agonist DHEA induced mechanical allodynia and thermal hyperalgesia when administered i.t., and the effects were reversed by BD 1047 (Kibaly et al., 2007). Moreover, recent studies show that the i.t. administration of the selective σ_1 agonist PRE-084 induced a significant upregulation of dorsal horn pNR1 (Kim et al., 2008). Altogether these results suggest that

σ_1 receptors might play an important role in the mechanisms underlying different experimental models of pain, specially when sensitization processes are involved. The results obtained in the behavioral models described above (summarized in Table 6) suggest that σ_1 receptors play an important role in nociception in the absence of opioid drugs.

Table 6. Summary of the involvement of σ_1 receptors in analgesia (see text and references for detailed information, as administration routes of drugs).

Involvement of σ_1 receptors on analgesia			
	Behavioral experimental models	**Effect of σ_1 agonists**	**Effect of σ_1 antagonism**
Modulation of opioid analgesia	Tail-flick test [a, b, c, d, e, f, g, h]	Inhibition	Enhancement
	Acetic acid-induced writhings [i, j]	Inactive (very low doses tested) *	Inactive (very low doses tested) *
Pain modulation in the absence of opioid drugs	Tail-flick test [a, b, c, e, f, g, k]	Inactive	Inactive
	Acetic acid-induced writhings [i, j]	Inactive (very low doses tested)*	Inactive (very low doses tested) *
	Nociceptive flexor response test [l]	Nociception	Reversion of the effects of σ_1 agonists
	Formalin-induced pain [k, m, n]	Reversion of the effects of σ_1 antagonists	Antinociception
	Plantar test [o]	Thermal hyperalgesia #	Reversion of the effects of σ_1 agonists
	von Frey test [o]	Mechanical allodynia #	Reversion of the effects of σ_1 agonists

* Additional experiments using higher doses of σ_1 ligands should be performed.

Selective σ_1 agonists should be tested.

[a] Chien et al., 1993, [b] Chien et al., 1994, [c] Chien et al., 1995, [d] King et al., 1997, [e] Marrazzo et al., 2006, [f] Mei and Pasternak, 2002, [g] Pan et al., 1998, [h] Rosisvalle et al., 2001, [i] Hiramatsu et al., 2002, [j] Hiramatsu et al., 2004, [k] Cendán et al., 2005a, [l] Ueda et al., 2001, [m] Cendán et al., 2005b, [n] Kim et al., 2006, [o] Kibaly et al., 2007.

In summary, σ_1 receptors are not only able to modulate opioid antinociception, at least in the tail-flick test, but may also play an active role in nociception in the absence of opioid drugs in some behavioral models (see Table 6).

3.4.5. Sigma$_1$ receptors and drugs of abuse

As shown before (Table 1), σ_1 receptors can bind several drugs of abuse. It is therefore not surprising that σ_1 ligands can modulate some of the effects of these drugs. Among the drugs of abuse studied to date, the involvement of σ_1 receptors in the actions of cocaine has been extensively investigated, but σ_1 receptors also appear to underlie the effects of other substances such as methamphetamine, MDMA (3,4-methylenedioxymethamphetamine) and ethanol, as will be described below.

3.4.5.1. Cocaine and σ_1 receptors

Cocaine is generally thought to act as a dopamine re-uptake inhibitor to produce its reinforcing effects, although other mechanisms might also be important (Mateo et al., 2004). Cocaine binds preferentially to σ_1 receptors rather than to σ_2 (Matsumto et al., 2002), and the affinity of cocaine for σ_1 receptors is in the micromolar range (Table 1), as is its affinity for its main pharmacological target, the dopamine transporter (DAT) (Rothman and Baumann, 2003). Cocaine levels in the post-mortem brain of addicts were estimated to be between 0.1 and 4 μM (Kalasinsky et al., 2000), which is close to the K_i value of cocaine for σ_1 receptors. In recent years several excellent and promising studies have been performed with σ_1 ligands against the effects of cocaine, as described below.

A) Modulation by σ_1 receptors of the acute effects of cocaine

The ability of compounds to attenuate the acute locomotor effects of cocaine is often used as an initial screening tool to identify agents able to block the psychostimulant activity of this drug of abuse. Convulsions and lethality, on the other hand, represent a measure of cocaine toxicity, and can result from exposure to acute large doses. Many σ_1 antagonists

have been reported to significantly prevent the acute locomotor stimulatory effects, convulsions or lethality induced by cocaine in rodents, including haloperidol, BD 1008 (and some of its analogs such as the selective σ_1 antagonists BD 1047 and BD 1063), BMY 14802, panamesine and rimcazole (and some of its analogs), among others (partially reviewed in Maurice et al., 2002 and Matsumoto et al., 2003; Matsumoto et al., 2004; Daniels et al., 2006; Liu et al., 2007). Furthermore, the administration of antisense oligodeoxynucleotides that knock down brain σ_1 receptors mimicked the effects of pharmacological σ_1 antagonism on the locomotor stimulatory effects or convulsions induced by cocaine (Matsumoto et al., 2001b and 2002). Particularly interesting are the studies in which post-treatment of mice with the novel σ receptor antagonists LR132 and YZ-011, after cocaine administration, also attenuated cocaine-induced lethality after an overdose. However, BD 1063 was unable to prevent death under these conditions, and the authors hypothesized that this result was due to differences in pharmacokinetics (Matsumoto et al., 2001b and 2002). The ability of σ receptor antagonists to prevent death after an overdose of cocaine in animals suggests a clinical application potentially worth further study. In contradistinction to the positive effects of σ_1 antagonists, the administration of DTG, the novel σ_1 agonists BD1031 and BD1052, or the selective σ_1 agonist SA4503 exacerbated locomotor stimulatory actions and the toxic effects (measured as convulsions and lethality rate) of the acute administration of cocaine (McCracken et al., 1999; Skuza, 1999; Matsumoto et al., 2001b and 2002) The results obtained in these behavioral models (summarized in Table 7) suggest that σ_1 receptors play an important role in the acute effects of cocaine. In addition to σ_1 receptors, it has been proposed that the σ_2 subtype might also be a good pharmacological target against cocaine-induced actions (partially reviewed by Matsumoto et al., 2004; Nawayhid and Werling, 2006; Matsumoto et al., 2007).

B) Modulation by σ_1 receptors of the effects of repeated cocaine administration

Several σ_1 antagonists have also been tested in behavioral models that used repeated doses of this drug of abuse. The σ_1 antagonists rimcazole and some of its analogs, and other putative σ antagonists did not alter or only slightly altered the discriminative stimulus of cocaine (Witkin et al., 1993; Katz et al., 2003; Liu et al., 2007), indicating that the interaction between cocaine and σ receptor ligands might be more complex than an exclusively competitive antagonism. Other studies that involved the repeated administration of cocaine found that σ receptor antagonists significantly prevented the development of cocaine-induced locomotor sensitization (Witkin et al., 1993; Ujike et al., 2006), which is considered a measurable index of nervous system plasticity resulting from repeated exposure to cocaine (Matsumoto et al., 2003). The effects of σ_1 antagonism on the rewarding properties of this drug of abuse have been explored with promising results. In the conditioned place preference test, the selective σ_1 receptor antagonists BD 1047 and NE-100 attenuated the acquisition (Romieu et al., 2000 and 2002) and also the expression of cocaine-induced conditioned place preference (Romieu et al., 2002). In addition, treatment with σ_1 antisense oligodeoxynucleotide was effective against the acquisition of conditioned place preference, indicating the specificity of these effects (Romieu et al., 2000). However, in cocaine self-administration experiments, Martin-Fardon and coworkers found that BD 1047 was inactive against the acute reinforcing effects of cocaine, supposedly because both the reinforcing quality and relevant neuroadaptive changes are likely to differ in rats subjected to involuntary administration (as in conditioned place preference) vs. self-administration of cocaine (Martin-Fardon et al., 2007). After extinction, cocaine addictive behavior can be reactivated by a discriminative stimulus associated with cocaine administration, or by a priming injection of cocaine (in self-administration or conditioned

place preference experiments, respectively). These processes were both blocked by BD 1047 (Romieu et al., 2004; Martin-Fardon et al., 2007), and the latter one was also blocked by σ_1 antisense oligodeoxynucleotides (Romieu et al., 2004). Interestingly, the σ_1 agonists PRE 084 and JO-1784 were unable to induce conditioned place preference (Romieu et al., 2002), but the administration of the latter σ_1 agonist, or even DHEA, was enough to reactivate conditioned place preference after extinction, in a BD 1047-sensitive manner (Romieu et al., 2004). The results in these behavioral models (summarized in Table 7) suggest that σ_1 receptors play an important role in neuronal plasticity after repeated cocaine administration, and that σ_1 antagonists could be useful to prevent craving and relapse of cocaine addiction.

It has been reported that σ_1 receptor density changes after repeated treatment with cocaine (Romieu et al., 2002; Romieu et al., 2004; Liu et al., 2005; Zhang et al., 2005; Silvers et al., 2006). Particularly interesting is the σ_1 receptor upregulation in the caudate putamen (an important area in the drug reward mechanism), which was not produced in dopamine D_1 receptor knockout mice (Zhang et al., 2005). Consistent with this finding was that cocaine treatment in the neuroblastoma cell line B-104 (lacking in dopamine transporter or receptors), was also unable to induce σ_1 receptor upregulation (Cormaci et al., 2007), suggesting a close relationship between dopamine and σ_1 receptors. In fact, it has been proposed that both D_1 receptors and σ_1 receptors are involved in cocaine-induced life-long alterations in neurons (Su and Hayashi, 2001).

Table 7. Summary of the involvement of σ_1 receptors in the behavioral effects induced by cocaine (see references and text for detailed information).

Involvement of σ_1 receptors in cocaine-induced behavioral effects					
			Behavioral experimental model	**Effect of σ_1 agonists**	**Effect of σ_1 antagonism**
Acute effects of cocaine	Psychostimulant effects		Locomotor activity [a, b, c, d, e, f]	Potentiation	Inhibition
	Toxicity		Convulsions [a, d, c, e, g, f]		
			Lethality [a, e, g, f]		
Repeated administration of cocaine	Self-reported effects of cocaine		Drug discrimination test [h, b, i]	Not tested	Slight or no effect
	Nervous system plasticity		Locomotor sensitization [j, i]	Not tested	Inhibition
	Rewarding properties	During addictive behavior	Conditioned place preference [k, l]	Not tested	Inhibition
			Self-administration [m]	Not tested	No effect
		After extinction of addictive behavior	Conditioned place preference after priming injection of drugs [n]	Reactivation	Inhibition
			Discriminative stimulus associated with cocaine availability for self-administration [m]	Not tested	Inhibition

[a] Daniels et al., 2006, [b] Liu et al., 2006, [c] Matsumoto et al., 2002, [d] Matsumoto et al., 2001b, e Matsumoto et al., 2003, [f] Maurice et al., 2002, [g] Matsumoto et al., 2004, [h] Katz et al., 2003; [i] Witkin et al., 1993, [j] Ujike et al., 1996; [k] Romieu et al., 2000, [l] Romieu et al., 2002, [m] Martin Fardon et al., 2007, [n] Romieu et al., 2007

C) Effects of σ_1 ligands on cocaine-induced immune system depression

Different experiments have been designed to investigate the effects of cocaine other than its acute toxicity or rewarding properties, specifically, modulation of the immune system by cocaine. It was recently reported that cocaine can enhance alveolar cell carcinoma growth in mice, and that this effect was mimicked by PRE 084 and reversed by BD 1047. Increased tumor growth induced by cocaine or PRE 084 was accompanied by an increase in IL-10 and a decrease in IFN-γ production (Gardner et al., 2004). In addition, the

selective σ_1 antagonist BD 1047 blocked enhancement of the replication of HIV-1 in mice with severe combined immunodeficiency implanted with HIV-1-infected human peripheral blood mononuclear cells (Roth et al., 2005), and also in human microglial cell cultures (Gekker et al., 2006). These reports suggest that σ_1 receptors are involved in the cocaine-induced depression of the immune system.

In summary, σ_1 antagonists appear to be potentially useful not only against acute cocaine toxicity or addiction, but also against the noxious modulation of the immune system in cocaine consumers. In addition, σ_1 agonists, as described in section 3.4.1., may be useful against some behavioral alterations induced by repeated cocaine exposure *in utero*. It therefore seems clear that cocaine produces its behavioral and biochemical effects, at least in part, through its interaction with σ_1 receptors, and that σ_1 ligands should be considered for the development of potential therapies to treat different aspects of cocaine abuse.

3.4.5.2. Other drugs of abuse and σ_1 receptors

Methamphetamine, like cocaine, also binds to σ_1 receptors in the micromolar range (Table 1), and with a 20-fold higher affinity than for σ_2 receptors (Nguyen et al., 2005), so it is not unexpected that σ_1 ligands modulate some effects of this psychostimulant. Early studies found that the σ_1 antagonists NE 100, BMY 10802 and MS-377 modulated the acute motor effects of methamphetamine only weakly, if at all (Ujike et al., 1992; Okuyama and Nakazato, 1996; Takahashi et al., 2000). However, more recent studies showed that the selective σ_1 antagonists BD 1063 and BD 1047, as well as σ_1 antisense oligodeoxynucleotide, inhibited methamphetamine-induced locomotor activity (Nguyen et al., 2005). It was also recently reported that like cocaine or methamphetamine, the compound MDMA ('ecstasy'), which is structurally related to methamphetamine, showed

preferential affinity for σ_1 receptors rather than for the σ_2 subtype, and that BD 1063 also attenuated the locomotor activity induced by this compound (Brammer et al., 2006). Furthermore, BMY 14802 and MS-377, two known σ_1 antagonists, inhibited the behavioral sensitization induced by the repeated administration of methamphetamine (Ujike et al., 1992; Akiyama et., 1994; Takahashi et al., 2000). As in studies with repeated cocaine administration, it was thought that σ_1 receptors might play a role in neuronal plasticity after the repeated administration of methamphetamine. $Sigma_1$ receptor levels were recently found to be unaltered in rats passively treated with this psychostimulant; however, in rats self-administered with methamphetamine, σ_1 receptors were upregulated in the rat midbrain, an area involved in learning and reward processes, but not in the cerebellum, frontal cortex, striatum and hippocampus. These observations underscored the role of σ_1 receptors in neuronal plasticity after the consumption of psychostimulants (Stefanski et al., 2004).

The role of σ_1 receptors in the behavior of other drugs of abuse has also been explored, and it was found that the σ_1 antagonist BD 1047 was effective against ethanol-induced locomotor stimulation, conditioned place preference, taste aversion and some symptoms of the abstinence syndrome after chronic ethanol consumption (Maurice et al., 2003; Meunier et al., 2006a). Interestingly, σ_1 receptor expression was increased in the hippocampus of mice after chronic ethanol consumption. However, both the σ_1 agonist JO-1784 and the antagonist BD 1047 shared some ameliorating properties against the abstinence syndrome after chronic ethanol consumption (Meunier et al., 2006a). These observations suggest a new pharmacological target for alleviating ethanol addiction and abstinence syndrome after withdrawal, although more behavioral tests should be performed. Interestingly, an association has been suggested between polymorphisms in the σ_1 receptor

gene and alcoholism (Miyatake et al., 2004), supporting the role of σ_1 receptors in chronic ethanol consumption.

3.5. Conclusions and perspectives of σ_1 receptors

At the present time it seems logical to attribute the neuropharmacological properties of σ_1 ligands to the neuromodulatory role of σ_1 receptors. They act as intracellular amplifiers for signal transductions involving IP_3 receptors, are clearly able to modulate neurotransmitter systems (mainly through NMDA receptors) and ion channels (such as K^+ channels), and may play an important role in neuroplasticity processes. Because of this typically modulatory nature of σ_1 receptors, σ_1 ligands are usually devoid of effect *per se* under control conditions in many experimental situations. In fact, and in agreement with the modulatory role of σ_1 receptors, σ_1 knockout mice do not display any overt phenotype. However, data showed that σ_1 ligands are highly active when a pharmacological or pathological imbalanced state arises. In addition, and also due to the modulatory role of σ_1 receptors, the combined administration of σ_1 receptor ligands and medications with a known therapeutic effect has been shown to improve the effects of the latter (at least in behavioral models of depression and in opioid-mediated analgesia), resulting in the need for lower doses to reach therapeutic concentrations. This synergistic action of σ_1 ligands and low doses of other known drugs merits further study in additional behavioral models. Of particular interest is the bell-shaped dose-response curve of σ_1 agonists in *in vitro* experiments, in behavioral tests in which σ_1 agonists are active (i.e., learning and memory processes, depression and anxiety), and even in some clinical trials. These data strongly suggest that researchers should take hormesis into account in order to design informative experiments or clinical trials with σ_1 agonists.

In the light of our current knowledge, it seems clear that σ_1 agonists are promising pharmacological tools against memory and learning disorders, and also against depression and anxiety. Although some previous findings suggest that σ_1 antagonists might be potentially useful tools against some symptoms of schizophrenia, currently the most promising therapeutic targets for σ_1 antagonism are nociception and some deleterious effects of certain drugs of abuse (such as cocaine, methamphetamine and ethanol). Importantly, many drugs used routinely in therapeutics show affinity for σ_1 receptors (see Table 1), and exert the same effects as other more selective σ_1 ligands in many behavioral tests and *in vitro* assays. Therefore, the therapeutical properties of these drugs might be due, at least in part, to their interaction with σ_1 receptors. Interestingly, several drugs have been proved to be effective against diseases (in behavioral animal models) different from those for which they are prescribed in clinical practice, through their interaction with σ_1 receptors. These findings raise the possibility of new therapeutic applications with drugs routinely used in therapeutics.

HYPOTHESIS AND GOALS

The σ_1 receptors are widely distributed in the central nervous system, including pain processing areas at both spinal (superficial layers of the spinal cord dorsal horn) and supraspinal locations (periaqueductal gray matter, locus coeruleus and rostroventral medulla) (Alonso et al., 2000; Kitaichi et al., 2000). Functional studies have postulated that an endogenous σ_1 system tonically inhibits the opioid system. This was initially proposed by Chien and Pasternak (1993), and later work concluded that inhibition occurred particularly at supraspinal levels (Pan et al., 1998; Marrazo et al., 2006; Mei and Pastenak, 2007). These studies demonstrated that σ_1 receptor antagonists and σ_1 receptor antisense oligodeoxynucleotides enhance the effect of opioid receptor agonists in a model of acute nociceptive pain induced by a thermal stimulus (tail-flick test). However, the effects of treatments in this model of nociceptive pain are not representative of their effects in other types of pain, particularly where central sensitization processes are activated.

Central sensitization occurs in pathological pain (e.g., inflammatory and neuropathic pain), and is triggered by sustained peripheral nociceptor input. This type of sensitization results in enhanced responsiveness of pain transmission neurons in the spinal cord dorsal horn and brain (Woolf and Salter, 2000; Ji and Woolf, 2001; Ji et al., 2003). Activation of the *N*-methyl-*D*-aspartic acid (NMDA) receptor plays a pivotal role in the development and maintenance of central sensitization (Willis et al., 2001; Ji and Woolf, 2001). This process involves the potentiation of NMDA receptor activity by the phosphorylation of NMDA receptor subunit 1 (pNR1), among other biochemical changes (Ji andWoolf, 2001; Willis, 2001; Gao et al., 2005). Because σ_1 receptors play an important modulatory role in the activity of glutamatergic (NMDA) neurotransmission (reviewed by Bermack and Debonnel, 2005b; Cobos et al., 2008a), it

can be hypothesized that σ_1 receptor-acting drugs might modulate central sensitization processes. Central sensitization can be induced by the intradermal administration of chemical algogens such as formalin (reviewed by Sawynok and Liu, 2004) or capsaicin (reviewed by Baron et al., 2000; Willis et al., 2001); consequently, σ_1 receptor-acting drugs might modulate the pain induced by these substances.

We recently reported that formalin-induced pain is diminished in σ_1 receptor knockout animals (Cendán et al., 2005a) or after systemic administration the σ_1 antagonists (Cendán et al., 2005b). Interestingly, the intrathecal administration of σ_1 receptor antagonists reduced both the formalin-induced increase in spinal cord pNR1 and pain behavior (Kim et al., 2006). Moreover, σ_1 receptor agonists increased NMDA-induced acute pain and pNR1 immunoreactivity in the spinal cord dorsal horn (Kim et al., 2008). It has therefore been suggested that spinal σ_1 receptors are involved in the central sensitization process through the modulation of NMDA receptors (Kim et al., 2008). However, to date no studies have evaluated the role of σ_1 receptors in one of the most characteristic signs of central sensitization and pathological pain: the sensitization to mechanical stimuli.

Capsaicin, the pungent component of the hot chili pepper, is reported to induce a patent central sensitization in both humans (e.g., Baumgartner et al., 2002; Klein et al., 2005) and experimental animals (e.g., Gilchrist et al., 1996; Joshi et al., 2006). Enhanced pain sensitivity in the area surrounding capsaicin injection (area of secondary mechanical hypersensitivity) results from this process (Baron, 2000; Willis, 2001). Indeed, changes in capsaicin-induced mechanical hypersensitivity have been used to study the behavioral consequences of drug treatment in central sensitization (e.g., Park et al., 1995; Baumgärtner et al., 2002; Gottrup et al., 2004; Bingham et al., 2005) and

capsaicin-induced mechanical hypersensitivity is considered a surrogate model of neuropathic pain (Baumgärtner et al., 2002; Klein et al., 2005). However, the possible role of σ_1 receptors in sensitization to the mechanical stimulus induced by capsaicin is unknown. In addition, the involvement of these receptors in pain induced by noxious mechanical stimulation in nonsensitized animals has not been studied.

The **main goal** of this Doctoral Thesis was to determine whether the σ_1 receptor can be considered a new pharmacological target for the treatment of mechanical allodynia or punctate mechanical nociceptive pain. To reach this goal we used three experimental tools: σ_1 receptor knockout mice, pharmacological antagonists and agonist for σ_1 receptor, and intracerebroventricular injections of σ_1 receptor antisense oligodeoxynucleotides (ASOs). Mechanical allodynia was assessed by measuring the threshold force necessary to induce paw withdrawal and the latency to paw withdrawal after applying an innocuous stimulus to the mouse paw following sensitization with an injection of capsaicin. Mechanical punctuate nociceptive pain was evaluated as the threshold force necessary to induce paw withdrawal and the latency time for paw withdrawal after stimulation with a painful stimulus of the nonsensitized paw (injected with capsaicin solvent).

The availability of σ_1 receptor knockout mice (developed by Langa et al., 2003) offers interesting possibilities for testing the role of σ_1 receptors in allodynia and nociception. Consequently, the **first specific goal** of this Doctoral Thesis was to compare the characteristics of mechanical nociceptive pain and capsaicin-induced mechanical allodynia in wild-type and σ_1 receptor knockout mice. For this purpose we evaluated the nocifensive responses produced by applying punctuate mechanical stimuli

at different intensities ranging from innocuous to painful in the paw of wild-type and σ_1 knockout mice previously treated with intraplantar capsaicin or its solvent.

The pharmacology of σ_1 receptors is well characterized. A wide variety of ligands bind to σ_1 receptors, including some neurosteroids, psychostimulants and antipsychotics (Maurice et al., 2001c; Monnet and Maurice, 2006; Cobos et al., 2008a). The antipsychotic drug haloperidol is mainly known as a dopamine D_2 receptor antagonist, but it shows the same affinity for D_2 and σ_1 receptors; furthermore, its metabolites display preferential activity at σ_1 receptors compared to D_2 receptors (Bowen et al., 1990; Matsumoto and Pouw, 2000). In addition, both selective agonists of σ_1 receptors, such as (+)-pentazocine and PRE-084, and antagonists, such as BD-1063, BD-1047 and NE-100 (Guitart et al., 2004; Hayashi and Su, 2004; Cobos et al., 2008a), are available. Accordingly, the **second specific goal** of this Doctoral Thesis was to evaluate whether pharmacological blockade of the σ_1 receptor with σ_1 selective antagonists (BD-1063, BD-1047 and NE-100) and σ_1 nonselective antagonists (haloperidol and its metabolites) modulates mechanical allodynia, nociceptive pain, or both, and whether coadministration with a σ_1 selective agonist (PRE-084) reverses the effects of σ_1 receptor antagonists. The effects of σ_1 antagonists in both experimental models were compare with those of several control drugs active against mechanical allodynia or nociceptive pain, such as gabapentin (Joshi et al., 2006), clonidine (Paqueron et al., 200), tetrodotoxin (Nieto et al., 2008; Hagen et al., 2008), pregabalin and mexiletine (Dworking et al., 2007). As a negative control we used rofecoxib, which is devoid of effect on mechanical allodynia and mechanical nociceptive pain (Bingham et al., 2005). In addition, to control for the influence of dopaminergic antagonism on the effects of haloperidol and its metabolites, we evaluated the effect of (–)-sulpiride, a dopamine

D_2/D_3 receptor antagonist devoid of activity on σ_1 receptors (Freedman et al., 1994; Matsumoto and Pouw, 2000; Cobos et al., 2007).

During mechanical stimulation of the area of secondary mechanical hypersensitivity induced by capsaicin, substantial changes in brain processing of nociceptive information have been described (Iadarola et al., 1998; Baron et al., 1999; Iannetti et al., 2005; Zambreanu et al., 2005). Because the possible supraspinal modulatory role of σ_1 receptors in this process remains unknown, the **third specific goal** of this Doctoral Thesis was to evaluate the behavioral consequences of supraspinal inhibition of σ_1 receptors in capsaicin-induced mechanical hypersensitivity by using intracerebroventricular injections of two different σ_1 receptor ASOs. In addition, we evaluated the effect of these ASOs on pain induced by a noxious mechanical punctate stimulus, to compare the role of supraspinal σ_1 receptors in mechanical nociceptive pain and mechanical allodynia. As controls for the specificity of the effects of ASOs, we tested the effects of the intracerebroventricular injection of their solvent and control oligodeoxynucleotides designed with the same length and base composition but in a different sequence.

PUBLISHED PAPERS

1. Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice

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1.1. ABSTRACT

We evaluated the role of σ_1 receptors on capsaicin-induced mechanical hypersensitivity and on nociceptive pain induced by punctate mechanical stimuli, using wild-type and σ_1 receptor knockout (σ_1-KO) mice and selective σ_1 receptor-acting drugs. Mutation in σ_1-KO mice was confirmed by PCR analysis of genomic DNA, and at the protein level by [^{3}H](+)-pentazocine binding assays. Both wild-type and σ_1-KO mice not treated with capsaicin showed similar responses to different intensities of mechanical stimuli (0.05 – 8 g force), ranging from innocuous to noxious, applied to the hind paw. This indicates that σ_1 gene inactivation does not modify the perception of punctate mechanical stimuli. The intraplantar (i.pl.) administration of capsaicin induced dose-dependent mechanical allodynia in wild-type mice (markedly reducing both the threshold force necessary to induce paw withdrawal and the latency to paw withdrawal induced by a given force). In contrast, capsaicin was completely unable to induce mechanical hypersensitivity in σ_1-KO mice. The high affinity and selective σ_1 antagonists BD-1063, BD-1047 and NE-100, administered subcutaneously (s.c.), dose-dependently inhibited mechanical allodynia induced by capsaicin (1 μg), yielding ED_{50} (mg/kg) values of 15.80 ± 0.93, 29.31 ± 1.65 and 40.74 ± 7.20, respectively. The effects of the σ_1 antagonists were reversed by the σ_1 agonist PRE-084 (32 mg/kg, s.c.). None of the drugs tested modified the responses induced by a painful mechanical punctate stimulus (4 g force) in nonsensitized animals. These results suggest that σ_1 receptors are essential for capsaicin-induced mechanical hypersensitivity, but are not involved in mechanical nociceptive pain.

1.2. INTRODUCTION

Sigma receptors exist as two distinct entities: sigma-1 (σ_1) and sigma-2 (σ_2) (Su and Hayashi, 2003; Cobos et al., 2008). The σ_1 receptor was first cloned in 1996 (Hanner et al., 1996), and is better characterized than the σ_2 subtype. Currently, σ_1 receptors are considered to play an important neuromodulatory role in the activity of several ion channels and neurotransmitter systems, mainly in glutamatergic (NMDA: *N*-methyl-*D*-aspartate) neurotransmission (Su and Hayashi, 2003; Bermack and Debonnel, 2005; Monnet and Maurice, 2006; Cobos et al., 2008)

$Sigma_1$ receptors are widely distributed in the central nervous system, including pain processing areas such as the superficial layers of the spinal cord dorsal horn, the periaqueductal gray matter, the locus coeruleus and rostroventral medulla (Alonso et al., 2000; Kitaichi et al., 2000). The enhancement of opioid-induced antinociception by σ_1 receptor antagonists has been extensively documented (Chien and Pasternak, 1994; Marrazzo et al., 2006; Mei and Pasternak, 2002 and 2007), but σ_1 receptors may also play a role in nociception in the absence of opioid drugs. In this regard, it has been reported that both genetic inactivation and pharmacological antagonism of σ_1 receptors produce an antinociceptive effect in the formalin test (Cendán et al., 2005a and b; Kim et al., 2006), which is associated with a reduction in the phosphorylation of NMDA receptor subunit 1 (pNR1) (Kim et al., 2006). Moreover, σ_1 receptor agonists increased NMDA-induced acute pain and pNR1 immunoreactivity in the spinal cord dorsal horn (Kim et al., 2008). Due to the importance of NMDA receptor phosphorylation in the central sensitization of pain pathway neurons (Zou et al., 2000; Gao et al., 2005; Ultenius et al., 2006), it has been suggested that σ_1 receptors may be involved in the central sensitization process through the modulation of NMDA receptors (Kim et al., 2008).

Central sensitization occurs in pathological pain (inflammatory and neuropathic pain) (Ji and Woolf, 2001). Therefore, research aimed at studying the effects of σ_1 receptor antagonism in models of pain that involve central sensitization seems likely to yield findings of relevance for treatment of these types of pain. The increase in sensitivity to pain in the area surrounding capsaicin injection (area of secondary mechanical hypersensitivity) results from central sensitization (Sang et al., 1996; Baron, 2000). Indeed, changes in capsaicin-induced mechanical hypersensitivity have been used to study the behavioral consequences of drug treatment in central sensitization (e.g. Park et al., 1995; Baumgärtner et al., 2002; Gottrup et al., 2004; Bigham et al., 2005). However, the possible role of σ_1 receptors in sensitization to the mechanical stimulus induced by capsaicin is unknown. Moreover, the involvement of these receptors in pain induced by noxious mechanical stimulation has not yet been studied.

Consequently, the aims of this study were: 1) to compare capsaicin-induced mechanical hypersensitivity and mechanical nociceptive pain in wild-type and σ_1 receptor knockout mice, and 2) to evaluate the effects on these experimental paradigms of several prototypical σ_1 receptor antagonists such as BD-1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide), BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) and NE-100 (*N*, *N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine hydrochloride), as well as the σ_1 receptor agonist PRE 084 [(2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride] (reviewed in Hayashi and Su, 2004; Cobos et al., 2008). In addition, since capsaicin-induced mechanical hypersensitivity is considered a surrogate model of neuropathic pain (Baumgärtner et al., 2002; Klein et al., 2005), we compared the effect of σ_1 receptor ligands in this behavioral model with that of several drugs active against

neuropathic pain such as pregabalin, mexiletine (used in humans as first-line and third-line medications, respectively) (Dworkin et al., 2007) and tetrodotoxin (effective against neuropathic pain in rodents and humans) (Hagen et al., 2008; Nieto et al., 2008).

1.3. METHODS

1.3.1. Experimental animals

Most experiments were performed in female wild-type (Charles River, Barcelona, Spain) and σ_1 receptor knockout CD-1 mice (Laboratorios Dr. Esteve, Barcelona, Spain S.A.) weighing 25 – 30 g. Animals were tested randomly throughout the phases of the estrus. Some experiments were also performed in male (wild-type and σ_1 receptor knockout) CD-1 mice to ensure that gender differences were not relevant variables in our results. To obtain CD-1 σ_1 receptor knockout mice, animals generated with a previously described method (Langa et al., 2003) were backcrossed onto the CD-1 background with selection for the mutant σ_1 gene at each generation. After 10 generations of successive backcrosses with pure CD-1 mice, which theoretically ensured that less than 1% of the genetic material from the original background remained (Wong, 2002), mice harboring the mutation were then bred to homozygosity and used in this study. Mice were housed in a temperature-controlled room (21 ± 1 °C) with air exchange every 20 min and an automatic 12-h light/dark cycle (08.00 to 20.00 h). They were fed a standard laboratory diet (Harlan Teklad Research diet, Madison, USA) and tap water *ad libitum* until the beginning of the experiments. The experiments were performed during the light phase (09.00–15.00 h). Mice were always handled in accordance with the European Communities Council Directive of 24 November 1986

(86/609/ECC). The experimental protocol was approved by the Ethical Research Committee of the University of Granada, Spain.

1.3.2. Drugs and drug administration

The σ_1 ligands and their providers were: BD-1063, BD-1047, PRE-084 (all from Tocris Cookson Ltd., Bristol, United Kingdom) and NE-100 synthesized as described previously (Nakazato et al., 1999). As a control for drugs active against neuropathic pain, we tested pregabalin (Laboratorios Dr. Esteve, S.A.), mexiletine (Sigma-Aldrich Química S.A., Madrid, Spain) and tetrodotoxin (Tocris Cookson Ltd.).

For binding assays, the radioligand used was [^{3}H](+)-pentazocine, with a specific activity of 32.2 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 HCl-Tris buffer pH 8 at 30 ºC). The cold drug used to define nonspecific binding was haloperidol (10 μM) (Sigma-Aldrich Química S.A.). For competition binding assays, the cold drugs were: BD-1047, BD-1063, NE-100, pregabalin, tetrodotoxin and mexiletine. All drugs were dissolved at a concentration of 1 mM in ultrapure water with the exception of haloperidol and mexiletine, which were dissolved in absolute ethanol. Further dilutions were prepared with incubation buffer. The final maximal concentration of ethanol in the incubation medium was 1% (vol/vol), when required. We previously verified that this concentration of ethanol did not affect [^{3}H](+)-pentazocine binding. Nonspecific binding was defined as the binding retained in the presence of haloperidol 10 μM, and was always less that 20% of the total binding.

For behavioral studies all drugs were dissolved in sterile physiological saline. Drugs or their solvent were administered subcutaneously (s.c., 5 ml/kg) into the

interscapular zone. In the experiments in which the selective σ_1 agonist PRE-084 was associated with other drugs, PRE-084 solution was injected immediately before and in a different area to the other drug to avoid any physicochemical interactions between the solutions. Capsaicin (Sigma-Aldrich Química S.A.) was dissolved in 1% dimethylsulfoxide (DMSO, Merck KGaA, Darmstadt, Germany) in physiological saline to a concentration of 0.2 µg/µl. Further dilutions of the capsaicin solution were performed to obtain the appropriate concentrations for different experiments. Capsaicin solution or its solvent (in a volume of 20 µl) was injected intraplantarly (i.pl.) into the right hind paw, using a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a $30^{1/2}$-gauge needle.

