UNIVERSIDAD DE GRANADA

FACULTAD DE MEDICINA

DEPARTAMENTO DE FARMACOLOGÍA

E INSTITUTO DE NEUROCIENCIAS

$SIGMA_1$ RECEPTORS: ALLOSTERIC MODULATION BY PHENYTOIN AND IRREVERSIBLE BLOCKADE BY HALOPERIDOL ADMINISTRATION

TESIS DOCTORAL PRESENTADA POR

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Licenciado en Bioquímica, para optar al grado de:

DOCTOR POR LA UNIVERSIDAD DE GRANADA

Habiendo obtenido la Suficiencia Investigadora dentro del Programa de Doctorado "*Neurociencias*" en el Área de Conocimiento de Farmacología

Granada, 2006

Editor: Editorial de la Universidad de Granada
Autor: Enrique José Cobos del Moral
D.L.: Gr. 2314 - 2006
ISBN: 84-338-4169-6

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Que el trabajo de investigación titulado, "SIGMA$_1$ RECEPTORS: ALLOSTERIC MODULATION BY PHENYTOIN AND IRREVERSIBLE BLOCKADE BY HALOPERIDOL ADMINISTRATION" ha sido realizado por D. Enrique José Cobos del Moral para optar al grado de Doctor por la Universidad de Granada, en el Departamento de Farmacología de la Facultad de Medicina de la Universidad de Granada, bajo mi dirección.

Y para que conste donde proceda se firma este certificado en Granada a 29 de Septiembre de 2006

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La realización de esta tesis ha sido posible gracias a una beca predoctoral del Ministerio de Educación y Ciencia y a la financiación de nuestro grupo de investigación por la Junta de Andalucía (CTS-109), por el CDTI (proyecto CENIT Genius Pharma) y por Laboratorios Dr. Esteve S.A.

A Teresa, por su cariño y apoyo incondicional, especialmente durante nuestro primer año de casados y mi último de tesis

Agradecimientos

Quiero expresar mi gratitud a todas las personas que con su apoyo y consejo me han ayudado durante todos estos años, tanto en el laboratorio como fuera de él.

A Jose y Esperanza. Se me quedan cortas las palabras. Gracias por haberme brindado la oportunidad de formarme como investigador al haberme aceptado en vuestro grupo de investigación, del que me he sentido desde el primer momento como un miembro útil y valorado. Creo sinceramente que ambos sois modelos a seguir, no sólo por vuestra calidad científica, sino también por vuestra calidad humana. Después de estos años no sólo habeis sido mis Directores de Tesis, además habeis demostrado continuamente ser mis amigos.

A Esperanza. Seguro que no muchos doctorandos pueden decir que su Director de Tesis les ha guiado tan dedicadamente, apoyado siempre y confiado en ellos como has hecho durante estos años conmigo; y no sólo en el laboratorio, sino también fuera de él. Gracias por todo lo que he aprendido de ti durante estos años, no sólo como investigador, sino también como persona. Estoy muy orgulloso de haber realizado esta Tesis bajo tu dirección.

A Jose. Por saber dirigir siempre a nuestro grupo no sólo con una calidad científica intachable, sino de una manera cordial y humana, por aceptar siempre de buen grado las sugerencias y opiniones de los demás y por pensar siempre en el bienestar de todos los que trabajamos en el laboratorio. Eres un Director de Tesis excepcional, y no sólo por tus vastos conocimientos, sino también por tu amabilidad.

A mis compañeros del Departamento: Cruzmi (Chusmi), José Miguel (Pujolete), José Manuel (Entrena), Paco (El grande), Gema, Anke, Luís y Willias. Siempre nos hemos apoyado y compartido nuestras inquietudes. Hemos trabajado duro, pero

también hemos disfrutado comiendo un buen chuletón en "lo de la hija de Aureliano" (El Calar). Ha sido un auténtico placer trabajar con todos y cada uno de vosotros.

A Agatángelo Soler, por ser un buen maestro, enseñarme e incentivarme a aprender técnicas de fluorimetría, ofrecerte siempre a que trabajemos juntos y por estar siempre disponible para lo que me hiciera falta (microcentrífugas, una cervecilla al mediodía…).

A Eduardo Fernández y Paco Arrébola por dejarme el material y las instalaciones para cultivos celulares del Departamento de Histología, y sobretodo por sus expertos consejos para la manipulación de líneas celulares establecidas.

A Elena Pita, por animar siempre el Departamento con ese tono de voz que te caracteriza, contigo en el Departamento no se aburre uno nunca.

A mis compañeros y amigos de otros laboratorios: Jesús y Raquel, Irene, Migue, Sergio (bueno, ya sé no trabajas en un laboratorio, pero como si lo hicieras), las melatoninas (Huda, Pepi, Inés y Francesca) y Victor (el super-reventado). Por contarme vuestras penas y escuchar las mías, y por las cervecillas que nos hemos tomado y las que nos tomaremos (no todo va a ser trabajar y hablar de ciencia, ¿no?).

A mis padres y a mi hermano, por apoyarme siempre durante estos años (a veces sé que no ha sido fácil) y por hacerme ver que siempre puedo contar con vosotros. A mis padres, particularmente, por enseñarme siempre con vuestro ejemplo a esforzarme para alcanzar mis objetivos, y a mi hermano por echarme una mano todas las veces que he tenido problemillas con el ordenador, que no han sido pocas.

A toda mi familia en general y a mi tía Mari Leo en particular, por estar tan orgullosa de mí y por hacer que me interesase por la Biología desde muy pequeño.

A los Mudarra, especialmente a Ana, por haber sido una compañera de piso tan genial durante los meses en que he estado escribiendo la tesis.

A mis amigos de Jaén: Juanito y muy especialmente a Pakito y Belén, gracias por escucharme cuando me ha hecho falta hablar y por ilusionaros tanto como yo con cada pequeño logro que iba consiguiendo durante estos años. Pakito y Belén, quiero agradeceros de antemano todos los ensayos en inglés de esta Tesis que vais a tener que soportar (eso os pasa por ser filólogos).

A mis amigos de Biología: Jorgito, Javi (el otro componente del "grupo de dos") y Ana, Miguel y Rocío, Javi (Sapi) e Isa, Raquelilla y Mercedes. Por interesaros siempre por cómo iba mi Tesis.

A Paqui, Juanmi (Juanmix), Puri, Cruz, Maribel, Manu, Sofía y muy especialmente a Aureliano, por ayudarme siempre de buen grado en todo lo que me hiciera falta, y sobretodo por hacerme sentir como si estuviera en mi casa.

A Teresa, a quien va dedicada esta Tesis. Al final hemos conseguido terminarla, ¿eh Chiqui? Lo digo en plural porque realmente todos los sacrificios que he hecho durante estos años, como los fines de semana de trabajo duro o las estancias en el extranjero, no los he hecho yo sólo, los hemos hecho los dos. Sin tu apoyo constante no sé cómo podría haber terminado esta Tesis. Gracias sobretodo por tu paciencia (sí, aunque te parezca mentira a veces tienes paciencia) y por tu cariño durante este último año, que ha sido además nuestro primer año de casados. No sé dónde me llevará esta carrera de investigador que he escogido, pero me lleve donde me lleve, lo que tengo claro es que estaremos siempre juntos apoyándonos.

A todos los becarios del resto de España que he tenido el placer de conocer, especialmente a Fernando (el Tron) de Galicia y a David Soto de Barcelona.

A Karen Shashok y Eva Colmenero, por revisar el inglés de esta Tesis.

A la Fundación Hospital Clínico y al Instituto de Neurociencias de la Universidad de Granada, por la ayuda recibida para los gastos de encuadernación de esta Tesis.

Por último, pero no por ello menos importante, quiero agradecer a todos los cobayas que han participado, aunque involuntariamente, en el desarrollo de este trabajo de investigación, especialmente a mi cobaya (Obi-wan, el de la página 107). Sin ellos no habría sido posible la realización de esta Tesis.

Acknowledgements

To Dr. Talvinder Sihra, for the guidance and all that I have learnt from him throughout the period that I spent in the UCL in London.

To Vivian, Phil and Jazmina. They made my stay in London very comfortable.

To Professor Bowen, for leading and guiding me. It was an honour to work in your lab in the Brown University during the last summer.

To Zhiping, Xue, Oakley, Hanae, Matthew, Cheng, Martha and Dioscaris. For being so friendly with me and for the time that we shared working in Dr. Bowen's lab.

To Elke and Jouliette, my house-mates in Providence, for making me feel like if I was at home during my stay in USA.

"Los hombres deberían saber que del cerebro, y nada más que del cerebro, vienen las alegrías, el placer, la risa, el ocio, las penas, el dolor, el abatimiento y las lamentaciones. A través del él pensamos, vemos, oímos y distinguimos lo feo de lo bonito, lo malo de lo bueno, lo agradable de lo desagradable"

Hipócrates – Siglo V A.C.

"Men ought to know that from the brain, and the brain alone, arise our pleasure, joy, laughter, just as well as our sorrow, pain, grief, and tears. Through it, in particular, we think, see, hear and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant"

Hippocrates –5th Century B.C.

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RESUMEN

Introducción: planteamiento y objetivos

La existencia de los denominados receptores sigma (σ) fue propuesta hace 30 años (Martin et al. 1976). Estudios posteriores revelaron la existencia de al menos dos subtipos de receptores σ, que fueron denominados σ_1 y σ_2 (Quirion et al., 1992). Farmacológicamente, los receptores σ_1 se distinguen de los σ_2 por su estereoselectividad por los isómeros dextrógiros de los benzomorfanos, mientras que los σ_2 muestran una estereoselectividad opuesta (Hellewell and Bowen, 1990; Quirion et al., 1992). Otras características farmacológicas que diferencian a los receptores σ_1 de los σ_2 son la modulación alostérica de los ligandos σ_1 por fenitoína (DPH) y la disminución de la fijación de radioligandos σ_1 tras la administración *in vivo* de haloperidol (Quirion et al. 1992).

Modulación alostérica del receptor σ_1 por DPH

La fenitoína modula alostéricamente la fijación de los ligandos σ_1 sin afectar a los ligandos σ_2 (Quirion et al. 1992), sin embargo se ha demostrado que esta modulación no afecta a todos los ligandos σ_1. DPH potencia la fijación de los siguientes radioligandos σ_1: [^{3}H]dextrometorfano, [^{3}H](+)-SKF-10,047, [^{3}H](+)-3-PPP y [^{3}H](+)-pentazocina (Musacchio et al., 1987, 1988, 1989a and b; Craviso and Musacchio, 1983; Karbon et al., 1991; Bailey and Karbon, 1993; McCann and Su, 1991; Culp et al., 1992; Rothman et al., 1991; Bonhaus et al., 1993; Chaki et al., 1996; DeHaven-Hudkins et al., 1993). Por el contrario, la fijación de otros radioligandos σ_1, tales como [^{3}H]haloperidol, [^{3}H]progesterona, [^{3}H]DTG, [^{3}H]DuP 734,

[^{3}H]RS-23597-190 o [^{3}H]NE-100, no es potenciada por este anticonvulsivo (Karbon et al., 1991; Meyer et al., 1998; Culp et al., 1992; Bonhaus et al., 1994; Tanaka et al., 1995; Chaki et al., 1996). Para explicar estos resultados, DeHaven-Hudkins y cols. (1993) propusieron que la modulación diferencial de los ligandos σ_1 por DPH podría producirse sólo en aquellos ligandos σ selectivos por el subtipo σ_1. Sin embargo, esta hipótesis no concuerda con el perfil de selectividad σ_1/σ_2 de los fármacos anteriormente nombrados.

Un análisis detallado de los datos anteriores sugiere que los radiofármacos modulados por DPH son habitualmente considerados como agonistas σ_1, por lo que nuestra hipótesis es que la DPH incrementa la afinidad por los receptores σ_1 de los agonistas, pero no de los antagonistas σ_1. Por tanto, nuestro **primer objetivo** fue estudiar si la DPH es capaz de modular diferencialmente la fijación de ligandos σ_1 en función de su carácter agonista o antagonista σ_1.

Inhibición irreversible del receptor σ_1 tras la administración de haloperidol

Como se ha mencionado anteriormente, otra característica farmacológica que distingue a los receptores σ_1 de los σ_2 es la disminución de la fijación de los radioligandos σ_1 tras la administración *in vivo* de haloperidol. El haloperidol es un antipsicótico ampliamente utilizado en terapéutica. Este psicofármaco es un conocido antagonista dopaminérgico, principalmente del subtipo D_2, sin embargo se une con una afinidad similar a los receptores σ (Bowen et al., 1990), mostrando cierta preferencia por la unión a los receptores σ_1 frente a los σ_2 (McCann et al., 1994; Matsumoto and Pouw, 2000), siendo sus acciones sobre estos receptores menos conocidas que sus

acciones dopaminérgicas. La administración aguda de este antipsicótico induce una reducción drástica y duradera de la fijación de radioligandos σ_1 sin afectar a los radioligandos σ_2 (Klein et al., 1994; Inoue et al., 2000), y sin modificar los niveles del ARNm del receptor σ_1 (Nakata et al., 1999; Inoue et al., 2000), de manera que se propuso que este proceso podría ser debido a la inactivación irreversible de este receptor tras la administración de haloperidol (Klein et al, 1994; Inoue et al., 2000). Pese a que la hipótesis de la inactivación irreversible del receptor σ_1 fue planteada en 1994 por Klein y cols., hoy en día se desconoce si el bloqueo irreversible de este receptor es producido también por otros antagonistas σ_1 o dopaminérgicos, o en cambio es una característica exclusiva del haloperidol. Teniendo en cuenta estos antecedentes, el **segundo objetivo** de esta Tesis Doctoral fue estudiar y comparar los cambios en la fijación de un radioligando selectivo σ_1 tras la administración aguda de haloperidol y de otros ligandos antagonistas dopaminérgicos o σ_1.

Los receptores σ_1 están presentes en diversas membranas celulares, como en membranas microsomales, nucleares y sinaptosomales (Itzhak et al., 1991; Cagnotto et al., 1994; DeHaven-Hudkins et al., 1994; Alonso et al., 2000). La inactivación irreversible de este receptor haría posible el estudio de los parámetros de recambio bioquímico y los procesos de redistribución de este receptor en estas membranas celulares, siendo éste nuestro **tercer objetivo**.

Klein y colaboradores (1994) demostraron que el metabolito reducido de haloperidol también producía la inactivación irreversible de los receptores σ_1, por lo que este efecto podría ser mediado o compartido por haloperidol y/o alguno de sus metabolitos. Teniendo en cuenta estos antecedentes, el **cuarto objetivo** de esta Tesis

Doctoral fue investigar si el bloqueo irreversible de los receptores σ_1 es producido por haloperidol *per se*, por alguno de sus metabolitos, o por ambos.

Métodos

Las membranas cerebrales (fracción nuclear cruda, fracción sinaptosomal cruda y fracción microsomal) se obtuvieron según los protocolos, ligeramente modificados, de González y cols. (2001) y de Gurd y cols. (1974).

Los ensayos de fijación de [^{3}H](+)-pentazocina y [^{3}H]NE-100 en membranas cerebrales de cobaya, así como aquellos ensayos de fijación de la [^{3}H](+)-pentazocina en celulas de neuroblastoma humano SH-SY5Y, se realizaron utilizando los protocolos descritos en la bibliografía (DeHaven-Hudkins et al., 1992; Tanaka et al., 1995; Hong et al., 2004, respectivamente), levemente modificados. Una vez terminada la reacción de fijación, las muestras fueron filtradas (Brandel cell harvester; Brandel Instruments, SEMAT Technical Ltd., UK) utilizando filtros de fibra de vidrio (Whatman GF/B), previamente humedecidos con una solución de polietileneimina 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).

Modulación alostérica del receptor σ_1 por DPH

Para cumplir el **primer objetivo**, que fue comprobar si la DPH es capaz de modular diferencialmente la fijación de ligandos agonistas o antagonistas σ_1, realizamos ensayos de fijación de radioligando en fracción sinaptosomal cruda de cerebro de

cobaya, utilizando dos aproximaciones experimentales diferentes. La primera consistió en la comparación de los posibles cambios en afinidad inducidos por DPH de diversos ligandos agonistas y antagonistas σ_1, mediante el uso de ensayos de competición, marcando al receptor σ_1 con el radioligando selectivo [^{3}H](+)-pentazocina y desplazando su fijación con concentraciones crecientes de varios ligandos fríos en presencia y ausencia de DPH. La segunda aproximación consistió en la comparación exhaustiva de la posible modulación de la fijación de los radioligandos [^{3}H](+)-pentazocina (agonista σ_1) y [^{3}H]NE-100 (antagonista σ_1), para lo que realizamos ensayos de fijación de estos radioligados en presencia y ausencia de DPH tanto en cinética de disociación como en situación de equilibrio.

Inhibición irreversible del receptor σ_1 tras la administración de haloperidol

Para cumplir con el **segundo objetivo**, y por lo tanto estudiar si otros ligandos antagonistas σ_1 o dopaminérgicos inhiben irreversiblemente al receptor σ_1, los cobayas fueron tratados de manera aguda con los ligandos antagonistas σ_1 BD 1047 (60 mg/kg), BD 1063 (60 mg/kg), NE-100 (30 mg/kg) y haloperidol (2 mg/kg), así como con el antagonista dopaminérgico (-)-sulpiride (100 mg/kg); a los tres días del tratamiento, para evitar contaminación de las muestras con fármaco residual, los cobayas fueron sacrificados y sus cerebros procesados para obtener la fracción nuclear cruda, sinaptosomal cruda y microsomal, en las que se realizaron los ensayos de fijación de la [^{3}H](+)-pentazocina.

Para estudiar los diferentes parámetros de recambio bioquímico y los procesos de redistribución del receptor σ_1 en distintas membranas subcelulares, y cumplir de esta

manera con el **tercer objetivo** de esta Tesis Doctoral, administramos haloperidol (2 mg/kg) de manera aguda a distintos grupos de cobayas para bloquear irreversiblemente al receptor σ_1, tras lo cual los animales fueron sacrificados a distintos tiempos, siendo sus cerebros procesados para obtener las tres fracciones subcelulares objeto de estudio. Estas soluciones de membranas cerebrales fueron incubadas con una concentración saturante del radioligando selectivo σ_1 [^{3}H](+)-pentazocina, para obtener las distintas curvas de recuperación del receptor σ_1 en estas tres preparaciones de membranas celulares.

Por último, para cumplir con el **cuarto objetivo** de esta Tesis Doctoral, que fue investigar si el bloqueo irreversible de los receptores σ_1 es producido por haloperidol *per se* o por alguno de sus metabolitos, realizamos preincubaciones *in vitro* con haloperidol y sus metabolitos con afinidad σ_1 (haloperidol metabolitos I y II), en distintas muestras: (1) membranas cerebrales de cerebro de cobaya, las cuales poseen una actividad metabólica muy limitada, (2) en homogenados de cerebro de cobaya, los cuales retienen los sistemas enzimáticos necesarios para el metabolismo del haloperidol, y (3) en células SH-SY5Y de neuroblastoma humano, utilizadas como modelo neuronal humano metabólicamente activo. Las concentraciones utilizadas de estos fármacos en el proceso de preincubación fueron proporcionales a las CI_{50} de cada ligando para la fijación de la [^{3}H](+)-pentazocina, con el objeto de mantener el mismo grado de inhibición de la fijación del radioligando con todos los fármacos. Posteriormente se procedió al lavado de las muestras para eliminar el fármaco con el que se preincubaron, siguiendo el protocolo de Bluth y cols (1989) con ligeras modificaciones, y se realizaron los ensayos de fijación de la [^{3}H](+)-pentazocina con el objeto de determinar si alguno de los fármacos ensayados se fija de manera irreversible al receptor σ_1, y

consecuentemente disminuye la fijación de nuestro radioligando marcador. Además, en algunas de las muestras de homogenado de cerebro de cobaya realizamos una preincubación con menadiona (1 mM), un inhibidor de la actividad cetona reductasa responsable de la producción de uno de los principales productos de la metabolización del haloperidol, el haloperidol reducido (también denominado haloperidol metabolito II).

Resultados y discusión

Modulación alostérica del receptor σ_1 por DPH

Experimentos de competición heterólogos en fracción sinaptosomal cruda de cerebro de cobaya, utilizando como radioligando selectivo σ_1 a la [^{3}H](+)-pentazocina, mostraron que la DPH incrementó de manera concentración-dependiente (250 μM y 1 mM) la afinidad de los ligandos agonistas σ_1: dextrometorfano, (+)-SKF-10,047, (+)-3-PPP, y PRE 084. Sin embargo, DPH 250 μM no incrementó la afinidad de los ligandos antagonistas: haloperidol, BD 1063, NE-100, progesterona, y BD 1047, incluso sus afinidades disminuyeron levemente con DPH 1 mM. De manera que la DPH parece discriminar a los ligandos agonistas σ_1 de los antagonistas utilizando esta aproximación experimental.

Utilizando una aproximación diferente, mediante la comparación de los cambios inducidos por DPH (250 μM) en la fijación de los radioligandos [^{3}H](+)-pentazocina y [^{3}H]NE-100 en fracción sinaptosomal cruda de cerebro de cobaya, demostramos que aunque ambos radioligandos marcan al mismo receptor, puesto que las afinidades

(medidas como K_i) por los sitios de fijación [^{3}H](+)-pentazocina y [^{3}H]NE-100 de 12 ligandos σ_1 fríos mostraron una buena correlación (r^2= 0,952), la fenitoína potenció la fijación del radioligando agonista σ_1 [^{3}H](+)-pentazocina, incrementando su afinidad (medida como el valor de K_D) más de dos veces y disminuyendo en 2,5 veces su tasa de disociación, mientras que el número máximo de receptores reconocidos por el radioligando (B_{max}) no fue significativamente modificado. En cambio, DPH disminuyó la fijación del radioligando antagonista σ_1 [^{3}H]NE-100 disminuyendo su valor de B_{max} en un 15% e incrementando su tasa de disociación al doble, mientras que su valor K_D permaneció constante.

Estos resultados se pueden explicar en base al modelo extendido de dos estados de activación de receptores propuesto por Hall (2000). Según este modelo, en situación de reposo los receptores muestran dos estados conformacionales que se encuentran en equilibrio: un estado inactivo (R) y otro activo (R*). Los ligandos agonistas promueven el estado R*, uniéndose a este estado con alta afinidad. Un modulador alostérico positivo, en nuestro caso la DPH, desplazaría el equilibrio entre los estados R y R* hacia el estado activo (R*), incrementando la fijación de los radioligandos agonistas. Esto, en nuestros experimentos, se traduciría en un incremento de la afinidad de los ligandos agonistas σ_1 en estudios de competición heterógos, y en el incremento de la afinidad y la disminución de la tasa de disociación de nuestro radioligando agonista [^{3}H](+)-pentazocina. En cambio, los ligandos antagonistas, según este modelo, no reconocen diferencialmente a los estados R y R*, por lo que su fijación no sería susceptible de ser modificada por un modulador alostérico, que es lo que ocurre en nuestros estudios de competición heterólogos cuando utilizamos como ligando desplazante a un ligando antagonista σ_1. Para explicar nuestros resultados con el

radioligando antagonista σ_1 [^{3}H]NE-100 utilizamos el mismo planteamiento basado en la teoría del modelo extendido de dos estados. Un modulador alostérico positivo, como hemos demostrado que es la fenitoína, estabiliza la conformación R* a expensas de la inactiva R, y por lo tanto la fijación de los radioligandos que se unan preferencialmente a la conformación inactiva R (agonistas inversos) debe verse disminuída, por lo que según nuestros resultados, [^{3}H]NE-100 se comportaría según este modelo como un agonista inverso, incrementando su tasa de disociación y disminuyendo su valor de B_{max} sin modificar su afinidad (K_D). Estudios previos en receptores adrenérgicos β_2 muestran que la fijación de radioligandos agonistas inversos es modulada de la misma manera que la fijación del [^{3}H]NE-100 en nuestras condiciones experimentales, disminuyendo el valor de B_{max} sin modificar su afinidad (K_D) (Azzi et al., 2001). Esta disminución de la fijación del [^{3}H]NE-100 no entra en contradicción con la ausencia de modulación en experimentos de competición utilizando a este ligando como ligando frío competidor, puesto que en esos experimentos la afinidad (K_i) del NE-100 no se modificó utilizando DPH 250 μM.

Inhibición irreversible del receptor σ_1 tras la administración de haloperidol

La administración aguda tres días antes de obtener los cerebros de BD 1047, BD 1063, NE-100 o (-)-sulpiride no indujo variación alguna en la fijación de la [^{3}H](+)-pentazocina en fracción nuclear cruda, sinaptosomal cruda o microsomal de cerebro de cobaya con respecto a animales controles. En cambio, la administración aguda de haloperidol indujo una abrupta disminución de la fijación del radioligando en las tres fracciones subcelulares, siendo por tanto la fijación irreversible al receptor σ_1

una cualidad exclusiva del haloperidol, no compartida por otros antagonistas dopaminérgicos o σ_1. La DE_{50} de este efecto en fracción sinaptosomal cruda fue de 0,017 ± 0,002 mg/kg.

La recuperación de la fijación de concentraciones saturantes de [^{3}H](+)-pentazocina (indicativa de la densidad de receptores σ_1) tras la administración aguda de haloperidol, se produjo progresivamente a lo largo del tiempo en las tres subfracciones estudiadas, alcanzándose el estado estacionario aproximadamente en 30 días. La densidad de receptores σ_1 en este estado estacionario fue inferior a la de los animales controles, incluso 64 días después de la administración de haloperidol, lo que podría ocasionar consecuencias funcionales. La constante de desaparición del receptor, indicativa de la tasa de degradación del receptor, fue la misma en las tres subfracciones estudiadas, mientras que la tasa de aparición fue tres veces más lenta en fracción nuclear cruda que en fracción sinaptosoma cruda, y esta levemente inferior o igual que en fracción microsomal, indicando que el receptor recién sintetizado ocupa más rápidamente el retículo endoplásmico, con una velocidad similar las membranas sinaptosomales, y de manera notablemente más lenta las membranas nucleares.

El orden de afinidad de diversos ligandos σ_1 en fracción sinaptosomal de cerebro de cobaya y en células SH-SY5Y de neuroblastoma humano mostró una buena correlación (0,991), indicando que ambas preparaciones poseen un receptor σ_1 farmacológicamente equivalente. La preincubación de los homogenados de cerebro de cobaya o de células SH-SY5Y de neuroblastoma humano con haloperidol indujo una inhibición resistente a lavado de la fijación de la [^{3}H](+)-pentazocina, lo que indicó una inactivación irreversible del receptor σ_1. Sin embargo cuando el haloperidol se preincubó con fracción sinaptosomal cruda de cerebro de cobaya, cuya capacidad

metabólica es notoriamente inferior a la del homogenado de cerebro, no indujo tal inhibición, lo que sugiere que haloperidol debe ser metabolizado para producir el bloqueo irreversible de los receptores σ_1. El haloperidol metabolito I no produjo efecto alguno en la fijación del radioligando marcador en ninguna de las tres muestras utilizadas, por lo que no es el metabolito responsable de la inactivación irreversible de los receptores σ_1. Sin embargo, la adición al medio de incubación de menadiona, un inhibidor de la actividad cetona reductasa responsable de la metabolización de haloperidol hacia haloperidol metabolito II (haloperidol reducido), inhibió el bloqueo irreversible de la fijación de la [^{3}H](+)-pentazocina en homogenado de cerebro de cobaya, lo que sugiere que el haloperidol debe metabolizarse a haloperidol reducido para bloquear irreversiblemente al receptor σ_1. Además, la preincubación de las tres muestras utilizadas (homogenado o fracción sinaptosomal de cerebro de cobaya, y células SH-SY5Y de neuroblastoma humano) con haloperidol reducido indujo una inhibición resistente a lavado de la fijación de la [^{3}H](+)-pentazocina.

Conclusiones

1. La fenitoina modula alostéricamente, y de manera diferencial, la fijación de los ligandos σ_1 en función de su actividad sobre los receptores σ_1, por lo que sería un método *in vitro* fiable para discriminar entre agonistas y antagonistas σ_1 de nueva síntesis.

2. La administración de haloperidol a cobayas induce, gracias a su metabolización a haloperidol reducido (haloperidol metabolito II), una inhibición irreversible del receptor σ_1 en cerebro; este efecto es duradero, no es compartido por otros ligandos σ_1 ni dopaminérgicos, se produce en un rango de dosis utilizado en terapéutica en humanos y es reproducible en un modelo neuronal humano, por lo que podría tener consecuencias funcionales de interés terapéutico.

SUMMARY

Introduction: hypothesis and goals

The actions of sigma (σ) receptors were first reported 30 years ago (Martin et al., 1976). Later studies exposed that there were at least two subclasses of σ receptors, called σ_1 and σ_2 receptors (reviewed by Quirion et al., 1992). Pharmacologically, the main difference between σ_1 and σ_2 binding sites is that the former display stereospecificity towards dextrorotatory isomers of benzomorphans, whereas the latter display reverse selectivity, i.e., levorotatory isomers show higher affinity than dextrorotatory isomers of σ ligands (Hellewell and Bowen, 1990; Quirion et al., 1992). Other pharmacological characteristics that distinguish σ_1 and σ_2 binding sites are the allosteric modulation of σ_1 ligand binding by phenytoin (DPH) and the decrease in σ_1 radioligand binding after *in vivo* haloperidol administration (Quirion et al., 1992).

Allosteric modulation of σ_1 receptors by DPH

Phenytoin allosterically modulates the binding of σ_1 radioligands without affecting σ_2 radioligands (Quirion et al., 1992). However, DPH not only discriminates between σ_1 and σ_2 ligands, but also distinguishes between different σ_1 ligands. In fact, DPH increases the binding of [^{3}H]dextromethorphan, [^{3}H](+)-SKF-10,047, [^{3}H](+)-3-PPP and [^{3}H](+)-pentazocine (Musacchio et al., 1987, 1988, 1989a and b; Craviso and Musacchio, 1983; Karbon et al., 1991; Bailey and Karbon, 1993; McCann and Su, 1991; Culp et al., 1992; Rothman et al., 1991; Bonhaus et al., 1993; Chaki et al., 1996; DeHaven-Hudkins et al., 1993), but not the binding of the following σ_1 radioligands: [^{3}H]haloperidol, [^{3}H]progesterone, [^{3}H]DTG, [^{3}H]DuP 734,

[^{3}H]RS-23597-190 and [^{3}H]NE-100 (Karbon et al., 1991; Meyer et al., 1998; Culp et al., 1992; Bonhaus et al., 1994; Tanaka et al., 1995; Chaki et al., 1996). DeHaven-Hudkins and co-workers (1993) proposed that the differential sensitivity of σ_1 compounds to allosteric modulation by phenytoin might be restricted to compounds that bind selectively to σ_1 binding sites. However, this hypothesis is not entirely consistent with the current knowledge of the selectivity patterns of these ligands.

A detailed analysis of the aforementioned data suggests that the radioligands modulated by DPH are typically considered σ_1 agonists, so our first hypothesis is that DPH differentially modulates the binding of σ_1 ligands depending on whether they act as agonists or antagonists of σ_1 receptors. Therefore, the **first goal** of this Doctoral Thesis was to test whether DPH is able to differentially modulate the binding of several σ_1 ligands depending on their agonistic or antagonistic activities on σ_1 receptors.

Irreversible inactivation of σ_1 receptors after haloperidol administration

As mentioned above, another pharmacological characteristic that distinguishes σ_1 from σ_2 receptors is the decrease in σ_1 radioligand binding after *in vivo* haloperidol administration. Haloperidol is an antypsychotic widely used in therapeutics; its antypsychotic activity and many other of its pharmacological effects result, at least in part, from its ability to act as an antagonist at dopamine D_2 receptors. However, this drug shows similar affinity for D_2 and σ receptors (Bowen et al. 1990), binding preferentially to σ_1 than to σ_2 receptors (McCann et al., 1994; Matsumoto and Pouw, 2000), although the consequences of its binding to σ receptors are less well-known.

The single administration of haloperidol produces a marked and long-lasting decrease in σ_1 radioligand binding without affecting σ_2 radioligands (Klein et al., 1994; Inoue et al., 2000). The treatment with haloperidol does not affect the levels of σ_1 receptor mRNA in brain (Nakata et al., 1999; Inoue et al., 2000), so it was proposed that the decrease in σ_1 radioligand binding could be due to the irreversible inactivation of σ_1 receptors after haloperidol administration (Klein et al., 1994; Inoue et al., 2000). Nowadays, it is still unknown if the inactivation of σ_1 receptors induced by a single administration of haloperidol is shared by other σ_1 receptor antagonists, or it is due to this drug's D_2 antagonist activity instead. Taking into account these antecedents, the **second goal** of this Doctoral Thesis was to study and compare the possible changes in the binding of a prototypic σ_1 receptor ligand after the single administration of haloperidol and other σ_1 antagonists or D_2 antagonists.

$Sigma_1$ receptors are located in microsomal, nuclear and synaptic membranes (Itzhak et al., 1991; Cagnotto et al., 1994; DeHaven-Hudkins et al., 1994; Alonso et al., 2000). The irreversible inactivation of σ_1 receptors by haloperidol would make it possible to evaluate the turnover of these receptors in the different subcellular membranes. As this is something which has not been previously investigated, the **third goal** of this Doctoral Thesis was to study the turnover and the redistribution processes of σ_1 receptors in guinea pig brain subcellular (nuclear, microsomal and synaptosomal) membranes.

Klein and co-workers (1994) reported that the administration of the reduced metabolite of haloperidol to guinea pigs also markedly decreased the number of brain σ_1 binding sites, so haloperidol-induced σ_1 receptor inactivation could be produced by the parent compound or by one or even more of its metabolites. In the light of these

antecedents, the **fourth goal** of this Doctoral Thesis was to investigate whether the irreversible blockade of σ_1 receptors is produced or shared by haloperidol and/or one of its metabolites.

Methods

Brain membranes (crude nuclear, crude synaptosomal and microsomal fractions) were obtained following the protocols of González et al., 2001; and Gurd et al., 1974, with slight modifications.

[^{3}H](+)-pentazocine and [^{3}H]NE-100 binding assays in guinea pig brain membranes, as well as [^{3}H](+)-pentazocine binding assays in SH-SY5Y human neuroblastoma cells, were performed according to the protocols, slightly modified, which are described in bibliographic references (DeHaven-Hudkins et al., 1992; Tanaka et al., 1995; Hong et al., 2004, respectively). The bound and free radioligands were separated by rapid filtration under a vacuum with a Brandel cell harvester (Brandel Instruments, SEMAT Technical Ltd., UK) over Whatman GF/B glass fiber filters (presoaked for at least 1 hour with 0.5% polyethylenimine). Afterwards, the radioactivity was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A).

Allosteric modulation of σ_1 receptors by DPH

To reach our **first goal** (to test if DPH is able to differentially modulate the binding of σ_1 agonist and antagonist ligands), we carried out σ_1 radioligand binding

assays in guinea pig brain crude synaptosomal fractions following two different approaches. (1) Radioligand competition assays were performed in the presence or absence of DPH, labelling σ_1 receptors with the selective σ_1 ligand [^{3}H](+)-pentazocine to test for possible DPH-induced changes in affinity of several known σ_1 cold agonists and antagonists for σ_1 receptors. (2) Equilibrium and kinetic radioligand binding assays were performed to compare the modulation by DPH of the binding of two selective σ_1 radioligands: the prototypical σ_1 agonist [^{3}H](+)-pentazocine and the prototypical σ_1 antagonist [^{3}H]NE-100.

Irreversible inactivation of σ_1 receptors after haloperidol administration

In order to study if the inactivation of σ_1 receptors induced by a single administration of haloperidol is shared by other σ_1 receptor antagonists, or it is due to this drug's D_2 antagonist activity instead, and, therefore, reach our **second goal**, we acutely administered the known σ_1 antagonists BD 1047 (60 mg/kg), BD 1063 (60 mg/kg), NE-100 (30 mg/kg) and haloperidol (2 mg/kg) and the known dopaminergic antagonist (–)-sulpiride (100 mg/kg) to guinea pigs. The animals were killed three days after drug administration in order to minimize the presence of residual drug in the brain. Afterwards, brain membranes were obtained (crude nuclear, crude synaptosomal and microsomal fractions) and [^{3}H](+)-pentazocine binding assays were performed.

As previously mentioned, the **third goal** of this Doctoral Thesis was to study the turnover of σ_1 receptors in guinea pig brain subcellular (nuclear, microsomal and synaptosomal) membranes. To this end, we performed single *in vivo* injections of

haloperidol (2mg/kg) and killed the animals after different times to study the time-course of recovery of [^{3}H](+)-pentazocine binding, by using a single saturating concentration of this radioligand, in crude nuclear, crude synaptosomal and microsomal brain membranes.

To achieve our **fourth goal**, which was to investigate whether haloperidol-induced σ_1 receptor inactivation is produced by the parent compound or by one or even more of its metabolites, we preincubated haloperidol and its metabolites with affinity for σ_1 receptors (haloperidol metabolites I and II) with several samples: (1) in guinea pig brain synaptosomal membranes, which have a limited metabolic activity; (2) in guinea pig brain homogenates, which retain all enzymatic systems that metabolize haloperidol; and (3) in whole SH-SY5Y human neuroblastoma cells, used as a metabolically active model of human neuronal cells. Drug concentrations used in preincubation processes were proportional to the IC_{50} of each ligand for [^{3}H](+)-pentazocine binding in order to reach the same inhibition degree of radioligand binding. Afterwards, samples were washed so as to minimize the presence of residual drug following a protocol similar to that of Bluth and co-workers (1989); then, [^{3}H](+)-pentazocine binding assays were carried out to study the capability of those cold ligands to bind irreversibly (wash-resistant) to σ_1 receptors, and therefore decreased radioligand binding. Moreover, some guinea pig brain homogenates were preincubated with menadione (1 mM), an inhibitor of the ketone reductase activity that leads to the production of reduced haloperidol (also called haloperidol metabolite II), which is one of the major haloperidol metabolites produced *in vivo*.

Results and discussion

Allosteric modulation of σ_1 receptors by DPH

Heterologous competition experiments in guinea pig brain synaptosomal fraction, using [^{3}H](+)-pentazocine to label selectively σ_1 receptors, showed that DPH (250 μM and 1 mM) concentration-dependently increased the affinity of the σ_1 agonists dextromethorphan, (+)-SKF-10,047, (+)-3-PPP and PRE 084. However, neither DPH 250 μM nor 1 mM increased (in fact, they slightly decreased) the affinity of the σ_1 receptor antagonists haloperidol, BD 1063, NE-100, progesterone and BD 1047. These findings suggest that, according to this experimental approach, allosteric modulation by DPH of the affinity of σ_1 receptor ligands depends on the agonist or antagonist characteristics of the ligand.

In order to extend the results described above, we evaluated the effects of DPH (250 μM) on the binding to synaptosomal fraction membranes from guinea pig brain of the prototypic σ_1 receptor agonist [^{3}H](+)-pentazocine and the putative σ_1 antagonist [^{3}H]NE-100. The order of affinity (measured as K_i) of twelve σ_1 ligands for binding sites labelled with [^{3}H](+)-pentazocine correlated well with their order of affinity for sites labelled with [^{3}H]NE-100 (r^2= 0.952). This suggests that both radioligands label the same receptor. DPH increased the binding of [^{3}H](+)-pentazocine, enhancing its affinity (K_D value) for σ_1 receptors, which was more than 2-fold higher than that of control conditions. DPH also altered kinetic [^{3}H](+)-pentazocine binding parameters, decreasing 2.5-fold its dissociation rate constant, but the maximal number of receptors (B_{max} value) labelled with [^{3}H](+)-pentazocine was not changed. In contrast, DPH

decreased by 15% the B_{max} value of [^{3}H]NE-100, and increased 2.5-fold its dissociation rate from σ_1 receptors, whereas its K_D value was not modified. In conclusion, phenytoin behaved as a positive allosteric modulator on the binding of [^{3}H](+)-pentazocine, whereas it negatively modulated the binding of [^{3}H]NE-100.

These results could be explained by the extension of the two-state model of receptor activation (Leff, 1995) proposed by Hall (2000). In this model, receptors display two conformational states: inactive (R) or active (R*). Agonists promote the R* state and bind to this state of the receptor with high affinity. The effect of a positive allosteric modulator (e.g., DPH in our experiments) in this model would consist of the induction of transition to R*, thereby increasing the binding of the agonist to the receptor. In our experiments, this event could explain the increase in σ_1 agonist affinity both in heterologous competition experiments and in [^{3}H](+)-pentazocine saturation assays, as well as the decrease of the [^{3}H](+)-pentazocine dissociation rate constant. On the other hand, antagonist ligands do not discriminate between R and R*, so their binding would not be able to be modulated by an allosteric modulator, which is what we found in heterologous competition experiments when the cold ligand used was a σ_1 antagonist. Our results with [^{3}H]NE-100 binding can also be explained with the extension of the two-state model of the receptor activation theory. Allosteric enhancers, by stabilizing the active conformation of receptors (R*) at the expense of the inactive conformation (R), decrease the binding of those ligands which bind preferentially to the inactive conformation of the receptor, i.e., inverse agonist ligands. In this study, we have shown that DPH reduced the binding of [^{3}H]NE-100, increasing its dissociation rate constant and decreasing its B_{max} value, but without modifying its affinity (K_D) for σ_1 receptors. Previous studies have shown that the binding of inverse agonist ligands for β_2

receptors was affected by an allosteric modulator in the same way as the interaction between [^{3}H]NE-100 and DPH described in this study: the allosteric modulator decreased B_{max} without changing K_D values (Azzi et al., 2001). The decrease in [^{3}H]NE-100 binding is not opposite to those results obtained in competition experiments using NE-100 as cold ligand, because in those experiments the affinity (K_i) of NE-100 was not modified in the presence of DPH 250 μM.

