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FACULTAD DE MEDICINA

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NEUROPATHIC PAIN INDUCED BY PACLITAXEL: ROLE OF VOLTAGE-GATED SODIUM CHANNELS AND SIGMA-1 (σ_1) RECEPTORS

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“La ciencia es el alma de la prosperidad de las naciones y la fuente de todo progreso”

Louis Pasteur

INDEX

RESUMEN

1. ANTECEDENTES Y OBJETIVOS

La aparición de dolor en pacientes cancerosos se debe tanto a la enfermedad como a algunos de los tratamientos que reciben. La mayoría de los fármacos antineoplásicos más eficaces (taxanos, alcaloides de la *Vinca* y derivados del platino) producen neuropatías periféricas que se caracterizan por una disfunción sensorial y dolor, que con frecuencia son factores limitantes del tratamiento (Sul y Deangelis, 2006; Park et al., 2008; Paice, 2010). La incidencia y gravedad de la neuropatía dolorosa depende, entre otros factores, del agente antineoplásico, de la pauta de administración y de la dosis acumulada (Gutiérrez-Gutiérrez et al., 2010). Estos fármacos antineoplásicos son el tratamiento de elección para un gran número de tumores sólidos y linfoides, por lo que cientos de miles de pacientes son afectados por estas neuropatías cada año, lo que constituye un grave problema para el que todavía no hay soluciones efectivas (Cleeland et al., 2010; Gutiérrez-Gutiérrez et al., 2010).

Dentro del grupo de los taxanos, el paclitaxel (Taxol®) es uno de los fármacos antineoplásicos más usados para el tratamiento del cáncer de mama y de ovario, entre otros (Balayssac et al., 2011; Argyriou et al., 2008). Los pacientes tratados con paclitaxel con frecuencia desarrollan una neuropatía sensorial que usualmente comienza de forma simétrica en los pies o en los pies y en las manos (con una distribución en forma de guante-calcetín), y que se manifiesta por parestesias, sensación de entumecimiento y hormigueo y dolor neuropático, (Dougherty et al., 2004; Jung et al., 2005; Argyriou et al., 2008). Desgraciadamente, no hay tratamientos sintomáticos o preventivos clínicamente eficaces (Argyriou et al., 2008), aunque se han desarrollado modelos de neuropatías inducidas por paclitaxel en roedores, que hacen posible probar tratamientos experimentales y clarificar su patogénesis (por ejemplo: Dina et al., 2001;

Flatters and Bennett, 2004; Liu et al., 2010). Estos modelos experimentales han demostrado que la administración repetida o aguda de paclitaxel a roedores produce hiperalgesia y/o alodinia a estímulos fríos, mecánicos y/o calientes (revisado en Authier et al., 2009). La mayoría de los modelos de esta neuropatía descritos previamente se habían caracterizado en ratas macho; sin embargo, el paclitaxel es fundamentalmente usado en mujeres (Rowinsky, 2010). Además, gran parte de los modelos de modificación genética para eliminar o sobreexpresar una determinada diana se desarrollan en ratones. Por tanto, el **objetivo general** de esta Tesis Doctoral fue desarrollar un modelo experimental de dolor neuropático inducido por paclitaxel en ratones hembra, y evaluar diferentes dianas terapéuticas potenciales en este modelo (mediante diversos abordajes experimentales, tales como estudios comportamentales y morfológicos).

Los canales de sodio dependientes de voltaje juegan un papel clave en la función neuronal en condiciones fisiológicas y patológicas (revisado por Dib-Hajj et al., 2010; Liu y Wood, 2011). Se han descrito alteraciones en la expresión de algunos de estos canales en neuronas sensoriales que contribuyen al dolor inflamatorio y neuropático (Lai et al., 2004; Wood et al., 2004; Amir et al., 2006). En concreto, se ha descrito que los canales de sodio sensibles a tetrodotoxina (TTX) están aumentados en neuronas nociceptivas de los ganglios de las raíces dorsales (Dib-Hajj et al. 1999; Black et al., 1999; Kim et al., 2001; Hong et al., 2004), del asta dorsal de la médula espinal (Hains et al., 2003, 2004) y núcleos talámicos (Zhao et al., 2006) en diferentes modelos de dolor neuropático. Esta regulación al alza de los canales de sodio sensibles a TTX produce cambios electrofisiológicos, como una respuesta exagerada de las neuronas a ciertos estímulos, y cambios comportamentales, como un incremento del dolor (Rizzo et al.,

1995; Cummins and Waxman, 1997; Black et al., 1999; Hains et al., 2004; Zhao et al., 2006). Por tanto, podría ser de interés terapéutico para controlar el dolor neuropático un fármaco que pudiera bloquear selectivamente estos subtipos de canales de sodio, y la TTX tiene este perfil.

La administración de TTX reduce las descargas ectópicas espontáneas y el dolor neuropático en ciertos modelos experimentales de ligadura de nervio periférico (Omana-Zapata et al., 1997; Lyu et al., 2000; Liu et al., 2001; Xie et al., 2005; Marcil et al., 2006). Sin embargo, la contribución de los canales de sodio sensibles a TTX, a la neuropatía dolorosa inducida por antineoplásicos nunca había sido investigada. En consecuencia, el **primer objetivo específico** de esta Tesis Doctoral, fue examinar el efecto del bloqueo de los canales de sodio sensibles a TTX en el dolor neuropático inducido por paclitaxel. Para ello, se evaluaron los efectos de la administración sistémica de dosis bajas de TTX en la *expresión* del dolor neuropático inducido por paclitaxel, una vez que la neuropatía estaba plenamente instaurada en los ratones. Además, puesto que hay precedentes de que el bloqueo de los canales de sodio durante la fase de inducción del dolor neuropático (en modelos de lesión mecánica), podría modificar el desarrollo de la neuropatía (Xie et al., 2005), también se probó el efecto preventivo de TTX, administrándola durante la fase de *desarrollo* del dolor neuropático inducido por paclitaxel.

Se han caracterizado dos subtipos de receptores sigma (σ), denominados σ_1 y σ_2, aunque solo el receptor σ_1 ha sido clonado en humanos y roedores (revisado por Cobos et al., 2008; Maurice y Su, 2009). Los receptores σ_1 están ampliamente distribuidos en el sistema nervioso central, incluidas áreas importantes para el procesamiento del dolor (Alonso et al., 2000; Kitaichi et al., 2000). Actualmente hay numerosas evidencias de

que los receptores σ_1 participan en la modulación del dolor (Cendán et al., 2005a; Cendán et al., 2005b; Kim et al., 2006; Entrena et al., 2009a; Entrena et al., 2009b), especialmente cuando participa el proceso de sensibilización central (Drews and Zimmer, 2009). Recientemente, se ha demostrado que los ratones knockout σ_1 (σ_1-KO) y que ratas tratadas con un antagonista σ_1, tienen reducido el dolor neuropático inducido por lesión mecánica del nervio ciático, en paralelo con una reducción de la fosforilación de la subunidad 1 del receptor de NMDA y de la fosforilación de ERK en el asta dorsal de la médula espinal (Roh et al., 2008; De la Puente et al., 2009), que son eventos importantes en el proceso de sensibilización central (Latremoliere and Woolf, 2009).

Por tanto, los receptores σ_1 parecen tener un papel relevante en el proceso de sensibilización central y en ciertos modelos de dolor neuropático. Sin embargo, hasta el momento, ningún estudio ha evaluado el papel del receptor σ_1 en ciertas neuropatías dolorosas de alta prevalencia como las neuropatías inducidas por la diabetes, por una infección por el virus del herpes o por fármacos antineoplásicos. En consecuencia, el **segundo objetivo específico** de esta Tesis Doctoral fue comprobar el papel del receptor σ_1 en el dolor neuropático inducido por el antineoplásico paclitaxel. Para conseguir este objetivo, se evaluó si la administración sistémica de antagonistas del receptor σ_1 era capaz de inhibir la expresión de los signos del dolor neuropático inducido por paclitaxel. Además, puesto que el bloqueo farmacológico o la inactivación genética del receptor σ_1, impide el desarrollo de dolor neuropático inducido por lesión mecánica nerviosa, también evaluamos si los antagonistas σ_1 administrados preventivamente o los animales sin el receptor σ_1 (σ_1-KO) desarrollaban el dolor neuropático inducido por paclitaxel.

En los últimos años, se han hecho grandes progresos en el conocimiento de la fisiología del receptor σ_1. Hoy se sabe que este receptor es una chaperona regulada por ligando, que se localiza en el retículo endoplasmático (Hayashi y Su, 2007) y se ha propuesto que es un modulador de varios receptores y canales iónicos (Maurice and Su, 2009). Normalmente el receptor σ_1 se localiza en una región del retículo endoplasmático relacionada funcionalmente con la mitocondria (mitochondrion-associated endoplasmic reticulum membrane, MAM), donde realiza su función de chaperona, ayudando al plegamiento de ciertas proteínas, con lo que modula el nivel del Ca^{2+} intramitocondrial, jugando un papel clave en el control de la homeostasis del Ca^{2+} celular (Su et al., 2010).

Por otro lado, se ha descrito un incremento en la incidencia de mitocondrias hinchadas y/o vacuolizadas en nervios periféricos sensoriales, que parecen ser relevantes para la patogénesis del dolor neuropático inducido por paclitaxel (Flatters y Bennett, 2006; Jin et al., 2008; Bennett, 2010). Se ha sugerido que tal incremento de mitocondrias atípicas se debe a la unión del paclitaxel con la β-tubulina asociada a la mitocondria, lo que produciría la liberación del Ca^{2+} almacenado en la misma y en consecuencia un desajuste de la homeostasis del Ca^{2+} celular (Flatters y Bennett, 2006; Siau y Bennett, 2006). Cabe destacar que alteraciones en la función mitocondrial y/o en los niveles del Ca^{2+} intracelular pueden afectar la excitabilidad de la membrana, la liberación de neurotransmisores y otros eventos celulares, que podrían contribuir a la neuropatía diabética (Verkhratsky y Fernyhough, 2008) y a otras neuropatías periféricas (Fernyhough y Calcutt, 2010).

Dado que los receptores σ_1 participan en la regulación del Ca^{2+} mitocondrial y celular, parece interesante investigar si los efectos beneficiosos del bloqueo de los receptores σ_1 en el dolor neuropático inducido por paclitaxel están asociados a una

reducción de las atipias mitocondriales inducidas por el antineoplásico, lo cual constituye el **tercer objetivo específico** de esta Tesis Doctoral. Para alcanzar dicho objetivo, se examinaron, con microscopía electrónica de transmisión, las características de las mitocondrias de los nervios safenos en días claves del curso temporal del dolor neuropático inducido por paclitaxel. Además, se estudió si el bloqueo farmacológico (antagonistas σ_1) o la inactivación genética (ratones σ_1-KO) del receptor σ_1, podrían prevenir los cambios mitocondriales inducidos por la administración de paclitaxel.

2. MÉTODOS

2.1. Animales de experimentación

Todos los experimentos fueron realizados en ratones hembra de la cepa CD-1 (Charles River, Barcelona, España) con un peso de 25-30 g. Para obtener los ratones σ_1-KO de la cepa CD-1, los animales previamente generados por Langa y colaboradores (2003) fueron cruzados durante 10 generaciones con ratones CD-1 puros, asegurando de esta manera una pureza de la cepa CD-1 de al menos un 99% (Wong et al., 2002); los ratones portadores de la mutación fueron posteriormente cruzados hasta obtener individuos knockout homocigotos, los cuales fueron utilizados en el curso de esta Tesis Doctoral. Los ratones fueron manipulados de acuerdo con la Directiva del Consejo de Comunidades Europeas de 24 de Noviembre de 1986 (86/609/ECC). El protocolo experimental fue aprobado por el Comité de Ética en Investigación de la Universidad de Granada.

2.2. Experimentos *in vivo*

Modelo experimental de dolor neuropático inducido por paclitaxel: protocolo y consideraciones generales

El paclitaxel (Taxol®) fue preparado para obtener una solución madre de 6 mg/ml en un solvente compuesto por 50% de Cremophor EL y 50% de etanol absoluto. Esta solución de paclitaxel fue diluida en salino, inmediatamente antes de la administración, hasta una concentración final de 0,2 mg/ml. El vehículo del paclitaxel también fue diluido con salino, justo antes de la inyección, en la misma proporción que la solución de paclitaxel. El paclitaxel (2 mg/kg) fue administrado intraperitonealmente (i.p.), en un volumen de 10 ml/kg, una vez al día durante cinco días consecutivos. Por tanto, la dosis acumulada fue de 10 mg/kg por ratón, dosis que producía una neuropatía dolorosa en ratas en otros estudios (Dina et al., 2001, 2004). En el grupo control el vehículo del paclitaxel fue administrado igualmente en un volumen de 10 ml/kg, una vez al día durante cinco días sucesivos.

El procedimiento general para comprobar que el paclitaxel producía dolor neuropático fue el siguiente. Se tomaron respuestas basales de cada animal en cada test nociceptivo, antes del comienzo del tratamiento con el antineoplásico o su vehículo (medidas pretratamiento). A continuación, 3 días después de las medidas pretratamiento, los animales recibieron una inyección al día de paclitaxel o su vehículo durante 5 días consecutivos (fase de tratamiento). Posteriormente, la respuesta de cada animal en cada test nociceptivo fue nuevamente evaluada (medidas postratamiento), durante 2 ó 3 semanas (dependiendo del test) después del tratamiento con el

antineoplásico o su vehículo. En los experimentos en los que se emplearon ratones σ_1-KO se siguió el mismo procedimiento.

Los efectos de la administración subcutánea (s.c.) del bloqueante de los canales de sodio dependientes de voltaje TTX, y de los antagonistas selectivos del receptor σ_1 (BD-1063 y S1RA) en el dolor neuropático inducido por paclitaxel fueron examinados de dos formas diferentes. Para evaluar el efecto de estos fármacos en el *desarrollo* del dolor inducido por paclitaxel, los animales recibieron 30 minutos antes de cada inyección i.p. de paclitaxel una inyección s.c. de TTX, BD-1063, S1RA o salino. Posteriormente, se evaluaron las manifestaciones de dolor neuropático (alodinia mecánica, alodinia al frío y/o hiperalgesia al calor) inducidas por paclitaxel durante las 2-3 semanas posteriores (dependiendo del test nociceptivo) a la administración del antineoplásico. Cada animal fue evaluado solamente en un único modelo de dolor y fue tratado con un solo fármaco. Para evaluar el efecto de TTX, BD-1063, S1RA o salino en la *expresión* del dolor inducido por paclitaxel, se realizó una sola inyección de uno de estos fármacos el día de máximo efecto de la expresión de cada uno de los signos del dolor neuropático evaluados (ver resultados para detalles). En este caso, se evaluó al animal antes y varias veces después, durante 3 horas, de la administración de cada uno de los fármacos en estudio. Cada animal recibió una única dosis de fármaco y fue evaluado en un sólo modelo.

Los animales que fueron utilizados para los estudios morfológicos con microscopía electrónica, también fueron sometidos a los test comportamentales, para cerciorarse de que habían desarrollado alodinia antes de proceder al sacrificio y obtención de las muestras para el estudio histológico. En estos experimentos, se siguió estrictamente la metodología usada para el resto de experimentos, con la única

excepción de que los test comportamentales usados (test de Von Frey y test de acetona) fueron realizados en los mismos animales y que las medidas postratamiento, fueron realizadas los días 7, 10 y 28.

Procedimiento para la valoración de la alodinia al frío

La evaluación de la alodinia al frío fue realizada mediante el método de la acetona que describió previamente Smith et. al., (2004). El método consiste en aplicar una gota de acetona en la piel de la planta de las patas traseras, mediante una jeringa conectada a un tubo de polietileno, y evaluar la respuesta del animal. Los animales fueron alojados (30 minutos antes de la evaluación) en los compartimentos donde se iban a realizar los experimentos, para que pudieran habituarse a ellos. Los compartimentos de evaluación tenían paredes de plástico transparente con un suelo de rejilla metálica. Después de este periodo de habituación, la acetona fue aplicada tres veces de forma alternativa en cada pata trasera a intervalos de 30 segundos. En cada una de las 6 medidas, se registró el tiempo de lamido o de mordisqueo de la pata estimulada mediante un cronómetro y se representó el tiempo acumulado del lamido o mordisqueo en las seis medidas realizadas. Dado que en nuestros experimentos previos el tiempo de lamido rara vez superaba los 10 segundos, se impuso un tiempo de corte de 10 segundos en cada aplicación de acetona. Para estudiar el curso temporal de la alodinia al frío inducida por paclitaxel, se realizaron medidas previas a la administración del antineoplásico (valor pretratamiento) y en días diferentes (días 7, 10, 14, 17, 21 y 24) después de la primera inyección de paclitaxel o su vehículo. Alrededor de un 33 % de los animales tratados con paclitaxel no mostraron alodinia al frío (ver resultados), por lo que en este test comportamental se diferenció entre animales ‘respondedores’ y ‘no respondedores’. Los ratones ‘no respondedores’ fueron fácilmente identificados debido

a que su tiempo de lamido/mordisqueo de la pata estimulada era inferior a 2 segundos en los días 7 y 10 después de la administración de paclitaxel. Cuando se valoró el efecto de los fármacos en estudio, en el desarrollo de la neuropatía inducida por paclitaxel se evaluaron todos los animales; sin embargo, los animales 'no respondedores' no fueron usados para estudiar el efecto de los fármacos en la expresión de la alodinia al frío ya que no expresaban la suficiente alodinia al frío como para identificar algún efecto antialodínico.

Como en los estudios de curso temporal se observó que el paclitaxel tenía su máximo efecto alodínico entre los días 10 y 14 después de la primera inyección (ver resultados), se decidió evaluar los efectos de la TTX en la expresión de la alodinia al frío el día 14. Por tanto, en estos animales se realizó una evaluación con acetona previa al tratamiento (tres días antes) y otra el día 14 postratamiento (de paclitaxel o su vehículo). Posteriormente, se trataron con TTX o su solvente (s.c.) y se registró la respuesta a la acetona de nuevo a los 60, 120 y 180 minutos después de la inyección. Para comprobar el efecto de los antagonistas σ_1 en la expresión del dolor neuropático inducido por paclitaxel, se realizó el mismo procedimiento descrito anteriormente para TTX, pero en este caso los animales se evaluaron el día 10. Este pequeño cambio de procedimiento se hizo con el objetivo de optimizar la duración de los experimentos, ya que realmente no existían diferencias en la expresión de la alodinia al frío entre el día 10 y 14. También se estudió el efecto de la TTX y de los antagonistas σ_1 en el desarrollo de la alodinia al frío. En este caso, se realizó la evaluación pretratamiento, tres días antes de la coadministración del paclitaxel con TTX, con los antagonistas σ_1 o con salino. Los fármacos o su solvente fueron inyectados 30 minutos antes de cada una de las cinco inyecciones de paclitaxel y posteriormente se evaluó el curso temporal de la alodinia al

frío inducida por paclitaxel los días 7, 10, 14, 17, 21 y 24 después de la primera inyección de paclitaxel o vehículo.

Procedimiento para la valoración de la alodinia mecánica

Para evaluar la alodinia mecánica, se registró el umbral de fuerza de retirada de la pata usando el aparato "Dynamic Plantar Aesthesiometer" (Ugo Basile, Italia) con ligeras modificaciones sobre el procedimiento previamente descrito (Kondo et al., 2006). El aparato electrónico de Von Frey utiliza un único filamento no flexible que es aplicado, en la planta de la pata trasera, con una fuerza que se va incrementado automáticamente (de 0 a 10 gramos, durante un periodo de 20 segundos). El reflejo de retirada hace que se corte el estímulo automáticamente y el valor de umbral mecánico es mostrado en una pantalla. El día del experimento, los ratones fueron colocados individualmente en los compartimentos de evaluación (con paredes de plástico y suelo de rejilla metálica) y se les dejó durante dos horas para que pudieran aclimatarse a ellos. Después de la habituación, cada ratón fue evaluado tres veces alternativas en cada pata trasera.

Para estudiar el curso temporal de la alodinia mecánica inducida por paclitaxel, los animales fueron evaluados previamente a la administración del paclitaxel (valor pretratamiento) y en diferentes días (días 7, 10, 14 y 17) después de la primera inyección de paclitaxel o su vehículo. La mayoría de los animales (96%) tratados con paclitaxel mostraron una reducción de su umbral doloroso mecánico; los animales que no mostraron alodinia mecánica no fueron usados para examinar el efecto de los fármacos en la expresión de la alodinia mecánica inducida por el antineoplásico. Como el día 10 fue el día que se observó un cambio máximo en el umbral mecánico, se evaluó este día el efecto del tratamiento agudo con los fármacos en la expresión de la alodinia

mecánica inducida por paclitaxel. Por tanto, el día 10, después del periodo de habituación al aparato, se midió el umbral mecánico y a continuación se inyectó s.c. el fármaco en estudio (TTX, antagonistas σ_1 o su solvente); posteriormente se midieron las latencias de retirada de la pata a los 30, 60, 90, 120 y 180 minutos, después de la inyección. Cuando se evaluó el efecto de los fármacos en el desarrollo de la alodinia mecánica inducida por paclitaxel, también se obtuvieron medidas pretratamiento tres días antes del tratamiento. En este caso, los fármacos o su solvente fueron inyectados s.c. 30 minutos antes de cada inyección diaria de paclitaxel o su vehículo (i.p.) durante 5 días. Las latencias postratamiento fueron evaluadas como se describió antes (días 7, 10, 14 y 17).