1.3.3. Mice brain membrane preparations

Crude synaptosomal membranes (P_2 fraction) were prepared for [^{3}H](+)-pentazocine binding with a previously described method (Cobos et al., 2007), with slight modifications. Mice were killed by cervical dislocation and brains were rapidly removed and homogenized in 15 volumes (wt/vol) of 0.32 M sucrose-10 mM Tris HCl pH 7.4 with a Polytron homogenizer (model PT10-35, Kinematica AG, Basel, Switzerland). The homogenates were centrifuged (Avanti 30, Beckman Coulter España S.A., Madrid, Spain) at 1000 *g* for 13 min, the resulting pellets were discarded and the supernatants were centrifuged again at 21 000 *g* for 15 min to obtain the P_2 pellets; each pellet, obtained from two whole brains, was re-suspended in 15 ml 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 10 mM Tris-HCl, pH 7.4, and frozen in aliquots (protein concentration 10.5 – 12 mg/ml) at –80 °C. Binding characteristics of

the tissue were stable for at least 1 month when stored at –80 °C. Protein concentrations were measured by the method of Lowry (Lowry et al., 1951) with some modifications, using bovine serum albumin as the standard.

1.3.4. [^{3}H](+)-Pentazocine binding assays

To confirm the absence of σ_1 receptors in mutant mice, we performed *in vitro* [^{3}H](+)-pentazocine saturation binding assays in wild-type and σ_1 knockout mouse brain membranes. We also tested the affinities of BD-1047, BD-1063, NE-100, PRE-084, pregabalin, mexiletine and tetrodotoxin for wild-type mouse brain σ_1 receptors with [^{3}H](+)-pentazocine competition binding assays. To perform these experiments, aliquots of mouse brain membranes were slowly thawed and resuspended in fresh incubation buffer, and [^{3}H](+)-pentazocine binding was tested as described previously (Cobos et al., 2007) with slight modifications. Resuspended membrane preparations (460 µl) in a final protein concentration of 0.80-1.02 mg/ml were incubated with 20 µl [^{3}H](+)-pentazocine (final concentration of 5 nM in competition assays and 0.1 – 37 nM in saturation experiments) and with 20 µl of the cold ligand or its solvent, at 30 °C, pH 8, for 240 min. The reaction was stopped with 5 ml ice-cold filtration buffer (Tris 10 mM pH 7.4). 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) over Whatman GF/B glass fiber filters (SEMAT Technical Ltd., St. Albans, UK), presoaked with 0.5% polyethylenimine in filtration buffer for at least 1 h prior to use, to reduce nonspecific binding. The filters were washed under vacuum twice with 5 ml-volumes of the ice-cold filtration buffer, and transferred to scintillation counting vials. After the addition of 4 ml liquid

scintillation cocktail (CytoScint scintillation counting solution, MP Biomedicals, Irvine, CA), the samples were allowed to equilibrate overnight. The radioactivity retained in the filter was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A.), with an efficiency of 52%. Each assay was conducted in triplicate.

1.3.5. PCR assays in wild-type and σ_1 knockout mice

Genomic DNA was obtained from tail tips with DNeasy Blood & Tissue kits (QIAGEN, Madrid, Spain) according to the manufacturer's instructions. Amplifications for PCR were done with Eppendorf HotMasterMix (Eppendorf, Hamburg, Germany) and 0.5 μM of each primer (Invitrogen Ltd, Paisley, UK). The PCR was performed with a thermal controller (iCycler, Bio-Rad Laboratories, Hercules, CA) using the following program. Initial template denaturation at 94 ºC, followed by 35 cycles: 30 s at 94 ºC, 45 s at 55 ºC and 2 min at 70 ºC; and, as a final extension step, 10 min at 70 ºC. The oligonucleotide primer (5′–3′) sequences specific for the genes examined were as follows: 5′-AAT TTT GCT CCC CTC CTC-3 and 5′-CGT TCA CAA ATA CCC ACT G-3 for the wild-type allele; 5′-GGA ACC AGA TGA CCC ACA GGT GC-3′ and 5′-CGC CAT TCA GGC TGC GCA ACT GTT GGG-3′ for the mutant allele (Langa et al., 2003). Different molecular weight markers (EZ Load Molecular Rulers, Bio-Rad Laboratories, Hercules, CA, USA) were also used. Amplified products were analyzed by electrophoresis on 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. The gels were then photographed with a UV transilluminator to visualize the ethidium bromide-stained bands. Genomic DNA was obtained from tail tips with DNeasy Blood & Tissue kits (QIAGEN, Madrid, Spain) according to the manufacturer's instructions. Amplifications for PCR were done with Eppendorf HotMasterMix (Eppendorf,

Hamburg, Germany) and 0.5 μM of each primer (Invitrogen Ltd, Paisley, UK). The PCR was performed with a thermal controller (iCycler, Bio-Rad Laboratories, Hercules, CA) using the following program. Initial template denaturation at 94 ºC, followed by 35 cycles: 30 s at 94 ºC, 45 s at 55 ºC and 2 min at 70 ºC; and, as a final extension step, 10 min at 70 ºC. The oligonucleotide primer (5′–3′) sequences specific for the genes examined were as follows: 5′-AAT TTT GCT CCC CTC CTC-3′ and 5′-CGT TCA CAA ATA CCC ACT G-3′ for the wild-type allele; 5′-GGA ACC AGA TGA CCC ACA GGT GC-3′ and 5′-CGC CAT TCA GGC TGC GCA ACT GTT GGG-3′ for the mutant allele (Langa et al., 2003). Different molecular weight markers (EZ Load Molecular Rulers, Bio-Rad Laboratories, Hercules, CA, USA) were also used. Amplified products were analyzed by electrophoresis on 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. The gels were then photographed with a UV transilluminator to visualize the ethidium bromide-stained bands.

1.3.6. Comparison of mechanical hypersensitivity induced by capsaicin and punctate nociceptive pain in wild-type and σ_1 knockout mice

Mice were habituated for 2 hours in individual black-walled test compartments placed on an elevated mesh-bottomed platform with a 0.5-cm^2 grid (to provide access to the ventral side of the mice hind paws). Animals were then removed from the compartment and given an i.pl. injection of capsaicin or its solvent into the right hind paw, and immediately returned to the compartment. Fifteen minutes later mechanical stimulation was applied with a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy).This device uses a fixed nonflexible metallic filament (0.5 mm diameter), that is applied with electronically adjustable forces of stimulation. Therefore the use of this

device makes it unnecessary to use more than one filament. This filament was electronically driven to the ventral side of the injected hind paw at least 5 mm apart from the site of injection of capsaicin or its solvent. When nocifensive paw withdrawal occurred, the stimulus was automatically terminated and the response latency time was automatically recorded. Each mouse was tested in three trials at 30-s intervals in each experimental session. A cut-off time of 50 s was used in each trial.

To compare the control response to innocuous and noxious punctate mechanical stimuli and the degree of capsaicin-induced mechanical hypersensitivity in σ_1 knockout and wild-type mice, we used two different experimental approaches. Firstly, in animals previously injected with a fixed dose of capsaicin (1 μg) or its solvent, we evaluated the effect of stimulation with the filament at a wide range of intensities (0.05 – 8 g force; i.e., 0.49 – 78.45 mN). This allowed us to construct a force-response curve (i.e., intensity of the stimulus vs. paw withdrawal latency time) and to quantify the force needed to induce a given behavioral response. Secondly, animals were administered i.pl. with different doses of capsaicin (0.125 – 4 μg) or its solvent, and 15 min later were stimulated with the filament at 0.5 g (4.90 mN) force. This intensity of the stimulus was innocuous and unable to induce paw withdrawal in wild-type nonsensitized animals (DMSO-treated), but produced rapid paw withdrawal in capsaicin-sensitized animals (see Figs. 2A and 3), and thus allowed us to evaluate the dose-dependency of capsaicin-induced mechanical allodynia.

1.3.7. Comparison of drug effects on capsaicin-induced mechanical allodynia and on mechanical punctate pain in nonsensitized animals

To evaluate the effect of drugs on mechanical allodynia induced by capsaicin, the drug under study or its solvent was administered s.c. 30 min before the i.pl. administration of capsaicin (1 μg). Fifteen minutes after the i.pl. injection (i.e., 45 min after drug administration), the effect of the drug was assessed by stimulation of the injected hind paw with the filament at 0.5 g force. As noted above, this intensity of mechanical stimulus did not induce paw withdrawal in DMSO-treated mice, but markedly reduced paw withdrawal latency time in capsaicin-sensitized wild-type mice (see Figs. 2A and 3 for details). To evaluate the effects of drugs on mechanical pain induced by a punctate stimulus in nonsensitized mice, drugs or their solvent were administered s.c.; 30 min later DMSO 1% (capsaicin solvent) was injected i.pl., and 15 min thereafter the animals were stimulated with the filament at 4 g (39.23 mN) force. This intensity of the mechanical stimulus was chosen because the withdrawal latency time it yielded in nonsensitized (DMSO-treated) animals was similar to that obtained in animals whose paw was sensitized with 1 μg capsaicin (i.pl.) and stimulated at 0.5 g force (see Results and Figs. 2A and 7 for details).

1.3.8. Data analysis

The equilibrium saturation binding parameters, dissociation constant (K_D) and maximum number of binding sites (B_{max}), were calculated by nonlinear regression analysis of the equation for a rectangular hyperbola. These parameters were also calculated from the linear regression obtained with Scatchard analysis as [*B/F*] vs. *B*, assuming *B* to be specific binding and *F* to be the free concentration of radioligand. Hill

plots were obtained from the saturation experiments by plotting the data as log [$B/(B_{max}$ - B)] vs. log [F], where the slope of the plot (n_H) represents the Hill coefficient. Data were analyzed with the SigmaPlot 2002 v. 8.0 program (SPSS Inc., Chicago, IL, USA). The IC_{50} (concentration of unlabeled drug that inhibited 50% of [^{3}H](+)-pentazocine specific binding) was estimated from the inhibition curves using nonlinear regression analysis of the equation for a sigmoid plot, assuming one-site competition, with the SigmaPlot v. 8.0 (2002) program. The K_i values for unlabeled ligands (which indicate the affinity of the inhibitor for the receptor) were 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 with nonlinear regression analysis from the control saturation experiments.

The degree of effect of drugs on mechanical hypersensitivity induced by capsaicin was calculated as: % reduction in mechanical hypersensitivity = [(LTD – LTS) / (CT – LTS)] × 100, where LTD is latency time in drug-treated animals, LTS is latency time in solvent-treated animals, and CT is the cut-off time (50 s). The ED_{50} (dose of drug producing half of the maximal response) and E_{max} values (maximum effect) were calculated from the dose–response curves using nonlinear regression analysis of the equation for a sigmoid plot. The EF_{50} values (force of mechanical stimuli applied producing half of the maximal reduction in paw withdrawal latency time) were calculated from the force-response curves using nonlinear regression analysis of the equation for a sigmoid plot. Nonlinear analysis were performed with the SigmaPlot 2002 v. 8.0 program. Parameters obtained from sigmoid plots and their standard errors were calculated as the best-fit values ± standard errors of regression; when required, they were compared with Snedecor's F test to check the goodness of fit of different

models that shared one or more parameters (DeLean et al., 1978), using the GraphPad Prism 3.00 program (GraphPad Software Inc., San Diego, CA, USA). The differences between values were considered significant when the *p* value was below 0.05.

The paw withdrawal latency times and percentage reductions in mechanical hypersensitivity were compared across experimental groups with one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test. The differences between means were considered significant when the value of *p* was below 0.05.

1.4. RESULTS

1.4.1. [^{3}H](+)-Pentazocine saturation binding assays and PCR analysis in wild-type and σ_1 knockout mice

Saturation assays in wild-type mice showed that the selective σ_1 receptor radioligand [^{3}H](+)-pentazocine bound in a saturable manner to only one population of specific binding sites in brain membranes. Replicates were fitted by nonlinear regression analysis to hyperbolic equations yielding an averaged equilibrium dissociation constant (K_D) of 1.70 ± 0.52 nM and a maximal number of receptors (B_{max}) of 381.64 ± 3.13 fmol/mg protein (Fig. 1A). Scatchard analysis of these results yielded a straight line, consistent with the existence of a single class of high-affinity σ_1 binding sites (Fig. 1B). The parameters K_D and B_{max} obtained with this analysis were 1.66 ± 0.39 nM and 383.81 ± 4.94 fmol/mg protein, respectively, which were very similar to those obtained with nonlinear regression analysis. The Hill analysis yielded a straight line whose slope (n_H) was very close to unity (1.02 ± 0.03). These results indicated the existence of a single population of [^{3}H](+)-pentazocine binding sites in wild-type mice. In expected contrast, when [^{3}H](+)-pentazocine saturation assays were performed in σ_1

knockout mice brain membranes, the level of σ_1 radioligand binding was undetectable (Figs. 1A and 1B).

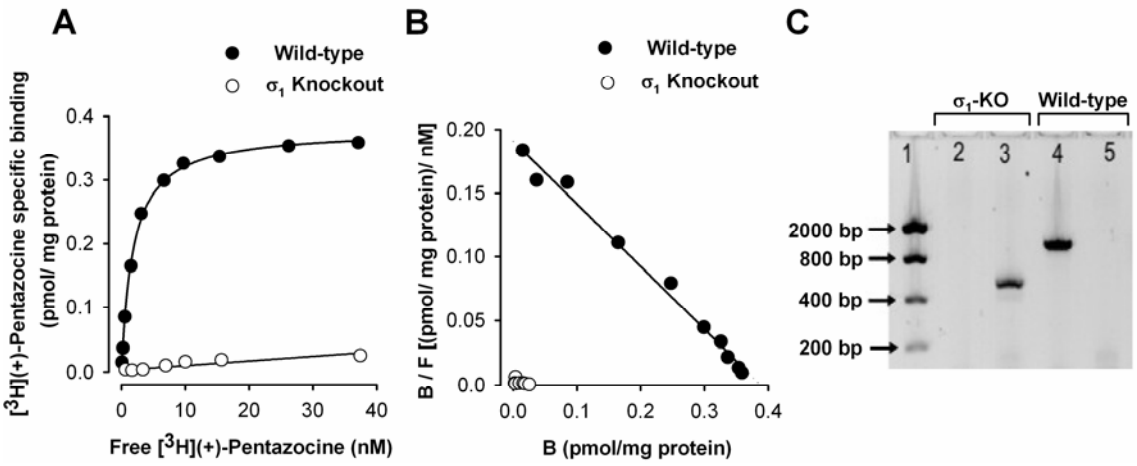

Figure. 1. (**A**) Representative saturation binding assays for the selective σ_1 receptor radioligand [^{3}H](+)-pentazocine in wild-type (●) and σ_1 knockout (○) mouse brain membranes (P_2 fraction). Membranes (0.86 – 1 mg/ml protein) were incubated at 30 °C, pH 8, for 240 min, with several concentrations of [^{3}H](+)-pentazocine (0.1 – 37 nM) in the presence of haloperidol 10 μM (for nonspecific binding) or its solvent. (**B**) Representative Scatchard plots of the binding data. **(C)** Electrophoresis of the PCR products: *lane 1*, molecular weight markers; *lane 2*, absence of product amplification with primers for the wild-type allele from homozygous mutant genomic DNA; *lane 3*, 529-bp product amplification with primers for the mutant allele from homozygous mutant genomic DNA; *lane 4*, 1160-bp product amplification with primers for the wild-type allele from wild-type genomic DNA; *lane 5*, absence of product amplification with primers for the mutant allele from wild-type genomic DNA.

The genotype of the mice used in the behavioral test was also analyzed by PCR analysis of tail genomic DNA. We were able to differentiate wild-type and knockout mice with two different sets of oligonucleotides used to amplify a 1160-bp DNA band (lane 4, Fig. 1C) corresponding to the wild-type allele, or a 529-bp DNA band (lane 3, Fig. 1C) corresponding to the mutant allele (see Langa et al., 2003). Lanes 2 and 5 are control experiments showing the absence of amplification product for the wild-type

allele in σ_1 knockout mice (lane 2) and for the mutant allele in wild-type animals (lane 5).

1.4.2. Comparison of capsaicin-induced mechanical hypersensitivity in wild-type and σ_1 knockout mice

To compare the degree of capsaicin-induced mechanical allodynia in wild-type and σ_1 knockout mice, we applied the filament at different intensities ranging from innocuous to noxious stimuli (0.05 – 8 g force) in mice treated with capsaicin (1 μg, i.pl.) or its solvent (Figs. 2A and 2B). As expected, in control (DMSO-treated) wild-type mice the paw withdrawal latency time decreased as the force applied increased (Fig. 2A). We used data from this experiment to calculate the EF_{50} value, which was 2.64 ± 0.08 g (Table 1). Capsaicin injection in wild-type animals induced displacement of the force-response curve to the left (Fig. 2A) and decreased the EF_{50} value to 0.28 ± 0.02 g, which was much lower and statistically different ($p < 0.01$) from that obtained in DMSO-treated mice (Table 1). This displacement to the left of the force-response curve clearly indicated capsaicin-induced mechanical hypersensitivity in wild-type animals. In contrast, the same experiments in σ_1 knockout mice showed that the paw withdrawal latency times at the different forces tested were similar in capsaicin- and DMSO-treated mice (Fig. 2B), i.e., capsaicin did not displace the force-response curve in these animals. The EF_{50} was 2.37 ± 0.05 g in capsaicin-treated σ_1 knockout mice, and the differences between this value and that calculated (2.40 ± 0.15 g) in DMSO-treated σ_1 knockout mice were not statistically significant (Table 1).

Interestingly, when we compared the force-response curves of wild-type and σ_1 knockout mice treated with capsaicin solvent (DMSO 1%), we found that the EF_{50}

values (2.64 ± 0.08 and 2.40 ± 0.15 g, respectively) were not statistically different (Table 1), indicating that nonsensitized wild-type and σ_1 knockout mice showed equivalent responses to mechanical punctate stimuli.

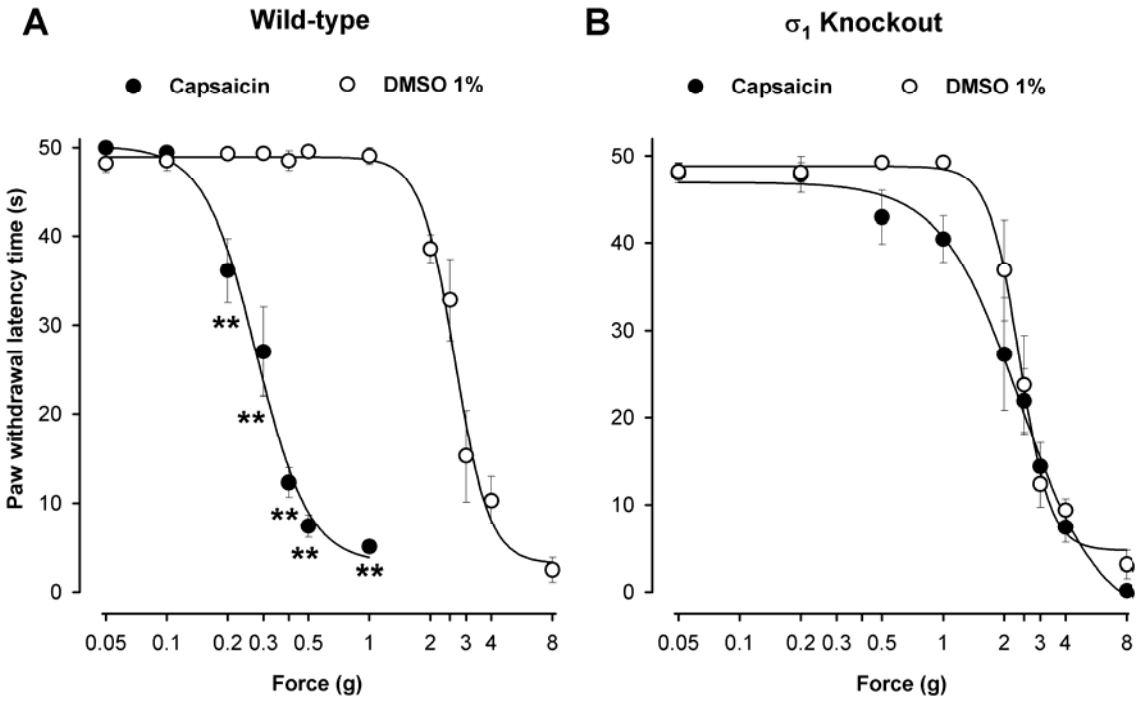

Figure. 2. Effects of different forces of stimulation with the filament on withdrawal latency time of the paw previously injected (15 min) with capsaicin (1 μg, ●) or its solvent (DMSO 1%, ○) in wild-type (**A**) and σ_1 knockout (**B**) mice. Each point and vertical line represent the mean ± SEM of the values obtained in 8 – 10 animals. **A:** Statistically significant differences between the latency time obtained with each value of applied force in DMSO- and capsaicin-treated wild-type mice: ** $p < 0.01$ (two-way ANOVA followed by Bonferroni test). **B:** No statistically significant differences between the values obtained in knockout animal groups treated with capsaicin or its solvent were found (two-way ANOVA).

Table 1: EF_{50} values (force of applied mechanical punctuate stimuli producing half of the maximal reduction in paw withdrawal latency time) in wild-type and σ_1 knockout mice treated intraplantarly with 1 μg capsaicin or 1% DMSO (capsaicin solvent). Data were calculated from the results of nonlinear regression analysis of the equation for sigmoid plots shown in Fig. 2.

	Intraplantar injection	**EF_{50} (g)**
Wild-type	1% DMSO	2.64 ± 0.08
	1 μg capsaicin	0.28 ± 0.02[a]
σ_1 knockout	1% DMSO	2.40 ± 0.15
	1 μg capsaicin	2.37 ± 0.05

Animals treated intraplantarly with capsaicin (1 μg) or its solvent (1% DMSO) were stimulated with a wide range of forces (0.05 – 8 g; 0.49 – 78.45 mN) applied with an electronically-driven monofilament. All results are given as the best-fit values ± standard error of regression. The results from the different force-response curves were compared by goodness of fit of simultaneous analyses to sigmoid plots with and without a set of constraints (same or different EF_{50}) with the F test (see text for details). [a] Statistically significant differences ($p < 0.01$) compared to 1% DMSO-treated wild-type mice (F test). There were no significant differences in EF_{50} values for paw withdrawal latency between knockout animals (treated or not with capsaicin) and DMSO 1%-treated wild-type mice (F test).

We also used a second approach to compare the responses to punctate mechanical stimuli in DMSO- and capsaicin-treated wild-type and σ_1 knockout mice. In this case we compared the effects of different doses of capsaicin (0.125-4 μg, i.pl.) on the paw withdrawal latency time in animals stimulated with the filament at 0.5 g force (Fig. 3).

In both DMSO-treated wild-type or σ_1 knockout mice, this force was an innocuous stimulus unable to induce any paw withdrawal (dose 0 in Fig. 3; see also Fig. 2). When capsaicin was administered i.pl. to wild-type mice, it induced a marked and dose-dependent reduction in paw withdrawal latency time, which was maximal at doses of capsaicin of 1 – 4 μg (Fig. 3), yielding an ED_{50} of 0.39 ± 0.036 μg of capsaicin. However, under the same experimental conditions σ_1 knockout mice showed only a modest, nonsignificant ($p > 0.1$) reduction in withdrawal latency time in the capsaicin-injected paw (Fig. 3), which was too small to calculate an ED_{50} value. In summary, these results with two different experimental approaches (Figs. 2 and 3) show that mechanical hypersensitivity induced by capsaicin was markedly reduced in mice lacking σ_1 receptors.

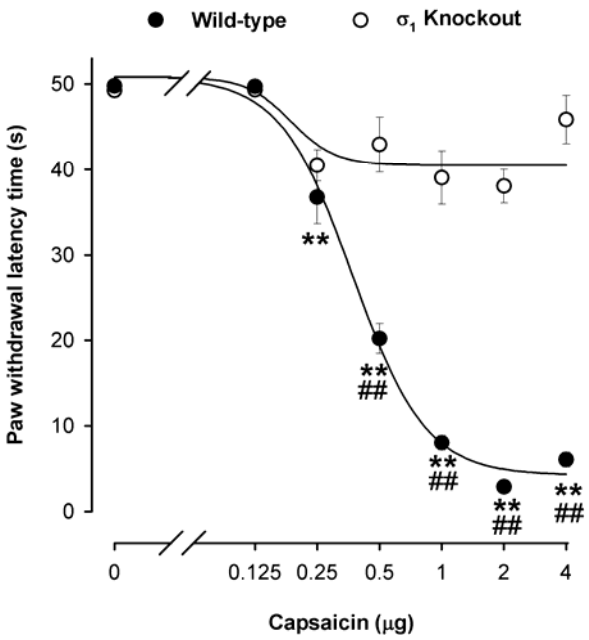

Figure. 3. Effects of the i.pl. administration of different doses of capsaicin (0.125 – 4 μg) or its solvent (DMSO 1% -dose 0-) on paw withdrawal latency time in wild-type (●) and σ_1 knockout mice (○). Animals were stimulated with the filament at 0.5 g (4.90 mN) force 15 min after the i.pl. injection. Each point and vertical line represent the mean ± SEM of values obtained in 8 – 10 animals. Statistically significant differences between the values obtained in capsaicin- and DMSO-treated animals: ** $p < 0.01$; and between the values obtained in wild-type and σ_1 knockout animals at the same doses of capsaicin: $^{\#\#}$ $p < 0.01$ (two-way ANOVA followed by Bonferroni test).

To rule out that gender differences may affect our results we compared the response of male and female animals in several experiments. We found that latency time for withdrawal of the paw stimulated with the filament (at 0.5 g force) was very similar in wild-type mice of both genders treated with DMSO i.pl. (46.93 ± 1.57 s and 48.97 ± 0.41 s in male and female, respectively), and was similarly reduced when capsaicin (1 μg, i.pl.) was injected (10.06 ± 0.91 s and 8.03 ± 0.82 s in male and female, respectively). Interestingly, the paw withdrawal latency time in σ_1 receptor knockout animals treated with capsaicin (1 μg, i.pl.) and stimulated with the filament (at 0.5 g force) was also similar in both genders (42.44 ± 2.69 s and 39.04 ± 3.11 s in male and female, respectively), but significantly ($p < 0.01$) much higher than in wild-type mice. These data indicate that gender differences did not affect the ability of capsaicin to induce mechanical allodynia or its inhibition by genetic inactivation of σ_1 receptors.

1.4.3. Affinity of drugs for [^{3}H](+)-pentazocine binding sites in mouse brain

We used competition binding assays to test the binding of the drugs to the σ_1 receptor, labeled with [^{3}H](+)-pentazocine, in wild-type mouse brain membranes (P_2 fraction). As expected, [^{3}H](+)-pentazocine specific binding (which always represented more than 80 % of the total binding) was concentration-dependently inhibited by the selective σ_1 antagonists BD-1047, BD-1063 and NE-100 (Fig. 4), which yielded affinity values (K_i) of 1.90 ± 0.48, 4.40 ± 1.04 and 14.56 ± 4.06 nM, respectively. The σ_1 agonist PRE-084 also displaced [^{3}H](+)-pentazocine specific binding, yielding a K_i value of 45.76 ± 11.50 nM. In contrast, pregabalin, tetrodotoxin and mexiletine showed negligible affinity for [^{3}H](+)-pentazocine binding sites (K_i > 10 000 nM) (Fig. 4).

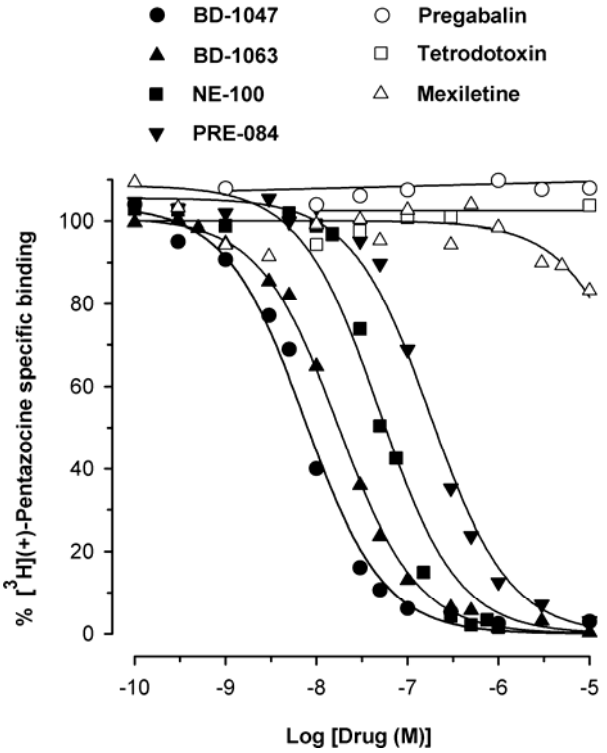

Figure. 4: Inhibition by unlabeled drugs of [^{3}H](+)-pentazocine specific binding to wild-type mouse brain membranes (P_2 fraction). [^{3}H](+)-pentazocine (5 nM) was incubated with 0.8 – 1 mg/ml brain membrane protein at 30 ºC, pH 8, for 240 min and increasing concentrations of BD-1047 (●), BD-1063 (▲), NE-100 (■), PRE-084 (▼), pregabalin (○), tetrodotoxin (□) or mexiletine (Δ). Data shown are the average of two experiments carried out in triplicate.

1.4.4. Antiallodynic effects induced by selective σ_1 antagonists

The effects of selective σ_1 antagonists on mechanical hypersensitivity induced by capsaicin i.pl. (1 μg) were assessed in wild-type animals stimulated with the filament at 0.5 g force. The s.c. administration of BD-1063 (8 – 64 mg/kg), BD-1047 (16 – 128 mg/kg) and NE-100 (16 – 128 mg/kg) induced a dose-dependent antiallodynic effect (Figs. 5A, 6A and 6B, respectively). Among the σ_1 antagonists tested, BD-1063 was the most potent in reversing the allodynic effect induced by capsaicin (ED_{50} = 15.80 ± 0.81 mg/kg), followed by BD-1047 (ED_{50} = 29.28 ± 1.16 mg/kg) and NE-100 (40.41 ± 5.08 mg/kg). We also calculated the maximum effect of each drug with nonlinear regression analysis of the equation for a sigmoid plot, and found that all three σ_1 antagonists yielded E_{max} values approaching 100% of the antiallodynic effect in this behavioral model (Figs. 5A, 6A and 6B).

To test whether gender differences may affect the antiallodynic activity of σ_1 receptor antagonists, we compared the effect of the selective σ_1 antagonist BD-1063 (16 mg/kg, s.c.) in male and female wild-type mice. We found that the administration of BD-1063 similarly inhibited the mechanical hypersensitivity induced by capsaicin in both genders (the percentage of reduction of mechanical hypersensitivity were 60.02 ± 8.72 and 63.54 ± 11.01 in female and male animals, respectively).

1.4.5. Reversal of the antiallodynic effects of selective σ_1 receptor antagonist by the prototype σ_1 receptor agonist PRE-084

In contradistinction to the effects induced by σ_1 antagonists, the selective σ_1 agonist PRE-084 (16 or 32 mg/kg) did not modify paw withdrawal latency time in wild-type animals treated with capsaicin (1μg) and stimulated with the filament at 0.5 g force (Figs. 5B and 6C). However, when PRE-084 (32 mg/kg, s.c.) was coadministered with any of several doses of the selective σ_1 antagonist BD-1063 (s.c.), the dose–response curve of the antiallodynic effect induced by this σ_1 antagonist was shifted to the right with no significant change in its E_{max} (Fig. 5A). This suggests competitive antagonism between these drugs at a common site.

In addition, the increase in paw withdrawal latency time in capsaicin-sensitized animals (i.e., the antiallodynic effect) induced by 20 mg/kg BD-1063 (s.c.) was reversed, in a dose-dependent manner, by the co-administration of PRE-084 (4, 16 and 32 mg/kg, s.c.) (Fig. 5B). The dose of 32 mg/kg PRE-084 (s.c.) completely reversed the antiallodynic effect induced by BD-1063 (20 mg/kg, s.c.), and the paw withdrawal

latency time of capsaicin-treated animals (7.90 ± 1.12 s) was similar to that in animals treated with the drug solvent (7.40 ± 1.26 s) (Fig. 5B).

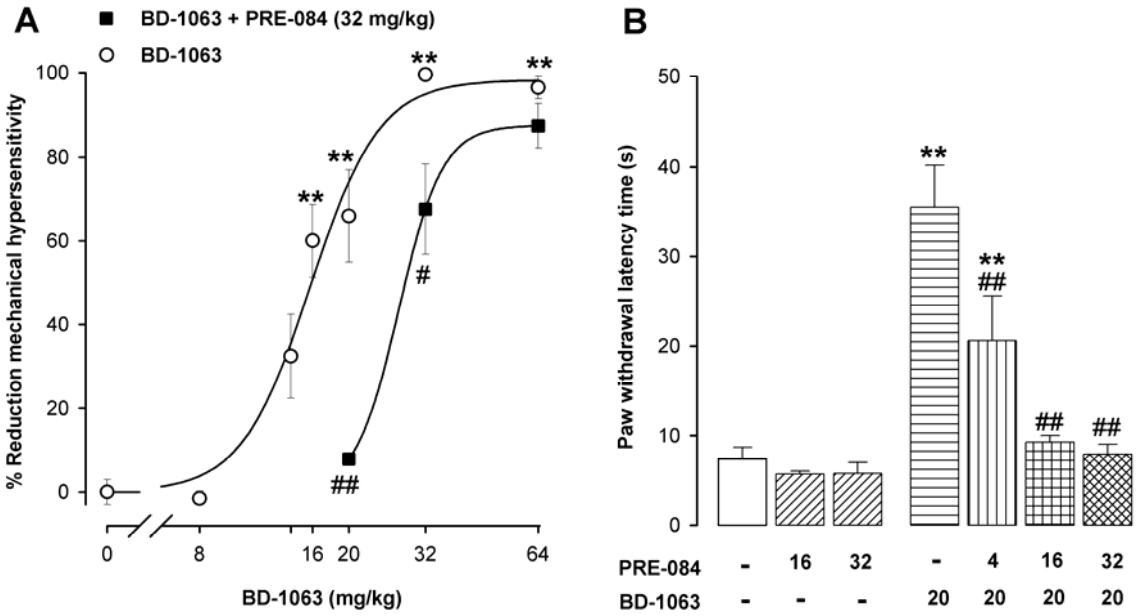

Figure. 5. Effects of BD-1063 on mechanical hypersensitivity induced by the i.pl. injection of capsaicin (1 µg) to the hind paw of wild-type mice. (**A**) Dose–response curves of the mechanical antiallodynic effect induced by s.c. BD-1063 (○) administered alone or associated to PRE-084 (32 mg/kg, s.c., ■). (**B**) Effect on paw withdrawal latency time of the s.c. administration of drug solvent, PRE-084 (16 and 32 mg/kg), BD-1063 (20 mg/kg) and the association of PRE-084 (4 – 32 mg/kg) with BD-1063 (20 mg/kg). Each point or bar and vertical line represent the mean ± SEM of the values obtained in 8 – 10 animals. **A**: Statistically significant differences between the values obtained in solvent- and BD-1063-injected mice: ** $p < 0.01$ (one-way ANOVA followed by Bonferroni test); and between the values obtained with the respective doses of BD-1063 associated or not with PRE-084: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (two way ANOVA followed by Bonferroni test). **B**: Statistically significant differences between the values obtained in solvent- (open column) - and drug-injected mice: ** $p < 0.01$; and between the values obtained in mice given BD-1063 associated with PRE-084 with respect to BD-1063 associated with PRE-084 solvent: $^{\#\#}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

This dose of PRE 084 (32 mg/kg, s.c.) was chosen for co-administration with the other σ_1 antagonists tested here, and we found that the antiallodynic effects induced by BD-1047 (32 mg/kg, s.c.) and NE-100 (64 mg/kg, s.c.) were also markedly reduced by the σ_1 agonist, yielding paw withdrawal latency times close to that obtained in solvent-treated animals (Fig. 6C).