Irreversible inactivation of σ_1 receptors after haloperidol administration

A single injection of haloperidol produced a marked inhibition of [^{3}H](+)-pentazocine binding in all the guinea pig brain subcellular fractions studied (crude nuclear, crude synaptosomal and microsomal fractions), which were measured three days after drug administration. This effect was not shared by other σ_1 antagonists (BD 1047, BD 1063 and NE-100) with high affinity for σ_1 receptors or by the dopaminergic antagonist (-)-sulpiride, so the irreversible inactivation of σ_1 receptors is a specific characteristic of haloperidol administration. The effect of haloperidol was dose-dependent, and its ED_{50} to induce σ_1 receptor inactivation in P_2 fraction was 0.017 ± 0.002 mg/kg. The recovery of [^{3}H](+)-pentazocine binding, by using saturating concentrations of radioligand (indicative of σ_1 receptor density), to steady state after haloperidol-induced inactivation required more than 30 days in the three subcellular fractions studied. The steady-state levels of σ_1 receptors estimated from recovery curves were lower, even 64 days after haloperidol administration, than the original steady-state concentration, which could produce functional implications. The disappearance rate constant, indicative of the receptor degradation rate, was not altered in any subcellular

fraction, whereas the receptor appearance rate constant was 3-fold lower in crude nuclear than in crude synaptosomal fraction, and this later one was slightly lower than in microsomal fraction, suggesting that the newly synthesized σ_1 receptors occupy microsomal membranes most rapidly than the other subcellular membranes, being the nuclear membranes the most slowly occupied.

The affinity of several drugs for σ_1 receptors in guinea pig brain P_2 fraction and SH-SY5Y human neuroblastoma cells correlated well ($r^2 = 0.991$), which suggests that guinea pig and human σ_1 receptors are pharmacologically equivalent. Haloperidol induced a wash-resistant inhibition of [^{3}H](+)-pentazocine binding when it was incubated *in vitro* with brain homogenate and SH-SY5Y cells, but not when incubated with P_2 fraction membranes. This suggests that haloperidol is metabolised in guinea pig brain homogenates and in SH-SY5Y cells, but not in P_2 fraction, to irreversibly inactivate σ_1 receptors. [^{3}H](+)-pentazocine maintained its ability to bind to σ_1 receptors to the same degree as in control assays in samples preincubated with haloperidol metabolite I, so this is not the haloperidol metabolite responsible for the irreversible inactivation of σ_1 receptors. On the other hand, the effect of haloperidol in guinea pig brain homogenates was reversed by menadione, an inhibitor of ketone reductase activity that leads to the production of reduced haloperidol, suggesting that haloperidol should be metabolised to reduced haloperidol to irreversibly inactivate σ_1 receptors. Moreover, preincubation of guinea pig brain homogenate, crude synaptosomal fraction or SH-SY5Y human neuroblastoma cells with reduced haloperidol, resulted in a wash-resistant inhibition of [^{3}H](+)-pentazocine binding.

Conclusions

1. The differential modulation by phenytoin (DPH) of σ_1 ligand binding to guinea pig brain membranes seems a reliable *in vitro* method to predict the pharmacological profile of newly synthesized σ_1 compounds.

2. The administration of haloperidol to guinea pigs induces, through its metabolism to reduced haloperidol (haloperidol metabolite II), the irreversible inactivation of brain σ_1 receptors. This is a long-lasting effect which is not shared by other σ_1 or dopaminergic antagonists, is produced at doses used in therapeutics in humans, can be reproduced in a human neuronal model, and it could have functional consequences of therapeutic interest.

INTRODUCTION

1.- MOLECULAR CHARASTERISTICS, DISTRIBUTION AND PHARMACOLOGICAL PROFILE OF SIGMA$_1$ RECEPTORS

Sigma (σ) receptors were first described in 1976 as a particular kind of opioid receptors, and they were later confused with the phencyclidine (PCP) binding site on *N*-methyl-D-aspartate (NMDA) receptors. In 1996 σ_1 receptor was cloned, and its amino acid sequence showed no homology with other mammalian proteins. Currently the pharmacological profile of σ_1 receptors is well defined, and they are considered a unique entity by the scientific community.

1.1. Historical overview

Sigma (σ) receptors were first described as a subclass of opioid receptors by Martin and co-workers in 1976 to account for the actions of (±)-SKF-10,047 (*N*-allylnormetazocine) and other racemic benzomorphans. Two findings demonstrated that σ receptors are not a subclass of opioid receptors: opioid receptors exhibit stereoselectivity for the (-)-isomers compounds, whereas σ receptors are stereoselective for the (+)-isomers (Zukin et al., 1982); in addition, the opioid receptor antagonist naloxone was ineffective against both the *in vivo* (Slifer and Balster, 1983; Vaupel, 1983; Shannon, 1982) and *in vitro* effects (Su, 1982; Tam, 1983; Young and Khazan, 1984) of (+)-SKF-10,047 and its binding to σ receptors. On the other hand, the (-)-isomer of SKF-10,047 binds mainly to μ and κ opioid receptors (Zukin et al., 1982). Displacement of [^{3}H]PCP binding sites by (+)-SKF-10,047 (Mendelshon et al., 1985; Zukin et al., 1982) added confusion to the identity of σ receptors, which were

sometimes designated 'σ opiate/PCP receptors' (Quirion et al., 1982). Further results (including autoradiographic studies with more selective radioligands) demonstrated that σ and PCP receptors were distinct sites, and their distribution in brain regions was found to differ (Gundlach et al., 1986a and b; Manallac et al., 1987; Sircar et al., 1986). In addition, the differences in the pharmacological profile of drugs such as haloperidol and (±)-pentazocine, two potent σ ligands which are weak or inactive against PCP receptor ligand binding (Tam et al., 1983; Tam and Cook, 1984; Gundlach et al., 1986b), soon convinced the scientific community that σ receptors were different from PCP binding sites.

1.2. Sigma receptor subtypes: σ_1 and σ_2 receptors

Sigma receptors can bind with high to moderate affinity a wide spectrum of known compounds of very different structural class, such as butyrophenones (haloperidol), benzomorphans (pentazocine, SKF-10,047), piperidines ((+)-3-PPP), guanidines (DTG), morphinans (dextromethorphan), and others, such as phenothiazines (perphenazine, chlorpromazine), tricyclic antidepressants (imipramine), monoamine oxidase inhibitors (clorgyline), serotonin-uptake inhibitors (sertraline), cytochrome P450 inhibitors (proadifen), drugs of abuse (cocaine), polyamines (ifenprodil) and certain steroids (progesterone) (reviewed in Walker et al., 1990 and in Hayashi and Su, 2004a, among others). Currently, the number of pharmacological agents derived from σ receptors is increasing with the development of new compounds (Berardi et al., 2003, 2004 and 2005; Matsumoto et al., 2001a, b and c; Kim et al., 2006b; Shiba et al., 2006;

Arrington et al., 2004; Nakazato et al., 1999b; Constantino et al., 2005; Ronsisvalle et al., 1998, 2000 and 2001b; Marrazzo et al., 2001 and 2002, among 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; Quirion et al. 1992). It was reported that σ_1 binding sites display stereospecificity towards dextrorotatory isomers of benzomorphans and other opioids, whereas σ_2 binding sites display reverse selectivity, i.e., levorotatory isomers display higher affinity than dextrorotatory isomers of σ ligands. Other drugs such as haloperidol and 3-(+)-PPP showed more affinity for σ_1 receptors, but they are not as selective for σ_1 sites as the dextrorotatory benzomorphans. On the contrary, other typical σ ligands such as DTG showed slightly more affinity for σ_2 sites (Hellewell and Bowen, 1990; Quirion et al., 1992), as will be described in 1.4 *"Pharmacological profile of σ_1 receptors*.

The presence of σ binding sites (later known as σ_2 receptors) in pheochromocytoma PC12 cells was first suggested by the presence of binding sites for the σ radioligands [^{3}H]DTG and [^{3}H](+)-3-PPP, but these binding sites differed from the known guinea pig brain σ site in their markedly lower affinity for (+)-benzomorphans such as (+)-pentazocine and (+)-SKF-10,047. However, when other prototypic σ ligands were taken into account, the marked overlap in their pharmacological profile suggested a close relationship (Hellewell and Bowen, 1990). The binding profile of σ ligands to rat hepatic membranes was found to be similar to that observed in PC12 cells when σ sites were labelled with [^{3}H]DTG, but when the σ radioligand [^{3}H](+)-pentazocine was used, the results showed the typical σ ligand selectivity observed in guinea pig brain. It was therefore concluded that [^{3}H]DTG

labelled two different σ sites with high and low affinities for (+)-benzomorphans (σ_1 and σ_2 receptors, respectively), and that [^{3}H](+)-pentazocine selectively labelled the σ_1 binding site (Hellewell and Bowen, 1990; Walker et al., 1990).

Molecular weight was found to differ between the two σ receptors subtypes: the σ_1 receptor is a 29-kDa single polypeptide which has been cloned in several animal species and humans (Kekuda et al., 1996; Prasad et al., 1998; Hanner et al., 1996; Pan et al., 1998b; Seth et al., 1997, 1998), whereas σ_2 receptors have not yet been cloned and have an apparent molecular weight of 21.5 kDa, detected with [^{3}H]azido-DTG photo-affinity labelling in the presence of dextrallorphan to mask σ_1 binding site (Hellewell et al., 1994).

Other characteristics that classically distinguishes σ_1 and σ_2 binding sites are the allosteric modulation of σ_1 ligand binding by phenytoin (DPH), which will be discussed later in section 1.4.2 *Allosteric modulation of σ_1 receptors*, and the decrease in σ_1 radioligand binding after *in vivo* haloperidol administration, which was proposed to be due to the formation of an irreversible or slowly reversible complex of haloperidol or some of its metabolites with σ_1 receptors, but not with σ_2 subtype (Klein et al., 1994, Inoue et al., 2000).

Some researchers, particularly Debonnel and colleagues, among others, suggest that there exist different σ_1 receptor subtypes or a third subtype of σ receptor. In the CA_3 region of the rat dorsal hippocampus the σ_1 receptor agonists (+)-pentazocine and JO-1784 (igmesine) potentiate the pyramidal neuron firing activity induced by microiontophoretic application of NMDA; however, the effect of (+)-pentazocine was observed both in intact rats and in rats pre-treated with pertussis toxin or colchicine, whereas both treatments abolished the effect of JO-1784 (Monnet et al., 1994; Debonnel

et al., 1996b). Similarly, the NMDA-evoked [^{3}H]noradrenaline release from preloaded rat hippocampal slices was potentiated by the σ_1 receptor agonists (+)-pentazocine and BD 737. The effect of both drugs was reverted by NE-100, whereas haloperidol and BD 1063 reverted the effect of (+)-pentazocine but not that of BD 737 (Gonzalez-Alvear and Werling, 1995; Monnet et al., 1996). Radioligand binding studies with the prototypical σ_1 radioligand [^{3}H](+)-pentazocine showed that this drug labelled a high- and a low-affinity site in several cell lines, but the lower-affinity site did not correspond to σ_2 receptors (Vilner et al., 1995). Further evidence of the existence of other σ binding sites was provided by the finding of a [^{3}H]DTG binding site in the mouse spinal cord that was found to differ from σ_1 or σ_2 sites (Kovacs and Larson, 1998). Moreover, Tsao and Su (1997) purified from the rat liver and brain a [^{3}H](+)-SKF-10,047-binding protein with a molecular mass of 31 kDa which binds opioids (such as (-)-morphine, (-)-naloxone and DADLE), benzomorphans (such as (+)-pentazocine and (+)-SKF-10,047), and haloperidol with nanomolar affinity. Furthermore, other σ receptor ligands such as DTG (+)-3-PPP and progesterone bound poorly to the protein. Because the protein resembles the σ opioid site originally proposed by Martin and co-workers in 1976, the existence of a 'σ/opiate' receptor was again proposed. In addition, naloxone reverted the potentiation of NMDA neuronal response induced by (+)-pentazocine, a specific σ_1 ligand, but not the potentiation induced by other σ_1 ligands such as BD 737, L 687-384 and JO-1784 (Couture and Debonnel, 2001). (±)-Cyclazocine, which presents high affinity for the 'σ/opiate' binding site mentioned above, acted as an antagonist suppressing the effect of JO-1784 and (+)-pentazocine. These results suggest that the effects induced by some σ_1 ligands may, in fact, be sensitive to naloxone while others may not (Couture and Debonnel, 2001). The original

classification of σ receptors as opioids might thus have been partly accurate, and the findings suggests that in addition to the conventional classification of σ_1 and σ_2 receptors, a third subtype of σ receptor sensitive to naloxone should be considered. In addition, it was proposed that there might be two subtypes of σ_1 receptor: a metabotropic and a non-metabotropic σ_1 receptor (Maruo et al., 2000). Currently, the conventional classification of σ receptors still comprises only σ_1 and σ_2 receptors.

1.2.1. Cloning and structure of σ_1 receptors

A significant progress in the σ receptor knowledge was the cloning of the σ_1 receptor. The σ_1 receptor was cloned from guinea-pig liver (Hanner et al., 1996), mouse kidney (Seth et al., 1997), a JAR human choriocarcinoma cell line (Kekuda et al., 1996; Prasad et al., 1998), and from the rat and mouse brain (Seth et al., 1998; Mei and Pasternak, 2001; Pan et al., 1998b). The protein is composed by 223 amino acid, and shows the typical σ_1 binding profile (Seth et al., 1998; Kekuda et al., 1996; Hanner et al., 1996). The amino acid sequence of the σ_1 receptor cloned from the human placental cell line was highly homologous to the σ_1 receptor cloned from guinea pig liver, mouse kidney and rat brain, in spite of that they proceed from different species and organs (Fig. 1).

The amino acid sequence of the cloned σ_1 receptor shows no homology with other mammalian proteins, but shares approximately 30% identity with the yeast gene that encodes the sterol C8–C7 isomerase necessary for cholesterol synthesis (Moebius et al. 1997). However, a mammalian sterol C8–C7 isomerase has been recently cloned (Hanner et al., 1995), but this protein shows no similarity with either the yeast

isomerase or with the σ_1 receptor, and in addition, σ_1 receptors do not possess sterol isomerase activity (Hanner et al., 1996).

```
1          10          20          30          40          50
MQWAVGRRWA WAALLLAVAA VLTQVVWLWL GTQSFVFQRE EIAQLARQYA
MQWAVGRRWL WVALFLAAVA VLTQIVWLWL GTQNFVFQRE EIAQLARQYA
MPWAAGRRWA WITLILTIIA VLIQAAWLWL GTQNFVFSRE EIAQLARQYA
MPWAVGRRWA WITLFLTIVA VLIQAVWLWL GTQSFVFQRE EIAQLARQYA

          60          70          80          90          100
GLDHELAFSR LIVELRRLHP GHVLPDEELQ WVFVNAGGWM GAMCLLHASL
GLDHELAFSK LIVELRRLHP VHVLPDEELQ WVFVNAGGWM GAMCLLHASL
GLDHELAFSR LIVELRRLHP GHVLPDEELQ WVFVNAGGWM GAMCILHASL
GLDHELAFSR LIVELRRLHP GHVLPDEELQ WVFVNAGGWM GAMCLLHASL

         110         120         130         140         150
SEYVLLFGTA LGSRGHSGRY WAEISDTIIS GTFHQWREGT TKSEVFYPGE
SEYVLLFGTA LGSPRHSGRY WAEISDTIIS GTFHQWREGT TKSEVFYPGE
SEYVLLFGTA LGSHGHSGRY WAEISDTIIS GTFHQWKEGT TKSEVFYPGE
SEYVLLFGTA LGSHGHSGRY WAEISDTIIS GTFHQWREGT TKSEVYYPGE

         160         170         180         190         200
TVVHGPGEAT AVEWGPNTWM VEYGRGVIPS TLAFALADTV FSTQDFLTLF
TVVHGPGEAT AVEWGPNTWM VEYGRGVIPS TLGFALADTV FSTQDFLTLF
TVVHGPGEAT ALEWGPNTWM VEYGRGVIPS TLFFALADTF FSTQDYLTLF
TVVHGPGEAT DVEWGPNTWM VEYGRGVIPS TLAFALSDTI FSTQDFLTLF

         210         220  223
YTLRSYARGL RLELTTYLFG QDP      Human
YTLRVYARAL QLELTTYLFG QDP      Guinea pig
YTLRAYARGL RLELTTYLFG QDS      Mouse
YTLRAYARGL RLELTTYLFG QDP      Rat
```

Figure 1. Amino acid sequence alignment for the σ_1 receptors from human JAR choriocarcinoma cells (Kekuda et al., 1996), guinea pig liver (Hanner et al., 1996), mouse kidney (Seth et al., 1997) and rat brain (Seth et al., 1998). Highlighted amino acids indicate the differences among the sequences of the cloned rodent species and human sequence. The alignment was performed with the ClustalX ver. 1.64b program.

Analysis of the amino acid sequence led to the conclusion that the σ_1 receptor protein possesses an endoplasmic reticulum retention signal at the NH_2 terminus (Hanner et al., 1996; Seth et al., 1997). The structure of σ_1 receptors has not yet been fully defined, but initial hydropathy analysis of the deduced amino acid sequence of the σ_1 receptor suggested a single transmembrane segment (Hanner et al., 1996; Seth et al., 1997; Kekuda et al., 1996) with the COOH terminus on the inner side of the membrane (Fig. 2A). Dussossoy and co-workers (1999) proposed a similar model with one transmembrane domain, but with the COOH terminus on the luminal side of the endoplasmic reticulum or nuclear envelope. A recent study of Aydar and co-workers (2002) presented evidence that the σ_1 receptor has two transmembrane segments that are

located in the plasma membrane (when expressed in *Xenopus laevis* oocytes) with the NH_2 and COOH termini on the cytoplasmic side of the membrane (Fig. 2B).

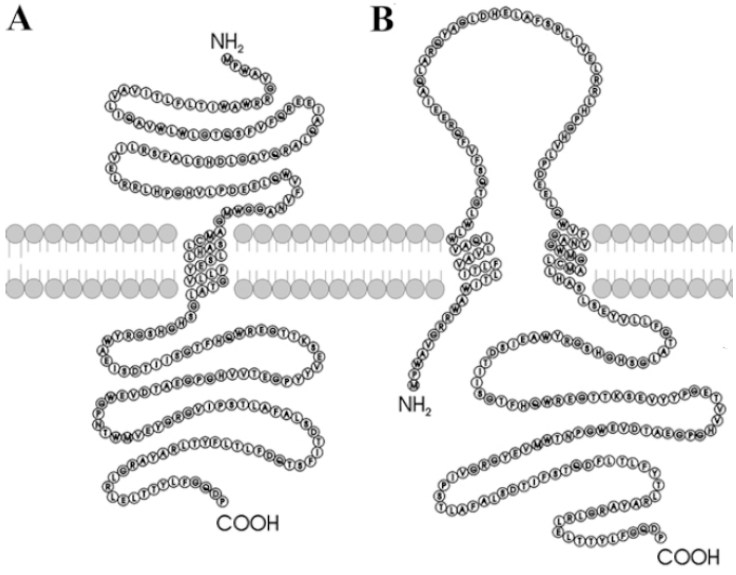


Figure 2. Two of the different models for σ_1 receptor structure that have been proposed. Receptor model with a single putative transmembrane domain (A) and two putative transmembrane domains (B)

Another important finding in σ_1 receptor biology was the discovery of the cDNA sequence and the gene encoding the receptor (Prasad et al., 1998; Seth et al., 1997). Four exons of 207, 201, 93, and 1132 bp with three intercalated introns of 126, 130 and approximately 1250 bp were found to encode σ_1 receptor cDNA. A few years later, a splice variant lacking the 93-bp exon was found in the Jurkat human T lymphocyte cell line. This variant of σ_1 receptors lacked the ability to bind [^{3}H](+)-3-PPP, [^{3}H](+)-pentazocine and [^{3}H]haloperidol, so the ligand-binding domain is likely to be in or around the region coded by exon 3 (Ganapathy et al., 1999). Studies of point mutations in exon 3 expressed in MCF-7 cells identified two anionic amino acids, D126 and E172, that were required for ligand binding. Although the ligand-binding function was abolished by either of these two mutations, expression of the mutant protein was normal (Seth et al., 2001).

Cloning of the σ_1 receptor has led to the development of σ_1-receptor knockout mice (Langa et al., 2003). Homozygous mutant mice are viable, fertile and do not display any overt phenotypic differences compared with wild-type mice.

1.3. Distribution of σ_1 receptors

1.3.1. Anatomical distribution of σ_1 receptors

Receptors for σ_1 are widely distributed in peripheral organs and different areas of the central nervous system (CNS), where they have been more thoroughly studied because of their potential role in several CNS disorders (see Hayashi and Su, 2004a for review).

Initial binding studies using the non-selective σ ligand [^{3}H](+)-3-PPP found the highest densities of binding in the spinal cord, pons-medulla, cerebellum, central grey, red nucleus, and hippocampus with moderate densities in the hypothalamus and cerebral cortex and low densities in the basal ganglia and thalamus (Gundlach et al., 1986a). Further studies have focused on the anatomical distribution of σ_1 receptors, which has been extensively described in the rodent brain in research with autoradiographic methods and selective σ_1 ligands (Okuyama et al., 1995b; Walker et al., 1992; Ishiwata et al., 1998 and 2006; Kawamura et al., 2000a and b), with *in situ* hybridization (Kitaichi et al., 2000; Zamanillo et al., 2000), or with immunohistochemical techniques (Alonso et al., 2000). In these studies, σ_1 receptor is particularly concentrated in specific areas in limbic systems and brainstem motor structures. The highest levels of σ_1 receptor can be observed in olfactory bulb, hypothalamic nuclei, different areas of the

hippocampus such as the pyramidal and non-pyramidal layers, and especially in the granular layer of the gyrus dentatus (Alonso et al., 2000). In addition, intense to moderate σ_1 receptors density is seen in various other areas such as the midbrain or motor nuclei of the hind brain. Sigma receptors are also located at high density in the spinal cord (Kovacs and Larson, 1995, 1998; Alonso et al., 2000), mainly in the dorsal horn (Alonso et al., 2000).

Studies that compared σ_1 versus σ_2 receptor distributions found that σ_1 sites were more abundant in the dentate gyrus of the hippocampus, facial nucleus, thalamic, and hypothalamic nuclei, with moderate densities in the striatum, cerebellum, dorsal raphe nucleus and locus coeruleus (Bouchard and Quirion 1997; Inoue et al., 2000). Only a few areas in the brain involved in the control of posture and movement are particularly enriched with σ_2 sites (Bouchard and Quirion 1997).

The σ_1 receptor is also widely distributed in peripheral organs and structures (Stone et al., 2006) such as the digestive tract (Samovilova and Vinogradov 1992), vas deferens (DeHaven-Hudkins et al., 1991), kidney (Hellewell et al., 1994), liver (McCann and Su, 1991; Hellewell et al., 1994; DeHaven-Hudkins et al., 1994), lungs (Stone et al., 2006), heart (Ela et al., 1994; Monassier and Bousquet, 2002; DeHaven-Hudkins et al., 1994), adrenal medulla (Rogers et al., 1990; Wolfe et al., 1989), testis (Wolfe et al., 1989; DeHaven-Hudkins et al., 1994), pituitary and ovaries (Wolfe et al., 1989), and also in blood mononuclear cells (Wolfe et al., 1888). Like the σ_1 receptor, the σ_2 receptor has also been found in different peripheral organs, particularly the liver and kidney (Hellewell et al. 1994).

1.3.2. Subcellular distribution of σ_1 receptors

Early studies of the subcellular distribution of σ receptors in subcelullar fractions of mouse brain indicated that the order of density of [^{3}H](+)-SKF-10,047 and [^{3}H](+)-3-PPP binding sites, was: microsomal > mitochondrial > synaptosomal > myelin > nuclear fraction (Itzhak et al., 1991). Studies of the selective ligand [^{3}H](+)-pentazocine in binding experiments with subcellular fractionation also found that σ_1 receptors are more abundant in microsomal membranes, but they are also present in nuclear, mitochondria and synaptic fractions (DeHaven-Hudkins et al., 1994; Cagnotto et al., 1994). Immunohistochemical studies further confirmed the existence of σ_1 receptors in the endoplasmic reticulum not only in neurones, but also in oligodendrocytes, lymphocytes and certain cancer cells (Dussossoy et al., 1999; Alonso et al., 2000; Palacios et al., 2004; Hayashi and Su, 2003a and b, 2004b).

Microscopy studies localized the highest density of σ_1 receptors in the endoplasmic reticulum and nuclear envelope in different cell lines, such as THP1 and NG-108 cells, and also in neurones from rat brain (Dussossoy et al., 1999; Hayashi and Su, 2003a and b; Alonso et al., 2000), which is consistent with the endoplasmic reticulum retention signal present in the NH_2 terminus of the cloned σ_1 receptor (Hanner et al., 1996; Seth et al., 1997) (as described in section 1.2.1 *Cloning and structure of σ_1 receptors*). Studies performed in NG-108 neuroblastoma × glioma 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 (Hayashi and Su, 2003a and b). In neurones from the rat hypothalamus and hippocampus, two regions of the CNS containing high concentrations of σ_1 receptors (Alonso et al., 2000), electron

microscopy studies showed that σ_1 receptor immunostaining was mostly associated with neuronal perikarya and dendrites, where it was localized in the limiting plasma membrane, the membrane of mitochondria and some cisternae of the endoplasmic reticulum. At the synaptic contacts, intense immunostaining was associated with postsynaptic structures including the postsynaptic thickening and some polymorphous vesicles, whereas presynaptic axons were devoid of immunostaining (Alonso et al., 2000).

1.4. Pharmacological profile of σ_1 receptors

During the early years of research, σ receptors were labelled with non-selective ligands such as (+)-3-PPP or DTG. Binding of the first selective prototypic σ_1 receptor ligand, [^{3}H](+)-pentazocine, was firstly described by Bowen and co-workers (1993b), who showed that this ligand exhibited 200-fold higher affinity for σ_1 than for σ_2 receptors. More ligands are now available that can discriminate between the σ receptors subtypes, such as NE-100, which exhibits 55-fold more affinity for σ_1 than for the σ_2 subtype (Bucolo et al., 1999), and SA4503, which exhibits 100-fold more affinity for σ_1 than for σ_2 receptors (Matsuno et al., 1996b) (Table I). BD 1047 and BD 1063 exhibit 50-fold higher affinity for σ_1 than for σ_2 receptors, although BD 1047 also binds to β-adrenoceptors in the high nM range (Matsumoto et al., 1995), (Table I, see pp. 18-19). However, the affinity of BD 1047 and BD 1063 for σ_1 receptors, compared to their affinity for other binding sites, is high enough to consider them selective σ_1 ligands. Other compounds that exhibit preferential affinity for σ_1 are shown in Table I. Some of them show only slightly higher affinity for σ_1 receptors, such as BD 1008 (Maeda et al.,

2000), which exhibits 4-fold higher affinity for σ_1, or cocaine, which has about 10-fold better affinity for σ_1 compared to the σ_2 subtype (Matsumoto et al., 2002). Some σ ligands, such as 4-IBP (John et al,. 1994) or (+)-MR 200 (Ronsisvalle et al., 2001a), are not able to distinguish between σ_1 and σ_2 subtypes, but they are not reported to label other receptors, at least with high affinity in comparison to σ receptors. The selectivity between σ subtypes of other ligands, such as panamesine or donepezil, has not been yet determined, and the only data available were obtained by labelling σ sites with non-discriminant σ radioligands (Gründer et al., 1999; Kato et al., 1999).

Haloperidol and its metabolites deserve special consideration among the σ ligands. Haloperidol binds with high affinity to dopamine D_2 receptors (Table I) and σ receptors, although its affinity was 17-fold higher for σ_1 than for σ_2 receptors (McCann et al., 1994; Matsumoto and Pouw, 2000). Haloperidol is metabolized by cytochrome P450 (CYP), specifically by CYP3A4, and by a cytosolic ketone reductase activity; metabolism by the latter results in several metabolites (Usuki et al., 1998) (Fig. 3), with different affinities for σ receptors.

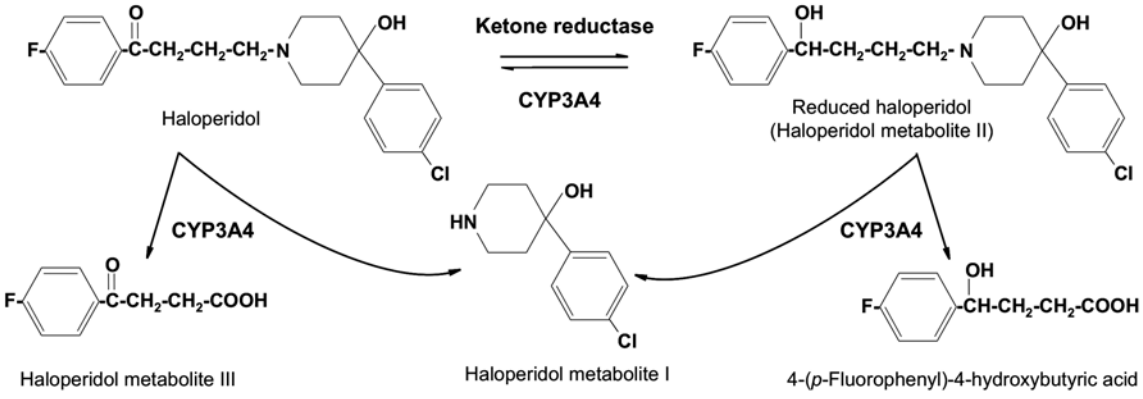


Figure 3. Metabolic pathways of haloperidol and its metabolites.

Reduced haloperidol (also called haloperidol metabolite II) 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., 1990a; Jaen et al., 1993). Haloperidol metabolite I has a lower affinity for σ_1 receptors than haloperidol and reduced haloperidol, but no affinity for σ_2 or D_2 receptors; whereas metabolite III has no affinity for either σ or D_2 receptors (Bowen et al., 1990; Matsumoto and Pouw, 2000). It was proposed that haloperidol or one of its metabolites could bind to σ_1 receptors, but not to σ_2 receptors, in an irreversible way *in vivo* in guinea pigs and *in vitro* in guinea pig brain homogenates (Klein et al., 1994, Inoue et al., 2000).

1.4.1. Putative σ_1 receptor endogenous ligands

Roman and co-workers (1989) reported that two endogenous peptides, NPY and peptide YY, have high affinity (9.8 and 4.9 nM, respectively) for rat brain σ receptors labelled with [^{3}H](+)-SKF-10,047, although these results could not be confirmed later by other researchers (McCann and Su, 1991; Tam and Mitchell, 1991). Neurosteroids are now considered the most probable endogenous σ_1 receptor ligands. This group was named by Baulieu to categorize steroids that are synthesized in the central and peripheral nervous systems, and includes pregnenolone, progesterone, dehydroepiandrosterone (DHEA), allopregnenolone, and their sulfate esters (reviewed by Baulieu, 1998). The physiological actions of neurosteroids have been demonstrated from embryogenesis through adult life. They include genomic actions mediated by steroid receptors translocating into the nucleus, and non-genomic neuromodulatory actions that directly affect several ion channels, neurotransmitter receptors, and second

messenger systems (Table I; see Monnet and Maurice, 2006 for a detailed review). Regarding non-genomic actions, the activity of neurosteroids on σ_1 receptors has been extensively documented. The interaction between neurosteroids and the σ_1 receptor was first suggested by Su and co-workers (1988) from *in vitro* experiments in guinea pig brain and spleen. Among the steroids tested, progesterone was the most potent inhibitor of σ_1-specific radioligand binding. Other steroids such as dehydroepiandrosterone sulfate, pregnenolone sulfate, testosterone or deoxycorticosterone exhibited lower affinities (Su et al., 1988 and 1990; Hayashi and Su, 2004a). This issue remains controversial, because the affinity of progesterone for σ_1 receptors, which is in the μM range (Cobos et al., 2005 and 2006), does not appear to be very high for an endogenous ligand. In fact, it was proposed that the progesterone concentration in free serum is insufficient to occupy the σ_1 receptors in the brain, even during late pregnancy (Schwarz et al., 1989). However, some reports show that endogenous levels of neurosteroids after adrenalectomy, castration or ovariectomy and also during pregnancy can affect σ_1 responses (Debonnel et al., 1996; Bergeron et al., 1996 and 1999; Unani et al., 2001). Moreover, the exogenous administration of neurosteroids leads to a dose-dependent inhibition of *in vivo* [^{3}H](+)-SKF-10,047 binding to σ_1 in mice, with progesterone being the most potent inhibitor in comparison to pregnenolone sulfate and DHEA sulfate (Maurice et al., 1996a and 2001a). Furthermore, there are many experimental paradigms in which neurosteroids share σ_1 ligand properties (see Monnet and Maurice, 2006 for an extensive review).

So at this point, the literature certainly supports the hypothesis that the endogenous σ_1 antagonist could be progesterone, and the endogenous agonists could be DHEA and pregnenolone sulfate.

1.4.2. Allosteric modulation of σ_1 receptors

It has been conventionally assumed that a characteristic difference between σ_1 and σ_2 binding sites is that the binding of σ_1 ligands is allosterically enhanced by DPH, whereas this drug does not modulate σ_2 binding (see Walker et al., 1990; Quirion et al., 1992; and Maurice et al., 2001a for reviews). This assumption has now been questioned, because DPH seems to discriminate between σ_1 ligands. Phenytoin was shown to enhance the binding of some σ_1 radioligands such as [^{3}H]dextromethorphan (Musacchio et al., 1988, 1989a and b; Craviso and Musacchio, 1983), [^{3}H](+)-SKF-10,047 (Karbon et al., 1991; McCann and Su, 1991; Culp et al., 1992), [^{3}H](+)-3-PPP (Bailey and Karbon, 1993; Culp et al., 1992; Bonhaus et al., 1993; Musacchio et al., 1989b; Chaki et al., 1996) and [^{3}H](+)-pentazocine (DeHaven-Hundkins et al., 1993; Cobos et al., 2006). Saturation assays showed that the increase in binding of these radioligands was due to an increase in the radioligand affinity without an increase in the maximal number of receptors recognized by the radioligands (Craviso and Musacchio, 1983; Musacchio et al., 1988; McCann and Su, 1991; DeHaven-Hudkins et al., 1993; Cobos et al., 2006). Furthermore, the addition of DPH to the incubation medium decreased the dissociation rate constant of [^{3}H]dextromethorphan (Musacchio et al., 1987; Craviso and Musacchio, 1983), [^{3}H](+)-3-PPP (Bonhaus et al., 1994) and [^{3}H](+)-pentazocine (Cobos et al., 2006).

However, DPH did not modify the binding of other σ_1 radioligands such as [^{3}H]haloperidol (Karbon et al., 1991), [^{3}H]progesterone (Meyer et al., 1998), [^{3}H]DTG (Karbon et al., 1991), [^{3}H]DuP 734 (Culp et al., 1992) or [^{3}H]RS-23597-190 (Bonhaus et al., 1994) and [^{3}H]NE-100 (Tanaka et al., 1995; Chaki et al., 1996), which in some

studies in fact decreased (Cobos et al., 2006). Those ligands modulated by DPH are typically considered σ_1 agonists, whereas ligands that are not modulated by DPH are generally considered as antagonists (see Maurice et al., 2001a and Hayashi and Su, 2004a for reviews). Whether DTG is an agonist or an antagonist at the σ_1 binding site is unclear (Hayashi and Su, 2004a).

Using a different approach with competition assays in the presence or absence of DPH, DeHaven-Hudkins and co-workers (1993) evaluated several σ_1 cold ligands and found that modulation of the radioligands described above was related to modulation of the ligands. Recent work by our group with the competition assays approach showed clear evidence that DPH is able to modulate the affinity of σ_1 cold ligands depending on their pharmacological profile (Cobos et al., 2005). The σ_1 agonists (+)-3-PPP, dextromethorphan, (+)-SKF-10,047, PRE 084, and (+)-pentazocine exhibited an increase in affinity, whereas the σ_1 antagonists haloperidol, BD 1063, BD 1047, NE-100, DTG and progesterone showed no increase, or a slight decrease.

Other allosteric modulators of σ_1 receptors have been identified but are less well characterized than DPH: ropizine (Mussacchio et al., 1988, 1989b), noscapine (Craviso and Musacchio, 1983) and nicotine (Paul et al., 1993). Ropizine and noscapine, like DPH, increased the binding of σ_1 radioligands [^{3}H](+)-PPP andr [^{3}H]dextromethorphan (Mussacchio et al., 1988, 1989b; Craviso and Musacchio, 1983); moreover, noscapine, like DPH, is able to increase the affinity and decrease the dissociation rate constant of [^{3}H]dextromethorphan (Craviso and Musacchio, 1983). Nicotine has been also proposed as a positive σ_1 receptor allosteric modulator, because it enhanced the association rate constant of the selective σ_1 ligand [^{3}H](+)-pentazocine (Paul et al., 1993).

Table I. 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 [d]	+++ [d]	Agonist [a]	
(-)-Pentazocine	σ_1/σ_2 [d]	++ [d]	Agonist [ac,h]	κ_1 agonist, μ_1, μ_2, ligand, low affinity δ, and κ_3 opioid ligand [h]
(+)-SKF-10,047	σ_1 [a]	+++ [a]	Agonist [a]	NMDA receptor ligand [a]
(–)-SKF-10,047	σ_1/σ_2 [d]	+ [c]	?	μ and κ opioid agonist [g]
Antipsychotics				
Chlorpromazine	? [a]	++ [a]	? [a]	Dopamine D_2 antagonist [a]
Haloperidol	σ_1/σ_2 [a]	+++ [a]	Antagonist [a] Irreversible? [aj]	Dopamine D_2 and D_3 antagonist [l] σ_2 agonist [b]
Nemonapride	σ_1/σ_2? [a]	+++ [a]	? [a]	Dopamine D_2 antagonist [a]
Antidepressants				
Fluoxetine	σ_1 [a]	++ [a]	Agonist [a]	SSRI [a]
Fluvoxamine	σ_1 [a]	+++ [a]	Agonist [a]	SSRI [a]
Imipramine	σ_1 [a]	++ [a]	Agonist [a]	Monoamine reuptake inhibitor [a]
Other compounds				
BD 737	σ_1/σ_2 [az]	+++ [x]	Agonist [x]	-
BD 1008	σ_1/σ_2 [ae]	+++ [ae]	Antagonist [a]	σ_2 agonist [u]
BD 1047	σ_1 [aa]	+++ [aa]	Antagonist [aa]	β adrenoceptor ligand [aa]
BD 1063	σ_1 [aa]	+++ [aa]	Antagonist [aa]	-
BMY 14802	σ_1/σ_2 [k]	++ [k]	Antagonist [x]	5-HT_{1A} agonist [w]
Carbetapentane	σ_1/σ_2 [aw]	+++ [aw]	Agonist [b]	ORL1 antagonist [q], muscarinic M1 antagonist [ai]
Clorgyline	σ_1 [ad]	+++ [ad]	Agonist? [s]	Irreversible MAO-A inhibitor [ad]
Cocaine	σ_1/σ_2 [z]	+ [a]	Agonist [a]	Dopamine, norepinephrine and 5-HT transporter inhibitor [j]
Dextromethorphan	σ_1 [al]	++ [ar]	Agonist [b]	NMDA receptor allosteric antagonist [ax], inhibitor of voltage-operated Ca^{2+} and Na^+ channels [ay]
Donepezil	σ_1/σ_2? [am]	+++? [am]	Agonist? [an]	Cholinesterase inhibitor [am]
DTG	σ_1/σ_2 [a]	+++ [a]	? [a]	σ_2 agonist [b]
Dup 734	σ_1 [a]	+++ [a]	Antagonist [ap]	5-HT_2 antagonist [aq]
E-5842	σ_1 [y]	+++ [y]	Antagonist [x]	Low to moderate affinity for dopamine, 5-HT and glutamate receptors [y]
Haloperidol Metabolite I	σ_1 [k]	++ [k]	Antagonist [ak] Irreversible? [aj]	-
Haloperidol Metabolite II	σ_1/σ_2 [k]	+++ [k]	Antagonist [ak] Irreversible? [aj]	Dopamine D_2 and D_3 ligand [l]
4-IBP	σ_1/σ_2 [at]	+++ [at]	Agonist [au]	Dopamine D_2 ligand [at]
JO-1784 (Igmesine)	σ_1 [a]	+++ [a]	Agonist [a]	-
Metaphit	σ_1/σ_2 [t]	++ [v]	Irreversible antagonist [t]	Acylator of PCP and σ_2 binding sites [t]
(+)-MR 200	σ_1/σ_2 [m]	+++ [m]	Antagonist [ao]	-

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 [x]	5-HT_{1A} agonist [a], 5-HT_{1B} agonist [b]
Panamesine (EMD 57445)	σ_1/σ_2? [av]	+++? [av]	Antagonist [x]	-
(+)-3-PPP	σ_1/σ_2 [d]	++ [ar]	Agonist [a]	σ_2 agonist [b], NMDA receptor ligand [e], dopaminergic agonist [i]
PRE 084	σ_1 [a]	+++ [a]	Agonist [a]	-
Rimcazole	σ_1/σ_2 [f]	+ [a]	Antagonist [a]	DAT inhibitor [ab]
SA4503	σ_1 [as]	+++ [a]	Agonist [a]	-
DPH	σ_1 [c]	-	Allosteric modulator [c]	Delayed rectifier K^+ channels blocker [n], T-type Ca^{2+} current inhibitor [o], Na^+ current inhibitor [p]
Ropizine	σ_1 [c]	-	Allosteric modulator [c]	DAT inhibitor [r]
Neurosteroids				
Progesterone	σ_1 [a]	+ [ar]	Antagonist [a]	NMDA negative/$GABA_A$ positive modulator [b]
Pregnenolone sulfate	σ_1 [a]	+ [a]	Agonist [a]	NMDA positive/$GABA_A$ negative modulator [b]
DHEAS	σ_1 [a]	+ [a]	Agonist [a]	$GABA_A$ negative modulator [b]

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

[a] Reviewed in Hayashi and Su, 2004; [b] Reviewed in Maurice et al., 2001; [c] Reviewed in Walker et al., 1990; [d] Bowen et al, 1993b; [e] Hofner et al., 2000; [f] Matsumoto et al., 2001c; [g] Zukin et al., 1982; [h] Chien and Pasternak, 1995b; [i] Coldwell et al., 1999; [j] Blakely and Bauman, 2000; [k] Matsumoto and Pouw, 2000; [l] Jaen et al., 1993; [m] Ronsisvalle et al., 2001a; [n] Nobile and Lagostena, 1998; [o] Todorovic and Lingle, 1998; [p] Rush and Elliott, 1997; [q] Shah et al, 1998; [r] Izenwasser et al., 1993; [s] Debonnel and Montigny, 1996; [t] Bluth et al., 1989;[u] Maurice et al., 1999a; [v] Cobos et al., 2006b (unpreviously published observation); [w] Matos et al., 1996; [x] Guitart et al., 2004; [y] Guitart et al., 1998. [z] Matsumoto et al., 2002; [aa] Matsumoto et al., 1995; [ab] Husbands et al., 1999; [ac] Hiramatsu and Hoshino, 2005; [ad] Itzhak et al., 1991; [ae] Maeda et al., 2000; [ai] Hudkins and DeHaven-Hudkins, 1991; [aj] Klein et al., 1994; [ak] Cendán et al., 2005a; [al] McCann et al., 1994; [am] Kato et al., 1999; [an] Maurice et al., 2006; [ao] Marrazzo et al., 2006; [ap] Ault and Werling, 1999; [aq] Tam et al., 1992; [ar] Cobos et al., 2005; [as] Matsuno et al., 1996b; [at] John et al., 1994; [au] Bermack and Debonnel., 2001; [av] Gründer et al., 1999; [aw] Calderon et al., 1994; [ax] LePage et al., 2005; [ay] Trube and Netzer, 1994; [az] Hellewell et al., 1994.