Procedimiento para la valoración de la hiperalgesia al calor

La hiperalgesia al calor fue evaluada usando el método de Hargreaves et al. (1988), ligeramente modificado. Los ratones fueron habituados en los compartimentos donde se iba a realizar la evaluación durante dos horas. Estos compartimentos individuales estaban hechos de plástico transparente y se colocaron encima de una superficie de cristal. Después del periodo de habituación, se procedió a realizar las medidas para examinar la hiperalgesia al calor usando el aparato "Plantar Test" (Ugo Basile, Italia); para ello se dirigía un foco de luz que producía calor hacia la planta de la pata trasera del animal hasta que retiraba la pata. El reflejo de retirada interrumpía la luz que se reflejaba desde la pata hasta una célula fotoeléctrica y automáticamente el aparato cortaba la luz y el contador de tiempo que se había activado al encender el foco luminoso. Por tanto, el valor de latencia para retirada de la pata (que indica el umbral doloroso al calor) era automáticamente recogido. La intensidad de la luz fue ajustada al comienzo de los experimentos para obtener un valor pretratamiento de latencia de

retirada promedio de aproximadamente 8 segundos. Esta intensidad no fue cambiada a lo largo del experimento. Cada ratón fue evaluado tres veces alternativamente en cada pata y posteriormente se promediaron las seis medidas. Se impuso un tiempo de corte de 16 segundos en cada evaluación y se dejó un minuto entre cada medida en la misma pata, con el objetivo de evitar lesiones al animal.

Para evaluar el curso temporal de la hiperalgesia inducida por paclitaxel, se obtuvieron latencias basales pretratamiento tres días antes del tratamiento con paclitaxel o su vehículo. Las latencias postratamiento se registraron en los días 7, 10, 14 y 17 después de la primera inyección de paclitaxel. Puesto que la máxima hiperalgesia al calor se obtuvo el día 7, consideramos este día como el más adecuado para examinar los efectos del tratamiento con TTX en la *expresión* de la hiperalgesia al calor inducida por paclitaxel. Por tanto, el día 7, después del periodo de evaluación, se obtuvieron latencias basales e inmediatamente después se inyectó s.c. la TTX o su solvente. Posteriormente se registraron las latencias de retirada de la pata a los 30, 60, 90, 120 y 180 minutos después de dicha inyección. Alrededor de un 9% de los animales tratados con paclitaxel no desarrollaron hiperalgesia al calor el día 7 después de la administración del paclitaxel. Estos animales no fueron usados para evaluar los efectos de la TTX en la expresión de la hiperalgesia al calor. Cuando se examinó el efecto del tratamiento con TTX en el *desarrollo* de la hiperalgesia al calor inducida por paclitaxel, también se obtuvieron latencias basales pretratamiento tres días antes del tratamiento con paclitaxel o su vehículo. En este caso, la TTX o su solvente fueron inyectados s.c. 30 minutos antes de cada inyección diaria de paclitaxel o su vehículo (i.p.) durante 5 días consecutivos. Las latencias postratamiento fueron evaluadas en los días 7, 10, 14 y 17 después de la primera inyección de paclitaxel.

Procedimiento para la valoración de la incoordinación motora

Para comprobar que los fármacos en estudio no producían alteraciones motoras que pudieran dar lugar a falsos positivos en los test de dolor, se estudió si dichos fármacos producían incoordinación motora con una aparato denominado Rotarod (Cibertec, España), como se describió previamente (Patel et al., 2001), con ligeras modificaciones. Los animales se colocaban encima de un cilindro que va incrementando su velocidad de 4 a 40 revoluciones por minuto, durante 5 minutos. Cuando el animal se cae, al no poder permanecer encima del cilindro, el tiempo de latencia de permanencia queda registrado automáticamente. Con el objetivo de acostumbrar al animal al aparato, 24 horas antes del test con los fármacos, los animales recibieron 3 sesiones de entrenamiento, separadas por 30 minutos. El día del ensayo con los fármacos, antes del tratamiento se tomaban medidas basales (tiempo 0), posteriormente se inyectaba el fármaco o su solvente y a continuación se realizaban varias medidas a lo largo del tiempo (30, 60, 90, 120 y 180 minutos después de la inyección). Se estableció un tiempo de corte de 300 segundos en cada medida.

2.3. Experimentos *in vitro*

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

Para estudiar las características de la unión de BD-1063 y S1RA a los receptores σ_1 realizamos ensayos de fijación de radioligando, marcando los receptores σ_1 con [^{3}H](+)-pentazocina (un radioligando selectivo del receptor σ_1). Los experimentos se realizaron en fracción sinaptosomal cruda (fracción P2) de cerebro completo de ratón, obtenida siguiendo el protocolo descrito por Entrena y cols. (2009a; b). Para estudiar la afinidad por los receptores σ_1 de cerebro de ratón de los ligandos σ_1 evaluados en los

experimentos *in vivo*, se realizaron ensayos de competición con el radioligando. Estos ensayos fueron realizados usando los protocolos previamente descritos (Entrena et al., 2009a; b). Brevemente, las suspensiones de membranas cerebrales (en una concentración final de proteína de 0.79-1.10 mg/ml) fueron incubadas con [^{3}H](+)-pentazocina 5 nM y con el fármaco frío (a varias concentraciones 10^{-10}-10^{-5} M) o su solvente, a 30 ºC, pH 8, durante 240 minutos. Para determinar si los ligandos σ_1 tenían una interacción competitiva o no competitiva con el receptor σ_1 se usó un protocolo previamente descrito (Bowen et al., 1989; Brammer et al., 2006), con ligeras modificaciones. Así, los estudios de saturación con [^{3}H](+)-pentazocina (0.06-33 nM) se realizaron en ausencia o en presencia de concentraciones fijas de los ligandos en estudio (12.5 nM para BD-1063 y 75 nM para S1RA). Estas concentraciones fueron seleccionadas (a partir de los datos de los estudios de competición) para obtener aproximadamente el mismo desplazamiento de [^{3}H](+)-pentazocina. La fijación no específica fue definida como la fijación retenida en presencia de 10 μM de haloperidol. La reacción se detuvo con 5 ml de Tris 10 mM pH 7,4 enfriado en hielo, y el radioligando unido fue separado del libre mediante filtración con un harvester M-12 T (Brandel Instruments, SEMAT Technical Ltd., Reino Unido). Los filtrados de las muestras se realizaron a través de filtros de fibra de vidrio Whatman GF/B (SEMAT Technical Ltd., Reino Unido), previamente humedecidos con una solución de polietilenimina al 5% durante una hora. Posteriormente la radioactividad contenida en los filtros se midió en un contador de centelleo líquido (Beckman Coulter España S.A.).

Estudio ultraestructural con microscopía electrónica de transmisión

Para evaluar la morfología ultraestructural de los nervios safenos, los animales fueron anestesiados con isofluorano y perfundidos intracardiacamente con suero salino

seguido de una solución de glutaraldehído al 2% y paraformaldehído al 1%, en tampón fosfato 0.1 M, pH 7.4 durante 15 minutos. Posteriormente se procedió a la disección de los nervios safenos derecho e izquierdo, tomando aproximadamente 4-5 mm de la parte media de los mismos, y se procedió como se expone a continuación, de acuerdo con las pautas de procesamiento establecidas previamente por Flatters y Bennett (2006), con ligeras modificaciones.

La muestra de nervio safeno se mantuvo en la solución fijadora durante la noche a 4 ºC. Después de la fijación, las muestras fueron transferidas a una solución de sucrosa al 10%, en tampón fosfato 0.1 M, pH 7.4 durante 24 h a 4 ºC. A continuación se realizó una posfijación en tetróxido de osmio al 1% en tampón fosfato 0.1 M, pH 7.4, conteniendo ferrocianuro potásico al 1%, durante 60 minutos a 4 ºC, en oscuridad. Posteriormente, la muestra fue deshidratada en una serie graduada de alcoholes e incrustada en resina Epoxi. A continuación se realizaron cortes transversales semifinos (1 μm), los cuales fueron teñidos con azul de toluidina y examinados con microscopia óptica para comprobar la calidad de la muestra. Posteriormente se realizaron cortes ultrafinos (70 nm) con un ultramicrotomo y se montaron las muestras sobre rejillas para microscopia electrónica de transmisión, realizándose el contrastado de las mismas mediante citrato de plomo y acetato de uranilo. Las secciones ultrafinas fueron examinadas en un microscopio electrónico de transmisión equipado con una cámara monocroma con la que se obtuvieron las fotografías. Las medidas morfométricas se realizaron con el programa ImageJ (http://rsb.info.nih.gov/ij/index.html), con el que se analizaron los siguientes parámetros: área (A), perímetro (P), circularidad ($4\pi[A/P^2]$, el valor 1 indica un círculo perfecto) y el diámetro de feret (la distancia más larga entre 2 puntos cualquiera a lo largo de los límites seleccionados).

Para llevar a cabo el análisis ultraestructural y morfométrico se tomaron imágenes de 30 axones mielínicos de cada nervio safeno de cada ratón (15 de safeno derecho y 15 del izquierdo). Los cortes ultrafinos se estudiaron a diferentes aumentos: 4400x, 7000x y 20000x. A **4400x**, se delimitó el contorno de las fibras mielínicas mediante ImageJ (Fig. A) y se midió su diámetro de feret, lo que permitió clasificarlas en fibras tipo Aβ (6-12 µm) y Aδ (< 6 µm) (Fig. B) (Gardner et al., 2000).

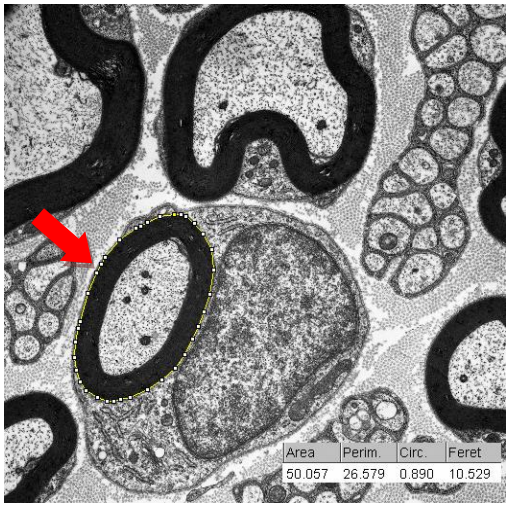

Fig. A. Delimitación del contorno de una fibra mielínica (4400 X).

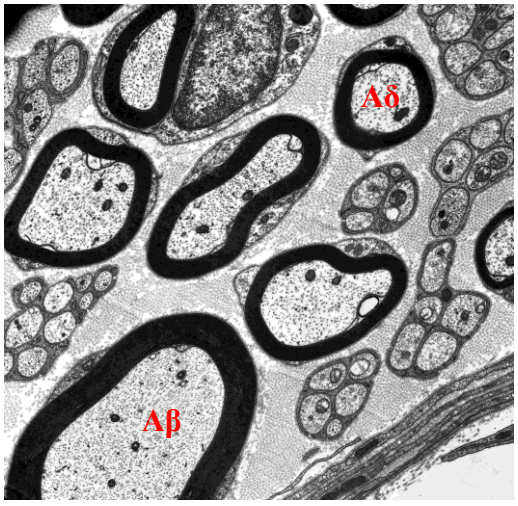

Fig. B. Fibras mielínicas Aβ y Aδ de nervio safeno (4400 X).

Las fibras amielínicas (fibras C) fueron identificadas a **7000x** de aumento. Al igual que con las fibras mielínicas se tomaron imágenes de al menos 30 axones amielínicos de cada ratón (Fig. C).

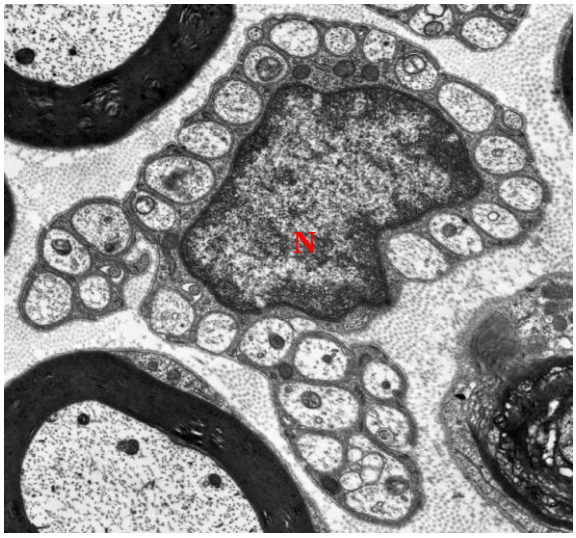

Fig.C. Fibras amielínicas de nervio safeno situadas alrededor del núcleo (N) de una célula de Schwam (7000 X)

El análisis morfométrico del área, perímetro, circularidad y diámetro de feret de las mitocondrias de las fibras mielínicas (Fig. D) y amielínicas (Fig. E) fue realizado a **20000x** de aumento. Para ello, se delimitó el contorno de cada una de las mitocondrias mediante ImageJ (Fig. F) y se calcularon los distintos parámetros. Sin embargo el análisis detallado de los datos se realizó exclusivamente con los valores del área, puesto que el resto de parámetros están estrechamente relacionados al área y fueron afectados por los tratamientos del mismo modo.

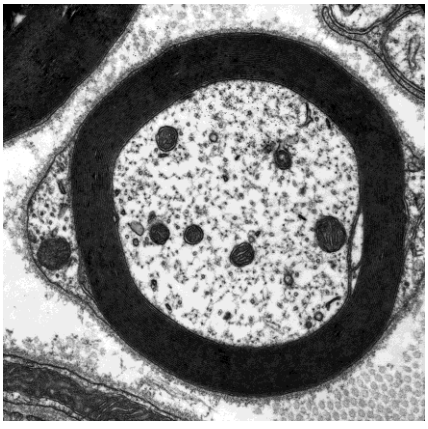

Fig. D. Fibras mielínicas (20000 X)

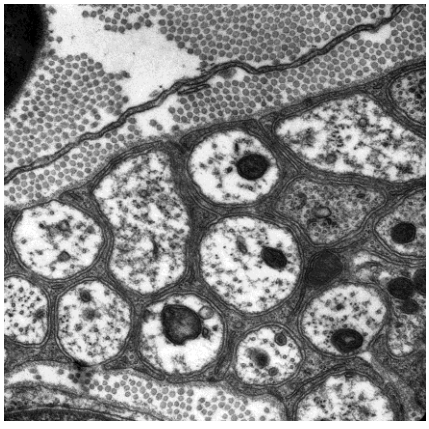

Fig. E. Fibras amielínicas (20000 X)

El análisis del área mitocondrial (n=10679) de los animales controles (incluidos los ratones σ_1-KO) permite distinguir mitocondrias de tamaños muy variados y que nosotros clasificamos en 3 grupos: pequeñas (área $\leq 9 \times 10^{-2}$ μm^2), medianas (9,1-18 x 10^{-2} μm^2) y grandes (>18 x 10^{-2} μm^2) (ver sección de resultados).

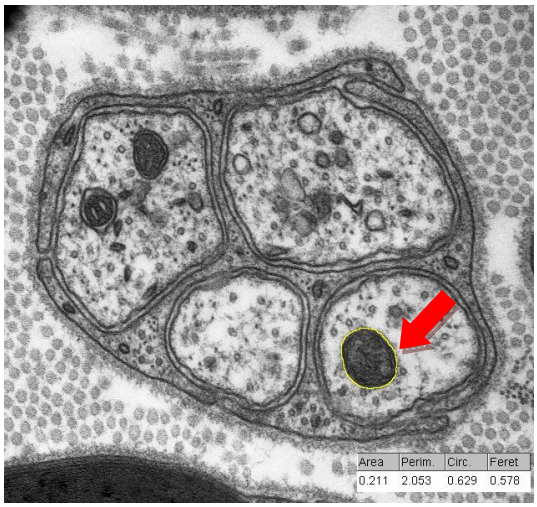

Fig. F. Delimitación del contorno de una mitocondria en una fibra amielínica (20000 X).

3. RESULTADOS Y DISCUSIÓN

3.1. Modelo de dolor neuropático inducido por paclitaxel en ratones hembra: curso temporal de la alodinia al frío, la alodinia mecánica y la hiperalgesia al calor

Los valores pretratamiento en cada test nociceptivo: test de la acetona (alodinia al frío), test de Von Frey electrónico (alodinia mecánica) y test de Hargreaves (hiperalgesia al calor) fueron similares en todos los animales. La administración durante 5 días consecutivos de paclitaxel (2 mg/kg/día) modificó las respuestas a estos tests de

manera significativa (medidas postratamiento), mientras que el vehículo del paclitaxel no tuvo ningún efecto significativo.

El tratamiento con paclitaxel incrementó de manera significativa el tiempo de lamido o mordisqueo de la pata en respuesta a la aplicación de acetona, de la mayoría de los animales (animales respondedores), durante las 3 semanas posteriores al tratamiento. Sin embargo, aproximadamente una tercera parte de los animales no desarrollaron alodinia al frío, es decir, su respuesta a la acetona después del tratamiento con paclitaxel fue similar a sus valores pretratamiento. El grupo tratado con el vehículo del paclitaxel tampoco desarrolló alodinia al frío. La alodinia al frío inducida por paclitaxel fue máxima 10-14 días después de la primera inyección del antineoplásico. Por tanto, estos días eran los más adecuados para evaluar el efecto de los fármacos en estudio en la expresión de la alodinia al frío inducida por paclitaxel.

El tratamiento con paclitaxel también indujo alodinia mecánica en los ratones, puesto que redujo de manera estadísticamente significativa el umbral de fuerza necesario para provocar la retirada de la pata al ser estimulada con el Von Frey electrónico. Esta alodinia mecánica desapareció el día 17 y fue máxima el día 10 después de la primera inyección del antineoplásico. Por tanto el día 10 postratamiento fue considerado el día más adecuado para evaluar el efecto de los fármacos en estudio en la alodinia mecánica inducida por paclitaxel.

Las inyecciones de paclitaxel también redujeron de manera significativa, la latencia de retirada de la pata tras la estimulación con un foco calorífico (test de Hargreaves). Por tanto, el paclitaxel indujo hiperalgesia al calor en los ratones que fue máxima 7 días después de la primera inyección del antineoplásico, siendo éste el día

idóneo para evaluar el efecto de los fármacos en estudio en la expresión de dicha hiperalgesia al calor.

3.2. Efectos del bloqueante de canales de sodio dependientes de voltaje TTX en el dolor neuropático inducido por paclitaxel en el ratón

Para comprobar el efecto de la TTX en la expresión de los signos del dolor neuropático inducido por paclitaxel, la TTX fue administrada cuando el dolor neuropático estaba plenamente desarrollado (el día de máxima expresión de cada signo). La administración s.c. de una dosis de TTX inhibió, de manera temporal, la expresión de la alodinia al frío, la alodinia mecánica y la hiperalgesia al calor, mientras que los animales inyectados con salino siguieron mostrando dolor neuropático. Por el contrario, la TTX no tuvo ningún efecto en la respuesta a los test nociceptivos de los animales controles (tratados con el vehículo del paclitaxel). La administración s.c. repetida de TTX previa a cada dosis de paclitaxel (es decir, durante la fase de inducción del dolor neuropático) previno por completo el desarrollo de la alodinia mecánica y al frío, pero no así el de la hiperalgesia al calor. Como cabría esperar, el grupo control tratado con salino antes de cada una de las 5 inyecciones del antineoplásico, si desarrolló los 3 signos del dolor neuropático evaluados. Aunque la TTX es una potente neurotoxina, con las dosis empleadas en este estudio, los animales no mostraron incoordinación motora en el test del rotarod y tampoco observamos ningún tipo de toxicidad durante los experimentos.