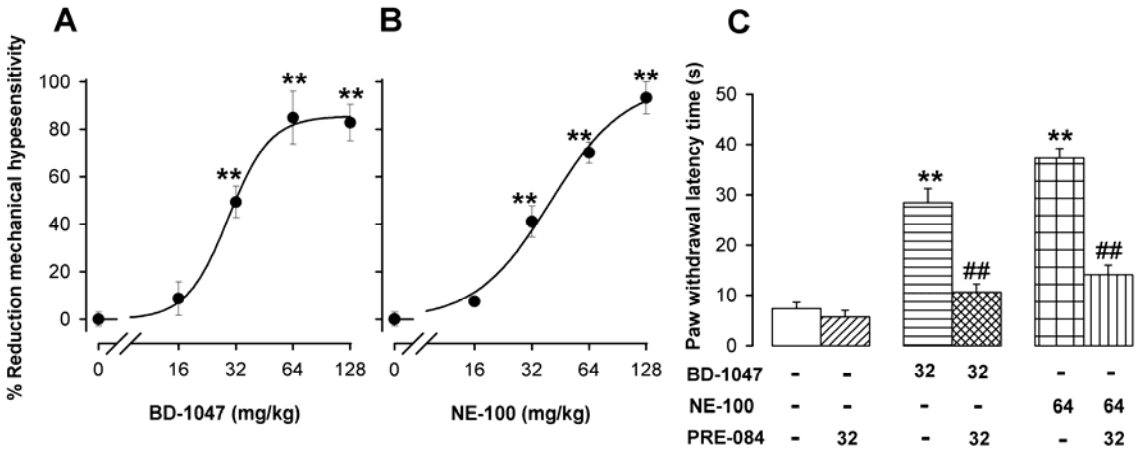

Figure. 6. Effects of the s.c. administration of BD-1047 or NE-100 on mechanical hypersensitivity induced by the i.pl. injection of capsaicin (1 µg) to the hind paw of wild-type mice. **A** and **B**: dose–response curves of the mechanical antiallodynic effects of BD-1047 (16 – 128 mg/kg) and NE-100 (16 – 128 mg/kg), respectively. **C**: Effects on paw withdrawal latency time of the association of PRE-084 (32 mg/kg, s.c.) with BD-1047 (32 mg/kg) or NE-100 (64 mg/kg). Each point or bar and vertical line represent the mean ± SEM of the values obtained in 8 – 10 animals. **A** and **B**: Statistically significant differences between the values obtained in solvent- and drug-injected mice: ** $p < 0.01$ (one-way ANOVA followed by Bonferroni test). (**C**) Statistically significant differences between the values obtained in solvent- and drug-injected mice: ** $p < 0.01$; and between the values obtained in mice given each drug (BD-1047 or NE-100) associated with PRE-084 with respect to each drug associated with PRE-084 solvent: $^{\#\#} p < 0.01$ (two-way ANOVA followed by Bonferroni test).

1.4.6. Comparison of the effects of σ_1 receptor antagonists and control drugs on mechanical allodynia and mechanical nociception.

Paw withdrawal latency time in capsaicin (1 μg)-treated wild-type animals stimulated with the filament at 0.5 g force reached values close to the cut-off time (50 s) after the s.c. administration of BD-1063 (32 mg/kg), BD-1047 (64 mg/kg) and NE-100 (128 mg/kg) (Fig. 7, left panel). As a control for the reliability of this model, we tested the effects on mechanical allodynia of pregabalin (64 mg/kg), mexiletine (32 mg/kg) and tetrodotoxin (6 μg/kg), which are non-σ_1 drugs (Fig. 4) with reported antiallodynic effects in humans (Dworkin et al., 2007) and experimental animals (Nieto et al., 2008). These drugs markedly increased paw withdrawal latency time in capsaicin-treated animals (Fig. 7, left panel), exerting a maximal antiallodynic effect in this surrogate model of neuropathic pain. Interestingly, their antiallodynic effect was not reversed by PRE-084 (32 mg/kg, s.c.) (data not shown).

To explore whether the drugs we tested had antinociceptive effects on mechanical punctate pain in nonsensitized mice, we applied the filament at 4 g force (noxious stimulus) in the paw of mice injected with 1% DMSO (capsaicin solvent). Importantly, in control animals the withdrawal latency time obtained after stimulation with 4 g force in nonsensitized (DMSO-treated) animals was similar to that obtained in capsaicin (1 μg)-treated mice and stimulated at 0.5 g force (7.56 ± 0.89 s and 7.40 ± 1.26 s, respectively; Fig. 7). The three selective σ_1 antagonists BD-1063, BD-1047 and NE-100 (at the same doses that exhibited maximal antiallodynic effects) failed to affect paw withdrawal latencies in nonsensitized animals stimulated at 4 g force (Fig. 7, right panel); therefore, they had no antinociceptive effect in experiments with a noxious punctate mechanical stimulus. Interestingly, pregabalin (64 mg/kg) and tetrodotoxin (6

μg/kg) were also devoid of effect against a noxious punctate mechanical stimulus (Fig. 7, right panel). However, mexiletine (32 mg/kg) increased the paw withdrawal latency time markedly in these nonsensitized mice, inducing a clear antinociceptive effect (Fig. 7, right panel).

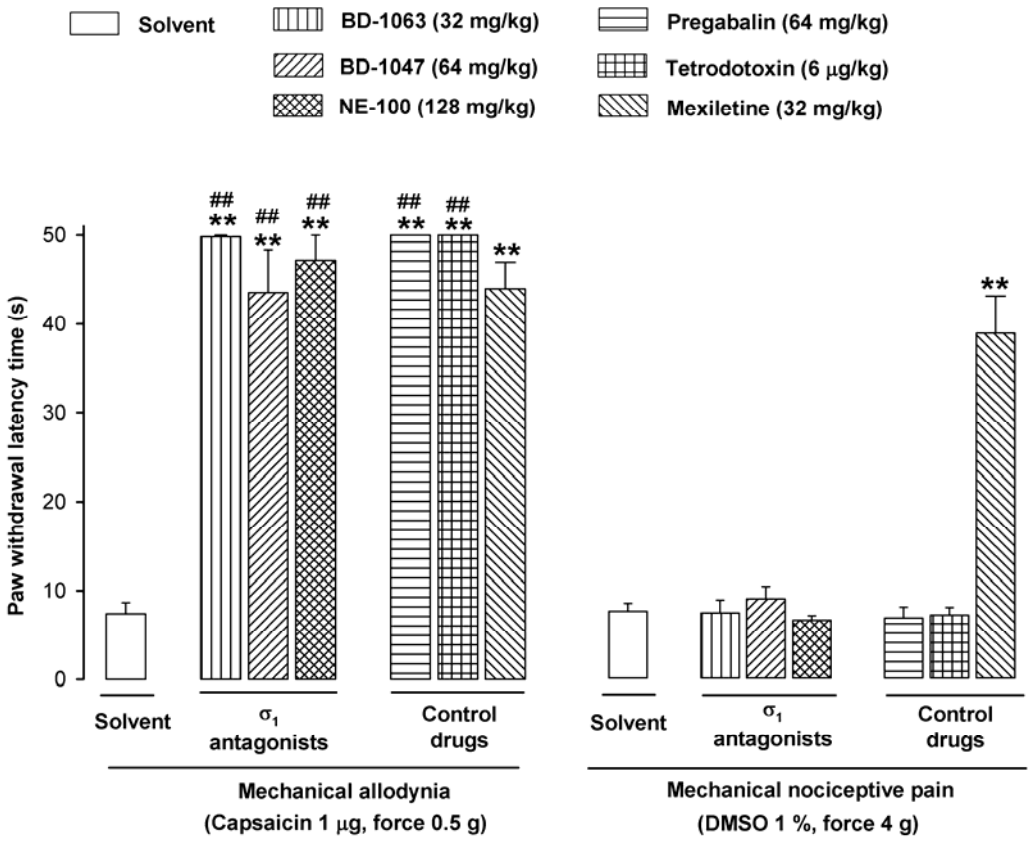

Figure. 7. Comparison of the effects of s.c. administration of σ_1 receptor selective antagonists and control drugs (non-σ_1 drugs used to treat neuropathic pain) on mechanical allodynia (left panel) and mechanical nociceptive pain (right panel). Paw withdrawal latency time was recorded in wild-type mice under two experimental conditions: capsaicin-treated paw stimulated at 0.5 (4.90 mN) g force (mechanical allodynia) and 1% DMSO-treated paw stimulated at 4 g (39.23 mN) force (mechanical pain). Each bar and vertical line represent the mean ± SEM of the values obtained in 6 – 8 animals. Statistically significant differences between the values obtained in drug- and solvent-treated mice under the same experiments conditions: ** $p < 0.01$; statistically significant differences between the effects of each drug tested under the two experimental conditions: $^{\#\#}$ $p< 0.01$ (two-way ANOVA followed by Bonferroni test).

1.5. DISCUSSION

In this study we found that selective antagonism of σ_1 receptors by genetic inactivation or drug treatment completely inhibited the mechanical hypersensitivity induced by capsaicin to a mechanical punctate stimulus, without modifying mechanical punctate nociceptive pain. These effects have not been reported previously.

Both PCR and [^{3}H](+)-pentazocine binding studies demonstrated the presence of the σ_1 receptor in wild-type mice and its inactivation in σ_1 receptor knockout animals. These results, obtained in CD-1 mice, were identical to those previously reported in chimeric (A129-C57BL/6J) animals (Langa et al., 2003). We found that nonsensitized (control) σ_1 receptor knockout mice responded to a wide range of mechanical punctate stimulus intensities (from innocuous to noxious) in a way similar to wild-type mice. Consequently, genetic inactivation of the σ_1 receptor did not affect normal mechanical stimulus perception or the motor response necessary to produce paw withdrawal. However, mutant mice were immune to sensitization by capsaicin: they did not show allodynia or hyperalgesia in response to a mechanical punctate stimulus when they were injected with capsaicin. Methodological limitations did not account for these results, since under our experimental conditions wild-type animals were sensitized to the mechanical punctate stimulus by capsaicin, as previously reported (Gilchrist et al., 1996; Joshi et al., 2006). Our results were not affected by gender differences because the responses of male and female CD-1 mice were almost identical in all the experiments performed. Capsaicin (1 μg, i.pl.) induced a similar mechanical hypersensitivity in both genders and the sensitization induced by capsaicin was

similarly inhibited in male and female mice by genetic inactivation of the σ_1 receptor or by s.c. administration of the σ_1 receptor antagonist BD-1063.

In addition, we found that capsaicin induced a dose-dependent (ED_{50} = 0.34 ± 0.015 μg) mechanical sensitization in wild-type C57BL/6J mice, which was almost completely abolished in σ_1 receptor knockout C57BL/6J mice, similarly to CD-1 mice (see section 3.2. above). Therefore, our findings cannot be attributed to the genetic background of the mice used (either CD-1 or C57BL/6J), but to the inactivation of the σ_1 receptor gene.

The effects in σ_1 receptor knockout mice were mimicked in wild-type CD-1 animals treated with BD-1063, BD-1047 or NE-100, as all three drugs dose-dependently inhibited capsaicin-induced mechanical allodynia without modifying mechanical nociceptive pain. Several facts suggest that these drugs inhibited the sensitization induced by capsaicin through antagonism of σ_1 receptors. Firstly, the inhibition of capsaicin-induced mechanical hypersensitivity by these drugs was prevented by the coadministration of PRE-084, which is a known selective σ_1 agonist (Hayashi and Su, 2004). Inhibition of the antiallodynic effect of BD-1063 by PRE-084 was apparently competitive in nature, since PRE-084 shifted the dose–response curve of BD-1063 to the right without modifying its maximum antiallodynic efficacy. Moreover, all three σ_1 antagonists tested here are considered selective σ_1 ligands because of their ability to completely displace binding by [^{3}H](+)-pentazocine (widely considered a selective σ_1 radioligand), but not the binding of many other radioligands for other receptors (Okuyama et al., 1993; Chaki et al., 1994; Matsumoto et al., 1995). The presence of [^{3}H](+)-pentazocine binding sites different from the cloned σ_1 receptor and able to interact with the drugs used in this study in wild-type mice can be ruled out, because of

the absence of [^{3}H](+)-pentazocine binding in brain tissues of the σ_1 receptor knockout CD-1 mice used in the present study. Therefore, the similarities between the effects of capsaicin in σ_1 knockout and wild-type mice treated with σ_1 antagonists, together with the clear σ_1 pharmacology of these effects, strongly suggest that the inhibition of sensitization induced by capsaicin is a σ_1-mediated effect.

Intradermal capsaicin administration induces secondary mechanical hypersensitivity through a central mechanism (Sang et al., 1996; Baron, 2000) involving an activation of NMDA receptors with an increase in pNR1 (Zou et al., 2000), and the phosphorylation of extracellular signal-regulated kinase (pERK) (Kawasaki et al., 2004). Therefore, modulation of NMDA-mediated responses or phosphorylation of NR1 or ERK by σ_1 receptors could explain our results. Several facts indirectly support this hypothesis. Sigma-1 receptors play an important role in the modulation of glutamatergic NMDA-mediated responses (reviewed in Su and Hayashi, 2003; Bermack and Debonnel, 2005; Monnet and Maurice, 2006; Cobos et al., 2008). It has also been reported that the NMDA-induced increase in pNR1 is enhanced by σ_1 receptor agonists, and that this effect was reversed by the σ_1 antagonist BD-1047 (Kim et al., 2008). Moreover, σ_1 receptor antagonists inhibited both the enhancement of pNR1 in spinal cord dorsal horn neurons produced by formalin and the second phase of formalin-induced pain (Kim et al., 2006). Since the mechanisms underlying the second phase of the formalin test and the mechanical hypersensitivity produced by capsaicin are similar, involving an increase in pNR1 and pERK in spinal cord dorsal horn neurons (Zou et al., 2000; South et al., 2003; Choi et al., 2006), the antagonism of capsaicin-induced mechanical allodynia that we found in σ_1 knockout animals and after treatment with σ_1 receptor antagonists in wild-type animals may be due to a reduction in capsaicin-induced pNR1 or pERK in spinal

cord dorsal horn neurons. In this context it is interesting to note that σ_1 knockout animals did not show elevated levels of pERK1/2 in the spinal cord after sciatic nerve ligation, whereas wild-type mice did (Zamanillo et al., 2008). Obviously, further studies are needed to demonstrate the role of modulation of NMDA-receptor activation and levels of pNR1 and pERK in the inhibition of capsaicin-induced sensitization induced by σ_1 receptor antagonism.

We show that the absence of a functional σ_1 receptor in σ_1 knockout mice or its pharmacological blockade inhibits capsaicin-induced mechanical hypersensitivity but not mechanical nociceptive pain. This apparent absence of activity of σ_1 receptors in nonsensitized animals, and their activity in mice sensitized with capsaicin, is in agreement to the widely reported neuromodulatory role of σ_1 receptors in several biochemical and behavioral responses in which σ_1 ligands are often ineffective in naïve animals but highly active in states of pharmacological or pathological imbalance (reviewed in Hayashi and Su, 2004 and 2005; Monnet and Maurice, 2006; Cobos et al., 2008). This modulatory role of σ_1 receptors has also been studied in opioid-mediated analgesia. Sigma-1 antagonism does not affect tail-flick latency in naïve mice; however, it enhances the analgesic effects of opioid receptor agonists in this test (Cendán et al., 2005a, Chien and Pasternak, 1993; Chien and Pasternak, 1994; Marrazzo et al., 2006; Mei and Pasternak, 2002 and 2007). In light of these results, it was suggested that σ_1 receptors are tonically active in decreasing opioid analgesia (first proposed in Chien and Pasternak, 1993). The effectiveness of σ_1 antagonism in other behavioral models of pain such as formalin-induced pain (Cendán et al., 2005a and b, Kim et al., 2006), and in capsaicin-induced mechanical hypersensitivity (present study), may indicate that the hypothesis of a tonically active σ_1 system able to facilitate pain perception is not limited

to the modulation of opioid pathways, but might represent a wider mechanism of pain processing modulation.

Capsaicin-induced mechanical hypersensitivity is considered a surrogate model of some of the physiopathological mechanisms underlying neuropathic pain in humans (Baumgärtner et al., 2002; Klein et al., 2005). Moreover, the activity of drugs against capsaicin-induced mechanical hypersensitivity is considered potentially predictive of their action against neuropathic pain (e.g., Park et al., 1995; Gottrup et al., 2004; Joshi et al., 2006). We therefore compared the effect of σ_1 receptor antagonists to that of several antineuropathic drugs, and found that both pregabalin and tetrodotoxin inhibited capsaicin-induced mechanical allodynia without modifying mechanical nociceptive pain. These effects were expected since previous studies reported that both drugs inhibited mechanical allodynia in models of neuropathic pain (Han et al., 2007; Nieto et al., 2008) at doses that did not exert antinociceptive effects in control animals (Field et al., 1997; Nieto et al., 2008). Interestingly, gabapentin (an analogue of pregabalin), also showed a similar profile, inhibiting mechanical allodynia induced by capsaicin or neuropathy, but not mechanical nociceptive pain (Field et al., 1997; Joshi et al., 2006). We also found that mexiletine, an orally active congener of lidocaine used to treat neuropathic pain (Dworkin et al., 2007), inhibited both capsaicin-induced mechanical allodynia and mechanical nociceptive pain in nonsensitized animals. Another study also showed that mexiletine was almost equipotent in inhibiting mechanical allodynia and mechanical nociceptive pain (noxious pinch) (Khandwala et al., 1997).

Interestingly, the antineuropathic drugs pregabalin, mexiletine and tetrodotoxin inhibited capsaicin-induced mechanical hypersensitivity, but were devoid of affinity for σ_1 receptors and their antiallodynic effects were not reversed by PRE-084. These

findings suggest that the σ_1 system is not involved in the ability of these drugs to inhibit sensitization induced by capsaicin. Therefore, σ_1 receptor blockade may be a novel pharmacological target against mechanical hypersensitivity not shared by other known antineuropathic drugs. In fact, it was recently reported that the blockade of σ_1 receptors inhibited mechanical and cold allodynia in an experimental model of neuropathic pain (Zamanillo et al., 2008).

In summary, we found that σ_1 receptor antagonism (produced pharmacologically or through genetic inactivation) inhibited capsaicin-induced mechanical hypersensitivity but not mechanical nociceptive pain. These findings suggest that σ_1 receptors can facilitate, through a tonically active mechanism, the capsaicin-induced amplification of the pain signaling system. Further studies to evaluate the role of σ_1 receptors in other models of pain that activate central sensitization processes may yield findings of relevance to treat pathological pain.

1.6. REFERENCES

All references indicated in all sections of this manuscript are listed in the section *Bibliography*

2. Antagonism by haloperidol and its metabolites of mechanical hypersensitivity induced by intraplantar capsaicin in mice: role of sigma-1 receptors

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Informative title: Antiallodynic effects of haloperidol and its metabolites

2.1. ABSTRACT

We evaluated the effects of haloperidol and its metabolites on capsaicin induced mechanical hypersensitivity (allodynia) and on nociceptive pain induced by punctate mechanical stimuli in mice. Subcutaneous administration of haloperidol or its metabolites I or II (reduced haloperidol) dose-dependently reversed capsaicin-induced (1 μg, intraplantar) mechanical hypersensitivity of the hind paw (stimulated with a nonpainful, 0.5 g force, punctate stimulus). The order of potency of these drugs to induce antiallodynic effects was the order of their affinity for brain sigma-1 (σ_1) receptor ([^{3}H](+)-pentazocine-labeled). Antiallodynic activity of haloperidol and its metabolites was dose-dependently prevented by the selective σ_1 receptor agonist PRE-084, but not by naloxone. These results suggest involvement of σ_1 receptors, but discard any role of the endogenous opioid system, on the antiallodynic effects. Dopamine receptor antagonism also appears unlikely to be involved in these effects, since the D_2/ D_3 receptor antagonist (-)-sulpiride, which had no affinity for σ_1 receptors, showed no antiallodynic effect. None of these drugs modified hind-paw withdrawal after a painful (4 g force) punctate mechanical stimulus in non-capsaicin-sensitized animals. As expected, the control drug gabapentin showed antiallodynic but not antinociceptive activity, whereas clonidine exhibited both activities and rofecoxib, used as negative control, showed neither. These results show that haloperidol and its metabolites I and II produce antiallodynic but not antinociceptive effects against punctate mechanical stimuli, and suggest that their antiallodynic effect may be due to blockade of σ_1 receptors but not to dopamine receptor antagonism.

2.2. INTRODUCTION

Sigma (σ) receptors, initially considered a subtype of opioid receptors and later confused with phencyclidine binding sites in NMDA (*N*-methyl-*D*-aspartate) receptors, are now described as a distinct pharmacological entity (Guitart et al., 2004; Monnet and Maurice, 2006, for reviews). Two subtypes (σ_1 and σ_2) have been pharmacologically characterized. The σ_1 receptor, cloned in several animal species and humans, has been described as a unique protein with no homology with known mammalian proteins (Guitart et al., 2004; Monnet and Maurice, 2006). Drug binding to σ_1 receptors is allosterically modulated by phenytoin (Quirion et al., 1992), and testing for this modulation has been proposed as a method to discriminate between σ_1 receptor agonists and antagonists *in vitro* (Cobos et al., 2005, 2006). The pharmacology of σ_1 receptors is now well characterized, and selective agonists, such as (+)-pentazocine and PRE-084 [2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride], and antagonists, such as BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) and NE-100 (*N*, *N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine hydrochloride), are both available (Guitart et al., 2004; Hayashi and Su, 2004). Some neurosteroids, psychostimulants, and antipsychotics also bind to σ_1 receptors (Maurice et al., 2001c; Monnet and Maurice, 2006; Cobos et al., 2008a). Among the antipsychotics, haloperidol (HP) is mainly known as a D_2 receptor antagonist, although it shows the same affinity for D_2 and σ_1 receptors (Bowen et al., 1990; Matsumoto and Pouw, 2000) and exhibits σ_1 receptor antagonistic activity (Maurice et al., 2001c; Hayashi and Su, 2004). Two major metabolic pathways for HP have been identified in experimental animals and humans (see Cobos et al., 2007 for

references). One is a reversible reductive pathway that produces HP metabolite II (HP-Met-II), also called reduced HP [4-(4-chlorophenyl)-α-(4-fluorophenyl)-4-hydroxy-1-piperidinebutanol]. The other is an oxidative *N*-dealkylation pathway that leads to HP metabolites I (HP-Met-I, 4-(4-chlorophenyl)-4-hydroxypiperidine) and III (HP-Met-III, *p*-fluorobenzoylpropionic acid). Studies performed in rodent brain membranes and human neuroblastoma cells showed that metabolites I and II of HP bind to σ_1 receptors with less affinity than HP, but show much lower (HP-Met-II) or no affinity (metabolite I) for D_2 receptors, whereas metabolite III has no affinity for either σ_1 or D_2 receptors (Bowen et al., 1990; Matsumoto and Pouw, 2000; Cobos et al., 2007).

Sigma-1 receptors are involved in nociception, among other processes. They are distributed in the central nervous system in areas of great importance for pain control, such as the superficial layers of the spinal cord dorsal horn, the periaqueductal gray matter, the locus coeruleus, and rostroventral medulla (Alonso et al., 2000; Kitaichi et al., 2000). Functional studies have postulated that an endogenous σ_1 system tonically modulates the opioid system. The antinociception induced by agonists of opioid receptors in the tail-flick test is antagonized by systemic administration of the selective σ_1 agonist (+)-pentazocine, whereas it is enhanced by the σ_1 antagonist HP (Chien and Pasternak, 1993, 1994; Mei and Pasternak, 2002; Mei and Pasternak, 2007). New σ_1 ligands such as the σ_1 antagonist (+)-MR 200 [(+)-methyl (1R,2S)-2-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate] and the proposed σ_1 agonist (+/-)-PPCC [(1R,2S/1S,2R)-2-[4-hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl) cyclopropanecarboxylate] also modulate opioid receptor agonist-induced antinociception (Marrazzo et al., 2006; Prezzavento et al., 2008). Sigma ligands are also able to modulate nociception *per se* (i.e., not associated to opioid agonists). Selective σ_1

agonists induce nociception when used alone in the nociceptive flexor response test, and the effects of (+)-pentazocine are reversed by selective σ_1 receptor antagonists (Ueda et al., 2001). Moreover, both phases of pain behavior in the formalin test are diminished in σ_1 receptor knockout mice (Cendán et al., 2005a) and after the systemic administration of the σ_1 receptor antagonists HP and reduced HP (Cendán et al., 2005b). Pain behavior in the second phase of the formalin test is also reduced after intrathecal administration of the σ_1 receptor antagonists BD-1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino ethylamine dihydrobromide) and BMY-14802 (α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazinebutanol) (Kim et al., 2006). However, the possible role of σ_1 receptors in mechanical stimulus-induced pain is unknown.

The intradermal injection of capsaicin induces an immediate pain behaviorresponse followed by longer-lasting secondary mechanical hypersensitivity (Gilchrist et al. 1996; Joshi et al., 2006). The mechanisms underlying the mechanical hypersensitivity (allodynia) produced by the intradermal injection of capsaicin and the second phase of the formalin test are comparable, involving a phenomenon of central sensitization produced and maintained mainly by NMDA receptor stimulation (South et al., 2003; Zou et al., 2000; Soliman et al., 2005). Sigma-1 receptors play an important modulatory role in NMDA receptor activity (Debonnell and de Montigny, 1996; Kim et al., 2006) and even modulate acute pain induced by NMDA (Kim et al., 2008). Therefore, we hypothesized that σ_1 receptor ligands might be able to modify the mechanical allodynia induced by capsaicin.

The main aim of this study was to evaluate the effects of the sigma ligands HP and its metabolites on mechanical hypersensitivity induced by the intraplantar injection of capsaicin, and to determine whether these effects are due to their antagonism of σ_1

receptors. To this end, we correlated the effect of drugs in behavioral tests with their affinity for brain σ_1 receptors labeled with [^{3}H](+)-pentazocine and attempted to prevent the effects of HP and its metabolites by administering the prototype σ_1 receptor agonist PRE-084 (Su et al., 1991; Cobos et al., 2008b). To control for the influence of dopaminergic antagonism on the effects of interest, we evaluated the effect of (-)-sulpiride, a D_2 and D_3 receptor antagonist devoid of activity on σ_1 receptors (Freedman et al., 1994; Matsumoto and Pouw, 2000). Involvement of endogenous opioid system modulation in the antiallodynic effect of HP and its metabolites was tested by evaluating the possible antagonism of this effect by the opioid receptor antagonist naloxone. We also tested the effect of HP and its metabolites on pain induced by mechanical punctate stimuli in animals not sensitized with capsaicin. The activity of these drugs was compared with that of control drugs (gabapentin, clonidine and rofecoxib) with known effects on capsaicin-induced mechanical hypersensitivity or mechanical pain. Finally, we used the rotarod test to explore the possible role of motor incoordination in the effects of the drugs tested.

2.3. MATERIAL AND METHODS

2.3.1. Experimental animals

Female CD-1 mice (Charles River, Barcelona, Spain) weighing 25 – 30 g were used for all experiments. The animals were housed in a temperature-controlled room (21 ± 1 °C) with air exchange every 20 min and an automatic 12-h light/dark cycle (08.00 to 20.00 h). They were fed a standard laboratory diet and tap water *ad libitum* until the beginning of the experiments. The experiments were performed during the light phase (09.00 – 15.00 h). Naive animals were used throughout the study. Mice were always

handled in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC). The experimental protocol was approved by the Research Ethics Committee of the University of Granada, Spain.

2.3.2. Drugs and drug administration

The σ ligands used (and their suppliers) were the nonselective σ_1 antagonists haloperidol (HP), haloperidol metabolite I [HP-Met-I, 4-(4-chlorophenyl)-4-hydroxypiperidine], haloperidol metabolite II [HP-Met-II, 4-(4-chlorophenyl)-α-(4-fluorophenyl)-4-hydroxy-1-piperidinebutanol] and haloperidol metabolite III (HP-Met-III, *p*-fluorobenzoylpropionic acid) (all from Sigma-Aldrich Química S.A., Madrid, Spain), as well as the selective σ_1 antagonist BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) and the selective σ_1 agonist PRE-084 [2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride] (both from Tocris Cookson Ltd., Bristol, United Kingdom). As a control of dopaminergic antagonism we used the D_2 and D_3 antagonist (-)-sulpiride (Sigma-Aldrich Química S.A., Madrid, Spain). We also used naloxone hydrochloride (Sigma-Aldrich Química S.A.) and morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health) to evaluate the involvement of opioidergic system modulation in the antiallodynic effect of HP and its metabolites. Three additional drugs (all from Sigma-Aldrich Química S.A.) were used as controls in the behavioral assays: a) clonidine, which has antinociceptive and antiallodynic effects (Paqueron et al., 2003); b) gabapentin, which has antiallodynic but not antinociceptive effects (Joshi et al., 2006, Tanabe et al., 2005); and c) rofecoxib, an antiinflammatory drug (Moore et al., 2005),

which is devoid of antinociceptive and antiallodynic activity in animals without inflammation (Bingham et al., 2005; Padi and Kulkarni, 2004).

The radioligand used for binding assays was [^{3}H](+)-pentazocine with a specific activity of 33.6 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 HCl-Tris buffer pH 8 at 30 ºC). Haloperidol, HP metabolites I, II, III, and (-)-sulpiride were dissolved in absolute ethanol to make up a stock solution of 1 mM, from which further dilutions were prepared with incubation buffer to yield a final maximal concentration of ethanol in the incubation medium of 1% (vol/vol). We previously verified that this final concentration of ethanol did not affect the binding of [^{3}H](+)-pentazocine. The other cold drugs (PRE-084, gabapentin, clonidine, and rofecoxib) used in competition binding assays were dissolved in deionized ultrapure water.

The drugs were suspended in 5% gum arabic (Sigma-Aldrich Química S.A.) in water for *in vivo* assays, and all were injected subcutaneously (s.c.) into the interscapular region. An equal volume of vehicle was used in control animals. When PRE-084 was used to reverse the effects of HP or its metabolites, it was s.c. injected immediately before the other drug solution. Each injection was performed in different areas to avoid mixture of the drug solutions and any interference with results due to physicochemical interaction. The chemical algogen used was capsaicin (Sigma-Aldrich Química S.A.), which was dissolved in 1% dimethylsulfoxide (DMSO, Merck KGaA, Darnstadt, Germany) in physiological sterile saline to a concentration of 0.05 µg/µl. Capsaicin solution was injected intraplantarly (i.pl.) into the right-hind paw in a volume of 20 µl, using a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain)

with a $30^{1/2}$-gauge needle. Control animals were injected with the same volume of capsaicin solvent (DMSO 1% in saline).

2.3.3. Mice brain membrane preparations

Crude synaptosomal membranes (P_2 fraction) were prepared for [^{3}H](+)-pentazocine binding as previously described (Cobos et al., 2006) with slight modifications. Mice were killed by cervical dislocation and the brain was rapidly removed and homogenized in 15 volumes (wt/vol) of 0.32 M sucrose-10 mM Tris HCl, pH 7.4, with a Polytron homogenizer (model PT10-35, Kinematica AG, Basel, Switzerland). The homogenates were centrifuged (Avanti 30, Beckman Coulter España S.A., Madrid, Spain) at 1000 *g* for 13 min, the resulting pellets were discarded, and the supernatants were centrifuged at 21 000 *g* for 15 min to obtain the P_2 pellets; each pellet, obtained from two whole brains, was resuspended in 15 ml 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 (protein concentration 12 – 14 mg/ml) at –80 °C. Binding characteristics of the tissue were stable for at least 1 month when stored at –80 °C. Protein concentrations were measured by the method of Lowry et al. (1951) with some modifications, using bovine serum albumin as the standard.

2.3.4. [^{3}H](+)-Pentazocine binding assays

To test the affinities of drugs for mice brain σ_1 receptors, we performed [^{3}H](+)-pentazocine competition binding assays. Aliquots of mice brain membranes were slowly thawed and resuspended in fresh incubation buffer and [^{3}H](+)-pentazocine

binding assays were performed as previously described (Cobos et al., 2007) with slight modifications. Resuspended membrane preparations (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 with 20 µl [^{3}H](+)-pentazocine (final concentration of 5 nM) for 240 min at 30 °C, pH 8. Nonspecific binding was defined as the binding retained in the presence of HP 10 µM, and was always less that 20% of the total binding.

To stop [^{3}H](+)-pentazocine binding to the mouse brain membranes, 5 ml ice-cold filtration buffer (Tris 10 mM pH 7.4) was added to the tubes. 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) over Whatman GF/B glass fiber filters (SEMAT Technical Ltd., St. Albans, Hertfordshire, UK) presoaked with 0.5% polyethylenimine in Tris 10 mM, pH 7.4, for at least 1 h prior to use, to reduce nonspecific binding. The filters were washed under a vacuum twice with 5-ml volumes of the ice-cold filtration buffer, and transferred to scintillation counting vials. Then 4 ml liquid scintillation cocktail (CytoScint scintillation counting solution, MP Biomedicals, Irvine, CA, USA) was added to each vial and the mixture was equilibrated for at least 20 h. The radioactivity retained in the filter was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A.), with an efficiency of 52%. Each assay was conducted in triplicate.

2.3.5. Evaluation of mechanical punctate nociceptive pain and capsaicin-induced mechanical hypersensitivity

Animals were placed in the experimental room (under low illumination) to allow them to acclimatize to the study room for 1 h before the experiments were begun. After that time the animals were placed into individual test compartments for 2 h before the test to habituate them to the test conditions. The test compartments had black walls and were situated on an elevated mesh-bottomed platform with a 0.5-cm^2 grid to provide access to the ventral surface of the hind paws. After this period, the animals were carefully removed from the compartment, injected i.pl. with 1 μg capsaicin (or its solvent) in the right-hind paw proximate to the heel, and immediately returned to the compartment. In all experiments, punctate mechanical stimulation was applied with a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) at 15 min after the administration of capsaicin (time to maximum effect, data not shown) or its solvent. Briefly, a nonflexible filament (0.5 mm diameter) was electronically driven into the ventral side of the paw previously injected with capsaicin or solvent (i.e., the right-hind paw), at least 5 mm away from the site of the injection towards the fingers. When a paw withdrawal response occurred, the stimulus was automatically terminated and the response latency time was automatically recorded. A cut-off time of 50 s was used. In all experiments, the filament was applied to the right-hind paw of each mouse three times, separated by intervals of 0.5 min, and the mean value of the three trials was considered the withdrawal latency time of the animal.

Responses to mechanical stimuli were compared between control (DMSO treated) and capsaicin-sensitized mice by applying the filament at a wide range of electronically controlled intensities from 0.05 to 8 g force, recording the paw withdrawal latency time

for each force applied as described above. Each animal was tested using only one intensity of mechanical stimulation in order to maintain a strictly constant time (15 min) between administration of capsaicin or its solvent and the behavioral evaluation. As expected, shorter withdrawal latency times were obtained as higher forces were applied (see Fig. 2 and Results section). This approach allowed us to construct a force-response curve (i.e., intensity of the stimulus vs. paw withdrawal latency time) and to quantify the degree of mechanical punctate nociceptive pain in DMSO-treated animals and the mechanical sensitization in capsaicin-treated animals.