2. SIGNAL TRANSDUCTION AND CELLULAR EFFECTS OF SIGMA$_1$ RECEPTORS

The classical second messenger systems have been widely explored to identify σ_1 receptor signal transduction mechanisms; moreover, the modulatory role of σ_1 receptors in the activity of some ion channels and in different kinds of neurotransmission has also been reported.

2.1. Coupling of σ_1 receptors to G-proteins

Despite the fact that the structure of σ_1 receptors (Fig. 2) is not the typical structure of a G-protein-coupled receptor (with seven transmembrane domains), it has been reported that G-proteins mediate some electrophysiological and behavioural responses of σ_1 ligands (Soriani et al., 1998, 1999a; Bergeron et al., 1996; Ueda et al., 2001a).

Early radioligand binding studies in rat brain membranes showed that guanine nucleotides (GTP or Gpp(NH)p) altered binding parameters such as the affinity and the association rate constant, of the non-selective σ_1 agonist [^{3}H](+)-3-PPP (Beart et al., 1989; Itzhak et al., 1989; Itzhak and Stein, 1991), whereas the binding of several σ_1 antagonists such as DuP 734, NE-100 and DTG was not modified by guanine nucleotides (Culp et al., 1992; Tanaka et al., 1995; Itzhak and Stein, 1991). In addition, competition binding assays that labelled σ receptors with [^{3}H](+)-3-PPP or [^{3}H](+)-pentazocine in brain membranes showed that the inhibition curves of the agonists (+)-SKF-10,047, (±)-cyclazocine, (±)-pentazocine, (+)-pentazocine, (+)-3-PPP

and SA4503 were displaced to the right in the presence of guanine nucleotides (indicating decreasing affinity) (Matsuno et al., 1996b; Itzhak et al., 1989), whereas these nucleotides had no effect on the affinity of haloperidol and chlorpromazine (Itzhak et al., 1989). Subsequent experiments showed that the selective σ_1 agonist (+)-pentazocine stimulated [^{35}S]GTPγS binding in several mouse and guinea pig preparations (Tokuyama et al., 1999; Ueda et al., 2001b; Maruo et al., 2000). Furthermore, the putative σ_1 agonists DHEAS and pregnenolone sulfate also increased [^{35}S]GTPγS binding in an NE-100- or progesterone-reversible manner (Ueda et al., 2001b). GTPase activity assays also provided evidence of the coupling of σ_1 receptors to G-proteins, in view of the finding that some σ_1 agonists, such as (+)-3-PPP and the selective σ_1 agonists (+)-pentazocine and SA4503, were found to stimulate GTPase activity in a NE-100-reversible manner (Tokuyama et al., 1997).

The results summarized above indicate that σ_1 receptors are coupled to G-proteins, but the data in the literature are contradictory about the subtype of G-protein involved: some studies suggested it might be a G-protein sensitive to pertussis toxin ($G_{i/o}$) (Bergeron et al., 1996; Tokuyama et al., 1999; Ueda et al., 2001a; Mtchedlishvili and Kapur, 2003), whereas other researchers found evidence that it could be a G-protein sensitive to cholera toxin (G_s) (Soriani et al., 1998; 1999a).

In spite of these results, the coupling of σ_1 receptors to G-proteins remains controversial because of studies that reported results different to those described above. In fact, the binding of some σ_1 agonists radioligands, such as [^{3}H](+)-SKF-10,047 and [^{3}H](+)-pentazocine, to rat and guinea pig brain and liver membranes was not modulated by guanine nucleotides, or by cholera or pertussis toxin (Selley et al., 1988; Hong and Werling, 2000; Basile et al., 1992; DeHaven-Hudkins et al., 1992).

Furthermore, studies based on a different approach with competition experiments in the presence or absence of GTPγS found that the affinity of σ_1 agonists (+)-pentazocine and BD 737 was not modulated in the presence of the guanine nucleotide (Hong and Werling, 2000). In addition, (+)-pentazocine, (+)-SKF-10,047 and DTG were unable to stimulate [^{35}S]GTPγS binding in rat brain membranes (Odagaki et al., 2005), whereas 3-(+)-PPP-induced enhancement of [^{35}S]GTPγS binding was found to be due to its dopaminergic agonist properties, since it was reverted by the dopaminergic antagonist (-)-sulpiride but not by the selective σ_1 antagonist BD 1047 (Odagaki et al., 2005). Negative results were also found in the modulation of GTPase activity: some σ_1 agonists such as (+)-pentazocine, (+)-SKF-10,047, (+)-3-PPP and BD 737 were unable to increase GTPase activity in rat brain membranes (Hong and Werling, 2000; Odagaki et al., 2005). Thus whether σ receptors are directly associated with G-proteins or not remains controversial.

The existence of both metabotropic and non-metabotropic σ_1 receptor subtypes was proposed by Maruo and co-workers (2000), who found no significant correlation between the number of σ_1 receptors labelled with the selective σ_1 agonist [^{3}H](+)-pentazocine and (+)-pentazocine-stimulated [^{35}S]GTPγS binding in different brain areas and in peripheral organs from mice and guinea pigs. This was suggested to be due to the presence of at least two subtypes (metabotropic and non-metabotropic) of σ_1 receptor, with the former being abundant in some guinea pig peripheral organs such as spleen. The existence of metabotropic and non-metabotropic σ_1 receptors may partially explain why controversy has arisen over whether σ_1 receptors are coupled to G-proteins or not.

2.2. Modulation of cellular effectors by σ_1 receptors

2.2.1. Modulation of the phospholipase C-protein kinase C and $InsP_3$-Ca^{2+} release system

The modulation of classical second messenger systems by σ_1 receptors, specifically the phospholipase C (PLC) system, and the subsequent activation of $InsP_3$ receptors and protein kinase C (PKC), have been widely explored. $Sigma_1$ receptors can modulate the activity of different substances through the PLC-PKC system in different experimental situations such as cholinergic-stimulated phosphoinositide metabolism (Candura et al., 1990; Bowen et al., 1990b, 1992 and 1993a), NMDA-induced increase in $[Ca^{2+}]_i$ (Monnet et al., 2003), NMDA-induced dopamine release (Nuwayhid and Werling, 2003a and 2003b) and modulation of the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) after stimulation of $InsP_3$ receptors at the endoplasmic reticulum (Hong et al., 2004; Peeters et al., 2004; Yagasaki et al., 2006, Su and Hayashi, 2003). The mechanism of modulation of classical second messenger systems by σ_1 receptors appears to be a complex one involving the translocation of σ_1 receptors from their intracellular location to the plasma membrane, and the subsequent recruitment of membrane-bound second messenger cascade involving PLC and PKC. This was proposed as a mechanism by which an intracellular receptor modulates metabotropic responses (Morin-Surun et al., 1999).

Assays by Morin Surun and co-workers (1999) which measured spontaneous rhythmic activity in hypoglossal nucleus neurones (a single burst of action potentials followed by low-amplitude tonic activity) showed that the selective σ_1 agonist

(+)-pentazocine induced a robust and rapid decrease in hypoglossal activity, which was reverted by the selective σ_1 antagonist NE-100 and the selective PLC inhibitor U-73,122. The subsequent activation of PKC β_1 and β_2 isoforms (detected by confocal microscopy) and the phosphorylation of σ_1 receptors lead to desensitization of the response, which was reversible by PKC inhibitors but not by PKA (protein kinase A) inhibitors. Upon the activation of PKC, σ_1 receptors can be phosphorylated and translocated from inside the cell to the plasma membrane (Morin-Surun et al., 1999). An unexplained result of the experiments performed by Morin-Surun and co-workers is the fact that the activity of hypoglossal neurones was inhibited not only by σ_1 agonists such as (+)-pentazocine and (+)-SKF-10,047, but also by σ_1 antagonist such as BD 1047, BD 1063 and haloperidol (Morin-Surun et al., 1999).

Bradykinin increases $InsP_3$ production through the activation of the PLC system, which induces the release of Ca^{2+} from intracellular stores. This latter process was enhanced by σ_1 agonists such as (+)-pentazocine and PRE 084, and by the putative agonists pregnenolone and DHEA, and was reverted by σ_1 antagonists such as haloperidol, NE-100 or BD 1047, by the putative antagonist progesterone (Hayashi et al, 2000; Hong et al., 2004; Peeters et al., 2004), and also by σ_1 receptor antisense oligodeoxynucleotides (Hayashi et al., 2000). It was postulated that activation of the σ_1 receptor might act directly on $InsP_3$ receptors, after dissociation of the σ_1 receptor-ankyrin complex (specifically the ANK220 isomer) from the $InsP_3$ receptor in NG-108 cells (Hayashi and Su, 2001). ANK2020 is a member of a family of cytoskeletal adapter proteins that interconnect membrane proteins with the cell cytoskeleton (Bennett and Stenbuck, 1979) and is present on the endoplasmic reticulum, plasma membrane and Golgi complex (De Matteis and Morrow, 1998; Tuvia et al., 1999). The dissociation of

ANK220 from $InsP_3$ receptors correlated with the efficacy of each ligand in potentiating the efflux of Ca^{2+} induced by bradykinin (Hayashi and Su, 2001). In the presence of a σ_1 receptor antagonist, this effect is prevented and σ_1 receptors are dissociated from ankyrin and $InsP_3$ receptors, which remain on the endoplasmic reticulum (Hayashi and Su, 2001; Su and Hayashi, 2001). Under basal conditions σ_1 ligands did not affect $[Ca^{2+}]_i$ (Hayashi et al., 2000, Hong and Werling, 2004), and the cells need to be stimulated to make appropriate levels of $InsP_3$ available for the modulation of $[Ca^{2+}]_i$ by σ_1 receptor agonists. Enhancement of the PLCγ/$InsP_3$/Ca^{2+} pathway by σ_1 receptors has also been recently demonstrated as a response to activation of TrkB receptors (receptors for brain-derived neurotrophic factor) (Yagasaki et al., 2006). Activation of PLCγ by brain-derived neurotrophic factor (BDNF) and the subsequent increase in $[Ca^{2+}]_i$ were potentiated by imipramine or fluvoxamine (two antidepressants with σ_1 receptor affinity). Furthermore, enhancement of the effect of BDNF by imipramine was blocked by the σ_1 antagonist BD1047 (Yagasaki et al., 2006).

The results described above showed that the σ_1 receptor might act as a modulator for intracellular Ca^{2+} mobilizations after activation of the PLC/PKC/$InsP_3$ system, so σ_1 receptors might also enhance the cellular effects of different receptors through calcium signalling.

2.2.2. Modulation of calcium channels

In addition to the effects of $\sigma_1$ligands on the regulation of $[Ca^{2+}]_i$ through the PLC-PKC-$InsP_3$ system, these receptors also modulate plasmalemmal voltage-dependent calcium channels. Interaction between σ receptors and Ca^{2+} channels was

suggested when the increase in $[Ca^{2+}]_i$ mediated by high-voltage-activated calcium channels was blocked by σ ligands (the order of potency being BD 737 > rimcazole > haloperidol > ifenprodil > opipramol > carbetapentane > caramiphen > dextromethorphan) in cultured hippocampal pyramidal neurone preparations (Church and Fletcher, 1995). Sigma ligands were reported to inhibit depolarisation-induced increases in $[Ca^{2+}]_i$ in primary cultures of neurones and brain synaptosomes (Klette et al., 1997; Brent et al., 1996 and 1997). Klette and co-workers (1997) reported that σ-ligands such as (+)-pentazocine, (+)-cyclazocine, (+)-SKF-10,047, carbetapentane and haloperidol inhibited KCl-induced increases in $[Ca^{2+}]_i$ in primary cultures of neurones. Although the effects described above are produced by known σ_1 ligands, known σ_1 agonists and σ_1 antagonists produced the same effect. Other experiments were performed in rat forebrain synaptosomes, where intra-synaptosomal free calcium levels where measured under basal conditions and after depolarization with KCl, veratridine and 4-aminopyridine (Brent et al., 1996 and 1997). In these assays, (+)-pentazocine, (-)-pentazocine, BD 1008 and DTG inhibited the rise in Ca^{2+} levels induced by depolarization, but also decreased basal $[Ca^{2+}]_i$, suggesting that σ receptor activation *per se* affects $[Ca^{2+}]_i$ in rat forebrain synaptosomes. This effect was inhibited by the σ antagonist rimcazole (Brent et al., 1996 and 1997).

Zhang and Cuevas (2002) used a whole-cell patch-clamp recording technique, and showed that σ receptor ligands rapidly depressed peak calcium channel currents in isolated neurones from neonatal rat intracardiac and superior cervical ganglia. The rank order potency of the inhibition was haloperidol > ibogaine > (+)-pentazocine > DTG, which was consistent with the order of affinity of these ligands for σ_2, but not for σ_1 receptor. Maximum inhibition of calcium channel currents was around 95%, suggesting

that σ receptors block all calcium channel subtypes found on the cell body of these neurones, which includes N-, L-, P/Q-, and R-type calcium channels. These effects were not modified by cell dialysis or the intracellular application of $GDP_{\beta}S$, suggesting that a possible metabotropic σ receptor was not involved. A role for σ receptors, however, is supported by the observation that metaphit, an irreversible σ antagonist, blocked the effects of DTG in these cells.

Recently, several lines of evidence have added support to arguments for the involvement of σ_1 receptors in Ca^{2+} signalling. Specifically, the selective σ_1 agonists (+)-pentazocine and PRE 084 modulated Ca^{2+} signalling in NG108 cells via σ_1 receptors by two different modes of action. Firstly, (+)-pentazocine was shown to inhibit the increase in $[Ca^{2+}]_i$ induced by depolarization with KCl, in agreement with the effects observed in forebrain synaptosomes by Brent and co-workers in 1996 and 1997. By contrast, PRE 084 potentiated the effect of KCl. Both effects were reverted by σ_1 receptor antisense oligodeoxynucleotide (Hayashi et al., 2000), suggesting that σ_1 receptors modulate voltage-dependent Ca^{2+} channels at the plasma membrane. A second mode of action involves σ_1 receptors at the intracellular level: as described in the section 2.2.1 *Modulation of phospholipase C-protein kinase C and $InsP_3$-Ca^{2+} release system by σ_1 receptors*, σ_1 agonists potentiated the $InsP_3$ receptor-induced increase in $[Ca^{2+}]_i$ in the endoplasmic reticulum. It therefore seems clear that more studies are necessary to clarify the role of these receptors in plasmalemmal voltage-dependent calcium channels.

Paul and co-workers (1993) showed that the σ ligands (+)-pentazocine, (+)-SKF-10,047 and haloperidol, in the μM concentration range, selectively inhibited the increase in $[Ca^{2+}]_i$ after the addition of nicotine to adrenal chromaffin cells. In addition, since nicotine interfered with the equilibrium constants of [^{3}H](+)-pentazocine

binding, it was suggested that the σ_1 receptor was likely coupled to the Ca^{2+} ionophore associated to the nicotine receptor (Paul et al., 1993). The modulation of Ca^{2+} currents by σ_1 receptors, as with K^+ currents, suggests that σ_1 receptors might form complexes with other proteins; these complexes might explain the wide variety of actions produced by σ_1 ligands in the CNS, and might also partially explain some of the discrepancies found in the literature (Bermack and Debonell, 2005).

2.2.3. Modulation of potassium channels

Potassium channels have been shown to constitute an important target for σ drugs in rat cortical synaptosomes and C6 glioma cells (Jeanjean et al., 1993), NCB-20 cells (Morio et al., 1994), rat neurohypophysial terminals (Wilke et al., 1999; Lupardus et al., 2000) and frog melanotropic cells (Soriani et al., 1998, 1999a and b), among others. Soriani and co-workers were the first to report that the selective σ_1 agonist (+)-pentazocine reduced several K^+ currents in frog melanotropic cells in experiments with perforated patches (Soriani et al., 1998, 1999a and b), and noted that some of these effects were mediated by a G_s-protein (Soriani et al., 1998, 1999a). The connection between K^+ channels, σ_1 receptors and G-proteins has not always been clear, however. Modulation of voltage-gated K^+ channels by (±)-pentazocine or (±)-SKF-10,047 in rat neurohypophysial terminals persisted even after nerve terminals were internally perfused with a GTP-free solution, $GDP_\beta S$ or $GTP\gamma S$, suggesting that G-proteins do not play a role in these responses (Lupardus et al. 2000). The inhibition of different types of voltage-activated K^+ channels by σ ligands was recently reported in intracardiac neurones by Zhang and Cuevas (2005), with the following rank order potency:

(+)-pentazocine > ibogaine > DTG. These findings suggested that the effects are mediated by σ_1 receptor activation. Moreover, the effects of DTG were blocked by preincubation of the irreversible σ antagonist metaphit, confirming that the effect is mediated by σ receptor activation (Zhang and Cuevas, 2005). However, several σ_1 receptor antagonists such as haloperidol, BD 1047 and BD 1063 also depressed the activity of intracardiac neurone voltage-activated K^+ channels, although because the concentrations of σ ligands used in that study were in the μM range, these ligands might have been binding to other receptors such as σ_2 receptors (Zhang and Cuevas, 2005). Interestingly, neither cell dialysis nor the application of $GDP_\beta S$ affected the blockade of intracardiac neurone voltage-activated K^+ channels induced by σ ligands, which suggested that G-proteins were not involved in this effect (Zhang and Cuevas, 2005).

Some recent reports showed that σ_1 receptors could modulate K^+ conductance by a direct interaction between σ_1 receptor protein and K^+ channels. A significant finding was the modulation by (±)-SKF-10,047 of K^+ channels in excised outside-out patches in rat neurohypophysial terminals (Lupardus et al., 2000; Wilke et al., 1999), which ruled out a role for any soluble cytoplasmic factors in this effect. Moreover, K^+ channels present in cell-attached patches were not modulated by the σ drug applied outside the patch, indicating that σ_1 receptors and the K^+ channels under investigation must be in close proximity for any functional interaction to occur (Lupardus et al., 2000). Further investigation of this modulation also suggested that a protein-protein interaction is the likely mechanism of σ_1 receptor action on K^+ channels (Aydar et al., 2002). In these experiments, Aydar and co-workers (2002) studied the heterologous expression, in *Xenopus* oocytes, of the σ_1 receptors with the potassium channels Kv 1.4 and 1.5, which resulted in modulation of the channel function in the absence of any σ ligand, and

greater modulation in the presence of σ ligands. Moreover, Kv 1.4 and 1.5 channel co-immunoprecipitated with σ_1 receptor proteins, suggesting that σ_1 receptors are directly associated with these K^+ channels. Therefore, σ_1 receptors and K^+ channels probably form a stable macro-molecular complex with functional implications.

2.3. Neurotransmitter systems and σ_1 receptors

The widespread presence of σ_1 receptors in the nervous system supports a modulatory role for σ_1 receptors in the activity of several neurotransmitter systems.

2.3.1. Modulation of GABAergic neurotransmission

There are few studies that addressed the effects of σ_1 ligands on GABAergic neurotransmission. Early studies showed that the non-selective σ ligand DTG inhibited the firing rate of GABAergic interneurons of anaesthetized rats (Zhang et al., 1993a). More recent studies, using more selective ligands, showed that (+)-SKF-10,047, 5-HT, and trazodone, an antidepressant drug that acts mainly on serotonergic systems (Owens et al., 1997; Pazzagli et al., 1999) with a moderate affinity for σ_1 receptors (Garrone et al., 2000), inhibited KCl-evoked GABA release in rat mossy fibre cerebellar synaptosomes. The effect of (+)-SKF-10,047 and trazodone, but not that of 5-HT, were reverted by the selective σ_1 receptor antagonist BD 1047 and by the non-selective σ ligand (+)-3-PPP (Garrone et al., 2000). Some recent studies related the action of neurosteroids and σ_1 receptors on GABAergic neurotransmission. Low concentrations (in the nM range) of the putative σ_1 agonist pregnenolone sulfate reduced the frequency

on $GABA_A$ receptor-mediated spontaneous and miniature inhibitory postsynaptic currents; these effect were mimicked by the σ_1 receptor agonist (+)-SKF-10,047 and blocked by the σ_1 receptor antagonists BD 1063 and haloperidol (Mtchedlishvili and Kapur, 2003). These findings indicate that σ_1 receptors can negatively modulate the GABAergic system, but more studies are necessary to determine whether a clear connexion exists between σ_1 and the GABAergic system, and to elucidate the effectors mediating these effects.

2.3.2. Modulation of glutamatergic neurotransmission

Several lines of evidence strongly suggest that the activation of σ_1 receptors modulates glutamatergic neurotransmission. Of the three subtypes of glutamate-gated ion channels (NMDA, kainite and AMPA-kainate receptors), the connection between σ_1 and NMDA receptors has been widely explored, given that some σ ligands have the same central effects as substances targeting NMDA receptors: they alter mood and motricity, and also have an impact on memory and cerebro-protective activities (Maurice and Lockhart, 1997).

It has been shown that the selective σ_1 agonists (+)-pentazocine and JO-1784 enhanced NMDA-induced firing activity in the dorsal hippocampus, specifically in the CA_3 region, as did other σ_1 ligands such as DTG and BD 737, whereas (+)-3-PPP, NE-100, BMY 14802 and haloperidol blocked the effect of the agonists (reviewed in Debonnel and Montigny, 1996). The effect of JO-1784 and DTG was blocked by pre-treatment with pertussis toxin, whereas pre-treatment did not modify the (+)-pentazocine potentiation of neuronal activation by NMDA (Monnet et al., 1994). It

therefore seems that the effect of only some σ_1 ligands is mediated by $G_{i/o}$ proteins. Potentiation of the NMDA response in the CA_1 region of the dorsal hippocampus was also studied, and it was found that (+)-pentazocine, as in the CA_3 region, potentiated the firing activity induced by NMDA, whereas JO-1784 and DTG did not (Debonnel et al., 1996b). So despite the fact that (+)-pentazocine and JO-1784 are considered selective σ_1 agonists, their effect are not equivalent.

The role of neurosteroids in the potentiation of NMDA responses in the CA_3 region has been extensively studied. The putative σ_1 receptor agonist DHEA, at low doses, potentiated the NMDA response in extracellular recordings from the CA_3 region of the rat, and these effects were blocked by the σ_1 antagonists NE-100 and haloperidol, and also by the putative σ_1 antagonist progesterone (Bergeron et al., 1999; Debonnel et al., 1996a), which was also able to reverse the NMDA potentiation induced by (+)-pentazocine, JO-1784 and DTG (Bergeron et al., 1996). Some studies related steroidal tonus with the σ-mediated potentiation of glutamatergic neurotransmission in the hippocampus (Debonnel et al., 1996a; Bergeron et al., 1996 and 1999). Potentiation of the NMDA response by DTG in the CA_3 region of the rat dorsal hippocampus was significantly greater in ovariectomized rats than in males and non-ovariectomized females (Debonnel et al., 1996a; Bergeron et al., 1996). In addition, in pregnant rats 10-fold higher doses of DTG, (+)-pentazocine and DHEA were required to potentiate the neuronal response to NMDA in the CA_3 region of the dorsal hippocampus in comparison to control females (Bergeron et al., 1999). These results suggest that endogenous progesterone, by acting as a σ_1 antagonist, may produce a tonic inhibition of the function of σ receptors and consequently a decrease in NMDA receptor function.

Glutamate receptor-induced $[Ca^{2+}]_i$ increases have also been reported to be modulated by σ_1 ligands. It has been shown that the σ_1 agonists (+)-SKF-10,047, (+)-pentazocine, and JO-1784, but not DTG, initially potentiated the glutamate response in pyramidal neurones. This effect was sensitive to the selective σ_1 antagonist NE-100 and to the cPKC inhibitor Gö-6976, but rapidly faded. Potentiation was followed by a reduction in the glutamate response, which was also sensitive to NE-100 but not to Gö-6976, suggesting that the modulation of NMDA responses by σ_1 drugs is due at least in part to the recruitment of the PLC–PKC cascade (Monnet et al., 2003).

Other studies documented potentiation of glutamatergic neurotransmission through the enhancement of glutamate release by σ_1 ligands. It is known that BDNF enhanced the glutamatergic transmission in the developing cortical network by inducing glutamate release (Numakawa et al., 2002). Recent studies showed that stimulation of the PLCγ pathway by BDNF (and subsequent glutamine release) was potentiated by pre-treatment with imipramine or fluvoxamine, two antidepressants with high affinity for σ_1 receptors. Interestingly, BD 1047, a potent σ_1 antagonist, blocked the imipramine-dependent potentiation of glutamate release. In addition, over-expression of σ_1 receptors *per se*, without antidepressant pre-treatment, enhanced the induction of the PLCγ pathway by BDNF, and hence glutamate release (Yagasaki et al., 2006). Furthermore, the putative σ_1 agonists DHEA and pregnenolone sulfate also showed a positive action on glutamate release. DHEA, a putative σ_1 agonist which is also a positive allosteric modulator of NMDA receptors (Mellon and Griffin, 2002), increased spontaneous glutamate release in rat synaptosomes (Lhullier et al., 2004). Pregnenolone sulfate, which, like DHEA, is also a positive modulator of NMDA receptors (Park-Chung et al., 1997), induced an increase in glutamate release in primary mixed hippocampal cell

cultures (Meyer et al., 2002). Moreover, pregnenolone sulfate, DHEA sulfate and (+)-pentazocine induced a robust potentiation of the frequency of miniature excitatory postsynaptic currents (mEPSCs) mediated by AMPA receptors in cultured hippocampal neurones, and this effect was reverted by haloperidol, BD 1063 and pertussis toxin, which suggests that pregnenolone sulfate increases spontaneous glutamate release via activation of a presynaptic $G_{i/o}$-coupled σ_1 receptor (Meyer et al., 2002). A similar enhancement of mEPSC frequency by pregnenolone sulfate was seen in CA_1 neurones, but only before postnatal day 6 (Mameli et al., 2005). However, in CA_1 neurones from mature hippocampi, pregnenolone sulfate had a little effect on basal release, although it clearly enhanced facilitated glutamate release (Partridge and Valenzuela, 2001), which was also reversed by the selective σ_1 antagonist BD 1063 and by pertussis toxin (Schiess and Partridge, 2005). It therefore seems that pregnenolone sulfate produces different effects in mature and immature neurones through metabotropic σ_1 receptors.

Recent studies showed that neurosteroids are also able to enhance glutamatergic neurotransmission through σ_1 receptors in other experimental models. The repeated administration of DHEAS has a prominent facilitating effect on the induction of high-frequency stimulation-dependent long-term potentiation in rat hippocampal CA_1 pyramidal cells, an effect that was reversed by the σ_1 antagonists NE-100 and haloperidol (Chen et al., 2006).

All studies described above strongly suggest that σ_1 receptors act as positive modulators of glutamatergic neurotransmission, mainly in the hippocampus, whereas in the cortex the opposite results were found (Hayashi et al., 1995; Klette et al., 1997). Early studies showed that the σ_1 ligands JO-1784, (+)-pentazocine, dextromethorphan, (+)-cyclazocine, (+)-SKF-10,047, carbetapentane, haloperidol and DTG, attenuated

NMDA-induced increases in $[Ca^{2+}]_i$ in primary cultures of rat cortical neurones (Hayashi et al., 1995; Klette et al., 1997).However, very high concentrations of σ ligands were used in these experiments, and both σ_1 agonists and antagonists exhibited similar effects. Currently is commonly accepted that σ_1 receptors modulate NMDA effects in a positive manner.

In addition to the effects documented above, the role of σ_1 receptors in the modulation of adrenergic, dopaminergic, cholinergic and serotonergic neurotransmission through NMDA receptors has also been extensively reported, as will be described below.

2.3.3. Modulation of dopaminergic neurotransmission

Modulation of the dopaminergic system by σ_1 receptors has been studied with different approaches, which include electrophysiological techniques and the determination *in vivo* and *in vitro* of the levels of dopamine and its major metabolites. In many cases contradictory results have been reported, making it difficult to reach solid conclusions.

Electrophysiological studies have evaluated both the firing activity and the number of active neurones in substantia nigra pars compacta (A_9) or ventral tegmental area (A_{10}) dopaminergic neurones. It was reported that DTG and the σ_1 agonists JO-1784 and (+)-pentazocine had no effect on the firing rate of A_9 neurones (Gronier and Debonnel, 1999; Zhang et al., 1992). On the contrary, other reports showed that DTG and the σ_1 agonists (+)-pentazocine and SA4503 decreased the firing rate of A_9 neurones (Steinfelds et al., 1989; Minabe et al., 1999), whereas the σ antagonist

BMY 14802 increased the firing rate of these neurones (Zhang et al., 1992; Steinfelds et al., 1989). This antagonist also increased the firing rate of A_{10} neurones (Zhang et al., 1992; Steinfelds et al., 1989), whereas the σ_1 agonists SA4503 and JO-1784 were inactive in modulating the firing rate of A_{10} neurones (Gronier and Debonnel, 1999; Minabe et al., 1999). A particular case is the non-selective σ_1 agonist (+)-3-PPP, which decreased the firing rate both in A_9 and A_{10} neurones (Steinfelds et al, 1989, Steinfelds and Tam, 1989; Zhang et al., 1992), probably due to its dopaminergic agonist properties, because its effects were reversed by (+)-butaclamol (Zhang et al., 1992).

It was reported that the acute administration of the selective σ_1 agonist SA4503 decreased the number of spontaneously active dopaminergic A_9 neurones, but increased that of spontaneously active A_{10} neurones, and that these effects were reversed by NE-100 (Minabe et al., 1999). In contrast, other studies showed that the acute administration of the σ_1 agonists (+)-pentazocine and JO-1784, as well as that of DTG or the σ antagonist E-5842, did not alter the number of spontaneously active A_9 or A_{10} dopaminergic neurones (Zhang et al., 1993b; Sanchez-arroyos and Guitart, 1999). Interestingly, different results were obtained after repeated administration of the σ ligands. The repeated administration of DTG or the σ_1 agonists (+)-pentazocine and SA4503 (but not JO-1784) increased the number of spontaneously active dopaminergic A_{10} neurones (Zhang et al., 1993b; Minabe et al., 1999), whereas the σ_1 antagonist E-5842 induced the opposite effect, decreasing the number spontaneously active dopaminergic A_{10} neurones (Sanchez-arroyos and Guitart, 1999). In addition, the repeated administration of JO-1784 and SA4503 decreased the number of spontaneously active A_9 neurones (Zhang et al., 1993b; Minabe et al., 1999) whereas the σ_1 antagonist E-5842 was inactive (Sanchez-arroyos and Guitart, 1999). In summary, experiments

with the σ ligands obtained different responses depending on the area evaluated and the type of administration (single or repeated).

The levels of dopamine metabolites in the cortex and striatum have been measured, after the administration of σ_1 ligands, at the extracellular and tissue level with *in vivo* brain microdialysis techniques and post-mortem determinations, respectively. Studies with *in vivo* brain microdialysis techniques found that some σ_1 agonist such as (±)-pentazocine and (+)-SKF-10,047 increased extracellular dihydroxy phenylacetic acid (DOPAC) levels in the striatum but not in the frontal cortex (Matsuno et al., 1995b), whereas the σ_1 antagonist panamesine increased extracellular DOPAC levels in the prefrontal cortex (Skuza et al., 1998) and the σ antagonists MR 200 and BMY 14802 did not modify extracellular DOPAC levels in the striatum (Moison et al., 2003; Kanzaki et al., 1992). Since the major proportion of extracellular DOPAC derives from an intraneuronal pool of newly synthesized dopamine (Zetterstrom et al., 1988; Soares-Da-Silva and Garrett, 1990), it seems that σ_1 ligands could modulate dopamine metabolism.

Conflicting reports have been published regarding the role of DTG in the modulation of extracellular DOPAC levels. Some authors described an increase in extracellular DOPAC levels after intra-nigral administration of DTG (Bastianetto et al., 1995), whereas other authors reported that DOPAC levels were not modified after intra-striatal administration of this drug (Moison et al., 2003). The levels of extracellular homovanillic acid (HVA), another dopamine metabolite, have also been measured: the σ antagonist BMY 14802 did not modify HVA levels in the striatum (Kanzaki et al., 1992), whereas other σ ligands, such as DTG and the σ_1 antagonist panamesine, increased extracellular HVA levels in the striatum and medial prefrontal cortex,

respectively (Skuza et al., 1998; Bastianetto et al., 1995). The levels of dopamine metabolites have also been measured post-mortem in the brain cortex and striatum. The σ_1 agonists (+)-pentazocine and (+)-SKF-10,047 increased tissue levels of DOPAC and HVA in the striatum (Iyengar et al., 1990), whereas SA4503 increased DOPAC levels in the frontal cortex, but not in the striatum (Kobayashi et al., 1997). Although all three compounds are known σ_1 ligands with high affinity, and both (+)-pentazocine and SA4503 are known selective σ_1 agonists, they have different effects in the cortex and striatum. The σ_1 antagonist panamesine increased DOPAC and HVA levels, but this effect was observed in both the brain cortex and striatum (Skuza et al., 1998). In summary, all σ_1 ligands described above that showed activity in the modulation of DOPAC or HVA levels induced an increase in extracellular or tissue levels of dopamine metabolites, suggesting an enhancement of dopamine metabolism, but both σ_1 agonist and antagonists have been shown to produce this effect.

The only study to date that has examined the direct effect of a σ ligand on tyrosine hydroxylase activity (which is responsible for the synthesis of DOPA, a precursor of dopamine) in the striatum found that the intra-nigral injection of DTG increased this enzymatic activity (Weiser et al., 1995). However, no other, more selective σ_1 ligand were tested to clarify the role of σ_1 receptors in this enzyme activity.

Several reports have related the effects of σ_1 ligands with extracellular dopamine levels in the brain cortex and striatum. The selective σ_1 agonist (+)-pentazocine increased extracellular dopamine levels in the medial prefrontal cortex (Gudelsky et al., 1995). In addition, the acute systemic administration of the selective σ_1 agonists (+)-pentazocine and (+)-SKF-10,047 (but not its (–)-enantiomer) increased the extracellular concentration of dopamine in the striatum, but this effect was shared by the

σ_1 antagonist Dup 734 (Gudelsky et al., 1995). In contrast, dopamine concentration in the striatum was unaffected by the systemic administration of DTG, and was markedly suppressed by the non-selective σ_1 agonist (+)-3-PPP (Gudelsky et al., 1995; Kanzaki et al., 1992); this latter effect was reversed by the σ antagonist BMY 14802 (Kanzaki et al., 1992). On the contrary, other studies using the same technique with intra-striatal administration of σ ligands showed that (+)-pentazocine, DTG and MR 200 induced a decrease in extracellular dopamine concentration (Gudelsky et al., 1999; Patrick et al., 1993; Moison et al., 2003), but higher doses of the same ligands induced a biphasic effect consisting of a brief stimulatory effect followed by a prolonged inhibitory effect on dopamine release (Gudelsky et al., 1999; Moison et al., 2003). It was suggested that this biphasic effect was due to the involvement of different σ subtypes, or that it reflected a non-specific effect that did not involve σ receptors, because the excitatory effect occurred only after the infusion of very high concentrations of σ ligands (Gudelsky et al., 1999; Moison et al., 2003). Thus the negative modulation of extracellular dopamine concentration by σ_1 ligands appears to be a more specific effect of σ_1 than the increase in dopamine concentration. From these data it seems that the use of drugs with different degrees of selectivity for σ_1 receptors, and also different administration procedures, led to marked differences among studies. However, studies in rat nucleus accumbens slices and SH-SY5Y cells confirmed the negative modulatory role of σ_1 receptors on dopamine release. It has been shown that the σ_1 agonists (+)-pentazocine and BD 737 decreased by 40% the dopamine release induced by KCl; the effect of these agonists was prevented by the σ_1 antagonists DuP 734 and BD 1008, but not by the σ_2 antagonist BIMU-8 (Ault and Werling, 1999 and 2000). The negative modulation of extracellular dopamine levels by σ_1 receptors is also consistent with a

recent study of Peeters and co-workers, who showed that the repeated administration of the selective σ_1 agonist PRE 084 induced, as did chronic treatment with a dopaminergic antagonist, an increase in dopamine-stimulated GTPγS binding in striatal membranes. This effect of PRE 084 appears to be selective for σ_1 receptors, because it was reversed by the σ_1 antagonist BD 1047. Sensitization of dopamine receptors by repeated PRE 084 administration was explained by the decrease in extracellular dopamine levels after treatment with a σ_1 agonist (Peeters et al., 2004).

In other neurotransmitter systems, σ_1 receptors have been shown to be effective in regulating dopaminergic neurotransmission by modulating the effect of other agents, particularly NMDA receptors, but as in the experiments described above, the direction of this modulation could not be clarified because of differences in the findings. Takahasi and co-workers, using *in vivo* microdialysis techniques, showed that the systemic administration of the σ_1 antagonist ligand MS-377, which had no effect under control conditions, attenuated the increase in dopamine levels induced by PCP (Takahasi et al., 2001). Another study using patch clamp whole cell recording showed that the σ_1 antagonists MS-377, haloperidol, BD 1063 and NE-100 inhibited the currents evoked by NMDA in dopamine neurones of the rat ventral tegmental area (Yamazaki et al., 2002). Werling's group have made substantial contributions to our knowledge of the role of σ_1 receptors on NMDA-stimulated [^{3}H]dopamine release. They have published several reports on the inhibition of NMDA-stimulated [^{3}H]dopamine release from striatum slices by σ_1 receptor agonists, including (+)-pentazocine, (+)-SKF-10,047, BD 737, and the putative agonist pregnenolone sulfate, but found that the putative σ_1 antagonist progesterone also inhibited dopamine release in their experimental model. The effect induced by low concentrations of the σ_1 ligands tested, including the effect of

progesterone, were reversed by the σ_1 antagonists DuP 734 and BD 1008 (Gonzalez-Alvear and Werling, 1994, 1995, 1997; Nuwayhid and Werling, 2003a and b) and by pre-treatment with the PKC_β selective inhibitor LY379196 and the PLC inhibitor U-73,122 (Nuwayhid and Werling, 2003a and b), which indicates the participation of these classical second messenger systems in these σ_1 ligand-mediated effects. The modulation of NMDA-stimulated [^{3}H]dopamine release has been also demonstrated in nucleus accumbens and cortical slices. In both preparations the σ_1 agonists (+)-pentazocine and BD 737 inhibited [^{3}H]dopamine release (Ault et al., 1998; Ault and Werling 1998). This inhibitory effect of σ_1 agonists was reversed by the known σ_1 antagonists DuP 734 and BD 1008 (Ault et al., 1998; Ault and Werling 1998), and also by DTG (Ault and Werling 1998), but not by the σ_2 antagonist BIMU-8 (Ault et al., 1998; Ault and Werling 1998), suggesting a specific σ_1-mediated effect.

In summary, the studies described above, which showed that the effect of σ_1 agonist can be reverted by known σ_1 antagonists, often revealed an inhibitory role of σ_1 receptors on dopamine release (Kanzaki et al., 1992; Peeters et al., 2004; Gonzalez-Alvear and Werling, 1994, 1995, 1997; Nuwayhid and Werling, 2003a and b; Ault et al., 1998; Ault and Werling 1998, 1999 and 2000). Nevertheless, more experiments using highly selective σ_1 and σ_2 agonists and antagonists should be performed to clarify the role of σ receptors on *in vitro* and *in vivo* dopamine release.

In addition, the σ_2 subtype has been demonstrated to be active in modulating extracellular levels of dopamine. Interestingly, the known selective σ_1 agonist (+)-pentazocine enhanced amphetamine-stimulated [^{3}H]dopamine release in rat caudate putamen slices and in PC12 cells, acting as a σ_2 agonist in these experimental models (Izenwasser et al., 1998; Weatherspoon and Werling, 1999). Enhancement of the

amphetamine-mediated response was reversed by the non-selective σ antagonist BD 1008, but also by the selective σ_2 antagonists BIMU-8 (Izenwasser et al., 1998; Weatherspoon and Werling, 1999) and Lu28-179 (Izenwasser et al., 1998). However, the response was not reversed by the selective σ_1 antagonist DuP 734 (Izenwasser et al., 1998; Weatherspoon and Werling, 1999). Similar results were obtained with (-)-pentazocine (Izenwasser et al., 1998). These results indicate that σ_2 receptors can mediate the effects of putative selective σ_1 ligands if the concentration used is high enough. This finding could partially explain the controversy and the marked inconsistencies between known σ_1 agonists and σ_1 antagonists in their ability to modulate the dopaminergic system.