Estos resultados sugieren que los canales de sodio sensibles a TTX juegan un papel relevante en la generación y mantenimiento del dolor neuropático inducido por paclitaxel. Nuestros resultados coinciden en gran medida con los descritos previamente por otros autores, en modelos de lesión mecánica de los nervios periféricos, donde la

TTX también inhibió la expresión (Lyu et al., 2000; Marcil et al., 2006) y el desarrollo (Xie et al., 2005) del dolor neuropático. Llama la atención que la TTX inhibió la expresión de la alodinia mecánica a dosis más bajas (1-3 µg/kg) que las necesarias para inhibir la expresión de la alodinia al frío (3-6 µg/kg) o la de la hiperalgesia al calor (solo con la dosis más alta 6 µg/kg), inducidas por paclitaxel. Además la TTX solo pudo prevenir el desarrollo de ambos tipos de alodinias, mientras que fue incapaz de impedir el desarrollo de la hiperalgesia al calor. Estos efectos diferenciales de TTX podrían explicarse por la distinta participación que tienen las fibras mielínicas y/o amielínicas en la patogénesis de cada uno de estos signos de dolor. Así en la alodinia mecánica participan fundamentalmente fibras mielinizadas (sobre todo fibras Aβ), mientras que en la hiperalgesia al calor intervienen principalmente fibras C amielínicas (Ossipov et al., 1997; Khan et al., 2002). En el caso de la alodinia al frio intervienen tanto las fibras mielínicas grandes Aβ, como las pequeñas Aδ (Hao et al., 1996; Kim et al., 1999). Esta aparente preferencia funcional de la TTX por inhibir signos mediados por fibras mielínicas es consistente con los estudios electrofisiológicos que demuestran que la TTX aplicada directamente a un nervio periférico bloquea preferentemente la conducción de las fibras A sin afectar a las fibras C (Clarke et al., 2003). Además, las neuronas grandes y medianas de los ganglios de las raíces dorsales, que poseen fundamentalmente fibras Aβ y Aδ, mostraron una mayor regulación al alza de los canales y corrientes de sodio sensibles a TTX que las neuronas pequeñas de los ganglios de las raíces dorsales, que poseen fibras amielínicas C (Everill et al., 2001; Kim et al, 2001; Craner et al., 2002; Hong et al., 2004). Por tanto, la mayor eficacia de TTX para inhibir la alodinia al frío y mecánica parece indicar una mayor contribución de los

canales de sodio dependientes de voltaje sensibles a TTX en la patogénesis de estos signos del dolor neuropático.

3.3. Efectos del bloqueo o ausencia de los receptores σ_1 en el dolor neuropático inducido por paclitaxel en el ratón

Los ensayos de fijación con el radioligando selectivo para el receptor σ_1, [^{3}H](+)-pentazocina, ponen de manifiesto que tanto el antagonista σ_1 prototipo (BD-1063), como el nuevo antagonista σ_1 (S1RA), tienen una gran afinidad por el receptor σ_1 (valores de K_i de 4.43 y 29.99 nM, respectivamente) e interaccionan con él competitivamente.

En este bloque experimental no se hicieron experimentos para evaluar el papel del receptor σ_1 en la hiperalgesia al calor inducida por paclitaxel, puesto que datos experimentales nuestros y los descritos previamente por otros autores, indicaban que el bloqueo con un antagonista σ_1 (Roh et al., 2008) o la ausencia (De la Puente et al., 2009) del receptor σ_1 no inhibía dicha hiperalgesia al calor.

Los antagonistas σ_1 fueron administrados s.c. cuando los signos del dolor neuropático eran máximos para comprobar el efecto del bloqueo del receptor σ_1 en la expresión del dolor neuropático inducido por paclitaxel. La administración aguda de BD-1063 y de S1RA redujo la duración del tiempo de lamido/mordisqueo de la pata inducido por la acetona, y aumentó el umbral de fuerza necesario para provocar una respuesta de retirada de la pata estimulada con el filamento de Von Frey, siendo ambos efectos dosis-dependientes. Sin embargo, el grupo tratado con salino siguió mostrando alodinia, durante las 3 horas de evaluación. Por el contrario, ni el BD-1063 ni el S1RA tuvieron ningún efecto en la respuesta a la acetona ni al filamento de Von Frey en los animales controles, tratados con el vehículo del paclitaxel. Para estudiar el efecto del antagonismo farmacológico del receptor σ_1 en el desarrollo de la alodinia al frío y

mecánica inducida por paclitaxel, el BD-1063 y el S1RA, así como su solvente (salino), fueron administrados s.c. antes de cada una de las inyecciones de paclitaxel. Ambos antagonistas σ_1, pero no su solvente, bloquearon por completo tanto el desarrollo de la alodinia al frío como el de la alodinia mecánica. Por último, ni el BD-1063 ni el S1RA, a dosis que inhiben la expresión de la alodinia, produjeron efectos de incoordinación motora. Estos resultados ponen de manifiesto que el bloqueo del receptor σ_1 impide tanto la expresión como el desarrollo de la alodinia al frío y la alodinia mecánica inducidas por paclitaxel y, por tanto, que el receptor σ_1 debe estar activado para que se desarrolle este tipo de dolor neuropático. En consecuencia, los antagonistas del receptor σ_1 podrían tener interés terapéutico para contrarrestar el dolor inducido por paclitaxel, siendo especialmente interesante el efecto profiláctico. Ello es así puesto que (a diferencia de las neuropatías inducidas por lesiones mecánicas o enfermedades metabólicas) en las neuropatías inducidas por fármacos neurotóxicos, se conoce perfectamente cuando empieza el riesgo de la lesión neurotóxica, por lo que sería posible aplicar un tratamiento preventivo.

También empleamos una herramienta no farmacológica, como son los animales σ_1-KO, para comprobar que efecto tiene la completa ausencia del receptor σ_1 en el dolor neuropático inducido por paclitaxel, comparando el curso temporal del dolor neuropático de estos animales con el de los animales salvajes. En primer lugar, comprobamos que los animales σ_1-KO tenían una respuesta basal a la aplicación de acetona y al filamento de Von Frey, prácticamente idéntica a la de los animales salvajes. Igualmente, los ratones σ_1-KO tampoco tuvieron ninguna deficiencia en la coordinación motora en el test del rotarod. Sin embargo, cuando se compararon los cursos temporales de la alodinia al frío y mecánica de los ratones salvajes y σ_1-KO, fue posible comprobar

que mientras que los animales salvajes desarrollaban ambos tipos de alodinia, los animales desprovistos del receptor σ_1 mantuvieron unos valores y umbrales de respuesta prácticamente iguales que antes del tratamiento con el antineoplásico, es decir, no desarrollaron dolor neuropático. Como era de esperar el vehículo del paclitaxel no tuvo ningún efecto en ningún tipo de animal. Por consiguiente, estos datos ponen de manifiesto que el receptor σ_1 tiene que estar presente para que se desarrolle el dolor neuropático inducido por paclitaxel, confirmando el papel relevante de dicho receptor en esta neuropatía.

Estos datos coinciden en general con investigaciones previas, en modelos de neuropatía por lesión mecánica de nervio periférico, en las cuales animales salvajes tratados preventivamente con un antagonista σ_1 (Roh et al., 2008) y animales σ_1-KO (De la Puente et al., 2009) tampoco desarrollan dolor neuropático, y podrían explicarse por el hecho de que los receptores σ_1 parecen ser necesarios para el proceso de sensibilización central (Drews and Zimmer, 2009). En el proceso de sensibilización central que ocurre en el dolor neuropático se produce un incremento en la función de las neuronas que forman las vías del dolor, provocado por un aumento en la excitabilidad y eficacia sináptica, así como una reducción de su inhibición (Latremoliere and Woolf, 2009). La neuropatía inducida por paclitaxel ha sido asociada con alteraciones electrofisiológicas de los nervios periféricos y médula espinal (Cata et al., 2006; Xiao and Bennett, 2008) que son características de dicho proceso. Además, también se ha descrito que el paclitaxel produce una regulación a la baja de los transportadores de glutamato en la médula espinal de ratas neuropáticas (Weng et al., 2005; Cata et al., 2006), que podría producir una estimulación excesiva y prolongada de los receptores de NMDA, y sus rutas moleculares asociadas (como la fosforilación de la ERK), que

podría desencadenar sensibilización central que generaría y mantendría el estado de dolor neuropático (Ji et al., 2009; Chen et al., 2010). Es interesante resaltar, que algunas de las anomalías electrofisiológicas típicas del proceso de sensibilización central están significativamente reducidas en los animales σ_1-KO con respecto a animales salvajes (De la Puente et al., 2009). También se ha descrito que la fosforilación de ERK, que se produce tras la lesión del nervio ciático, no ocurre en animales σ_1-KO (De la Puente et al., 2009), y que la regulación al alza de la subunidad NR1 del receptor NMDA y de su forma fosforilada (pNR1), asociada al dolor neuropático inducido por lesión mecánica del nervio periférico fue prevenida por un antagonista σ_1 (Roh et al., 2008). Por tanto, nuestros datos podrían ser explicados por una posible inhibición, debida al bloqueo farmacológico o a la ausencia del receptor σ_1, de los procesos electrofisiológicos y bioquímicos relacionados con la sensibilización central inducidos por paclitaxel.

3.4. Efectos del bloqueo o la ausencia de los receptores σ_1 en las anomalías mitocondriales en el nervio safeno asociadas al dolor neuropático inducido por paclitaxel

Para comprobar si el modelo experimental de dolor neuropático inducido por paclitaxel descrito en esta Tesis Doctoral, estaba relacionado con anomalías mitocondriales similares a las descritas por otros autores (Flatters and Bennett, 2006; Jin et al., 2008), se recogieron muestras de nervio safeno en 3 puntos clave del curso temporal del dolor neuropático: antes del tratamiento (día pretratamiento), el día de máxima expresión del dolor (día 10 postratamiento) y un día en el que el dolor ya no se manifestaba (día 28 postratamiento). Antes de sacrificar al animal para la obtención de los nervios safenos, todos los animales fueron sometidos a los tests comportamentales. En este caso se realizó tanto el test de la acetona como el de Von Frey electrónico en el

mismo animal, con el objetivo de utilizar el menor número de animales posible y para estar seguros de que el mismo animal manifestaba ambas alodinias. Para llevar a cabo el objetivo principal de este bloque experimental (comprobar si el bloqueo o la ausencia de los receptores σ_1 impedía la aparición de las anomalías mitocondriales), también se realizó el mismo procedimiento experimental en animales σ_1-KO, y se llevó a cabo un tratamiento preventivo con el antagonista selectivo σ_1 BD-1063, 30 minutos antes de cada dosis de paclitaxel.

En primer lugar, se comprobó que el paclitaxel, pero no su vehículo, produjo alodinia mecánica y al frío en los animales salvajes y que los animales σ_1-KO y los animales salvajes tratados preventivamente con BD-1063, no desarrollaron dolor neuropático inducido por el antineoplásico. Al analizar las imágenes de microscopía electrónica de transmisión del nervio safeno de animales salvajes y σ_1-KO controles (sin tratamiento), se puso de manifiesto que no existían diferencias significativas en las características ultraestructurales tanto de las fibras mielínicas como amielínicas. Las mitocondrias tenían una estructura oval o circular envuelta por una doble membrana lipídica y en su interior se apreciaban las crestas y el material denso amorfo típicos.

El análisis morfométrico del área mitocondrial mostró un amplio rango de valores (desde 2 hasta más de 60 x 10^{-2} μm^2) y al realizar un análisis detallado de su distribución, fue posible distinguir 3 grupos diferentes: mitocondrias pequeñas (área $\leq$ 9 x 10^{-2} μm^2), medianas (9.1-18 x 10^{-2} μm^2) y grandes (> 18 x 10^{-2} μm^2), en todas las fibras de los nervios safenos de animales controles salvajes y σ_1-KO. Cuando se analizaron las mitocondrias según el tipo de fibra se observó que en las fibras mielínicas Aβ y Aδ, la mayoría de las mitocondrias eran pequeñas y medianas. En las fibras C amielínicas ocurría lo mismo, sin embargo, el porcentaje de mitocondrias grandes era

significativamente más alto (5-6 veces más) que el de las fibras mielínicas, tanto en animales salvajes como en σ_1-KO. Todas las mitocondrias incluidas en el grupo de "mitocondrias grandes" (>18 x 10^{-2} μm^2) tenían la doble membrana y se caracterizaban por un hinchamiento pronunciado y/o vacuolización, lo que implicaba un agrandamiento de 2-6 veces del área media mitocondrial y un diámetro superior a 480 nm. Con estos criterios, similares (e incluso más restrictivos) a los usados previamente por otros autores (Flatters and Bennett, 2006; Jin et al., 2008), se consideraron a estas mitocondrias como **"mitocondrias atípicas"**.

Tras el estudio con microscopía electrónica de transmisión de los nervios safenos de ratones salvajes y knockout σ_1 a los 10 días del inicio del tratamiento con paclitaxel, no se observaron en ninguno de los grupos alteraciones significativas de las células de Schwann o fenómenos de degeneración mielínica, ni agregados neurotubulares. Sin embargo, el tratamiento con paclitaxel indujo en los animales salvajes un incremento significativo (6.4 veces, con respecto a los animales salvajes sin tratamiento) en el porcentaje de mitocondrias atípicas de la fibras Aβ; mientras que en los animales σ_1-KO se produjo un incremento bastante menor (1.8 veces, con respecto a los animales σ_1-KO sin tratamiento), que no llegó a ser significativo. En las fibras Aδ el tratamiento con paclitaxel también produjo un incremento del porcentaje de mitocondrias atípicas (que no llegó a ser significativo) en los animales salvajes (3.8 veces), pero no modificó dicho porcentaje en los animales σ_1-KO (1.33 veces). En las fibras amielínicas el paclitaxel no produjo ningún cambio significativo en el porcentaje de mitocondrias atípicas, en ambos tipos de ratones. También se comprobó que a los 10 días de tratamiento, el vehículo del paclitaxel no produjo ningún cambio estadísticamente significativo en ninguno de los 3 tipos de fibras. Por el contrario, cuando se analizaron las imágenes

tomadas de nervios safenos, obtenidos 28 días después del tratamiento con paclitaxel o su vehículo, se puso de manifiesto que no existían diferencias significativas en el porcentaje de mitocondrias atípicas, ni en las fibras mielínicas ni en las amielínicas, de ratones salvajes y σ_1-KO. El tratamiento preventivo con el antagonista del receptor σ_1 BD-1063, a una dosis que previene el dolor neuropático inducido por paclitaxel, previno por completo el incremento en el porcentaje de mitocondrias atípicas que provoca el paclitaxel en las fibras mielínicas de ratones salvajes.

El hecho de que el curso temporal de las atipias mitocondriales coincida con el curso temporal del dolor neuropático inducido por paclitaxel, y que cuando se bloquea la aparición del dolor (mediante BD-1063 o en ratones σ_1-KO) también se reduzcan considerablemente las atipias mitocondriales, hace pensar que ambos fenómenos estén relacionados. Hasta hace poco se desconocía si estas mitocondrias atípicas tenían alguna consecuencia funcional, sin embargo, recientemente, se ha descrito que las mitocondrias de los nervios ciáticos de animales con dolor neuropático inducido por paclitaxel y por oxaliplatino (otro antineoplásico) tienen reducida la respiración mitocondrial y su producción de ATP, y por tanto deben estar funcionalmente dañadas (Zheng et al., 2011). El mecanismo mitotóxico por el cual el paclitaxel produce este fenómeno no está del todo claro. Se ha descrito que la tubulina (proteína a la que se une el paclitaxel) se encuentra asociada a una proteína mitocondrial, el canal aniónico dependiente de voltaje (en inglés VDAC) (Carre et al., 2002), que es la proteína más abundante de la membrana externa mitocondrial y que controla el flujo de ciertos metabolitos e iones, como el Ca^{2+} (Tan y Colombini, 2007). Se ha sugerido que los fármacos que se unen a los microtúbulos (como el paclitaxel) podrían modular la interacción de la β-tubulina con VDAC y por tanto modificar la permeabilidad de la mitocondria (Rostovtseva y

Bezrukov, 2008). De hecho VDAC podría regular al poro de transición de permeabilidad mitocondrial (en inglés mPTP), que es un poro no específico que se abre en la membrana externa mitocondrial, bajo ciertas condiciones como un exceso del Ca^{2+} almacenado en la mitocondria, y que permitiría la entrada de moléculas de diámetro menor de 1.5 kDa. La apertura de mPTP provocaría el hinchamiento y vacuolización mitocondrial, pero también la liberación del Ca^{2+} mitocondrial (revisado por Halestrap, 2009). En base a ésto, se ha propuesto que el paclitaxel se uniría a VDAC, lo que aumentaría la permeabilidad mitocondrial (apertura de mPTP), que provocaría el hinchamiento y vacuolización mitocondrial, y la salida del Ca^{2+} almacenado en la mitocondria. Esta salida de Ca^{2+} llevaría a un aumento del Ca^{2+} intracelular que podría provocar hiperexcitabilidad neuronal, lo que explicaría la hipersensibilidad que induce el paclitaxel (Flatters and Bennett, 2006; Jin et al., 2008). Apoyando esta teoría, ha sido descrito por varios autores que ciertos agentes que reducen el Ca^{2+} intracelular inhiben y/o previenen el dolor neuropático inducido por paclitaxel (Flatters y Bennett, 2004; Matsumoto et al., 2006; Siau y Bennett, 2006; Xiao et al., 2007; Xiao et al., 2008; Gauchan et al., 2009; Okubo et al., 2011).

Se ha descrito que el receptor σ_1 es una chaperona regulada por ligando que se encuentra normalmente en la membrana del retículo endoplasmático (en una región asociada funcionalmente a la mitocondria) y que participa en la modulación de la homeostasis del Ca^{2+} (Su et al., 2010). En concreto, el receptor σ_1 interviene en el plegamiento del receptor de inositol trifosfato (IP_3), incrementando la entrada de Ca^{2+} en la mitocondria (Hayashi and Su, 2007). Quizás, la ausencia o el bloqueo del receptor σ_1 pueda prevenir el exceso de Ca^{2+} mitocondrial responsable de la apertura de mPTP y por tanto, de esta forma, prevenir el aumento de mitocondrias atípicas. Se ha propuesto

que el efecto global que conlleva la activación de los receptores σ_1 es un incremento del Ca^{2+} citosólico, debido a la potenciación de la entrada de Ca^{2+} a la célula a través del receptor de NMDA en la membrana plasmática, y a la liberación de Ca^{2+} inducido por IP_3 desde las reservas del retículo endoplasmático (De la Puente et al., 2009). Por tanto, la ausencia o bloqueo de los receptores σ_1 podría compensar el aumento del Ca^{2+} citosólico derivado de la apertura del mPTP, inducida por paclitaxel, impidiendo que la concentración de Ca^{2+} citosólico alcance el umbral necesario para desencadenar el proceso de sensibilización central y por tanto, el dolor neuropático.

4. CONCLUSIONES

4.1. Conclusiones específicas

1. La administración repetida de paclitaxel indujo dolor neuropático en ratones hembra, que se caracterizó por alodinia al frío y mecánica, e hiperalgesia al calor.

2. La administración sistémica del bloqueante de canales de sodio TTX, inhibió la expresión de la alodinia mecánica, la alodinia al frío y la hiperalgesia al calor inducidas por paclitaxel. En cambio cuando la TTX se administró como tratamiento profiláctico, solo previno el desarrollo de ambos tipos de alodinias.

3. Los antagonistas selectivos del receptor σ_1, BD-1063 y S1RA (administrados s.c.), inhibieron la expresión de la alodinia mecánica y al frío inducidas por paclitaxel.

4. El antagonismo farmacológico (con BD-1063 o S1RA) o la inactivación genética de los receptores σ_1 bloqueó por completo el desarrollo de la alodinia mecánica y al frío inducidas por paclitaxel.

5. El dolor neuropático inducido por paclitaxel está asociado con un aumento en el porcentaje de mitocondrias atípicas en las fibras mielínicas del nervio safeno. En

paralelo con la prevención del dolor, la inactivación genética o el antagonismo farmacológico de los receptores σ_1 también previno el incremento de atipias mitocondriales producido por el antineoplásico.

4.2. Conclusiones generales

1. La administración repetida de paclitaxel a ratones imita algunos de los signos del dolor neuropático inducido por paclitaxel en humanos y representa un modelo adecuado para explorar dianas terapéuticas potenciales para combatir el dolor neuropático derivado de su administración.

2. Los canales de sodio dependientes de voltaje y los receptores σ_1 juegan un papel importante en el desarrollo y la expresión del dolor neuropático inducido por paclitaxel. Por tanto, el bloqueo de estas dianas podría constituir una nueva estrategia terapéutica para el tratamiento de la neuropatía inducida por paclitaxel.

INTRODUCTION

1. PACLITAXEL IN THE TREATMENT OF CANCER

1.1. Overview of cancer and its treatment

The World Health Organization (WHO) defined *neoplasia* (Greek for "new growth") as "the abnormal and uncontrolled proliferation of cells in a tissue or organ" and *cancer* (Latin for "cancer") as "a general term for many diseases that are characterized by uncontrolled, abnormal growth of cells" (International agency for research on Cancer, World Cancer Report 2008). Most neoplasias proliferate to *tumors* (distinct masses of cells) that may be benign or malignant. The term *cancer* is typically used only for malignant tumors. Cancer cells can invade nearby tissues and spread (*metastasize)* to other parts of the body through the blood and lymph systems. The consequence is a complex array of clinical presentations that can result in the death of patients.

Cancer is a group of diseases that affects millions of people each year and is one of the major causes of death. According to WHO, 12.4 million of people were diagnosed with cancer worldwide in 2008, including 3.4 million in Europe, and it is expected that by 2030 there will be approximately 26.4 million of cases of cancer in the world (International agency for research on Cancer, World Cancer Report 2008). Cancer can cause almost any sign or symptom according to its localization, size, and the degree of its effect on organs or tissues. When a cancer has spread, signs or symptoms may appear in different parts of the body (International Agency for Research on Cancer, World Cancer Report 2008).