When the effects of drugs were tested, the drug under study (or its solvent) was administered s.c. 30 min before the i.pl. administration of capsaicin or DMSO 1% (i.e., 45 min before we evaluated the response to the mechanical punctate stimulus). The antinociceptive effects of drugs were assessed in DMSO-treated animals using a mechanical stimulation of 4 g force, which induced a marked reduction in paw withdrawal latency time in these nonsensitized mice (see Fig. 2 and Results for details). The antiallodynic effects of the drugs were evaluated in capsaicin-sensitized mice using a mechanical stimulation of 0.5 g force. This intensity of the mechanical stimulus did not induce paw withdrawal in DMSO-treated mice, but markedly reduced paw withdrawal latency time in capsaicin-sensitized mice. We chose these forces because the latency time in capsaicin-sensitized animals stimulated with 0.5 g was similar to that in nonsensitized animals (DMSO-treated) stimulated at 4 g force (see Figs. 2 and 6).

2.3.6. Rotarod test

Changes in motor coordination were tested with an accelerating rotarod (Cibertec, Madrid, Spain) as previously described (Nieto et al., 2008), with slight modifications.

Briefly, mice were required to walk against the motion of an elevated rotating drum at speeds increasing from 4 to 40 revolutions/min over a 300-s period. The latency to fall-down was recorded automatically, with a cut-off time of 300 s. Twenty-four hours prior to each experiment with drugs, mice were acclimatized to the apparatus in 3 training sessions on the rotarod separated by 30-min intervals. On the day of the test, rotarod latencies were measured immediately before (time 0) and 45 min after the drug or vehicle was given. This time was chosen because it was the time used to test the effects of drugs on mechanical hypersensitivity.

2.3.7. Data analysis

We estimated the IC_{50} (concentration of unlabeled drug that inhibited 50% of [^{3}H](+)-pentazocine specific binding) values and their standard errors from the inhibition curves with nonlinear regression analysis of the equation for a sigmoid plot, assuming one-site competition; the SigmaPlot v. 8.0 (2002) program was used for all estimates. The EF_{50} values (force of mechanical stimulus applied that produced half the maximal reduction in paw withdrawal latency time) were calculated from the force-response curves using nonlinear regression analysis of the equation for a sigmoid plot. The degree of effect on capsaicin-induced mechanical hypersensitivity was calculated as: % reduction mechanical hypersensitivity = [(LTD – LTS) / (CT – LTS)] × 100, where LTD is the latency time for paw withdrawal in drug-treated animals, LTS is the latency time in solvent-treated animals, and CT is the cut-off time (50 s). The ED_{50} (dose of drug that produced half the maximal inhibition of mechanical allodynia) and E_{max} values (maximum antiallodynic effect) were calculated from the dose-response curves using nonlinear regression analysis of the equation for a sigmoid plot. The EF_{50},

ED_{50} and E_{max} values obtained from sigmoid plots and their standard errors were calculated as the best-fit values ± standard errors of regression with the SigmaPlot v. 8.0 (2002) program.

The values obtained in several experimental groups were compared with one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test. Differences between two means were assessed by the Student's *t*-test. The differences were considered significant when the value of P was below 0.05.

2.4. RESULTS

2.4.1. Affinity of drugs for [^{3}H](+)-pentazocine binding sites in the mouse brain

We used competition binding assays to test the affinities of the drugs under study for the σ_1 receptor, labeled with [^{3}H](+)-pentazocine, in mouse brain membranes (P_2 fraction). Specific binding of [^{3}H](+)-pentazocine (which always represented more than 80% of the total binding) was concentration-dependently inhibited by the unlabeled ligands with the following order of potency (IC_{50} values): HP (5.45 ± 0.48 nM) > HP-Met-II (12.80 ± 0.69 nM) > PRE-084 (171.40 ± 18.36 nM) > HP-Met-I (254.18 ± 18.40 nM) >>> HP-Met-III or (-)-sulpiride (the last two compounds had negligible affinity for [^{3}H](+)-pentazocine binding sites, with IC_{50} > 10 000 nM) (Table 1 and Fig. 1). The drugs used as controls in the behavioral tests (gabapentin, clonidine, and rofecoxib) also did not substantially decrease [^{3}H](+)-pentazocine binding, showing IC_{50} values higher than 10 000 nM (data not shown).

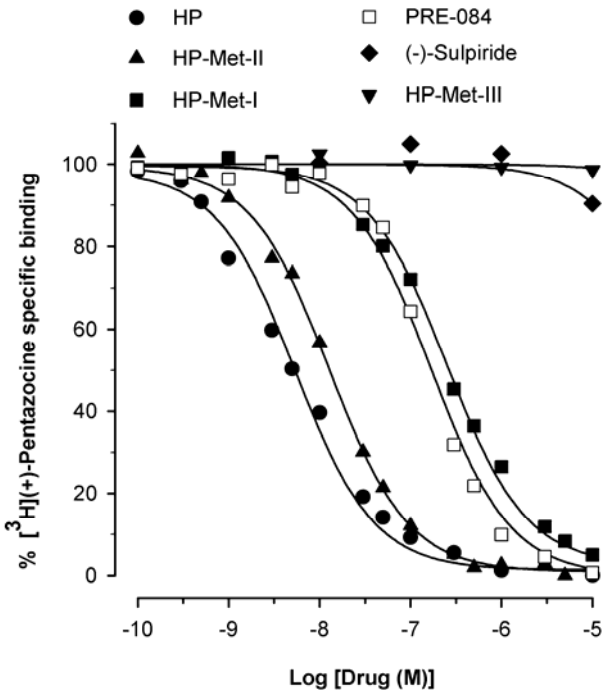

Figure 1. Inhibition by unlabeled drugs of [^{3}H](+)-pentazocine specific binding to membranes (P_2 fraction) obtained from whole mouse brain. [^{3}H](+)-pentazocine (5 nM) was incubated with 0.8 mg/ml brain membrane protein at 30 ºC, pH 8, for 240 min and increasing concentrations of haloperidol (HP, ●), haloperidol metabolite I (HP-Met-I, ■), haloperidol metabolite II (HP-Met-II,▲), haloperidol metabolite III (HP-Met-III, ▼), PRE-084 (□) or (-)-sulpiride (♦). Data shown are the average of two experiments carried out in triplicate.

2.4.2. Evaluation of mechanical punctate nociceptive pain and capsaicin-induced mechanical hypersensitivity

We applied the filament at different intensities ranging from innocuous to noxious stimuli (0.05 – 8 g force) in mice treated with capsaicin (1 µg, i.pl.) or its solvent. As expected, paw withdrawal latency time decreased as the force applied increased in both experimental situations (Fig. 2). Forces higher than 1 g were needed to induce paw withdrawal in nonsensitized (DMSO-treated) mice, and the shortest withdrawal latency time was obtained at 8 g force (Fig. 2). In contrast, a weak mechanical stimulus (0.2 g) was sufficient to induce paw withdrawal in capsaicin-sensitized mice, and withdrawal latency time was minimal at forces as low as 0.5 – 1 g (Fig. 2). Treatment with capsaicin produced a 9.4-fold decrease in the EF_{50} value for inducing paw withdrawal in comparison to control mice (EF_{50} values of 2.59 ± 0.08 g and 0.30 ± 0.02 g for DMSO-

and capsaicin-treated animals, respectively), which clearly indicates that capsaicin induced mechanical hypersensitivity to punctate stimuli.

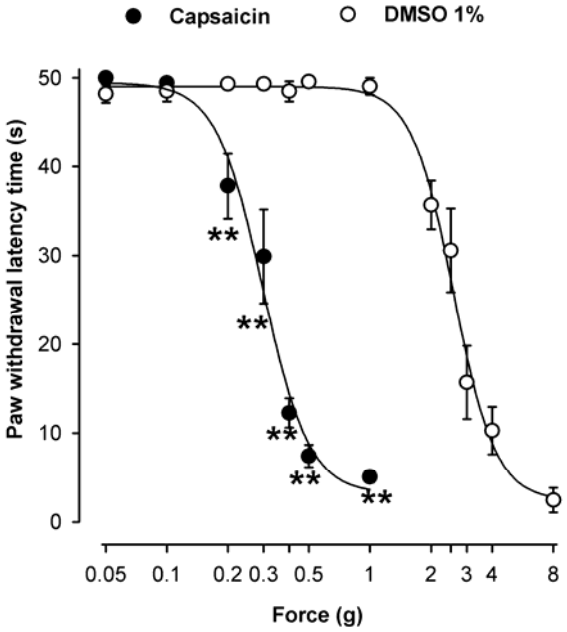

Figure 2. Withdrawal latency time of the hind paw stimulated with a filament at different forces, 15 min after the intraplantar injection of capsaicin (1 μg, ●) or its solvent (DMSO 1%, ○). Each point and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (n = 6 – 8 per group). Each group was tested with only one stimulation force. Statistically significant differences between the latency time values obtained with each applied force under the two experimental conditions: ** P < 0.01 (two-way ANOVA followed by Bonferroni test).

2.4.3. Effect of haloperidol and its metabolites on capsaicin-induced mechanical hypersensitivity

The effects of s.c. administration of drugs was investigated as the change in mechanical hypersensitivity induced by intraplantar capsaicin. Injection of the σ_1 ligands HP (0.004 – 0.25 mg/kg), HP-Met-II (0.04 – 2 mg/kg) and HP-Met-I (8 – 128 mg/kg) induced a dose-dependent increase in withdrawal latency in the capsaicin-injected hind paw when stimulated with a filament at 0.5 g force, i.e., they produced antiallodynic effects (Fig. 3). The order of potency in these effects (ED_{50} values) was: HP (0.026 ± 0.006 mg/kg) > HP-Met-II (0.135 ± 0.03 mg/kg) >> HP-Met-I (31.05 ±

7.83 mg/kg), the same as the order of potency of these compounds for displacing [^{3}H](+)-pentazocine binding in mouse brain membranes (Table 1).

The maximum effect (E_{max}) for each drug was calculated by regression analysis. Haloperidol and its metabolite II produced the maximum possible effect in this model (E_{max} = 100 %); however, the E_{max} for HP-Met-I was lower (62.27 ± 6.99 %).

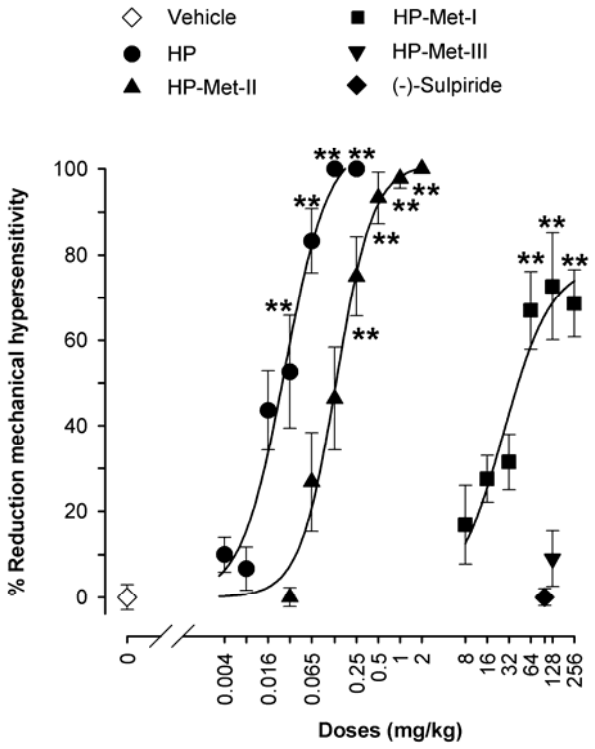

Figure 3. Effects of different doses of subcutaneously administered haloperidol (HP, ●), haloperidol metabolite II (HP-Met-II, ▲), haloperidol metabolite I (HP-Met-I, ■), haloperidol metabolite III (HP-Met-III, ▼), (-)-sulpiride (♦) or their solvent (◇) on mechanical hypersensitivity (allodynia) induced by intraplantar injection of capsaicin (1 μg) to mouse hind paw. The results represent the percentage reduction in capsaicin-induced mechanical hypersensitivity (calculated as explained in Methods). Each point and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (n = 8–10 per group). Each group was treated with only one dose of drug or solvent. Statistically significant differences between the values obtained in solvent- and drug-injected groups: ** $P < 0.01$ (one-way ANOVA followed by Bonferroni test).

Interestingly, HP-Met-III and (-)-sulpiride, which had no affinity for [^{3}H](+)-pentazocine-labeled σ_1 receptor (Fig. 1; Table 1), showed no antiallodynic effects even at very high doses (128 and 100 mg/kg, s.c., respectively) (Fig. 3; Table 1).

Table 1. Potencies of several drugs in inhibiting the specific binding of [^{3}H](+)-pentazocine to mouse brain membranes and mechanical hypersensitivity (allodynia) induced by intraplantar capsaicin in mice.

	Drug	IC_{50} (nM)	ED_{50} (mg/kg, s.c)
Sigma-1 antagonists	Haloperidol	5.45 ± 0.48	0.026 ± 0.006
	Haloperidol metabolite II	12.80 ± 0.69	0.135 ± 0.030
	Haloperidol metabolite I	254.18 ± 18.40	31.05 ± 7.83
Sigma-1 agonist	PRE-084	171.40 ± 18.36	Inactive[a]
Non-sigma-1 ligands	Haloperidol metabolite III	> 10 000	Inactive[a]
	(-)-Sulpiride	> 10 000	Inactive[a]

The IC_{50} values (concentration of unlabeled drug that inhibited the specific binding of [^{3}H](+)-pentazocine by 50%) were obtained with competition assays performed in mouse brain membranes (P_2 fraction). The ED_{50} values (dose of drug producing half of the maximal antiallodynic effect) were obtained from dose-response curves of the drug's effects on withdrawal response latency time of the capsaicin-sensitized paw after stimulation with a punctate mechanical stimulus at 0.5 g force. See Methods for details.

[a] The effect of PRE-084 was tested at 32-64 mg/kg, s.c. The dose of haloperidol metabolite III was 128 mg/kg, s.c., and the dose of (-)-sulpiride was 100 mg/kg, s.c.

2.4.4. Reversion of the antiallodynic effects of haloperidol and its metabolites by the selective σ_1 receptor agonist PRE-084

The known σ_1 receptor agonist PRE-084 did not modify paw withdrawal latency in capsaicin-treated animals at either dose (32 or 64 mg/kg s.c.) (Fig. 4; Table 1). However, PRE-084 (16 – 64 mg/kg, s.c.) produced a dose-dependent reversion of the antiallodynic effect of HP (0.06 mg/kg, s.c.) (Fig. 4). The highest dose of PRE-084 abolished the effect of HP, and paw withdrawal latency was similar to that in animals

treated with the drug solvent (Fig. 4). PRE-084 (32 – 64 mg/kg, s.c.) also completely and dose-dependently prevented the antiallodynic effects of HP-Met- II (0.25 mg/kg, s.c.) and HP-Met-I (64 mg/kg, s.c.) (Fig. 4). As a control, we evaluated the ability of PRE-084 to reverse the effect of BD-1063, a selective antagonist of σ_1 receptors (Matsumoto et al., 1995; Hayashi and Su, 2004). We found that BD-1063 (20 mg/kg, s.c.) produced an increase in paw withdrawal latency similar to that induced by HP and its metabolites I and II, and that the antiallodynic effect of BD-1063 was completely antagonized by PRE-084 (32 mg/kg, s.c.) (Fig. 4).

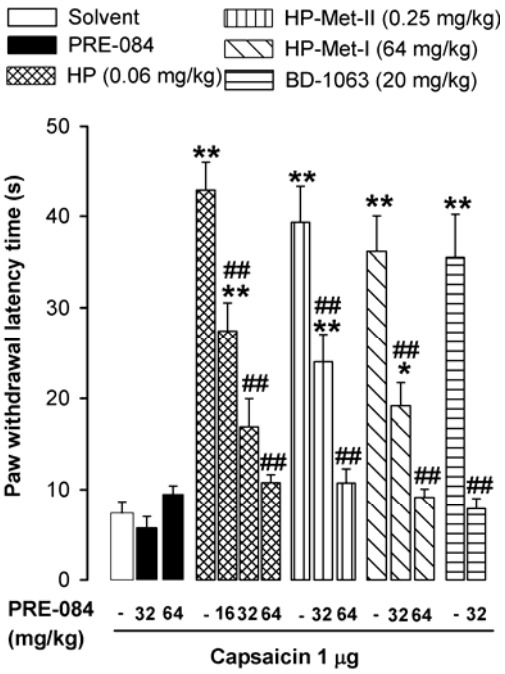

Figure 4. Effects of the association of subcutaneous administration of PRE-084 (16-64 mg/kg) or its solvent with haloperidol (HP, 0.06 mg/kg), haloperidol metabolite II (HP-Met-II, 0.25 mg/kg), haloperidol metabolite I (HP-Met-I, 64 mg/kg), BD-1063 (20 mg/kg) or their solvent (all injected s.c.) on mechanical hypersensitivity induced by the intraplantar injection of capsaicin (1 μg) to the mouse hind paw. The results represent the latency to hind-paw withdrawal of the capsaicin-sensitized paw after ipsilateral stimulation with a filament at 0.5 g force (see Methods for details). Each bar and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (n = 8–10 per group). Each group was treated with only one of the drugs (or their solvent) associated to one dose of PRE-084 or its solvent. Statistically significant differences between the values obtained in each drug treated group with respect to the solvent-treated group: * $P < 0.05$; ** $P < 0.01$. Statistically significant differences between the values obtained in the groups treated with each drug (HP, HP Met-II, HP Met-I or BD-1063) associated with PRE-084 with respect to those in the groups treated with each drug associated with PRE-084 solvent: ## $P < 0.01$ (two-way ANOVA followed by Bonferroni test).

2.4.5. Effect of naloxone on the antiallodynic activity of morphine, haloperidol and its metabolites

As expected, morphine (0.25 mg/kg, s.c.) inhibited mechanical hypersensitivity induced by capsaicin, and its effect was reversed by the opioid receptor antagonist naloxone (1 mg/kg, s.c.) (Fig. 5). In contrast, the same dose of naloxone did not significantly modify the antiallodynic effect of HP (0.06 mg/kg, s.c.), HP-Met-II (0.25 mg/kg, s.c.), or HP-Met-I (64 mg/kg, s.c.) (Fig. 5).

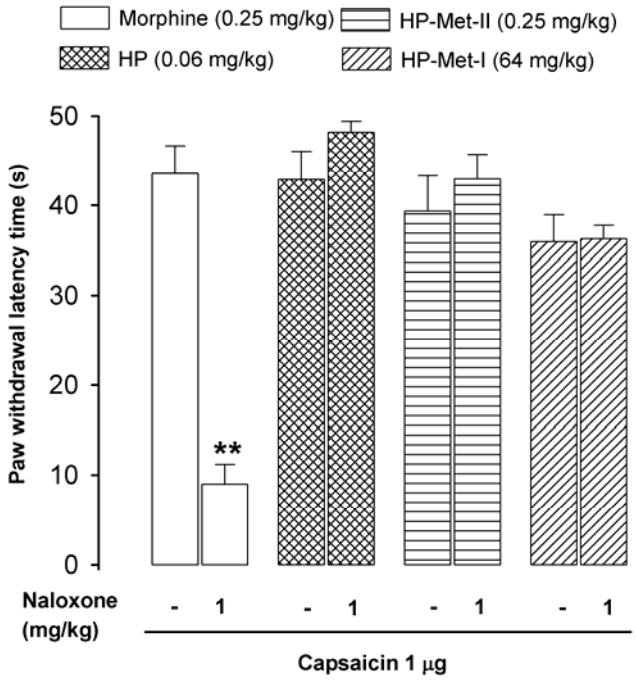

Figure 5. Effects of the association of subcutaneous administration of naloxone (1 mg/kg) or its solvent with morphine (0.25 mg/kg), haloperidol (HP, 0.06 mg/kg), haloperidol metabolite II (HP-Met-II, 0.25 mg/kg) and haloperidol metabolite I (HP-Met-I, 64 mg/kg) on mechanical hypersensitivity induced by the intraplantar injection of capsaicin (1 μg) to the mouse hind paw. The results represent the latency to hind-paw withdrawal of the capsaicin-sensitized paw after ipsilateral stimulation with a filament at 0.5 g force (see Methods for details). Each bar and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (n = 8–10 per group). Each group was treated with only one of the drugs under study associated to naloxone or its solvent. Statistically significant differences in values were only found between the morphine and morphine + naloxone treated groups: ** $P < 0.01$ (non-paired Student's *t*-test).

2.4.6. Comparison of the antiallodynic and antinociceptive effects of haloperidol, its metabolites and control drugs

To compare the effects of drugs on mechanical punctate stimuli-induced nociceptive pain (in nonsensitized animals) and mechanical allodynia (in capsaicin-treated animals), we chose mechanical intensity stimuli that produced similar withdrawal latency times in both experimental situations. Thus, when animals were pretreated with drug solvent, the withdrawal latency time obtained after stimulation with a 4 g force in nonsensitized (1% DMSO-treated) mice (6.58 ± 1.17 s) was very similar to that obtained in animals treated with capsaicin (1 µg, i.pl.) and stimulated at 0.5 g force (8.03 ± 0.84 s; Fig. 6).

The subcutaneous administration of HP, HP-Met-II or HP-Met-I (0.125, 0.5 or 64 mg/kg, respectively) markedly increased paw withdrawal latency time in capsaicin-sensitized animals stimulated with the filament at 0.5 g force. Therefore they exerted antiallodynic effects, which were maximal for HP and HP-Met-II (Fig. 6, left panel). The control drugs gabapentin (64 mg/kg, s.c.) and clonidine (0.5 mg/kg, s.c.) also induced a marked antiallodynic effect in capsaicin-treated animals (Fig. 6, left panel). However, as expected, rofecoxib was inactive in this behavioral test (Fig. 6, left panel).

Nonsensitized (DMSO-treated) mice treated with the σ_1 receptor antagonists HP, HP-Met-I, and HP-Met-II (at the same doses that showed maximal antiallodynic effects) did not increase paw withdrawal latencies after stimulation with a filament at 4 g force, i.e., these drugs did not exert any antinociceptive effect on punctate mechanical nociceptive pain (Fig. 6, right panel). Gabapentin and rofecoxib were also devoid of effect against this noxious punctate mechanical stimulus (Fig. 6, right panel). However, clonidine (0.5 mg/kg, s.c.) increased the paw withdrawal latency time up to the cut-off

time in nonsensitized mice stimulated with the filament at 4 g force (Fig. 6, right panel), inducing a clear antinociceptive effect.

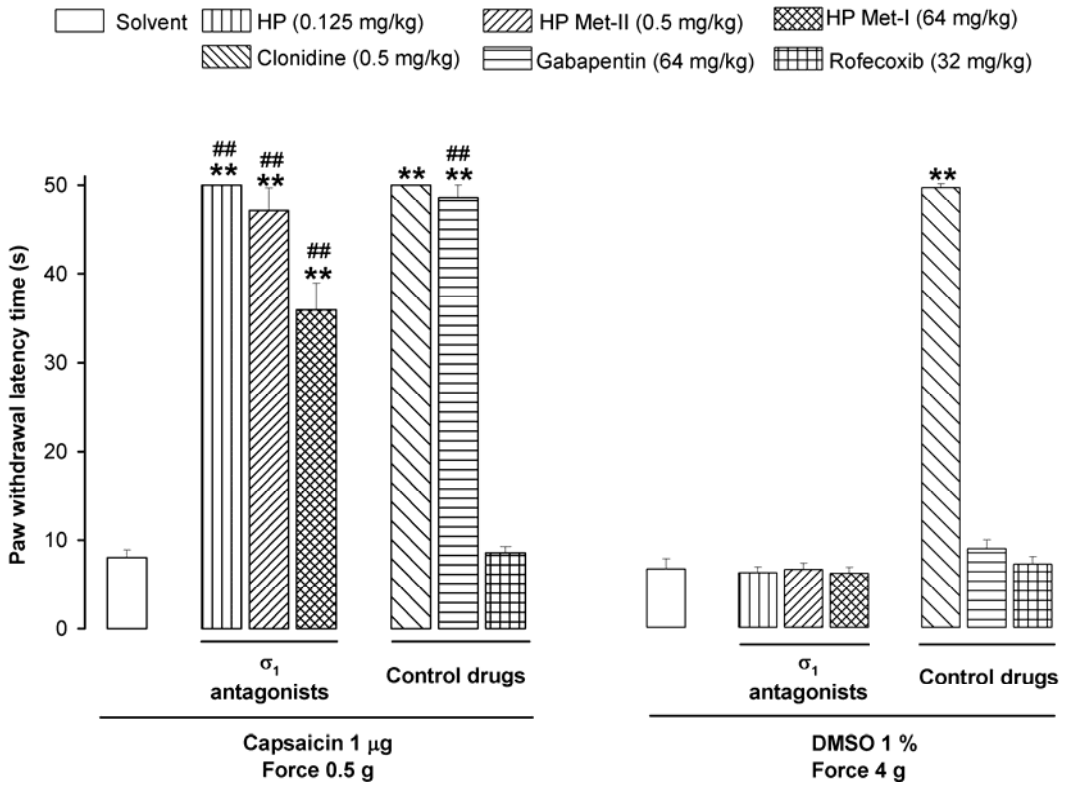

Figure 6. Effects of s.c. administration of haloperidol (HP, 0.125 mg/kg), haloperidol metabolite II (HP-Met-II, 0.5 mg/kg), haloperidol metabolite I (HP-Met-I, 64 mg/kg), clonidine (0.5 mg/kg), gabapentin (64 mg/kg), rofecoxib (32 mg/kg) or their solvent on latency time to paw withdrawal under two experimental conditions: capsaicin-treated paw stimulated at 0.5 g force; i.e., mechanical allodynia (left panel), and 1% DMSO-treated paw stimulated at 4 g force, i.e., mechanical nociceptive pain (right panel) (see Methods for details). Each bar and vertical line represent the mean ± SEM of the values obtained an independent group of animals (n = 8 – 10 per group). Each group was treated with only one of the drugs (or their solvent) under study and tested under only one of the two experimental conditions. Statistically significant differences between the values obtained in the drug- and solvent-treated group under the same experimental conditions: ** $P < 0.01$; statistically significant differences between the effects of each drug tested under the two experimental conditions (0.5 g force in capsaicin-treated mice versus 4 g force in DMSO-treated mice): $^{\#\#} P < 0.01$ (two-way ANOVA followed by Bonferroni test).

2.4.7. Effect of drugs on the rotarod test

The latency to fall-down from the rotarod before the drug or solvent was injected (time 0) was similar in all experimental groups (data not shown). The s.c. administration of HP (0.06 mg/kg), HP-Met-I (64 mg/kg), and HP-Met-II (0.25 mg/kg) had no effect on rotarod latency in comparison to control (solvent-treated) animals 45 min after administration (Fig. 7A), i.e., they did not interfere with motor coordination at times and doses that produced around 70% of the maximum antiallodynic effect (compare Fig. 4 and 7A). The s.c. administration of (-)-sulpiride (100 mg/kg), HP-Met-III (128 mg/kg) or PRE-084 (64 mg/kg) was also devoid of any effect on rotarod latency (Fig. 7A and 7B).

On the other hand, animals treated with higher doses of HP (0.125 mg/kg, s.c.) or HP-Met-II (1 mg/kg, s.c.), which produced 100% of the antiallodynic effect (see Fig. 3), showed a significant decrease in rotarod latencies in comparison to both their own values at time 0 (data not shown) and the values in solvent-treated animals at 45 min after administration (Fig. 7B). Interestingly, the effects of HP and HP-Met-II in the rotarod test were not prevented by PRE-084 at a dose (64 mg/kg, s.c.) that completely prevented the antiallodynic effect of both drugs (compare Fig. 4 and Fig 7B).

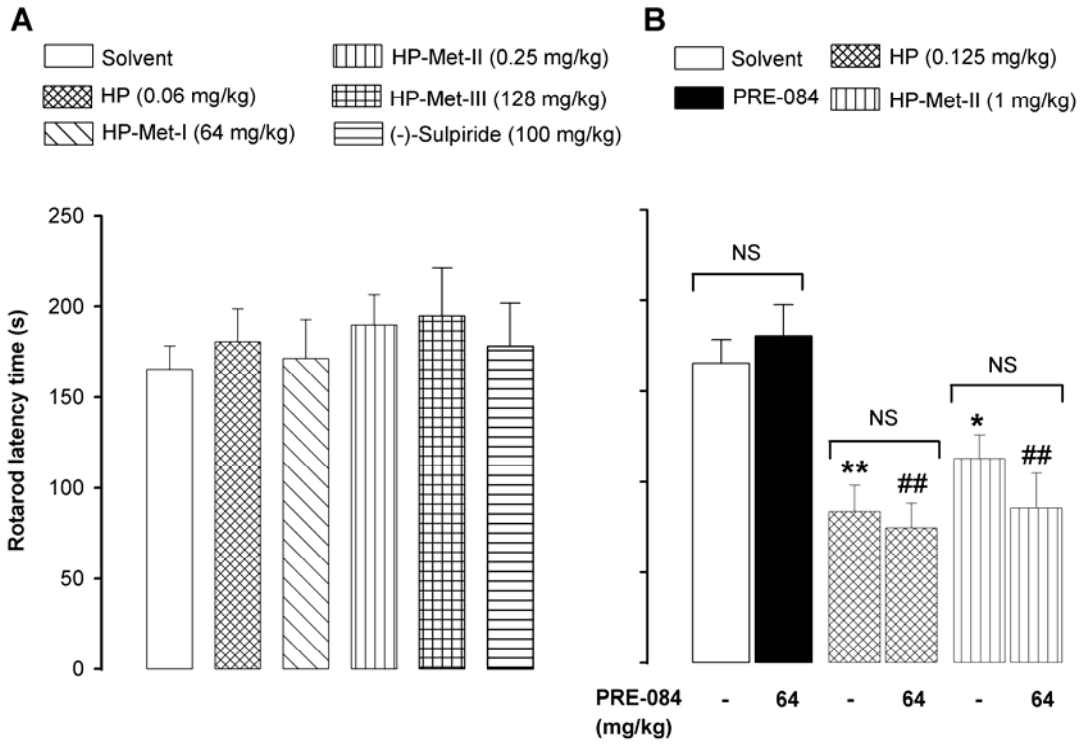

Figure 7. Rotarod latency times obtained 45 min after s.c. administration of drugs or their solvent. **(A)** Effects of the solvent, haloperidol (HP, 0.06 mg/kg), haloperidol metabolite I (HP-Met-I, 64 mg/kg), haloperidol metabolite II (HP-Met-II, 0.25 mg/kg), haloperidol metabolite III (HP-Met-III, 128 mg/kg) or (-)-sulpiride (100 mg/kg). Each bar and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (n = 8 per group). Each group was treated with only one of the drugs (or their solvent) under study. There were no statistically significant differences between the values obtained in solvent- and drug-treated groups (one-way ANOVA). **(B)** Effects of solvent, PRE-084 (64 mg/kg), and haloperidol (HP, 0.125 mg/kg) or haloperidol metabolite II (HP-Met-II, 1 mg/kg), both alone or associated with PRE-084 (64 mg/kg). Each bar and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (n = 8 per group). Each group was treated with only one of the associations of drugs (or their solvent) with PRE-084 (or its solvent). Statistically significant differences between the values obtained in the drug- and solvent-treated groups: * $P < 0.05$; ** $P < 0.01$. Statistically significant differences with respect to the group treated with PRE-084 alone: $^{\#\#}$ $P < 0.01$. NS = non-significant differences (two-way ANOVA followed by Bonferroni test).

2.5. DISCUSSION

The main findings of this study were that the subcutaneous administration of HP or HP metabolites I or II dose-dependently antagonized capsaicin-induced mechanical allodynia (probably by blocking σ_1 receptors) but did not modify nociceptive pain induced by a painful punctate stimulus in nonsensitized animals. These effects have not been previously reported.

We found that the intraplantar injection of capsaicin produced mechanical allodynia (sensitization to an innocuous mechanical punctate stimulus), which was manifested by both a decrease in the force necessary to induce hind-paw withdrawal and a decrease in the latency time for paw withdrawal when a determined force was applied. These results agree with those of previous studies that also reported capsaicin-induced mechanical hypersensitivity to punctate stimulus in rodents (Gilchrist et al. 1996; Joshi et al., 2006, Brenchat et al., 2008). Under our experimental conditions, HP, HP-Met-I and HP-Met-II exerted dose-dependent antiallodynic effects. It is noteworthy that the antiallodynic effect of HP is produced at a range of doses similar to those producing positive effects in animal models predictive of antipsychotic activity (Natesan et al., 2008; Millan et al., 2008). The control drugs gabapentin and clonidine were able to inhibit capsaicin-induced mechanical allodynia, whereas rofecoxib was inactive. These results agree with those previously described for gabapentin and clonidine, both of which exerted antiallodynic effects (Joshi et al., 2006; Paqueron et al., 2003), and with the lack of effect of rofecoxib against capsaicin-induced mechanical hypersensitivity (Bingham et al., 2005).

We also evaluated the effects of the drugs on the response to mechanical stimuli in nonsensitized (DMSO-treated) animals. The punctate stimulus at 4 g force is painful in

nonsensitized animals, since it exceeds the threshold for activation of Aδ- and C-nociceptors in mouse glabrous skin (Cain et al., 2001), and induces paw withdrawal. We chose this force (4 g) in nonsensitized animals because it produced a paw withdrawal latency similar to that found in capsaicin-sensitized animals stimulated with an innocuous force (0.5 g), and allowed us to evaluate the effect of drugs on nociceptive pain induced by a type of stimulus qualitatively similar to that used in capsaicin-sensitized animals. Haloperidol and its metabolites did not affect the response to punctate stimuli in nonsensitized animals, which suggests that the drugs tested did not alter mechanical stimulus perception or the motor mechanisms necessary to produce paw withdrawal. Previous studies have also found that HP and its metabolites did not modify heat-induced nociceptive pain (tail flick test) (Chien and Pasternak, 1994; Cendán et al., 2005b), but data on their effect on mechanical nociceptive pain have not been previously reported. Regarding the drugs used as controls, our results fully agree with those previously reported, since neither gabapentin nor rofecoxib affected mechanical nociception in nonsensitized animals (Tanabe et al., 2005; Padi and Kulkarmi, 2004), whereas clonidine reduced mechanical pain in these animals (Honda et al., 2002; Paqueron et al., 2003).

Haloperidol and HP-Met-II showed affinity for σ_1 receptors at nanomolar concentrations (Cobos et al., 2007, this study), and for D_2 and D_3 receptors at nanomolar (HP) and low micromolar (HP-Met-II) concentrations (Bowen et al. 1990; Jaen et al., 1993). Therefore, their antiallodynic activity might be due to their effects on σ_1 or dopamine receptors or on both. Interestingly, (-)-sulpiride, which shows nanomolar affinity for D_2 and D_3 receptors but no affinity for σ_1 receptors (Freedman et al., 1994; Matsumoto and Pouw, 2000; this study), is devoid of antiallodynic effects,

whereas HP-Met-I, which has affinity for σ_1 receptors but not for D_2 receptors (Bowen et al., 1990; Cobos et al., 2007), shows antiallodynic activity. Therefore dopamine receptor antagonism seems unlikely to be involved in the antiallodynic effects of HP and its metabolites. Because HP also has nanomolar affinity for α_1-adrenoceptors (Millan et al., 2000), these receptors could play a role in its antiallodynic effect. However, the highly selective agonist of σ_1 receptors PRE-084 has no affinity for α_1-adrenoceptors and many other receptors (Su et al., 1991) but totally inhibits the antiallodynic effects of HP and its metabolites. This strongly suggests that α_1-adrenoceptors are not involved in their antiallodynic effects.