2.3.4. Modulation of adrenergic neurotransmission

It has been shown that some σ_1 ligands (both agonists and antagonists) such as haloperidol, (+)-3-PPP, (+)-SKF-10,047 and DTG inhibited [^{3}H]norepinephrine presynaptic re-uptake in rat brain synaptosomes and cultured bovine adrenal chromaffin cells, in an order of potency that correlated with the affinity for σ_1 receptors (Rogers and Lemaire 1991). However, the σ_1 antagonist DuP 734 did not inhibit the synaptosomal uptake of norepinephrine (Tam et al., 1992). Clearer results were obtained in the isolated rabbit iris-ciliary body, where the selective σ_1 agonist (+)-pentazocine inhibited [^{3}H]norepinephrine release in a NE-100-sensitive manner (Campana et al., 2002).

The role of σ_1 receptors as modulators of NMDA-induced responses was also studied in NMDA-induced [^{3}H]norepinephrine release. The selective σ_1 agonists JO-1784 and (+)-pentazocine, like the non-selective σ_1 agonist (+)-3-PPP, were shown

to potentiate, in a concentration-dependent manner, NMDA-induced [^{3}H]norepinephrine release in rat hippocampal slices without affecting the basal outflow; on the other hand, DTG acted as an inverse agonist in this experimental paradigm. The σ_1 antagonists haloperidol or BD 1063, which did not modify NMDA-evoked [^{3}H]norepinephrine release, completely prevented the effects of JO-1784, (+)-pentazocine, (+)-3-PPP and DTG (Monnet et al., 1992a, 1995 and 1996). The NMDA-induced release of [^{3}H]norepinephrine can be modulated not only by typical σ_1 receptor agonists but also by the putative σ_1 receptor ligands, the neurosteroids. The putative σ_1 agonist DHEAS also potentiated NMDA-induced [^{3}H]norepinephrine release, whereas pregnenolone sulfate, like DTG, acted as an inverse agonist. The effects of neurosteroids were reversed by the σ_1 antagonists BD 1063, haloperidol, and also by the putative σ_1 antagonist progesterone (Monnet et al., 1995). In summary, stimulation of σ_1 receptors seems to exert a positive modulatory role in noradrenergic neurotransmission, at least in the NMDA-induced response.

2.3.5. Modulation of cholinergic neurotransmission

Early studies showed that haloperidol, reduced haloperidol, (+)-pentazocine and DTG potently inhibited the phosphoinositide response to muscarinic agonists (Bowen et al., 1990b); moreover, other studies have shown that some σ ligands inhibited carbachol-stimulated phosphoinositide turnover (Hudkins and DeHaven-Hudkins, 1991; Vargas and Pechnick, 1991), and that this effect correlated with the affinity of these drugs for σ receptors (Hudkins and DeHaven-Hudkins, 1991). These observations led to a deeper investigation of the role of σ receptors on cholinergic neurotransmission.

Some studies demonstrated that evoked and spontaneous acetylcholine release is increased by σ_1 receptor agonist. *In vivo* microdialysis studies demonstrated that several σ_1 ligands, such as SA4503, (±)-pentazocine, (+)-SKF-10,047, (+)-3-PPP and DTG, increased spontaneous acetylcholine release in the rat frontal cortex and hippocampus (Matsuno et al., 1993b, 1995a; Kobayashi et al., 1996a and b) with an order of potency that correlated with the affinity of the drugs for σ_1 receptors (Matsuno et al., 1993b). In addition, the effect of the dextroisomer of (±)-SKF-10,047 was greater than that of the levoisomer, which also agrees with σ_1 receptor pharmacology (Matsuno et al., 1993b, 1995a). Furthermore, some σ_1 antagonists such as haloperidol or NE-100 reversed the effects of σ_1 agonists (Matsuno et al., 1993b, 1995a; Kobayashi et al., 1996a). In addition, the σ_1 agonists (+)-SKF-10,047, JO-1784 and SA4503 potentiated KCl-evoked [^{3}H]acetylcholine release from rat hippocampal slices (Junien et al. 1991; Horan et al., 2002); the effects of the σ_1 agonists were stereoselective and reversed by haloperidol, whereas DTG showed an inhibitory effect on KCl-induced [^{3}H]acetylcholine release (Junien et al. 1991).

However, σ_1 receptor ligands may also modulate cholinergic systems indirectly, through their interaction with the glutamatergic systems. Indeed, NMDA receptors regulate acetylcholine release in several brain structures (Lodge and Johnston 1985; Snell and Johnson 1986; Nishimura and Boegman 1990), and σ compounds may indirectly facilitate cholinergic receptor functions through their NMDA-potentiating properties (Monnet and Maurice, 2006).

Most of the effects of σ ligands on the cholinergic system were obtained with *in vivo* experimental models of amnesia (reviewed in Monnet and Maurice 2006), and will be discussed later in section 3.3 *Role of σ_1 receptors in learning and memory*.

2.3.6. Modulation of serotonergic neurotransmission

Several recent reports have documented the modulation of serotonergic neurotransmission by σ_1 ligands. The σ ligands 4-IBP, (+)-pentazocine, and DTG, after two days of treatment, induced a significant increase (more than 50%) in the firing activity of 5-HT neurones of the dorsal raphe nucleus (Bermack and Debonnel, 2001). A similar effect was obtained with OPC-14523 (Bermack et al., 2004), which binds both σ_1 and 5-HT_{1A} receptors (Oshiro et al., 2000; Tottori et al., 2001). The increase in the firing activity induced by OPC-14523 was prevented by the co-administration of NE-100, suggesting that this activity was mediated by σ_1 receptors (Bermack et al., 2004). Neurosteroids were also related to modulation of the serotonergic system through σ_1 receptors. Subchronic treatment with the putative σ_1 agonist DHEA increased the firing activity of 5-HT neurones in the dorsal raphe nucleus in an NE-100 sensitive manner, whereas the putative σ_1 antagonist progesterone alone had no effect (Robichaud and Debonnel, 2004). In summary, several lines of evidence show that σ_1 receptor stimulation induces a positive effect on 5-HT neurone firing activity in dorsal raphe nucleus.

The precise mechanisms of the effects described above have not been established. They may be indirect effects that occur through the influence of σ_1 ligands on glutamatergic neurotransmission, given that AMPA and NMDA receptors have been shown to mediate the glutamatergic excitatory input in the dorsal raphe nucleus (Celada et al., 2001). In fact, some reports have proposed a relationship between the activity of NMDA receptors, the σ system and serotonin release. The selective σ_1 antagonist MS-377 had no effect on extracellular serotonin levels *per se*, but attenuated the

increase in serotonin levels in the rat medial prefrontal cortex induced by PCP (Takahasi et al., 2001).

2.4. Role of σ_1 receptors in lipid rafts

As described in section 2.2 *Subcellular distribution of σ_1 receptors*, σ_1 receptor binding sites are enriched in microsomal membranes. $Sigma_1$ receptors in NG108 cells are localized on both the endoplasmic reticulum reticular network and the nuclear envelope, but they are seen mostly as highly clustered globular structures associated with the endoplasmic reticulum (Hayashi and Su, 2003a and b). These σ_1 receptor-enriched globules contain moderate amounts of free cholesterol and neutral lipids (Hayashi and Su, 2003a, and 2004b), so it has been hypothesized that they work as lipid storage sites on the endoplasmic reticulum (i.e., lipid droplets associated with the endoplasmic reticulum) (Hayashi and Su, 2005). Endoplasmic reticulum lipid droplets are formed by coalescence of neutral lipids into discs inside the bilayer of the endoplasmic reticulum membranes. When they reach a critical size, they bud off to form cytosolic lipid droplets (Murphy and Vance, 1999), which were proposed to be a new transport pathway of lipids between the endoplasmic reticulum and Golgi apparatus or plasma membrane (Ohashi et al., 2003) that can be guided by σ_1 receptors (Hayashi and Su, 2003a and 2005). This would explain how σ_1 receptors can interact with neurotransmitter or growth factor receptors, which are located in lipid microdomains in the plasma membrane (Bruses et al., 2001; Hering et al., 2003). In addition, over-expression of σ_1 receptors increased cholesterol contents in lipid rafts in NG108 and

PC12 cells, suggesting that upregulation of σ_1 receptors potentiated lipid raft formation (Takebayashi et al., 2004a; Hayashi and Su, 2005).

The over-expression of σ_1 receptors also alters the proportion of gangliosides in lipid rafts (Takebayashi et al., 2004a), which have been proposed to play a crucial role in regulating the localization of growth factor receptors, among other proteins, in lipid rafts (Simons and Ikonen, 1997). $Sigma_1$ receptors could alter the function of some tropic factors, and in fact, it has been demonstrated that σ_1 receptor agonists enhanced the action of BDNF on excitatory glutamatergic neurotransmission (Yagasaki et al., 2006) and on the differentiation (e.g., neurite sprouting) of PC12 cells caused by nervous and epidermal tropic factors at a very early stage (Takebayashi et al., 2002). This latter effect was reverted by the σ_1 receptor antagonist NE-100 and by the σ_1 antisense oligodeoxynucleotide; furthermore, over-expression of σ_1 receptors enhanced nervous growth factor-induced neurite sprouting (Takebayashi et al., 2002). In addition, the transfection of rat primary hippocampal cultures with functionally dominant-negative σ_1 receptors suppressed oligodendrocyte differentiation (Hayashi and Su, 2004b), a finding which reinforces the role of σ_1 receptors on cell differentiation (see Hayashi and Su, 2005 for a more complete review).

2.5. Biphasic effects of σ_1 agonists

It has been reported that some σ ligands produce a biphasic bell-shaped effect in different behavioural, biochemical and electrophysiological models. In animal models of amnesia, some selective σ_1 agonists induced attenuation of the amnesia induced by *p*-chloroamphetamine (PCA), carbon monoxide and $\beta_{25\text{-}35}$-amyloid related peptide, but

in a bell-shaped manner (Matsuno et al., 1993a; Maurice et al. 1994c, 1998). In addition, it was reported that the intrastriatal infusion of the selective σ_1 agonist (+)-pentazocine through a microdialysis probe resulted in a biphasic effect on extracellular dopamine concentration, i.e., a brief increase followed by a prolonged decrease (Gudelsky et al., 1999). Moreover, the enhancement of the BDK-induced increase in $[Ca^{2+}]_i$ by σ_1 agonists (PRE 084, (+)-pentazocine and pregnenolone sulfate) in NG-108 cells also showed a bell-shaped concentration-effect curve for all agonists tested (Hayashi et al., 2000). Another dual effect of (+)-pentazocine was reported in cardiac myocytes, where this drug decreased contractility at 1 nM, whereas a 10-fold higher concentration produced a marked increase (Novakova et al., 1995). Another interesting finding was that low doses of σ agonists induced potentiation of neuronal activation induced by NMDA (Monnet et al., 1990 and 1992b), but at higher doses, the effects of selective σ_1 agonists such as JO-1784 progressively decreased and disappeared. At high doses these agonists acted as antagonists, preventing the potentiation induced by low doses of other σ agonists (Bergeron et al., 1995; Bergeron and Debonnel, 1997). Similar findings was reported with the selective σ_1 ligand SR31742A (Liang and Wang., 1998). The biphasic effect of JO-1784 is consistent with some results from clinical trials in which its efficacy as an antidepressant was higher at a dose of 20 mg/day than at 100 mg/day (Pande et al., 1998). These dual effects of σ ligands have been hypothesized to be due to the presence of two subtypes of σ_1 receptor (Bergeron and Debonnel, 1997; Novakova et al., 1995; Bermack and Debonnel, 2005), or to the existence of two different conformations of the same receptor (Novakova et al., 1995). The different (and sometimes opposite) results obtained in different experimental models could be due to the bell-shaped responses of σ_1 ligand. Thus the biphasic

responses of σ_1 agonists may constitute an important factor to take into account in attempts to explain much of the controversy in the literature about σ_1 receptors (Bermack and Debonnel, 2005).

3. THERAPEUTIC POTENTIAL OF SIGMA$_1$ RECEPTORS

Given the widespread distribution of σ_1 receptors in the central nervous system and their modulatory role in different cellular and biochemical effects (see Su and Hayashi, 2003 for review), σ_1 ligands have been proposed to be useful in different therapeutic fields such as analgesia, depression and anxiety, amnesic and cognitive deficits, psychosis and treatment for drugs of abuse (see Guitart et al., 2004; Bermack and Debonell, 2005; Skuza et al., 2003; Maurice and Lockhart, 1997; Maurice et al., 2001a; Matsumoto et al., 2003; Hayashi and Su, 2004a; Monnet and Maurice, 2006 and for reviews). These potential therapeutic applications are reviewed briefly below.

3.1. Sigma$_1$ receptors and analgesia

Several σ_1 ligands such as (+)-pentazocine, haloperidol, haloperidol metabolite I, haloperidol metabolite II and (+)-MR 200 have been proved to be inactive in the tail-flick test (Chien and Pasternak, 1993, 1994, 1995b; Cendán et al., 2005a; Ronsisvalle et al., 2001a; Marrazzo et al., 2006), with the exception of (–)-pentazocine, which showed analgesic activity due to its κ_1 agonist properties (Chien and Pasternak 1995b). Despite the inactivity of pharmacological blockade and activation of σ_1 receptors in tail-flick tests, this receptor has been extensively documented as a modulator of opioid analgesia

in this behavioural model. The subcutaneous (s.c.) administration of the selective σ_1 agonist (+)-pentazocine antagonized the antinociception induced by morphine administered systemically, supraspinally or spinally in the tail-flick test (Chien and Pasternak, 1993, 1994; Mei and Pasternak, 2002), but did not modify other non-analgesic effects of morphine, such as inhibition of gastrointestinal transit or lethality (Chien and Pasternak, 1994). These effects probably occur at the supraspinal level, because the intrathecal (i.t.) administration of (+)-pentazocine did not reverse the spinal analgesic effect of morphine (i.t.) (Mei and Pasternak, 2002). Other σ ligands such as (–)-pentazocine and DTG, when administered systemically, also diminished analgesia induced by the s.c. administration of morphine (Chien and Pasternak, 1993, 1994). In addition, systemic administration of the σ_1 antagonist haloperidol not only reversed the effects of (+)-pentazocine, (-)-pentazocine and DTG, but also increased morphine-induced antinociception, whereas the D_2 antagonist (-)-sulpiride was inactive (Chien and Pasternak, 1993, 1994, 1995a). Furthermore, the enhanced analgesia induced by the co-administration of haloperidol and morphine was reverted with the irreversible μ opioid receptor antagonist β-funaltrexamine (Chien and Pasternak, 1994), confirming the modulatory role of σ_1 receptors in μ-induced analgesia. More recent studies have confirmed the supraspinal modulation of μ-opioid antinociception by σ_1 receptors. Blockade of σ_1 receptors by the non-selective σ_1 antagonist (+)-MR 200 (s.c.) enhanced the analgesia induced by the intracerebroventricular (i.c.v.) administration of the selective μ-opioid agonist DAMGO (Marrazzo et al., 2006); furthermore, the selective blockade of σ_1 receptor synthesis by the i.c.v. administration of specific anti-sense oligodeoxynucleotides also enhanced the antinociception induced by morphine administered s.c. or i.c.v. (Mei and Pasternak, 2002).

In addition, σ_1 receptors were shown to modulate δ-and κ-opioid analgesia. The selective σ_1 agonist (+)-pentazocine diminished the δ-opioid antinociception induced by DPDPE (Chien and Pasternak, 1994; Mei and Pasternak, 2002). Furthermore, the analgesia induced by DPDPE was enhanced after administration of the σ_1 antagonists haloperidol and (+)-MR 200 (Chien and Pasternak, 1994; Marrazzo et al., 2006), and also by down-regulation of σ_1 receptors by the i.c.v. administration of σ_1 antisense oligodeoxynucleotides (Mei and Pasternak, 2002). Analgesia induced by κ-opioid administration was also modulated by σ_1 receptors. As in μ- and δ-opioid antinociception, the σ_1 agonist (+)-pentazocine decreased the antinociception induced by the κ_3-opioid agonist naloxone benzoylhydrazone (NalBzoH) (Chien and Pasternak, 1994; Mei and Pasternak, 2002) or by the κ_1-opioid agonist U50,488H (Chien and Pasternak, 1994; Mei and Pasternak, 2002), whereas pharmacological blockade of σ_1 receptors by the σ_1 antagonists haloperidol or (+)-MR 200 increased the κ-opioid antinociceptive effect (Chien and Pasternak, 1994, 1995a; Ronsisvalle et al., 2001a), as did the i.c.v. administration of σ_1 antisense oligodeoxynucleotides (King et al., 1997; Mei and Pasternak, 2002).

The synthetic analgesic (–)-pentazocine is an interesting compound, because is acts as both a σ_1 and a κ_1-opioid agonist. Because of its κ_1 opioid agonist activity it is able to produce analgesia alone, and it can also inhibit its own analgesic effect through σ_1 receptors. Thus the analgesic effect of (–)-pentazocine was potentiated by the administration of the σ_1 antagonist haloperidol in the absence of other opioid drugs. Moreover, the inhibition of (-)-pentazocine-induced analgesia by nor-binaltorphimine (Nor-BNI) confirmed the involvement of κ_1 receptors (Chien and Pasternak, 1995b). In addition, (+)-pentazocine (i.c.v.) decreased the analgesic effect of the agonists for κ and

μ opioid receptors nalorphine and nalbuphine, and this analgesia was enhanced by the i.c.v. administration of σ_1 antisense oligodeoxynucleotides (Mei and Pasternak, 2002).

In summary, σ_1 receptors tonically inhibit all analgesic opioid systems. The efficacy of the supraspinal administration of σ_1 antisense oligodeoxynucleotides in enhancing the analgesia induced by μ, δ and κ agonists suggests that the supraspinal location of σ_1 receptors plays a pivotal role in this modulation.

Some recent reports showed that σ_1 receptors are able to modulate nociception in the absence of any opioid drug in some behavioural tests. Ueda and co-workers (2001a) showed that the σ_1 agonists (+)-pentazocine and SA4503, (+)-3-PPP, and also the putative σ_1 agonists DHEAS and pregnenolone sulfate, can even induce nociception when used alone in the nociceptive flexor response test. However, the σ_1 antagonists BD 1063, NE-100 and the putative σ_1 antagonist progesterone were inactive. In addition, the effect of the selective σ_1 receptor agonist (+)-pentazocine was reverted by all three antagonists, and the effect of DHEAS through σ_1 receptors was reverted by the selective σ_1 antagonist NE-100 (Ueda et al., 2001a).

Studies with the formalin test in mice by Cendán and co-workers (2005a) found that the systemic administration, in the absence of any opioid drug, of the non-selective σ_1 antagonists haloperidol and haloperidol metabolite II, like the selective σ_1 antagonist haloperidol metabolite I, showed an analgesic effect in formalin-induced nociception. The order of potency for the antinociception induced by haloperidol and its metabolites correlated with their affinity for σ_1 receptors, but not for σ_2 or D_2 receptors. The antinociception induced by haloperidol and its metabolites was found to be insensitive to naloxone, which indicates that the widely described modulation of opioid receptors (Chien and Pasternak, 1993, 1994, 1995a and b; King et al., 1997; Ronsisvalle et al.,

2001a; Mei and Pasternak 2002; Marrazzo et al., 2006) does not mediate these effects. The generation of σ_1 receptor knock-out mice (Langa et al., 2003) facilitated research on σ_1 receptor functions. Sigma$_1$ receptor knock-out mice showed antinociception both in the first and second phase of formalin-induced pain (Cendán et al., 2005b), but interestingly, the antinociception induced by haloperidol and haloperidol metabolite II was higher than in σ_1 receptor knock-out mice (Cendán et al., 2005a). These results suggested that in addition to σ_1 receptors, other mechanisms (possibly σ_2 receptors) may be involved in the analgesia induced by these non-selective σ_1 antagonists. Recent experiments with the same behavioural test found that in contradistinction to the supraspinal action of σ_1 antagonists on the modulation of opioid analgesia (King et al. 1997; Mei and Pasternak, 2002), the i.t. administration of the σ_1 receptor antagonists BD 1047 and BMY 14802 dose-dependently reduced formalin-induced pain behaviours through the blockade of σ_1 receptors in the second phase but not in the first phase of the formalin test (Kim et al., 2006a), underscoring the importance of σ_1 receptors in the second phase of formalin-induced pain. These results were consistent with the results previously reported by Cendán and co-workers, 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 (Cendán et al., 2005a).

In summary, σ_1 receptors are not only able to modulate opioid antinociception, but they also play an active role in nociception in the absence of any opioid drugs.

3.2. Role of σ_1 receptors in depression and anxiety

Several neurotransmitter systems that are important in the pathophysiology of depression and anxiety can be modulated by σ_1 receptors. Specifically, adrenergic and serotonergic neurotransmission appear to play an important role on these disorders (Delgado and Moreno, 2000; Goodnick and Goldstein, 1998). As described above, *in vitro* experiments suggested that σ_1 ligands inhibit the re-uptake and increase the release of norepinephrine, and modify the firing activity of 5-HT neurones (see sections 2.3.4 *Modulation of adrenergic system* and 2.3.6 *Modulation of serotonergic neurotransmission*).

3.2.1. Depression and σ_1 receptors

The effects of σ_1 ligands were tested in the forced swimming test, the tail suspension test and evaluation of the NMDA receptor-mediated behaviour after olfactory bulbectomy (OBX), which are behavioural tests classically used to predict the antidepressant activity of drugs. It was shown that some selective σ_1 receptor agonists such as SA4503, (+)-pentazocine and JO-1784 dose-dependently decreased immobility in the forced swimming test, and that these effects were blocked by the selective σ_1 antagonists NE-100 and BD 1047 and by the administration of σ_1 antisense probe (Matsuno et al., 1996a; Urani et al., 2001; Maurice et al., 2001a and 2006). Donepezil, a potent cholinesterase inhibitor that binds to σ receptors with high affinity (Kato et al., 1999), also showed antidepressant-like activity in forced swimming tests in mice. This effect seems to be produced by σ_1 receptor stimulation, because it was not seen with

other cholinesterase inhibitors such as rivastigmine and tacrine, and was blocked by the pre-administration of the σ_1 receptor antagonist BD 1047 and by *in vivo* treatment with antisense probe (Maurice et al., 2006). In addition, SA4503 and (+)-pentazocine also decreased immobility time in the tail suspension test, and this effect was antagonized by NE-100 (Ukai et al., 1998). Some studies showed a relationship between the antidepressant-like effect of σ_1 agonists and NMDA antagonists. The antidepressant-like effect of SA4503 in the forced swimming test was potentiated by competitive and non-competitive NMDA antagonists (Skuza and Rogoz., 2002; Skuza, 2003), suggesting that the antidepressant-like effect of σ_1 agonists was produced, at least in part, through the modulation of NMDA receptors. Because the pharmacological effect of σ_1 receptors is typically modulatory, compounds that posses a high affinity for both σ_1 receptors and receptors related with depression may offer reasonable expectations of efficacy. OPC-14523, a newly synthesized compound with possible antidepressant activity, exhibits affinity for σ, 5-HT_{1A} and 5-HT_{1B} receptors (Oshiro er al., 2000; Tottori et al., 2001) and produced a marked antidepressant-like effect in the forced swimming test after a single oral administration. Its maximum effect was higher than that for fluoxetine and imipramine, which have known antidepressant activity but require at least 4 days of treatment to be effective). That antidepressant-like effect was reversed by the σ_1 receptor antagonist NE-100 and also by the selective 5-HT_{1A} antagonist WAY-100635 (Tottori et al., 2001). Moreover, pre-treatment for one week with *p*-chloroamphetamine (PCA), which depletes brain 5-HT, failed to diminish the antidepressant effects of OPC-14523 in this behavioural test (Yamada et al., 2000), suggesting that σ receptors alone can mediate the antidepressant effects produced by OPC-14523. The potent

antidepressant-like action of OPC-14523 was mimicked by the co-administration of σ and 5-HT_{1A}-receptor agonists (Yamada et al., 2000).

It was reported that (+)-pentazocine and the antidepressants imipramine and fluvoxamine, which exhibit affinity for σ_1 receptors, enhanced nerve growth factor-induced neurite sprouting in PC12 cells via σ_1 receptors (Takebayashi et al. 2002). This is of interest because it has been proposed that the therapeutic actions of these antidepressants may involve neurotropic actions (Takebayashi et al., 2002 and 2004b; Nestler et al., 2002).

In the OBX model of depression, OBX rats show increased dizocilpine-induced behavioural modifications, including locomotor and circling activity. Repeated treatments with low doses of JO-1784 reversed the effect of olfactory bulbectomy such that the behaviour of OBX rats was not significantly different from control rats (Bermack et al., 2002).

A few clinical trials in humans have already been published. These studies found that JO-1784, at doses of 20 mg/day, exhibited a stronger antidepressant effect than the known antidepressant fluoxetine at the same dose (Pande et al., 1988). One possible advantage of the therapeutic use of σ_1 ligands as antidepressants is that chronic treatment with σ_1 agonists was not accompanied by side effects such as increased body weight, unlike classic antidepressants such as desipramine and fluoxetine (Ukai et al., 1998).

There are many reports of the role of neurosteroids in depression through σ_1 receptors (reviewed in Maurice et al., 2001a and Van Broekhoven and Verkes, 2003). The administration of DHEAS and pregnenolone sulfate, putative σ_1 agonist ligands, had antidepressant-like effects in the mouse forced swimming test model of depression,

and its effect was prevented by the co-administration of NE-100 or BD 1047 (Reddy et al., 1998; Urani et al., 2001). In addition, the antidepressant-like effects of neurosteroids and some σ_1 agonists have been shown to be dependent on endogenous neurosteroidal levels. In adrenalectomized and castrated mice, the effect of JO-1784 in the forced swimming test was enhanced compared to control animals, whereas the selective σ_1 agonist PRE 084 demonstrated a significant antidepressant-like effect only in adrenalectomized and castrated mice. These effects were blocked by the selective σ_1 antagonist BD 1047 (Urani et al., 2001). The antidepressant efficacy of the selective agonist JO-1784 in the forced swimming test was enhanced in 12-month-old senescence-accelerated (SAM) mice; this finding was thought to be due to the decrease in neurosteroid levels in these mice, particularly of the putative σ_1 antagonist progesterone (Phan et al., 2005).

3.2.2. 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 attenuated the motor suppression induced by previous electric footshock (Kamei et al., 1997; Noda et al., 2000). The effect of (+)-SKF-10,047 was reversed by NE-100, which indicates that this effect was mediated by σ_1 receptors (Noda et al., 2000). Moreover, Gue and co-workers showed that JO-1784 suppressed the stress-induced colonic motor disturbances induced by fear stress in rats, in a model that mimicked the gastrointestinal tract disorders frequently present in anxiety (Gue et al., 1992).

In humans, JO-1784 also showed good results in a phase-1 model of functional diarrhoea (Volz and Stoll, 2004). Opipramol, which apart from its σ_1 and σ_2 receptor affinity also possesses histamine H_1-antagonistic properties in connection with lower affinities for D_2 and 5-HT_{2A}, showed efficacy in generalized anxiety disorder and somatoform disorders in humans. The receptor profile of opipramol, and the results of studies of the selective σ_1 ligand JO-1784 in pre-clinical animal models, suggest that opipramol may act pharmacologically and clinically via σ receptors (Volz and Stoll, 2004).

Neurosteroids were also effective in stress models. DHEAS and pregnenolone sulfate were able to attenuate the conditioned fear stress motor suppression, and this effect was antagonized by the selective σ_1 antagonist NE-100 and the putative σ_1 antagonist progesterone (Noda et al., 2000). Interestingly, the concentration of DHEAS in the plasma of stressed mice was lower than in non-stressed mice (Noda et al., 2000). In addition, it was reported recently that DHEA attenuated stress-induced sexual dysfunction in rats, and that this effect of DHEA was reverted by the selective σ_1 ligand NE-100 (Mizuno et al., 2006).

3.3. 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 were observed (Drachman and Leavitt, 1974; Davies and Maloney, 1976; Bartus et al., 1982; Decker and McGaugh, 1991); moreover,

the administration of muscarinic or nicotinic receptor antagonists induced amnesia in animals and humans (Glick and Zimmerberg 1972, Levin et al. 1989; Flood and Cherkin, 1986). On the other hand, NMDA receptors are involved in the induction of different forms of synaptic plasticity which are thought to play a role in learning and memory processes (Collingridge, 1987; Izquierdo, 1991). In fact, NMDA receptor blockade by competitive or non-competitive antagonists produces impairments in learning (Maurice and Lockhart, 1997).

As described previously in sections 2.3.2 *Modulation of glutamatergic neurotransmission* and 2.3.5 *Modulation of cholinergic neurotransmission*, σ_1 receptors are able to modulate glutamatergic and cholinergic neurotransmission. However, the activation or blockade of σ_1 receptors failed to improve learning capacities in control animals; in fact, 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 down-regulation of σ_1 receptor expression by antisense oligodeoxynucleotides, failed to show any effect on learning in control animals. These results suggest that σ_1 receptors are not involved in normal memory functions (for reviews see Maurice et al., 1999b and 2001a; Monnet and Maurice, 2006). Bearing in mind the modulatory role of σ_1 receptors in different processes, it is not surprising that they have been found to modulate memory and learning processes under pathological conditions.

3.3.1. Anti-amnesic effects in cholinergic models of amnesia

In contradistinction to the absence of effect by σ_1 ligands under control conditions, they have a marked effect on amnesia induced in rodents by the cholinergic muscarinic antagonist scopolamine. The learning impairment induced by scopolamine, measured as spontaneous alternation, passive avoidance or performance in a water-maze, was attenuated or reversed by the σ_1 ligands DTG, (+)-3-PPP, (+)-SKF-10,047, (±)-pentazocine, (+)-pentazocine, (–)-pentazocine, dimemorfan, JO-1874, and SA4503 (Earley et al., 1991; Matsuno et al., 1997; Maurice and Privat, 1997; Maurice et al., 2001b; Hiramatsu et al., 2004 and 2005; Wang et al., 2003) The effects of σ_1 agonists were reversed by σ_1 antagonists such as haloperidol or NE-100, or by the down-regulation of σ_1 receptor expression by specific antisense oligodeoxynucleotides (Maurice and Privat, 1997; Maurice et al., 2001b; Hiramatsu et al., 2004 and 2005; Wang et al., 2003). The putative endogenous σ_1 ligands pregnenolone sulfate and DHEAS also reversed the amnesic effect of scopolamine in several behavioural models (Mathis et al., 1999; Meziane et al., 1996; Li et al., 1995; Urani et al., 1998). These results may be due, at least in part, to the participation of σ_1 receptors, because their effects were reversed by the putative σ_1 antagonist progesterone and the selective σ_1 receptor antagonist NE-100 (Urani et al., 1998).

In other cholinergic models of amnesia, it has been demonstrated that the administration of the selective σ_1 agonist SA4503 attenuates the impairment of spatial learning performance in rats with cortical cholinergic dysfunction induced by ibotenic acid injection on the basal forebrain (Senda et al., 1998). Furthermore, the selective σ_1 agonist PRE 084 was effective reversing the amnesic effects induced by the nicotinic

antagonist mecamylamine. These data indicate that σ_1 receptors may also modulate nicotinic cholinergic receptor-mediated behaviours (Maurice et al., 1994a).

The memory impairments induced by the 5-HT depleter *p*-chloroamphetamine (PCA) in the passive avoidance test were also assessed. This model of amnesia also involves cholinergic dysfunction, since the immediate post-training administration of the acetylcholinesterase inhibitors tetrahydroaminoacridine (tacrine) and physostigmine attenuated the PCA-induced deficits (Matsuno et al. 1993a). The amnesia induced by PCA amnesia was attenuated, in a bell-shaped manner, by the administration of (±)-pentazocine, (+)-3-PPP, DTG, and (+)-SKF-10,047, and this last effect was stereoselective (Matsuno et al., 1993a and 1994). These effects were observed when the σ ligands were administered before and after training and before retention, which indicates that these ligands improved cholinergic-dependent memory processes during the acquisition, consolidation and retention phases (Matsuno et al., 1994).

The anti-amnesic effects of σ_1 agonists have been also tested against learning deficits associate with physiological or pathological ageing. Cholinergic systems in the basal forebrain are known to be altered during ageing, and the degenerative changes in cholinergic nuclei are correlated with memory impairment in aged rats. $Sigma_1$ agonist ligands attenuated the learning deficits in SAM, aged mice and aged rats (Maurice et al., 1996b; Phan et al., 2003; Tottori et al., 2002).

3.3.2. Anti-amnesic effects in glutamatergic (NMDA) models of amnesia

The σ ligands (+)-SKF-10,047, (+)-pentazocine, JO-1784, DTG, PRE 084, and SA4503 attenuated the learning deficits induced by dizocilpine, a non-competitive

NMDA-receptor antagonist, in rats and mice presented with different mnesic tasks such as spontaneous alternation, passive avoidance, place learning in the water-maze, a three-panel runway, or 8 radial-arm maze (Maurice and Privat, 1997; Maurice et al., 1994a and b, 2001c; Ohno and Watanabe., 1995; Zou et al., 1998 and 2000). The anti-amnesic effect of σ_1 agonists was reverted by the σ_1 antagonists haloperidol, NE-100 and BD 1047 (Maurice and Privat, 1997; Zou et al., 1998 and 2000; Maurice et al., 2006), and by the administration of antisense oligodeoxynucleotides against σ_1 receptors (Maurice et al., 2001b and c, 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) was blocked by BD 1047 or antisense treatment. Therefore, donepezil behaved as an effective σ_1 receptor agonist, reversing the dizocilpine-induced impairments (Maurice et al., 2006).

The involvement of σ_1 receptors in the anti-amnesic effect induced by the steroids DHEAS and pregnenolone sulfate has been extensively documented. These neurosteroids were effective against the impairments induced by different NMDA antagonists such as the competitive antagonists 3-((±)2-carboxypiperazin- 4-yl)-propyl-1 phosphonic acid (CPP) and (−)-2-amino-5-phosphonopentanoic acid (D-AP5) and the non-competitive antagonist dizolcipine (see Maurice et al., 2001a and Monnet and Maurice 2006 for reviews), and these anti-amnesic effects were reversed by NE-100 and BMY 14802 (Maurice et al., 1997 and Zou et al., 2000). Furthermore, progesterone, the endogenous putative σ_1 antagonist, reversed the effect of σ_1 agonists (including DHEAS and pregnenolone sulfate) after dizocilpine-induced impairments (Maurice and Privat, 1997; Zou et al., 2000).

3.3.3. Anti-amnesic effects in glutamatergic-cholinergic mixed models

In mice, repetitive exposures to carbon monoxide (CO) gas induce long-lasting but delayed amnesia, that could be measured about one week after exposure (Nabeshima et al. 1991). Like models of ischemia, this model involves the neurotoxicity of excitatory amino acids, and the hippocampal cholinergic system appears markedly affected by the hypoxic toxicity (Nabeshima et al. 1991). Competitive and non-competitive NMDA antagonists have been reported to efficiently prevent CO-induced amnesia and the neurodegeneration that occurs in the hippocampus (Ishimaru et al. 1992; Nabeshima et al. 1991). The σ ligands (+)-SKF-10,047, PRE 084, JO-1784 and DTG reversed CO-induced amnesia, and their effects were prevented by NE-100, BMY 14802 and BD 1047 (Maurice et al., 1994c, 1999a; Meunier et al., 2006a). Some cholinesterase inhibitors (such as tacrine, rivastigmine, galanthamine and donepezil) where also tested in the CO-induced amnesia model, and it was found that all drugs showed anti-amnesic properties, but the pre-administration of BD 1047 block only the effect of donepezil (Meunier et al., 2006a). 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 was suggested that these drugs might produce their effects through their σ_2 agonistic activity (Maurice et al., 1999a).

The amnesia induced by $\beta_{25\text{-}35}$-amyloid related peptide is an animal model of Alzheimer's disease-type amnesia that involves both cholinergic and glutamatergic neurotransmission through NMDA receptors (Maurice et al., 1996c and d; Wang et al., 2003). The selective σ_1 receptor agonists (+)-pentazocine, PRE 084 and SA4503, and the putative σ_1 agonists DHEAS and pregnenolone sulfate, attenuated the amnesia

induced by the central administration of β_{25-35}-amyloid related peptide, and this effect was reverted by haloperidol and the putative σ_1 antagonist progesterone (Maurice et al., 1998). Similar results were found with the σ_1 agonists (+)-SKF-10,047 and dimemorfan (Wang et al., 2003). These findings are consistent with the neuroprotective action of PRE 084, which attenuated cell death in cultured cortical neurones after incubation with β_{25-35}-amyloid related peptide in the presence of dizocilpine. This effect was reversed by the selective σ_1 antagonist NE-100 (Marrazzo et al., 2005).

3.4. Schizophrenia and σ_1 receptors

The dopamine hypothesis of schizophrenia, which implicates enhanced mesolimbic dopamine function, was first proposed in the 1960s and remains a dominant hypothesis for the pathophysiology of this disorder, particularly regarding the genesis of positive symptoms (Depatie and Lal, 2001). In addition to the role played by the central dopaminergic system in schizophrenia, it is also important to consider the glutamatergic system (Tamminga et al., 1998; Olney and Farber, 1995) and the intricate relationship between dopamine, glutamate, and σ receptors. Amphetamine and PCP administration are usually employed as behavioural models of schizophrenia. Amphetamine and other dopaminergic agonists, such as methamphetamine and apomorphine, mimic acute positive symptoms of schizophrenia, whereas PCP not only emulates positive symptoms, but also mimics the long-lasting psychosocial dysfunction similar to the negative symptoms of this disease, and can also induce schizophrenia-like psychosis in humans. Another commonly employed pharmacological model of schizophrenia is the

evaluation of the development of behavioural sensitization to cocaine (Guitart et al., 2004).

The first suggestion that σ receptors might be involved in the pathophysiology of schizophrenia arose from the finding that synthetic ligands such as (+)-SKF-10,047 exhibited psychotomimetic effects, and from the fact that many typical antipsychotic drugs interact with σ receptors, in addition to acting through dopamine receptors (Tam and Cook, 1984; Walker et al., 1990; Matsumoto and Pouw, 2000). However, unlike the correlation between the affinities of classical neuroleptics for dopamine receptors and their therapeutic doses in humans, a comparable relationship does not exist for σ receptors (Walker et al., 1990).

Many descriptions of novel putative antipsychotic compounds with high affinity for σ receptor have been published (Okuyama, 1999; Okuyama et al., 1993; Bartoszky et al. 1996; Takahashi et al. 1999; Nakazato et al., 1999a; Guitart and Farre, 1998). Among these compounds, the non-selective σ_1 antagonist BMY 14802 was first identified as an antipsychotic candidate on the basis of classical neuropharmacological tests. BMY 14802 inhibits apomorphine-induced climbing and stereotypy, and the development of sensitization to methamphetamine and cocaine (Taylor et al. 1993; Ujike et al. 1992b and 1996); furthermore, other studies showed that BMY 14802 reversed the amphetamine-induced decline in DOPAC in the neostriatum (Pierce and Rebec, 1992). In addition, the novel putative antipsychotic σ_1 ligand E-5842 also inhibits apomorphine-induced climbing and amphetamine-induced locomotor activity (Guitart et al., 1998). Other σ ligands such as DTG and SR 31742A decreased the amphetamine-induced enhancement of locomotion (Ruckert and Schmidt, 1993; Poncelet et al., 1993); however, the selective σ_1 antagonist MS-377 failed in influence

the locomotor activity induced by methamphetamine, but attenuated the development of methamphetamine-induced behavioural sensitization in a dose-dependent manner (Takahasi et al., 2000). Recently, it was shown that the σ_1 receptor antagonists BD 1063 and BD 1047, as well as σ_1 antisense oligodeoxynucleotide, significantly attenuated the locomotor stimulatory effects of methamphetamine in mice, suggesting that σ_1 receptors are involved in the acute actions of methamphetamine and that antagonism of this receptor is sufficient to prevent the locomotor stimulatory effects of methamphetamine (Nguyen et al., 2005). It has also been shown that σ_1 receptor antagonists inhibit hyper-locomotion induced by cocaine (see 3.5.1.1 *Effects of σ_1 ligands on acute effects of cocaine*).

One potential mechanism of action of σ_1 receptors is the modulation of NMDA receptor function, as was described in section 2.3.2 *Modulation of glutamatergic neurotransmission*. A dysfunction in glutamatergic neurotransmission, specifically in the frontal cortex, was implicated in schizophrenia (Tamminga et al., 1998; Jentsch and Roth, 1999), so σ_1 receptors may affect some schizophrenic symptoms related to a dysfunction in glutamatergic neurotransmission. PCP-induced behaviour, which is insensitive to selective D_2 antagonists, was attenuated by low doses of the selective σ_1 antagonist NE-100 (< 0.1 mg/kg), with the advantage that this did not produce any extrapyramidal effect (Okuyama et al. 1993, 1994, 1995a). On the other hand, recent studies shown that PCP-induced cognitive deficits were significantly improved by the subchronic administration of the selective σ_1 agonist SA4503, the putative σ_1 agonist DHEAS, and fluvoxamine (a SSRI with high affinity for σ_1 receptors), but not by paroxetine (an SSRI without affinity for σ_1 receptors). Furthermore, the effect of those σ_1 ligands on PCP-induced cognitive deficits was antagonized by the co-administration

of the selective σ_1 receptor antagonist NE-100, suggesting that σ_1 receptor agonists are potentially useful in therapy for the cognitive deficits of schizophrenia (Hashimoto et al., 2006).