Cancer treatment is a complex task that involves coordinating the efforts of multiple medical specialties. Most patients require surgery to remove and/or establish the diagnosis of the primary tumor, but cancer treatment also includes chemotherapy

and/or irradiation. Unfortunately, the therapy used to fight against cancer usually induces severe side effects. In particular, chemotherapeutic agents, usually cause serious toxicity problems that lead to important adverse effects. This is because cancer and normal cells are very similar in most respects, and their differentiation is highly challenging (International Agency for Research on Cancer, World Cancer Report 2008).

1.2. The history of taxol

Taxol is an antineoplastic agent belonging to the group of taxanes or taxoids. They are a structurally homogeneous class of compounds present in two genera of the yew family: *Taxus* and *Austrotaxus*. The yew family (*Taxaceae)* is exclusively found in the northern hemisphere, with the exception of the genus *austrotaxus*, endemic to the island of New Caledonia. The genus *Taxus* is the most important and includes several species of yew found in Eurasia and North America (Appendino, 1995).

Pharmaceutical interest in taxanes goes back to the second half of the 20th century, but the medicinal and poisonous properties of the yew have been known for centuries. Thus, Julius Caesar recorded in *On the Gallic Wars*, written in 51 B.C., that the leader Catuvolcus committed suicide by drinking tea made from yew bark, and native tribes of North America used the bark, foliage, and fruits of yew as disinfectant and abortifacient and for stomach ache (Appendino, 1996; Small and Catling, 1999).

The chemical components of several *Taxus* species possess various biological properties and have been studied for more than 100 years (reviewed by Parmar et al., 1999). However, a major interest in the study of these components arose in the 1960s, when the National Cancer Institute (NCI) of the United States was carrying out a screening program of plant extracts to identify substances with anti-cancer activity. This

program demonstrated the cytotoxicity of stem bark extract of the Pacific yew tree, *Taxus brevifolia*, in KB assay (cell line derived from a human nasopharyngeal carcinoma) and its activity against carcinosarcoma in rats and against leukemia in mice (Guenard et al., 1993). Under this NCI screening program, in 1969, Wall and collaborators isolated the most active ingredient from this extract, designated taxol, (Cragg et al., 1993; Guenard et al., 1993), and subsequently clarified and published its structure (Wani et al., 1971).

Taxol initially showed only moderate activity against P388 and L1210 murine leukemia models and was not considered a promising candidate for development as an anti-tumor drug at that time (Cragg et al., 1993). In 1975, however, the NCI published interesting results demonstrating the strong activity of taxol against murine B16 melanoma (Cragg et al., 1998). Subsequently, taxol was found to be a strong inhibitor of cell division, with a single mechanism of action at tubulin level that was different from the mode of action of other "tubulin drugs", such as vinblastine or colchicine (Schiff et al., 1979). As a result of these two major findings, the compound became a new lead structure for further pharmacological studies, and the NCI began to consider optimization of its production (reviewed by Cragg, 1998 and by Wall, 1998). As a natural product, taxol production is limited by the number of yew trees and involves their large-scale destruction. Besides, the compound accumulates in the trunk bark of yew tree at a very low concentration (about 0.02% of dry weight) and its extraction is extremely expensive (Expósito et al., 2009). In fact, the shortage of raw material threatened the development of phase I trials in 1983, which required progressively larger amounts of the compound (Cragg et al., 1993).

In 1991, in order to obtain adequate supplies of taxol and accelerate its development as a drug, NCI entered into a Cooperative Research and Development Agreement with Bristol-Myers Squibb, selected from among various pharmaceutical companies expressing interest in the development of taxol (Wall, 1998). The company focused its efforts on developing an alternative and renewable source of taxol in other more common species of yew tree. Bristol-Myers Squibb found that the European yew (*Taxus baccata*), which is widely grown as an ornamental tree, contains 10-deacetyl baccatin III. This substance can be converted to taxol by chemical processes, is more abundant than taxol, and can be obtained from the needles of the plant. This renewable source of the drug, obtained through a semi-synthetic procedure, solved the problem of taxol supply. A key role in this development was played by the pioneering research of the group led by Poitier (Denis et al., 1988), who discovered the semi-synthetic procedure that permitted the large-scale production of taxol and derived compounds, such as docetaxel (Taxotere®). The search is ongoing for novel and more efficient methods of taxol production, including the use of cell cultures (reviewed by Cragg et al., 1993; Cragg, 1998; Expósito et al., 2009).

Finally, after the promising results obtained in the Phase II clinical trials of taxol (Holmes et al., 1991), the FDA (US Food and Drug Administration) approved its marketing to treat refractory metastatic ovarian cancer and metastatic and refractory or anthracycline-resistant breast cancer in 1992 and 1994, respectively. It is important to note that, although the name "taxol" had been used without any restriction, Bristol-Myers Squibb registered it as a trademark (Taxol®) in 1992. Since that time, it has been known generically as “paclitaxel”. This generic term proved controversial in some sectors of the scientific community but is used in the present thesis because it was

approved by the WHO and the British Pharmacopoeia (Anonymous, 1995; Chesnoff, 1995; Khan, 1995).

1.3. Microtubules and microtubule-binding agents

Microtubules are ubiquitous fibrillar structures present in eukaryotic cells and play a key role in a variety of cellular processes, including transport, signaling, and mitosis. Along with actin microfilaments and intermediate filaments, microtubules are the main components of the cytoskeleton. Microtubules are polymers formed by the homogeneous association of heterodimers of α- and β-tubulin combined with microtubular-associated proteins. Tubulin heterodimers are associated in a head-to-tail manner to form protofilaments, which associate in a lateral manner to form hollow rods with an overall diameter of about 25 nm (Fig. 1). Microtubules are highly dynamic and are in continuous dynamic rearrangement, growing and shrinking, due to the association and dissociation, respectively, of tubulin dimers from the microtubule ends. This dynamic process, which is essential to perform microtubule functions, is driven by GTP binding and hydrolysis at the exchangeable (E-site) of β-tubulin. Microtubule assembly requires β-tubulin loaded with GTP, which is hydrolyzed upon addition of the tubulin dimer to the elongating microtubule (reviewed by Joshi, 1998 and Orr et al., 2003).

In mammalian cells, these structures are present in both interphase and mitotic cells. In dividing cells, microtubules that form the mitotic spindle are highly dynamic and extremely sensitive to microtubule-targeted drugs. For this reason, compounds that alter microtubule function have proved to be highly active in patients with cancer. Many of these microtubule-binding agents are natural products, and some of them, such as *Vinca* alkaloids and taxanes, have been the most commonly prescribed anticancer

therapies for some decades (reviewed by Dumontet and Jordan, 2010 and Yue et al., 2010).

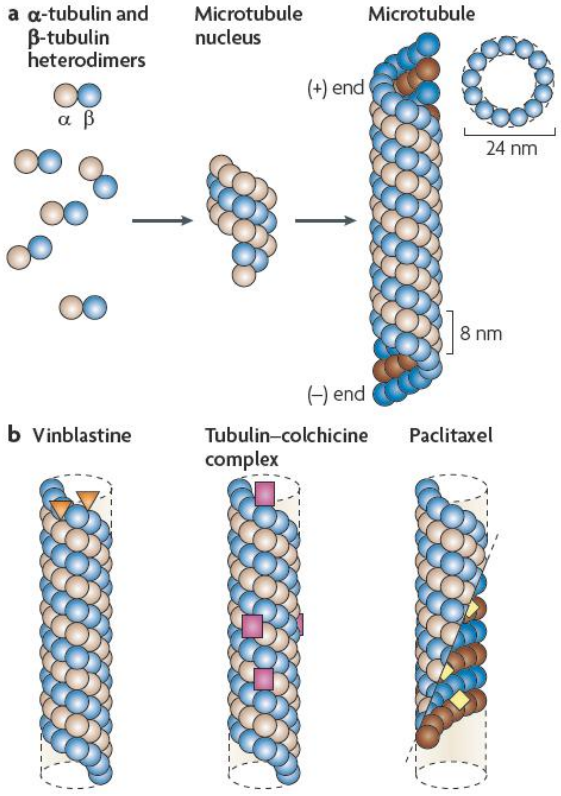

Fig. 1. Microtubule formation and the binding sites of microtubule inhibitors. **a)** Soluble tubulin dimers, containing one α-tubulin peptide and one β-tubulin peptide, polymerize to form a microtubule nucleus. Additional dimers are added head-to-tail and the resulting microtubules are highly dynamic structures containing a (+) end, characterized by an exposed β-tubulin peptide and a (–) end, characterized by an exposed α-tubulin peptide. **b)** Binding sites of microtubule inhibitors are shown (orange triangles, purple squares and yellow diamonds). *Vinca* alkaloids, such as vinblastine, bind to microtubule ends; colchicine binds to soluble dimers, which can be incorporated in the microtubules; taxanes, such as paclitaxel, bind along the interior surface of microtubules (Figure taken from Dumontet and Jordan, 2010).

Classically, drugs that interfere with microtubular structure and function are divided into stabilizers and destabilizers, according to their effects on microtubule polymer mass at high concentrations (Yue et al., 2010). Another classification is based on their binding sites on microtubules and distinguishes 4 subgroups: (1) agents binding to the *Vinca* alkaloid-binding site, close to the exchangeable GTP site on β-tubulin; (2)

agents binding to the colchicine-binding site, located at the interface between α and β subunits of the tubulin dimer, adjacent to the GTP-binding site of α-tubulin; (3) agents binding to the taxane-binding site, which is the NH_2 terminal 31 amino acid of β-tubulin, a deep hydrophobic pocket; and (4) agents binding to other binding sites (Yue et al., 2010).

Microtubule destabilizing compounds destabilize microtubules by inhibiting the assembly of tubulin heterodimers into microtubule polymers or by depolymerizing existing polymers. They mainly act at *Vinca* alkaloid- or colchicine-binding sites. *Vinca*-site binders include the *Vinca* alkaloids (vinblastine, vincristine, vinorelbine, vindesine and vinflunine), the cryptophycins, and the dolastatins. Colchicine-site binders include colchicine and its analogues. Some of these destabilizing products, including herbicides, psychoactive drugs and food components, bind to other sites on tubulin (reviewed by Dumontet and Jordan, 2010 and Yue et al., 2010).

Microtubule-stabilizing agents stabilize tubulin polymers by starting tubulin polymerization or stabilizing existing microtubules under normally destabilizing conditions. Most of these compounds bind to a taxane-binding site or an overlapping site. This group include the taxanes, paclitaxel and docetaxel, the epothilones, ixabepilone and patupilone, discodermolide, eleutherobins, sarcodictyins, cyclostreptin, dictyostatin, laulimalide, rhazinilam, peloruside A, certain steroids and polyisoprenyl benzophenones (reviewed by Dumontet and Jordan, 2010 and Yue et al., 2010).

At a basic mechanistic level, both stabilizers and destabilizers of microtubules have a similar final result: blocking mitosis. In fact, taxanes and *Vinca* alkaloids can be combined in a clinical regimen with no apparent antagonism and even revealed synergism *in vitro* (reviewed by Dumontet and Jordan, 2010). Both types of

microtubule-binding agent cause interruption of microtubule dynamics followed by mitotic arrest and cell death in cancer cells. Moreover, the division between stabilizers and destabilizers is somewhat simplistic and does not account for all their mechanisms of action, such as the effects on tumor vasculature. In fact, the targets of these compounds in cancer therapy include not only cancer cells but also vascular endothelial cells, given that endothelial cells are highly dependent for their normal functions (motility, invasion, attachment, alignment and proliferation) on the tubulin cytoskeleton. Microtubule-binding compounds may display both anti-angiogenic and vascular-disrupting actions, inducing a reduction in the blood flow of tumors, with tumor-related endothelial cells being considerably more sensitive than are normal endothelial cells (reviewed by Yue et al., 2010).

Another important aspect to consider is that, the utilization of microtubule-binding agents is often limited by the development of drug resistance, as observed with antimitotic drugs. One major cause of drug resistance to microtubule-active agents is multidrug resistance (MDR) mediated by ATP-binding cassette (ABC) transporters and alterations of the tubulin-microtubule complex. MDR proteins such as P-glycoprotein, product of the MDR1 gene, are transporters that act as drug efflux pumps, producing the efflux of drugs such as taxanes and *Vinca* alkaloids from tumor cells, preventing the accumulation of therapeutic intracellular concentrations of the active drug. Another relevant factor is that P-glycoprotein is expressed on endothelial cells of the capillaries of the central nervous system (CNS), which may explain, in part, why the brain is excluded from the distribution of many chemotherapeutic agents. Enhanced or decreased expression by cancer cells of some isoforms of tubulin, such as βIII-tubulin,

has been associated with a reduced response rate to taxane and *Vinca* alkaloids, respectively (reviewed by Dumontet and Jordan, 2010).

1.4. Pharmacology of paclitaxel

1.4.1. Chemistry of taxanes

Taxoids or taxanes are a group of naturally occurring diterpene compounds characterized by the presence of the taxane skeleton (Fig. 2). The chemical structure of more than 100 compounds in this group is now known (Expósito et al., 2009). The first agent of this class with antitumor properties to be identified was paclitaxel (Taxol®; $C_{47}H_{51}NO_{14}$; 5β,20-epoxy-1,2β,4,7β,-10β,13α-hexahydroxy-tax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester), the prototypic taxane.

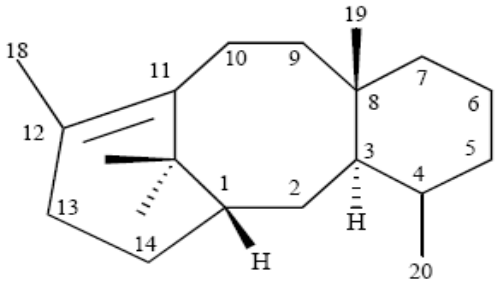

Figure 2. Chemical structure of the taxane ring (Figure taken from Expósito et al., 2009).

Paclitaxel is a poly-oxygenated diterpene alkaloid with a complex structure (Fig. 3), which can be isolated naturally or produced by a semi-synthetic procedure (Münster and Hudis, 2000). Another semi-synthetic taxane, with antitumor activity, is docetaxel (Taxotere®), which differs from paclitaxel in the C_{10} taxane ring position and in the ester side chain attached at C_{13} (Fig. 3). Both have a large fused taxane ring system with

a rare four-member oxetan ring at positions C_4 and C_5 and an ester side chain at C_{13} (Sparreboom et al., 1998).

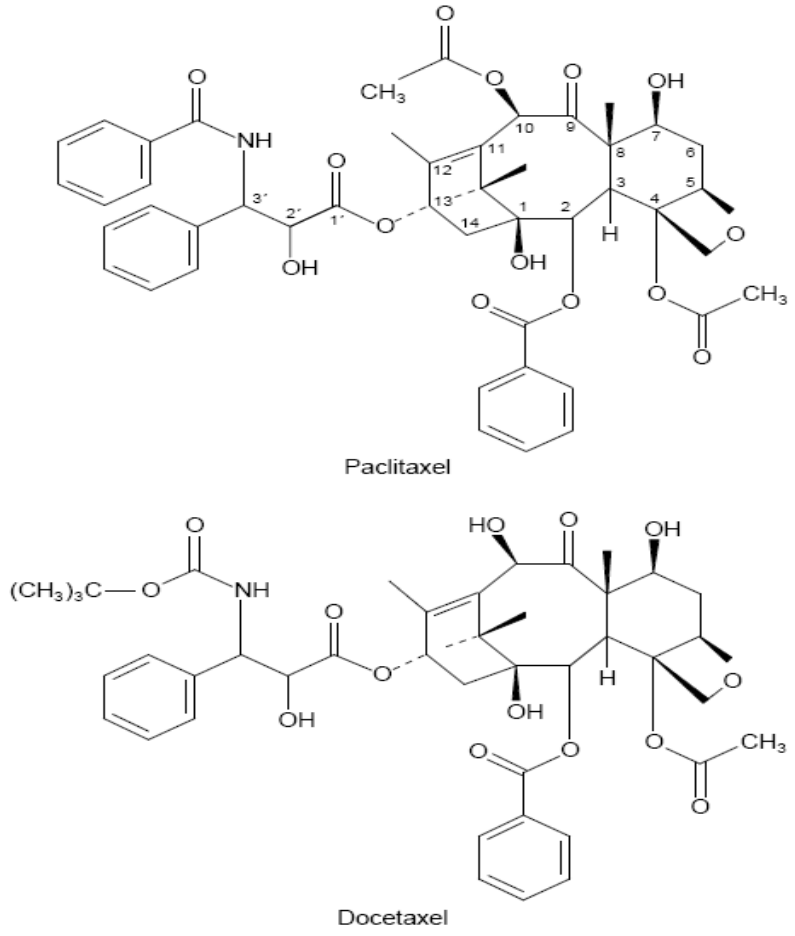

Fig. 3. Structures of paclitaxel and docetaxel (Figure taken from Eisenhauer and Vermorken, 1998).

Both paclitaxel and docetaxel are white powders and insoluble in water and, because they do not have acidic or basic moieties charged in a pharmaceutically useful pH range, manipulation of the pH does not improve the water solubility. Paclitaxel can be dissolved at millimolar concentrations in a variety of alcohols, e.g., methanol or ethanol, and it is also soluble in polyoxyethyleneglycerol triricinoleate 35 (Cremophor EL), polyethylene glycols (PEGs) 300 and 400, chloroform, acetone, methylene

chloride, or acetonitrile, and it is freely soluble in dimethylacetamide (DMA) and dimethylsulfoxide (DMSO). However, docetaxel is sparingly soluble in acetone, soluble in chloroform, and freely soluble in dimethylformamide, 96% (v/v) of aqueous ethanol and methanol (Sparreboom et al., 1998).

1.4.2. Mechanisms of the antineoplastic action of paclitaxel

In 1979, the group led by Susan B. Horwitz demonstrated that paclitaxel killed cancer cells in a completely different manner to that of any other drug known at that time, i.e., by promoting microtubule assembly (Schiff et al., 1979). The discovery of this novel mechanism of action was crucial for advancing the development of paclitaxel (see section 1.2). Over the following years, Horwitz's group investigated the detailed action mechanism of paclitaxel at molecular level (see Orr et al., 2003 for references). In 1993, they discovered that paclitaxel binds to N-terminal 31 amino acids of the β-tubulin subunit of tubulin polymers (Rao et al., 1994), the taxane-binding site (see section 1.3). Paclitaxel is placed in a pocket formed by several hydrophobic residues, situated on the luminal side of the microtubule (Orr et al., 2003). However, other sites of interaction may also be involved (Magnani et al., 2009; Xiao et al., 2006).

In vitro, paclitaxel binds to the microtubule polymer, enhancing the polymerization of tubulin. Microtubules formed in the presence of the drug have great stability and are resistant to depolymerization by Ca^{2+} and to cold temperature and dilution. In the absence of GTP, which is a requisite for microtubule polymerization under normal conditions, paclitaxel even promotes tubulin polymerization. Paclitaxel also modifies the structure of the microtubule polymer by reducing the number of protofilaments from a normal average of 13 to 12 (reviewed by Orr et al., 2003).

In cells, high concentrations of paclitaxel increase polymer mass and induce microtubule bundle formation in interphase cells. However, the main effect of lower concentrations of the drug is the suppression of microtubule dynamics without altering the polymer mass (reviewed by Orr et al., 2003).

When paclitaxel binds to microtubule, it is believed to induce a conformational change in the dimers of tubulin, strengthening both longitudinal and lateral interdimer interactions and thereby stabilizing microtubules (reviewed by Downing, 2000). It was also speculated by Horwitz's group that paclitaxel causes a loss of flexibility of the involved regions, which prevents a ‘‘roll out’’ of lateral contacts in microtubules that would otherwise open up their walls (Xiao et al., 2006). Therefore, this mode of action leads to the suppression of microtubule dynamics required for a correct mitotic function, effectively blocking cell cycle progression in the G2/M phase and resulting in apoptosis. Cancer cells, with their high mitotic activity, are therefore especially susceptible (see section 1.3).

Apoptosis is activated by paclitaxel-induced perturbations of mitotic processes and mitotic arrest. However, the precise mechanisms by which these perturbations result in cell death have not been fully elucidated (Rowinsky, 2010). At any rate, paclitaxel is known to kill tumor cells through a p53-independent mechanism and to kill cells with a mutation in p53 more efficiently than cells with normal p53. These are findings of major interest, because mutations of p53, a well-known tumor suppressor gene (Cowell, 1990), are present in more than 60% of human cancers (reviewed by Wang et al., 2000). Paclitaxel is also known to interact with numerous proteins that regulate mitosis and various mechanisms that potentially link mitotic arrest with apoptosis. Thus, it has been reported that paclitaxel induces the activation of the pro-apoptotic effectors, Bax, Bad,

and Apaf-1 and the inactivation of anti-apoptotic effectors Bcl-2 and Bcl_{xl} (reviewed by Perez, 2009).