In contrast, there are several arguments indicating that the antiallodynic effects of HP and its metabolites are due to their activity on σ_1 receptors. Firstly, their order of potency for inducing antiallodynic effects correlates with their order of affinity for σ_1 binding sites in mouse brain (this study), rat brain (Bowen et al., 1990; Matsumoto and Pouw, 2000), and guinea pig brain and human neuroblastoma cells (Cobos et al., 2007). Secondly, HP-Met-III and (-)-sulpiride, which had negligible affinity for σ_1 receptors in this and other studies (Bowen et al., 1990; Cobos et al., 2007; Matsumoto and Pouw, 2000), exerted no antiallodynic activity in this model. Thirdly, the selective σ_1 receptor agonist PRE-084 (Su et al., 1991; Hayashi and Su, 2004), at doses that do not produce any effect *per se* on capsaicin-induced mechanical sensitization, reversed the antiallodynic effects of HP, HP-Met I, and HP-Met II. Fourthly, the selective σ_1 receptor antagonist BD-1063 (Matsumoto et al., 1995; Hayashi and Su, 2004) produced a response pattern very similar to that of HP and its metabolites, i.e., an antiallodynic effect that was fully reversed by the σ_1 receptor agonist PRE-084. Therefore, the

antiallodynic effects of the drugs studied seem to be due to their antagonistic activity on σ_1 receptors.

Haloperidol and other σ_1 receptor antagonists increase opioid receptor agonist-induced antinociception (see Introduction for references). Hence, their ability to inhibit capsaicin-induced mechanical hypersensitivity might be due to an increase in the effects of endogenous opioids. We found that a dose of naloxone that reversed the antiallodynic effect of morphine was unable to modify the antiallodynic effects of HP and its metabolites. These experiments rule out any influence on our results of modulation of the endogenous opioid system by HP or its metabolites.

Previous studies have reported that HP and its metabolites I and II, as well as other antagonists of σ_1 receptors such as BD-1047 and BMY-14802, inhibited the second phase of formalin-induced pain (Cendán et al., 2005b; Kim et al., 2006). The mechanisms underlying the second phase of the formalin test and the mechanical hypersensitivity induced by capsaicin involve a process of central sensitization, in which the activity of glutamatergic NMDA receptors plays a pivotal role (see Baron, 2000 and Introduction for references). Therefore, either a direct effect on NMDA receptors or an indirect modulation of NMDA-mediated effects could explain our results. Haloperidol and reduced HP show affinity for NMDA receptors, but their affinity for NMDA receptors is very low (K_i in the μM range) (Whittemore et al. 1997; Coughenour and Cordon 1997) in comparison to their affinity for σ_1 receptors, which is 1000 times higher (nM range) (Matsumoto and Pouw 2000, Cobos et al., 2007, and present study). Therefore, a direct effect of HP or its metabolites on NMDA receptors might be ruled out as an explanation for their antiallodynic effect in the present study. On the other hand, it has been reported that σ_1 receptor agonists facilitated several

NMDA receptor-mediated electrophysiological effects, and that HP and other σ_1 receptor antagonists inhibited these effects of σ_1 receptor agonists (Debonnel and Montigny, 1996; Chen et al., 2006; Martina et al., 2007). Interestingly, low doses of HP (0.01-0.1 mg/kg), within the range of doses used in the present study, antagonized the potentiation induced by σ_1 receptor agonists of NMDA-mediated response *in vivo* (Monnet et al., 1992; Maurice and Privat, 1997; Bermack and Debonnel, 2005b). Therefore, the antiallodynic effects of HP and its metabolites might be explained by their ability to indirectly modulate NMDA-mediated effects trough an action σ_1 receptors.

Unspecific interference on motor coordination does not appear to explain the antiallodynic effects, because doses of HP, HP-Met-I and HP-Met-II that lacked activity in the rotarod test exerted around 70% of the maximum antiallodynic activity. Higher doses of HP and HP-Met-II (that produced 100% of the antiallodinic effect) affected the response in the rotarod test but these motor effects were not reversed by PRE-084 (i.e. were *not* due to antagonism of σ_1 receptors), whereas PRE-084 reversed the antiallodynic effects. Moreover, neither HP nor HP-Met-II affected paw withdrawal latency in nonsensitized animals (stimulated at a force of 4 g) at doses that interfered with rotarod test responses. Taken together, these results indicate dissociation between the motor and neurochemical mechanisms involved in the effects of HP and its metabolites in paw withdrawal induced by mechanical stimulation and the rotarod tests.

It is known that σ_1 receptor agonists and antagonists modulate pain processing, acting at both spinal (Kim et al., 2006 and 2008) and supraspinal sites (Chien and Pasternak, 1994; Mei and Pasternak, 2002, 2007; Marrazo et al., 2006). Therefore, HP and its metabolites might act at any of these sites to produce their antiallodynic effects.

Since we administered the drugs subcutaneously, our data do not shed light on the level of central pain processing (spinal or supraspinal) at which HP and its metabolites produce their antiallodynic effects, and new experiments will be needed to identify their level of action.

Regarding the therapeutic potential of our findings, it has been reported that HP (2–5 mg) is useful in humans to treat migraine (Honkaniemi et al., 2006), chronic pain (Raft et al., 1979) and reduce nalbuphine-induced hyperalgesia (Gear et al., 2006). Moreover, a case report described complete acute relief of neuropathic pain with a 2-mg dose of HP (Shir et al., 1990). The affinity of HP for σ_1 receptors is high enough to bind to them in humans at therapeutic doses. In fact, a dose of 3 mg HP occupied around 80% of σ_1 receptors in different areas of the human brain (Ishiwata et al., 2006). Interestingly, this dose of HP occupies more σ_1 than D_2 receptors in the human brain (Ishiwata et al., 2006). This raises the possibility that the analgesic effects observed with HP in humans might be related to its ability to bind to σ_1 receptors, and that the antiallodynic activity of this compound may have clinical applications. New studies are necessary to test these possibilities.

In summary, our results show that haloperidol and its metabolites I and II inhibit capsaicin-induced mechanical hypersensitivity and suggest that the antagonism of σ_1 receptors produced by these drugs is responsible for their antiallodynic effects in this model. These findings raise the possibility of new therapeutic applications for this antipsychotic drug, which is routinely used for other indications in humans.

2.6. REFERENCES

All references indicated in all sections of this manuscript are listed in the section *Bibliography*.

3. Supraspinal sigma-1 receptors modulate capsaicin-induced mechanical hypersensitivity in mice: studies with antisense oligodeoxynucleotides

José Manuel Entrena, Cruz Miguel Cendán, Francisco Rafael Nieto, Daniel Zamanillo, Esperanza del Pozo, José Manuel Baeyens and Enrique José Cobos.

(SUBMITTED, 2009)

Informative title : Supraspinal modulation of allodynia by sigma-1 receptors

3.1. ABSTRACT

We evaluated the effects of supraspinal sigma-1 (σ_1) receptor inhibition by antisense oligodeoxynucleotides (ASOs) on capsaicin-induced mechanical hypersensitivity (allodynia) and on nociceptive pain induced by punctate mechanical stimulation. Two antisense oligodeoxynucleotides (ASO-A and ASO-B), targeting different areas of the σ_1 receptor mRNA, were administered to the mouse brain by intracerebroventricular (i.c.v.) injection once daily during four consecutive days. Both σ_1 ASOs (2.5-10 μg/mouse/day) dose-dependently prevented capsaicin-induced mechanical allodynia when animals were evaluated 24 h after the last i.c.v. injection; i.e., they inhibited the withdrawal of the hind paw stimulated with a normally innocuous mechanical punctate stimulus (0.5 g force) after sensitization with capsaicin (1 μg, intraplantar). This antiallodynic effect was not found in animals treated with control oligodeoxynucleotides unable to target the σ_1 receptor mRNA (a mismatch oligodeoxynucleotide derived from ASO-A, and a scrambled sequence of the ASO-B), a result that supports the specificity of the effects induced by the ASOs. The antiallodynic effects of both σ_1 ASOs completely disappeared one week after their administration, which indicates that animals were able to recover normal σ_1 receptor function. None of the oligodeoxynucleotides tested modified hind paw withdrawal after stimulation with a painful (4 g force) punctate mechanical stimulus in animals not sensitized with capsaicin. These results suggest that supraspinal σ_1 receptors play a role in the mechanisms underlying capsaicin-induced mechanical allodynia, but not in those involved in mechanical punctate nociceptive pain.

3.2. INTRODUCTION

Sigma-1 (σ_1) receptors, which have been cloned in humans and rodents, including mice (Hanner et al., 1996; Prasad et al., 1998; Pan et al., 1998), shows no homology with other mammalian proteins (Monnet and Maurice, 2006). These receptors play an important neuromodulatory role in glutamatergic (NMDA: *N*-metyl-*D*-aspartate) neurotransmission (Bermack and Debonnel, 2005b; Cobos et al., 2008a).

It has been proposed (initially by Chien and Pasternak, 1993) that σ_1 receptors are involved in nociception. We recently reported that formalin-induced pain is reduced in σ_1 knockout mice (Cendán et al., 2005a), and also found that systemic σ_1 antagonism decreased both formalin-induced pain and the mechanical hypersensitivity that develops in the area surrounding capsaicin injection (area of secondary mechanical hypersensitivity) (Cendán et al., 2005b; Entrena et al., 2009a and b). Importantly, both responses are thought to result from central sensitization (Sawynok and Liu, 2004; Baron et al., 2000), an enhancement of pain transmission at the spinal cord dorsal horn, triggered by sustained peripheral nociceptor input (Woolf and Salter, 2000; Ji and Woolf, 2001; Ji et al., 2003). This process involves the potentiation of spinal NMDA receptor activity by the phosphorylation of NMDA receptor subunit 1 (pNR1) (Ji et al., 2003; Gao et al., 2005). Sigma-1 receptors are highly concentrated in the spinal cord dorsal horn (Alonso et al., 2000), and it was recently found that the intrathecal (i.t.) administration of σ_1 antagonists reduced both the formalin-induced increase in spinal cord pNR1 and pain behavior (Kim et al., 2006). Furthermore, the i.t. administration of σ_1 agonists increased pNR1 in the spinal cord, as well as NMDA-induced pain (Kim et al., 2008). These results suggest that spinal σ_1 receptors play an important role in pain hypersensitivity resulting from central sensitization.

Central sensitization in the spinal cord is accompanied by substantial changes in the brain processing of nociceptive information, which lead to central sensitization-like phenomenon at the supraspinal level that contribute to enhanced pain sensitivity (Porreca et al., 2002; Ji et al., 2003). Sigma-1 receptors are also highly concentrated in some supraspinal areas involved in pain processing, including several brainstem regions (Alonso et al., 2000; Kitaichi et al., 2000). However, the possible supraspinal modulatory role of σ_1 receptors in a behavioral model that involves prominently central sensitization has not been investigated. Because changes in pain-related supraspinal activity have been extensively documented during mechanical stimulation of the area of secondary mechanical hypersensitivity induced by capsaicin (Iadarola et al., 1998; Baron et al., 1999; Iannetti et al., 2005; Zambreanu et al., 2005), this study was designed to evaluate the behavioral consequences of the supraspinal inhibition of σ_1 receptors on capsaicin-induced mechanical hypersensitivity. To inhibit supraspinal σ_1 receptors, we injected mice intracerebroventricularly with σ_1 receptor antisense oligodeoxynucleotides (ASO), which are effective tools for studying supraspinal σ_1 receptor functions (Matsumoto et al., 2003; Monnet and Maurice, 2006; Mei and Pasternak, 2007). To test the specificity of the possible effects of σ_1 ASO on mechanical hypersensitivity, we also studied their effects on pain induced by noxious mechanical stimulation in nonsensitized animals. Furthermore, we tested, in both experimental paradigms, the effects of control oligodeoxynucleotides unable to target the σ_1 receptor mRNA.

3.3. MATERIAL AND METHODS

3.3.1. Experimental animals

Female CD-1 mice (Charles River, Barcelona, Spain) weighing 25–30 g were used for all experiments. Mice were housed in a temperature-controlled room (21 ± 1 °C) with air exchange every 20 min and an automatic 12-h light/dark cycle (08.00 to 20.00 h). They were fed a standard laboratory diet (Harlan Teklad Research diet, Madison, WI, USA) and tap water *ad libitum* until the beginning of the experiments. The experiments were performed during the light phase (09.00–15.00 h), and were designed to minimize the number of animals used and their suffering. Mice were always handled in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC). The experimental protocol was approved by the Research Ethics Committee of the University of Granada, Spain.

3.3.2. Drugs and drugs administration

We used two different phosphorothioate-modified antisense oligodeoxynucleotides (ASOs) against the mouse σ_1 receptor cDNA sequence. The σ_1 ASO sequences and targets were: ASO-A (5′-GAG TGC CCA GCC ACA ACC AGG-3′), targeted downstream from the start codon of the mouse σ_1 mRNA (from +77 to +97) as previously reported (King et al., 1997; Pan et al., 1998); and ASO-B (5′-CCA CGG CAT TCT AGC GGG CA-3′), targeting an area spanning the start codon (from –11 to +9), as previously described (Ueda et al., 2001). We used two additional phosphorothioate-modified control oligodeoxynucleotides (COs) to check the specificity of the effects of the two σ_1 ASOs. As control for ASO-A we used a mismatch oligodeoxynucleotide in which three pairs of bases were switched to obtain CO-A (5′-

GAG **GT**C CC**G** **A**CC ACA **CA**C AGG-3´), designed previously (King et al., 1997; Pan et al., 1998). As a control for ASO-B we completely scrambled its sequence to obtain CO-B (5´-GTT GGA CCA ATC CGG CGC CA-3´). This sequence shows no possible hybridization to the target or to any other known cDNA sequences in the GenBank Database (NIH, Bethesda, MD, USA). The σ_1 ASOs and COs used in this study were synthesized and provided by Eurogentec (Liège, Belgium).

Oligodeoxynucleotides were dissolved in physiological sterile saline to a concentration of 5 µg/µl, and stored at -20 ºC until use. Further dilutions of the oligodeoxynucleotide solutions in saline were prepared to obtain the appropriate concentrations for different experiments. Sigma-1 ASOs, their respective COs or their solvent (saline) were administered daily as intracerebroventricular (i.c.v.) bolus injections (2 µl) during four consecutive days (Pan et al., 1998; Mei and Pasternak, 2002). The i.c.v. injections were performed according to a previously described method (Robles et al., 1992; Ocaña et al., 1995). Briefly, the injection site was identified according to the method of Haley & McCormick (1957), and the drug solution was injected with a 701 RN Hamilton microsyringe (Teknokroma, Barcelona, Spain) and a 26-gauge needle, with a sleeve around the needle to prevent the latter from penetrating more than 3 mm into the skull. After the experiments were done, the position of the injection was evaluated in each brain, and the results from animals in which the tip of the needle did not reach the lateral ventricle were discarded (less than 3% of the animals were discarded due to errors in the injection site).

The chemical algogen used was capsaicin (Sigma-Aldrich Química SA, Madrid, Spain), which was dissolved in 1% dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) in physiological sterile saline to a concentration of 0.05 µg/µl. Capsaicin

solution was injected intraplantarly (i.pl.) into the right hind paw in a volume of 20 µl (1 µg/paw), using a 1710 TLL Hamilton microsyringe (Teknokroma) with a $30^{1/2}$-gauge needle. Control animals were injected i.pl. with the same volume of capsaicin solvent (DMSO 1% in saline).

3.3.3. Evaluation of mechanical allodynia and mechanical nociceptive pain

The mice were habituated for 2 h in individual black-walled test compartments situated on an elevated mesh-bottomed platform with a 0.5-cm^2 grid to provide access to the ventral side of the mice hind paws. The animals were removed from the compartment, given an i.pl. injection of capsaicin (1 µg) or its solvent in the right hind paw, and were immediately returned to the compartment. Fifteen minutes after the i.pl injection, mechanical stimulation was performed using a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) as previously described (Entrena et al., 2009a and b). Briefly, a nonflexible filament (0.5 mm diameter) was electronically driven to the ventral side of the injected hind paw at least 5 mm away from the site of the injection of capsaicin or its solvent. When nocifensive paw withdrawal occurred, response latency time was automatically recorded by the apparatus. Each mouse was tested in three trials separated by an interval of 30 s in each experimental session. A cut-off time of 50 s was used. All tests were performed by an experimenter who was blinded to the treatment the mice had received.

Mechanical allodynia and mechanical punctate pain were evaluated as previously described (Entrena et al., 2009a and b) on days 5 and 11 after the first administration of σ_1 ASO, CO or its solvent (i.e., 24 h and one week after treatment had ended). Briefly, the antiallodynic effects of i.c.v. treatments were evaluated in capsaicin-sensitized mice

by applying a mild mechanical stimulation at 0.5 g force to the injected paw. This mechanical stimulation did not produce paw withdrawal in DMSO-treated (capsaicin solvent) mice (i.e., it was innocuous in control animals), but induced a clear paw withdrawal in capsaicin-sensitized mice (see Fig. 1 and Results for details). This protocol allowed us to evaluate mechanical allodynia as pain in response to a normally innocuous stimulus. The antinociceptive effects of oligodeoxynucleotide treatments were assessed in DMSO-treated (control, nonsensitized) animals with a stronger mechanical stimulation of 4 g force. This force was chosen because it induced paw withdrawal in the nonsensitized mice (i.e., it was a noxious stimulus), with a paw withdrawal latency similar to that observed in capsaicin-sensitized animals stimulated at 0.5 g force (see Fig. 1 and Results for details). To control for the possible effects on the behavioral tests of repeated i.c.v. injections *per se*, we compared the results from saline i.c.v.-treated animals with those in naïve animals which did not receive any i.c.v. injections.

3.3.4. Data analysis

The degree of effect of treatment on capsaicin-induced mechanical hypersensitivity was calculated as the percent reduction in mechanical hypersensitivity = [(LTO – LTS) / (CT – LTS)] × 100, where LTO is the latency time for paw withdrawal in oligodeoxynucleotide-treated animals, LTS is the latency time in solvent-treated animals and CT is the cut-off time (50 s). The values obtained in the different experimental groups were compared with two-way analysis of variance (ANOVA) followed by the Bonferroni test. In all cases, the differences between means were

considered significant when the value of P was below 0.05. All results are given as the mean ± SEM.

3.4. RESULTS

3.4.1. Evaluation of mechanical allodynia and mechanical nociceptive pain in naïve and saline i.c.v.-treated animals

To determine whether the repeated i.c.v. administration procedure modified the response to punctate mechanical stimulation of the hind paw, we compared the response in naïve (non-i.c.v.-treated) animals and i.c.v.(saline)-treated mice under different experimental conditions. Mechanical punctate stimulation at 0.5 g force was unable to induce significant paw withdrawal in nonsensitized (1% DMSO-treated, i.pl.) naïve mice (i.e., it was an innocuous mechanical stimulus), and paw withdrawal latency time (48.97 ± 0.41 s) was close to the cut-off time (Fig. 1). However, when naïve animals were injected i.pl. with capsaicin, mild stimulation at 0.5 g force induced rapid paw withdrawal, decreasing the paw withdrawal latency time to 7.4 ± 1.26 s. Therefore i.pl. treatment with capsaicin induced mechanical allodynia in naïve mice (Fig. 1). To assess mechanical nociceptive pain in nonsensitized naïve animals, we stimulated them with the filament at 4 g force. This stimulus induced clear paw withdrawal nonsensitized naïve animals (i.e., it was a noxious stimulus). The latency time for paw withdrawal in these animals (10.28 ± 2.68 s) was similar ($P > 0.1$) to that obtained in capsaicin-sensitized animals stimulated with a much lower force (0.5 g) (Fig. 1).

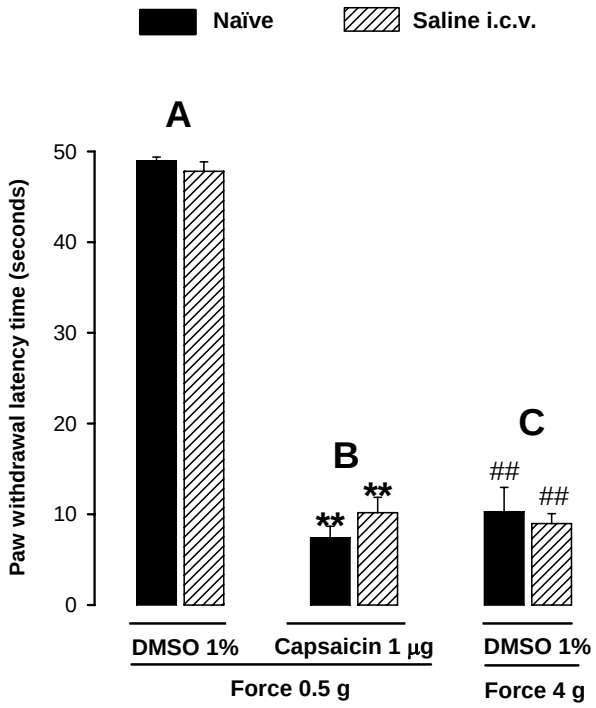

Figure. 1. Comparison of hind paw withdrawal latency time after punctate mechanical stimulation between nontreated (naïve) mice and mice treated i.c.v with saline (once daily during four consecutive days) under three experimental conditions: (A) stimulation with the filament at a normally innocuous intensity, (B) mechanical allodynia, and (C) nociceptive pain. Experiments were performed 24 h after the last i.c.v. injection. Latency time for paw withdrawal was evaluated after: (A) stimulation with the filament at 0.5 g force in animals in which 1% DMSO was administered intraplantarly (i.pl.) (capsaicin solvent), (B) stimulation with the filament at 0.5 g force in animals in which1 µg capsaicin was administered i.pl., and (C) stimulation with the filament at 4 g force in animals treated with 1% DMSO i.pl. Each bar and vertical line represent the mean ± SEM of the values obtained in an independent groups of animals (n = 8–10 per group). Statistically significant differences were found between condition B *versus* condition A (** $P < 0.01$) and between condition C *versus* condition A ($^{\#\#} P < 0.01$), but not between condition B *versus* condition C ($P > 0.1$). We found no statistically significant differences between the values obtained in naïve and saline i.c.v.-treated animals tested under the same experimental conditions. All statistical comparisons were performed with a two-way ANOVA followed by a Bonferroni test.

In animals treated daily with saline (oligodeoxynucleotides solvent) i.c.v. from days 1 to 4 and evaluated on day 5, the responses were similar ($P > 0.1$) to naïve animals under all experimental conditions: a) stimulation at an innocuous intensity (0.5 g force) in nonsensitized animals, b) capsaicin-induced mechanical allodynia (stimulation with 0.5 g force in capsaicin-sensitized animals), and c) mechanical nociceptive pain (stimulation with 4 g force in nonsensitized animals) (Fig. 1). Therefore the i.c.v. injection procedure did not produce brain alterations able to modify the response to the mechanical stimulus under different experimental conditions.

It is remarkable that both naïve and saline i.c.v.-treated animals showed similar reductions in paw withdrawal latency time after capsaicin-induced mechanical allodynia (and stimulation with 0.5 g force) and in tests of mechanical nociceptive pain (4 g force stimulation) in nonsensitized animals. Therefore, this protocol allowed us to evaluate the effects of i.c.v. treatments under conditions that reproduced pathological and physiological states with quantitatively similar behavioral responses.

3.4.2. Effects of supraspinally administered σ_1 ASOs on capsaicin-induced mechanical allodynia

Antisense treatment with ASO-A or ASO-B (2.5–10 μg/mice/day, i.c.v.), during 4 days inhibited capsaicin-induced mechanical hypersensitivity in animals evaluated 24 h after the last ASO administration. We found a dose-dependent increase in withdrawal latency time in the capsaicin-injected hind paw stimulated at 0.5 g force, i.e., both ASOs produced antiallodynic effects (Fig. 2).

Interestingly, ASO-B was much more potent than ASO-A in preventing capsaicin-induced mechanical sensitization (Fig. 2). Importantly, the antiallodynic effect attained

by both σ_1 ASOs at a dose of 10 µg/mouse/day approached the maximum effect under our experimental conditions (Fig. 2). In contrast, repeated i.c.v. administration of CO-A or CO-B (controls for ASO-A and ASO-B, respectively), at the same doses as their respective ASO, did not exert statistically significant antiallodynic effects in capsaicin-sensitized animals (Fig. 2).

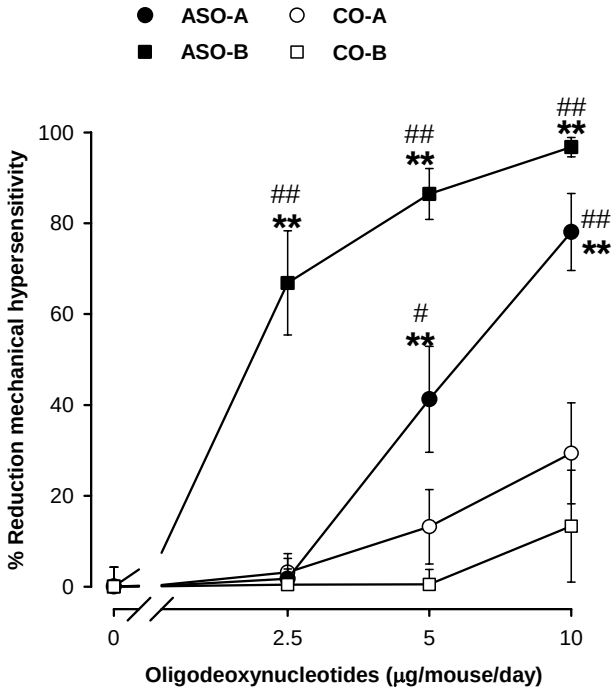

Figure. 2. Effects of repeated intracerebroventricular (i.c.v.) administration of σ_1 antisense (ASO) and control oligodeoxynucleotides (CO) on mechanical hypersensitivity induced by the intraplantar injection of capsaicin to the mouse hind paw. Animals were treated once daily during four consecutive days with several doses of one of the following oligodeoxynucleotides: ASO-A (●), ASO-B (■), CO-A (○), CO-B, (□) or their solvent (saline –dose 0–), and the behavioral test was performed 24 h after the last i.c.v. injection. The results represent the changes in mechanical hypersensitivity (calculated as explained in the Methods) in the capsaicin (1 µg)-treated paw after stimulation with the filament at 0.5 g force Each point and vertical line represent the mean ± SEM of the values obtained in an independent groups of animals (n = 8–10 per group). Each group was treated with only one dose of each ASO or solvent. Statistically significant differences between the values obtained in the solvent- and σ_1 ASO -injected groups (** $P < 0.01$), and between the values obtained with each dose of σ_1 ASO and their respective CO ($^{\#}$ $P < 0.05$; $^{\#\#}$ $P < 0.01$). There were no statistically significant differences among values in solvent- and CO-injected groups. All statistical comparisons were performed with a two-way ANOVA followed by a Bonferroni test.

3.4.3. Effects of supraspinally administered σ_1 ASOs on mechanical nociceptive pain

To explore the possible effects of the treatments on mechanical punctate nociceptive pain, we applied the filament at 4 g force (noxious stimulus) to the hind paw of nonsensitized mice (1% DMSO-treated mice, i.pl.). Animals were evaluated 24 h after the last i.c.v administration with 10 μg/mouse/day of each σ_1 ASO (a dose which induced a patent antiallodynic effect, as noted above), or their respective CO. In contradistinction to the marked antiallodynic effect induced by ASO-A or ASO-B (Fig. 3, left panel), neither σ_1 ASOs nor COs affected the latencies for paw withdrawal in nonsensitized animals stimulated at 4 g force (Fig. 3, right panel). Therefore, they showed no antinociceptive effect against a noxious punctate mechanical stimulus.

3.4.4. Recovery of the capsaicin-induced mechanical hypersensitivity one week after the supraspinal administration of σ_1 ASOs

To evaluate whether the antiallodynic effects induced by repeated supraspinal treatments with ASO-A or ASO-B were permanent or not, we allowed a period of 1 week after the last administration of the oligodeoxynucleotides before performing behavioral assays. Capsaicin-induced mechanical hypersensitivity was compared in mice that previously received 10 μg/mouse/day ASO-A or ASO-B during four consecutive days, 1 day and 7 days after the last i.c.v. injection. We found a clear antiallodynic effect 24 h after the last injection of both ASOs, but mechanical hypersensitivity recovered completely one week after the last i.c.v. injection (Fig. 4).Paw withdrawal latency times after sensitization with capsaicin one week after the end of i.c.v. treatment with both ASOs were similar to the times in i.c.v. saline-treated

animals (Fig. 4). In contrast, treatment with both COs had no significant effect at any of the times tested (Fig. 4).Neither ASO-A nor ASO-B induced any permanent change in capsaicin-induced mechanical hypersensitivity.

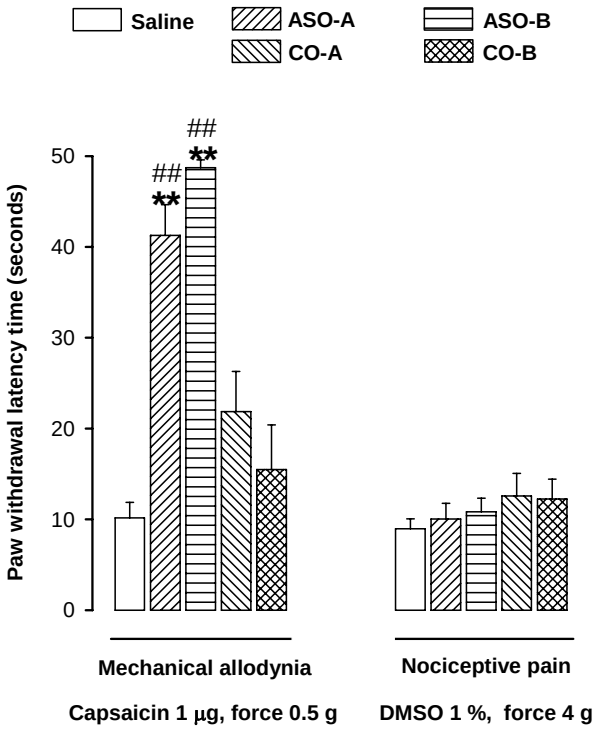

Figure. 3. Effects of repeated intracerebroventricular administration of σ_1 antisense oligodeoxynucleotides (ASO-A and ASO-B), their respective controls (CO-A and CO-B) or their solvent (saline) on latency time for paw withdrawal after punctate mechanical stimulation under two experimental conditions: allodynia and nociceptive pain. Mechanical allodynia (left panel) was evaluated in the capsaicin (1 µg)-treated paw stimulated at 0.5 g force, and mechanical punctuate nociceptive pain (right panel) was evaluated in the 1% DMSO-treated paw stimulated at 4 g force. Animals were treated once daily from days 1 to 4 with the oligodeoxynucleotides (10 µg/mouse/day) or their solvent (saline), and the behavioral test was performed on the 5th day. Each bar and vertical line represent the mean ± SEM of the values obtained in an independent group (n = 8–10 per group). Each group was treated with only one each ASO and CO (or their solvent) under study and tested under only one of the two experimental conditions. Statistically significant differences between the values obtained in oligodeoxynucleotide- and solvent-treated groups under the same experimental conditions: ** $P < 0.01$; and between the effects of each oligodeoxynucleotide under the two experimental conditions: $^{\#\#} P < 0.01$ (two-way ANOVA followed by Bonferroni test).

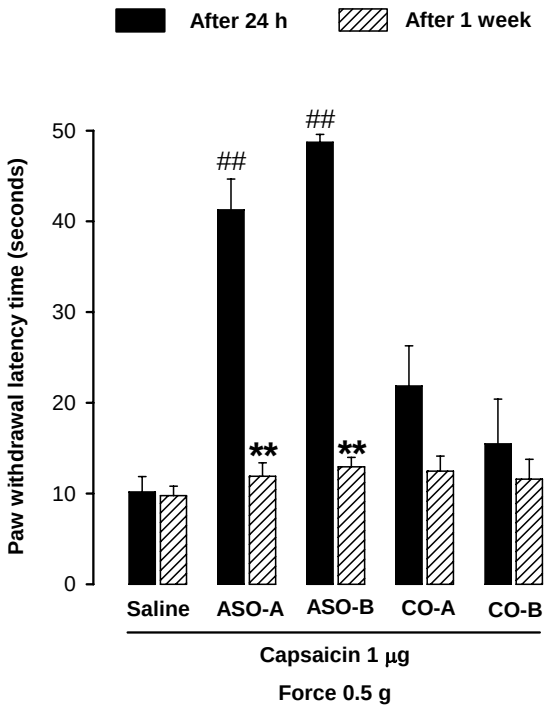

Figure. 4. Comparison of the antiallodynic effects of σ_1 antisense oligodeoxynucleotides (ASO-A and ASO-B), their respective controls (CO-A and CO-B) or their solvent (saline), one day or seven days after the end of i.c.v. treatment. Animals were treated intracerebroventricularly (i.c.v.) once daily during four consecutive days with saline, 10 µg/mouse/day of ASO-A, ASO-B, or their respective controls (CO-A or CO-B). Antiallodynic effects were manifested as an increase in paw withdrawal latency time after stimulation with a filament at 0.5 g force of the paw previously sensitized with capsaicin (1 µg, i.pl.). Animals were evaluated 24 h and one week after the last i.c.v. injection (see Methods for details). Each bar and vertical line represent the mean ± SEM of the values obtained in an independent group of animals (8–10 per group). Each group was treated with only one of ASO or CO (or their solvent) under study. Statistically significant differences were found between the values obtained 24 h and one week after the last i.c.v. administration of ASOs (** $P < 0.01$), and between the values obtained 24 h after the last administration of saline *versus* ASO ($^{\#\#} P < 0.01$). No statistically significant differences were found among the values obtained one week after treatment with saline, ASO or CO. All statistical comparisons were performed with two-way ANOVA followed by a Bonferroni test.

3.5. DISCUSSION

The main finding of this study was that the i.c.v. administration of σ_1 ASOs produced a marked, dose-dependent inhibition of capsaicin-induced mechanical allodynia (a model of central sensitization) without modifying mechanical nociceptive pain induced by a painful punctate stimulus in nonsensitized animals. This is the first report to document the effect of supraspinal σ_1 receptor inactivation in a model of central sensitization.

Currently, the use of antisense oligodeoxynucleotides to knock down specific proteins is considered a key strategy for drug target validation (Dean, 2001; Wang et al., 2004). We previously reported that the systemic administration of σ_1 receptor antagonists, including the selective σ_1 antagonist BD-1063, BD-1047 and NE-100 and also haloperidol and its σ_1-active metabolites, inhibited capsaicin-induced mechanical allodynia (Entrena et al., 2009a and b). Here we have extended upon this previous work by using σ_1 ASOs as selective tools to interfere with σ_1 receptor function. We injected ASOs i.c.v. to inhibit σ_1 receptor supraspinally. The results of the present study agree with those of our previous study with σ_1 receptor antagonist drugs (Entrena et al., 2009a and b), but also extend them to document the significant contribution of supraspinal σ_1 receptors to the mechanical hypersensitivity induced by capsaicin. Further experiments with microinjections of σ_1 ASOs or σ_1 antagonists in different brain nuclei would be useful to determine the exact anatomical location of this effect.