However, the results of clinical trials showed no significant improvement in psychiatric symptoms in response to BMY 14802 treatment (Gewirtz et al. 1994), whereas panamesine, a selective σ ligand, showed efficacy in open clinical studies of patients with schizophrenia (Frieboes et al., 1997). An association was suggested between polymorphisms in the σ_1 receptor gene and schizophrenia (Ishiguro et al. 1998), but these results were in contradistinction with other more recent studies (Ohmori et al. 2000; Uchida et al. 2003; Satoh et al., 2004), so the involvement of σ_1 receptors in psychosis currently remains controversial.

Anatomical studies have provided evidence of the role of σ receptors in the motor side effects of neuroleptics. Neuroleptic-induced motor side effects such as buccal, oral, lingual and facial movements (as in tardive dyskinesia), eye movements (as in oculogyric crises), and dystonia involve brain areas that are rich in σ receptors, such as hypoglossal, facial, motor trigeminal, oculomotor, abducens and trochlear cranial nerve nuclei, the substantia nigra pars compacta, cerebellum and red nucleus (Bouchard and Quirion, 1997; McLean and Weber, 1988; Gundlach et al., 1986a). Thus, it has been suggested that σ receptors may mediate the undesirable motor side effects of antipsychotic drugs (Walker et al. 1990). In early studies it was shown that some σ receptor ligands such as DTG, haloperidol and (+)-SKF-10,047 may elicit dystonic reactions when they were directly injected into the red nucleus, whereas antipsychotics such as clozapine and sulpiride, which have no σ_1 affinity, failed in induced any dystonic reaction (Matsumoto et al. 1990; Walker et al., 1988). Moreover, some σ_1

receptor antagonists such as BD 1047 or BD 1063 were reported to attenuate orofacial dyskinesias and dystonic reactions after microinjection of haloperidol or DTG into the facial nucleus, spinal trigeminal nucleus or red nucleus, suggesting that these drugs might prevent such undesired movements (Matsumoto et al. 1995; Tran et al. 1998). DTG and haloperidol have been reported to be σ_2 agonists, and BD 1047 and 1063 have been reported to be σ_1 antagonists (Table I), so there may be a connexion between σ_1 and σ_2 with regard to dystonic reactions. In fact, the neck dystonia induced by DTG was also prevented by the administration of the selective σ_2 antagonist SM-21 (Ghelardini et al. 2000). More recently it was found that the affinities of neuroleptics for σ (both σ_1 and σ_2) receptors correlated well with their risk for producing acute dystonic reactions (Matsumoto and Pouw, 2000).

3.5. Sigma$_1$ receptors and drugs of abuse

It has been reported that σ_1 receptors seem to be involved in the effects of several drugs of abuse. The involvement of σ_1 receptors in cocaine's actions has been extensively studied (reviewed in Maurice et al., 2002 and Matsumoto et al., 2003), but σ_1 receptors appear to underlie the effects of other drugs of abuse, such as methamphetamine (reviewed in Guitart et al., 2004; Stefanski et al., 2004), ethanol (Maurice et al., 2003; Meunier et al., 2006b) and nicotine (Horan et al., 2001).

3.5.1. Cocaine and σ_1 receptors

In 1988 Sharkey and co-workers described the interaction of cocaine with σ binding sites, reporting that the affinity of cocaine for σ_1 receptors was compatible with blood concentrations reported in human volunteers. It was therefore proposed that cocaine produced its effects, at least in part, through its interaction with σ_1 receptors (reviewed in Maurice et al., 2002; Matsumoto et al., 2003).

3.5.1.1. Modulation by σ_1 ligands of the acute effects of cocaine

Cocaine possesses psychomotor stimulant effects that contribute to its addiction potential. The ability of compounds to attenuate the acute locomotor effects of cocaine is often used as an initial screening tool to identify agents that have the ability to block the psychostimulant activity of this drug of abuse. There are many σ_1 antagonists that have been reported to attenuate the locomotor stimulatory effects of cocaine in rodents, such as haloperidol, BD 1008, BD 1047, BD 1063, BMY 14802, panamesine and rimcazole, among others (reviewed in Matsumoto et al., 2003). The ability of these compounds to attenuate the locomotor stimulatory effects of cocaine involves, at least in part, σ_1 receptors, because the administration of antisense oligodeoxynucleotides that knock down brain σ_1 receptors in mice also diminished motor activity (Matsumoto et al., 2002).

Convulsions represent a measure of cocaine toxicity, and the incidence of convulsions in individuals who abuse cocaine is significant. Cocaine-induced convulsions can result from exposure to large doses of cocaine, typically in an overdose

situation. The effects of σ receptor antagonists on cocaine-induced convulsions have been widely explored and reported. $Sigma_1$ antagonists such as BMY 14802 and haloperidol have been reported to significantly attenuate cocaine-induced convulsions in mice (Matsumoto et al., 2001b; Ushijima et al., 1998). In addition, the σ_1 antagonist analogues of BD 1008 (including the selective σ_1 antagonists BD 1063 and BD 1047) have been reported to attenuate cocaine-induced convulsions in mice (see Matsumoto et al., 2003 and Maurice et al., 2002 for reviews; Matsumoto et al., 2004; Daniels et al., 2006). Moreover, several analogues of rimcazole were also successful in attenuating cocaine-induced convulsions (Katz et al., 2003; Matsumoto et al., 2001c), and the ability of these compounds to prevent cocaine-induced convulsions correlated with their affinities for σ receptors rather than for dopamine transporters (Matsumoto et al., 2001c). Several new putative σ antagonists were also tested in this behavioural model, and the results showed that both preferential σ_1 ligands and preferential σ_2 ligands were able to attenuate cocaine-induced convulsions. These findings suggest that both subtypes can be targeted to attenuate the convulsive effects of cocaine (reviewed by Matsumoto et al., 2003). In addition to the pharmacological approach, the blockade of σ_1 receptor synthesis with specific oligodeoxynucleotides was also tested; these findings showed that a reduction of about 40% in σ_1 receptors in the brain attenuated the convulsive effects of cocaine (Matsumoto et al., 2001a and 2002).

The role of σ receptors on lethality induced by cocaine has also been studied. Pre-treatment with some σ_1 ligands (including the selective σ_1 antagonists BD 1047 and BD 1063, among others) has been reported to attenuate cocaine-induced lethality (Matsumoto et al., 2001a and b, 2002; McCracken et al., 1999a; Matsumoto et al., 2004; Daniels et al., 2006). These experiments demonstrated that blocking the access of

cocaine to σ receptors reduced its toxic effects. In addition, the post-treatment of mice with other σ receptor antagonists (LR132, YZ-011) after cocaine administration significantly attenuated cocaine-induced lethality after an overdose (Matsumoto et al., 2001a, 2002). The ability of σ receptor antagonists to prevent death after an overdose of cocaine in animals suggest a clinical application potentially worth further study.

In addition, the administration to mice of the putative σ_2 antagonist DTG, the putative σ_1 agonists BD1031 and BD1052, or the selective σ_1 agonist SA4503 exacerbated the locomotor stimulatory actions, and the toxic effects (measured as convulsions and lethality rate) of cocaine (Matsumoto et al., 2001a and b, 2002; McCracken et al.,1999; Skuza, 1999).

3.5.1.2. Effects of σ_1 ligands after repeated administration of cocaine

Repeated administration of cocaine to animals can result in behavioural sensitization or reverse tolerance if the animals develop an enhanced response to a given dose of cocaine. This phenomenon serves as a measurable index of nervous system plasticity that results upon repeated exposure to cocaine. Several σ ligands (such as BMY 14802, NPC 16377, rimcazole and SR 31742A) significantly attenuated the development of cocaine-induced locomotor sensitization (Ujike et al., 1996; Witkin et al., 1993). Early experiments with σ_1 agonists showed that 3-(+)-PPP administered in the abstinence period for cocaine enhanced stereotypy in rats (Ujike et al., 1992a). These enhanced response was attenuated by the σ antagonist BMY 14802 and also by the D_2 antagonist (±)-sulpiride, which suggested a relationship between the dopaminergic and σ systems in the response to cocaine (Ujike et al., 1992a).

Studies with the conditioned place preference paradigm, which is used to evaluate the rewarding properties of drugs of abuse after their repeated administration, showed that the selective σ_1 receptor antagonists BD 1047 and NE-100 reduced the rewarding properties of cocaine in this experimental model (Romieu et al., 2000). Moreover, the treatment of animals with a specific σ_1 antisense oligodeoxynucleotide attenuated cocaine-induced conditioned place preference (Romieu et al., 2000). Furthermore, σ_1 antagonists were effective in reducing not only the development, but also the expression of cocaine-induced conditioned place preference. Thus, σ_1 receptor antagonists appear to be able to prevent alterations that occur in response to the repeated administration of cocaine, and to reverse the functional consequences of these changes once they have occurred (Romieu et al., 2002). In addition to the beneficial effects of σ_1 antagonists on cocaine-induced effects, σ_1 receptor agonists such as JO-1784 or PRE 084, like the putative σ_1 agonists PREGS and DHEAS, potentiated cocaine rewarding properties measured as cocaine-induced conditioned place preference acquisition, and this effect was blocked by the selective σ_1 antagonist BD 1047 (Romieu et al., 2002).

Recent experiments disclosed the role of σ_1 receptors in the cocaine-induced enhancement of human immunodeficiency virus (HIV) infection (Roth et al., 2005; Gekker et al., 2006). Cocaine is associated with an increased risk for, and progression of, clinical disease associated with HIV infection. Studies with a human xenograft model, in which human peripheral blood mononuclear cells were implanted into severe combined immunodeficient mice (huPBL-SCID) which were thn infected with a HIV reporter virus, showed that the systemic administration of cocaine increased the percentage of HIV-infected mice two- or three-fold, and increased the viral load 100- to 300-fold. A selective σ_1 antagonist, BD 1047, blocked the effects of cocaine on HIV

replication. So it seems that systemic exposure to cocaine can enhance HIV infection in vivo, at least in part, by activating σ_1 receptors (Roth et al., 2005). Moreover, the cocaine-mediated stimulation of HIV expression in microglial cells was blocked by treatment of microglia with inhibitors of TGF-β-1, and also with the σ_1 antagonist BD 1047 (Gekker et al., 2006).

In contradistinction to the noxious effects of σ_1 agonists on the rewarding properties and toxic effects of cocaine, σ_1 agonists showed a beneficial role in the *in utero* cocaine-induced memory alterations in offspring rats. Repeated cocaine exposure *in utero* resulted in behavioural alterations that particularly affected learning and memory processes in juvenile offspring rats. Activation of the σ_1 receptors with the selective agonist JO-1784 or the putative σ_1 endogenous ligand DHEA allowed complete behavioural recovery of the memory functions in rats exposed to cocaine prenatally, which was reversed by the σ_1 antagonist BD 1063 (Meunier and Maurice, 2004).

3.5.2. Other drugs of abuse and σ_1 receptors

Some actions of other drugs of abuse also appear to be linked with σ_1 receptors. As described above in section 5.4 *Schizophrenia and σ_1 receptors*, several σ_1 ligands generally considered antagonists, such as BMY 14802, E-5842, DTG, SR 31742A, MS-377, BD 1063 or BD 1047, and σ_1 antisense oligodeoxynucleotides, attenuated or reversed several *in vitro* and *in vivo* actions of methamphetamine, such as the enhancement of locomotor activity, the development of methamphetamine-induced behavioural sensitisation, and the amphetamine-induced decline in DOPAC in the

neostriatum (Taylor et al. 1993; Ujike et al. 1992b; Guitart et al., 1998; Ruckert and Schmidt, 1993; Poncelet et al., 1993; Takahasi et al., 2000; Nguyen et al., 2005).

The links between the actions of σ_1 receptors and ethanol addiction have also been studied (Maurice et al., 2003; Meunier et al., 2006). Administration of ethanol dose-dependently induced locomotor stimulation, and conditioned place preference and taste aversion. All effects evaluated were reverted by the σ_1 receptor antagonist BD 1047 (Maurice et al., 2003). By contrast, pre-treatment with the selective σ_1 receptor agonist PRE 084 before ethanol failed to affect the resulting locomotor stimulation, but dose-dependently enhanced the conditioned place preference (Maurice et al., 2003). The effect of σ_1 ligands was also tested in the abstinence syndrome after chronic ethanol consumption (Meunier et al., 2006). Animals treated with chronic ethanol consumption, upon withdrawal, showed increased locomotion, anxiety and object exploration, which impeded correct reactions to object habituation, spatial change or novelty. Both the σ_1 agonist JO-1784 and the antagonist BD 1047 decreased hyper-responsiveness and restored habituation. However, correct reactions to spatial change and novelty were only produced by JO-1784 treatment (Meunier et al., 2006). These observations suggest a new pharmacological target for alleviating ethanol addiction and abstinence syndrome after withdrawal.

In addition, an association has been suggested between polymorphisms in the σ_1 receptor gene and alcoholism (Miyatake et al., 2004). The role of σ_1 receptors in the responses to other drugs of abuse such as nicotine were also studied, and it was found that pre-treatment with the selective σ_1 agonist SA4503 significantly attenuated the conditioned place preference response to(–)-nicotine (Horan et al., 2001).

3.5.3. Stimulant drugs of abuse and σ_1 receptor plasticity

3.5.3.1. Cocaine and neuro-adaptive changes in σ_1 receptors

Cocaine is generally thought to act as a dopamine re-uptake inhibitor to produce its reinforcing effects (Kuhar et al., 1991), although other mechanisms such as σ_1 receptors might also be important, as described above. The affinity of cocaine for σ receptors is in the μM range (Sharkey et al., 1988; Matsumoto et al., 2001c, 2002; Ritz and George, 1993; Ramamoorthy et al., 1995) which is only about 3-10 fold higher than the concentration needed to inhibit its main pharmacological target, the DAT (Ritz and George, 1993; Rothman and Baumann, 2003). In addition, cocaine binds preferentially to σ_1 receptors rather than to σ_2, with a 10-higher affinity for the σ_1 subtype (Matsumoto et al., 2002). Despite the relatively low affinity of cocaine for σ_1 receptors, this drug has been shown to achieve micromolar concentrations in the body (Mittleman and Wetli, 1984; Spiehler and Reed, 1985), suggesting that its affinity for σ_1 receptors may be clinically relevant.

In addition, repeated treatment with cocaine produced up-regulation of σ_1 receptors detectable in whole mouse brain (Liu et al., 2005). This up-regulation was not produced in the cerebellum (Romieu et al., 2002 and 2004; Liu et al., 2005), but was seen in the cortex, olfactory bulb, hippocampus, hypothalamus and striatum (Romieu et al., 2004; Liu et al., 2005). Of particular interest concerning the drug reward mechanism is σ_1 receptor up-regulation in the nucleus accumbens (Zhang et al., 2005; Romieu et al., 2002). The up-regulation of σ_1 receptors after repetitive treatment with cocaine was not produced in dopamine D_1 receptor knockout mice (Zhang et al., 2005), and was also

blocked by treatment with the selective σ_1 antagonist BD 1063 (Liu et al., 2005). These findings suggest that the interaction of cocaine with both D_1 and σ_1 receptors is necessary for the up-regulation of σ_1 receptors. Interestingly, when pregnant rats period were repeatedly given cocaine, male offspring 5 weeks after birth displayed decreased D_2 receptor binding in the nucleus accumbens and increased D_3 and σ receptor binding (labelled with [^{3}H]DTG, which does not differentiate between σ_1 and σ_2 receptors) in the nucleus accumbens and striatum. By contrast, female offspring displayed no differences in receptor binding in either region, so these alterations in dopamine and σ receptor binding are sex-specific and may have effects on the development of behaviour (Silvers et al., 2006).

The actions of psychostimulants such as cocaine in animals or humans involve short-lived, long-term and life-long effects. The short-lived effects of psychostimulants (such as cocaine) include immediate euphoria, whereas the long-term and life-long effects of psychostimulants encompass the craving for and relapse to consumption of the psychostimulant even after a prolonged period of withdrawal from psychostimulant intake (Su and Hayashi, 2001). It was proposed that cocaine might cause life-long alterations in neurones by interacting with both D_1 receptors and σ_1 receptors (Su and Hayashi, 2001; Liu et al., 2005). As described by Hayashi and Su (2001), σ_1 receptors are coupled to $InsP_3$ receptors and to the cytoskeletal protein ANK220 on the endoplasmic reticulum as a trimeric complex, After activation of σ_1 receptors by agonists such as the selective σ_1 agonist (+)-pentazocine, or even cocaine, σ_1 receptors dissociate as a σ_1 receptor–ANK220 complex from the $InsP_3$ receptors on the endoplasmic reticulum (Hayashi and Su, 2003a). The σ_1 receptor–ANK220 complex translocates to other cellular structures, including the nucleus and plasma membrane

(Hayashi et al., 2003a), as explained in section 2.2.1 *Modulation of phospholipase C-protein kinase C and $InsP_3$-Ca^{2+} release system by σ_1 receptors*. Thus, because cytoskeletal proteins are important for the structural organization and activities of proteins in altered neurones, and because cocaine can affect the dynamics of cytoskeletal proteins, it was suggested that cocaine might exert its life-long effects by causing structural alteration of cells at least in part via its interaction with σ_1 receptors (see Su and Hayashi, 2001 for a more comprehensive report).

3.5.3.2. Methamphetamine and neuro-adaptive changes in σ_1 receptors

Methamphetamine, like cocaine, also binds to σ receptors in the μM range, and with a 20-fold higher affinity for σ_1 than for σ_2 receptors (Nguyen et al., 2005). In addition, is known that the repeated exposure to metamphetamine (4 mg/kg/day) for 10 days induces up-regulation of σ_1 receptors in rats in the substantia nigra, frontal cortex and cerebellum, suggesting that methamphetamine-induced behaviour may be associated with the up-regulation of σ receptors in critical brain regions (Itzhak, 1993 and 1994). However, no association between σ_1 receptor gene polymorphisms and methamphetamine abuse was found (Inada et al., 2004). In a recent study, Stefanski and co-workers (2004) found that σ_1 receptors were up-regulated in the rat midbrain, an area involved in learning and reward processes, but not in the cerebellum, frontal cortex, striatum and hippocampus of rats in a methamphetamine self-administration experiment (average daily dose of 1.3 mg/kg for 25 days). By contrast, in rats which passively received methamphetamine (yoked controls) no differences in comparison to untreated controls were found in any brain area tested. The differences between the Stefanski's

and Itzhak's studies were thought to be due to differences in the experimental protocols. Another interesting finding by Stefanski and co-workers (2004) was that levels of σ_1 receptor mRNA were altered in rats which actively self-administered methamphetamine. Levels of mRNA were significantly reduced in the frontal cortex and increased in the hippocampus, and were not modified in midbrain, whereas levels of σ_1 receptor protein were not modified in the frontal cortex and hippocampus were increased in midbrain, suggesting a lower turnover rate of σ_1 receptor proteins in the midbrain and frontal cortex. Conversely, there appeared to be a higher turnover rate of σ_1 receptors in the hippocampus. Unfortunately, the biochemical turnover parameters of σ_1 receptor have not yet been investigated.

HYPOTHESIS AND GOALS

Although the actions of σ_1 receptor were first reported in 1976 (Martin et al., 1976), several aspects of σ_1 receptor pharmacology remain unclear. This Doctoral Thesis was undertaken to deepen our knowledge of two characteristics of σ_1 receptors that are not shared by the σ_2 subtype: their allosteric modulation by phenytoin (DPH) and the reduction in radioligand binding induced by the acute *in vivo* administration of haloperidol.

It has been conventionally assumed that a characteristic difference between σ_1 and σ_2 receptors is that the binding of σ_1 ligands is allosterically enhanced by DPH, whereas this anticonvulsant drug does not modulate σ_2 binding (see Walker et al., 1990; Quirion et al., 1992; and Maurice et al., 2001 for reviews). However, close analysis of the data obtained in this research suggests that DPH not only discriminates between σ_1 and σ_2 ligands, but also distinguishes between different σ_1 ligands. In fact, DPH increases binding of the σ_1 ligands [^{3}H]dextromethorphan, [^{3}H](+)-SKF-10,047, [^{3}H](+)-3-PPP and [^{3}H](+)-pentazocine (Musacchio et al., 1987, 1988, 1989a and b; Craviso and Musacchio, 1983; Karbon et al., 1991; Bailey and Karbon, 1993; McCann and Su, 1991; Culp et al., 1992; Rothman et al., 1991; Bonhaus et al., 1993; Chaki et al., 1996; DeHaven-Hudkins et al., 1993), but not binding of the σ_1 radioligands [^{3}H]haloperidol, [^{3}H]progesterone, [^{3}H]DTG, [^{3}H]DuP 734, [^{3}H]RS-23597-190 and [^{3}H]NE-100 (Karbon et al., 1991; Meyer et al., 1998; Culp et al., 1992; Bonhaus et al., 1994; Tanaka et al., 1995; Chaki et al., 1996).

DeHaven-Hudkins and co-workers (1993) proposed that the differential sensitivity of σ_1 compounds to allosteric modulation by phenytoin might be restricted to compounds that bind selectively to σ_1 binding sites. However, this hypothesis is not

entirely consistent with current knowledge of the selectivity patterns of these ligands. For instance, (+)-3-PPP binding is modulated by DPH but is not a selective σ_1 ligand, given that its affinity is only around 1.5-fold higher for σ_1 than for σ_2 receptors (Bowen et al., 1993b), whereas NE-100 and DuP 734 are known selective σ_1 ligands (see Table I, page 18), but their binding is not enhanced by DPH (Tanaka et al., 1995; Chaki et al., 1996; Culp et al., 1992). An alternative explanation based on chemical structure is not supported by evidence, because the ligands modulated by DPH do not share a common chemical structure. For example, some piperidines such as (+)-3-PPP are modulated by DPH, whereas others such as DuP 734 or RS-23597-190 are not. Therefore earlier hypotheses do not fully explain the differential modulation of σ_1 ligand binding by DPH.

Bearing in mind that the radioligands modulated by DPH are typically considered σ_1 agonists, whereas the radioligands not modulated by DPH are generally considered σ_1 antagonists (see Maurice et al., 2001a and Hayashi and Su, 2004a for reviews), we hypothesized that DPH differentially modulates the binding of σ_1 ligands depending on whether they act as agonists or antagonists of σ_1 receptors. The **first goal** of this Doctoral Thesis was therefore to test whether DPH is able to differentially modulate the binding of several σ_1 ligands depending on their agonistic or antagonistic activities on σ_1 receptors.

To test this hypothesis we performed σ_1 radioligand binding assays in guinea pig brain crude synaptosomal fractions with two different approaches. (1) Radioligand competition assays were performed in the presence or absence of DPH, labelling σ_1 receptors with the selective σ_1 ligand [^{3}H](+)-pentazocine to test for possible

DPH-induced changes in affinity of several known σ_1 cold agonists and antagonists for σ_1 receptors. (2) Equilibrium and kinetic radioligand binding assays were performed to compare the modulation by DPH of the binding of two selective σ_1 radioligands: the prototypical σ_1 agonist [^{3}H](+)-pentazocine and the prototypical σ_1 antagonist [^{3}H]NE-100.

Some reports showed that the repeated administration of haloperidol to rats or humans decreased the number of σ binding sites (Jansen et al., 1992; Kizu et al., 1991; Reynolds et al., 1991). It was suggested that these results were due to the down-regulation of σ receptors (Itzhak and Stein, 1991; Kizu et al., 1991; Reynolds et al., 1991). Later studies showed that the repeated administration of haloperidol only affected the binding of σ_1 radioligands, whereas σ_2 radioligand binding was not modified in rat brain membranes (Nakata et al., 1999; Inoue et al., 2000; Bailey and Karbon, 1993; Itzhak and Stein, 1991). However, the levels of brain σ_1 receptor mRNA were not altered by the repeated administration of haloperidol (Nakata et al., 1999; Inoue et al., 2000). This latter finding ruled out the σ_1 receptor down-regulation hypothesis.

It has been reported that a single administration of haloperidol to guinea pigs produced a long-lasting decrease in the number of brain σ_1 binding sites (Klein et al., 1994; Inoue et al., 2000). This effect was hypothesized to be due to the irreversible inactivation of σ_1 receptors induced by the administration of haloperidol (Klein et al, 1994; Inoue et al., 2000). Thus the irreversible inactivation of σ_1 receptors by haloperidol makes it possible to evaluate the turnover of these receptors. $Sigma_1$ receptors are located in microsomal, nuclear and synaptic membranes (Itzhak et al.,

1991; Cagnotto et al., 1994; DeHaven-Hudkins et al., 1994; Alonso et al., 2000). The biochemical turnover parameters of σ_1 receptors in the different subcellular membranes and the redistribution processes of these receptors have not previously been investigated, so the **second goal** of this Doctoral Thesis was to study the turnover of σ_1 receptors in guinea pig brain subcellular (nuclear, microsomal and synaptosomal) membranes. To this end we performed single *in vivo* injections of haloperidol and killed the animals after different times to study the time-course of recovery of [^{3}H](+)-pentazocine binding in three different membrane preparations: crude nuclear, crude synaptosomal and microsomal membranes.

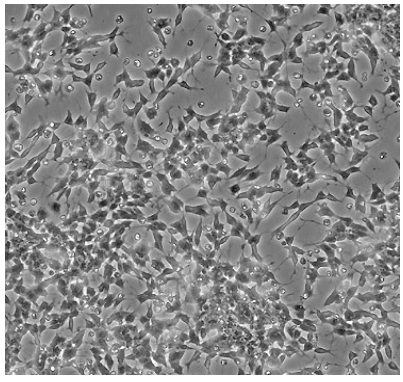
It is unknown whether the inactivation of σ_1 receptors induced by a single administration of haloperidol is shared by other σ_1 receptor antagonists, or whether it is due to this drug's D_2 antagonist activity. Accordingly, the **third goal** of this Doctoral Thesis was to test whether the irreversible inhibition of σ_1 receptors is produced by a single *in vivo* administration of prototypic σ_1 receptor antagonists or by other D_2 antagonists. For these experiments we acutely administered several known σ_1 antagonists (BD 1047, BD 1063, NE-100 and haloperidol) and the known dopaminergic antagonist (–)-sulpiride to guinea pigs, and observed the changes in binding of the selective σ_1 receptor ligand [^{3}H](+)-pentazocine in crude nuclear, crude synaptosomal and microsomal guinea pig brain membranes.

Klein and co-workers (1994) reported that, as seen after haloperidol administration, a single injection of reduced haloperidol to guinea pigs also markedly decreased the number of brain σ_1 binding sites, so haloperidol-induced σ_1 receptor inactivation could be produced by the parent compound or by one or more of its

metabolites. Interestingly, guinea pigs but not rats showed evidence of an effect on haloperidol metabolism similar to that seen in humans (Korpi et al., 1985). In the light of these antecedents, the **fourth goal** of this Doctoral Thesis was to investigate whether the irreversible blockade of σ_1 receptors is produced by haloperidol or by one of its metabolites *in vitro* in guinea pig brain and also in a model of human neuronal cells (SH-SY5Y human neuroblastoma cells).

In this final set of experiments we tested the ability of haloperidol and its commercially available metabolites to produce wash-resistant inhibition of σ_1 receptors labelled with the selective σ_1 ligand [^{3}H](+)-pentazocine under different experimental conditions: (1) in guinea pig brain synaptosomal membranes, which have limited metabolic activity, (2) in guinea pig brain homogenates, which retain all enzymatic systems that metabolize haloperidol, and (3) in whole SH-SY5Y human neuroblastoma cells, used as a metabolically active model of human neuronal cells.

Guinea pig



SH-SY5Y human neuroblastoma cells

PUBLISHED PAPERS

1. Phenytoin differentially modulates the affinity of agonist and antagonist ligands for σ_1 receptors of guinea pig brain

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SYNAPSE 55: 192-195 (2005)

Running title: Modulation by phenytoin of sigma$_1$ ligand affinity.

1.1. ABSTRACT

We evaluated the effects of phenytoin (DPH) on the affinity for sigma$_1$ (σ_1) receptors of agonist or antagonist σ_1 ligands in guinea pig brain. Heterologous competition experiments showed that DPH (250 μM and 1 mM) concentration-dependently increased the affinity of the σ_1 agonists dextromethorphan, (+)-SKF-10,047, (+)-3-PPP and PRE 084. However, neither DPH 250 μM nor 1 mM increased (in fact, they slightly decreased) the affinity of the σ_1 receptor antagonists haloperidol, BD 1063, NE-100, progesterone and BD 1047. These findings suggest that allosteric modulation by DPH of the affinity of σ_1 receptor ligands depends on the agonist or antagonist characteristics of the ligand. Therefore, determining in vitro the differential modulation by DPH of σ_1 ligand affinity appears to constitute a procedure that can predict the pharmacological profile of different σ_1 ligands.

1.2. INTRODUCTION

Sigma receptors, now considered to be unique receptors different from opiate and phencyclidine binding sites, exist in two distinct entities denoted σ_1 and σ_2 (Walker et al., 1990; Quirion et al., 1992; Maurice et al., 2001a). From a pharmacological point of view, σ_1 and σ_2 receptors differ in their stereoselectivity for benzomorphans, with the drugs (+)-pentazocine and (+)-SKF-10,047 showing higher affinity for σ_1 binding sites (Quirion et al., 1992; Maurice et al., 2002).

It is conventionally assumed that σ_1 and σ_2 binding sites also differ in that the binding of σ_1 ligands is allosterically enhanced by phenytoin, whereas this drug does not

enhance σ_2 binding (for reviews see Walker et al., 1990; Quirion et al., 1992; Maurice et al., 2001a). However, a careful review of earlier studies reveals inconsistencies in the data, since some studies failed to show any increase by DPH in the binding of the preferential σ_1 ligands [^{3}H]NE-100 (Tanaka et al., 1995) and [^{3}H]progesterone (Meyer et al., 1998). These studies may mean that not all σ_1 ligands share the same characteristics in terms of their modulation by DPH, and suggest that DPH discriminates not only between σ_1 and σ_2 ligands but also between different σ_1 ligands. To search for an explanation for these unclear findings, we hypothesized that DPH might differentially modulate the binding of σ_1 ligands depending on their agonist or antagonist action on σ_1 receptors. To test this hypothesis we performed binding competitions assays to compare the effects of DPH on the affinity of several known agonist and antagonist ligands for σ_1 receptors labeled with [^{3}H](+)-pentazocine.

1.3. MATERIALS AND METHODS

Adult (8-weeks-old) male Dunkin Hartley guinea pigs were handled in accordance with the ethical principles of the European Communities Council Directive (86/609/ECC). Binding experiments were carried out in the crude synaptosome fraction (or P_2 fraction) obtained from brain with a method described previously (González et al., 2001), with slight modifications (DeHaven-Hudkins et al., 1992). In brief, 440 µl membrane preparation suspended in incubation buffer (50 mM HCl Tris, pH 7.44) was incubated with 20 µl [^{3}H](+)-pentazocine (final concentration of 0.5 nM in competition assays, and 0.40 to 48 nM in saturation experiments), 20 µl cold ligand or its solvent, and 20 µl DPH or its solvent (0.3 M NaOH) for 150 min at 37°C. The final volume was

0.5 ml, the final protein concentration was 0.43-0.46 mg/ml (which was within the linear range of the relation of specific binding and protein concentration of the tissue, data not shown) and the final pH in the incubation medium was 7.44. Nonspecific binding was defined with 1 μM of haloperidol, except in the competition assays, where different concentrations of cold ligand were used. Incubations were stopped with 5 ml of cold (4°C) filtration buffer (Tris 10 mM, pH 7.4). The bound and free radioligand were separated by rapid filtration under a vacuum with a Brandel cell harvester (Brandel Instruments, SEMAT Technical Ltd., UK) over Whatman GF/B glass fiber filters (presoaked for at least 1 h with 0.5% polyethylenimine). Radioactivity was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A) with an efficiency of 52%. Each experiment was repeated at least three times, and each assay was conducted in triplicate.

The radioligand used in the assays was [^{3}H](+)-pentazocine (PerkinElmer Life Sciences, Boston, 34 Ci/mmol). Dilutions from the stock solution were prepared with incubation buffer. The cold ligands used were (+)-pentazocine, (+)-SKF-10,047 hydrochloride, (+)-3-PPP hydrochloride, dextromethorphan hydrobromide, DTG, haloperidol, progesterone (all from Sigma-Aldrich Química S.A.); PRE 084 hydrochloride, BD 1063 dihydrochloride, BD 1047 dihydrobromide (Tocris Cookson Ltd.) and NE-100 chlorhydrate (synthesized as described previously by Nakazato et al., 1999a). Haloperidol, (+)-pentazocine, DTG and progesterone were dissolved in absolute ethanol to make up a 1-mM or 10-mM solution, depending on the final concentration of drug to be used for competition experiments. The maximum final concentration of ethanol in the incubation medium was 1% (vol/vol), which had no effect on binding. The rest of the unlabeled drugs were dissolved in deionized water. We also used

phenytoin (DPH; Sigma-Aldrich Química SA) dissolved in NaOH 0.3 M to make a 25-mM solution. In all cases, further dilutions were prepared with incubation buffer. The final solvent and pH of the incubation medium in all the assay tubes was NaOH 12 mM, with pH 7.44 at 37°C.

Data were analyzed with the SigmaPlot 2002 v. 7.0 program (SPSS Inc., Il, USA). The IC_{50} values (concentration of unlabeled drug that inhibited 50% of binding) were estimated from the inhibition curves using nonlinear regression analysis of the equation for a sigmoid plot, and the K_i values for the unlabeled ligands were calculated with the Cheng-Prussoff equation. Statistical analysis consisted of one-way analysis of variance (ANOVA) followed by Dunnett´s test when multiple comparisons against a single control were performed. Differences were considered significant when $P < 0.05$.

1.4. RESULTS

Saturation experiments performed under control conditions showed that [^{3}H](+)-pentazocine binds in a saturable manner to only one population of specific binding sites. Data fitted by nonlinear regression analysis to a hyperbolic equation ($r^2 = 0.99$) yielded an equilibrium dissociation constant (K_D) of 2.45 ± 0.22 nM and a maximal number of receptors (B_{max}) of 1.48 ± 0.03 pmol [^{3}H](+)-pentazocine/mg protein (data not shown). The specific binding of [^{3}H](+)-pentazocine (which always represented more than 95% of the total binding) was concentration-dependently inhibited by the unlabeled σ_1 ligands tested, with the following order of potency: haloperidol (K_i = 1.03 ± 0.04 nM) > (+)-pentazocine (K_i = 2.56 ± 0.10 nM) >BD 1047 (K_i = 5.30 ± 0.26 nM) > NE 100 (K_i = 12.69 ± 2.63 nM) > BD 1063 (K_i = 16.37 ±

0.63 nM) > DTG (K_i = 64.32 ± 3.42 nM) > (+)-3-PPP (K_i = 74.97 ± 3.68 nM) > (+)-SKF-10,047 (K_i = 149.88 ± 10.14 nM) ≈ PRE 084 (K_i = 150.85 ± 14.77 nM) > dextromethorphan (K_i = 227.43 ± 9.84 nM) > progesterone (K_i = 1440.64 ± 92.96 nM).

The incubation with DPH 250 μM or 1 mM enhanced, in a concentration-dependent way, the affinity for σ_1 receptor of the ligands dextromethorphan, (+)-SKF-10,047, (+)-3-PPP and PRE 084, displacing the inhibition curves to the left and significantly decreasing ($P < 0.01$) their IC_{50} and K_i values (Fig. 1A and Fig. 2). Phenytoin 1 mM slightly decreased the K_i value of (+)-pentazocine. The ratios of K_i values in the presence of DPH solvent to values in the presence of DPH 1 mM were higher than unity in all cases (Fig. 2). In contrast, DPH at 250 μM and at 1 mM failed to increase the affinity for the σ_1 receptor of the ligands haloperidol, NE-100, BD 1063, BD 1047 and progesterone, nor did it increase that of DTG (Fig. 1B and Fig. 2). Phenytoin 1 mM slightly decreased the affinity of these ligands for σ_1 receptors, displacing the inhibition curves to the right and slightly increasing IC_{50} and K_i values; therefore, the ratios of K_i from control competition assays to K_i from competition curves in the presence of DPH 1 mM were always lower than unity (Fig. 1B and Fig. 2). Hill analysis of all competition curves yielded straight lines (r^2 = 0.97 - 0.99) with slopes or pseudo-Hill coefficients (n'_H) close to unity for all the ligands, both in the absence and in the presence of DPH. This confirms the existence of a single population of binding sites whose n'_H values did not change in the presence of DPH. The only exception was NE-100, whose n'_H values were around 1.73 both in the absence and in the presence of DPH.

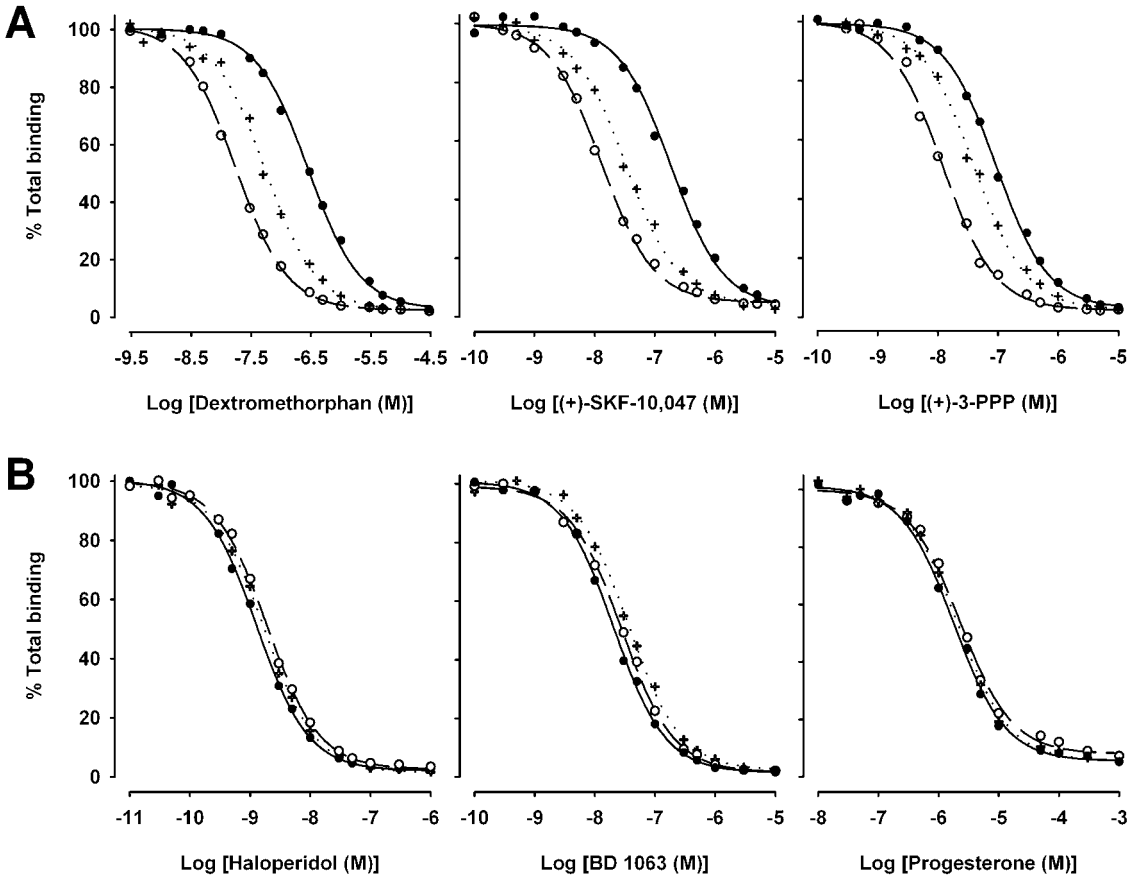

Figure 1. Inhibition by unlabeled σ_1 ligands of [^{3}H](+)-pentazocine binding to synaptosome crude fraction obtained from guinea pig brain. [^{3}H](+)-pentazocine (0.5 nM) was incubated with 0.46 mg/ml membrane protein and increasing concentrations of ligands in the presence of DPH solvent (●), DPH 250 µM (**+**) or DPH 1 mM (○), for 150 min at 37°C. (A) Effects of the σ_1 agonists dextromethorphan, (+)-SKF-10,047 and (+)-3-PPP. (B) Effects of the σ_1 antagonists haloperidol, BD 1063 and progesterone. Data shown are representative of at least three experiments done in triplicate. Standard errors of each data point did not exceed the 5% of the mean. Protein concentrations were measured by the Lowry method with some modifications, using bovine serum albumin as the standard.

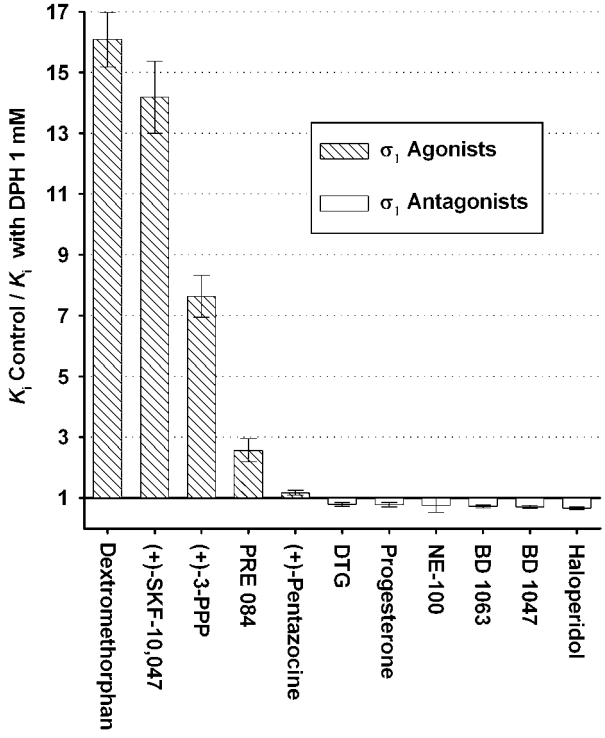

Figure 2. Ratios of the K_i value for the inhibitor drug in the absence of DPH (control) to the value obtained in the presence of DPH 1 mM, for several σ_1 ligands. The values of K_i were obtained with the Cheng-Prusoff equation from IC_{50} values from competition experiments.