Taxanes also induce other much less well-studied molecular pathways that may or may not be related to their effects on microtubule dynamics. Paclitaxel induces the transcription of factors and enzymes that intervene in proliferation, apoptosis, and inflammation (see Rowinsky, 2010 for references). Thus, it exerts an influence on the immune system by stimulating anti-tumor and anti-autoimmunity effects. The drug possesses lipopolysaccharide (LPS)-mimetic properties, stimulating macrophages and inducing the secretion of cytokines, such as TNF-α and interleukins, which activate other immune cells (Javeed et al., 2009). Paclitaxel also promotes the transcription of the cyclooxygenase (COX)-2 gene and the stabilization of the COX-2 messenger RNA transcript, leading to increased production of prostaglandins, which generate mediators of inflammation but have also been associated with tumorigenesis (Olsen, 2005). These findings support the idea that paclitaxel suppresses tumors *via* several mechanisms and not solely by the inhibition of cell division.

1.4.3. Administration, dose, and schedule of paclitaxel

The search for the optimal paclitaxel dose and schedule has been the focus of considerable research over the past decade. Taken together, reported data indicate that the drug has major antitumor activity with multiple schedules and that no specific schedules are more effective than others. However, the toxicity profiles are quite different, and differences in efficacy among some schedules derive from their toxicity. The oral bioavailability of taxanes is highly reduced, which is in part due to the constitutive overexpression of p-glycoprotein and the CYP450 metabolizing capability

of enterocytes and/or first-pass metabolism in the liver and/or intestines. Therefore, paclitaxel is generally administered intravenously every 3 weeks at a dosage of 175 mg/m^2 over 3 h or, less frequently, at 135-175 mg/m^2 over 24 h. Paclitaxel has also been administered into the pleural and peritoneal cavities (reviewed by Rowinsky, 2010). However, it was recently reported that three 1-h infusions at weekly intervals (83-91 mg/m^2/week) provides higher dose-density and dose-intensity in comparison to 3-weekly cycles. The rationale for this approach is that the more frequent delivery of a moderate dose may be more efficacious than the 3-weekly standard dose through a more sustained exposure of dividing tumor cells to the cytotoxic drug. In addition, this weekly schedule results in a lower plasma concentration of paclitaxel, reducing some adverse effects such as neutropenia (Marchetti et al., 2002).

Paclitaxel is highly hydrophobic (see section 1.4.1), and commercially available formulations require polyoxyethylated castor oil (Cremophor EL®) and an ethanol vehicle for parental administration. However, the concentration of this vehicle in the therapeutic dose of parenteral paclitaxel is relatively high in comparison to other agents using this solvent and is considered responsible for some of the side-effects of paclitaxel therapy (Hennenfent and Govindan, 2006). The most frequent adverse effect is a hypersensitivity reaction, which usually requires premedication with antihistamines and corticosteroids. It has also been demonstrated that this solvent can alter the pharmacokinetics of intravenously administered paclitaxel, although this phenomenon is less well understood. In order to prevent these vehicle-related adverse effects, several strategies are in progress to develop alternative polyoxyethylated castor oil-free formulations of paclitaxel, including the use of albumin nanoparticles, prodrugs, polyglutamates, analogs, emulsions, and liposome (reviewed by Hennenfent and

Govindan, 2006).

1.4.4. Pharmacokinetics of paclitaxel

In common with all taxanes, paclitaxel has the following pharmacological characteristics: large volume of distribution, rapid binding to all tissues except for the CNS, long terminal half-life, significant hepatic metabolism, biliary excretion, and fecal elimination (reviewed by Rowinsky, 2010).

Although a two-compartment model was initially reported for paclitaxel, it appears that a three-compartment model may be more accurate. Its terminal plasma half-life ranges from 10 to 20 h. Paclitaxel has a large volume of distribution with an extensive binding of the drug to plasma proteins (>95% and reversible) and other cellular proteins, possibly including tubulin (see Rowinsky, 2010 for references). In fact, *in vitro* experiments showed tissue binding of 98-99%, with higher intracellular- than extracellular-bound paclitaxel (99.9% and 82%, respectively). Furthermore, the intracellular binding is virtually specific for tubulin with a high affinity, showing a dissociation constant of 5-10 nM (reviewed by Marchetti et al., 2002).

The drug generally shows a linear pharmacokinetics for 6- or 24-h infusions, but it becomes non-linear with shorter durations due to saturable distribution, metabolism, and elimination. Therefore, the drug exposure is not proportional to its dose. In addition, the traditional vehicle of paclitaxel, polyoxyethylated castor oil, may simulate nonlinearity (pseudo-nonlinearity) by binding to paclitaxel and inhibiting drug disposition (reviewed by Marchetti et al., 2002 and Rowinsky, 2010).

The main route of elimination of paclitaxel is by hepatic metabolism and excretion into the bile. The liver metabolizes and excretes paclitaxel and its metabolites into the

bile. In rats treated with radiolabeled paclitaxel, 98% of radioactivity was recovered from feces collected over 6 days. In humans, approximately 71% of the administered dose of paclitaxel is excreted in the feces over 5 days as either paclitaxel or its metabolites. Renal clearance of paclitaxel and its metabolites can be up to 14% of the administered dose (reviewed by Rowinsky, 2010).

Paclitaxel transport is mediated by the membrane-bound transporter proteins MDR1 and MRP-2 (ABC transporters), while the organic anion transporting polypeptide (OATP) 1B3 was identified as a key protein for the hepatic uptake of paclitaxel. The CYP450 enzymes involved in the metabolism are mainly CYP2C8, CYP3A4, and CYP3A5. Paclitaxel metabolites are 6α-hydroxypaclitaxel, p-hydroxy-C3´-paclitaxel and dihydroxypaclitaxel, which are less active than the parent compound. Patient exposure to paclitaxel may be changed by polymorphisms described in some of these genes (CYP2C8, CYP3A4, CYP3A5, P-glycoprotein/ABCB1, and OATP1B3) together with its pharmacological characteristics (reviewed by Gerritsen-van Schieveen et al., 2010 and Rodríguez-Antona, 2010).

1.4.5. Clinical use of paclitaxel

Taxanes, either as a single agent or in combination with other chemotherapeutic drugs, has demonstrated significant activity against several solid tumors, and it is one of the most potent anticancer compounds (reviewed by Gligorov and Lotz, 2004 and Rowinsky, 2010). Paclitaxel, initially approved for use in the palliative therapy of patients with ovarian and breast cancer resistant to chemotherapy (see section 1.1), has been tested (and in some cases approved) for the treatment of numerous cancers (reviewed Rowinsky, 2010).

Paclitaxel in combination with carboplatin or cisplatin is considered the treatment of choice for primary therapy of advanced ovarian cancer. It is also used alone or in combination as a second-line therapy for recurrent ovarian cancer. It was recently reported that weekly paclitaxel (83-91 mg/m^2 administered over 1 hour, every week) has a higher response rate and is better tolerated than the usual 3-weekly paclitaxel regimen (175 mg/m^2 administered over 3 hours, every 3 weeks). This enhanced efficacy of weekly paclitaxel may be due to greater drug exposure, a direct anti-angiogenic effect, or both (reviewed by Baird et al., 2010 and Liu and Matulonis, 2010).

Paclitaxel is also considered a fundamental drug in the treatment of patients with breast cancer. Its use in both metastatic and node-positive adjuvant settings is well established. Moreover, it is commonly used as neoadjuvant treatment in patients with locally advanced disease. For example, monotherapy with paclitaxel showed significant activity in both doxorubicin-naive and doxorubicin-refractory metastatic breast cancer (Saloustros et al., 2008). As in ovarian cancer therapy, paclitaxel was given every 3 weeks in most randomized trials for the treatment of breast cancer. However, weekly schedules may also favorably affect the therapeutic ratio (reviewed by Saloustros et al., 2008). A new formulation of paclitaxel (without Cremophor EL® vehicle), ABI-007 (Abraxane, from Abraxis BioScience), which is a nanoparticle albumin-bound paclitaxel, showed higher efficacy than the original formulation and has been approved by the FDA for the treatment of metastatic breast cancer (Morris and Fornier, 2008).

Paclitaxel in combination with cisplatin has also been approved by the FDA for the first-line treatment of non-small cell lung cancer in patients for whom potentially curative surgery and/or radiation therapy are not possible, and for second-line treatment of Kaposi sarcoma associated with the acquired immunodeficiency syndrome (see

Rowinsky, 2010 for references). With regard to gastric cancers, data from several phase I and phase II trials suggested that the adjuvant use of paclitaxel has potential effects in advanced disease and in curatively resected cases. Once more, weekly schedules appeared less toxic and more effective in comparison to 3-weekly administrations (reviewed by Sakamoto et al., 2009). Finally, paclitaxel in monotherapy or in combination with cisplatin or epidermal growth factor receptor inhibitors has also shown activity against recurrent and/or metastatic head and neck cancer (reviewed by Molin and Fayette, 2011).

1.4.6. Toxicity of paclitaxel

Although taxanes are in general moderately well tolerated, they can, either as single agents or in combination with chemotherapy regimens, produce toxicities such as alopecia, arthralgias, or myalgias and other potentially more serious effects, including hypersensitivity reactions, stomatitis/mucositis, peripheral neuropathy, or bone marrow suppression. These adverse effects have been extensively reviewed in several articles and are only summarized in the following paragraphs (see Rowinsky and Donehower, 1995; Markman, 2003; Rowinsky, 2010 for references).

Hypersensitivity reactions

Taxane therapy is associated with a high incidence of hypersensitivity reactions, which were especially problematic during the early development of paclitaxel, affecting up to 30% of patients. Researchers have generally reported them to be allergic reactions associated with the use of the solvent Cremophor EL®, although reactions directly related to paclitaxel cannot be ruled out. Most of these hypersensitivity reactions are characterized by dyspnea with bronchospasm, urticaria, and hypotension, generally

within the first 10 minutes after the first injection, although they can be more severe. Nowadays, however, most of them can be controlled by premedication with H-1 antagonists and corticosteroids.

Bone marrow toxicity

At the beginning of its clinical development, paclitaxel was commonly administered as a 24-h infusion to reduce the incidence of hypersensitivity reactions. However, this schedule was associated with an elevated frequency of bone marrow suppression, principally neutropenia. Its onset is usually on days 8 to 10 after treatment, and recovery is generally complete by days 15 to 21. Neutropenia is not cumulative, suggesting that paclitaxel does not irreversibly damage immature hematopoietic cells. However, the most critical determinant of the severity of neutropenia appears to be the time period for which plasma drug concentrations are higher than biologically active concentrations (0.05 to 0.1 μmol/L); therefore, paclitaxel is currently administered in infusions of shorter duration, which have shown similar levels of antineoplastic activity and the same incidence of hypersensitivity reactions but significantly less neutropenia.

When specific paclitaxel treatments are known to be associated with severe neutropenia, the prophylactic use of bone marrow colony-stimulatory factors (as G-CSF, GM-CSF) has become a usual therapeutic component. Severe thrombocytopenia and anemia are not common side effects of taxanes in monotherapy, but a significant platelet count depression can occasionally be observed when they are administered in combination with carboplatin.

Myalgias/arthralgias

Taxanes can induce myalgias/arthralgias, described as “flu-like symptoms”, with an occasional abdominal pain component. These adverse effects start at 24-48 h after

the end of paclitaxel treatment and generally last for 4-5 days. They are self-limiting and not associated with fever, and they do not appear to be associated with the more persistent and potentially disabling side effect of peripheral neuropathy. They can be controlled with nonsteroidal anti-inflammatory drugs, and the use of a narcotic is uncommon.

Cardiac toxicity

In the first clinical trials, it was reported that paclitaxel could alter the cardiac rhythm, with the most usual effect being a transient asymptomatic bradycardia. More important bradyarrhythmias were also observed, although less frequently. However, it was not clear at that time whether these events resulted from severe drug-related hypersensitivity or were induced by poorly-characterized paclitaxel-induced cardiac toxicity. Subsequent studies revealed that paclitaxel treatment alone was very rarely associated with serious cardiac side effects.

Neurotoxicity

Taxanes are potentially neurotoxic compounds. Paclitaxel can induce painful peripheral neuropathies, characterized by sensory symptoms such as numbness and paresthesia in a glove-and-stocking distribution. Motor and autonomic dysfunction may also occur, especially at high doses and in patients with preexisting neuropathies from diabetes mellitus or alcoholism. Optic-nerve disturbances can also be observed, characterized by scintillating scotomata. There is currently no effective treatment to prevent or ameliorate the neurotoxic effects of taxanes.

The next chapter of this thesis (section 2) contains an extensive review of paclitaxel-induced neuropathic pain.

Miscellaneous Toxic Effects

In common with other chemotherapeutic drugs, paclitaxel induces reversible alopecia of the scalp, and all body hair is often lost with cumulative therapy. However, gastrointestinal effects, such as vomiting and diarrhea, are rarely associated with paclitaxel use. Higher doses may cause mucositis, especially in patients with leukemia. Rare cases of neutropenic enterocolitis have been reported, usually after high doses of paclitaxel in combination with doxorubicin or cyclophosphamide.

2. NEUROPATHIC PAIN INDUCED BY PACLITAXEL

The International Association for the Study of Pain (IASP) defines neuropathic pain as "pain initiated or caused by a primary lesion or dysfunction in the nervous system". Neuropathic pain can be divided into peripheral or central pain according to the anatomical localization of the lesion. The IASP defines peripheral neuropathic pain as "pain initiated or caused by a primary lesion or dysfunction in the peripheral nervous system (PNS)". When it is a consequence of the toxicity of anticancer drugs, the lesion mainly targets the PNS (see section 2.2.1). Therefore, neuropathic pain in the setting of a peripheral neuropathy, such as that induced by paclitaxel, should be considered a peripheral neuropathic pain.

Clinical manifestations of neuropathic pain are often discussed in terms of positive and negative symptoms. Positive symptoms reflect an enhanced level of excitability in the nervous system and include pain, paresthesia, and dysesthesia. Negative symptoms imply reduced impulse conduction in the neural tissues and include hypoesthesia or anesthesia (Baron and Tölle, 2008; Gutiérrez-Gutiérrez et al., 2010). Typical positive signs of neuropathic pain evaluated in animals are allodynia (pain due to a stimulus that does not normally provoke pain [IASP]) and hyperalgesia (an increased response to a stimulus that is normally painful [IASP]), because hypersensitivity to mechanical, cold, and hot stimuli is relatively easy to record in rodents. In contrast, subjective symptoms (e.g., tingling, burning pain, etc) cannot be evaluated; therefore, the representation of neuropathic pain in animal models is unavoidably incomplete.

In 2000, Authier and coworkers published the first study that described hypersensitivity to pain stimuli produced by paclitaxel-induced neurotoxicity. Since then, many studies using different doses and schedules in rats or mice (repeat administration, single administration, weekly administration, etc) have been carried out in numerous

laboratories around the world. The time-course of the symptoms of paclitaxel-induced neuropathic pain, its pathophysiology, and experimental treatments are extensively reviewed below.

2.1. Chemotherapy-induced peripheral neuropathies in humans

Peripheral neuropathy induced by anti-cancer chemotherapy is considered the most common neurological complication of cancer treatment and is probably the most common toxic neuropathy in our setting (Velasco and Bruna, 2010). Chemotherapy-induced peripheral neuropathy is defined as the presence of signs or symptoms of peripheral nerve dysfunction (either somatic or autonomic) resulting from damage caused by chemotherapeutic agents to the peripheral or autonomic nervous system (Gutiérrez-Gutiérrez et al., 2010). The incidence of these neuropathies depends on the antineoplastic agent, and can vary widely (10-100%) according to the definition of neuropathy. In general, it is estimated that a neuropathy affects around 30-40% of patients receiving chemotherapy. The characteristics of chemotherapy-induced neuropathies depend on the anticancer drug in question, the dose intensity, and the cumulative dose (Gutiérrez-Gutiérrez et al., 2010).

For most chemotherapeutic agents, including the most widely used first and/or second-line cancer treatments, such as taxanes, *Vinca* alkaloids and the platinum-based drugs, these neuropathies are the side effects with the most significant negative impact on the patients´ quality of life, and they are often a cause of extreme pain and disabling dysfunctions (Paice, 2010). In many patients, the symptoms and functional limitations generated by these neuropathies are the most disagreeable and disabling of all adverse effects of chemotherapy. Furthermore, some chemotherapy-induced neuropathies are

chronic and leave lifelong sequelae. For these reasons, these neuropathies are major dose-limiting side effects of cancer treatment and influence the effectiveness of chemotherapy and patient survival. Thus, their onset produces delays in the administration of a new cycle, reductions in dose per cycle or even cessation of therapy. Therefore, neuropathies induced by anticancer drugs have long posed oncologists with a serious problem for which there has been no effective solution (Cleeland et al., 2010; Gutiérrez-Gutiérrez et al., 2010; Velasco and Bruna, 2010).

Furthermore, the incidence of these neuropathies has been increasing over recent years and is expected to continue to do so. The main factor responsible for this rise is the increased number of patients who are candidates for chemotherapy and the greater survival provided by more effective novel drugs and therapeutic regimens. Moreover, the use of hematopoietic factors (colony stimulating factors), which limit hematotoxicity, have enabled the use of higher doses of cytotoxic agents, which are also increasingly administered in combinations, exacerbating the risk of adverse neurotoxic effects (Gutiérrez-Gutiérrez et al., 2010; Velasco and Bruna, 2010). Table 1 exhibits the main anticancer agents that cause peripheral neuropathy.

2.2. Paclitaxel-induced peripheral neuropathy in humans

Neurotoxicity, usually manifesting as painful peripheral neuropathy, is considered the most important non-hematological toxicity associated with anti-cancer treatment using microtubule-stabilizing agents, such as taxanes or epothilones. Taxane-induced peripheral neuropathies have been more extensively studied than those induced by epothilones. Among taxanes, these neuropathies are more frequently observed in paclitaxel-based chemotherapy than in docetaxel-based chemotherapy (Lee and Swain, 2006).

Table 1. Clinical findings in commonly used antineoplastic drugs.

Drug	Sensory	Motor	Reflexes	Autonomic
Platinum compounds				
Cisplatin	30-40% of patients. Distal predominant, symmetric, upper and lower limb loss of all modalities (large fiber greater than small fiber). May progress for several months after drug is discontinued. Pain is common.	Normal	Reduced in proportion to sensory loss	Rare
Carboplatin	10-20% of patients. Similar but less prominent than cisplatin. Pain is less common than with cisplatin.	Normal	Similar to cisplatin	Rare
Oxaliplatin (acute)	80% of patients. Cold-induced dysesthesia in mouth, throat, and upper limbs.	Cramps and/or muscle spasms in throat muscles	No changes	None
Oxaliplatin (chronic)	Similar to cisplatin	Normal	Similar to cisplatin	Rare
***Vinca* alkaloids**				
Vincristine, vindesine, vinblastine, vinorelbine	30-40% of patients. Distal sensory loss to all modalities in the lower limbs. Uncommonly affects upper limbs	5-10% of patients. Distal symmetric weakness in lower limbs progressing to foot drop	Early reduction or absence	Constipation common; Orthostatic hypotension less common
Taxanes				
Docetaxel, paclitaxel	10-20% of patients. Mild distal loss of all modalities in feet.	Uncommon, mild weakness in foot muscles	Reduced ankle reflexes	Rare
Other agents				
Suramin	30% of patients. Distal symmetric loss of all modalities in the lower limbs	10-20% of patients. Mild distal weakness in lower limbs. Rarely, severe, subacute, distal, and proximal weakness.	Reduced in proportion to weakness	Rare
Bortezomid	30-40% of patients. Mild to moderate, distal symmetric loss of all modalities in the lower limbs	5-10% of patients. Mild distal weakness in lower limbs. Rare, severe distal weakness	Reduced in proportion to sensory loss	Rare
Thalidomide	20-40% of patients. Mild to moderate, distal symmetric loss of all modalities in the lower limbs	Weakness rare	Reduced in proportion to sensory loss	Rare

Note: table taken from Windebank and Grisold, 2008

2.2.1. Pathogenesis

The toxicity of anticancer drugs targets the PNS more frequently than the CNS.

Neurotoxicity in the CNS is occasionally reported, and its frequency is markedly increased when the blood-brain barrier is overwhelmed (high systemic dose, intracarotid infusion) or bypassed (intrathecal administration). However, PNS toxicity is more usual because the peripheral nerves are not protected by a blood-brain-like barrier (Hildebrand, 2006). In fact, toxic- or drug-induced neuropathies might have three sites of cellular involvement. Thus, axonal degeneration (axonopathy) is the most frequent type of pathological change observed after neurotoxic drug exposure, whereas demyelination (myelinopathy) is much rarer. Anticancer drugs can also affect cell bodies (ganglionopathy), especially those of dorsal root ganglia (DRG) (Cavaletti, 2007). However, the pathophysiological mechanisms directly involved in the pathogenesis of these neuropathies are only partially known, are probably multiple, and are not always related to the antitumor mechanism. Furthermore, most available data are from *in vitro* studies or experimental animal models (Velasco and Bruna, 2010).