Based on current standards, an ASO is generally considered selective if 1) it produces a functional effect, 2) mRNA or protein knockdown of the target is demonstrated, and 3) COs have no effect on function or levels of expression (Stone and Vulchanova, 2003). We found that the i.c.v. injection of σ_1 ASOs induced a marked antiallodynic effect in

capsaicin-sensitized animals. In addition, both σ_1 ASOs used in this study have been reported to decrease σ_1 receptor protein after their repeated administration in mice (Ueda et al., 2001; Mei and Pasternak, 2002). Furthermore, the antiallodynic effects induced by the σ_1 ASOs could not be replicated by their respective COs of the same length and the same base composition (although in a different sequence). Interestingly, CO-A was unable to decrease the levels of mRNA encoding σ_1 receptor or σ_1 protein (Pan et al., 1998; Mei and Pasternak, 2002). The design of CO-B by scrambling the sequence of ASO-B completely rules out possible hybridization with the target, as was confirmed in the GenBank database (see Methods).

It is known that phosphorothioate oligodeoxynucleotides, in addition to hybridization with the target mRNA sequence, can interact with proteins in a manner that may be sequence-dependent (Stone and Vulchanova, 2003; Crooke, 2004), and that this can have consequences that are independent of hybridization with the target mRNA sequence (Guvakova et al., 1995; Lai et al., 2006). It has been suggested that more than one ASO targeting the same gene should be used to control for possible sequence-dependent effects unrelated with the target mRNA (Stone and Vulchanova, 2003). In our study, the use of two different ASO sequences targeted to different areas of the σ_1 mRNA but sharing the same effect against capsaicin-induced mechanical hypersensitivity (together with the absence of functional effects of both COs) clearly showed that the effect we studied was not dependent upon any given single sequence, and argue strongly for the specificity of the antiallodynic effects of σ_1 receptor inhibition induced by σ_1 ASOs.

We found differences in potency between the effects of the two σ_1 ASOs, with ASO-B being more potent than ASO-A. These differences could be due to the different targets within the σ_1 mRNA sequence: ASO-A target a sequence in the coding region,

whereas ASO-B target a sequence spanning the start codon. Although targeting a region close to the start codon can not be considered an unalterable rule for the correct design of an active ASO (Stone and Vulchanova, 2003), it is thought that the coding region of some mRNA may be less targetable than the translation initiation codon (Dias and Stein, 2002; Stone and Vulchanova, 2003). This may be due to the lower complex stability required to prevent ribosome assembly (targeting the start codon) than to prevent ribosome elongation, which in turn may be attributable to the ability of the ribosomal machinery to unwind the oligonucleotide from its targeted mRNA (Dias and Stein, 2002).

We found that the antiallodynic effect induced by the supraspinal administration of σ_1 ASOs completely disappeared one week after the last administration. Therefore, as expected, σ_1 ASOs did not induce permanent changes in capsaicin-induced mechanical hypersensitivity as the generation of newly synthesized σ_1 receptors probably allowed the behavioral response to recover. These results indirectly indicate that the turnover speed of mouse brain σ_1 receptors is relatively fast, similar to that of the rat brain (Klein et al., 1994), but much faster than that of σ_1 receptors in the guinea pig brain, which took over a month to reach stable values after their irreversible inactivation with reduced haloperidol (Cobos et al., 2007). In fact, we recently reported that both the biochemical (reduction of [^{3}H](+)-pentazocine binding to brain membranes) and functional (antiallodynic effect) consequences of the inactivation of σ_1 receptors by reduced haloperidol, lasted less than one week (Cobos et al., 2008b). These findings indicate that the turnover of mouse brain σ_1 receptors is rapid, and are consistent with the recovery of σ_1 receptor function one week after the end of treatment with the ASO.

Sigma-1 receptor is a neuromodulatory protein which plays an important role in the modulation of several neurotransmitter systems, mainly in glutamatergic NMDA-mediated responses. It is known that σ_1 agonists enhance several NMDA receptor-mediated electrophysiological effects in the brain (reviewed by Debonnel and Montigny, 1996; Bermack and Debonnel, 2005b; Cobos et al., 2008a). It is also known that NMDA receptors are involved in the facilitation of painful inputs at supraspinal levels after capsaicin administration (Willis, 2001; Budai et al., 2007). Therefore the antiallodynic effects of the supraspinal administration of σ_1 ASOs that we observed might be explained by inhibition of the ability of σ_1 receptor to potentiate NMDA-mediated effects at supraspinal levels. Since supraspinal facilitation of nociceptive inputs by NMDA also occurs in pathological pain states (neuropathic and inflammatory pain) (Porreca et al., 2002), supraspinal σ_1 receptors could be relevant for these disorders. In fact, we recently reported that neuropathic pain (mechanical and cold allodynia) induced by sciatic nerve ligation was markedly reduced in σ_1 receptor knockout mice (Zamanillo et al., 2008).

In contrast to the σ_1 ASOs-induced inhibition of capsaicin-induced mechanical allodynia, we show that the supraspinal administration of σ_1 ASOs did not affect mechanical nociceptive punctate pain. This is consistent with previous results showing that haloperidol and its σ_1-active metabolites, selective σ_1 antagonists and knockout σ_1 were unable to modify mechanical nociceptive punctate pain (Entrena et al., 2009a and b). It is also known that σ_1 receptor antagonists did not affect tail-flick latency in experimental animals (Chien and Pasternak, 1993, 1994; Cendán et al., 2005b; Marrazzo et al., 2006). Therefore, the results of the present and previous studies suggest that σ_1 receptor antagonism does not play an important role in the control of nociceptive pain. However, σ_1 receptor antagonist drugs and σ_1 ASOs enhance the analgesic effects

of morphine and other opioid receptor agonists in the tail-flick test, suggesting a tonically active anti-opioid σ_1 system (Chien and Pasternak, 1993, 1994; King et al., 1997; Mei and Pasternak, 2002 and 2007; Marrazzo et al., 2006). Interestingly, the supraspinal location of the modulation of opioid analgesia by σ_1 receptors has been conclusively determined by experiments with the i.c.v. administration and microinjections in the brainstem of either opioids and σ_1 ligands or σ_1 ASOs (Mei and Pasternak, 2002 and 2007; Marrazzo et al., 2006; King et al., 1997; Pan et al., 1998). We previously reported that the effects of σ_1 receptor antagonism on capsaicin-induced mechanical hypersensitivity was not related to the modulation of the opioid system (Entrena et al., 2009b). However, both the enhanced analgesic action of opioids induced by the supraspinal inhibition of σ_1 receptors, as well as the inhibition of capsaicin-induced mechanical hypersensitivity by the supraspinal administration of σ_1 ASOs, suggest a tonic activity of the brain σ_1 system involved in pain control.

In summary, our results support that brain σ_1 receptors play a pivotal role in capsaicin-induced mechanical allodynia, but not in mechanical punctate nociceptive pain. These findings suggest that σ_1 receptors participate in the modifications that occur in brain processing after sensitization with capsaicin, without interfering in the physiological brain mechanisms involved in mechanical nociceptive pain.

3.6. REFERENCES

All references indicated in all sections of this manuscript are listed in the section *Bibliography*.

CONCLUSIONS

SPECIFIC CONCLUSIONS

1. Intraplantar injection of capsaicin produces a dose-dependent mechanical hypersensitivity to innocuous punctate stimulus (i.e. mechanical allodynia) in wild-type mice. However, the genetic inactivation of σ_1 receptors completely inhibited the sensitization induced by capsaicin in σ_1 receptor knockout mice.

2. The selective σ_1 receptor antagonists BD-1063, BD-1047 and NE-100 (s.c. administered) dose-dependently inhibited capsaicin-induced mechanical allodynia in wild-type mice. Their antiallodynic effect appears to be mediated through σ_1 receptor blockade, because it was prevented by the selective σ_1 receptor agonist PRE-084.

3. The nonselective σ_1 receptor antagonists haloperidol and its metabolites I or II (s.c. administered in wild-type mice) dose-dependently antagonized the mechanical allodynia induced by capsaicin. The order of their antiallodynic potency was that of their affinity for σ_1 receptors and their effect was prevented by the selective σ_1 receptor agonist PRE-084. Both results indicate that their antiallodynic effect appears to be due to a blockade of σ_1 receptors.

4. The antiallodynic activity of haloperidol and its σ_1–active metabolites seems unlikely to be due to dopamine receptor antagonism, since (-)-sulpiride (which shows similar affinity for dopamine receptors as haloperidol but was devoid of affinity for σ_1 receptors) does not produce an antiallodynic effect, whereas haloperidol metabolite I (which has affinity for σ_1 receptors but not for dopamine receptors) produces antiallodynia.

5. Supraspinal σ_1 receptors appear to play a pivotal role in capsaicin-induced mechanical hypersensitivity, since two different σ_1 receptor antisense oligodeoxynucleotides (ASOs) administered intracerebroventricularly (i.c.v.), produced a dose-dependent inhibition of capsaicin-induced mechanical allodynia. Their antiallodynic effects were transient and appear to be due to specific σ_1 receptor inhibition, because they were not observed in animals treated with control oligodeoxynucleotides unable to target the σ_1 receptor mRNA.

6. Sigma$_1$ receptor blockade may be a novel pharmacological target against mechanical hypersensitivity that is not shared by other known antineuropathic drugs, since gabapentin, pregabalin, tetrodotoxin, mexiletine and clonidine inhibited capsaicin-induced mechanical allodynia but were devoid of affinity for σ_1 receptors.

7. Sigma$_1$ receptors are not involved in the perception of innocuous mechanical stimulus or in mechanical nociceptive pain, because nonsensitized (control) σ_1 receptor knockout and wild-type mice responded similarly to a wide range of mechanical punctate stimulus intensities (from innocuous to noxious). In addition, treatments with σ_1 receptor antagonists (s.c.) or σ_1 receptor ASOs (i.c.v.) were also ineffective against mechanical nociceptive pain in wild-type mice**.**

GENERAL CONCLUSIONS

1. An endogenous σ_1 receptor-activating system appears to play a pivotal facilitatory role in the mechanisms underlying central sensitization induced by capsaicin, because the mechanical allodynia produced by capsaicin was inhibited by σ_1 receptor antagonists, σ_1 ASOs and genetic inactivation of σ_1 receptors. However, this endogenous system does not seem to participate in physiological nociceptive pain, which was not modified by any of these pharmacological or genetic interventions.

2. Sigma$_1$ receptors blockade may be a novel pharmacological target for the treatment of mechanical allodynia in pathologies in which central sensitization mechanisms are activated (such as neuropathic pain and other types of pathological pain), with the advantage of not interfering with physiological nociceptive pain. These findings open up an opportunity for developing new drugs to treat pathological pain and raise the possibility of new therapeutic applications for known drugs with σ_1 antagonist properties (such as haloperidol), used routinely for other indications in humans.

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LIST OF ABBREVIATIONS

(+)-3-PPP: (+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine

(+)-MR 200: [(+)-methyl (1*R*,2*S*)-*2*-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate]

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

$[Ca^{2+}]_i$: intracellular calcium concentration

4-IBP: 4-(*N*-benzylpiperidin-4-yl)-4-iodobenzamide

5-HT: 5-hydroxytryptamine or serotonin

AMH: A-polymodal nociceptors

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

ASO: antisense oligodeoxynucleotide

BD 737: *S*,2*R*-(-)-cis-*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)cyclohexylamine

BD 1008: *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine

BD-1031: 3*R*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo[4.3.0]nonane

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

BD-1052: *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-allyl-2-(1-pyrrolidinyl)ethylamine

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

BDNF: brain-derived neurotrophic factor

BK: bradykinin

BMY-14802: (α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazinebutanol)

CAMKII: Ca^{+2} – calmodulin-dependent kinase II

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CGRP: calcitonin gene-related peptide

CM: C mechano nociceptors

CMH: C mechano-heat nociceptors

CO: control oligodeoxynucleotide

DAG: 1,2-diacylglycerol

DAMGO: Tyr-*D*-Ala-Gly-*N*-methyl-Phe-Gly-ol

DAT: dopamine transporter

DCPS: dorsal column postsynaptic system

DHEA: dehydroepiandrosterone

DHEAS: dehydroepiandrosterone sulfate

DMSO: dimethylsulfoxide

DPH: phenytoin

DRG: dorsal root ganglion

DTG: 1,3-di-*o*-tolylguanidine

DuP 734: 1-(cyclopropylmethyl)-4-(20-(400-fluorophenyl)-20-oxoethyl)piperidine hydrobromide

E-5842: 4-(4-fluorophenyl)-1,2,3,6-tetrahydro-1-[4-(1,2,4-triazol-1-yl)butyl]piperidine citrate

ERK: extracellular signal-regulated kinase

fMRI: functional magnetic resonance imaging

GABA: gamma-amino butyric acid

GDNF: glial cell line-derived neurotrophic factor

HP-Met-I: Haloperidol metabolite I: 4-(4-chlorophenyl)-4-hydroxypiperidine)

HP-Met-II: Haloperidol metabolite II (reduced haloperidol): [4-(4-chlorophenyl)-α-(4-fluorophenyl)-4-hydroxy-1-piperidinebutanol]

HP-Met-III: Haloperidol metabolite III: *p*-fluorobenzoylpropionic acid)

HIV: human immunodeficiency virus

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

i.pl.: intraplantar

i.t.: intrathecal

IP_3: inositol 1,4,5-trisphosphate

$InsP_3$: inositol 1,4,5-trisphosphate

JO-1784: igmesine, (+)-*N*-cyclopropylmethyl-*N*-methyl-1,4-diphenyl-1-ethylbut-3-en-1-ylamine hydrochloride

KO: knockout

LR132: 1*R*,2*S*-(+)-cis-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl) cyclohexylamine)

LTM C nociceptors: low-threshold C-fiber mechanoreceptors

MDMA: 3,4-methylenedioxymethamphetamine

Metaphit: 1-[1-(3-isothiocyanato)phenyl]cyclohexylpiperidine

mGluR: metabotropic receptors

MIAs: mechanically insensitive afferents

MSAs: mechanically insensitive afferents

MK-801: dizocilpine

MS-377: (*R*)-(+)-1-(4-chlorophenyl)-3-[4-(2-methoxyethyl)piperazin-1-yl]me thyl-2-pyrrolidinone *L*-tartrate

MSAs: mechanically sensitive afferents

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

NGF: nerve growth factor

NK-1: neurokinin 1

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

NO: nitric oxide

NR1: NMDA receptor 1 subunit

OPC-14523: 1-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-5-methoxy-3,4-dihydro-2-quinolinone monomethanesulfonate

PAG: periaqueductal grey

PCA: *p*-chloroampheramine

PCP: phencyclidine

PCR: polymerase chain reaction

pERK: phosphorylated extracellular signal-regulated kinase

PET: positron emission tomography

PGE_2: prostaglandin E_2

PIP_2: phosphatidylinositol-4,5-bisphosphate

PKA: protein kinase A

PKC: protein kinase C

PKG: protein kinase G

PLC: phospholipase C

pNR1: phosphorylated NMDA receptor 1 subunit

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

PSDC: postsynaptic dorsal column

RVM: rostroventral medulla

s.c.: subcutaneuous

SA4503: 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride

SAM: senescence-accelerated mice

SERT: serotonin transporter

SK channels: calcium-activated K^+ channels

SKF-10,047: *N*-allylnormetazocine

SL-82.0715: eliprodil

SM-21: 3-α-tropanyl-2-(4-chlorophenoxy)butyrate

SP: substance P

SR31742A: cis-3-(hexahydroazepin-1-yl)1-(3-chloro-4- cyclohexylphenyl)propene-1

SRE: serum response element

SSRIs: selective serotonin reuptake inhibitors

STT : spinothalamic tract

TRPV-1: transient receptor potential vanilloid type 1

TRPV-2: transient-receptor potential vanilloid type 2

UMB23: (1-(3-phenylpropyl)piperidine oxalate)

VMpo: posterior portion of the ventromedial nucleus of the thalamus

VPL: ventroposterior lateral nucleus of the thalamus.

YZ-011: *N*-[2-(*m*-methoxyphenyl)ethyl]-*N*-methyl-2-(1-pyrrolodinyl)ethylamine

PARAMETERS

B: specific radioligand binding

B_{max}: maximum number of binding sites (receptors) labelled by a radioligand.

CT: cut-off time

ED_{50}: dose of drug producing half of the maximal antiallodynic effect

EF_{50}: force of mechanical stimuli applied producing half of the maximal reduction in paw withdrawal latency time

E_{max}: maximum antiallodynic effect

F: free concentration of radioligand.

IC_{50}: concentration of unlabeled drug that inhibited the specific binding of [^{3}H](+)-pentazocine by 50%

K_D: equilibrium dissociation constant, obtained from radioligand saturation assays. Concentration of radioligand that results in half-maximal specific binding. Indicative of the affinity of the radioligand for the receptor.

K_i: inhibition constant obtained from the radioligand K_D value and cold ligand IC_{50} value with the Cheng-Prussoff equation. Indicative of the affinity of the inhibitor for the receptor.

L: concentration of radioligand used in competition binding assays

LTD: latency time for paw withdrawal in drug-treated animals

LTO: latency time for paw withdrawal in oligodeoxynucleotide-treated animals

LTS is the latency time in solvent-treated animals

n_H: Hill coefficient, obtained from linear transformation of saturation assay data. Indicative of the population of binding sites bound by the radioligand.

BIBLIOGRAPHY

Aggleton JP, Keith AB, Rawlins JN, Hunt PR, Sahgal A. Removal of the hippocampus and transection of the fornix produce comparable deficits on delayed non-matching to position by rats. *Behav Brain Res* 52: 61-71, 1992

Akiyama K, Kanzaki A, Tsuchida K, Ujike H. Methamphetamine-induced behavioral sensitization and its implications for relapse of schizophrenia. *Schizophr Res* 12: 251-257, 1994

Ali Z, Meyer RA, Campbell JN. Secondary hyperalgesia to mechanical but not heat stimuli following a capsaicin injection in hairy skin. *Pain* 68: 401-411, 1996

Alonso G, Phan V, Guillemain I, Saunier M, Legrand A, Anoal M, Maurice T. Immunocytochemical localization of the $sigma_1$ receptor in the adult rat central nervous system. *Neuroscience* 97: 155-170, 2000

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

Bandler R, Keay KA. Columnar organization in the midbrain periaqueductal gray and the integration of emotional expression. *Prog Brain Res* 107: 285-300, 1996

Baron R, Baron Y, Disbrow E, Roberts TP. Brain processing of capsaicin-induced secondary hyperalgesia: a functional MRI study. *Neurology* 53: 548-557, 1999

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

Bartus RT. On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. *Exp Neurol* 163: 495-529, 2000

Baulieu EE. Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology* 23: 963-987, 1998

Baumann TK, Simone DA, Shain CN, LaMotte RH. Neurogenic hyperalgesia: the search for the primary cutaneous afferent fibers that contribute to capsaicin-induced pain and hyperalgesia. *J Neurophysiol* 66: 212-227, 1991

Baumgärtner U, Magerl W, Klein T, Hopf HC, Treede RD. Neurogenic hyperalgesia versus painful hypoalgesia: two distinct mechanisms of neuropathic pain. *Pain* 96: 141-151, 2002

Bergeron R, de Montigny C, Debonnel G. Pregnancy reduces brain sigma receptor function. *Br J Pharmacol* 127: 1769-1776, 1999

Bermack JE, Debonnel G. Modulation of serotonergic neurotransmission by short- and long-term treatments with sigma ligands. *Br J Pharmacol* 134: 691-699, 2001

Bermack JE, Debonnel G. The role of sigma receptors in depression. *J Pharmacol Sci* 97: 317-336, 2005a

Bermack JE, Debonnel G. Distinct modulatory roles of sigma receptor subtypes on glutamatergic responses in the dorsal hippocampus. *Synapse* 55: 37-44, 2005b

Bermack JE, Debonnel G. Effects of OPC-14523, a combined sigma and 5-HT1a ligand, on pre- and post-synaptic 5-HT1a receptors. *J Psychopharmacol* 21: 85-92, 2007

Bessou P, Perl ER. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J Neurophysiol* 32: 1025-1043, 1969

Bevan S, Szolcsanyi J. Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol Sci* 11: 330-333, 1990

Bevan S, Hothi S, Hughes G, James IF, Rang HP, Shah K, Walpole CS, Yeats JC. Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br J Pharmacol* 107: 544-552, 1992

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* 35: 721-731, 2002

Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci U S A* 100: 12480-12485, 2003

Bhave G, Gereau RW. Posttranslational mechanisms of peripheral sensitization. *J Neurobiol* 61: 88-106, 2004

Bingham S, Beswick PJ, Bountra C, Brown T, Campbell IB, Chessell IP, Clayton N, Collins SD, Davey PT, Goodland H, Gray N, Haslam C, Hatcher JP, Hunter AJ, Lucas F, Murkitt G, Naylor A, Pickup E, Sargent B, Summerfield SG, Stevens A, Stratton SC, Wiseman J. The cyclooxygenase-2 inhibitor GW406381X [2-(4-ethoxyphenyl)-3-[4-(methylsulfonyl)phenyl]-pyrazolo[1,5-b]pyridazine] is effective in animal models of neuropathic pain and central sensitization. *J Pharmacol Exp Ther* 312: 1161-1169, 2005

Bluth LS, Rice KC, Jacobson AE, Bowen WD. Acylation of σ receptors by Metaphit, an isothiocyanate derivative of phencyclidine. *Eur J Pharmacol* 161: 273-277, 1989

Bowen WD, Hellewell SB, McGarry KA. Evidence for a multi-site model of the rat brain σ receptor. *Eur J Pharmacol* 163: 309-318, 1989

Bowen WD, Moses EL, Tolentino PJ, Walker JM. Metabolites of haloperidol display preferential activity at sigma receptors compared to dopamine D-2 receptors. *Eur J Pharmacol* 177: 111-118, 1990

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

Brammer MK, Gilmore DL, Matsumoto RR. Interactions between 3,4-methylenedioxymethamphetamine and σ_1 receptors. *Eur J Pharmacol* 553: 141-145, 2006

Bucolo C, Marrazzo A, Ronsisvalle S, Ronsisvalle G, Cuzzocrea S, Mazzon E, Caputi A, Drago F. A novel adamantane derivative attenuates retinal ischemia-reperfusion damage in the rat retina through σ_1 receptors. *Eur J Pharmacol* 536: 200-203, 2006

Budai D, Khasabov SG, Mantyh PW, Simone DA. NK-1 receptors modulate the excitability of ON cells in the rostral ventromedial medulla. *J Neurophysiol* 97: 1388-1395, 2007

Burstein R, Potrebic S. Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. *J Comp Neurol* 335: 469-485, 1993

Byers MR, Bonica JJ. Peripheral pain mechanisms and nociceptor plasticity. *In: Bonica's Management of Pain, 3rd ed.,* edited by *Loeser JD, Butler SH, Chapman CR, Turk DC. Philadelphia: Lippincott, Williams & Wilkins*, p. 26-72, 2001

Cagnotto A, Bastone A, Mennini T. [^{3}H](+)-pentazocine binding to rat brain sigma$_1$ receptors. *Eur J Pharmacol* 266: 131-138, 1994

Cain DM, Khasabov SG, Simone DA. Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an in vivo study. *J Neurophysiol* 85: 1561-1574, 2001

Calabrese EJ, Baldwin LA. Hormesis: the dose-response revolution. *Annu Rev Pharmacol Toxicol* 43: 175-197, 2003

Calderon SN, Izenwasser S, Heller B, Gutkind JS, Mattson MV, Su TP, Newman AH. Novel 1-phenylcycloalkanecarboxylic acid derivatives are potent and selective σ_1 ligands. *J Med Chem* 37: 2285-2291, 1994

Calvino B, Grilo RM. Central pain control. *Joint Bone Spine* 73: 10-16, 2006

Campana G, Bucolo C, Murari G, Spampinato S. Ocular hypotensive action of topical flunarizine in the rabbit: role of σ_1 recognition sites. *J Pharmacol Exp Ther* 303: 1086-1094, 2002

Campbell JN, Meyer RA, LaMotte RH. Sensitization of myelinated nociceptive afferents that innervate monkey hand. *J Neurophysiol* 42: 1669-1679, 1979

Carpenter D, Engberg I, Lundberg A. Differential supraspinal control of inhibitory and excitatory actions from the fra to ascending spinal pathways. *Acta Physiol Scand* 63: 103-110, 1965

Castner SA, Goldman-Rakic PS, Williams GV. Animal models of working memory: insights for targeting cognitive dysfunction in schizophrenia. *Psychopharmacology (Berl)* 174: 111-125, 2004

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389: 816-824, 1997

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398: 436-441, 1999

Caterina MJ, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. *Annu Rev Neurosci* 24: 487-517, 2001

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

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

Chaki S, Tanaka M, Muramatsu M, Otomo S. NE-100, a novel potent σ ligand, preferentially binds to σ_1 binding sites in guinea pig brain. *Eur J Pharmacol* 251: R1-R2, 1994

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, Zhang D. Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* 415: 793-798, 2002

Chavez-Noriega LE, Marino MJ, Schaffhauser H, Campbell HUC, Conn PJ. Novel Potential Therapeutics for Schizophrenia: Focus on the Modulation of Metabotropic Glutamate Receptor Function. *Curr Neuropharmacol* 3: 9-34, 2005

Chen HS, Lipton SA. The chemical biology of clinically tolerated NMDA receptor antagonists. *J Neurochem* 97: 1611-1626, 2006

Chen L, Dai XN, Sokabe M. Chronic administration of dehydroepiandrosterone sulfate (DHEAS) primes for facilitated induction of long-term potentiation via sigma 1 (σ_1) receptor: optical imaging study in rat hippocampal slices. *Neuropharmacology* 50: 380-392, 2006

Chen Y, Hajipour AR, Sievert MK, Arbabian M, Ruoho AE. Characterization of the cocaine binding site on the sigma-1 receptor. *Biochemistry* 46: 3532-3542, 2007

Chien CC, Pasternak GW. Functional antagonism of morphine analgesia by (+)-pentazocine: evidence for an anti-opioid σ_1 system. *Eur J Pharmacol* 250: R7-R8, 1993

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

Chien CC, Pasternak GW. Sigma antagonists potentiate opioid analgesia in rats. *Neurosci Lett* 190: 137-139, 1995a

Chien CC, Pasternak GW. (-)-Pentazocine analgesia in mice: interactions with a sigma receptor system. *Eur J Pharmacol* 294: 303-308, 1995b

Choi SS, Seo YJ, Shim EJ, Kwon MS, Lee JY, Ham YO, Suh HW. Involvement of phosphorylated Ca^{2+}/calmodulin-dependent protein kinase II and phosphorylated extracellular signal-regulated protein in the mouse formalin pain model. *Brain Res* 1108: 28-38, 2006

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

Cobos EJ, Lucena G, Baeyens JM, del Pozo E. Differences in the allosteric modulation by phenytoin of the binding properties of the sigma1 ligands [^{3}H](+)-pentazocine and [^{3}H]NE-100. *Synapse* 59: 152-161, 2006

Cobos EJ, del Pozo E, Baeyens JM. 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, 2007

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

Cobos EJ, Entrena JM, Nieto FR, Sánchez C, Del Pozo E, Baeyens JM. Irreversible blockade of sigma-1 receptors by reduced haloperidol induced a long-lasting antiallodynic effect in mice. *Methods Find Exp Clin Pharmacol* 30: 179, 2008b

Collina S, Loddo G, Urbano M, Linati L, Callegari A, Ortuso F, Alcaro S, Laggner C, Langer T, Prezzavento O, Ronsisvalle G, Azzolina O. Design, synthesis, and SAR analysis of novel selective σ_1 ligands. *Bioorg Med Chem* 15: 771-783, 2007

Cormaci G, Mori T, Hayashi T, Su TP. Protein kinase A activation down-regulates, whereas extracellular signal-regulated kinase activation up-regulates σ-1 receptors in B-104 cells: Implication for neuroplasticity. *J Pharmacol Exp Ther* 320: 202-210, 2007

Coughenour LL, Cordon JJ. Characterization of haloperidol and trifluperidol as subtype-selective *N*-methyl-*D*-aspartate (NMDA) receptor antagonists using [^{3}H]TCP and [^{3}H]ifenprodil binding in rat brain membranes. *J Pharmacol Exp Ther* 280: 584-592, 1997

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

Crooke ST. Progress in antisense technology. *Annu Rev Med* 55: 61-95, 2004

Cuesta MC, Arcaya JL, Cano G, Sanchez L, Maixner W, Suarez-Roca H. Opposite modulation of capsaicin-evoked substance P release by glutamate receptors. *Neurochem Int* 35: 471-478, 1999

Dado RJ, Katter JT, Giesler GJ, Jr. Spinothalamic and spinohypothalamic tract neurons in the cervical enlargement of rats. I. Locations of antidromically identified axons in the thalamus and hypothalamus. *J Neurophysiol* 71: 959-980, 1994

Daniels A, Ayala E, Chen W, Coop A, Matsumoto RR. *N*-[2-(m-methoxyphenyl)ethyl]-*N*-ethyl-2-(1-pyrrolidinyl)ethylamine (UMB 116) is a novel antagonist for cocaine-induced effects. *Eur J Pharmacol 542: 61-68*, 2006

Davis KD, Meyer RA, Campbell JN. Chemosensitivity and sensitization of nociceptive afferents that innervate the hairy skin of monkey. *J Neurophysiol* 69: 1071-1081, 1993

De Biasi S, Rustioni A. Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci U S A* 85: 7820-7824, 1988

Dean NM. Functional genomics and target validation approaches using antisense oligonucleotide technology. *Curr Opin Biotechnol* 12: 622-625, 2001

Debonnel G, de Montigny C. Modulation of NMDA and dopaminergic neurotransmissions by sigma ligands: possible implications for the treatment of psychiatric disorders. *Life Sci* 58: 721-734, 1996

DeHaven-Hudkins DL, Lanyon LF, Ford-Rice FY, Ator MA. σ recognition sites in brain and peripheral tissues. Characterization and effects of cytochrome P450 inhibitors. *Biochem Pharmacol* 47: 1231-1239, 1994

DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* 235: E97-102, 1978

Delgado PL, Moreno FA. Role of norepinephrine in depression. *J Clin Psychiatry* 61 Suppl 1: 5-12, 2000

Depatie L, Lal S. Apomorphine and the dopamine hypothesis of schizophrenia: a dilemma? *J Psychiatry Neurosci* 26: 203-220, 2001

Dhir A, Kulkarni SK. Involvement of sigma-1 receptor modulation in the antidepressant action of venlafaxine. *Neurosci Lett* 420: 204-208, 2007

Dias N, Stein CA. Antisense oligonucleotides: basic concepts and mechanisms. *Mol Cancer Ther* 1: 347-355, 2002

Djouhri L, Lawson SN. Aβ-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. *Brain Res Brain Res Rev* 46: 131-145, 2004

Dong LY, Cheng ZX, Fu YM, Wang ZM, Zhu YH, Sun JL, Dong Y, Zheng P. Neurosteroid dehydroepiandrosterone sulfate enhances spontaneous glutamate release in rat prelimbic cortex through activation of dopamine D1 and sigma-1 receptor. *Neuropharmacology* 52: 966-974, 2007

Dougherty PM, Willis WD. Enhanced responses of spinothalamic tract neurons to excitatory amino acids accompany capsaicin-induced sensitization in the monkey. *J Neurosci* 12: 883-894, 1992

Dussossoy D, Carayon P, Belugou S, Feraut D, Bord A, Goubet C, Roque C, Vidal H, Combes T, Loison G, Casellas P. Colocalization of sterol isomerase and $sigma_1$ receptor at endoplasmic reticulum and nuclear envelope level. *Eur J Biochem* 263: 377-386, 1999

Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice AS, Stacey BR, Treede RD, Turk DC, Wallace MS. Pharmacologic management of neuropathic pain: evidence-based recommendations. *Pain* 132: 237-251, 2007

Egashira N, Harada S, Okuno R, Matsushita M, Nishimura R, Mishima K, Iwasaki K, Orito K, Fujiwara M. Involvement of the $sigma_1$ receptor in inhibiting activity of fluvoxamine on marble-burying behavior: comparison with paroxetine. *Eur J Pharmacol* 563: 149-154, 2007

Engelman HS, Allen TB, Macdermott AB. The distribution of neurons expressing calcium-permeable AMPA receptors in the superficial laminae of the spinal cord dorsal horn. *J Neurosci* 19: 2081-2089, 1999

Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Baeyens JM, Del Pozo E. Antagonism by haloperidol and its metabolites of mechanical hypersensitivity induced by intraplantar capsaicin in mice: role of sigma-1 receptor. *Psychopharmacol.* (In press), 2009a

Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Gris G, Del Pozo E, Zamanillo D, Baeyens JM, E. Sigma-1 receptors are Essentials for capsaicin-induced mechanical hypersensitivity: Studies with selective sigma-1 ligands and sigma-1 knockout mice. *Pain* (In press) 2009a

Erlanger J, Gasser HS. The compound nature of the action current of nerve as disclosed by the cathode ray oscillograph. *Am J Physiol* 70: 624-666, 1924

Erlanger J, Gasser HS. The action potential in fibers of slow conduction in spinal roots and somatic nerves. *Am J Physiol* 92: 43-82, 1930

Fang L, Wu J, Lin Q, Willis WD. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *J Neurosci* 22: 4196-4204, 2002

Fang L, Wu J, Lin Q, Willis WD. Protein kinases regulate the phosphorylation of the GluR1 subunit of AMPA receptors of spinal cord in rats following noxious stimulation. *Brain Res Mol Brain Res* 118: 160-165, 2003

Ferrer-Montiel A, Garcia-Martinez C, Morenilla-Palao C, Garcia-Sanz N, Fernandez-Carvajal A, Fernandez-Ballester G, Planells-Cases R. Molecular architecture of the vanilloid receptor. Insights for drug design. *Eur J Biochem* 271: 1820-1826, 2004

Field MJ, Holloman EF, McCleary S, Hughes J, Singh L. Evaluation of gabapentin and S-(+)-3-isobutylgaba in a rat model of postoperative pain. *J Pharmacol Exp Ther* 282: 1242-1246, 1997

Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci* 14: 219-245, 1991

Fields HL, Malick A, Burstein R. Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *J Neurophysiol* 74: 1742-1759, 1995

Fleckenstein AE, Volz TJ, Riddle EL, Gibb JW, Hanson GR. New insights into the mechanism of action of amphetamines. *Annu Rev Pharmacol Toxicol* 47: 681-698, 2007

Freedman SB, Patel S, Marwood R, Emms F, Seabrook GR, Knowles MR, McAllister G. Expression and pharmacological characterization of the human D_3 dopamine receptor. *J Pharmacol Exp Ther* 268: 417-426, 1994

Furuyama T, Kiyama H, Sato K, Park HT, Maeno H, Takagi H, Tohyama M. Region-specific expression of subunits of ionotropic glutamate receptors (AMPA-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociception. *Brain Res Mol Brain Res* 18: 141-151, 1993