1.5. DISCUSSION

In this study we show that DPH enhances the affinity for σ_1 receptors of the ligands (+)-3-PPP, dextromethorphan, (+)-SKF-10,047, PRE 084 and (+)-pentazocine which are all considered σ_1 agonists (Quirion et al., 1992; Matsuno et al., 1995a; Tokuyama et al., 1999; Maurice et al., 2001a; Brown et al., 2004). (+)-Pentazocine undergoes less modulation by DPH 1 mM than other agonists in these assays, which can be due to the fact that in homologous competition assays (experiments in which radioligand and unlabeled ligand are the same drug: [^{3}H](+)-pentazocine and (+)-pentazocine, in this case) the effect of a positive allosteric modulator can affect the affinity of both unlabeled and labeled ligands, counterbalancing the increased affinity of the unlabeled drug with the increased affinity of the labelled drug. On the other hand,

DPH did not increase, and even decreased, the affinity for σ_1 receptors of haloperidol, BD 1063, BD 1047, NE-100, DTG and progesterone. The characteristic these drugs share is their σ_1 antagonistic activity, which has been clearly demonstrated for haloperidol, BD 1063, BD 1047 and NE-100 (Matsumoto et al., 1995; Bergeron et al., 1996; Maurice et al., 2001a). The antagonist properties of DTG have not been consistently reported in the literature. Some studies identified DTG as an σ_1 agonist (Maurice et al., 2001a), whereas others reported σ_1 antagonist activity (Ault and Werling, 1997). The activity of progesterone reported in bioassay studies is mainly as σ_1 antagonist (Ueda et al., 2001a; Maurice et al., 2001a), albeit with exceptions (Nuwayhid and Werling 2003b). Our findings show that the effect of DPH on DTG and progesterone binding affinity is similar to its effect on the binding of some confirmed σ_1 antagonists.

The fact that DPH only enhances the binding of drugs with agonist activity on σ_1 receptors without increasing the binding of σ_1 receptor antagonists suggests an allosteric interaction, and rules out the possibility that DPH nonspecifically perturbs receptor conformation through (for example) effects on the surrounding lipid bilayer. Other authors have also found some drugs to selectively modulate agonist receptor binding without modifying the binding of antagonist ligands (Gao et al., 2002). This allosteric interaction can be explained on the basis of the extended two-state model of receptor activation (Hall, 2000). The conformational change in the receptor induced by the allosteric modulator (DPH) promotes the active state of the receptor, and this in turn increases the binding of a competitive agonist but not that of an antagonist. This is because the antagonist has the same affinity for both states (active and inactive) of the receptor, whereas the agonist has greater affinity for the active state (Hall, 2000).

Studies are currently underway with different experimental approaches to characterize the mechanisms involved in the differential modulation by DPH on the affinity of σ_1 receptor agonists and antagonists.

In conclusion, our data show that DPH increases the affinity for σ_1 receptor of agonist ligands. In contrast, it does not enhance, and may even decrease, the affinity of σ_1 antagonists. We therefore suggest that the use of heterologous competition binding assays to determine the differential modulation by DPH of sigma ligand affinity for σ_1 receptors provides a useful procedure to distinguish between σ_1 agonists and antagonists.

1.6. REFERENCES

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

2. Differences in the allosteric modulation by phenytoin of the binding properties of the σ_1 ligands [^{3}H](+)-pentazocine and [^{3}H]NE-100

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SYNAPSE 59: 152-161 (2006)

Running title: DPH differentially modulates sigma$_1$ ligand binding

2.1. ABSTRACT

The present study evaluated the effects of phenytoin (DPH) on the binding to synaptosomal fraction membranes from guinea pig brain of the prototypic sigma$_1$ (σ_1) receptor agonist [^{3}H](+)-pentazocine and the putative σ_1 antagonist [^{3}H]NE-100. Equilibrium and binding kinetics studies were done. The order of affinity of twelve σ_1 ligands for binding sites labelled with [^{3}H](+)-pentazocine correlated well with their order of affinity for sites labelled with [^{3}H]NE-100, suggesting that both radioligands label the same receptor. Phenytoin increased the binding of [^{3}H](+)-pentazocine, enhancing its affinity (K_D value) for σ_1 receptors and decreasing its dissociation rate from these receptors. The maximal number of receptors (B_{max} value) labelled with [^{3}H](+)-pentazocine was not changed. In contrast, phenytoin decreased the specific binding and maximal number of receptors labelled with [^{3}H]NE-100, and increased its dissociation rate from σ_1 receptors. The affinity of this radioligand for σ_1 receptors was not modified. In conclusion, phenytoin behaved as a positive allosteric modulator on the binding of [^{3}H](+)-pentazocine, whereas it negatively modulated the binding of [^{3}H]NE-100. These results add evidence in favour of the use of phenytoin *in vitro* to distinguish between agonists and antagonists of σ_1 receptors.

2.2. INTRODUCTION

Sigma receptors were first considered a subtype of opioid receptor, and later confused with the high-affinity phencyclidine binding sites on *N*-methyl-D-aspartate (NMDA) receptors. However, they are now defined as non-opioid, non-phencyclidine

sites, and are considered unique binding sites with high affinity for haloperidol, benzomorphans, some neurosteroids, and other drugs (Maurice et al., 2001; Quirion et al., 1992; Walker et al., 1990). The two distinct entities of sigma receptor are designated σ_1 and σ_2 (Quirion et al., 1992). To date only the σ_1 receptor has been cloned, firstly in guinea pig liver (Hanner et al., 1996), and later in other tissues including the rat and mouse brain (Pan et al., 1998b; Seth et al., 1998). The sequences of the different purified proteins are highly homologous, but different from any other mammalian proteins, indicating that σ_1 receptors constitute a distinct entity from any other known receptor (Maurice et al., 2001a; Maurice et al., 2002, for reviews).

Although the σ_1 receptors exist in peripheral organs, they are expressed most intensely in the central nervous system where they are ubiquitous and play a role in several neuronal processes. These receptors modulate ion channel activities at the plasma membrane, neuronal firing, and the release of several neurotransmitters. They also modify the postsynaptic responses to neurotransmitters by regulating intracellular calcium mobilization. Moreover, they have been reported to play a role in the mobilization of cytoskeletal adaptor proteins and intracellular lipid distribution (Guitart et al., 2004; Matsumoto et al., 2003; Maurice et al., 2001; Su and Hayashi, 2003, for reviews). The involvement of these receptors in the pathophysiology of certain psychiatric and neurological disorders has been postulated, and the possible therapeutic implications of σ_1 ligands is being investigated.The σ_1 receptor antagonists are able to antagonize not only the acute effects of cocaine but also the rewarding properties of this and other drugs of abuse (Matsumoto et al., 2003; Mauraice et al., 2001). The antagonists of σ_1 receptor also play a beneficial role in several models of nociception (Cendán et al., 2005; Mei and Pasternak, 2002), and they have been identified as

antipsychotic drugs based on classical neuropharmacological models of schizophrenia (Guitart et al., 2004). On the other hand, the σ_1 receptor agonists have beneficial effects in several models of amnesia, and they have also shown antidepressant-like activity in preclinical animal models (Guitart et al., 2004; Maurice et al., 2001a).

The main pharmacological characteristics of the σ_1 receptors are (1) their stereoselectivity for benzomorphans, with dextroisomers (such as (+)-pentazocine and (+)-SKF-10,047) showing higher affinity for σ_1 than σ_2 binding sites (Maurice et al., 2002; Quirion et al., 1992), and (2) the classical assumption that the binding of σ_1 ligands is allosterically enhanced by phenytoin, whereas this drug does not increase σ_2 binding (for reviews see Maurice et al., 2001a; Quirion et al., 1992 and Walker et al., 1990). This idea was based on the fact that phenytoin (DPH) increased the binding of the preferential σ_1 radioligands [^{3}H]dextromethorphan (Musacchio et al., 1988; Musacchio et al., 1989b), [^{3}H](+)-SKF-10,047 (Karbon et al., 1991; McCann and Su, 1991) and [^{3}H](+)-pentazocine (DeHaven-Hudkins et al., 1993), whereas it did not enhance the binding of [^{3}H]DTG, which has slightly more affinity for σ_2 than σ_1 receptors (Bailey and Karbon, 1993; Karbon et al., 1991).

Recent work by our group has shown that DPH not only distinguishes between σ_1 and σ_2 ligands but also differentially modulates the affinity of several unlabelled σ_1 ligands in guinea pig brain. The affinity of some σ_1 ligands (dextromethorphan, (+)-SKF-10,047, (+)-3-PPP, PRE 084, (+)-pentazocine) for σ_1 receptors labelled with [^{3}H](+)-pentazocine increased in the presence of DPH; however, the affinity of others (haloperidol, BD 1047, BD 1063, NE-100, progesterone) did not (Cobos et al., 2005). The clue seems to lie in the intrinsic efficacy of the σ_1 ligands studied to date: drugs with reported σ_1 ligand agonist properties undergo positive allosteric modulation,

whereas σ_1 antagonist drugs show no increase, or even a decrease, in their affinity for σ_1 receptors when incubated with DPH. The preliminary study by Cobos and colleagues (2005) was based on competition binding assays between cold σ_1 ligands and [^{3}H](+)-pentazocine.

The present study was intended to extend these findings by using additional binding techniques and two different radioligands. Here we used saturation and kinetics assays to characterize the allosteric modulation by DPH of the binding of the prototypic σ_1 agonist ligand [^{3}H](+)-pentazocine in guinea pig brain. Moreover, in order to confirm or refute the hypothesis that DPH modulates the binding of σ_1 ligands differently depending on their intrinsic activities, we extensively characterized the modulation by DPH of the binding of the putative antagonist σ_1 ligand [^{3}H]NE-100 in guinea pig brain.

2.3. MATERIALS AND METHODS

2.3.1. Animals

Male Dunkin Hartley guinea pigs (Charles River Laboratories España S.A., Barcelona, Spain) weighing 250 to 300 g were used. 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 (lights on from 08:00 h to 20:00 h), and were fed a standard laboratory diet and water ad libitum until the beginning of the experiments. They were handled and killed in accordance with the ethical principles of European Communities Council Directive 86/609/ECC and the University of Granada Ethics Committee.

2.3.2. Drugs and chemicals

The radioligands used in the assays and their providers were: [^{3}H](+)-pentazocine, specific activity 1.258 GBq/mmol (PerkinElmer Life Sciences, Boston, MA, USA), and [^{3}H]NE-100, specific activity 3.034 GBq/mmol (Amersham Biosciences Europe GmbH, Barcelona, Spain). The cold σ_1 ligands and their providers were: (+)-pentazocine, (-)-pentazocine, (+)-SKF-10,047 ((+)-*N*-allylnormetazocine) hydrochloride, (+)-3-PPP ((+)-3-(3-hydroxyphenyl)-N-propyl-piperidine) hydrochloride, dextromethorphan hydrobromide, DTG (1,3-di-o-tolylguanidine), haloperidol and progesterone (all from Sigma-Aldrich Química S.A., Madrid, Spain); PRE 084 (2-(4-morpholinethyl)1-phenylcyclo hexanecarboxylate) hydrochloride, BD 1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methyl-piperazine) dihydrochloride, BD 1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino) ethylamine) dihydrobromide (all from Tocris Cookson Ltd., Bristol, United Kingdom), and NE-100 (*N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy) phenyl]ethylamine) hydrochloride synthesized as described previously by Nakazato et al. (1999a).

Dilutions from the stock [^{3}H](+)-pentazocine or [^{3}H]NE-100 solutions were prepared with 50 mM HCl Tris buffer pH 7.4 at room temperature (incubation buffer A) or pH 7.55 (incubation buffer B) for [^{3}H](+)-pentazocine or [^{3}H]NE-100 binding assays, respectively. The pH of the incubation buffers was different in order to compensate for the variation in pH induced by the different temperatures of the incubation medium and the increase in pH induced by the DPH solvent. Haloperidol, (+)-pentazocine, (-)-pentazocine, DTG and progesterone were dissolved in absolute ethanol to make up a 1-mM or 10-mM solution, depending on the final concentration of drug used for

competition assays; further dilutions were prepared with incubation buffer yielding a final maximal concentration of ethanol in the incubation medium of 1% (vol/vol). We previously verified that this concentration of ethanol did not affect the binding. The rest of the unlabelled drugs were dissolved in deionized-ultrapure water at 1 mM or 10 mM, from which further dilutions were prepared with incubation buffer.

Phenytoin (diphenylhydantoin, DPH; Sigma-Aldrich Química SA, Madrid, Spain) was dissolved in NaOH 0.3 M to make up a 6.25-mM solution.

2.3.3. Membrane preparations

Guinea pigs were killed by decapitation and the brains minus the cerebellum were dissected (Matsumoto et al., 2002). Binding experiments were carried out in the crude synaptosome fraction or P_2 fraction, which was obtained with the method described previously (González et al., 2001) with slight modifications (DeHaven-Hudkins et al., 1992). Briefly, the tissue was homogenized in 5 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 at 4 °C, the resulting pellets were discarded and the supernatants were centrifuged again at 1000*g* for 10 min. The final supernatants were then centrifuged at 17 000 *g* for 20 min to obtain the P_2 pellets; each pellet was re-suspended in 10 ml 10 mM Tris-HCl, pH 7.4, and centrifuged again at 17 000 *g* for 20 min. The entire process was performed at 4 °C. Finally, each pellet obtained from one brain was re-suspended in 1 ml 10 mM Tris-HCl, pH 7.4, and frozen in aliquots (protein concentration 12-15 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. Radioligand binding assays

For [^{3}H](+)-pentazocine binding assays, membrane aliquots were slowly thawed and re-suspended in fresh incubation buffer A. We incubated re-suspended membrane preparations (440 µl) in a final protein concentration of 0.43-0.46 mg/ml with 20 µl [^{3}H](+)-pentazocine (final concentration of 0.5 nM in competition assays and 0.40-48 nM in saturation experiments), 20 µl DPH or its solvent and 20 µl of the cold ligand or its solvent for 150 min at 37 °C (DeHaven-Hudkins et al., 1992; Matsuno et al., 1996b). In binding kinetics experiments, different incubation times were used.

For [^{3}H]NE-100 binding assays, membrane aliquots were re-suspended in fresh incubation buffer B. The re-suspended membrane preparations, at a final protein concentration of 0.30-0.32 mg/ml, were incubated with 20 µl of [^{3}H]NE-100 (final concentration 1 nM in competition assays and 0.20-50 nM in saturation experiments), 20 µl DPH or its solvent and 20 µl cold ligand or its solvent for 45 min at 25 °C. In competition binding assays the incubation time was 150 min to allow steady state to be reached for the binding of the radioligand and the unlabelled drugs (Bylund and Yamamura, 1990). In binding kinetics assays, different incubation times were used. The final volume used for incubation was 500 µl.

In both [^{3}H]NE-100 and [^{3}H](+)-pentazocine binding assays the final pH in the incubation medium was 7.44 regardless of the incubation temperature, since this pH has

been reported to be the optimum for observing modulation by DPH of σ_1 ligand binding (Musacchio et al., 1988). In both assays, non-specific binding was defined as the binding retained on the filter and membranes in the presence of 1 μM haloperidol. In the competition assays, high concentrations (1 to 1000 μM depending on the drugs) of several unlabelled ligands were used to define non-specific binding.

The incubations were stopped with 5 ml cold (4 °C) filtration buffer (Tris 10 mM pH 7.4). The bound and free radioligand were separated by rapid filtration under a vacuum with a Brandel cell harvester (Model M-12 T, Brandel Instruments, SEMAT Technical Ltd., St. Albans, Hertfordshire, UK) over Whatman GF/B glass fibre filters (SEMAT Technical Ltd., UK) presoaked with 0.5% polyethylenimine in Tris 10 mM, pH 7.4, for at least 1 h prior to use, to reduce non-specific binding. The filters were washed twice with 5-ml volumes of ice-cold filtration buffer, transferred to scintillation counting vials containing 4 ml liquid scintillation cocktail (Optiphase Hisafe II, PerkinElmer Wallac, Loughborough, Leicestershire, UK), and equilibrated for at least 20 h. The radioactivity retained in the filter was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A., Madrid, Spain), with an efficiency of 52%. Each experiment was repeated at least three times, and each assay was conducted in triplicate.

2.3.5. Data analysis

Data were analysed with the SigmaPlot 2002 v. 8.0 program (SPSS Inc., IL, USA). The equilibrium saturation binding parameters, dissociation constant (K_D) and maximum number of binding sites (B_{max}) were calculated by non-linear regression

analysis of the equation for a rectangular hyperbola. These parameters were also calculated from the linear regression obtained with the Scatchard analysis as [*B/F*] versus *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 [$B/(B_{max} - B)$] versus log [*F*], where the slope of the plot (n_H) represents the Hill coefficient.

The IC_{50} (concentration of unlabelled drug that inhibited 50% of [^{3}H](+)-pentazocine or [^{3}H]NE-100 binding) was estimated from the inhibition curves using non-linear regression analysis of the equation for a sigmoid plot, assuming one-site competition. A pseudo-Hill coefficient (n'_H) from displacement assays was obtained. The K_i values for the unlabelled ligands (which indicates 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 the non-linear regression analysis from the control saturation experiments.

For binding kinetics assays, the *k* observed (k_{obs}) was obtained from non-linear regression to an exponential rise-to-maximum equation, and the dissociation rate constant (k_{-1}) was obtained from an exponential decay equation.

We used Student's *t*-test to compare pairs of independent means (SigmaStat 2.0; SPSS). Differences were considered significant when $P < 0.05$.

2.4. RESULTS

2.4.1. Sigma$_1$ ligand affinity for σ_1 binding sites labelled with [^{3}H](+)-pentazocine or [^{3}H]NE-100

We used competition binding assays to measure the affinity of a broad spectrum of unlabelled σ_1 ligands for the σ_1 receptor labelled with [^{3}H](+)-pentazocine or [^{3}H]NE-100. All unlabelled ligands tested inhibited, in a concentration-dependent way, the specific binding of [^{3}H](+)-pentazocine and [^{3}H]NE-100. The inhibition constants (K_i values) of these unlabelled ligands are shown in the Table I. The order of affinity of the different ligands for [^{3}H](+)-pentazocine binding sites agrees with their order of affinity for binding sites labelled with [^{3}H]NE-100 ($r^2 = 0.952$) (Fig. 1), which suggests that both radioligands label the same receptor.

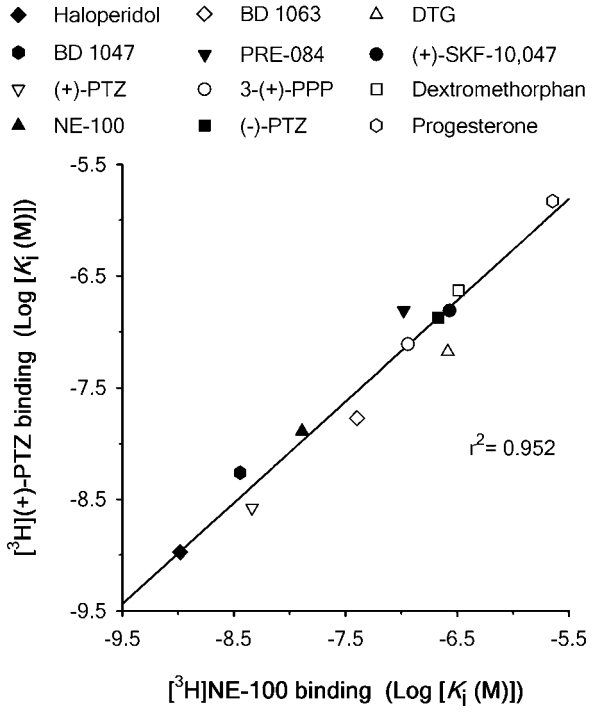

Figure 1. Relationship between the affinity of several unlabelled ligands for the σ_1 receptor labelled with [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) or [^{3}H]NE-100 in the crude synaptosome fraction obtained from guinea pig brain. Affinity is reported as the log of K_i (inhibitor constant) calculated with the Cheng-Prusoff equation from IC_{50} values of competition experiments performed as previously described (see Methods), and considering the K_D value of control saturation binding assays with [^{3}H](+)-PTZ and [^{3}H]NE-100. The data shown are representative of at least three experiments done in triplicate.

The Hill analysis of these competition assays yielded straight lines (r^2 = 0.97-0.99) whose slopes or pseudo-Hill coefficients (n'_H) were very close to unity for most ligands (Table I). This suggests the existence of a single population of binding sites.

Table I. Affinities of several cold ligands for σ_1 receptors labelled with [^{3}H](+)-pentazocine or [^{3}H]NE-100.

	***K*$_i$ (nM)**	
Ligand	**[^{3}H]NE-100**	**[^{3}H](+)-PTZ**
Haloperidol	1.05 ± 0.06 *(0.94 ± 0.04)*	1.06 ± 0.05 *(0.99 ± 0.01)*
BD 1047	3.62 ± 0.42 *(0.95 ± 0.05)*	5.49 ± 0.28 *(0.94 ± 0.02)*
(+)-Pentazocine	4.61 ± 0.39 *(0.92 ± 0.03)*	2.65 ± 0.11 *(0.95 ± 0.01)*
NE-100	13.19 ± 2.86 *(1.43 ± 0.13)*	13.14 ± 2.73 *(1.74 ± 0.13)*
BD 1063	40.02 ± 3.78 *(0.82 ± 0.03)*	16.94 ± 0.68 *(0.97 ± 0.03)*
PRE 084	105.76 ± 12.75 *(1.11 ± 0.06)*	156.15 ± 15.38 *(1.33 ± 0.01)*
(+)-3-PPP	114.91 ± 10.75 *(1.01 ± 0.04)*	77.60 ± 3.88 *(0.92 ± 0.02)*
(-)-Pentazocine	213.01 ± 19.15 *(0.98 ± 0.02)*	133.68± 6.63 *(0.99 ± 0.01)*
DTG	260.95 ± 24.47 *(0.93 ± 0.03)*	66.58 ± 3.61 *(0.95 ± 0.01)*
(+)-SKF-10,047	271.78 ± 20.08 *(0.97 ± 0.02)*	155.15 ± 10.61 *(0.89 ± 0.01)*
Dextromethorphan	325.84 ± 23.92 *(0.93 ± 0.03)*	235.42 ± 10.44 *(0.98 ± 0.02)*
Progesterone	2282.19 ± 358.15 *(1.08 ± 0.08)*	1491.24 ± 97.33 *(1.03 ± 0.08)*

The inhibitor constant values (K_i, nM), as a measure of affinity of cold ligands, were obtained with the Cheng-Prussoff equation considering the K_D value of [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) or [^{3}H]NE-100. The IC_{50} of cold ligands was obtained with competition assays performed in guinea pig brain membranes. The results are expressed as means ± SEM of three determinations. The values in parentheses are pseudo-Hill coefficients (n'_H) obtained from competition assays.

2.4.2. Effects of phenytoin on [^{3}H](+)-pentazocine or [^{3}H]NE-100 binding to σ_1 receptors from guinea pig brain

Phenytoin increased, in a concentration-dependent way, the specific binding of [^{3}H](+)-pentazocine to guinea pig brain membranes from 0.1 to 250 μM, (Fig. 2). This change reflected an increase in total binding. Non-specific binding did not change, and accounted for less than 5% of the total binding. In contrast, DPH decreased the specific binding of [^{3}H]NE-100 to guinea pig brain membranes in the same range of concentrations as mentioned above, and in a concentration-dependent way (Fig. 2).The specific binding of [^{3}H]NE-100 in the presence of DPH 250 μM was about 60% lower than in the presence of DPH solvent. This decrease in binding also reflected the change in total binding. Non-specific binding was unaltered, and accounted for around 20% of the total binding.

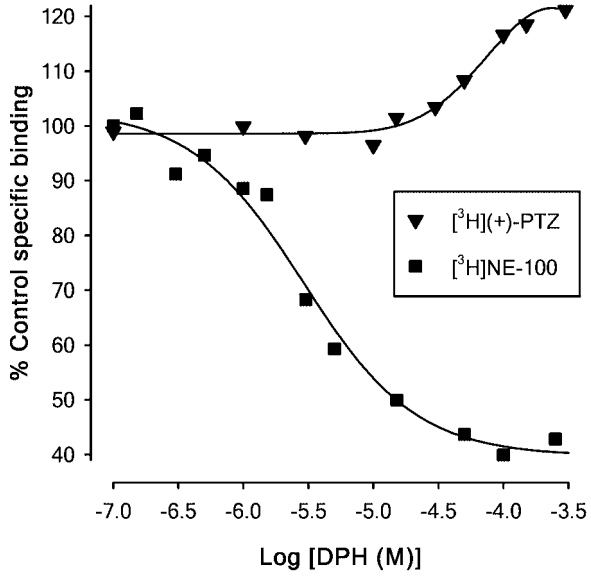

Figure 2. Differential modulation by phenytoin (DPH) of [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ, ▼) or [^{3}H]NE-100 (■) -specific binding to the crude synaptosome fraction obtained from guinea pig brain. Membranes were incubated with 0.5 nM [^{3}H](+)-PTZ at 37 °C for 150 min or with 1 nM [^{3}H]NE-100 at 25 °C for 45 min, in the presence of several concentrations of DPH or its solvent, at a final pH of 7.44. Haloperidol 1 μM was used to define non-specific binding in both kinds of experiment. The data shown are representative of three experiments done in triplicate.

2.4.3. Effects of phenytoin on the relationship between the specific binding of [^{3}H](+)-pentazocine or [^{3}H]NE-100 and the concentration of tissue proteins

Specific [^{3}H](+)-pentazocine binding in the presence of either DPH solvent or 250 μM DPH was linear (r^2 = 0.99) within the range of membrane protein concentrations of 0.153 to 0.800 mg/ml (Fig. 3A). Phenytoin at 250 μM increased the specific binding obtained under control conditions (DPH solvent), and the effect was greater at higher concentrations of protein (Fig. 3A).

Specific [^{3}H]NE-100 binding was also linear (r^2 = 0.99) within the range of membrane protein concentrations of 0.085 to 0.436 mg/ml, in the presence of both DPH solvent and 250 μM DPH. Phenytoin at 250 μM decreased the specific binding of [^{3}H]NE-100 at all concentrations of protein tested, and this effect was more pronounced when higher concentration of proteins were used (Fig. 3B).

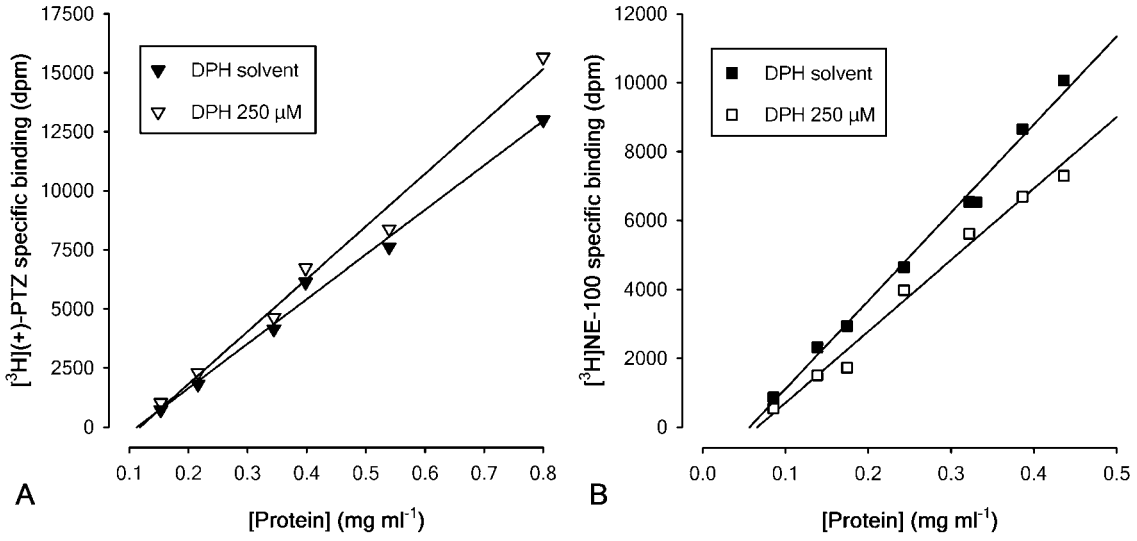

Figure 3. Influence of protein tissue concentration on specific binding of [^{3}H](+)-pentazocine -[^{3}H](+)-PTZ- (A) or [^{3}H]NE-100 (B) to the crude synaptosome fraction from guinea pig brain in the presence of DPH 250 µM or its solvent. (A) Several concentrations of protein membranes (0.153–0.800 mg/ml protein) were incubated at 37°C for 150 min with 0.5 nM [^{3}H](+)-PTZ in the presence of DPH 250 µM (▽) or its solvent (▼). (B) Several concentrations of protein membranes (0.085–0.436 mg/ml protein) were incubated at 25 °C for 45 min with 1 nM [^{3}H]NE-100 in the presence of DPH 250 µM (□) or its solvent (■). The final pH in all tubes was 7.44. Haloperidol 1 µM was used to define non-specific binding in both kinds of experiment. The data shown are representative of three assays done in triplicate.

2.4.4. Effects of phenytoin on [^{3}H](+)-pentazocine or [^{3}H]NE-100 saturation binding assays in guinea pig brain

Saturation assays under control conditions showed that [^{3}H](+)-pentazocine bound in a saturable manner to only one population of specific binding sites in the guinea pig brain. Data were fitted by non-linear regression analysis to a hyperbolic equation (r^2 = 0.992), yielding an equilibrium dissociation constant (K_D) of 2.451 ± 0.220 nM and a maximal number of receptors (B_{max}) of 1.479 ± 0.029 pmol/mg protein (Fig. 4A). Scatchard analysis of these results yielded a straight line (r^2 = 0.973),

consistent with the existence of a single class of high-affinity σ_1 binding sites (Fig. 4A). The parameters K_D and B_{max} obtained with this analysis were 2.030 ± 0.120 nM and 1.434 ± 0.105 pmol/mg protein, respectively, which were very similar to those obtained with non-linear regression analysis. The addition of DPH (250 μM) to the incubation medium clearly increased the affinity of [^{3}H](+)-pentazocine for its binding sites, increasing the slope of the line in the Scatchard plot (Fig. 4A). This drug significantly ($P < 0.01$) decreased the K_D value to 1.094 ± 0.096 nM (non-linear regression analysis). Phenytoin at 250 μM did not modify the maximal number of binding sites labelled with [^{3}H](+)-pentazocine, which was 1.467 ± 0.024 pmol/mg protein (Fig. 4A; Table II). The Hill analysis yielded straight lines (r^2 = 0.951 - 0.980) whose slopes (n_H) were always very close to unity under all these experimental conditions (Table II). These results confirmed the existence of a single population of binding sites both in the presence and in the absence of DPH.

The binding of [^{3}H]NE-100 to guinea pig brain was also saturable, and the values obtained with hyperbolic non-linear regression (r^2 = 0.999) were K_D = 8.738 ± 0.350 nM. and B_{max} = 1.705 ± 0.020 pmol/mg protein (Fig. 4B; Table II). The Scatchard analysis yielded a straight line (Fig. 4B), and the estimated values for the dissociation constant and maximal number of receptors were K_D = 8.333 ± 0.250 nM and B_{max} = 1.683 ± 0.058 pmol/mg protein, which were very similar to those obtained with non-linear regression analysis. Incubation with DPH 250 μM did not modify the affinity of the [^{3}H]NE-100 for its binding sites, and the K_D value of 8.735 ± 0.586 nM was very similar to that obtained under control conditions. However, DPH at 250 μM significantly decreased ($P < 0.05$) the maximal number of specific σ_1 binding sites labelled with [^{3}H]NE-100 to 1.475 ± 0.030 pmol/mg protein (Fig. 4B and Table II). Hill

analysis yielded straight lines with excellent correlations (r^2 = 0.999 - 0.997), and their slopes (n_H) were very close to unity (Table II).

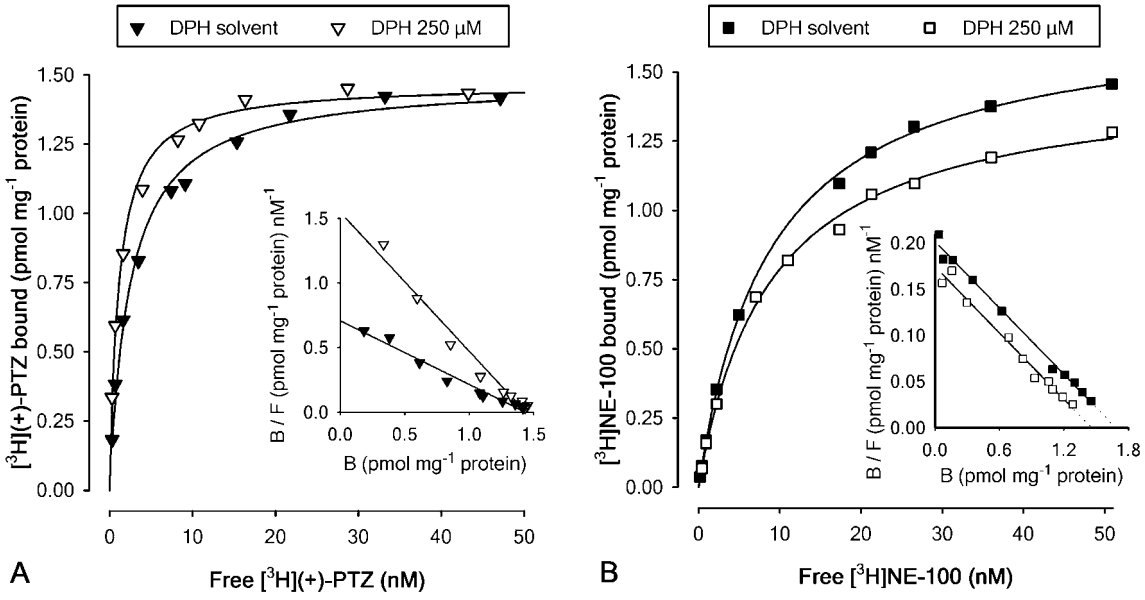

Figure 4. Effects of DPH 250 µM on binding saturation of σ_1 ligands to the crude synaptosome fraction obtained from guinea pig brain. (A) Membranes (0.43 mg/ml protein) were incubated for 150 min at 37 °C with several concentrations of [^{3}H](+)-pentazocine -[^{3}H](+)-PTZ- (0.4–48 nM) in the presence of DPH 250 µM (▽) or its solvent (▼). (B) Several concentrations of [^{3}H]NE-100 (0.20–50 nM) were incubated with brain membranes (0.32 mg/ml protein) and DPH 250 µM (□) or its solvent (■) for 45 min at 25 °C. In both kinds of saturation assay the final pH was 7.44 and haloperidol 1 µM was used to define non-specific binding. The data shown are representative of at least three experiments done in triplicate. Representative Scatchard plots of [^{3}H](+)-PTZ (A) and [^{3}H]NE-100 (B) binding are inserted.

Table II. Effects of DPH 250 μM on binding parameters of [^{3}H](+)-pentazocine or [^{3}H]NE-100 saturation binding assays.

	[^{3}H](+)-pentazocine			**[^{3}H]NE-100**		
	K_D	B_{max}	n_H	K_D	B_{max}	n_H
Control	2.451 ± 0.220	1.479 ± 0.030	1.016 ± 0.051	8.738 ± 0,350	1.705 ± 0,020	0.991 ± 0.008
DPH 250 μM	1.094 ± 0.096 **	1.467 ± 0.024	1.087 ± 0.094	8.735 ± 0.586	1.475 ± 0,030*	0.997 ± 0.018

The parameters K_D (dissociation constant of the radioligand, nM) and B_{max} (maximal number of receptors labelled, pmol/mg protein) were obtained from non-linear regression analysis of saturation assays performed in the presence of DPH 250 μM or its solvent (control condition) in the synaptosome fraction from guinea pig brains. The results are expressed as means ± SEM of three determinations obtained in triplicate. Statistically significant differences in comparison to controls: * $P < 0.05$; ** $P < 0.01$ (Student's *t* test).

2.4.5. Effects of phenytoin on the dissociation of [^{3}H](+)-pentazocine or [^{3}H]NE-100 from σ_1 receptors in guinea pig brain

Non-equilibrium kinetic binding assays of [^{3}H](+)-pentazocine and [^{3}H]NE-100 were performed to better characterize the effects of DPH on the binding of both σ_1 ligands. Both the association and dissociation of [^{3}H](+)-pentazocine were very slow processes that fit a monoexponential model ($r^2 = 0.99$). The steady state of association of [^{3}H](+)-pentazocine was reached at 150 min at 37 °C, with a k_{obs} value of 0.023 ± 0.001 min^{-1}. Dissociation of this compound from its receptor reached 91.52% after 450 min (Fig. 5A). Phenytoin at 250 μM slowed the dissociation kinetics of [^{3}H](+)-pentazocine and significantly ($P < 0.01$) decreased the dissociation rate from 0.0055 ± 0.0003 min^{-1} (control conditions, DPH solvent) to 0.0021 ± 0.0006 min^{-1} in the presence of 250 μM DPH (Fig. 5A).

On the other hand, the kinetics of [^{3}H]NE-100 at 25 °C under control conditions fit monoexponential functions ($r^2 = 0.961$ - 0.985) and indicated a very fast process. Complete [^{3}H]NE-100 association was reached 45 minutes after incubation of the radioligand with the tissue, and the k_{obs} value was 0.163 ± 0.010 min^{-1}. The specific [^{3}H]NE-100 bound was almost completely dissociated (98%) 30 minutes after haloperidol 1 μM was added (Fig. 5B). The addition of DPH at 250 μM together with 1 μM haloperidol to the incubation medium increased the dissociation rate of [^{3}H]NE-100 from 0.1323 ± 0.0095 min^{-1} (in the presence of DPH solvent) to 0.2516 ± 0.0266 min^{-1} (in the presence of DPH 250 μM) (Fig. 5B), and these values were significantly different ($P < 0.05$).

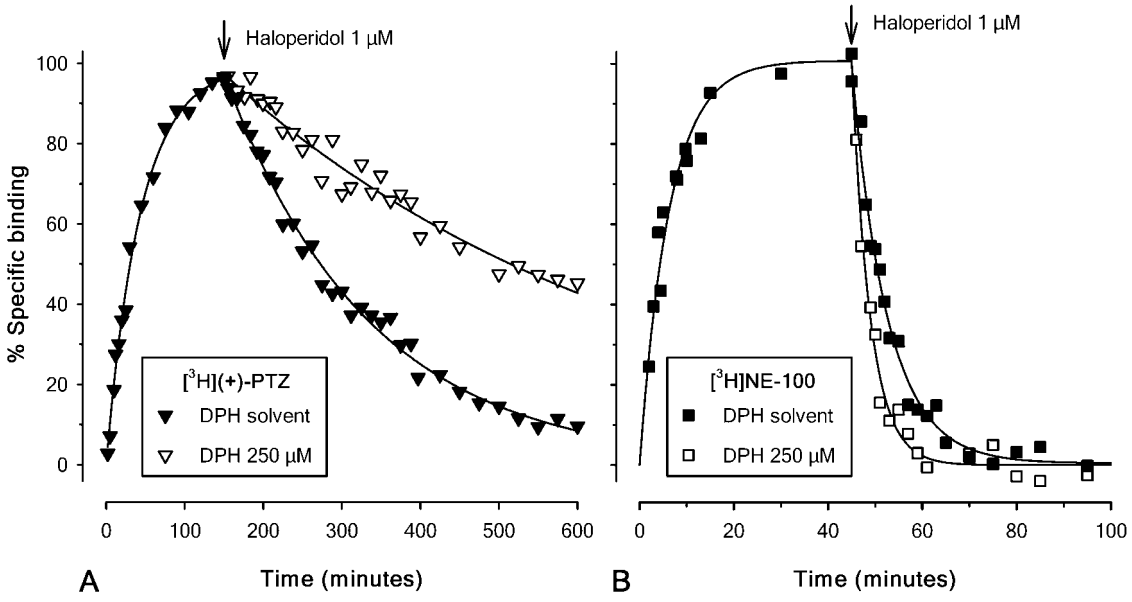

Figure 5. Effects of DPH 250 μM on dissociation kinetics of [^{3}H](+)-pentazocine -[^{3}H](+)-PTZ- (A) and [^{3}H]NE-100 (B) from guinea pig brain. (A) Membrane proteins (0.46 mg/ml) were incubated with 0.5 nM [^{3}H](+)-PTZ at 37 °C for several different periods (association assay) or for 150 min (dissociation assay). (B) Membrane proteins (0.30 mg/ml) were incubated with 1 nM [^{3}H]NE-100 at 25 °C for several different periods (association assay) or 45 min (dissociation assay). Dissociation was begun by the addition of 1 μM haloperidol with 250 μM DPH (open symbols) or its solvent (filled symbols). Samples were filtered after incubation at the indicated time points. The data shown were derived from one experiment performed in triplicate and are typical of the results obtained in three independent experiments. The dissociation constants (k_{-1} values) for [^{3}H](+)-PTZ binding were 0.0055 ± 0.0003 min^{-1} (DPH solvent, ▼) and 0.0021 ± 0.0006 min^{-1} (in the presence of 250 μM DPH, ▽). The dissociation constants (k_{-1} values) of [^{3}H]NE-100 binding were 0.1323 ± 0.0095 min^{-1} (DPH solvent, ■) and 0.2516 ± 0.0266 min^{-1} (in the presence of DPH 250 μM, □).