The precise mechanism by which paclitaxel causes peripheral neuropathy is not completely clear. It had been assumed that paclitaxel exerts its neurotoxicity through its antitumor mechanism, i.e., by interfering with neuronal microtubules that are essential for axonal transport. However, animal studies showed that other mechanisms may be involved (see section 2.3). It is well-documented that paclitaxel can affect all sensory neurons, with a preference for large myelinated fibers (mainly Aβ) conducting tactile sensation, vibration perception, and proprioception, and that this neuropathy is characterized by an ascending distal paresthesia and dysesthesia and pain, in a ‘glove and stocking’ distribution (Dougherty et al., 2004). The predominant distal loss of sensation in the large fibers implies a “dying back” process, starting from distal nerve endings and followed by effects on Schwann cells and neuronal body, or a disturbed axonal transport in the

affected neurons, which are the most widely-accepted mechanisms of paclitaxel-induced peripheral neuropathy (Argyriou et al., 2008; Balayssac et al., 2011). Supporting this hypothesis, vincristine, which also alters microtubule function, produces a peripheral neuropathy almost identical to that induced by paclitaxel and similar to that of other 'dying back'-type peripheral neuropathies (Park et al., 2008), such as diabetic peripheral neuropathy (Dobretsov et al., 2007) and HIV-associated neuropathy (Pardo et al., 2001). Nevertheless, some animal findings cast doubt on this hypothesis (Bennett et al., 2011; see section 2.3).

Like other chemotherapy-induced peripheral neuropathies, patients treated with taxanes have been investigated at clinical and neurophysiological level, but very few data are available on pathological changes in peripheral nerves. One of the two studies of human biopsies of the sural nerve after paclitaxel treatment revealed no evident disarray or atypical aggregation of microtubules in the axons or Schwann cell, although an occasional thinly myelinated axon suggestive of remyelination was observed (Wiernik et al., 1987). The other study evidenced severe nerve fiber loss, axonal atrophy, and secondary demyelination (Sahenk et al., 1994).

Similar results were found in nerve biopsies from patients with peripheral neuropathy induced by cisplatin. In addition, the highest concentration of cisplatin was found in DRG and the lowest in tissue protected by the blood-brain barrier. Interestingly, the clinical and histological severity of the neurotoxicity was associated with cisplatin levels found in the DRG. A reduced number of myelinated fibers and axonal and mitochondrial swelling were observed in nerve biopsies of patients with vincristine-induced neuropathy, but microtubule alterations were not detected. Other authors reported an increased number of neurofilaments in the DRG of patients with

vincristine neuropathy (reviewed by Cata et al., 2006). However, in most of these biopsies and postmortem examinations, the patients had been treated with more than one neurotoxic compound, and specimens were obtained at different times during or after chemotherapy, making it very difficult to draw clear conclusions from these data (Cavaletti and Marmiroli, 2010).

Other pathogenic mechanisms were reviewed by Velasco and Bruna (2010). Thus, a decrease in circulating levels of nerve growth factor (NGF) was found in patients treated with chemotherapy and may be related to the neuropathy induced. Peripheral nerve toxicity may also have a partially vascular origin, because cytostatics such as cisplatin or paclitaxel would induce apoptosis of the endothelial cells of the vasa nervorum, with consequent nerve fiber ischemia. Finally, the oxidative stress caused by the majority of antineoplastic drugs has also traditionally been associated with the activation of neuronal apoptosis mechanisms, which may be associated with the neuropathies.

2.2.2. Clinical characteristics

Neuropathy may be induced by chemotherapy early in the course of treatment or after repeated courses, and the severity of these neuropathies can sometimes progress for months after the discontinuation of treatment, a phenomenon known as “coasting”. In general, chemotherapy-induced neuropathies disappear after cessation of treatment. However, they are not always reversible, especially in the neurotoxicity induced by cisplatin. Symptoms and signs reported by patients with a chemotherapy-induced neuropathy can be sensory, motor, or autonomic. The earliest clinical signs in most of

these patients are usually a decrease in the sensation of vibration and loss of the Achilles jerk (Velasco and Bruna, 2010; Cavaletti et al., 2011).

Symmetric sensory distal neuropathy (as in most of the neuropathies induced by antineoplastic drugs) is the most common neurotoxic effect of taxane treatment, whereas motor and autonomic neuropathy is less frequent (Mielke et al., 2006; Argyriou et al., 2008). In general, taxane-induced neurotoxicity is cumulative and dose-dependent; however, there is no clear relationship between the onset and the dose or number of cycles of paclitaxel. Symptoms may arise after the first or second cycle of chemotherapy, or even in more advanced cycles of treatment with paclitaxel (Forsyth et al., 1997; Dougherty et al., 2004; Park et al., 2011). In addition, sensory symptoms may appear within 24-72 hours after the first administration of paclitaxel at a single high dose (Argyriou et al., 2008).

Primary clinical symptoms of paclitaxel-induced neuropathy include paresthesia, numbness, and/or neuropathic pain in the hands and feet with a stocking-and-glove distribution (figure 4), and they may affect the distal lower extremities first or start simultaneously in the toes and fingers. Moreover, there is a reduction in tendon reflexes, perception of vibration, and proprioception (Forsyth et al., 1997; Dougherty et al., 2004; Balayssac et al., 2011; Hershman et al., 2011; Park et al., 2011). In an old study, one patient also experienced perioral numbness (Wiernik et al., 1987). In addition, electrophysiological abnormalities, mostly involving the decrease or abolishment of sensory responses, corroborate the predominance of sensory fiber involvement (Argyriou et al., 2008).

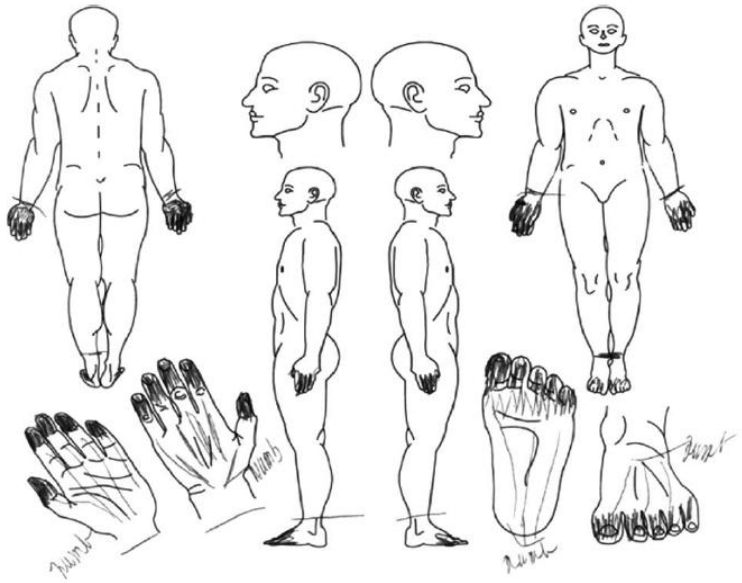

Fig. 4. A representative pain diagram completed by a patient with paclitaxel-induced neuropathic pain. The darkly shaded areas show where the patient complained of on-going pain. The patient described this zone as feeling like, 'rats biting on my fingers'. The lightly shaded area shows the border area of altered sensitivity. This area was described as 'numb' and 'cold'. A typically sharp boundary between the areas affected and 'normal' skin was the line across the wrist and ankles (figure taken from Dougherty et al., 2004).

Some authors reported that paclitaxel could induce motor neuropathy as well as sensory neuropathy (Freilich et al., 1996; Augusto et al., 2008), and it was even suggested that motor neuropathy may not be well recognized (Argyriou et al., 2008). Paclitaxel, like most chemotherapeutic drugs, is known to have poor blood-nerve barrier permeability (Kemper et al., 2004). In the sensory DRG, however, this blood-nerve barrier is relatively more permeable than elsewhere, due to dense vascularization and large fenestrations of endothelial cells that vascularize DRG (Jimenez-Andrade et al., 2008) and lower P-glycoprotein activity (Balayssac et al., 2005). This may result in higher concentrations of paclitaxel in the DRG than in other parts during treatment and may explain, at least in part, the predomination of sensory over motor symptoms.

The severity of most symptoms of paclitaxel-induced neuropathy is usually mild to moderate and can improve after the cessation of treatment (Forsyth et al., 1997). However,

severe symptoms, although less frequent, also affect a substantial percentage of patients and can persist for a long time (Argyriou et al., 2008). In fact, in a very recent study of women with early stage breast cancer who received paclitaxel-based adjuvant chemotherapy, around 80% experienced neuropathy symptoms up to 2 years after completion of treatment (Hershman et al., 2011).

2.2.3. Incidence and risk factors

The main risk factors associated with an increased incidence of taxane-induced peripheral neuropathies include the following: treatment schedule, single and cumulative dose level, duration of infusion, concomitant administration with other chemotherapeutics, age, and pre-existing peripheral neuropathy due to medical conditions (e.g., hereditary or associated with nutritional agents, diabetes mellitus, alcohol abuse, etc). It has also been reported that the incidence of peripheral neuropathy is lower in patients treated with docetaxel than in those treated with paclitaxel (Lee and Swain, 2006).

One of the most important factors appears to be the dose, either single or cumulative, with several clinical trials finding that severe neurotoxicity is less likely in patients receiving lower single doses of paclitaxel. The cumulative doses associated with severe neurotoxicity are variable depending on treatment schedules (see Lee and Swain, 2006; Argyriou et al., 2008; and Balayssac et al., 2011 for references).

The weekly administration and shorter infusion duration of paclitaxel, although linked to improved antitumor activity and a lesser incidence of neutropenia (see chapter 1), has been associated with a higher frequency of severe neurotoxicity. Paclitaxel concentrations higher than 0.05 μmol/L ($T_{>0.05}$) for ≥10.6 h were associated with a greater risk of paclitaxel-induced neuropathy (Mielke et al., 2006; Argyriou et al., 2008).

Several reports that demonstrated the synergistic anticancer effects of taxanes in combination with other chemotherapeutic agents, such as platinum compounds, also found additive effects in the production of peripheral neuropathy. Paclitaxel-induced peripheral neuropathy may also be more frequent and severe in patients with some predisposition to neuropathy, such as patients previously treated with chemotherapy or those with alcoholic or diabetic disease. Finally, there are contradictory data on the susceptibility of elderly patients to develop paclitaxel-induced peripheral neuropathy (Mielke et al., 2006; Argyriou et al., 2008).

2.2.4. Diagnosis

In common with all chemotherapy-induced neuropathies, the diagnosis of paclitaxel-induced peripheral neuropathy is normally based on clinical examination and quantitative methods, such as electrophysiological measurements (Mielke et al., 2006; Argyriou et al., 2008). Several comprehensive neurotoxicity grading scales have been used for the assessment of chemotherapy-induced peripheral neuropathy, including paclitaxel neuropathy. However, Cavaletti and co-workers recently concluded that the existing scales are not satisfactory for evaluating chemotherapy-induced peripheral neuropathy, including taxanes-induced neuropathy, because none of them has been designed or validated to assess neuropathic pain, among other reasons. In addition, most of these scales merge impairment, disability, and quality of life measures, leading to erroneous results interpretations and an uncertain under- or over-estimation of the effect. For example, one study found that two neurologists examining the same group of patients could only agree on the evaluation of 7 out of 37 patients (Postma et al., 1998). Nevertheless, they remain the most widely used instruments because they are quick to use and apparently easy to manage (see Cavaletti et

al., 2010 and Cleeland et al., 2010 for an extensive and critical review of assessment methods for chemotherapy-induced peripheral neuropathy and/or chemotherapy-induced neuropathic pain).

It has been suggested that neurophysiologic measures, including the assessment of distal latencies, conduction velocities, and amplitudes, are improving the sensitivity of evaluations of chemotherapy-induced peripheral neuropathy and may facilitate the identification of patients with subclinical peripheral neuropathy before the onset of clinically significant neurotoxicity (Mielke et al., 2006; Argyriou et al., 2008).

Although sural nerve biopsy has also been used for the diagnosis of paclitaxel-induced peripheral neuropathy (Sahenk et al., 1994), this invasive method appears to be inappropriate in comparison to less invasive, better-tolerated examinations. An alternative method may include the examination of intraepidermal fibers in skin biopsies, which has already been described as a diagnostic tool in other peripheral neuropathies (Sommer and Lauria, 2007; Botez and Herrmann, 2010). However, further studies are needed to establish whether skin biopsy is a useful method to assess peripheral neuropathy associated with treatment with taxanes or other chemotherapy drugs (Mielke et al., 2006; Argyriou et al., 2008).

Several clinical studies have assessed paclitaxel-induced peripheral neuropathies by using non-invasive quantitative sensory testing (QST), which measures the sensory threshold for a particular stimulus, such as touch or vibration, by repeatedly delivering a stimulus via a specific algorithm (Forsyth et al., 1997; Dougherty et al., 2004; Augusto et al., 2008; Hershman et al., 2011). Magnetic resonance imaging has also been proposed as a non-invasive method to diagnose sensory ganglionopathies but requires further validation before its utilization in clinical practice (Mielke et al., 2006; Argyriou et al., 2008).

2.2.5. Treatment

In general, the management of patients with neuropathic pain is complex, and the response to existing treatments is often inadequate. Even with well-established neuropathic pain medications, the effectiveness is unpredictable, dosing can be complicated, analgesic onset is delayed, and side effects are common (Dworkin et al., 2007). Neuropathic pain induced by chemotherapy is not an exception, and measures for its prevention or treatment remain inadequate, probably attributable to a lack of understanding of the cellular pathology responsible for the development of these painful peripheral neuropathies. In fact, there is no drug available to reliably prevent or cure chemotherapy-induced neuropathy (Cavaletti et al., 2011), and treatment modification or drug withdrawal is generally the only option for most patients. In recent years, however, considerable efforts have been made at preclinical and clinical level to find an effective treatment (Cavaletti et al., 2011).

Studies on the symptomatic treatment of taxane-induced peripheral neuropathy are limited, although amitriptyline, glutamine, prednisone, and gabapentin have been used with some measure of success to reducing pain, myalgia, and arthralgia. However, all of these compounds have to prove their effectiveness in further clinical studies. Various symptomatic and/or neuroprotective agents have also been tested in animal models and shown some efficacy (see section 2.3.4), but the clinical data remain controversial and further studies are required. These neuroprotective agents include thiols, neurotrophic factors, and antioxidants (reviewed by Argyriou et al., 2008).

The development of numerous experimental models of chemotherapy-induced peripheral neuropathy has allowed a large number of molecules to be tested. However, it should be borne in mind that the compound must be effective and produce acceptable

adverse effects while not interfering with the antitumor effect of the chemotherapeutic agent.

2.3. Experimental models of neuropathic pain induced by paclitaxel

Over the past 25 years, there has been growing interest in research on peripheral neuropathy induced by chemotherapy due to its increased incidence. To some extent, it is possible to replicate neuropathies experienced by chemotherapy-treated patients in laboratory animals. In particular, it is possible to induce neuropathic pain-like symptoms in mice and rats after the administration of chemotherapeutic agents. The major purpose of these animal models is to reliably reproduce the pathologic changes observed in humans and to quantify the extent of nervous system involvement. Consequently, these animal models are helping to discern the pathophysiological mechanisms responsible for these neuropathies and are allowing the testing of a multitude of compounds to treat and/or prevent these neuropathies. Moreover, these models of chemotherapy-induced neuropathic pain may be preferable to surgical models for the study of neuropathic pain, because they are easier to perform, especially in the mouse, whose small size hinders surgical procedures. Among existing animal models of neuropathic pain induced by antineoplastic drugs, the most frequent and widely studied are experimental models of neuropathic pain induced by taxanes, *Vinca* alkaloids, and platinum compounds (Authier et al., 2009; Colleoni and Sacerdote, 2010). Among these, the experimental model of paclitaxel-induced painful peripheral neuropathy is one of the most widely studied and is thoroughly reviewed below, being the model of chemotherapy-induced neuropathic pain used in the experimental part of this Doctoral Thesis.

Before discussing in depth the experimental models of neuropathic pain induced by

paclitaxel, it is important to bear in mind that the first studies of paclitaxel-induced neuropathy in animals did not provide an adequate description of the nociceptive symptoms generated by administration of this antineoplastic drug. In fact, it is possible to distinguish three different periods in the study of peripheral neuropathy induced by paclitaxel in animals. (1) In the mid-1980s, the pathology induced by paclitaxel was studied by administering the drug directly into the nerve of rats (e.g.: Röyttä et al., 1984). However, the usefulness of the intraneural injection model is limited in terms of comparison with the neuropathy induced by paclitaxel in patients, because it takes no account of the blood-nerve barrier. (2) Next, in the 1990s, experimental models of neuropathy induced by systemic paclitaxel administration to rodents were described (e.g.: Apfel et al., 1991). These models, which evaluated the motor-sensory status of the animal, are more representative of the developed neuropathy in humans but none of them documented positive signs of neuropathic pain. In fact, most of these studies found a loss of pain perception, probably due to very high cumulative doses of paclitaxel. (3) Finally, in 2000, Authier and colleagues described the first neuropathic pain model induced by paclitaxel with signs of hypersensitivity to pain stimuli. Since then, numerous studies have increased knowledge on the pathophysiological mechanisms of paclitaxel-induced neuropathic pain, allowing the testing of several compounds to alleviate and/or prevent this painful neuropathy in rodents.

2.3.1. Animal models of paclitaxel-induced neuropathy: morphological and electrophysiological abnormalities

The first studies on paclitaxel-induced neuropathy in animals, after systemic administration of the antineoplastic, were developed in the 1990s. These experimental animal models mainly described electrophysiological and histological changes, and

none of them yielded a complete description of the signs of pain induced by paclitaxel. Nevertheless, they provided relevant data on the main characteristics of this neuropathy. This section (2.3.1) reviews the main findings obtained using models of paclitaxel-induced neuropathies, which did not specifically study the neuropathic pain induced by this anticancer drug.

The first experimental model of systemic paclitaxel-induced peripheral neuropathy was reported in mice in 1991. In this early study, administration of high doses of paclitaxel (21.6 mg/kg x 6 weekly injections) induced a profound sensory neuropathy characterized by an elevated threshold to thermally induced pain (hypoalgesia), a drastic decrease in DRG content of substance P, and diminished amplitude of the compound action potential in the caudal nerve. However, light microscopy study showed no significant histological differences in DRG (Apfel et al., 1991). Two years later, Hamers and coworkers, using solely an electrophysiological approach, also found that paclitaxel induced a sensory neuropathy, which manifested as a decrease in sensory nerve conduction velocity with no motor impairment (Hamers et al., 1993). Subsequently, another laboratory reproduced the first model of Apfel but using older female mice, and the results were generally similar, although there was only mild substance P loss in the DRG and there was a moderate drop in the level of calcitonin gene-related peptide (CGRP) (Schmidt et al., 1995).

Cavaletti and colleagues (1995) coincided in general with the above studies, but they performed a further neurophysiological and morphological examination of the peripheral nerves, DRG, spinal rootlets, and spinal cord of rats treated weekly with paclitaxel (8 mg/kg x 5 weeks). They found an impairment of nerve conduction velocity as well as morphological and morphometric changes, which were mainly confined to the peripheral nerves, especially axonal and Schwann cell alterations; no pathological changes were

observed in DRG or motoneurons. They reported predominant damage to the largest myelinated fiber and axonal atrophy, which may explain the neurophysiological results obtained in this and earlier studies (Apfel et al., 1991; Hamers et al., 1993). Interestingly, a tendency of microtubules to surround mitochondria and intraaxonal neurotubular aggregates was also mainly reported in the largest myelinated fibers of paclitaxel-treated rats. These tubular alterations were similar but less intense than those observed after the intraneural administration of paclitaxel (Röyttä et al., 1984; Röytta and Raine, 1985; 1986), opening up the possibility of a common mechanism for paclitaxel-induced cytotoxicity and neurotoxicity. However, in these older studies, Röyttä and co-workers also reported abnormal neurotubular accumulations in unmyelinated fibers and Schwann cells, whereas Cavaletti and colleagues did not. In any case, data from intraneural delivery should be interpreted with care, because paclitaxel concentrations in the nerve would be much higher than those obtained during systemic administration.

Two years later, Cavaletti´s group performed a new study of paclitaxel neuropathy but administered the drug intravenously (5 mg/kg x 5, over a period of 10 days) (Cavaletti et al., 1997) to avoid the development of ascites found in the i.p. treated rats in their first study (Cavaletti et al., 1995) and to mimic the clinical administration route (see section 1.4.3). In general, the data confirmed the findings of the first study: impairment of nerve conduction velocity, intraaxonal neurotubular aggregates, and no pathological changes in the neuronal bodies of the spinal cord or DRG. They also expanded the scope of their investigation with some behavioral testing, finding that treated animals had significantly reduced pain perception (tail-flick test) and motor and proprioceptive activity (rotarod test). Interestingly, the vehicle used to dissolve the antineoplastic drug was not the common but troublesome Cremophor® EL (dissolved in saline with ethanol) but rather a mix of ethanol and Tween

80 dissolved in saline.