Galan A, Laird JM, Cervero F. In vivo recruitment by painful stimuli of AMPA receptor subunits to the plasma membrane of spinal cord neurons. *Pain* 112: 315-323, 2004

Gao X, Kim HK, Chung JM, Chung K. Enhancement of NMDA receptor phosphorylation of the spinal dorsal horn and nucleus gracilis neurons in neuropathic rats. *Pain* 116: 62-72, 2005

Garcia-Sanz N, Fernandez-Carvajal A, Morenilla-Palao C, Planells-Cases R, Fajardo-Sanchez E, Fernandez-Ballester G, Ferrer-Montiel A. Identification of a tetramerization domain in the C terminus of the vanilloid receptor. *J Neurosci* 24: 5307-5314, 2004

Gardner B, Zhu LX, Roth MD, Tashkin DP, Dubinett SM, Sharma S. Cocaine modulates cytokine and enhances tumor growth through sigma receptors. *J Neuroimmunol* 147: 95-98, 2004

Garrone B, Magnani M, Pinza M, Polenzani L. Effects of trazodone on neurotransmitter release from rat mossy fibre cerebellar synaptosomes. *Eur J Pharmacol* 400: 35-41, 2000

Gauriau C, Bernard JF. Pain pathways and parabrachial circuits in the rat. *Exp Physiol* 87: 251-258, 2002

Gauriau C, Bernard JF. A comparative reappraisal projections from the superficial laminae of the dorsal horn in rat: the forebrain. *J Comp Neurol* 468: 24-56, 2004

Gear RW, Aley KO, Levine JD. Pain-induced analgesia mediated by mesolimbic reward circuits. *J Neurosci* 19: 7175-7181, 1999

Gear RW, Lee JS, Miaskowski C, Gordon NC, Paul SM, Levine JD. Neuroleptics antagonize nalbuphine antianalgesia. *J Pain* 7: 187-191, 2006

Gekker G, Hu S, Sheng WS, Rock RB, Lokensgard JR, Peterson PK. Cocaine-induced HIV-1 expression in microglia involves sigma-1 receptors and transforming growth factor- β1. *Int Immunopharmacol* 6: 1029-1033, 2006

Georgopoulos AP. Functional properties of primary afferent units probably related to pain mechanisms in primate glabrous skin. *J Neurophysiol* 39: 71-83, 1976

Gewirtz GR, Gorman JM, Volavka J, Macaluso J, Gribkoff G, Taylor DP, Borison R. BMY 14802, a sigma receptor ligand for the treatment of schizophrenia. *Neuropsychopharmacology* 10: 37-40, 1994

Gilchrist HD, Allard BL, Simone DA. Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. *Pain* 67: 179-188, 1996

Gottrup H, Juhl G, Kristensen AD, Lai R, Chizh BA, Brown J, Bach FW, Jensen TS. Chronic oral gabapentin reduces elements of central sensitization in human experimental hyperalgesia. *Anesthesiology* 101: 1400-1408, 2004

Govindarajan VS. Capsicum--production, technology, chemistry, and quality--Part II. Processed products, standards, world production and trade. *Crit Rev Food Sci Nutr* 23: 207-288, 1986

Green AR, Mechan AO, Elliott JM, O'Shea E, Colado MI. The pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"). *Pharmacol Rev* 55: 463-508, 2003

Greene LA, Kaplan DR. Early events in neurotrophin signalling via Trk and p75 receptors. *Curr Opin Neurobiol* 5: 579-587, 1995

Gue M, Junien JL, Del Rio C, Bueno L. Neuropeptide Y and sigma ligand (JO 1784) suppress stress-induced colonic motor disturbances in rats through *sigma* and cholecystokinin receptors. *J Pharmacol Exp Ther* 261: 850-855, 1992

Guitart X, Codony X, Ballarín M, Dordal A, Farré AJ. E-5842: a new potent and preferential σ ligand: preclinical pharmacological profile. *CNS Drug Rev* 4: 201-224, 1998

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

Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL, Stein CA. Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J Biol Chem* 270: 2620-2627, 1995

Hagbarth KE, Kerr DI. Central influences on spinal afferent conduction. *J Neurophysiol* 17: 295-307, 1954

Hagen NA, du SP, Lapointe B, Ong-Lam M, Dubuc B, Walde D, Love R, Ngoc AH. Tetrodotoxin for moderate to severe cancer pain: a randomized, double blind, parallel design multicenter study. *J Pain Symptom Manage* 35: 420-429, 2008

Haley TJ, Mccormick WG. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol Chemother* 12: 12-15, 1957

Han DW, Kweon TD, Lee JS, Lee YW. Antiallodynic effect of pregabalin in rat models of sympathetically maintained and sympathetic independent neuropathic pain. *Yonsei Med J* 48: 41-47, 2007

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

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E, Glossmann H. Purification, molecular cloning, and expression of the mammalian $sigma_1$-binding site. *Proc Natl Acad Sci U S A* 93: 8072-8077, 1996

Hardy JC. Techniques of measuring intraoral air pressure and rate of airflow. *J Speech Hear Res* 10: 650-654, 1967

Harteneck C, Plant TD, Schultz G. From worm to man: three subfamilies of TRP channels. *Trends Neurosci* 23: 159-166, 2000

Hashimoto K, London ED. Interactions of *erythro*-ifenprodil, *threo*-ifenprodil, *erythro*-iodoifenprodil, and eliprodil with subtypes of σ receptors. *Eur J Pharmacol* 273: 307-310, 1995

Hashimoto K, Fujita Y, Shimizu E, Iyo M. Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of clozapine, but not haloperidol. *Eur J Pharmacol* 519: 114-117, 2005

Hashimoto K, Fujita Y, Iyo M . Phencyclidine-Induced Cognitive Deficits in Mice are Improved by Subsequent Subchronic Administration of Fluvoxamine: Role of sigma-1 Receptors. *Neuropsychopharmacology* 32: 514-522, 2006

Hayashi T, Maurice T, Su TP. Ca^{2+} signaling via σ_1 -receptors: novel regulatory mechanism affecting intracellular Ca^{2+} concentration. *J Pharmacol Exp Ther* 293: 788-798, 2000

Hayashi T, Su TP. Regulating ankyrin dynamics: Roles of sigma-1 receptors. *Proc Natl Acad Sci U S A* 98: 491-496, 2001

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

Hayashi T, Su TP. The potential role of sigma-1 receptors in lipid transport and lipid raft reconstitution in the brain: implication for drug abuse. *Life Sci* 77: 1612-1624, 2005a

Hayashi T, Su T. The sigma receptor: evolution of the concept in neuropsychopharmacology. *Curr Neuropharmacol* 3: 267-280, 2005b

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

Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. Rat liver and kidney contain high densities of σ_1 and σ_2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur J Pharmacol* 268: 9-18, 1994

Hendry IA, Stach R, Herrup K . Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. *Brain Res* 82: 117-128, 1974

Herbert H, Saper CB. Organization of medullary adrenergic and noradrenergic projections to the periaqueductal gray matter in the rat. *J Comp Neurol* 315: 34-52, 1992

Hiramatsu M, Hoshino T, Kameyama T, Nabeshima T. Involvement of *k*-opioid and σ receptors in short-term memory in mice. *Eur J Pharmacol* 453: 91-98, 2002

Hiramatsu M, Hoshino T. Involvement of kappa-opioid receptors and sigma receptors in memory function demonstrated using an antisense strategy. *Brain Res* 1030: 247-255, 2004

Hofner G, Wanner KT. [^{3}H]ifenprodil binding to NMDA receptors in porcine hippocampal brain membranes. *Eur J Pharmacol* 394: 211-219, 2000

Holzer P. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* 43: 143-201, 1991

Honda K, Koga K, Moriyama T, Koguchi M, Takano Y, Kamiya HO. Intrathecal α_2 adrenoceptor agonist clonidine inhibits mechanical transmission in mouse spinal cord via activation of muscarinic M_1 receptors. *Neurosci Lett* 322: 161-164, 2002

Hong W, Werling LL. Binding of σ receptor ligands and their effects on muscarine-induced Ca^{2+} changes in SH-SY5Y cells. *Eur J Pharmacol* 436: 35-45, 2002

Hong W, Nuwayhid SJ, Werling LL. Modulation of bradykinin-induced calcium changes in SH-SY5Y cells by neurosteroids and sigma receptor ligands via a shared mechanism. *Synapse* 54: 102-110, 2004

Honkaniemi J, Liimatainen S, Rainesalo S, Sulavuori S. Haloperidol in the acute treatment of migraine: a randomized, double-blind, placebo-controlled study. *Headache* 46: 781-787, 2006

Horan B, Gifford AN, Matsuno K, Mita S, Ashby CR, Jr. Effect of SA4503 on the electrically evoked release of ^{3}H-acetylcholine from striatal and hippocampal rat brain slices. *Synapse* 46: 1-3, 2002

Iadarola MJ, Berman KF, Zeffiro TA, Byas-Smith MG, Gracely RH, Max MB, Bennett GJ. Neural activation during acute capsaicin-evoked pain and allodynia assessed with PET. *Brain* 121 (Pt 5): 931-947, 1998

Iannetti GD, Zambreanu L, Wise RG, Buchanan TJ, Huggins JP, Smart TS, Vennart W, Tracey I. Pharmacological modulation of pain-related brain activity during normal and central sensitization states in humans. *Proc Natl Acad Sci U S A* 102: 18195-18200, 2005

Introini-Collison IB, McGaugh JL. Cocaine enhances memory storage in mice. *Psychopharmacology (Berl)* 99: 537-541, 1989

Ishiwata K, Oda K, Sakata M, Kimura Y, Kawamura K, Oda K, Sasaki T, Naganawa M, Chihara K, Okubo Y, Ishii K. A feasibility study of [^{11}C]SA4503-PET for evaluating sigma$_1$ receptor occupancy by neuroleptics: the binding of haloperidol to sigma1 and dopamine D_2-like receptors. *Ann Nucl Med* 20: 569-573, 2006

Ishikawa M, Ishiwata K, Ishii K, Kimura Y, Sakata M, Naganawa M, Oda K, Miyatake R, Fujisaki M, Shimizu E, Shirayama Y, Iyo M, Hashimoto K. High Occupancy of Sigma-1 Receptors in the Human Brain after Single Oral Administration of Fluvoxamine: A Positron Emission Tomography Study Using [^{11}C]SA4503. *Biol Psychiatry* 62: 878-883, 2007

Itzhak Y, Stein I, Zhang SH, Kassim CO, Cristante D. Binding of σ-ligands to C57BL/6 mouse brain membranes: effects of monoamine oxidase inhibitors and subcellular distribution studies suggest the existence of sigma-receptor subtypes. *J Pharmacol Exp Ther* 257: 141-148, 1991

Jaen JC, Caprathe BW, Pugsley TA, Wise LD, Akunne H. Evaluation of the effects of the enantiomers of reduced haloperidol, azaperol, and related 4-amino-1-arylbutanols on dopamine and σ receptors. *J Med Chem* 36: 3929-3936, 1993

Jancso N, Jancso-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br J Pharmacol Chemother* 31: 138-151, 1967

Jancso N, Jancso-Gabor A, Szolcsanyi J. The role of sensory nerve endings in neurogenic inflammation induced in human skin and in the eye and paw of the rat. *Br J Pharmacol Chemother* 33: 32-41, 1968

Ji RR, Woolf CJ. Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol Dis* 8: 1-10, 2001

Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 36: 57-68, 2002

Ji RR, Kohno T, Moore KA, Woolf CJ. Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci* 26: 696-705, 2003

Jiang G, Mysona B, Dun Y, Gnana-Prakasam JP, Pabla N, Li W, Dong Z, Ganapathy V, Smith SB. Expression, subcellular localization, and regulation of sigma receptor in retinal muller cells. *Invest Ophthalmol Vis Sci* 47: 5576-5582, 2006

John CS, Vilner BJ, Bowen WD . Synthesis and characterization of [^{125}I]-N-(N-benzylpiperidin-4-yl)-4- iodobenzamide, a new σ receptor radiopharmaceutical: high-affinity binding to MCF-7 breast tumor cells. *J Med Chem* 37: 1737-1739, 1994

Joshi SK, Hernandez G, Mikusa JP, Zhu CZ, Zhong C, Salyers A, Wismer CT, Chandran P, Decker MW, Honore P. Comparison of antinociceptive actions of standard analgesics in attenuating capsaicin and nerve-injury-induced mechanical hypersensitivity. *Neuroscience* 143: 587-596, 2006

Julius D, Basbaum AI. Molecular mechanisms of nociception. *Nature* 413: 203-210, 2001

Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, Oh U. Phosphorylation of vanilloid receptor 1 by Ca2+/calmodulin-dependent kinase II regulates its vanilloid binding. *J Biol Chem* 279: 7048-7054, 2004

Kalasinsky KS, Bosy TZ, Schmunk GA, Ang L, Adams V, Gore SB, Smialek J, Furukawa Y, Guttman M, Kish SJ. Regional distribution of cocaine in postmortem brain of chronic human cocaine users. *J Forensic Sci* 45: 1041-1048, 2000

Kamei H, Kameyama T, Nabeshima T. (+)-SKF-10,047 and dextromethorphan ameliorate conditioned fear stress through the activation of phenytoin-regulated σ_1 sites. *Eur J Pharmacol* 299: 21-28, 1996

Kamei H, Noda Y, Kameyama T, Nabeshima T. Role of (+)-SKF-10,047-sensitive sub-population of σ_1 receptors in amelioration of conditioned fear stress in rats: association with mesolimbic dopaminergic systems. *Eur J Pharmacol* 319: 165-172, 1997

Kangrga I, Randic M. Outflow of endogenous aspartate and glutamate from the rat spinal dorsal horn in vitro by activation of low- and high-threshold primary afferent fibers. Modulation by mu-opioids. *Brain Res* 553: 347-352, 1991

Katnik C, Guerrero WR, Pennypacker KR, Herrera Y, Cuevas J. Sigma-1 receptor activation prevents intracellular calcium dysregulation in cortical neurons during in vitro ischemia. *J Pharmacol Exp Ther* 319: 1355-1365, 2006

Kato K, Hayako H, Ishihara Y, Marui S, Iwane M, Miyamoto M. TAK-147, an acetylcholinesterase inhibitor, increases choline acetyltransferase activity in cultured rat septal cholinergic neurons. *Neurosci Lett* 260: 5-8, 1999

Katz JL, Libby TA, Kopajtic T, Husbands SM, Newman AH. Behavioral effects of rimcazole analogues alone and in combination with cocaine. *Eur J Pharmacol* 468: 109-119, 2003

Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der MC, Befort K, Woolf CJ, Ji RR. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J Neurosci* 24: 8310-8321, 2004

Kekuda R, Prasad PD, Fei YJ, Leibach FH, Ganapathy V. Cloning and functional expression of the human type 1 sigma receptor (hSigmaR1). *Biochem Biophys Res Commun* 229: 553-558, 1996

Kew JN, Kemp JA. Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl)* 179: 4-29, 2005

Khandwala H, Hodge E, Loomis CW. Comparable dose-dependent inhibition of AP-7 sensitive strychnine-induced allodynia and paw pinch-induced nociception by mexiletine in the rat. *Pain* 72: 299-308, 1997

Khasabov SG, Rogers SD, Ghilardi JR, Peters CM, Mantyh PW, Simone DA. Spinal neurons that possess the substance P receptor are required for the development of central sensitization. *J Neurosci* 22: 9086-9098, 2002

Kibaly C, Meyer L, Patte-Mensah C, Mensah-Nyagan AG. Biochemical and functional evidence for the control of pain mechanisms by dehydroepiandrosterone endogenously synthesized in the spinal cord. *Faseb J 22: 93-104,* 2007

Kim HW, Kwon YB, Roh DH, Yoon SY, Han HJ, Kim KW, Beitz AJ, Lee JH. 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, 2006

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

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

Kitaichi K, Chabot JG, Moebius FF, Flandorfer A, Glossmann H, Quirion R. 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, 2000

Klein M, Cooper TB, Musacchio JM. Effects of haloperidol and reduced haloperidol on binding to σ sites. *Eur J Pharmacol* 254: 239-248, 1994

Klein T, Magerl W, Rolke R, Treede RD. Human surrogate models of neuropathic pain. *Pain* 115: 227-233, 2005

Ko J, Na DS, Lee YH, Shin SY, Kim JH, Hwang BG, Min BI, Park DS. cDNA microarray analysis of the differential gene expression in the neuropathic pain and electroacupuncture treatment models. *J Biochem Mol Biol* 35: 420-427, 2002

Kofman O. The role of prenatal stress in the etiology of developmental behavioural disorders. *Neurosci Biobehav Rev* 26: 457-470, 2002

Kurtz MM. Neurocognitive impairment across the lifespan in schizophrenia: an update. *Schizophr Res* 74: 15-26, 2005

Lai JC, Tan W, Benimetskaya L, Miller P, Colombini M, Stein CA. A pharmacologic target of G3139 in melanoma cells may be the mitochondrial VDAC. *Proc Natl Acad Sci U S A* 103: 7494-7499, 2006

LaMotte RH, Shain CN, Simone DA, Tsai EF. Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. *J Neurophysiol* 66: 190-211, 1991

LaMotte RH, Lundberg LE, Torebjork HE. Pain, hyperalgesia and activity in nociceptive C units in humans after intradermal injection of capsaicin. *J Physiol* 448: 749-764, 1992

Langa F, Codony X, Tovar V, Lavado A, Gimenez E, Cozar P, Cantero M, Dordal A, Hernandez E, Perez R, Monroy X, Zamanillo D, Guitart X, Montoliu L. Generation and phenotypic analysis of sigma receptor type I (σ1) knockout mice. *Eur J Neurosci* 18: 2188-2196, 2003

Law AJ, Deakin JF. Asymmetrical reductions of hippocampal NMDAR1 glutamate receptor mRNA in the psychoses. *Neuroreport* 12: 2971-2974, 2001

Lawson SN. Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. *Exp Physiol* 87: 239-244, 2002

LePage KT, Ishmael JE, Low CM, Traynelis SF, Murray TF. Differential binding properties of [^{3}H]dextrorphan and [^{3}H]MK-801 in heterologously expressed NMDA receptors. *Neuropharmacology* 49: 1-16, 2005

Lewis RW. Less common lesions of the astragalus. *Ann Surg* 116: 891-897, 1942

Li D, Ren Y, Xu X, Zou X, Fang L, Lin Q. Sensitization of Primary Afferent Nociceptors Induced by intradermal capsaicin involves the peripheral release of calcitonin Gene-Related Peptide Driven by Dorsal Root Reflexes. *J Pain* , 2008

Li Z, Zhou R, Cui S, Xie G, Cai W, Sokabe M, Chen L. Dehydroepiandrosterone sulfate prevents ischemia-induced impairment of long-term potentiation in rat hippocampal CA1 by up-regulating tyrosine phosphorylation of NMDA receptor. *Neuropharmacology* 51: 958-966, 2006

Lin Q, Peng YB, Wu J, Willis WD. Involvement of cGMP in nociceptive processing by and sensitization of spinothalamic neurons in primates. *J Neurosci* 17: 3293-3302, 1997

Lin Q, Wu J, Willis WD. Dorsal root reflexes and cutaneous neurogenic inflammation after intradermal injection of capsaicin in rats. *J Neurophysiol* 82: 2602-2611, 1999

Lin YF, Angelotti TP, Dudek EM, Browning MD, Macdonald RL. Enhancement of recombinant alpha 1 beta 1 gamma 2L gamma-aminobutyric acidA receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both beta 1 and gamma 2L subunits. *Mol Pharmacol* 50: 185-195, 1996

Liu D. An experimental model combining microdialysis with electrophysiology, histology, and neurochemistry for studying excitotoxicity in spinal cord injury. Effect of NMDA and kainate. *Mol Chem Neuropathol* 23: 77-92, 1994

Liu M, Max MB, Robinovitz E, Gracely RH, Bennett GJ. The human capsaicin model of allodynia and hyperalgesia: sources of variability and methods for reduction. *J Pain Symptom Manage* 16: 10-20, 1998

Liu X, Banister SD, Christie MJ, Banati R, Meikle S, Coster MJ, Kassiou M. Trishomocubanes: novel σ ligands modulate cocaine-induced behavioural effects. *Eur J Pharmacol* 555: 37-42, 2007

Liu Y, Chen GD, Lerner MR, Brackett DJ, Matsumoto RR. Cocaine up-regulates Fra-2 and σ-1 receptor gene and protein expression in brain regions involved in addiction and reward. *J Pharmacol Exp Ther* 314: 770-779, 2005

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951

Lupardus PJ, Wilke RA, Aydar E, Palmer CP, Chen Y, Ruoho AE, Jackson MB. Membrane-delimited coupling between sigma receptors and K^+ channels in rat neurohypophysial terminals requires neither G-protein nor ATP. *J Physiol* 526 Pt 3: 527-539, 2000

Lynn B, Perl ER. A comparison of four tests for assessing the pain sensitivity of different subjects and test areas. *Pain* 3: 353-365, 1977

Lynn B, Carpenter SE. Primary afferent units from the hairy skin of the rat hind limb. *Brain Res* 238: 29-43, 1982

Lynn B, Schütterle S, Pierau SK. The vasodilator component of neurogenic inflammation is caused by a special subclass of heat-sensitive nociceptors in the skin of the pig. *J Physiol* 15: 587-593, 1996

Malinow R, Malenka RC. AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* 25: 103-126, 2002

Mannion RJ, Costigan M, Decosterd I, Amaya F, Ma QP, Holstege JC, Ji RR, Acheson A, Lindsay RM, Wilkinson GA, Woolf CJ. Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc Natl Acad Sci U S A* 96: 9385-9390, 1999

Marrazzo A, Prezzavento O, Pappalardo MS, Bousquet E, Iadanza M, Pike VW, Ronsisvalle G. Synthesis of (+)- and (-)-cis-2-[(1-adamantylamino)-methyl]-1-phenylcyclopropane derivatives as high affinity probes for σ_1 and σ_2 binding sites. *Farmaco* 57: 45-53, 2002

Marrazzo A, Caraci F, Salinaro ET, Su TP, Copani A, Ronsisvalle G. Neuroprotective effects of sigma-1 receptor agonists against beta-amyloid-induced toxicity. *Neuroreport* 16: 1223-1226, 2005

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

Martin-Fardon R, Maurice T, Aujla H, Bowen WD, Weiss F. Differential effects of σ_1 receptor blockade on self-administration and conditioned reinstatement motivated by cocaine vs natural reward. *Neuropsychopharmacology* 32: 1967-1973, 2007

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

Martina M, Turcotte ME, Halman S, Bergeron R. The sigma-1 receptor modulates NMDA receptor synaptic transmission and plasticity via SK channels in rat hippocampus. *J Physiol* 578: 143-157, 2007

Maruo J, Yoshida A, Shimohira I, Matsuno K, Mita S, Ueda H. Binding of [^{35}S]GTPγS stimulated by (+)-pentazocine sigma receptor agonist, is abundant in the guinea pig spleen. *Life Sci* 67: 599-603, 2000

Mateo Y, Budygin EA, John CE, Jones SR. Role of serotonin in cocaine effects in mice with reduced dopamine transporter function. *Proc Natl Acad Sci U S A* 101: 372-377, 2004

Matos FF, Korpinen C, Yocca FD. 5-HT$_{1A}$ receptor agonist effects of BMY-14802 on serotonin release in dorsal raphe and hippocampus. *Eur J Pharmacol* 317: 49-54, 1996

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

Matsumoto RR, Pouw B. Correlation between neuroleptic binding to σ_1 and σ_2 receptors and acute dystonic reactions. *Eur J Pharmacol* 401: 155-160, 2000

Matsumoto RR, Hewett KL, Pouw B, Bowen WD, Husbands SM, Cao JJ, Hauck NA. Rimcazole analogs attenuate the convulsive effects of cocaine: correlation with binding to sigma receptors rather than dopamine transporters. *Neuropharmacology* 41: 878-886, 2001a

Matsumoto RR, McCracken KA, Friedman MJ, Pouw B, De Costa BR, Bowen WD. Conformationally restricted analogs of BD1008 and an antisense oligodeoxynucleotide targeting σ_1 receptors produce anti-cocaine effects in mice. *Eur J Pharmacol* 419: 163-174, 2001b

Matsumoto RR, McCracken KA, Pouw B, Zhang Y, Bowen WD. Involvement of sigma receptors in the behavioral effects of cocaine: evidence from novel ligands and antisense oligodeoxynucleotides. *Neuropharmacology* 42: 1043-1055, 2002

Matsumoto RR, Liu Y, Lerner M, Howard EW, Brackett DJ. σ receptors: potential medications development target for anti-cocaine agents. *Eur J Pharmacol* 469: 1-12, 2003

Matsumoto RR, Gilmore DL, Pouw B, Bowen WD, Williams W, Kausar A, Coop A. Novel analogs of the σ receptor ligand BD1008 attenuate cocaine-induced toxicity in mice. *Eur J Pharmacol* 492: 21-26, 2004

Matsumoto RR, Pouw B, Mack AL, Daniels A, Coop A. Effects of UMB24 and (+/-)-SM 21, putative σ_2 -preferring antagonists, on behavioral toxic and stimulant effects of cocaine in mice. *Pharmacol Biochem Behav* 86: 86-91, 2007

Matsuno K, Senda T, Matsunaga K, Mita S, Kaneto H. Similar ameliorating effects of benzomorphans and 5-HT$_2$ antagonists on drug-induced impairment of passive avoidance response in mice: comparison with acetylcholinesterase inhibitors. *Psychopharmacology (Berl)* 112: 134-141, 1993

Matsuno K, Senda T, Matsunaga K, Mita S. Ameliorating effects of σ receptor ligands on the impairment of passive avoidance tasks in mice: involvement in the central acetylcholinergic system. *Eur J Pharmacol* 261: 43-51, 1994

Maurice T, Roman FJ, Privat A. Modulation by neurosteroids of the in vivo (+)-[^{3}H]SKF-10,047 binding to σ_1 receptors in the mouse forebrain . *J Neurosci Res* 46: 734-743, 1996

Maurice T, Lockhart BP. Neuroprotective and anti-amnesic potentials of sigma (σ) receptor ligands. *Prog Neuropsychopharmacol Biol Psychiatry* 21: 69-102, 1997

Maurice T, Privat A. SA4503, a novel cognitive enhancer with σ_1 receptor agonist properties, facilitates NMDA receptor-dependent learning in mice. *Eur J Pharmacol* 328: 9-18, 1997

Maurice T, Su TP, Privat A. Sigma$_1$ (σ_1) receptor agonists and neurosteroids attenuate B_{25-35}-amyloid peptide-induced amnesia in mice through a common mechanism. *Neuroscience* 83: 413-428, 1998

Maurice T, Phan VL, Urani A, Kamei H, Noda Y, Nabeshima T. Neuroactive neurosteroids as endogenous effectors for the sigma1 (σ_1) receptor: pharmacological evidence and therapeutic opportunities. *Jpn J Pharmacol* 81: 125-155, 1999

Maurice T, Phan VL, Privat A . The anti-amnesic effects of sigma1 (σ_1) receptor agonists confirmed by in vivo antisense strategy in the mouse. *Brain Res* 898: 113-121, 2001a

Maurice T, Phan VL, Urani A, Guillemain I. Differential involvement of the sigma$_1$ (σ_1) receptor in the anti-amnesic effect of neuroactive steroids, as demonstrated using an in vivo antisense strategy in the mouse. *Br J Pharmacol* 134: 1731-1741, 2001b

Maurice T, Urani A, Phan VL, Romieu P. The interaction between neuroactive steroids and the sigma1 receptor function: behavioral consequences and therapeutic opportunities. *Brain Res Brain Res Rev* 37: 116-132, 2001c

Maurice T, Martin-Fardon R, Romieu P, Matsumoto RR. Sigma$_1$ (σ_1) receptor antagonists represent a new strategy against cocaine addiction and toxicity. *Neurosci Biobehav Rev* 26: 499-527, 2002

Maurice T, Casalino M, Lacroix M, Romieu P. Involvement of the sigma$_1$ receptor in the motivational effects of ethanol in mice. *Pharmacol Biochem Behav* 74: 869-876, 2003

Maurice T, Meunier J, Feng B, Ieni J, Monaghan DT. Interaction with σ_1 protein, but not *N*-methyl-*D*-aspartate receptor, is involved in the pharmacological activity of donepezil. *J Pharmacol Exp Ther* 317: 606-614, 2006

Maurice T, Gregoire C, Espallergues J. Neuro(active)steroids actions at the neuromodulatory sigma$_1$ (σ_1) receptor: biochemical and physiological evidences, consequences in neuroprotection. *Pharmacol Biochem Behav* 84: 581-597, 2006

Mavlyutov TA, Ruoho AE. Ligand-dependent localization and intracellular stability of sigma-1 receptors in CHO-K1 cells. *J Mol Signal* 2: 8, 2007

McCarthy PW, Lawson SN. Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience* 28: 745-753, 1989

McCracken KA, Bowen WD, De Costa BR, Matsumoto RR. Two novel σ receptor ligands, BD1047 and LR172, attenuate cocaine-induced toxicity and locomotor activity. *Eur J Pharmacol* 370: 225-232, 1999

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

Mei J, Pasternak GW. Modulation of brainstem opiate analgesia in the rat by sigma$_1$ receptors: a microinjection study. *J Pharmacol Exp Ther* 322: 1278-1285, 2007

Melzack R, Wall PD. Pain mechanisms: a new theory. *Science* 150: 971-979, 1965

Mennini T, Gobbi M. The antidepressant mechanism of Hypericum perforatum. *Life Sci* 75: 1021-1027, 2004

Meunier J, Maurice T. Beneficial effects of the sigma$_1$ receptor agonists igmesine and dehydroepiandrosterone against learning impairments in rats prenatally exposed to cocaine. *Neurotoxicol Teratol* 26: 783-797, 2004

Meunier J, Gue M, Recasens M, Maurice T. Attenuation by a sigma$_1$ (σ_1) receptor agonist of the learning and memory deficits induced by a prenatal restraint stress in juvenile rats. *Br J Pharmacol* 142: 689-700, 2004

Meunier J, Demeilliers B, Celerier A, Maurice T. Compensatory effect by sigma1 (σ_1) receptor stimulation during alcohol withdrawal in mice performing an object recognition task. *Behav Brain Res* 166: 166-176, 2006a

Meunier J, Ieni J, Maurice T. The anti-amnesic and neuroprotective effects of donepezil against amyloid beta$_{25-35}$ peptide-induced toxicity in mice involve an interaction with the σ_1 receptor. *Br J Pharmacol* 149: 998-1012, 2006b

Meyer DA, Carta M, Partridge LD, Covey DF, Valenzuela CF. Neurosteroids enhance spontaneous glutamate release in hippocampal neurons. Possible role of metabotropic σ_1-like receptors. *J Biol Chem* 277: 28725-28732, 2002

Meyer RA, Ringkamp M, Campbell JN, Raja SN. Peripheral mechanisms of cutaneous nociception. In: *Wall and Melzack's Textbook of pain 5th ed,* edited by McMahon SB, Koltzenburg M, London, p: 3-34, 2007

Meyer RA, Ringkamp M, Campbell JM, Raja SN. Section 1: Neurobiology of pain. Wall and Melzack's T*ext book of Pain,* 2007.