2.5. DISCUSSION

The results we obtained with different experimental approaches show that DPH positively modulated the binding of [^{3}H](+)-pentazocine, whereas it had a negative effect on [^{3}H]NE-100 binding to σ_1 receptors in guinea pig brains. We also show that

both radioligands labelled the same receptor, as deduced from the good correlation (r^2 = 0.952) between the affinity of twelve σ_1 ligands of different chemical structure for σ_1 receptors labelled with [^{3}H](+)-pentazocine and [^{3}H]NE-100. As a possible explanation for the interaction between DPH and the two radioligands, we can rule out non-selective interference of DPH with σ_1 receptor binding through an unspecific alteration in the plasma membrane or membrane-associated proteins, because of the differential modulation of [^{3}H]NE-100 and [^{3}H](+)-pentazocine binding by DPH. We can also rule out that the different temperatures used for [^{3}H](+)-pentazocine and [^{3}H]NE-100 binding assays (37 °C and 25 °C, respectively) might explain the different effect of DPH on binding assays, because previous studies based on competition experiments and performed at the same temperature reported that DPH differentially modulated the affinity of several unlabelled σ_1 agonists and antagonists for σ_1 receptors labelled with [^{3}H](+)-pentazocine (Cobos et al., 2005). To explain our previous findings we formulated a hypothesis based on the intrinsic efficacy of the unlabelled ligands as the key to these differences (Cobos et al., 2005). The present study further supports this hypothesis, as [^{3}H](+)-pentazocine is considered a prototypic σ_1 agonist (Walker et al., 1990), whereas [^{3}H]NE-100 is considered an antagonist of σ_1 receptors (Maurice et al., 2001a).

In equilibrium assays using several concentrations of DPH, we found that DPH increased the binding of [^{3}H](+)-pentazocine to guinea pig brain membranes (Fig. 2). This is consistent with the previously reported increase in the binding of other σ_1 agonist ligands such as [^{3}H](+)-3-PPP (Chaki et al., 1996; Tanaka et al., 1995), [^{3}H]dextromethorphan (Musacchio et al., 1987; Musacchio et al., 1988) and [^{3}H]SKF-10,047 (McCann and Su, 1991). In addition, we showed that the effect of DPH

250 μM on [^{3}H](+)-pentazocine binding increased with the concentration of membrane proteins, a relationship not reported previously.

The results of the dissociation binding assays constitute the first reported evidence of the positive allosteric interaction between DPH and [^{3}H](+)-pentazocine. An alteration in the dissociation of a radioligand from the primary orthosteric site is considered the most reliable indicator of an allosteric action via a secondary allosteric site of a receptor (Kostenis and Mohr, 1996); in particular, a reduction in the dissociation rate of the radioligand is indicative of a positive allosteric interaction (Gao et al., 2002). We show that 250 μM DPH decreased the dissociation rate of [^{3}H](+)-pentazocine from its binding sites by approximately 50% (Fig. 5A), a finding that confirms the positive nature of the allosteric interaction.

Interestingly, saturation experiments showed that DPH at 250 μM increased the affinity of [^{3}H](+)-pentazocine for σ_1 receptors (decreasing the K_D value by half) without significantly changing the maximum number of receptors (B_{max}) or the number of sites that the drug labels (Hill coefficient close to unity). Two possible explanations might account for these results. First, DPH (allosteric ligand) might induce a conformational change in the receptor that enhances the affinity of the orthosteric ligand [^{3}H](+)-pentazocine for its site on the σ_1 receptor—a phenomenon called conformational induction (Chirstopoulos and Kenakin, 2002). Another possible explanation derives from the extension of the two-state model of receptor activation (Leff, 1995) proposed by Hall (2000). This model makes no assumptions about signal transduction steps, and is potentially applicable to any type of receptor (Hall, 2000). In this model, receptors display two conformational states: inactive (R) or active (R*). Agonists promote the R* state and bind to this state of the receptor with high affinity.

The effect of a positive allosteric modulator (e.g., DPH in our experiments) in this model would consist of the induction of transition to R*, thereby increasing the affinity and binding of the orthosteric agonist (e.g., [^{3}H](+)-pentazocine) to the receptor. In saturation assays the high concentrations of the radioligand agonist used to define B_{max} bind all of the active receptors of the system. In this situation the effect of a positive allosteric modulator might not be to increase the maximal number of active receptors in the system (B_{max}). However, it would affect the likelihood that the radioligand binds to the active receptors of the system at non-saturating concentrations (i.e., concentrations that label less than the totality of the receptors of the system), thereby increasing the affinity and decreasing the K_D value of the radioligand for the receptor.

On the other hand, we provide here initial evidence that DPH clearly decreased the specific binding of [^{3}H]NE-100 in equilibrium assays (Fig. 2, 3B and 4B). Other authors have previously reported that DPH did not modify the binding of [^{3}H]NE-100 in competition assays (Tanaka et al., 1995). The discrepancy between these findings and ours can be explained by differences in the experimental conditions, specifically, in incubation time, protein concentration and membrane preparation. Moreover, it is important to note that we observed a negative modulation by DPH of [^{3}H]NE-100 binding not only in competition experiments, but also in saturation and binding kinetics experiments; thus the data reported here are internally consistent. The maximal decrease observed in displacement assays was about 60%, and was produced by a concentration of 250 μM DPH. Theoretically, these findings could be the result of competition between DPH and [^{3}H]NE-100 for the same binding site (same epitope) of the σ_1 receptors, or of negative allosteric modulation (non-competitive inhibition) by DPH of [^{3}H]NE-100 binding. The findings that maximal inhibition of [^{3}H]NE-100 specific

binding was above non-specific binding levels and that DPH decreased the apparent maximal number of receptors labelled with [^{3}H]NE-100 without inducing change in affinity—as discussed below—are evidence of non-competitive allosteric antagonism (Kenakin, 2004).

We also performed binding kinetics assays to look for further evidence of the allosteric effect of DPH, since it has been reported that allosteric inhibitors enhanced the dissociation rate of the radioligand modulated (Kenakin, 2004). The fact that DPH 250 μM significantly increased (by almost 100%) the dissociation rate of [^{3}H]NE-100 from σ_1 binding sites constitutes an important finding which provides further support for the notion that DPH behaves as an allosteric inhibitor of [^{3}H]NE-100 binding.

To explain the differential modulation by DPH of [^{3}H](+)-pentazocine and [^{3}H]NE-100 binding, the extended two-state model of receptor activation (Hall, 2000) is a useful starting point. According to this model the effects of a positive allosteric modulator on radioligand binding depend on the pharmacological activity of the radioligand. Allosteric enhancers, by stabilizing the active conformation of receptors (R*) at the expense of the inactive conformation (R), increase the binding of the agonist radioligand, whereas they decrease the binding of those ligands which bind preferentially to the inactive conformation of the receptor, i.e., inverse agonist ligands. In this study we show that DPH reduced the binding of [^{3}H]NE-100, decreasing its B_{max} value but without modifying its affinity for σ_1 receptors. Previous studies have shown that the binding of inverse agonist ligands for β_2 receptors was affected by an allosteric modulator in the same way as the interaction between [^{3}H]NE-100 and DPH described in this study: the allosteric modulator decreased B_{max} without changing K_D values (Azzi et al., 2001). In light of the two-state model theory and the findings of Azzi and

colleagues, it is tempting to propose that NE-100 behaves as an inverse agonist ligand; however, in the absence of functional data to support this hypothesis, further experiments will be needed.

Regarding the possible physiological or neurological outcomes of the interaction between DPH and sigma ligands, it should be remembered that the concentration of DPH that produced the effects reported in this study— 250 μM—is equivalent to a concentration in human plasma of roughly 60 μg/ml. Because this concentration is above the normal therapeutic range of 10-20 μg/ml (Garg et al., 2000), DPH is unlikely to modulate the therapeutic effects of σ_1 ligands in patients.

In summary, we show that the nature of the allosteric modulation by DPH of σ_1 binding differs depending on the ligand's pharmacological profile. Positive allosteric interaction is observed with [^{3}H](+)-pentazocine, the ligand that showed agonistic activity at the σ_1 receptors, whereas DPH negatively modulated the binding of [^{3}H]NE-100, a putative antagonist of σ_1 receptors. These findings suggest a way to predict the intrinsic efficacy of newly synthesized σ_1 receptor ligands *in vitro*.

2.6. REFERENCES

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

3. Irreversible blockade of σ_1 receptors by haloperidol and its metabolites in guinea pig brain and SH-SY5Y human neuroblastoma cells

ENRIQUE J. COBOS, ESPERANZA DEL POZO AND JOSÉ M. BAEYENS

SUBMITTED

Running title: Inactivation of sigma$_1$ receptors by reduced haloperidol

3.1. ABSTRACT

We evaluated the effect of haloperidol and its metabolites on [^{3}H](+)-pentazocine binding to σ_1 receptors in SH-SY5Y human neuroblastoma cells and guinea pig brain P_1, P_2 and P_3 subcellular fractions. Affinity of the drugs for σ_1 receptors in brain membranes and SH-SY5Y cells correlated well ($r^2 = 0.991$), which suggests that guinea pig and human σ_1 receptors are pharmacologically equivalent. Three days after a single i.p. injection in guinea pigs of haloperidol (but not of BD 1047, BD 1063, NE-100 or (-)-sulpiride), [^{3}H](+)-pentazocine binding to brain membranes was markedly decreased. Recovery of σ_1 receptor density to steady state after haloperidol-induced inactivation required more than 30 days in all brain subcellular fractions. Haloperidol metabolite II (reduced haloperidol) or metaphit, but not haloperidol metabolite I or BD 1063, irreversibly (wash-resistant) inhibited [^{3}H](+)-pentazocine binding to σ_1 receptors in guinea pig brain homogenate and P_2 fraction *in vitro*. We found similar results in human SH-SY5Y cells, which suggests that irreversible binding of reduced haloperidol to σ_1 receptors may also take place in humans. Haloperidol irreversibly inactivated σ_1 receptors when it was incubated *in vitro* with brain homogenate and SH-SY5Y cells, but not when incubated with P_2 fraction membranes, which suggests that haloperidol is metabolized to inactivate σ_1 receptors. Menadione, an inhibitor of the ketone reductase activity that leads to the production of reduced haloperidol, completely reverted haloperidol-induced inactivation of σ_1 receptors in brain homogenates. These results suggest that haloperidol may irreversibly inactivate σ_1 receptors in guinea pig and human cells, probably after metabolism to reduced haloperidol.

3.2. INTRODUCTION

Sigma receptors, defined as nonopioid and nonphencyclidine sites, are considered a unique pharmacological entity. Two subtypes of sigma receptor, $sigma_1$ (σ_1) and $sigma_2$ (σ_2), are distinguished (Quirion et al., 1992). σ_1 receptors have been cloned in several species, including guinea pigs and humans (Kekuda et al., 1996; Hanner et al., 1996), and are located in the brain and different peripheral tissues such as the testes, heart and liver (DeHaven-Hudkins et al., 1994). At the subcellular level they are located in nuclear, synaptic and microsomal membranes, and they are most abundant in the latter (Itzhak et al., 1991; DeHaven-Hudkins et al., 1994). To date, no studies have compared the turnover of σ_1 receptors in different subcellular locations.

$Sigma_1$ receptors bind a wide variety of drugs, such as dextrorotatory benzomorphans (e.g., (+)-pentazocine), antipsychotics (e.g., haloperidol), neurosteroids (e.g., progesterone) and psychostimulants (such as cocaine and methamphetamine) with high to moderate affinities (Quirion et al., 1992; Maurice et al., 2002). These receptors appear to be involved in anxiety, depression, psychosis, learning and memory, and nociception, as well as in the acute and chronic effects of cocaine and other drugs of abuse (Hayashi and Su, 2004a; Guitart et al., 2004; Cendan et al., 2005b; Maurice et al., 2002).

Haloperidol was introduced in therapeutics over 40 years ago and is still widely used for the treatment of acute and chronic psychosis; its antipsychotic activity is thought to be mediated, at least in part, by its dopamine D_2 receptor antagonistic properties (Usuki et al., 1988). Haloperidol is also a σ_1 antagonist, showing similar affinity for D_2 and σ_1 receptors (Bowen et al., 1990a; Matsumoto and Pouw, 2000), but

the consequences of its binding to σ_1 receptors are less well known. Several metabolic pathways are involved in the metabolism of haloperidol, as summarized in Figure 1. Reduction of the ketone group of haloperidol leads to the formation of reduced haloperidol (also called haloperidol metabolite II) in both guinea pigs and humans, but not in rats (Korpi et al., 1985; Eyles and Pond, 1992); thus the guinea pig constitutes a model for human haloperidol metabolism. Cytochrome P450 (CYP), mainly CYP3A4, is responsible for the oxidative *N*-dealkylation of haloperidol, which is the major *in vivo* metabolic pathway and produces haloperidol metabolites I and III (Usuki et al., 1998). A similar process can ensue from reduced haloperidol (Pan et al., 1998a; Fang et al., 2001). CYP3A4 is also responsible for the back-oxidation of reduced haloperidol to haloperidol (Pan et al., 1998a; Usuki et al., 1998), and the formation of the pyridinium metabolites HPP^+ and, at a lower rate, $RHPP^+$ (Igarashi et al., 1995; Eyles et al., 1996; Usuki et al., 1998; Fang et al., 2001), which display neurotoxic properties resembling those of their structural analogue MPP^+ (Bloomquist 1994; Fang et al., 1995). In addition, haloperidol is also converted to HPP^+ without the participation of CYP3A4 by the monoamino oxidase A (MAO-A) (Usuki et al, 2002).

A single administration of haloperidol or reduced haloperidol to guinea pigs produced a long-lasting decrease in the number of brain σ_1 binding sites (Klein et al., 1994). This effect was hypothesized to be due to the formation of an irreversible or slowly reversible complex of haloperidol, reduced haloperidol or some of their metabolites with σ_1 receptors (Klein et al., 1994; Inoue et al., 2000). However, it is unknown whether the effect (1) is similar at different subcellular locations of the σ_1 receptor, (2) is shared by prototypical σ_1 receptor antagonists (such as BD 1047,

BD 1063 or NE-100), and (3) whether haloperidol *per se* or any of its metabolites is responsible for σ_1 receptor inactivation.

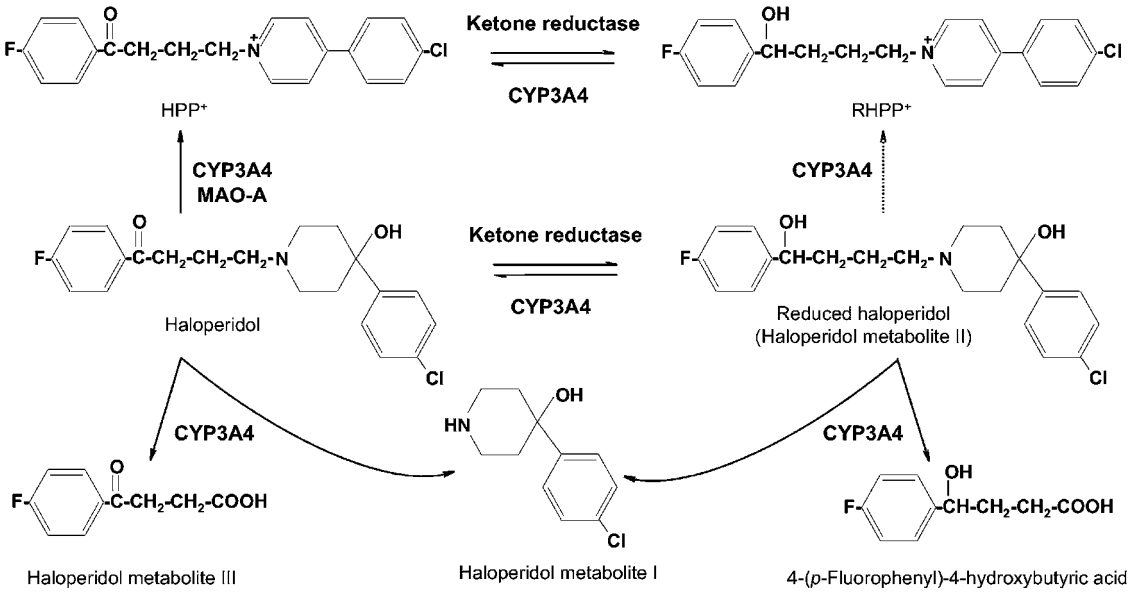

Figure 1. Enzymes responsible for the different metabolic pathways of haloperidol and its metabolites. Dotted arrows indicates a minor pathway (see text for details and references)

In the light of these antecedents the goals of this study were (1) to compare the reversibility of the effects on [^{3}H](+)-pentazocine binding to guinea pig brain subcellular fractions (P_1, P_2 and P_3) induced by a single administration of haloperidol and different prototypic σ_1 receptor antagonists (BD 1047, BD 1063 and NE-100); (2) to study the turnover of σ_1 receptors in P_1, P_2 and P_3 fractions from guinea pig brain (using a single administration of haloperidol to irreversibly block them, and [^{3}H](+)-pentazocine to specifically mark them); and (3) to compare the ability of haloperidol and some haloperidol metabolites to bind irreversibly to σ_1 receptors from guinea pig brain and SH-SY5Y human neuroblastoma cells.

3.3. MATERIALS AND METHODS

3.3.1. Animals

Male Dunkin Hartley guinea pigs (Charles River Laboratories España S.A., Barcelona, Spain) weighing 250 to 300 g at the beginning of the experiments were used. 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 (lights on from 08:00 h to 20:00 h), and were fed a standard laboratory diet and water *ad libitum*. Animals were handled and killed in accordance with the ethical principles of European Communities Council Directive 86/609/ECC and the University of Granada Ethics Committee.

3.3.2. Chemicals, drugs and drug treatments

The radioligand used in the assays was [^{3}H](+)-pentazocine, with a specific activity of 33.7 Ci/mmol (PerkinElmer Life Sciences, Boston, MA, USA). The other compounds used (and their suppliers) were: haloperidol, haloperidol metabolites I, II and III, (-)-sulpiride, metaphit methanesulfonate salt, MPP^+, menadione sodium bisulfite, NADPH (all from Sigma-Aldrich Química S.A., Madrid, Spain), BD 1063 dihydrochloride and BD 1047 dihydrobromide (Tocris Cookson Ltd., Bristol, United Kingdom), and NE-100 (which was synthesized as described previously by Nakazato et al., 1999).

For binding assays, dilutions from the stock [^{3}H](+)-pentazocine solution were prepared with incubation buffer (50 mM HCl-Tris buffer pH 7.44 at 37 ºC).

Haloperidol, haloperidol metabolites I, II and III, and (–)-sulpiride were dissolved in absolute ethanol to make up a stock solution, from which further dilutions were prepared with incubation buffer yielding 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. Metaphit, BD 1063, BD 1047 and MPP^+ were prepared in deionized ultrapure water from which further dilutions were prepared with incubation buffer.

For the *in vivo* treatments, the drugs BD 1063, BD 1047, NE-100 and haloperidol were administered to the guinea pigs in a 6% DMSO solution in physiological saline (0.9% NaCl). In order to facilitate solubilization, haloperidol solutions contained 0.0075% HCl. (-)-Sulpiride was dissolved in tartrate 10 mM with 1% glacial acetic acid. The pH of drug solutions used for *in vivo* treatments was adjusted to approximately 7.0. The drugs (haloperidol, BD 1047, BD 1063, NE-100 and (–)-sulpiride) were administered by single intraperitoneal (i.p.) injections in a volume of 5 ml/kg except (–)-sulpiride, which was injected in a volume of 2 ml/kg. The animals were killed three days after drug administration in order to minimize the presence of residual drug in the brain (Inoue et al., 1997).

3.3.3. Guinea pig brain membrane preparation

Guinea pigs were killed by decapitation and the brains minus the cerebellum were dissected (Cobos et al., 2005, 2006). Binding experiments were carried out in the crude nuclear fraction, crude synaptosome fraction and microsomal fraction, obtained with methods described previously (Gurd et al., 1974) with slight modifications.

Briefly, the tissue was homogenized in 5 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 homogenate was centrifuged (Avanti 30, Beckman Coulter España S.A., Madrid, Spain) at 1000 *g* for 13 min, the supernatant (S_1) was collected in a clean tube and the pelleted nuclear fraction was resuspended with 6.5 ml of 10 mM Tris-HCl pH 7.4 (buffer A), and spun again at 1000 *g* for 10 min to obtain the washed nuclear fraction (P_1). Supernatant 1 (S_1) was also centrifuged under the same conditions to remove the remaining nuclear fraction; the resulting supernatant was centrifuged at 17 000 *g* for 20 minutes to obtain the crude cytosol fraction (S_2) and crude synaptosomal/mitochondrial fraction. This latter fraction was resuspended with 10 ml buffer A and centrifuged under the same conditions to yield the washed crude synaptosomal/mitochondrial fraction (P_2). Supernatant 2 (S_2) was also spun at 17 000 *g* for 20 min to remove the remaining synaptosomal fraction, and the supernatant was then ultracentrifuged at 149 000 *g* for 90 min (L-70 Ultracentrifuge, Beckman Coulter España S.A.) to obtain the microsomal fraction (P_3). The entire process was performed at 4 °C. The P_1, P_2 and P_3 fractions (approximately 28, 12 and 22 mg/ml of protein, respectively) were resuspended in buffer A and frozen in aliquots at −80 °C. Binding characteristics of the tissue were stable for at least 1 month when samples were stored at −80 °C.

3.3.4. [^{3}H](+)-pentazocine binding assays in guinea pig membranes

For radioligand binding assays in guinea pig brain membranes, aliquots of membranes were slowly thawed and resuspended in fresh incubation buffer to obtain a

final protein concentration of 0.66–1.08 mg/ml for P_1, 0.30–-0.66 mg/ml for P_2 and 0.20–0.42 mg/ml for P_3. Protein concentration was measured by the method of Lowry et al. (1951) with some modifications, using bovine serum albumin as the standard. Membrane solutions were incubated with 20 µl of [^{3}H](+)-pentazocine solution and 20 µl of the cold ligand solution or its solvent at 37 °C for 240 min. [^{3}H](+)-Pentazocine binding was stable during at least 600 min (data not shown), and the final volume was 500 µl (Cobos et al., 2005, 2006). Non-specific binding was defined as the binding retained on the filter and membranes in the presence of 1 µM haloperidol. In competition assays, high concentrations (1 to 1000 µM depending on the drugs) of several unlabelled ligands were used to define non-specific binding.

To stop the [^{3}H](+)-pentazocine binding to the guinea pig 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 fibre filters (SEMAT Technical Ltd., St. Albans, UK) pre-soaked with 0.5% polyethylenimine in Tris 10 mM, pH 7.4, for at least 1 h prior to use, to reduce non-specific binding. The filters were washed twice with 5 ml-volumes of the ice-cold filtration buffer and transferred to scintillation counting vials, and then 4 ml liquid scintillation cocktail (Optiphase Hisafe II, PerkinElmer Wallac, Loughborough, UK) was added and the mixture was equilibrated for at least 20 h. The radioactivity retained in the filter was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A.), with an efficiency of 52%. Each assay was conducted in triplicate.

To study the capability of some cold ligands to produce irreversible binding, we used a protocol similar to that previously described (Klein et al., 1994; Bluth et al. 1989; Ronsisvalle et al., 2002). P_2 membranes (1.2 mg protein/ml) were incubated at 37 ºC for 90 min with different cold ligands in a concentration 50-fold higher than their IC_{50} for [^{3}H](+)-pentazocine binding. Membranes were washed to minimize the presence of residual drug; for this purpose, the tubes with the samples (1 ml) were filled up to 15 ml with incubation buffer at 37 ºC and centrifuged for 20 min at 17 000 *g*. The pellets were resuspended in 15 ml incubation buffer and incubated during 30 min at 37 ºC to dissociate the non-covalently bound drug. Membranes were then pelleted under the same conditions as before and immediately distributed in aliquots of 0.23 – 0.35 mg of protein/ml for binding assays.

To evaluate the ability of brain homogenate *in vitro* to produce metabolites of haloperidol that irreversibly bind to σ_1 receptors, guinea pig brains were homogenized with a Polytron homogenizer as previously described. Aliquots of brain homogenate in 0.32 M sucrose-10 mM Tris-HCl pH 7.4 were incubated with several drugs at a concentration 50 times higher than their IC_{50}, with or without NADPH 1 mM and menadione 0.5 mM. After incubation, the tubes with the samples (1 ml) were filled up to 15 ml with the same buffer, and the P_2 fraction was obtained as described in the section *Guinea pig brain membrane preparation*. The crude synaptosomal fraction was resuspended in 15 ml buffer A and incubated during 30 min at 37 ºC to dissociate non-covalently bound drug. The samples were then pelleted (20 min at 17 000 *g*) and distributed in aliquots of 0.35 – 0.68 mg of protein/ml for binding assays.

3.3.5. Cell culture of SH-SY5Y human neuroblastoma cells

The cell line SH-SY5Y (passage 12) was obtained from cell culture collection ECACC number 94030304 (human neuroblastoma cell line) through the Centro de Instrumentación Científica, University of Granada. Cells were cultured in 75 mm^2 cell culture flasks with Ham's F12: EMEM (EBSS) (1:1) modified medium containing 1% nonessential amino acids and supplemented with 2 mM glutamine and 15% foetal bovine serum. Cell cultures were maintained at 37 °C in humidified conditions under 5% CO_2. The medium was changed twice weekly and cultures were harvested by trypsinization using trypsin-EDTA. Cells were pelleted by centrifugation at 350 *g* for 8 min (Heraeus Sepatech Omnifuge 2.0RS, Osterode, Germany), resuspended in growth medium and split at ratio of 1:4 each week and a half.

For SH-SY5Y neuroblastoma cells, all drugs used (haloperidol, haloperidol metabolites I and II, BD 1063 and metaphit) were dissolved in DMSO to make up a highly concentrated solution; further dilutions were prepared with growth medium to yield a final maximum concentration of DMSO of 0.001%.

For binding assays, cells (passage 16 – 18) were harvested by nonenzymatic dissociation using Cell Dissociation Solution. Cells were pelleted as describe above, resuspended in Tris-HCl 10 mM pH 7.4, and frozen in aliquots at –80 °C. All reagents used to maintain, split and harvest the cells were provided by Sigma-Aldrich Química S.A.

3.3.6. [^{3}H](+)-pentazocine binding assays in SH-SY5Y neuroblastoma cells

[^{3}H](+)-pentazocine binding assays in SH-SY5Y neuroblastoma cells were performed with the method previously described by Hong and cols. (2004) with slight modifications. Briefly, cells aliquots were slowly thawed and resuspended in fresh incubation buffer to a final protein concentration of 0.13 – 0.22 mg/ml protein. The whole cell suspension was incubated with 20 µl [^{3}H](+)-pentazocine (final concentration 5 nM) and 20 µl of different concentrations of cold ligands or their solvents for 180 min at 37 °C, with a final volume of 500 µl. [^{3}H](+)-pentazocine binding was stopped with ice-cold filtration buffer and rapid filtration under a vacuum, as for binding assays with guinea pig membranes.

To determine whether the cold σ_1 ligands used bound irreversibly σ_1 receptors in SH-SY5Y neuroblastoma cells, the cells were incubated in their own 75-mm^2 cell culture flasks with 10 ml of different drug solutions (at a concentration 50-fold higher than their IC_{50} for the [^{3}H](+)-pentazocine binding) for 90 min at 37 ºC. Then each flask was washed twice with 13 ml PBS (phosphate-buffered saline), and 10 ml growth medium was added to the flasks, which were incubated during 30 min at 37 ºC in order to dissociate non-covalently drug. The growth medium was removed and cells were prepared for binding experiments as described in the section *Cell culture of SH-SY5Y neuroblastoma cells*.

3.3.7. Data analysis

Data were analysed with the SigmaPlot 2002 v. 6.0 program (SPSS Inc., IL, USA). The equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}) from saturation assays were calculated by non-linear regression analysis of the results fitted to a rectangular hyperbola equation. These parameters were also calculated using a linear regression from the Scatchard analysis. The Hill coefficient (n_H) was also obtained from the Hill plots.

The IC_{50} (concentration of unlabelled drug that inhibited 50% of [^{3}H](+)-pentazocine-specific binding) was estimated from the inhibition curves using non-linear regression analysis of the equation for a sigmoid plot, assuming one-site competition.

To calculate the receptor turnover parameters we used non-linear regression to an exponential rise-to-maximum equation: $R_t = r/k(1-e^{-kt}) + R_0e^{-kt}$, where R_t represents the number of receptors at a given discrete time (t), and R_0 is the number of receptors at time 0 (both expressed as pmol of bound radioligand/mg protein), r is the receptor appearance rate constant (expressed as pmol of bound radioligand/mg protein/day), and k is the disappearance rate constant (in units of day^{-1}), with the r/k ratio representing the number of receptors at steady state. The half-life of receptor recovery ($t_{1/2}$) was obtained from the expression Ln2/k.

The value of ED_{50} (dose of haloperidol that produced half of the maximal σ_1 receptor inactivation) was calculated from the dose-response curve using non-linear regression analysis, so that the data could be fitted to a sigmoidal equation.

For multiple comparisons, the values in the control group were compared against those obtained in the treated groups using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. Further ANOVAs were used to compare parameters from linear regressions with the program Statgraphics Plus 5.1. (StatPoint Inc., Herdon, VA, USA). Parameters obtained from non-linear regressions 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. All results are given as the mean ± S.E.M. or the best-fit values ± standard error of regression.

3.4. RESULTS

3.4.1. Characteristics of [^{3}H](+)-pentazocine binding to guinea pig brain P_1, P_2 and P_3 subcellular fractions

Saturation assays showed that [^{3}H](+)-pentazocine bound in a saturable manner to only one population of specific binding sites in P_1, P_2 or P_3 subcellular fractions from guinea pig brain. Data were fitted by non-linear regression analysis to a hyperbolic equation (r^2 = 0.996, 0.997 or 0.998 for P_1, P_2 or P_3 fractions, respectively), and similar equilibrium dissociation constants (K_D) were obtained for all three subcellular fractions (3.046 ± 0.267 nM, 4.245 ± 0.322 nM or 3.610 ± 0.277 nM for P_1, P_2 or P_3 fractions respectively). However, the maximal number of receptors (B_{max}) was slightly higher in fraction P_3 than in fraction P_2 (2.937 ± 0.042 versus 2.582 ± 0.050 pmol/mg protein, p <

0.05), and was much higher in both these fractions than in fraction P_1 (0.927 ± 0.018 pmol/mg protein, $p < 0.001$) (Fig. 2A). Hill analysis yielded straight lines with slopes (n_H) very close to unity (0.998 ± 0.030, 0.979 ± 0.025 and 1.001 ± 0.023 for fraction P_1, P_2 and P_3 respectively), indicating a single population of binding sites in all fractions. Scatchard analysis of these results yielded straight lines, consistent with the existence of a single class of σ_1 binding site (Fig. 2B). The values of K_D and B_{max} (Table II) were close to those obtained with non-linear regression analysis.

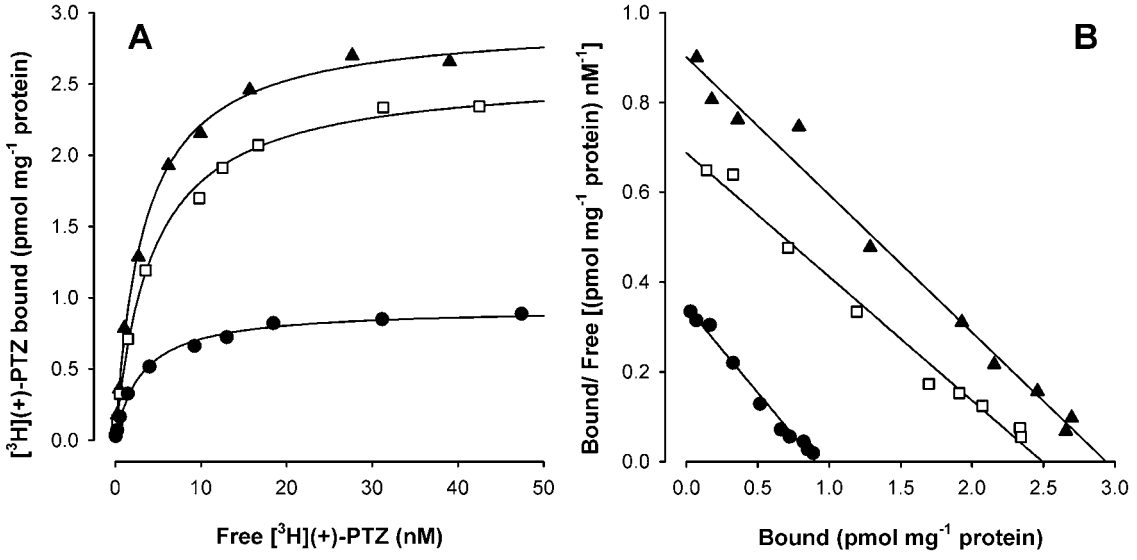

Figure 2. (A) [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) saturation assays in guinea pig brain subcellular fractions. P_1 fraction (●, 0.99 mg/ml protein), P_2 fraction (□, 0.51 mg/ml protein), and P_3 fraction (▲, 0.42 mg/ml protein) membranes were incubated for 240 min at 37 °C with several concentrations of [^{3}H](+)-PTZ (0.4–48 nM) and haloperidol 1 μM (to define non-specific binding) or its solvent. (B) Scatchard plots from saturation assays for P_1 (●), P_2 (□) and P_3 (▲) fractions. The data shown are representative of three experiments done in triplicate.

3.4.2. Effect of acute administration *in vivo* of σ_1 antagonists and (–)-sulpiride on [^{3}H](+)-pentazocine binding to guinea pig brain membranes

In animals killed three days after single i.p. injections of the σ_1 antagonists BD 1063 (60 mg/kg), BD 1047 (60 mg/kg) or NE-100 (30 mg/kg), the level of specific [^{3}H](+)-pentazocine binding in brain P_1, P_2 or P_3 subcellular fractions was not significantly different to control animals (Fig. 3.). By contrast, in animals killed three days after haloperidol administration (2 mg/kg, i.p.), there was a marked decrease in σ_1 binding sites labelled with [^{3}H](+)-pentazocine in all three subcellular fractions (Fig. 3).

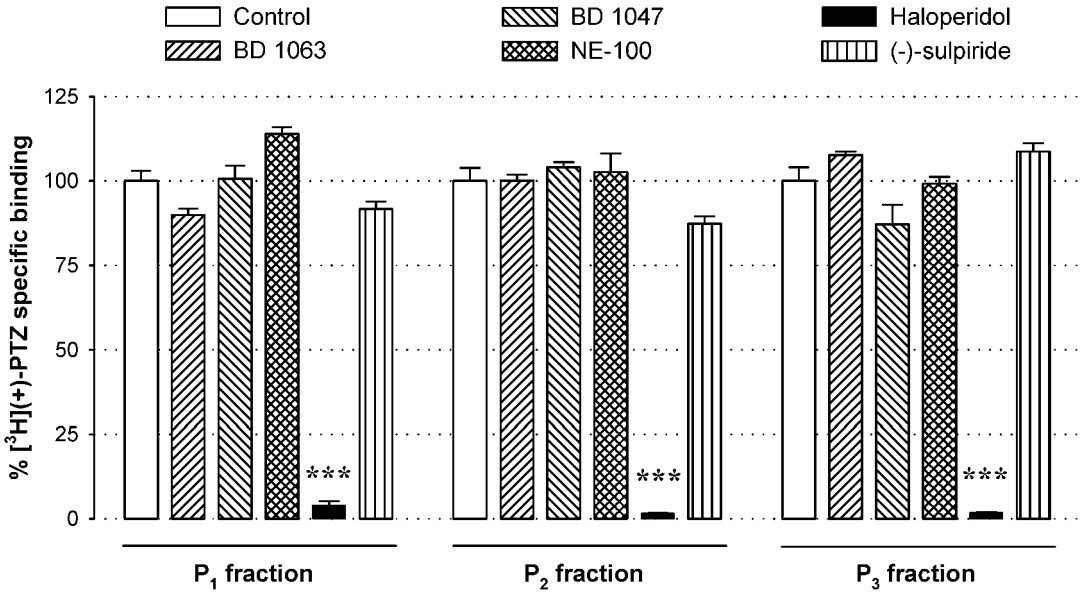

Figure 3. Comparison of [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) specific binding in P_1, P_2 and P_3 fractions obtained from brains of guinea pig treated three days previously with a single i.p. injection of BD 1063 (60 mg/kg), BD 1047 (60 mg/kg), NE-100 (30 mg/kg), haloperidol (2 mg/kg) or (-)-sulpiride (100 mg/kg). P_1 (0.66 – 1.08 mg/ml protein), P_2 (0.35 – 0.66 mg/ml protein) and P_3 (0.20 – 0.32 mg/ml protein) fractions were incubated at 37 °C with 20 nM [^{3}H](+)-PTZ for 240 min. Haloperidol 1 μM was used to define non-specific binding. Statistically significant differences between the solvent and drug-injected groups: *** $p < 0.001$ (one-way ANOVA followed by Newman-Keuls test). The data shown are the average of three experiments done in triplicate.

The effect of haloperidol was dose-dependent, and its ED_{50} to induce σ_1 receptor inactivation in fraction P_2 was 0.017 ± 0.002 mg/kg (Fig. 4.). On the other hand, [^{3}H](+)-pentazocine binding assays performed in P_1, P_2 and P_3 brain subcellular fractions from guinea pigs treated with a single injection of (–)-sulpiride (100 mg/kg, i.p.), a known D_2/D_3 antagonist whose affinity for these receptors is similar to that of haloperidol (Freedman et al., 1994), yielded values that did not differ significantly from the values obtained in control assays (Fig. 3).

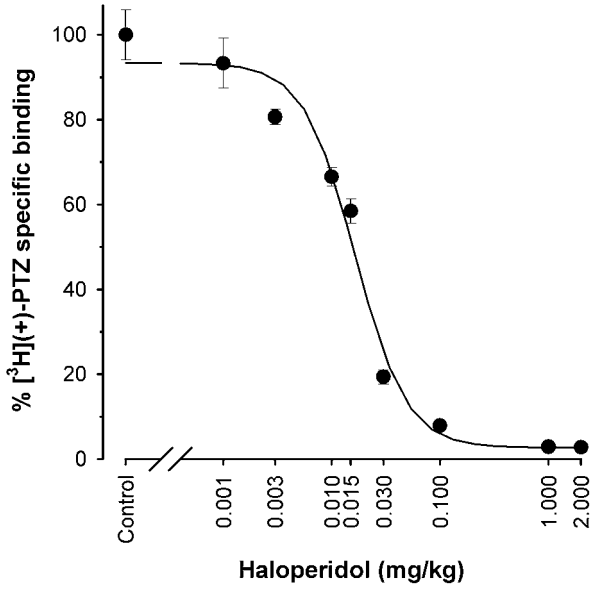

Figure 4. Specific binding of [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) in guinea pig brain P_2 fraction membranes obtained three days after the i.p. administration of single injections with different doses of haloperidol. Membranes (0.38 – 0.43 mg/ml protein) were incubated at 37 °C with 0.5 nM [^{3}H](+)-PTZ for 240 min. Haloperidol 1 μM was used to define non-specific binding. The data shown are the average of three experiments performed in triplicate. The values of [^{3}H](+)-PTZ binding obtained in animals treated with doses of 0.003 and 0.01 – 2 mg/kg of haloperidol were significantly different ($p < 0.01$ and $p < 0.001$, respectively) from the control value (one-way ANOVA followed by Newman-Keuls test).

3.4.3. Recovery of σ_1-receptor density in guinea pig brain membranes after *in vivo* haloperidol-induced receptor inactivation

The initial loss of brain σ_1 binding sites induced by the acute administration of haloperidol (2 mg/kg, i.p.) was followed by a steady, time-dependent recovery (Fig. 5). Experiments using a single saturating concentration of [^{3}H](+)-pentazocine (40 nM)

provided an estimate of the B_{max} values for analysis of the exponential recovery function (Barturen and García-Sevilla, 1992) (Fig. 5 A, B and C), whose parameters are summarized in Table I.