In 1998, an interesting study tested several schedules and doses of paclitaxel (see table 2) and concluded that not only the cumulative dose but also the dose frequency and individual dose are important for the severity of the neuropathy and other general health aspects (Cliffer et al., 1998). They found that paclitaxel induced large-fiber sensory neuropathy in rats that was characterized by robust sensory electrophysiological deficits (with mild motor involvement), reductions in axon caliber and density, degenerated myelin profiles in lumbar dorsal root, and impairment of proprioceptive function (reduced ability to remain balanced on a narrow beam), consistent with deficits preferentially in large sensory fiber structure and function. However, they found no change in the tail-flick test, unlike other authors (Apfel et al., 1991; Cavaletti et al., 1997; Campana et al., 1998; Boyle et al., 1999).

In 1999, a new rat study on paclitaxel-induced neuropathy found a mixed sensorimotor neuropathy manifested by gait disturbance, impaired rotarod performance, and elevation of tail-flick threshold. However, the authors probably used a more severe schedule than did others, because rats were treated with paclitaxel until they were able to tolerate the treatment (Boyle et al., 1999).

A more recent study (Mimura et al., 2000), using only a morphological approach, found that high doses of paclitaxel in mice induced axonal degeneration, myelin fragmentations, and phagocytosis in the nerve fibers of the dorsal funiculus, spinal roots, and peripheral nerves (sciatic and tibial). However, no drug-related degeneration was observed in motor neurons in the spinal cord or ventral spinal roots, supporting the hypothesis of a predominantly sensory neuropathy. They also found that the severity of the neuropathy is more dependent on the treatment schedule than on the cumulative dose.

Table 2. Rat models of paclitaxel-induced neuropathy: behavioral and electrophysiological findings

Animals	Schedules	CD (mg/kg)	EP anomalies		Motor anomalies	Pain behavior						References
			Sensorial	Motor		MA	MH	CA	CH	TH	HH	
Rats	**Repeated treatment**											
M W	24 mg/kg x 5 W, IP	120	—	NT	NT	NT	NT	NT	NT	NT	NT	Kilpatrick et al., 2001
F W	12.5 mg/kg x 9 W, IP	112.5	+	—	NT	NT	NT	NT	NT	NT	NT	Jamieson et al., 2007
F W	16 mg/kg x 5 W, IP	80	+	NT	NT	NT	NT	NT	NT	NT	NT	Cavaletti et al., 1995
M W	16 mg/kg x 5 W, IP	80	—	NT	NT	NT	NT	NT	NT	NT	NT	Kilpatrick et al., 2001
M SD	16 mg/kg x 5 W, IP	80	+	—	Not found	NT	+	NT	NT	—	+	Authier et al., 2000
M W	1.2 mg/kg x 5D x7 W + 2.4 mg/kg x 5D x 2 W	66	+	—	NT	NT	NT	NT	NT	NT	NT	Hamers et al., 1993
F W	16 mg/kg x 4W, IP	64	+	NT	NT	NT	NT	NT	NT	NT	NT	Persohn et al., 2005
M W	9 mg/kg x 6W, IP	54	+	—	NT	NT	NT	NT	NT	NT	NT	Hamers et al., 1993
M SD	25, 12.5, 12.5 mg/kg every third day, IP	50	NT	NT	NT	NT	NT	NT	NT	—	+	Campana et al., 1998
F W	12.5 mg/kg x 4W, IV	50	+	NT	NT	NT	NT	NT	NT	NT	NT	Persohn et al., 2005
F W	9 mg/kg x 5W, IP	45	+	NT	NT	NT	NT	NT	NT	—	+	Chentanez et al., 2003
F W	8 mg/kg x 5 W, IP	40	+	NT	NT	NT	NT	NT	NT	NT	NT	Cavaletti et al., 1995
F W	10 mg/kg x 4W, IV	40	+	NT	NT	NT	NT	NT	NT	NT	NT	Persohn et al., 2005; Carozzi et al., 2010
F DA	9 mg/kg x 2D x W (until max tolerated dose) 5% dextrose	37±8.8	NT	NT	Gait disturbance, Reduced coordination	NT	NT	NT	NT	—	+	Boyle et al., 1999
M SD	18 mg/kg x 3 D apart, IV	36	NT	NT	Reduced coordination	+	NT	NT	+	NT	NT	Jimenez-Andrade et al., 2006; Peters et al., 2007
M SD	16 mg/kg x 3 D apart, IP	32	NT	NT	NT	+	NT	+	NT	—	NT	Nishida et al., 2008
M SD	8 mg/kg x 4 D alternative, IP	32	NT	NT	NT	+	+	NT	NT	NT	NT	Bennett et al., 2011
F SD	15 mg/kg x 2 W, IV	30	+	—	Reduced coordination	NT	NT	NT	NT	NT	—	Cliffer et al., 1998
F DA	5 mg/kg x 6 consecutive D, IP (after tumor implantation)	30	NT	NT	Gait disturbance	NT	NT	NT	NT	—	+	Boyle et al., 2001
F W	5 mg/kg x 5 D alternative, IV(Tween80)	25	+	NT	Reduced coordination	NT	NT	NT	NT	—	+	Cavaletti et al., 1997
F W	5 mg/kg x 5 D alternative, IV	25	+	NT	NT	NT	NT	NT	NT	NT	NT	Pisano et al., 2003; Lauria et al., 2005
M W	5 mg/kg x 5 D alternative, IP	25	+	—	NT	NT	NT	NT	NT	NT	NT	Bardos et al., 2003
F SD	12 mg/kg x 2 W, IV	24	+	—	Not found	NT	NT	NT	NT	NT	—	Cliffer et al., 1998
M SD	12 mg/kg x 2 W, IV	24	+	+	Not found	NT	NT	NT	NT	NT	NT	Kirchmair et al., 2007

M SD	6 mg/kg x 4 W, IP	24	NT	NT	Reduced coordination, normal muscle strength	+	NT	NT	+	NT	NT	Kawashiri et al., 2009; Tatsushima et al., 2011
M SD	8 mg/kg x 3 D apart, IP	24	NT	NT	NT	+	NT	NT	NT	NT	NT	Liu et al., 2010
M SD	6 mg/kg x 4 D alternative, IP	24	NT	NT	NT	+	+	NT	NT	NT	NT	Bennett et al., 2011
F W	5 mg/kg x 4W, IV	50	+	NT	NT	NT	NT	NT	NT	NT	NT	Persohn et al., 2005
M SD	1.2 mg/kg x 5D x 3W, IP	18	NT	NT	NT	NT	NT	NT	NT	—	+	Campana et al., 1998
M SD	1 mg/kg x12 D, IP	12	NT	NT	NT	NT	+	NT	NT	NT	NT	Dina et al., 2001
M SD	1 mg/kg x10 D, IP	10	NT	NT	NT	+	+	NT	NT	+	—	Dina et al., 2001; Dina et al., 2004; Alessandri—Haber et al., 2004; Alessandri—Haber et al., 2008
M SD	2 mg/kg x 4 D alternative, IP	8	+	—	Not found	+	+	+	+	+/—	—	Polomano et al., 2001; Flatters and Bennett, 2004; Flatters et al., 2006; Flatters and Bennett, 2006; Siau and Bennett, 2006; Siau et al., 2006; Ledeboer et al., 2007; Xiao et al., 2007; Xiao and Bennett, 2008; Jin et al., 2008; Rahn et al., 2008; Xiao et al., 2008; Xiao et al., 2009; Kiya et al., 2010; Bennett et al., 2011; Boyette—Davis et al., 2011; Zheng et al., 2011
			Spontaneous discharges in A- and C-fiber;									
M SD	2 mg/kg x 4 D alternative, IP (DMSO + TWEEN)	8	NT	NT	Ssedation not found	+	NT	NT	NT	NT	NT	Kim et al., 2010
M W	2 mg/kg x 4 D alternative, IP	8	NT	NT	NT	NT	+	NT	NT	NT	NT	Okubo et al., 2011
M LE	1 mg/kg x 6 D alternative, IP	6	NT	NT	NT	+	NT	NT	NT	NT	NT	Boyette—Davis et al., 2011
M SD	0.5 mg/kg x10 D, IP	5	NT	NT	NT	NT	+	NT	NT	NT	NT	Dina et al., 2001
M SD	1 mg/kg x 4 D alternative, IP	4	—	NT	Not found	+	+	+	+	+/—	—	Polomano et al., 2001; Ledeboer et al., 2006; Ledeboer et al., 2007; Naguib et al., 2008; Gracias et al., 2010
M SD	1 mg/kg x 4 D alternative, IP DMSO	4	Spontaneous activity, after-discharges		NT	+	+	NT	NT	+	—	Weng et al., 2005; Cata et al., 2006; Cata et al., 2008
M W	1 mg/kg x 4 D alternative, IP	4	NT	NT	Reduced spontaneous motility	+	NT	NT	NT	+	—	Pascual et al., 2005; Pascual et al., 2010
F SD	1 mg/kg x 4 D alternative, IP	4	NT	NT	Not found	+	NT	NT	NT	+	—	Jolivalt et al., 2001
Rats	**Acute treatment**											
M SD	1 mg/kg , IP	1	NT	NT	NT	NT	+	NT	NT	NT	NT	Dina et al., 2001

EP: electrophysiological; NT: not tested; CD: cumulative dose; M: male; F: female; W: Wistar; SD: Sprague-Dawley; DA:Dark Agouti; LE: Long Evans; W: weeks; D: days; MA. Mechanical allodynia; MH: mechanical hyperalgesia; CA: cold allodynia; CH: cold hyperalgesia; TH: thermal (heat) hyperalgesia; HH: heat hypoalgesia

Table 3. Mouse models of paclitaxel-induced neuropathy: behavioral and electrophysiological findings

Animals	Schedules	CD (mg/kg)	EP anomalies		Motor anomalies	Pain behavior						References
			Sensorial	Motor		MA	MH	CA	CH	TH	HH	
Mice	**Repeated treatment**											
F C57Bl/6	[60 mg/kg x 3 D alternative, IV] x 2 3 W apart	360	+	—	Reduced coordination	NT	NT	NT	NT	NT	NT	Wang et al., 2004
F BALB/c	70 mg/kg x 4W, IV	280	+	+	NT	NT	NT	NT	NT	NT	NT	Carozzi et al., 2010
F BALB/c	50 mg/kg x 4W, IV	200	+	+	NT	NT	NT	NT	NT	NT	NT	Carozzi et al., 2010
F C57Bl/6, Wld^s	60 mg/kg x 3D alternative, IV	180	+	—	Reduced coordination	NT	NT	NT	NT	NT	NT	Wang et al., 2002; Wang et al., 2004; Callizot et al., 2008
M F CD1	21.6 mg/kg x 6D consecutive, IP	129.6	+	NT	NT	NT	NT	NT	NT	—	+	Apfel et al., 1991
F C57Bl/6	8 mg/kg x 4D alternative, IP	32	NT	NT	NT	+	NT	+	NT	NT	NT	Ward et al., 2011
M ddY	4 mg/kg x 4D alternative, IP	16	NT	NT	NT	+	NT	NT	NT	+	—	Matsumoto et al., 2006
F C57Bl/6	4 mg/kg x 4D alternative, IP	16	NT	NT	NT	+	NT	+	NT	NT	NT	Ward et al., 2011
F CD1	2 mg/kg x 5D consecutive, IP	10	NT	NT	NT	+	NT	+	NT	+	—	Nieto et al., 2008
MF C57Bl/6	2 mg/kg x 4D alternative, IP	8	NT	NT	NT	NT	NT	+(F)	NT	NT	NT	Ward et al., 2011
MF (10 strains)	1 mg/kg x 4D alternative, IP	4	NT	NT	NT	+	NT	+	NT	—	—	Smith et al., 2004
MF C57Bl/6	1 mg/kg x 4D alternative, IP	4	NT	NT	NT	NT	NT	+(F)	NT	NT	NT	Ward et al., 2011
Mice	**Acute treatment**											
M C57Bl/6	5 mg/kg , IP	5	NT	NT	NT	+	NT	NT	NT	NT	NT	Gauchan et al., 2009a and b
M ddY	4 mg/kg , IP	4	Hypersensitization of Aδ- and Aβ-fiber, not C-fiber		NT	+	NT	NT	NT	+	—	Matsumoto et al., 2006
M ddY	4 mg/kg x, IV	4	NT	NT	NT	+	NT	NT	NT	+	—	Matsumoto et al., 2006
M ddY	0.1-40 mg/kg, IP	0.1-40	NT	NT	NT	+	+	NT	NT	NT	NT	Hidaka et al., 2009

EP: electrophysiological; NT: not tested; CD: cumulative dose; M: male; F: female; W: weeks; D: days; MA. Mechanical allodynia; MH: mechanical hyperalgesia; CA: cold allodynia; CH: cold hyperalgesia; TH: thermal (heat) hyperalgesia; HH: heat hypoalgesia.

Since 2000, similar research has continued to be published, largely to test the capacity of compounds to prevent or inhibit the consequences of systemic paclitaxel administration in rodents (see tables 2 and 3). Most findings are in agreement with previous studies, using high doses and reporting that paclitaxel induced a predominantly sensorial peripheral neuropathy manifested by: (1) a reduced sensory nerve conduction velocity (Wang et al., 2002; Bardos et al., 2003; Chentanez et al., 2003; Pisano et al., 2003; Wang et al., 2004; Lauria et al., 2005; Persohn et al., 2005; Kirchmair et al., 2007; Jamieson et al., 2007; Callizot et al., 2008); (2) a generalized myelinated nerve fiber degeneration, usually more prominent in the largest fibers (Kilpatrick et al., 2001; Ogawa et al., 2001; Wang et al., 2002; Chentanez et al., 2003; Pisano et al., 2003; Wang et al., 2004; Persohn et al., 2005; Callizot et al., 2008); and (3) thermal hypoalgesia (Boyle et al., 2001; Chentanez et al., 2003). In addition, Cavaletti´s group quantified for the first time the density of intraepidermal nerve fibers (IENFs) in paclitaxel-treated rats and found that the antineoplastic induced a significant reduction in IENF density that was correlated with the reduction in nerve conduction velocity (Lauria et al., 2005). It was also reported that paclitaxel neuropathy could be caused, at least in part, by vascular damage to the vasa nervorum, because the drug reduced the blood flow of the sciatic nerve (Kirchmair et al., 2007).

Therefore, despite the different schedules, doses, and methodologies used, it is possible to draw some common conclusions from the above findings. The severity of paclitaxel-induced peripheral neuropathy depends both on the dose and frequency of paclitaxel administration. Most reports agree that systemic treatment with high doses of paclitaxel (see tables 2 and 3), regardless of the vehicle used, produces a sensory neuropathy with no clear motor impairment (Apfel et al., 1991; Hamers et al., 1993;

Cavaletti et al., 1995; Cliffer et al., 1998; Mimura et al., 2000; Wang et al., 2002; Bardos et al., 2003; Chentanez et al., 2003; Pisano et al., 2003; Wang et al., 2004; Lauria et al., 2005; Persohn et al., 2005; Kirchmair et al., 2007; Jamieson et al., 2007) or a mixed sensorimotor neuropathy (Cavaletti et al., 1997; Boyle et al., 1999; Callizot et al., 2008). Most pathological alterations were found only at the level of the axons or Schwann cells, and the damage was mainly observed in the largest myelinated fibers (Cavaletti et al., 1995; Cliffer et al., 1998; Kilpatrick et al., 2001; Wang et al., 2002; 2004; Persohn et al., 2005). All articles, except one (Jamieson et al., 2007) failed to detect any morphological changes in neuronal bodies of spinal cord or ganglia; however, several report biochemical changes (Apfel et al., 1991; Schmidt et al., 1995; Kilpatrick et al., 2001).

Wang and coworkers (2002, 2004) used very high cumulative doses of paclitaxel (180-360 mg/kg) and confirmed the marked selectivity of the drug for sensory nerve fibers and the lack of a pathological effect on DRG neurons. This selectivity for the sensorial tract is in agreement with the preponderance of sensory symptoms over motor symptoms in paclitaxel neuropathy and can be explained by the lack of a true blood-nerve barrier in the DRG. In fact, cell bodies of sensory neurons have a dense vascularization, large fenestrations of endothelial cells that vascularize them (Jimenez-Andrade et al., 2008), and lower P-glycoprotein activity (Balayssac et al., 2005). However, these peculiarities in the vascularization of the DRG, which permit the easy access and accumulation of paclitaxel (Cavaletti et al., 2000), are contradicted by the lack of damage to neurons of the DRG itself.

Therefore, the lack of involvement of the soma of motoneuron or DRG neurons, together with the predominant damage observed in peripheral nerves, may suggest that

paclitaxel directly damages axons in the PNS. However, based on these data, it is not possible to rule out primary damage to the cellular bodies of peripheral nerves during the development of paclitaxel-induced neuropathy, because these reports were mainly morphological studies that did not examine alterations in the expression of proteins such as cell injury markers. Moreover, many authors did not study the DRG, probably on the assumption that the damage was localized in axons. Nevertheless, a recent study looked specifically for changes at DRG level in rat (Jamieson et al., 2007) and found, in association with paclitaxel-induced sensory neuropathy, a significant nucleolar enlargement and nuclear eccentricity, but only in lumbar DRG neurons with larger cell bodies. In addition, they found a small but significant increase in the transcription factors ATF3 and c-Jun and in neuropeptide Y (NPY), whose DRG expression is known to increase in response to peripheral nerve injury.

Finally, in many of the above articles, authors used the tail-flick test to check the sensitivity to pain. All of them, with only one exception (Cliffer et al., 1998), showed a deficit in pain perception that manifested as thermal hypoalgesia. This fact contrasts with the above-reported morphological findings of paclitaxel-induced damages mainly in the largest myelinated fibers, since heat nociception is mainly due to the activation of C-fiber nociceptors (Ossipov et al., 1999; Khan et al., 2002). Another study with shorter schedules of paclitaxel found that thermal hypoalgesia was not accompanied by morphological alterations to the sciatic nerve or changes in nerve conduction velocity (Campana et al., 1998).

In general, most data from these animal models on the causes of paclitaxel-induced neuropathy were largely in agreement with existing knowledge in humans. However, none of them performed a complete study on neuropathic pain signs induced by this

antineoplastic.

2.3.2. Animal models of paclitaxel-induced neuropathy: behavioral abnormalities

Although it is accepted that paclitaxel induces a painful peripheral neuropathy in rodents, there is no general agreement on the symptoms and intensity, which depend on the paclitaxel doses and schedules, nociceptive tests, animal strains, and, probably, on minor differences in laboratory methodologies (see tables 2 and 3).

The first report on neuropathic pain symptoms induced by paclitaxel demonstrated that either one single or five repeated weekly i.p. injections, with dose and schedules similar to those used by Hamers et al. (1993) and Cavaletti et al. (1995), induced a nociceptive peripheral neuropathy in male rats with no significant motor impairment (Authier et al., 2000). The authors found mechanical hyperalgesia and thermal hypoalgesia but not mechanical or thermal allodynia. Mechanical hyperalgesia observed after the repeat treatment appeared 1 week after the first administration and persisted until 1 week after the end of the treatment, coinciding with the day of maximum effect. In the single injection group, the onset of mechanical hyperalgesia was dose-dependent and was observed with the highest dose from days 5 to 14, finding the maximum effect on day 7. Thermal hypoalgesia was less persistent or evident, being significant from the 3rd injection to 1 week after the last injection of paclitaxel, whereas, in the single injection group, it was only present at the highest dose and persisted for 3 days from the 4th day after paclitaxel injection. Importantly, the group administered repeatedly with the solvent of paclitaxel (Cremophor EL and absolute ethanol in equal parts diluted in saline) also showed mechanical hyperalgesia, although it was less evident. Likewise, it is also important to note that the doses of paclitaxel used by Authier´s group produced a

lower body weight gain and, in the repeat schedule, some degree of mortality. In fact, these doses are significantly higher than those used in most subsequent studies of paclitaxel-induced positive signs of neuropathic pain, which have rarely shown a general toxicity of this nature.

A year later, the group led by Professor Bennett and coworkers published the most widely used and replicated animal model of paclitaxel-induced neuropathic pain (Polomano et al., 2001). In this article, authors described the pain behaviors induced by four intraperitoneal injections of low doses of paclitaxel (0.5, 1 and 2 mg/kg) to rats on alternate days. None of the doses of paclitaxel affected the animals´ general health during the experiment, and all groups of animals gained weight normally. However, all three doses of the antineoplastic, with no indication of a dose-response relationship, produced a painful neuropathy that manifested as mechanical and cold allodynia and mechanical and heat hyperalgesia, both in the hind paws and tail. These neuropathic pain sensations began within days and lasted for several weeks. However, no motor impairment was detected. In this study, the paclitaxel vehicle (Cremophor EL + ethanol dissolved in saline) had no effect. Therefore, these data are consistent with the usual clinical assumptions about paclitaxel neuropathy, i.e., a symmetric sensory distal (length-dependent) painful neuropathy with a less frequent motor neuropathy (see section 2.2.2). They also attempted to elucidate whether this painful neuropathy in rat is length-dependent by measuring mechanically evoked pain threshold at three locations on the tail: distal, mid, and proximal. However, this experiment provided no clear evidence of a distal process. The authors suggested that the development of this neuropathy might be so rapid that any length-dependence is hidden and that a similar

effect may explain clinical observations of the simultaneous appearance of symptoms in hands and feet (see section 2.2.2).