Meyerson JD, Vasko MR. Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther* 302: 839-845, 2002

Mezey E, Toth ZE, Cortright DN, Arzubi MK, Krause JE, Elde R, Guo A, Blumberg PM, Szallasi A. Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc Natl Acad Sci U S A* 97: 3655-3660, 2000

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

Millan MJ, Brocco M, Rivet JM, Audinot V, Newman-Tancredi A, Maiofiss L, Queriaux S, Despaux N, Peglion JL, Dekeyne A. S18327 (1-[2-[4-(6-fluoro-1, 2-benzisoxazol-3-yl)piperid-1-yl]ethyl]3-phenyl imidazolin-2-one), a novel, potential antipsychotic displaying marked antagonist properties at α_1- and α_2-adrenergic receptors: II. Functional profile and a multiparametric comparison with haloperidol, clozapine, and 11 other antipsychotic agents. *J Pharmacol Exp Ther* 292: 54-66, 2000

Millan MJ, Loiseau F, Dekeyne A, Gobert A, Flik G, Cremers TI, Rivet JM, Sicard D, Billiras R, Brocco M. S33138 (N-[4-[2-[(3aS,9bR)-8-cyano-1,3a,4,9b-tetrahydro[1] benzopyrano[3,4-c]pyrrol-2(3H)-yl)-ethyl]phenyl-acetamide), a preferential dopamine D_3 versus D_2 receptor antagonist and potential antipsychotic agent: III. Actions in models of therapeutic activity and induction of side effects. *J Pharmacol Exp Ther* 324: 1212-1226, 2008

Miyatake R, Furukawa A, Matsushita S, Higuchi S, Suwaki H. Functional polymorphisms in the sigma$_1$ receptor gene associated with alcoholism. *Biol Psychiatry* 55: 85-90, 2004

Mizuno T, Yotsuyanagi S, Nagasaka Y, Namiki M. Dehydroepiandrosterone alleviates copulatory disorder induced by social stress in male rats. *J Sex Med* 3: 612-618, 2006

Mochizuki-Oda N, Nakajima Y, Nakanishi S, Ito S. Characterization of the substance P receptor-mediated calcium influx in cDNA transfected Chinese hamster ovary cells. A possible role of inositol 1,4,5-trisphosphate in calcium influx. *J Biol Chem* 269: 9651-9658, 1994

Moebius FF, Reiter RJ, Hanner M, Glossmann H. 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, 1997

Monassier L, Manoury B, Bellocq C, Weissenburger J, Greney H, Zimmermann D, Ehrhardt JD, Jaillon P, Baro I, Bousquet P. σ_2-receptor ligand-mediated inhibition of inwardly rectifying K^+ channels in the heart. *J Pharmacol Exp Ther* 322: 341-350, 2007

Monnet FP, Debonnel G, de Montigny C. In vivo electrophysiological evidence for a selective modulation of *N*-methyl-*D*-aspartate-induced neuronal activation in rat CA_3 dorsal hippocampus by sigma ligands. *J Pharmacol Exp Ther* 261: 123-130, 1992

Monnet FP, Debonnel G, Bergeron R, Gronier B, de Montigny C. The effects of sigma ligands and of neuropeptide Y on *N*-methyl-*D*-aspartate-induced neuronal activation of CA_3 dorsal hippocampus neurones are differentially affected by pertussin toxin. *Br J Pharmacol* 112: 709-715, 1994

Monnet FP, Morin-Surun MP, Leger J, Combettes L. Protein kinase C-dependent potentiation of intracellular calcium influx by σ_1 receptor agonists in rat hippocampal neurons. *J Pharmacol Exp Ther* 307: 705-712, 2003

Monnet FP. Sigma-1 receptor as regulator of neuronal intracellular Ca^{2+}: clinical and therapeutic relevance. *Biol Cell* 97: 873-883, 2005

Monnet FP, Maurice T. The $sigma_1$ protein as a target for the non-genomic effects of neuro(active)steroids: molecular, physiological, and behavioral aspects. *J Pharmacol Sci* 100: 93-118, 2006

Moore RA, Derry S, Makinson GT, McQuay HJ. Tolerability and adverse events in clinical trials of celecoxib in osteoarthritis and rheumatoid arthritis: systematic review and meta-analysis of information from company clinical trial reports. *Arthritis Res Ther* 7: R644-R665, 2005

Morin-Surun MP, Collin T, Denavit-Saubie M, Baulieu EE, Monnet FP. Intracellular σ_1 receptor modulates phospholipase C and protein kinase C activities in the brainstem. *Proc Natl Acad Sci U S A* 96: 8196-8199, 1999

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S. Molecular cloning and characterization of the rat NMDA receptor. *Nature* 354: 31-37, 1991

Mtchedlishvili Z, Kapur J. A presynaptic action of the neurosteroid pregnenolone sulfate on GABAergic synaptic transmission. *Mol Pharmacol* 64: 857-864, 2003

Nakazato A, Kumagai T, Ohta K, Chaki S, Okuyama S, Tomisawa K. 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, 1999

Narita N, Hashimoto K, Tomitaka S, Minabe Y. Interactions of selective serotonin reuptake inhibitors with subtypes of σ receptors in rat brain. *Eur J Pharmacol* 307: 117-119, 1996

Natesan S, Reckless GE, Barlow KB, Odontiadis J, Nobrega JN, Baker GB, George SR, Mamo D, Kapur S. The antipsychotic potential of l-stepholidine--a naturally occurring dopamine receptor D_1 agonist and D_2 antagonist. *Psychopharmacology (Berl)* 199: 275-289, 2008

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. *Neuron* 34: 13-25, 2002

Nguyen EC, McCracken KA, Liu Y, Pouw B, Matsumoto RR. Involvement of sigma (σ) receptors in the acute actions of methamphetamine: receptor binding and behavioral studies. *Neuropharmacology* 49: 638-645, 2005

Nieto FR, Entrena JM, Cendan CM, Pozo ED, Vela JM, Baeyens JM. Tetrodotoxin inhibits the development and expression of neuropathic pain induced by paclitaxel in mice. *Pain* 137: 520-531, 2008

Nobile M, Lagostena L. A discriminant block among K^+ channel types by phenytoin in neuroblastoma cells. *Br J Pharmacol* 124: 1698-1702, 1998

Noda Y, Kamei H, Kamei Y, Nagai T, Nishida M, Nabeshima T. Neurosteroids ameliorate conditioned fear stress: an association with $sigma_1$ receptors. *Neuropsychopharmacology* 23: 276-284, 2000

Novakova M, Bruderova V, Sulova Z, Kopacek J, Lacinova L, Kvetnansky R, Vasku A, Kaplan P, Krizanova O, Jurkovicova D. Modulation of expression of the sigma receptors in the heart of rat and mouse in normal and pathological conditions. *Gen Physiol Biophys* 26: 110-117, 2007

Nudmamud-Thanoi S, Reynolds GP. The NR1 subunit of the glutamate/NMDA receptor in the superior temporal cortex in schizophrenia and affective disorders. *Neurosci Lett* 372: 173-177, 2004

Nuseir K, Heidenreich BA, Proudfit HK. The antinociception produced by microinjection of a cholinergic agonist in the ventromedial medulla is mediated by noradrenergic neurons in the A7 catecholamine cell group. *Brain Res* 822: 1-7, 1999

Nuwayhid SJ, Werling LL. $Sigma_2$ (σ_2) receptors as a target for cocaine action in the rat striatum. *Eur J Pharmacol* 535: 98-103, 2006

Ocana M, del Pozo E, Barrios M, Baeyens JM. Subgroups among mu-opioid receptor agonists distinguished by ATP-sensitive K^+ channel-acting drugs. *Br J Pharmacol* 114: 1296-1302, 1995

Okuyama S, Imagawa Y, Ogawa S, Araki H, Ajima A, Tanaka M, Muramatsu M, Nakazato A, Yamaguchi K, Yoshida M, . NE-100, a novel sigma receptor ligand: in vivo tests. *Life Sci* 53: L285-L290, 1993

Okuyama S, Imagawa Y, Tomisawa K. Behavioral evidence for modulation by sigma ligands of (+)MK-801-induced hyperlocomotion in monoamine-depleted mice. *Neuropharmacology* 35: 467-474, 1996

Okuyama S, Nakazato A. NE-100: a novel sigma receptor antagonist. *CNS Drug Rev* 2: 226-237, 1996

Olausson H, Wessberg J, Morrison I, McGlone F, Vallbo A. The neurophysiology of unmyelinated tactile afferents. *Neurosci Biobehav Rev* In press, 2008

Padi SS, Kulkarni SK. Differential effects of naproxen and rofecoxib on the development of hypersensitivity following nerve injury in rats. *Pharmacol Biochem Behav* 79: 349-358, 2004

Pal A, Hajipour AR, Fontanilla D, Ramachandran S, Chu UB, Mavlyutov T, Ruoho AE. Identification of regions of the σ-1 receptor ligand binding site using a novel photoprobe. *Mol Pharmacol* 72: 921-933, 2007

Palacios G, Muro A, Vela JM, Molina-Holgado E, Guitart X, Ovalle S, Zamanillo D. Immunohistochemical localization of the σ_1-receptor in oligodendrocytes in the rat central nervous system. *Brain Res* 961: 92-99, 2003

Palecek J. The role of Dorsal Columns Pathway in Visceral Pain. *Psysiology Res.* 53: S125-S130, 2004

Pan YX, Mei J, Xu J, Wan BL, Zuckerman A, Pasternak GW. Cloning and characterization of a mouse σ_1 receptor. *J Neurochem* 70: 2279-2285, 1998

Paoletti P, Neyton J. NMDA receptor subunits: function and pharmacology. *Curr Opin Pharmacol* 7: 39-47, 2007

Paqueron X, Conklin D, Eisenach JC. Plasticity in action of intrathecal clonidine to mechanical but not thermal nociception after peripheral nerve injury. *Anesthesiology* 99: 199-204, 2003

Park KM, Max MB, Robinovitz E, Gracely RH, Bennett GJ. Effects of intravenous ketamine, alfentanil, or placebo on pain, pinprick hyperalgesia, and allodynia produced by intradermal capsaicin in human subjects. *Pain* 63: 163-172, 1995

Peeters M, Romieu P, Maurice T, Su TP, Maloteaux JM, Hermans E. Involvement of the sigma$_1$ receptor in the modulation of dopaminergic transmission by amantadine. *Eur J Neurosci* 19: 2212-2220, 2004

Petralia RS, Wang YX, Wenthold RJ. The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. *J Neurosci* 14: 6102-6120, 1994

Phan VL, Miyamoto Y, Nabeshima T, Maurice T. Age-related expression of σ_1 receptors and antidepressant efficacy of a selective agonist in the senescence-accelerated (SAM) mouse. *J Neurosci Res* 79: 561-572, 2005

Planells-Cases R, Garcia-Sanz N, Morenilla-Palao C, Ferrer-Montiel A. Functional aspects and mechanisms of TRPV1 involvement in neurogenic inflammation that leads to thermal hyperalgesia. *Pflugers Arch* 451: 151-159, 2005

Poncelet M, Santucci V, Paul R, Gueudet C, Lavastre S, Guitard J, Steinberg R, Terranova JP, Breliere JC, Soubrie P, . Neuropharmacological profile of a novel and selective ligand of the sigma site: SR 31742A. *Neuropharmacology* 32: 605-615, 1993

Porreca F, Ossipov MH, Gebhart GF. Chronic pain and medullary descending facilitation. *Trends Neurosci* 25: 319-325, 2002

Prasad PD, Li HW, Fei YJ, Ganapathy ME, Fujita T, Plumley LH, Yang-Feng TL, Leibach FH, Ganapathy V. Exon-intron structure, analysis of promoter region, and chromosomal localization of the human type 1 σ receptor gene. *J Neurochem* 70: 443-451, 1998

Prasad BC, Gururaj HB, Kumar V, Giridhar P, Ravishankar GA. Valine pathway is more crucial than phenyl propanoid pathway in regulating capsaicin biosynthesis in Capsicum frutescens mill. *J Agric Food Chem* 54: 6660-6666, 2006

Prezzavento O, Parenti C, Marrazzo A, Ronsisvalle S, Vittorio F, Arico G, Scoto GM, Ronsisvalle G. A new sigma ligand, (+/-)-PPCC, antagonizes kappa opioid receptor-mediated antinociceptive effect. *Life Sci* 82: 549-553, 2008

Quirion R, Bowen WD, Itzhak Y, Junien JL, Musacchio JM, Rothman RB, Su TP, Tam SW, Taylor DP. A proposal for the classification of σ binding sites. *Trends Pharmacol Sci* 13: 85-86, 1992

Raft D, Toomey T, Gregg JM. Behavior modification and haloperidol in chronic facial pain. *South Med J* 72: 155-159, 1979

Ren Y, Zou X, Fang L, Lin Q. Sympathetic modulation of activity in Adelta- and C-primary nociceptive afferents after intradermal injection of capsaicin in rats. *J Neurophysiol* 93: 365-377, 2005

Rexed B. The cytoarchitectonic organization of the spinal cord in the cat. *J Comp Neurol* 96: 414-495, 1952

Riedel G, Platt B, Micheau J. Glutamate receptor function in learning and memory. *Behav Brain Res* 140: 1-47, 2003

Ringkamp M, Peng YB, Wu G, Hartke TV, Campbell JN, Meyer RA. Capsaicin responses in heat-sensitive and heat-insensitive A-fiber nociceptors. *J Neurosci* 21: 4460-4468, 2001

Rizvi TA, Ennis M, Behbehani MM, Shipley MT. Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. *J Comp Neurol* 303: 121-131, 1991

Robles I, Barrios M, Baeyens JM. Centrally administered aminoglycoside antibiotics antagonize naloxone-precipitated withdrawal in mice acutely dependent on morphine. *Neurosci Lett* 145: 189-192, 1992

Rogoz Z, Skuza G. Mechanism of synergistic action following co-treatment with pramipexole and fluoxetine or sertraline in the forced swimming test in rats. *Pharmacol Rep* 58: 493-500, 2006

Romieu P, Martin-Fardon R, Maurice T. Involvement of the sigma$_1$ receptor in the cocaine-induced conditioned place preference. *Neuroreport* 11: 2885-2888, 2000

Romieu P, Phan VL, Martin-Fardon R, Maurice T. Involvement of the sigma$_1$ receptor in cocaine-induced conditioned place preference: possible dependence on dopamine uptake blockade. *Neuropsychopharmacology* 26: 444-455, 2002

Romieu P, Meunier J, Garcia D, Zozime N, Martin-Fardon R, Bowen WD, Maurice T. The sigma$_1$ (σ_1) receptor activation is a key step for the reactivation of cocaine conditioned place preference by drug priming. *Psychopharmacology (Berl)* 175: 154-162, 2004

Romieu P, Lucas M, Maurice T. σ_1 receptor ligands and related neuroactive steroids interfere with the cocaine-induced state of memory. *Neuropsychopharmacology* 31: 1431-1443, 2006

Ronsisvalle G, Marrazzo A, Prezzavento O, Pasquinucci L, Falcucci B, Di Toro RD, Spampinato S. Substituted 1-phenyl-2-cyclopropylmethylamines with high affinity and selectivity for sigma sites. *Bioorg Med Chem* 8: 1503-1513, 2000

Ronsisvalle G, Marrazzo A, Prezzavento O, Cagnotto A, Mennini T, Parenti C, Scoto GM. Opioid and sigma receptor studies. New developments in the design of selective sigma ligands. *Pure and Applied Chemistry* 73: 1499-1509, 2001

Roth MD, Whittaker KM, Choi R, Tashkin DP, Baldwin GC. Cocaine and σ-1 receptors modulate HIV infection, chemokine receptors, and the HPA axis in the huPBL-SCID model. *J Leukoc Biol* 78: 1198-1203, 2005

Rothman RB, Baumann MH. Monoamine transporters and psychostimulant drugs. *Eur J Pharmacol* 479: 23-40, 2003

Rückert NG, Schmidt WJ. The σ receptor ligand 1,3-di-(2-tolyl)guanidine in animal models of schizophrenia. *Eur J Pharmacol* 233: 261-267, 1993

Ruda MA, Bennett GJ, Dubner R. Neurochemistry and neural circuitry in the dorsal horn. *Prog Brain Res* 66: 219-268, 1986

Rush AM, Elliott JR. Phenytoin and carbamazepine: differential inhibition of sodium currents in small cells from adult rat dorsal root ganglia. *Neurosci Lett* 226: 95-98, 1997

Sagen J, Proudfit HK. Hypoalgesia induced by blockade of noradrenergic projections to the raphe magnus: reversal by blockade of noradrenergic projections to the spinal cord. *Brain Res* 223: 391-396, 1981

Sang CN, Gracely RH, Max MB, Bennett GJ. Capsaicin-evoked mechanical allodynia and hyperalgesia cross nerve territories. Evidence for a central mechanism. *Anesthesiology* 85: 491-496, 1996

Sang CN, Hostetter MP, Gracely RH, Chappell AS, Schoepp DD, Lee G, Whitcup S, Caruso R, Max MB. AMPA/kainate antagonist LY293558 reduces capsaicin-evoked hyperalgesia but not pain in normal skin in humans. *Anesthesiology* 89: 1060-1067, 1998

Sasaki YF, Rothe T, Premkumar LS, Das S, Cui J, Talantova MV, Wong HK, Gong X, Chan SF, Zhang D, Nakanishi N, Sucher NJ, Lipton SA. Characterization and comparison of the NR3A subunit of the NMDA receptor in recombinant systems and primary cortical neurons. *Neurophysiol* 87: 2052-2063, 2002

Sawyork J, Liu XJ. The formalin test: Characteristics and usefulness of the model. *Rev. Analgesia* 7: 145-163, 2004

Schaible HG, Grubb BD. Afferent and spinal mechanisms of joint pain. *Pain* 55: 5-54, 1993

Schmidt R, Schmelz M, Forster C, Ringkamp M, Torebjork E, Handwerker H. Novel classes of responsive and unresponsive C nociceptors in human skin. *J Neurosci* 15: 333-341, 1995

Schmidt R, Schmelz M, Ringkamp M, Handwerker HO, Torebjork HE. Innervation territories of mechanically activated C nociceptor units in human skin. *J Neurophysiol* 78: 2641-2648, 1997

Schmidt R, Schmelz M, Torebjork HE, Handwerker HO. Mechano-insensitive nociceptors encode pain evoked by tonic pressure to human skin. *Neuroscience* 98: 793-800, 2000

Scholz J, Woolf CJ. Can we conquer pain? *Nat Neurosci* 5 Suppl: 1062-1067, 2002

Schwarz S, Pohl P, Zhou GZ. Steroid binding at sigma-"opioid" receptors. *Science* 246: 1635-1638, 1989

Seth P, Leibach FH, Ganapathy V. Cloning and structural analysis of the cDNA and the gene encoding the murine type 1 sigma receptor. *Biochem Biophys Res Commun* 241: 535-540, 1997

Seth P, Fei YJ, Li HW, Huang W, Leibach FH, Ganapathy V. Cloning and functional characterization of a σ receptor from rat brain. *J Neurochem* 70: 922-931, 1998

Seth P, Ganapathy ME, Conway SJ, Bridges CD, Smith SB, Casellas P, Ganapathy V. 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, 2001

Sherrington CS. Observations on the scratch-reflex in the spinal dog. *J Physiol* 34: 1-50, 1906

Shigemoto R, Ohishi H, Nakanishi S, Mizuno N. Expression of the mRNA for the rat NMDA receptor (NMDAR1) in the sensory and autonomic ganglion neurons. *Neurosci Lett* 144: 229-232, 1992

Shin EJ, Nah SY, Kim WK, Ko KH, Jhoo WK, Lim YK, Cha JY, Chen CF, Kim HC. The dextromethorphan analog dimemorfan attenuates kainate-induced seizures *via* σ_1 receptor activation: comparison with the effects of dextromethorphan. *Br J Pharmacol* 144: 908-918, 2005

Shir Y, Shenkman Z, Kaplan L. [Neuropathic pain unrelieved by morphine, alleviated by haloperidol]. *Harefuah* 118: 452-454, 1990

Silvers JM, Wallace DR, Harrod SB, Mactutus CF, Booze RM. Prenatal cocaine alters dopamine and sigma receptor binding in nucleus accumbens and striatum in dams and adolescent offspring. *Neurotoxicol Teratol* 28: 173-180, 2006

Skuza G. Effect of sigma ligands on the cocaine-induced convulsions in mice. *Pol J Pharmacol* 51: 477-483, 1999

Skuza G, Rogoz Z. A potential antidepressant activity of SA4503, a selective σ_1 receptor agonist. *Behav Pharmacol* 13: 537-543, 2002

Skuza G, Rogoz Z. The synergistic effect of selective sigma receptor agonists and uncompetitive NMDA receptor antagonists in the forced swim test in rats. *J Physiol Pharmacol* 57: 217-229, 2006a

Skuza G, Rogoz Z. Effect of BD 1047, a $sigma_1$ receptor antagonist, in the animal models predictive of antipsychotic activity. *Pharmacol Rep* 58: 626-635, 2006b

Slugg RM, Meyer RA, Campbell JN. Response of cutaneous A- and C-fiber nociceptors in the monkey to controlled-force stimuli. *J Neurophysiol* 83: 2179-2191, 2000

Sluka KA. Activation of the cAMP transduction cascade contributes to the mechanical hyperalgesia and allodynia induced by intradermal injection of capsaicin. *Br J Pharmacol* 122: 1165-1173, 1997

Soliman AC, Yu JS, Coderre TJ. mGlu and NMDA receptor contributions to capsaicin-induced thermal and mechanical hypersensitivity. *Neuropharmacology* 48: 325-332, 2005

Soriani O, Foll FL, Roman F, Monnet FP, Vaudry H, Cazin L. A-Current down-modulated by *σ* receptor in frog pituitary melanotrope cells through a G protein-dependent pathway. *J Pharmacol Exp Ther* 289: 321-328, 1999a

Soriani O, Le Foll F, Galas L, Roman F, Vaudry H, Cazin L. The σ-ligand (+)-pentazocine depresses M current and enhances calcium conductances in frog melanotrophs. *Am J Physiol* 277: E73-E80, 1999b

Sorkin LS, McAdoo DJ, Willis WD. Raphe magnus stimulation-induced antinociception in the cat is associated with release of amino acids as well as serotonin in the lumbar dorsal horn. *Brain Res* 618: 95-108, 1993

Sorkin LS, McAdoo DJ. Amino acids and serotonin are released into the lumbar spinal cord of the anesthetized cat following intradermal capsaicin injections. *Brain Res* 607: 89-98, 1993

Sorkin LS, Yaksh TL, Doom CM . Mechanical allodynia in rats is blocked by a Ca2+ permeable AMPA receptor antagonist. *Neuroreport* 10: 3523-3526, 1999

South SM, Kohno T, Kaspar BK, Hegarty D, Vissel B, Drake CT, Ohata M, Jenab S, Sailer AW, Malkmus S, Masuyama T, Horner P, Bogulavsky J, Gage FH, Yaksh TL, Woolf CJ, Heinemann SF, Inturrisi CE. A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. *J Neurosci* 23: 5031-5040, 2003

Stefanski R, Justinova Z, Hayashi T, Takebayashi M, Goldberg SR, Su TP. $Sigma_1$ receptor upregulation after chronic methamphetamine self-administration in rats: a study with yoked controls. *Psychopharmacology (Berl)* 175: 68-75, 2004

Stephenson FA. Structure and trafficking of NMDA and GABAA receptors. *Biochem Soc Trans* 34: 877-881, 2006

Sterner O, Szallasi A. Novel natural vanilloid receptor agonists: new therapeutic targets for drug development. *Trends Pharmacol Sci* 20: 459-465, 1999

Stone LS, Vulchanova L. The pain of antisense: in vivo application of antisense oligonucleotides for functional genomics in pain and analgesia. *Adv Drug Deliv Rev* 55: 1081-1112, 2003

Stone JM, Arstad E, Erlandsson K, Waterhouse RN, Ell PJ, Pilowsky LS. [^{123}I]TPCNE--a novel SPET tracer for the sigma-1 receptor: first human studies and in vivo haloperidol challenge. *Synapse* 60: 109-117, 2006

Su TP, London ED, Jaffe JH. Steroid binding at sigma receptors suggests a link between endocrine, nervous, and immune systems. *Science* 240: 219-221, 1988

Su TP, Wu XZ, Cone EJ, Shukla K, Gund TM, Dodge AL, Parish DW. Sigma compounds derived from phencyclidine: identification of PRE-084, a new, selective sigma ligand. *J Pharmacol Exp Ther* 259: 543-550, 1991

Su TP, Hayashi T. Cocaine affects the dynamics of cytoskeletal proteins via sigma$_1$ receptors. *Trends Pharmacol Sci* 22: 456-458, 2001

Su TP, Hayashi T. Understanding the molecular mechanism of sigma-1 receptors: towards a hypothesis that sigma-1 receptors are intracellular amplifiers for signal transduction. *Curr Med Chem* 10: 2073-2080, 2003

Szallasi A, Blumberg PM. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience* 30: 515-520, 1989

Szallasi A, Blumberg PM. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol Rev* 51: 159-212, 1999

Szolcsanyi J, Jancso-Gabor A . Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain-producing potency of pungent agents. *Arzneimittelforschung* 25: 1877-1881, 1975

Szolcsanyi J, Jancso-Gabor A . Sensory effects of capsaicin congeners. Part II: Importance of chemical structure and pungency in desensitizing activity of capsaicin-type compounds. *Arzneimittelforschung* 26: 33-37, 1976

Takahashi S, Sonehara K, Takagi K, Miwa T, Horikomi K, Mita N, Nagase H, Iizuka K, Sakai K. Pharmacological profile of MS-377, a novel antipsychotic agent with selective affinity for σ receptors. *Psychopharmacology (Berl)* 145: 295-302, 1999

Takahashi S, Miwa T, Horikomi K. Involvement of σ_1 receptors in methamphetamine-induced behavioral sensitization in rats. *Neurosci Lett* 289: 21-24, 2000

Takahashi S, Horikomi K, Kato T. MS-377, a novel selective σ_1 receptor ligand, reverses phencyclidine-induced release of dopamine and serotonin in rat brain. *Eur J Pharmacol* 427: 211-219, 2001

Takebayashi M, Hayashi T, Su TP. Nerve growth factor-induced neurite sprouting in PC12 cells involves *σ*-1 receptors: implications for antidepressants. *J Pharmacol Exp Ther* 303: 1227-1237, 2002

Takebayashi M, Hayashi T, Su TP. σ-1 receptors potentiate epidermal growth factor signaling towards neuritogenesis in PC12 cells: potential relation to lipid raft reconstitution. *Synapse* 53: 90-103, 2004

Tam SW, Steinfels GF, Gilligan PJ, Schmidt WK, Cook L. DuP 734 [1-(cyclopropylmethyl)-4-(2'(4"-fluorophenyl)-2'-oxoethyl)- piperidine HBr], a *sigma* and 5-hydroxytryptamine2 receptor antagonist: receptor-binding, electrophysiological and neuropharmacological profiles. *J Pharmacol Exp Ther* 263: 1167-1174, 1992

Tanabe M, Takasu K, Kasuya N, Shimizu S, Honda M, Ono H. Role of descending noradrenergic system and spinal α_2-adrenergic receptors in the effects of gabapentin on thermal and mechanical nociception after partial nerve injury in the mouse. *Br J Pharmacol* 144: 703-714, 2005

Taylor CW, Merritt JE, Putney JW, Jr., Rubin RP. Effects of Ca2+ on phosphoinositide breakdown in exocrine pancreas. *Biochem J* 238: 765-772, 1986

Taylor DP, Eison MS, Moon SL, Schlemmer RF, Jr., Shukla UA, VanderMaelen CP, Yocca FD, Gallant DJ, Behling SH, Boissard CG. A role for σ binding in the antipsychotic profile of BMY 14802? *NIDA Res Monogr* 133: 125-157, 1993

Theriault E, Otsuka M, Jessell T. Capsaicin-evoked release of substance P from primary sensory neurons. *Brain Res* 170: 209-213, 1979

Todd AJ, Sullivan AC. 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, 1990

Todd AJ, Watt C, Spike RC, Sieghart W. Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. *J Neurosci* 16: 974-982, 1996

Todorovic SM, Lingle CJ. Pharmacological properties of T-type Ca^{2+} current in adult rat sensory neurons: effects of anticonvulsant and anesthetic agents. *J Neurophysiol* 79: 240-252, 1998

Tohda C, Kuraishi Y. Visualization of glutamate release from rat spinal cord with a confocal laser scanning microscope. *Neurosci Res* 24: 183-187, 1996

Tominaga M, Julius D. Capsaicin receptor in the pain pathway. *Jpn J Pharmacol* 83: 20-24, 2000

Tominaga M, Tominaga T. Structure and function of TRPV1. *Pflugers Arch* 451: 143-150, 2005

Torebjork HE. Afferent C units responding to mechanical, thermal and chemical stimuli in human non-glabrous skin. *Acta Physiol Scand* 92: 374-390, 1974

Torebjork HE, Lundberg LE, LaMotte RH. Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J Physiol* 448: 765-780, 1992

Tottori K, Miwa T, Uwahodo Y, Yamada S, Nakai M, Oshiro Y, Kikuchi T, Altar CA. Antidepressant-like responses to the combined sigma and 5-HT_{1A} receptor agonist OPC-14523. *Neuropharmacology* 41: 976-988, 2001

Treede RD, Meyer RA, Campbell JN. Myelinated mechanically insensitive afferents from monkey hairy skin: heat-response properties. *J Neurophysiol* 80: 1082-1093, 1998

Ueda M, Kuraishi Y, Satoh M. Detection of capsaicin-evoked release of glutamate from spinal dorsal horn slices of rat with on-line monitoring system. *Neurosci Lett* 155: 179-182, 1993

Ueda M, Kuraishi Y, Sugimoto K, Satoh M. Evidence that glutamate is released from capsaicin-sensitive primary afferent fibers in rats: study with on-line continuous monitoring of glutamate. *Neurosci Res* 20: 231-237, 1994

Ueda H, Inoue M, Yoshida A, Mizuno K, Yamamoto H, Maruo J, Matsuno K, Mita S. Metabotropic neurosteroid/ *σ* -receptor involved in stimulation of nociceptor endings of mice. *J Pharmacol Exp Ther* 298: 703-710, 2001

Ujike H, Kanzaki A, Okumura K, Akiyama K, Otsuki S. Sigma (σ) antagonist BMY 14802 prevents methamphetamine-induced sensitization BBB. *Life Sci* 50: L129-L134, 1992

Ujike H, Kuroda S, Otsuki S. *σ* Receptor antagonists block the development of sensitization to cocaine. *Eur J Pharmacol* 296: 123-128, 1996

Ukai M, Maeda H, Nanya Y, Kameyama T, Matsuno K. Beneficial effects of acute and repeated administrations of σ receptor agonists on behavioral despair in mice exposed to tail suspension. *Pharmacol Biochem Behav* 61: 247-252, 1998

Ultenius C, Linderoth B, Meyerson BA, Wallin J. Spinal NMDA receptor phosphorylation correlates with the presence of neuropathic signs following peripheral nerve injury in the rat. *Neurosci Lett* 399: 85-90, 2006

Urani A, Roman FJ, Phan VL, Su TP, Maurice T. The antidepressant-like effect induced by σ_1-receptor agonists and neuroactive steroids in mice submitted to the forced swimming test. *J Pharmacol Exp Ther* 298: 1269-1279, 2001

Urani A, Romieu P, Portales-Casamar E, Roman FJ, Maurice T. The antidepressant-like effect induced by the sigma$_1$ (σ_1) receptor agonist igmesine involves modulation of intracellular calcium mobilization. *Psychopharmacology (Berl)* 163: 26-35, 2002a

Urani A, Romieu P, Roman FJ, Maurice T. Enhanced antidepressant effect of sigma$_1$ (σ_1) receptor agonists in $\beta_{25\text{-}35}$-amyloid peptide-treated mice. *Behav Brain Res* 134: 239-247, 2002b

Urani A, Romieu P, Roman FJ, Yamada K, Noda Y, Kamei H, Manh TH, Nagai T, Nabeshima T, Maurice T. Enhanced antidepressant efficacy of σ_1 receptor agonists in rats after chronic intracerebroventricular infusion of β-amyloid-(1-40) protein. *Eur J Pharmacol* 486: 151-161, 2004

Van Berckel BNM. Glutamate and schizophrenia. *Curr Neuropharmacol* 1: 351-370, 2003

Van Der SM, Di M, V. Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. *Eur J Biochem* 271: 1827-1834, 2004

Volz HP, Stoll KD. Clinical trials with sigma ligands. *Pharmacopsychiatry* 37 Suppl 3: S214-S220, 2004

Walker JM, Bowen WD, Walker FO, Matsumoto RR, De Costa B, Rice KC. Sigma receptors: biology and function. *Pharmacol Rev* 42: 355-402, 1990

Walker JM, Bowen WD, Patrick SL, Williams WE, Mascarella SW, Bai X, Carroll FI. A comparison of (-)-deoxybenzomorphans devoid of opiate activity with their dextrorotatory phenolic counterparts suggests role of σ_2 receptors in motor function. *Eur J Pharmacol* 231: 61-68, 1993

Walker SM, Meredith-Middleton J, Lickiss T, Moss A, Fitzgerald M. Primary and secondary hyperalgesia can be differentiated by postnatal age and ERK activation in the spinal dorsal horn of the rat pup. *Pain* 128: 157-168, 2007

Wall PD. Neurophysiological studies on the mechanism of anesthesia. *Anesthesiology* 28: 799-800, 1967

Walpole CS, Bevan S, Bovermann G, Boelsterli JJ, Breckenridge R, Davies JW, Hughes GA, James I, Oberer L, Winter J, . The discovery of capsazepine, the first competitive antagonist of the sensory neuron excitants capsaicin and resiniferatoxin. *J Med Chem* 37: 1942-1954, 1994

Wang D, Noda Y, Tsunekawa H, Zhou Y, Miyazaki M, Senzaki K, Nitta A, Nabeshima T. Role of *N*-methyl-*D*-aspartate receptors in antidepressant-like effects of σ_1 receptor agonist 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (SA-4503) in olfactory bulbectomized rats. *J Pharmacol Exp Ther* 322: 1305-1314, 2007

Wang HH, Chien JW, Chou YC, Liao JF, Chen CF. Anti-amnesic effect of dimemorfan in mice. *Br J Pharmacol* 138: 941-949, 2003

Wang J, Mack AL, Coop A, Matsumoto RR. Novel sigma (σ) receptor agonists produce antidepressant-like effects in mice. *Eur Neuropsychopharmacol* 17: 708-716, 2007

Wang S, Sim TB, Kim YS, Chang YT. Tools for target identification and validation. *Curr Opin Chem Biol* 8: 371-377, 2004

Waterhouse RN, Chang RC, Atuehene N, Collier TL. In vitro and in vivo binding of neuroactive steroids to the sigma-1 receptor as measured with the positron emission tomography radioligand [^{18}F]FPS. *Synapse* 61: 540-546, 2007

Whittemore ER, Ilyin VI, Woodward RM. Antagonism of *N*-methyl-*D*-aspartate receptors by σ site ligands: potency, subtype-selectivity and mechanisms of inhibition. *J Pharmacol Exp Ther* 282: 326-338, 1997

Wiberg M, Westman J, Blomqvist A. Somatosensory projection to the mesencephalon: an anatomical study in the monkey. *J Comp Neurol* 264: 92-117, 1987

Wilke RA, Lupardus PJ, Grandy DK, Rubinstein M, Low MJ, Jackson MB. K^+ channel modulation in rodent neurohypophysial nerve terminals by sigma receptors and not by dopamine receptors. *J Physiol* 517 (Pt 2): 391-406, 1999

Willcockson H, Valtschanoff J. AMPA and NMDA glutamate receptors are found in both peptidergic and non-peptidergic primary afferent neurons in the rat. *Cell Tissue Res* 334: 17-23, 2008

Willis WD, Coggeshall RE. Sensory mechanisms of the Spinal Cord 2nd *New York Plenum Press*, 1991

Willis WD, Al Chaer ED, Quast MJ, Westlund KN. A visceral pain pathway in the dorsal column of the spinal cord. *Proc Natl Acad Sci U S A* 96: 7675-7679, 1999

Willis WD. Role of neurotransmitters in sensitization of pain responses. *Ann N Y Acad Sci* 933: 142-156, 2001

Willis WD, Coggeshall RE. Sensory mechanisms of the Spinal Cord 3rd ed. *New York. Kluwer Academic/Plenum Publishers,* 2004

Witkin JM, Terry P, Menkel M, Hickey P, Pontecorvo M, Ferkany J, Katz JL. Effects of the selective *sigma* receptor ligand, 6-[6-(4-hydroxypiperidinyl)hexyloxy]-3-methylflavone (NPC 16377), on behavioral and toxic effects of cocaine. *J Pharmacol Exp Ther* 266: 473-482, 1993

Wong GT. Speed congenics: applications for transgenic and knock-out mouse strains. *Neuropeptides* 36: 230-236, 2002

Wood JN, Winter J, James IF, Rang HP, Yeats J, Bevan S. Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci* 8: 3208-3220, 1988

Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience* 62: 327-331, 1994

Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* 288: 1765-1769, 2000

Wu J, Fang L, Lin Q, Willis WD. Fos expression is induced by increased nitric oxide release in rat spinal cord dorsal horn. *Neuroscience* 96: 351-357, 2000

Yagasaki Y, Numakawa T, Kumamaru E, Hayashi T, Su TP, Kunugi H. Chronic antidepressants potentiate via sigma-1 receptors the brain-derived neurotrophic factor-induced signaling for glutamate release. *J Biol Chem* 281: 12941-12949, 2006

Yao Y, Mayer ML. Characterization of a soluble ligand binding domain of the NMDA receptor regulatory subunit NR3A. *J Neurosci* 26: 4559-4566, 2006

Zahm DS, Jensen SL, Williams ES, Martin JR, III. Direct comparison of projections from the central amygdaloid region and nucleus accumbens shell. *Eur J Neurosci* 11: 1119-1126, 1999

Zamanillo D, de la Puente B, Nadal X, Ovalle S, Sanchez R, Portillo E, Romero L, Muro A, Palacios G, Baeyens JM, Lopez-Garcia JA, Buschmann H, Maldonado R, Vela JM Sigma 1 receptor blocking: A new therapeutic approach for neuropathic pain. 12th World Congress of Pain: ph247, 2008

Zambreanu L, Wise RG, Brooks JC, Iannetti GD, Tracey I. A role for the brainstem in central sensitisation in humans. Evidence from functional magnetic resonance imaging. *Pain* 114: 397-407, 2005

Zhang D, Zhang L, Tang Y, Zhang Q, Lou D, Sharp FR, Zhang J, Xu M. Repeated cocaine administration induces gene expression changes through the dopamine D_1 receptors. *Neuropsychopharmacology* 30: 1443-1454, 2005

Zhang H, Cuevas J. σ Receptor activation blocks potassium channels and depresses neuroexcitability in rat intracardiac neurons. *J Pharmacol Exp Ther* 313: 1387-1396, 2005

Zhang X, Bao L. The development and modulation of nociceptive circuitry. *Curr Opin Neurobiol* 16: 460-466, 2006

Zhuang ZY, Gerner P, Woolf CJ, Ji RR. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 114: 149-159, 2005

Zou X, Lin Q, Willis WD. Enhanced phosphorylation of NMDA receptor 1 subunits in spinal cord dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats. *J Neurosci* 20: 6989-6997, 2000.