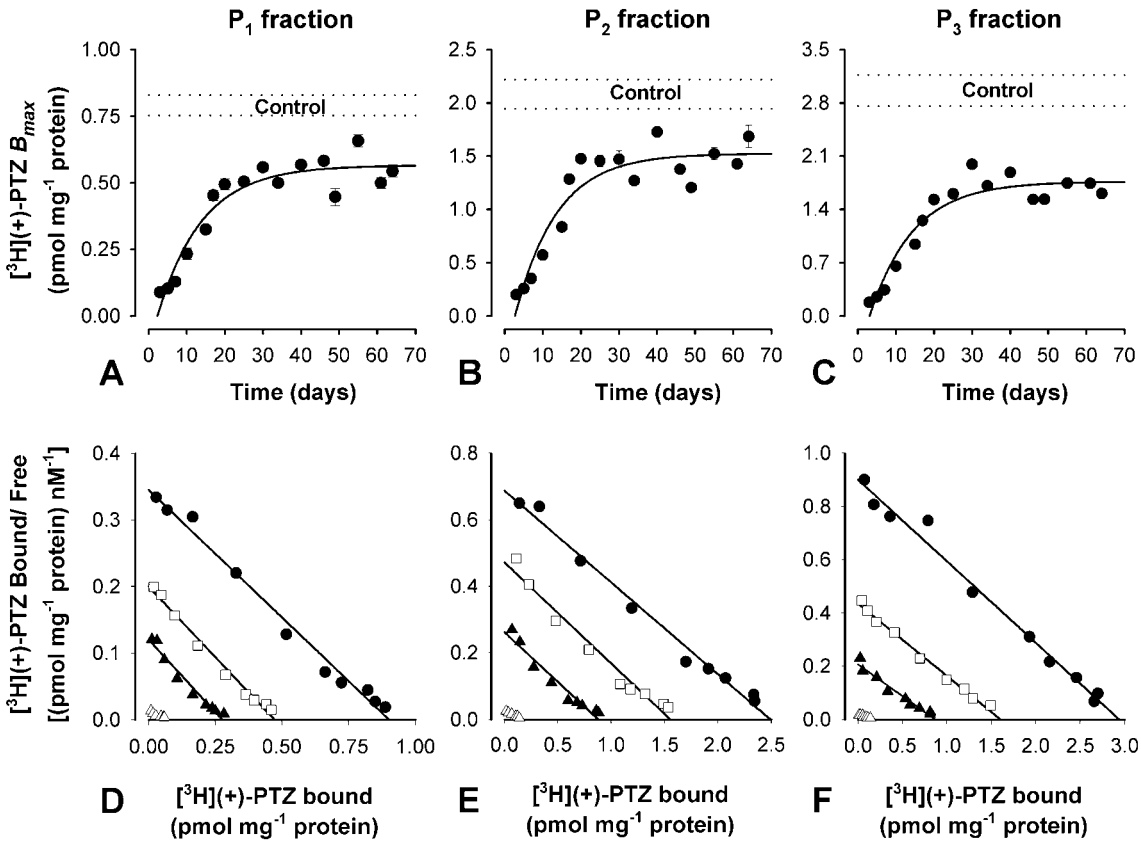

Figure 5. Time course of the recovery of σ_1 receptor density after inactivation by haloperidol (2 mg/kg, i.p.) in the P_1 (A), P_2 (B) and P_3 (C) subcellular fractions of guinea pig brain. The B_{max} values were determined with a single saturating concentration of [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ, 40 nM), which was incubated at 37 °C for 240 min with guinea pig brain membranes. Haloperidol 1 µM was used to define non-specific binding. The dashed lines represent the B_{max} mean ± standard error values of 5 control animals. The turnover parameters obtained from the non-linear regression to an exponential rise-to-maximum equation are summarized in Table 1. Scatchard analysis from saturation experiments in the P_1 (D), P_2 (E) and P_3 (F) subcellular fractions of guinea pig brain for the control group (●), and for animals killed 5 (△), 15 (▲) and 25 (□) days after the acute administration of haloperidol (2 mg/kg, i.p.). K_D and B_{max} values obtained are summarized in Table 2.

The disappearance rate constant (k), and, therefore, the apparent half-life ($t_{1/2}$), were very similar in all three subcellular fractions ($t_{1/2}$ = 8.116 ± 1.834; 7.559 ± 1.929 and 7.886 ± 1.678 days for fractions P_1, P_2 and P_3, respectively). On the other hand, the appearance rate constant (r) was significantly lower in fraction P_1 in comparison to fractions P_2 or P_3, which did not differ significantly (Table I). The steady-state levels of σ_1 receptors estimated from recovery curves (r/k) in the different fractions were P1 << P2 < P3 (Table I). In all cases these values were lower than the original steady-state concentration observed in untreated guinea pigs (Fig. 5A, B and C).

Table I. Turnover parameters of σ_1 receptor labelled with [^{3}H](+)-pentazocine in guinea pig brain subcellular fractions

	Turnover parameters		
Subcellular Fraction	***r*** (pmol mg^{-1} of protein) day^{-1}	***k*** day^{-1}	***r/k*** pmol mg^{-1} of protein
P_1	0.048 ± 0.009[a]	0.085 ± 0.019	0.565 ± 0.028[a]
P_2	0.140 ± 0.031	0.092 ± 0.023	1.524 ± 0.086[b]
P_3	0.155 ± 0.028	0.088 ± 0.019	1.764 ± 0.088

Guinea pigs were injected with a single dose of haloperidol (2 mg/kg, i.p.) and killed after intervals of 3 to 64 days to assess the reappearance of specific binding of [^{3}H](+)-pentazocine to brain membranes. Receptor turnover parameters were calculated from data shown in Figure 5 A, B and C. Parameters were calculated from non-linear regression analysis according to the equation $R_t = r/k(1-e^{-kt}) + R_0e^{-kt}$ where r is the appearance rate constant, k is the disappearance rate constant and r/k is the number of receptors at steady state. The results for subcellular fractions were compared by goodness of fit of simultaneous analyses with and without a set of constraints (same or different r, k and r/k values) with the F test (see text for details). [a] $p < 0.001$, compared to P_2 and P_3 subcellular fractions (F test); [b] $p < 0.05$, compared to P_3 subcellular fraction (F test)

Several saturation assays were performed in the different subcellular fractions to assess the possible variations in affinity (K_D) and the number of populations of binding sites (n_H) of the radioligand at different recovery times (5, 15 and 25 days after haloperidol administration). Hill coefficients (n_H) were close to one (0.904 – 1.001) in the control group and at all different recovery times in fractions P_1, P_2 or P_3. Scatchard analysis of these results yielded straight lines, consistent with the existence of a single class of σ_1 binding site (Fig. 5 D, E and F). Parameters from these analysis are summarized in Table II; the K_D for the control groups did not differ significantly in comparison to the K_D for the assays performed at different recovery times. It is interesting to note that the B_{max} values calculated with the Scatchard analysis (Table II) and with a single saturating concentration of [^{3}H](+)-pentazocine were very similar (Fig. 5 A, B and C).

Table II. Affinity and number of σ_1 receptors labelled with [^{3}H](+)-pentazocine in P_1, P_2 and P_3 fractions obtained from guinea pig brain after acute administration of haloperidol

		P_1 Fraction	**P_2 Fraction**	**P_3 Fraction**
Control	K_D	2.612 ± 0.104	3.627 ± 0.158	3.263 ± 0.134
	B_{max}	0.902 ± 0.043	2.494 ± 0.130	2.940 ± 0.141
Days after haloperidol administration (2 mg/kg)				
5 days		Negligible binding	Negligible binding	Negligible binding
15 days	K_D	2.300 ± 0.172	3.353 ± 0.300	4,196 ± 0,329
	B_{max}	0.277 ± 0.025[a]	0.880 ± 0.095[a]	0.870 ± 0,080[a]
25 days	K_D	2.364 ± 0.104	3.299 ± 0.225	3.685 ± 0.138
	B_{max}	0.504 ± 0,007[a]	1.454 ± 0.054[a]	1.604 ± 0.058[a]

The equilibrium dissociation constant values (K_D, nM) and maximal number of [^{3}H](+)-pentazocine binding sites (B_{max}, pmol of radioligand bound/mg of protein) were calculated from Scatchard analysis of data from saturation experiments. The values for control animals and those killed 15 and 25 days after administration of haloperidol (2 mg/kg, i.p.) were compared by ANOVA. [a] $p < 0.001$, compared with control animals (ANOVA)

3.4.4. Comparison of drugs affinity for σ_1 receptors labelled with [^{3}H](+)-pentazocine in guinea pig brain fraction P_2 and SH-SY5Y neuroblastoma cells

We used competition binding assays to compare the affinity of unlabelled drugs for the σ_1 receptor labelled with [^{3}H](+)-pentazocine in the guinea pig brain P_2 fraction and SH-SY5Y cells. The unlabelled ligands tested inhibited, in a concentration-dependent way, the binding of [^{3}H](+)-pentazocine in guinea pig brain P_2 fraction and in SH-SY5Y neuroblastoma cells with the following order of potency: haloperidol >

haloperidol metabolite II > BD 1063 >> haloperidol metabolite I > metaphit > MPP^+ > (-)-sulpiride > haloperidol metabolite III, which was inactive (Fig. 6 and Table III).

The order of affinity of the different ligands for [^{3}H](+)-pentazocine binding sites in guinea pig brain P_2 fraction agrees with their order of affinity for binding sites labelled with [^{3}H](+)-pentazocine in SH-SY5Y neuroblastoma cells ($r^2 = 0.991$) (Fig. 6 C). These results suggest that the radioligand labels the same receptor in guinea pig brain synaptosomal membranes and SH-SY5Y human neuroblastoma cell preparations.

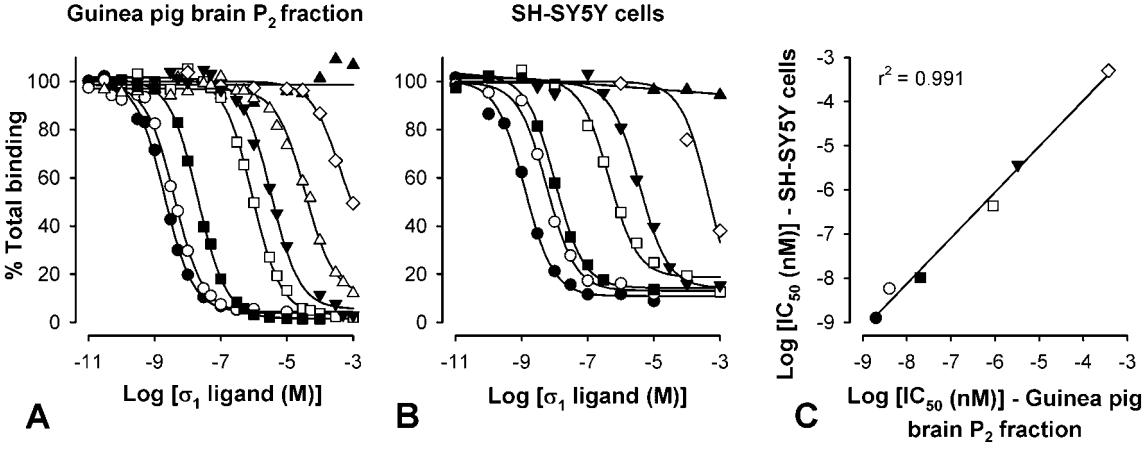

Figure 6. Inhibition by unlabelled σ_1 ligands of [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) binding to P_2 fraction obtained from guinea pig brain P_2 fraction (A) and to SH-SY5Y human neuroblastoma cells (B). [^{3}H](+)-pentazocine (0.5 nM for guinea pig brain P_2 fraction or 5 nM for SH-SY5Y cells assays) was incubated at 37 ºC for 240 min with guinea pig brain membranes or for 180 min with SH-SY5Y whole cell preparations, and increasing concentrations of haloperidol (●), haloperidol metabolite II (○), BD 1063 (■), haloperidol metabolite I (□), metaphit (▼), MPP^+ (△), (–)-sulpiride (◇) and haloperidol metabolite III (▲). Data shown are the average of at least three experiments carried out in triplicate. (C) Correlation between drug affinities measured as IC_{50} (showed in Table 3) for [^{3}H](+)-PTZ binding sites in SH-SY5Y neuroblastoma cells and guinea pig brain P_2 fraction.

Table III. IC_{50} of several cold drugs for [^{3}H](+)-pentazocine binding sites in the P_2 fraction from guinea pig brain and SH-SY5Y human neuroblastoma cells

Drug	IC_{50} (nM)	
	Guinea pig brain P_2 fraction	**SH-SY5Y neuroblastoma**
Haloperidol	1.98 ± 0.11	1.25 ± 0.16
Haloperidol metabolite II	4.04 ± 0.31	5.78 ± 0.76
BD 1063	20.40 ± 0.75	10.34 ± 1.60
Haloperidol metabolite I	900.09 ± 54.03	443.19 ± 78.85
Metaphit	3292.06 ± 251.18	3720.46 ± 532.59
MPP^+	35716.24 ± 3638.77	not determined
(–)-Sulpiride	387410.92 ± 104402.08	513396.52 ± 121203.38
Haloperidol metabolite III	negligible	negligible

The IC_{50} (concentration of unlabelled drug that inhibited 50% of specific [^{3}H](+)-pentazocine binding) was estimated from the inhibition curves with non-linear regression analysis of the equation for a sigmoid plot, assuming one-site competition.

3.4.5. Reversibility by washing of the binding of haloperidol and its metabolites in guinea pig brain P_2 fraction and SH-SY5Y cells

To determine whether haloperidol *per se* or any of its metabolites with affinity for [^{3}H](+)-pentazocine binding site (metabolites I and II) were responsible for the inactivation of σ_1 receptor observed *in vivo,* we tested *in vitro* whether the binding of these drugs to σ_1 receptor was reversible by washing in the guinea pig brain P_2 fraction and in SH-SY5Y cells. We also evaluated the effect of MPP^+, a structural analogue of haloperidol pyridinium metabolites. We used BD 1063 and metaphit as controls for

reversible and an irreversible σ_1 ligand, respectively. All drugs were used at a concentration 50-fold higher that their IC_{50} for [^{3}H](+)-pentazocine binding to obtain an equivalently high degree of inhibition of radioligand binding. After preincubation (90 min) of the guinea pig brain P_2 fraction with BD 1063, haloperidol, haloperidol metabolite I and MPP^+, and washing of the membranes (as described in the section *[^{3}H](+)-pentazocine binding assays in guinea pig membranes),* [^{3}H](+)-pentazocine maintained its ability to bind to σ_1 receptors to the same degree as in control membranes incubated with the drug solvent (Fig. 7). By contrast, preincubation with haloperidol metabolite II and metaphit produced a marked and statistically significant wash-resistant inhibition of [^{3}H](+)-pentazocine binding (61.417 ± 6.594% and 52.017 ± 8.524% inhibition, respectively) (Fig. 7).

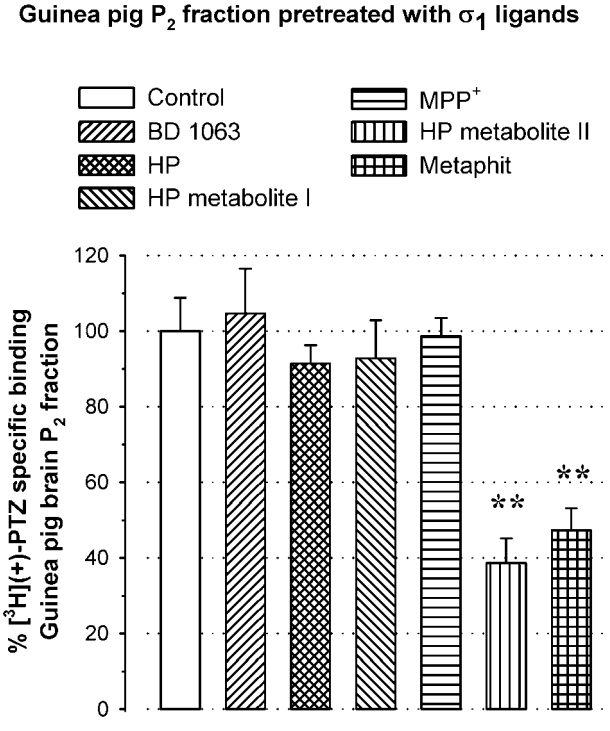

Figure 7. [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) specific binding to guinea pig brain P_2 fraction after *in vitro* pre-treatment with several σ_1 cold ligands. Brain membranes (0.31 – 0.47 mg/ml protein) were incubated for 90 min with a high concentration (50 times the IC_{50} for [^{3}H](+)-PTZ binding) of the following σ_1 ligands: BD 1063, haloperidol (HP), HP metabolite I, MPP^+, HP metabolite II, and metaphit. Then they were washed as described in *Material and methods* and incubated with [^{3}H](+)-pentazocine (0.5 nM) at 37 °C for 240 min. Statistically significant differences between the solvent and drug-treated groups: ** $p < 0.01$ (one-way ANOVA followed by Newman-Keuls test). Data shown are the average of three experiments carried out in triplicate.

We also performed similar experiments in SH-SY5Y human neuroblastoma cells, which were incubated with several ligands at a concentration 50-fold higher that their IC_{50} for σ_1 receptors in these cells. After 90 min of preincubation with BD 1063 and haloperidol metabolite I, and subsequent washing of the samples (as described in the section *[^{3}H](+)-pentazocine binding assays in SH-SY5Y neuroblastoma cells)*, [^{3}H](+)-pentazocine maintained its ability to bind to σ_1 receptors to the same degree as in control cells, whereas the preincubation with haloperidol and haloperidol metabolite II produced a washing-resistant inhibition of [^{3}H](+)-pentazocine binding (29.738 ± 1.252% and 57.394% ± 2.246% of inhibition, respectively) (Fig. 8). The ability of metaphit to bind irreversibly to σ_1 receptors in SH-SY5Y cells could not be studied because after 90 min of preincubation with this ligand the cells became spherical and died.

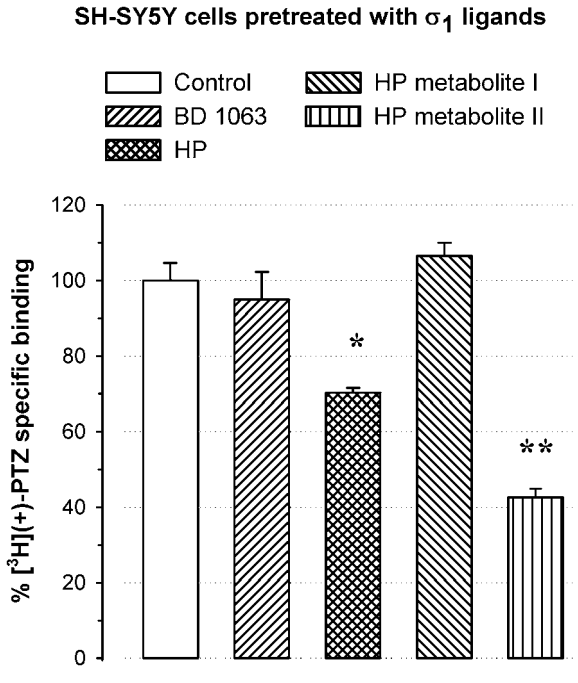

Figure 8. [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) specific binding to SH-SY5Y neuroblastoma cells after *in vitro* pre-treatment with several σ_1 cold ligands. Cell homogenates (approximately 0.175 mg/ml protein) were incubated for 90 min with a high concentration (50 times the IC_{50} for [^{3}H](+)-PTZ binding) of the following σ_1 ligands: BD 1063, haloperidol (HP), HP metabolite I and HP metabolite II. Then they were washed as described in Material and methods and incubated with [^{3}H](+)-pentazocine (5 nM) at 37 °C for 180 min. Statistically significant differences between the solvent and drug-treated groups: * $p < 0.05$; ** $p < 0.01$ (one-way ANOVA followed by Newman-Keuls test). Data shown are the average of three experiments carried out in triplicate.

3.4.6. Reversibility by washing of the binding of haloperidol and its metabolites to fraction P_2 obtained from guinea pig brain homogenates

To test whether the irreversible inactivation of σ_1 receptors induced by haloperidol in SH-SY5Y cells and the absence of this inactivation in guinea pig brain fraction P_2 was due to the inability to metabolize haloperidol in guinea pig brain P_2 membranes, we performed similar experiments in guinea pig brain homogenate. After 90 min of preincubation of guinea pig brain homogenates with cold σ_1 ligands (at a concentration 50 times higher than their IC_{50} for [^{3}H]-(+)-pentazocine specific binding), homogenate aliquots were washed to obtain P_2 fraction as described in the section *[^{3}H](+)-pentazocine binding assays in guinea pig membranes*. In these experiments [^{3}H](+)-pentazocine maintained its ability to bind to σ_1 receptors to the same degree as in control assays in samples preincubated with BD 1063 and haloperidol metabolite I (Fig. 9A). By contrast, preincubation with haloperidol, haloperidol metabolite II and metaphit markedly inhibited [^{3}H](+)-pentazocine binding (40.147 ± 4.823%, 76.766 ± 7.834% and 54.212 ± 3.637% of inhibition respectively; $p < 0.01$ in all cases) (Fig. 9A). Because NADPH is a cofactor necessary in most metabolic processes involving haloperidol (Usuki et al., 1998), we added 1 mM NADPH to the incubation medium with the σ_1 ligands that bind irreversibly to the [^{3}H](+)-pentazocine binding site. There were no statistically significant differences in comparison to the results obtained in the absence of NADPH (Fig. 9 B). Moreover, we found non-statistically significant differences ($p > 0.05$; one-way ANOVA) in the inhibition of [^{3}H](+)-pentazocine binding induced by haloperidol metabolite II preincubation in guinea pig brain P_2

fraction (Fig. 7) in comparison to the inhibition seen in homogenates not supplemented or supplemented with NADPH (Fig. 9).

We also tested the effect of menadione (an inhibitor of the haloperidol ketone reductase activity, which leads to the formation of haloperidol metabolite II or reduced haloperidol, see Fig. 1) on the inhibition of [^{3}H](+)-pentazocine binding induced by different drugs in guinea pig brain homogenate. Menadione (0.5 mM) produced a statistically significant ($P < 0.05$) reversion of the ability of haloperidol to irreversibly inactivate [^{3}H](+)-pentazocine binding sites, and a highly significant ($P < 0.01$) reversion of the effect of haloperidol in the presence of NADPH (Fig. 9B). By contrast, menadione did not statistically modify the irreversible inactivation of σ_1 receptors produced by haloperidol metabolite II and metaphit (Fig. 9 B).

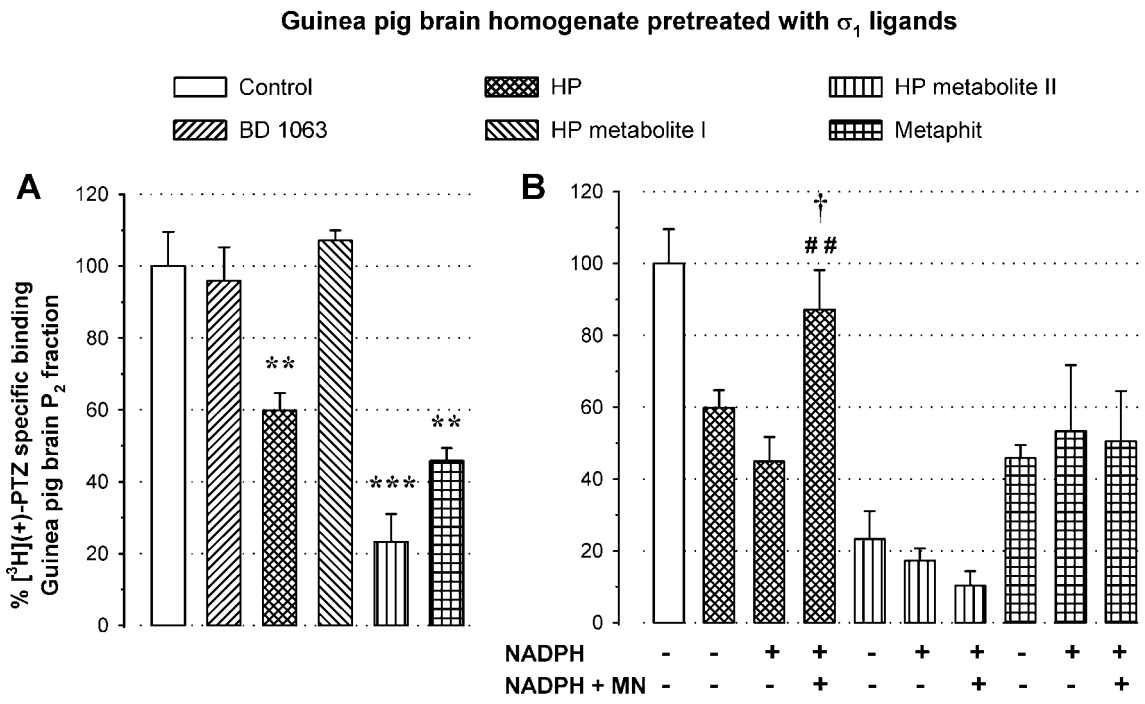

Figure 9. [^{3}H](+)-pentazocine ([^{3}H](+)-PTZ) specific binding to washed P_2 fraction obtained from guinea pig brain homogenates preincubated with several σ_1 cold ligands. (A) Guinea pig brain homogenates were incubated with high concentrations (50 times the IC_{50} for [^{3}H](+)-PTZ binding) of BD 1063, haloperidol (HP), HP metabolite I, HP metabolite II, and metaphit, and homogenates were washed to obtain P_2 fractions as described in Material and methods. Brain P_2 fraction (0.30 – 0.66 mg/ml protein) was incubated with [^{3}H](+)-pentazocine (0.5 nM) at 37 °C for 240 min. Statistically significant differences between the solvent and drug-treated groups: ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA followed by Newman-Keuls test). (B) Guinea pig brain homogenates were incubated with HP, HP metabolite II and metaphit in the presence or absence of NADPH 1 mM and menadione (MN, 0.5 mM), then P_2 fractions were obtained and incubated with [^{3}H](+)-pentazocine as before. Statistically significant differences were found between the groups pre-treated with σ_1 cold ligands + NADPH + menadione versus the groups pre-treated with σ_1 cold ligands + NADPH (## $p < 0.01$) or with no pre-treatment († $p < 0.05$) (one-way ANOVA followed by Newman-Keuls test). Data shown are the average of at least three experiments carried out in triplicate.

3.5. DISCUSSION

We found that [^{3}H](+)-pentazocine binds to only one population of σ_1 receptors, with similar affinity but different B_{max}, in guinea pig brain P_1, P_2 and P_3 subcellular

fractions. Receptors were more abundant in microsomal than in synaptosomal or nuclear membranes, as previously reported (Itzhak et al., 1991; DeHaven-Hudkins et al., 1994). A single injection of haloperidol produced long-lasting inhibition of [^{3}H](+)-pentazocine binding in all subcellular fractions. This effect was not shared by other σ_1 antagonists (BD 1047, BD 1063 and NE-100) with high affinity for σ_1 receptors (Cobos et al., 2005 and 2006) or by (-)-sulpiride, a D_2/D_3 receptor antagonist (Freedman et al., 1994).

We took advantage of this exclusive characteristic of haloperidol to study the turnover of σ_1 receptors in P_1, P_2 and P_3 fractions, where we found that the recovery of σ_1 receptors was slow in all three subcellular fractions. The receptor appearance rate constant (*r*) denoted a multistep process comprising both the synthesis and insertion of receptors into the membranes. Our results indicate that the microsomal fraction is occupied the most rapidly, which is expected considering that σ_1 receptors have a putative signal for retention in the endoplasmic reticulum (Hanner et al., 1996). The *r* value for synaptosomal (P_2) membranes was close to that for microsomal membrane receptors, whereas nuclear membrane receptors were occupied only one-third as rapidly as receptors in the other fractions. As expected, the disappearance rate constant (*k*), indicative of the receptor degradation rate, was not altered in any subcellular fraction. The steady-state levels of σ_1 receptors estimated from recovery curves (*r/k*) were lower than the original steady-state concentration. This has been described previously for other receptors such as D_2 (Norman et al., 1987), 5-HT_2 (Battaglia et al., 1987) and α_2-adrenoceptors (Barturen and García-Sevilla, 1992) after their irreversible inactivation by EEDQ. These results were explained by hypothesizing a subpopulation of receptors subject to very slow metabolism or even exempt from turnover, but our

results show that [^{3}H](+)-pentazocine binds to only one population in both control and treated animals ($n_H \approx 1$). Another explanation proposed was that treatment with an irreversible agent modified the kinetics of the processes that determine receptor steady-state levels. Whatever the reason, our results demonstrate that σ_1-receptor levels are lower in all three subcellular fractions 64 days after a single administration of haloperidol. New experiments will be needed to determine the functional implications of these findings, since the recovery of function after receptor inactivation does not necessarily correlate at a 1:1 proportion with the percentage of recovered receptors (Pineda et al., 1997).

To assess whether haloperidol by itself, one of its metabolites or both together produced the irreversible blockade of σ_1 receptors, we performed experiments *in vitro* in guinea pig brain and SH-SY5Y cells. The affinity of several drugs for σ_1 receptors in guinea pig brain P_2 fraction and SH-SY5Y human neuroblastoma cells correlated well ($r^2 = 0.991$), which suggests that guinea pigs and humans have pharmacologically equivalent σ_1 receptors. The affinity of haloperidol and its metabolites for guinea pig brain σ_1 receptors agrees with the data of Matsumoto and Pouw (2000), but no such studies have previously been performed in SH-SY5Y cells, although these cells express σ_1 receptors that bind other σ_1 ligands (Hong and Werling, 2002; Hong et al., 2004). When guinea pig brain P_2 membranes were washed after preincubation with haloperidol, BD 1063 or haloperidol metabolite I, we found no differences in [^{3}H](+)-pentazocine binding in comparison to controls. By contrast, the same experiments showed that metaphit, a known irreversible σ_1 ligand (Bluth et al., 1989), and reduced haloperidol produced a marked and wash-resistant inhibition of [^{3}H](+)-pentazocine binding. These data indicate that haloperidol (1) is not able *per se*

to irreversibly block σ_1 receptors, (2) is probably metabolized *in vivo* to a reactive compound that irreversibly inactivates σ_1 receptors, and (3) is not metabolized *in vitro* in our P_2 fraction preparation to this putative reactive compound.

In SH-SY5Y cells and in brain homogenates, as in P_2 fraction experiments, BD 1063 and haloperidol metabolite I reversibly inhibited [^{3}H](+)-pentazocine binding, whereas reduced haloperidol and metaphit induced wash-resistant inhibition. However, in contrast to our results in the P_2 fraction, haloperidol irreversibly inhibited [^{3}H](+)-pentazocine binding in SH-SY5Y cells and brain homogenate, which indicates that haloperidol is converted *in vitro* in these preparations to a metabolite able to irreversibly block σ_1 receptors. As described in the *Introduction* section, cytochrome P450 (mainly CYP3A4), located in microsomes, produces haloperidol metabolites I and III, whereas cytosolic ketone reductase activity leads to the formation of haloperidol metabolite II. CYP3A4 is also responsible for the formation of the pyridinium metabolites and the back-oxidation of reduced haloperidol to haloperidol. Considering that haloperidol is probably metabolized to irreversibly inactivate σ_1 receptors, removal of the crude cytosol fraction (which includes cytosolic and microsomal proteins) in the process of obtaining the P_2 membranes could explain the absence of effect of haloperidol in these samples. Haloperidol metabolites I and III induced reversible inhibition of [^{3}H](+)-pentazocine binding (metabolite I) or had no affinity for σ_1 receptors (metabolite III). Consequently, they can not explain the irreversible inactivation of these receptors.

We did not test the effect of pyridinium metabolites of haloperidol, but several findings suggest that they are probably not the haloperidol derivatives that irreversibly inactivate σ_1 receptors. Firstly, MPP^+, a structural analogue of haloperidol pyridinium

derivatives, has affinity for σ_1 receptors but did not produce wash-resistant inhibition of [^{3}H](+)-pentazocine binding, which indicates that the presence of the pyridinium does not necessarily induce the irreversible blockade of the σ_1 receptors. Secondly, the rate of HPP^+ formation from haloperidol by rodent brain homogenates or human liver microsomes *in vitro* is very slow (Igarashi et al., 1995; Fang et al., 2001) and insufficient to explain our results. In fact, the brain concentration of HPP^+ after a single dose of haloperidol is one hundred times lower than that of haloperidol (Igarashi et al., 1995). By contrast, reduced haloperidol is highly concentrated (2–10 times more than haloperidol) in different brain areas after haloperidol administration to guinea pigs (Usuki et al., 1998). Interestingly, the haloperidol-induced σ_1-receptor inactivation in brain homogenate was completely reversed by menadione, an inhibitor of cytosolic ketone reductase activity (Usuki et al., 1998; Eyles and Pond, 1992). This suggests that haloperidol is metabolized to reduced haloperidol to produce long-lasting inactivation of σ_1 receptors. The effect of menadione against the effect of haloperidol in brain homogenates appears to be specific, since menadione did not modify the effects of metaphit or reduced haloperidol. In previous studies haloperidol in the brain was not reduced in the absence of NADPH, whereas in blood, haloperidol reduction was evident, was unaffected by the addition of NADPH, and was completely inhibited by menadione (Eyles and Pond, 1992). Thus the ketone reductase activity seen in our brain homogenate preparation may be at least partially due to the blood contained in the brain homogenate.

Several additional facts suggest that reduced haloperidol *per se* is the main metabolite responsible for the irreversible inactivation of σ_1 receptors induced by haloperidol. Firstly, preincubation of P_2 fraction membranes, brain homogenates and

SH-SY5Y cells with reduced haloperidol produced a wash-resistant inhibition of [^{3}H](+)-pentazocine binding, as did metaphit, a known irreversible blocker of σ_1 receptors (Bluth et al., 1989). Secondly, the effect of haloperidol in guinea pig brain homogenates, but not that of reduced haloperidol, was reversed by menadione. In addition, our results suggest that metabolites of reduced haloperidol are not involved in the irreversible inactivation of σ_1 receptors. The metabolism of reduced haloperidol to its pyridinium derivatives was produced mainly by CYP3A4, which is located in microsomes, but the inhibition of [^{3}H](+)-pentazocine binding produced by reduced haloperidol in brain P_2 fraction (which has no CYP450 activity) and brain homogenate was similar.

These findings may have therapeutic implications. (1) The inactivation of σ_1 receptors *in vivo* by the administration of haloperidol was produced at doses ($\geq$ 0.003 mg/kg) used in therapeutics in humans. In fact, the density of brain σ receptors was diminished in patients treated with haloperidol, but not in patients treated with phenothiazine antipsychotics (Reynolds et al., 1991). (2) The formation of reduced haloperidol occurs in humans (Usuki et al., 1988), and this compound has high affinity (in the nanomolar range) for σ_1 receptors (Matsumoto and Pouw, 2000 and present study), acting as an irreversible antagonist (present study). (3) Antagonists of σ_1 receptors in pre-clinical studies were effective in blocking the acute toxicity and the rewarding effects of cocaine (Maurice et al., 2002), and as antinociceptive agents (Guitart et al., 2004; Cendan et al., 2005a). Therefore a single administration of haloperidol seems able to induce long-lasting σ_1. antagonism in humans and animal species able to reduce haloperidol, and would be useful to offer prolonged protection against the adverse effects of cocaine and nociception.

In conclusion, our results show that haloperidol, through its metabolism to reduced haloperidol, irreversibly inactivates σ_1 receptors in guinea pigs and humans. Additional studies should be done to evaluate the functional consequences of this effect.

3.6. REFERENCES

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

CONCLUSIONS

SPECIFIC CONCLUSIONS

1. Phenytoin (DPH) increases the affinity of several agonist ligands for σ_1 receptors (labelled with [^{3}H](+)-pentazocine) in synaptosomal fraction from guinea pig brain. In contrast, DPH does not enhance, and may even decrease, the affinity of σ_1 receptor antagonists for such receptors.

2. Although [^{3}H](+)-pentazocine and [^{3}H]NE-100 label the same receptor in the crude synaptosomal fraction from guinea pig brain, DPH differentially modulates the binding (measured as affinity, B_{max} and dissociation rate constant) of both radioligands to σ_1 receptors.

3. A single administration of haloperidol *in vivo* induces irreversible inactivation of guinea pig brain σ_1 receptors in the crude nuclear (P_1), crude synaptosomal/mitochondrial (P_2) and microsomal (P_3) fractions. This effect is produced at doses used in therapeutics in humans, exhibits dose-dependency, and is not shared by other dopaminergic or σ_1 receptor antagonists.

4. The turnover of guinea pig brain σ_1 receptors is a very slow process in all subcellular fraction studied (P_1, P_2 or P_3 fractions). The rate of disappearance is similar in all three subcellular fractions, whereas the rate of appearance is significantly lower in nuclear membranes than in synaptosomal and microsomal membranes. The steady-state levels of σ_1 receptors, after their recovery from inactivation by a single administration of haloperidol, are lower than the original steady-state concentration in all subcellular fractions studied.

5. The σ_1 receptors in guinea pig brain P_2 fraction and in SH-SY5Y human neuroblastoma cells bind several σ_1 ligands with the same order of potency, which suggests that guinea pig and human σ_1 receptors are pharmacologically equivalent.

6. Pre-treatment *in vitro* with haloperidol induces irreversible inactivation of σ_1 receptors in guinea pig brain homogenate and SH-SY5Y human neuroblastoma cells, but not in guinea pig brain P_2 fraction, which suggests that haloperidol irreversibly inactivates σ_1 receptors through one of its metabolites produced by cytosolic or microsomal enzymes.

7. The irreversible blockade of σ_1 receptors induced by incubation *in vitro* with haloperidol of guinea pig brain homogenate can be prevented by menadione, an inhibitor of cytosolic ketone-reductase activity that leads to the production of haloperidol metabolite II (reduced haloperidol). In addition, reduced haloperidol induces irreversible inactivation of σ_1 receptors not only in guinea pig brain homogenate and SH-SY5Y human neuroblastoma cells, but also in guinea pig brain P_2 fraction. These results suggest that haloperidol irreversibly inactivates σ_1 receptors through its metabolism to reduced haloperidol.

GENERAL CONCLUSIONS

1. The differential modulation by phenytoin (DPH) of σ_1 ligand binding to guinea pig brain membranes seems a reliable *in vitro* method to predict the pharmacological profile of newly synthesized σ_1 compounds.

2. The administration of haloperidol to animal species able to metabolize it to reduced haloperidol (such as guinea pigs and humans) is expected to irreversibly inactivate σ_1 receptors, an effect which could have functional consequences of therapeutic interest.

DEFINITIONS OF RADIOLIGAND BINDING PARAMETERS

B: specific radioligand binding.

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

ED_{50}: dose of drug that produced half of the maximal effect.

F: free concentration of radioligand.

IC_{50}: concentration of unlabelled drug that inhibited 50% of radioligand-specific binding, obtained from competition experiments.

k: disappearance rate constant of receptors after their irreversible inactivation.

k_{-1}: radioligand dissociation rate constant, obtained from radioligand dissociation assays.

k_{obs}: k observed, obtained from radioligand association assays. Related to the association rate constant.

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.

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

n'_H: pseudo-Hill coefficient, obtained from linear transformation of competition assay data. Indicative of the population of binding sites bound by the cold ligand.

r: appearance rate constant of receptors after their irreversible inactivation.

R_0: number of receptors at time 0 after their irreversible inactivation.

R_t: number of receptors at a given discrete time after their irreversible inactivation.

$t_{1/2}$: half-life of receptor recovery after their irreversible inactivation.

LIST OF ABBREVIATIONS

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

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

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

5-HT: serotonine

Allopregnenolone: 3-hydroxy-5-pregnan-20-one

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

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

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

BDK: bradikynin

BDNF: brain-derived neurotrophic factor

BIMU-8: *endo*-*N*- 8-methyl-8-azabicyclo[3.2.1.]oct-3-yl) -2,3-dihydro-(1-methyl)ethyl-2-oxo-1*H*-benzimidazole-1-carboxamidehydrochloride

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

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

CNS: central nervous system

CO: carbon monoxide

CPP: 3-((±)2-carboxypiperazin- 4-yl)-propyl-1 phosphonic acid

CYP: cytochrome P450

DADLE: Tyr-D-Ala-Gly-Phe-D-Leu

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

D-AP5: (−)-2-amino-5-phosphonopentanoic acid

DAT: dopamine transporter

DHEA: dehydroepiandrosterone

DHEAS: dehydroepiandrosterone sulfate

Dizocilpine: MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cicloheptен5,10-imine

DMSO: dimethyl sufoxide

DOPAC: 3,4-dihydroxyphenylacetic acid

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

DPH: phenytoin

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

GABA: γ-aminobutyric acid

GDP$_\beta$S: M guanosine 5'-*O*-(2-thiodiphosphate)

Glu: glutamate

Gö-6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo [2,3-a] pyrrolo [3,4-c] carbazole

Gpp(NH)p: 5'-guanylylimidodiphosphate

GTPγS: guanosine-5'-*O*-γ-thio-triphosphate

HP: Haloperidol

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

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

Haloperidol metabolite III: *p*-fluorobenzoylpropionic acid

HPP^+: 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium;

HIV: human immunodeficiency virus

HVA: homovanillic acid

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

i.t.: intrathecal

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

L 687-384: 1-benzylspiro(1,2,3,4-tetrahydronaphthalene-1,4-piperidine)

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

Lu28-179: 1'-[4-[1-94-fluorophenyl)-1*H*-indol-3-yl]-1-butyl]spiro[isobenzofuran-1(3*H*), 4'-piperidine]

LY379196: 5,21:12,17-dimetheno-18H-dibenzo[i,o]pyrrolo[3,4-1][1,8] diacyclohexadecine-8,20(19H)-dione,8-[(dimethylamino)methyl]-6,7,8,9,10,11-hexahydro-monomethanesulfonate (9Cl)

MAO: monoamino oxidase

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

mEPSCs: miniature excitatory postsynaptic currents

MN: menadione

MPP^+, *N*-methyl-4-phenylpyridinium dihydrochloride;

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

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

NalBzoH: naloxone benzoylhydrozone

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

Nor-BNI: nor-binaltorphimine

NPY: neuropeptide Y

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

OBX: olfactory bulbectomy

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

P_1 fraction: crude nuclear fraction

P_2 fraction: crude synaptosomal/ mitochondrial fraction

P_3 fraction: microsomal fraction

Panamesin: EMD 57445, (*S*)-(-)-[4-hydroxy-4-(3,4-benzodioxol-5-yl)-piperidin-1-ylmethyl]-3-(4-methoxyphenyl) oxazolidin-2-one

PCA: *p*-chloroampheramine

PCP: phencyclidine

PKC: protein kinase C

PKA: protein kinase A

PLC: phospholipase C

PRE 084: 2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate

(+)-PTZ: (+)-pentazocine

$RHPP^+$: 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]pyridinium

RS-23597-190: 3-(piperidine-1-yl)propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride

σ_1: $sigma_1$

σ_2: $sigma_2$

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

SAM: senescence-accelerated

s.c.: subcutaneous

SSRIs: Selective serotonin reuptake inhibitors

SKF-10,047: *N*-allylnormetazocine

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

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

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

U-73,122: 1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexy]-1H-pyrrole-2,5-dione

WAY 100635: *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide

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

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