This early report (Polomano et al., 2001) showed that paclitaxel induced a marked and prolonged cold and mechanical hyperalgesia/allodynia, with a less prominent heat hyperalgesia. However, they halted the evaluation at one month after the first injection of the antineoplastic, when the painful neuropathy remained apparent. In subsequent reports, using the same paclitaxel schedule (the highest dose) but with a slight variation in the method of testing mechanical hypersensitivity, they showed that these symptoms, especially the mechanical hyperalgesia, persisted at three (Flatters and Bennett, 2004) and even five months (Flatters and Bennett, 2006) after the first paclitaxel injection. They also confirmed that the drug induced cold hypersensitivity and mild and short-lasting heat hypersensitivity.

Since then, Bennett's group, either alone (Flatters et al., 2006; Siau and Bennett, 2006; Siau et al., 2006; Xiao and Bennett, 2008; Xiao et al., 2008; Zheng et al., 2011; Bennett et al., 2011) or in collaboration with other groups (Xiao et al., 2007; Jin et al., 2008; Xiao et al., 2009), has continued to use this animal model (paclitaxel 2 mg/kg i.p., injected to rats every other day for 4 days). These articles have represented a significant advance in knowledge on the physiopathology of the neuropathic pain induced by paclitaxel (see section 2.3.3) and on its treatment (see section 2.3.4). Various authors used this experimental rat model (paclitaxel 1 or 2 mg/kg) for the same purpose, making slight changes in the method of testing behavioral abnormalities. Some of these offered novel insights into the manifestations of neuropathic pain.

Thus, a Spanish group confirmed that paclitaxel produces a marked reduction in the mechanical threshold response and a less evident reduction in the latency to heat

stimuli (Pascual et al., 2005; 2010). They also showed that paclitaxel did not modify the immobility index or body temperature, but they contributed the first report that paclitaxel reduced spontaneous motility (Pascual et al., 2005). This finding deserves special attention, because a reduction in spontaneous motility may indicate some degree of spontaneous pain, which is especially difficult to recognize in animal models (Colleoni and Sacerdote, 2010) and is considered worthy of further investigation (Costigan et al., 2009; Colleoni and Sacerdote, 2010). Another group from the University of Texas also used Bennett´s model but dissolved the paclitaxel in dimethyl sulfoxide (DMSO) rather than Cremophor EL. They obtained similar behavioral results but found a longer-lasting thermal hyperalgesia (Cata et al., 2007; 2008).

Several laboratories, using the experimental model with no changes (Jolivant, et al., 2006; Rahn et al., 2008; Kiya et al., 2011), with small schedule modifications (4 daily consecutive injections) (Naguib et al., 2008; Xu et al., 2010), or without cremophor EL as vehicle (Favre-Guilmard et al., 2009; Kim et al., 2010) found that paclitaxel produced similar neuropathic pain sensations to those reported by Bennet et al., although two of them (Naguib et al., 2008; Rahn et al., 2008) did not observe thermal hyperalgesia. Paclitaxel-induced neuropathic pain was initially not reported to be dose-dependent (Polomano et al., 2001), but it was subsequently found that the duration of mechanical allodynia after the highest studied dose (2 mg/kg i.p., every other day for 4 days) was more than double the duration (16 vs. 6 weeks) after the lowest dose (1 mg/kg, i.p., every other day for 4 days) (Ledeboer et al., 2007).

At about the same time as Bennett's group, researchers at the University of California described another model of painful neuropathy induced by paclitaxel in the rat (Dina et al., 2001). In their model, 0.1, 0.5 and 1 mg/kg paclitaxel (dissolved in

Cremophor EL and ethanol) was injected i.p. once a day (Monday through Friday) for 2 weeks. After the 1st injection of paclitaxel (1 mg/kg), rats acutely developed mechanical hyperalgesia, which was significant at 1 h, maximal at 6 h, and resolved at 24 h. After chronic treatment, paclitaxel produced a dose- and time-dependent mechanical hyperalgesia that was significant from the 1st injection and maximal after the 5th and persisted for several days after the last dose, followed by a gradual recovery. Interestingly, they administered the drug for 2 additional days (i.e., 12 doses) and observed that mechanical hyperalgesia was markedly prolonged for a further 3 weeks. Chronic paclitaxel (1 mg/kg) treatment also induced mechanical allodynia and thermal hyperalgesia. In subsequent reports, authors replicated this model and tested only mechanical hyperalgesia, with similar results (Dina et al., 2004; Alessandri-Haber et al., 2004; 2008). It was also found that paclitaxel-treated rats increased nociception to hyposmotic stimuli (Alessandri-Haber et al., 2004).

Other authors replicated one of the schedules used by Cliffer et al. (1998) (2x18 mg/kg, 3 days apart), who had not tested pain behavior (apart from the tail flick test), and they found mechanical allodynia and cold hyperalgesia but not thermal hyperalgesia in the rats at 10 days after the start of paclitaxel administration. Interestingly, paclitaxel also reduced the ability of the rats to ambulate on a rotarod apparatus, but it should be noted that the doses were relatively elevated (Jimenez-Andrade et al., 2006; Peters et al., 2007). Other authors used the same schedule and a similar dose (2x16 mg/kg, 3 days apart) and found similar results: mechanical hyperalgesia and cold allodynia but not thermal hyperalgesia (Nishida et al., 2008). Authors using a different schedule and dose (8 mg/kg, i.p., on 3 alternate days) also found mechanical hypersensitivity in rats, which was not completely evaluated over time (Liu et al., 2010). Other researchers reported

that the repeat weekly administration of paclitaxel (6 mg/kg) induced mechanical allodynia and cold hyperalgesia, but they stopped the experiment at 4 weeks after the first administration despite the evident persistence of neuropathic pain (Kawashiri et al., 2009; Tatsushima et al., 2011). They also tested motor function and found motor incoordination but normal motor strength (Kawashiri et al., 2009).

We draw attention to the study that first explored whether paclitaxel neuropathy is accompanied by cognitive deficits (Boyette-Davis and Fuchs, 2009). The authors used a new schedule (6 i.p. injection of 2 mg/kg on alternate days) and observed an evident mechanical hypersensitivity in the paclitaxel-treated rats that started at 24 h after the 1st injection and was still present on day 19, when they stopped testing. However, they detected no disturbances in general cognitive functioning.

All of the above studies were performed in rats, and some authors developed models of paclitaxel-induced neuropathic pain in mice or replicated schedules previously used in rats. In 2004, the group led by Prof. Mogil attempted to reproduce Bennett´s rat model in mice (Smith et al., 2004). They treated several mouse strains with paclitaxel and found that all developed statistically significant mechanical allodynia with a significant effect of strain. They selected two strains with high- and low-mechanical allodynia effects to test the response to cold and heat stimuli. Both strains developed a similar degree of cold allodynia and neither strain showed thermal hyperalgesia. The authors suggested that paclitaxel-induced mechanical allodynia is genotype-dependent.

In another mouse study, using three different schedules of 4 mg/kg paclitaxel (single i.p. injection, 4 i.p. injections on alternate days and single i.v. injection), the authors found no significant difference in mechanical allodynia (onset at 2 weeks after

treatment) as a function of the number of injections or administration route. They also found a less pronounced thermal hyperalgesia, which appeared later in the single i.p. schedule. They concluded that the protocol using a single administration is a proper and simple approach to study paclitaxel-induced neuropathic pain (Matsumoto et al., 2006). Another group took note of this suggestion and found that when paclitaxel was acutely administered, it also induced a dose-dependent mechanical hypersensitivity; however, this was short-lived, in disagreement with Matsumoto et al. (2006), peaking at 24 h post-administration and then gradually decreasing until it disappeared by 96 h (Hidaka et al., 2009). Both studies used the same mice strain (ddY mice). In other reports, a single paclitaxel (5 mg/kg) injection induced a long-lasting mechanical hypersensitivity with maximal effects at 2 weeks post-administration (Gauchan 2009a and b), in agreement with Matsumoto et al. (2006) but not with Hidaka et al. (2009).

Therefore, the different experimental models described above (acute or repeat treatment) generally reproduce a reasonably robust bilateral neuropathic pain state that is usually long-lasting, with no observable impairment of general health, except in the study by Authier´s group, which used the highest cumulative dose (80 mg/kg). It is interesting to note that all of the studies, without exception, tested for and found mechanical hypersensitivity. In fact, some authors only evaluated mechanical sensitivity as a control to test that the developed neuropathy is a painful neuropathy (Xiao and Bennett, 2008; Favre-Guilmard et al., 2009). Whenever the sensitivity to a cold stimulus has been tested, it has been found that paclitaxel also causes cold hypersensitivity. However, thermal hyperalgesia has usually been found to be less prominent, shorter-lasting, and sometimes absent.

It is unclear whether paclitaxel-induced neuropathic pain is dose-dependent, given that Polomano et al., (2001) reported that it was not, whereas others evidenced a clear dose-dependency (Authier et al., 2000; Dina et al., 2001; Hidaka et al., 2009). Nevertheless, the last article published by Bennett´s group (Bennett et al., 2011) showed that with the same schedule but higher doses, paclitaxel-induced mechanical hypersensitivity appeared dose-dependent, with more rapid onset of neuropathic pain at higher doses. Probably, as in the clinical setting, the dose, frequency of treatment, and cumulative dose are all involved in the development of paclitaxel-induced neuropathy in rodents (see section 2.2).

As in patients, motor alterations have been less frequent, but this may be because they have been little studied. Two different relatively high dose schedules reduced motor coordination (Peters et al., 2007; Kawashiri et al., 2009) but did not impair motor strength (Kawashiri et al., 2009). Authier et al. (2000) used the highest dose and found no motor strength impairment but a non-significant reduction in motor activity. One study that used Bennett´s model found reduced spontaneous motility (Pascual et al., 2005) that could result from a hypothetical spontaneous pain. Therefore, paclitaxel may possibly produce a deficit in propioception but not in motor function. A comprehensive study of the effect of paclitaxel on locomotor activity would be very helpful.

It should also be noted that most of the above studies (with the exception of Smith et al., 2004 and Jolivant et al., 2006) were performed in male animals, contrasting with the clinical reality of taxanes, which play an important role in the treatment of breast and ovarian cancer in females (see section 1.4.5).

2.3.3. Pathophysiology

The mechanisms underlying paclitaxel-evoked painful peripheral neuropathy are not fully understood. However, the development of animal models has enabled a significant advance in the understanding of its pathophysiology.

2.3.3.1. Electrophysiological findings

Unlike the earlier studies of paclitaxel neuropathy, where electrophysiological evaluation was frequently performed (see section 2.3.1), this approach has been very uncommon in detailed reports on the neuropathic pain associated with this neuropathy. In one investigation, Authier et al. (2000) only found a significant decrease in nerve conduction velocity with chronic paclitaxel treatment, in agreement with studies using similar schedules and doses of paclitaxel (see section 2.3.1). A recent report using Bennett´s model (2 mg/kg x 4 alternative days) also found a decrease in sensory nerve conduction velocity (Kiya et al. 2011).

However, other authors have reported contradictory results. One study in paclitaxel-treated rats identified a sub-population of C-fibers that was markedly hyper-responsive (although statistical significance was not reached) to sustained threshold and suprathreshold mechanical stimuli but showed normal spontaneous activity, conduction velocity, and mechanical threshold (Dina et al., 2001). In contrast, another mouse study found that paclitaxel specifically induced hypersensitization of myelinated Aδ- and Aβ-fiber but not of unmyelinated C-fiber (Matsumoto et al., 2006).

A study using Bennett´s model (1 mg/kg, instead of 2 mg/kg, x 4 alternative days) found that paclitaxel-evoked neuropathic pain was associated with electrophysiological irregularities in peripheral nerves and spinal cord, including spontaneous discharges in A- and C-fiber nociceptors and in spinal wide dynamic range neurons (Cata et al., 2006;

Xiao and Bennett, 2008), prolonged after-discharges to mechanical and thermal stimuli, and increased wind-up to cutaneous stimuli (Cata et al., 2006).

2.3.3.2. Morphological and immunohistochemical findings

A) Axonal degeneration

Consistent with previous studies that used high doses (see section 2.3.1), Authier and colleagues (2000) found that treatment with paclitaxel (both chronic and acute) produced degenerative changes in the sciatic nerve, in the nerve fibers in paw subcutaneous tissue, and in the white matter of lumbar spinal cord. All nervous fibers of subcutaneous tissue were affected but only large myelinated fibers in the sciatic nerve. In the repeat treatment group, the neurons in spinal cord gray matter remained unaffected but Schwann cells in the nerves of paw subcutaneous tissue were affected, whereas, in the single injection group, only axonal degeneration was found. There were no changes in the single injection vehicle group, whereas all alterations were found in both paclitaxel- and vehicle-treated groups with the repeat schedule. These findings suggest that the primary lesion may be axonal and due to paclitaxel alone and not the vehicle, whereas the remaining alterations, found with the repeat schedule, cannot be exclusively attributed to the effect of paclitaxel. This is not surprising, because it is known that the solvent Cremophor EL® is not totally innocuous (Gelderblom et al., 2001). In agreement with Authier´s study, a recent report found axonal degeneration in the sciatic nerve of rats treated weekly with paclitaxel at much smaller doses, but no histological abnormalities were observed in the sciatic nerve of vehicle-treated rats (Kawashiri et al., 2009).

In contrast, Bennett´s group, using their smaller dose protocol, found no evidence of degeneration of sensory or motor axons in the sciatic nerve, dorsal or ventral roots,

spinal cord dorsal columns, or Lissauer´s tract, and they observed no damage in the DRG or spinal motor neurons. The only morphological change detected was an endoneurial edema in the peripheral nerves of paclitaxel-treated rats (Polomano et al., 2001). In a later report (Flatters and Bennett, 2006), they confirmed that paclitaxel-induced neuropathic pain was not associated with axonal or DRG cell degeneration or with axonal microtubule anomalies during a period of more than 5 months after treatment. However, they found that paclitaxel-induced neuropathic pain was associated with an increase in atypical mitochondria in C-fibers and myelinated axons. These abnormal mitochondria were swollen and vacuolated, the cristae were collapsed, and the intermembrane space was expanded (Flatters and Bennett, 2006; Jin et al., 2008).

B) Changes in ATF3 expression and macrophage infiltration

Several studies have used immunohistochemical techniques to identify the type of cells (neurons or glia) activated or injured by paclitaxel treatment. Thus, Jimenez-Andrade et al. (2006) found signs of injury (ATF3 expression) in neurons at 10 days after paclitaxel treatment, mainly in large, medium, and, to a lesser extent, small cell bodies of trigeminal, thoracic, and lumbar DRG. In lumbar DRG, they found a significantly higher percentage of large-diameter neurons expressing ATF3 in comparison to medium- and small-diameter neurons. They also observed increases in activated satellite cells and macrophages and in the nodules of Nageotte (clusters of ATF3-expressing satellite cells) that occur in sensory ganglia after neuronal cell loss, which were more prominent in lumbar ganglia than in trigeminal or thoracic ganglia. They also found an abnormal accumulation of neurofilaments within cell soma and proximal axons in medium and large sensory neurons.

In subsequent reports, the same authors (Peters et al., 2007a, b) focused on the lumbar DRG, lumbar spinal cord, and sciatic nerve of paclitaxel-treated rats. They confirmed their previous results in the lumbar DRG and, in a time course study, found significantly increased neuronal injury (higher ATF3) in paclitaxel-treated versus vehicle-treated rats at all time points evaluated, reporting a greater increase at days 4 and 6 than at days 1 and 10 after paclitaxel infusion. A significant increase in the activation and hypertrophy of satellite cells surrounding the sensory neurons was observed from days 6 to 10. Paclitaxel treatment also increased the ATF3 expression in these satellite cells, but only on day 10, and most of them formed nodules of Nageotte. The authors suggested that the significant decrease in neuronal ATF3 between days 6 and 10 (26% to 5% of total DRG neurons) may result from the death of a subset of ATF3 expressing neurons, because this fall on day 10 coincided with an increase in the nodules of Nageotte. Nodules of Nageotte have been reported in sensory ganglia after neuronal cell loss and are characterized as "tombstones" of sensory neurons in several human peripheral neuropathies (see Jimenez-Andrade et al., 2006 for references). The number of activated macrophages in DRG was also significantly increased from days 6 to 10.

Peters et al. (2007a, b) examined the sciatic nerve at mid-level and found increased ATF3 expression on days 6 and 10, almost exclusively detected in myelinating Schwann cells, and intense infiltration of macrophages on day 10. In the lumbar spinal cord, there was no evidence of ATF3 expression in neurons or non-neuronal cells in the dorsal or ventral spinal cord after paclitaxel administration. However, with respect to glia, paclitaxel only induced a significant increase in the density of activated microglia in deep (III-VI) laminae, which is the area of the dorsal

spinal cord where most large myelinated fibers terminate (Todd and Koerber, 2006). In contrast, the greatest increase in activated astrocytes occurred in superficial (I-II) laminae, where the central terminals of most small C-fibers terminate (Peters et al., 2007a, b). Therefore, these findings suggest that both A and C fibers are impacted by paclitaxel but that the extent and severity of injury may vary between different populations of sensory neurons, as evidenced by the distinct glial response after injury to small versus large sensory fibers. Moreover, given that paclitaxel has very limited ability to cross the normal blood brain barrier, the authors suggested that this glial activation may result from the degeneration of central terminals of injured primary afferent fibers or from the spinal release of factors from injured sensory neurons rather than from a direct effect of paclitaxel on spinal cord neurons (Peters et al., 2007b). Based on their investigations, they drew an elegant schematic diagram of the cellular and molecular events that occur over time after paclitaxel administration (Figure 5).

A recent report partially agreed with the findings of Peter et al., associating paclitaxel-induced neuropathic pain with an upregulation of ATF3 in large and medium but not small DRG neurons (Liu et al., 2010). However, they detected no ATF3 expression in satellite cells. They also found an increase in macrophages in DRG but not in the spinal cord. They suggested that the infiltration of macrophages into the DRG might be due to a paclitaxel-induced increase in the DRG level of a chemotactic factor (Liu et al., 2010). Moreover, it has been reported that doses of paclitaxel that evoked neuropathic pain and increased ATF3 expression also induced the upregulation of inflammation- and/or immune response-related genes in lumbar DRG. Among these genes, the authors focused on the expression of matrix metalloproteinase-3 (MMP-3), which they found to be exclusively increased in large neurons of lumbar DRG. This

upregulation of MMP-3 occurred prior to the accumulation of macrophages, suggesting that it may be a key molecule that induces accretion and activation of macrophages in the DRG after paclitaxel treatment; the authors proposed that this may be one of the first events enabling development of the neuropathy (Nishida et al., 2008).

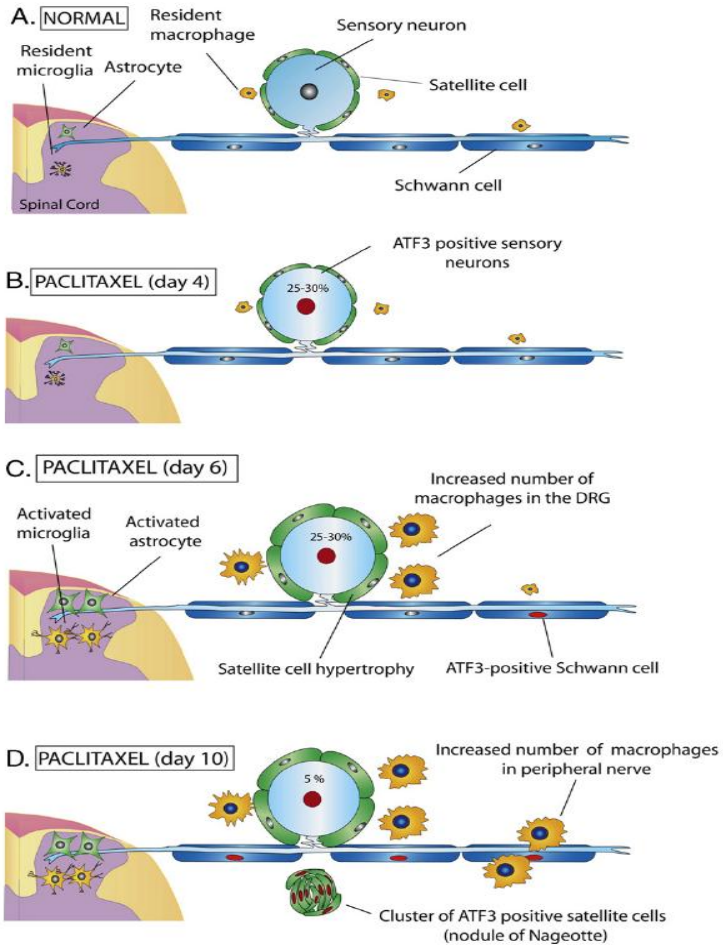

Fig. 5. Schematic diagram describing a primary afferent neuron and supporting cells, under normal conditions and after treatment with paclitaxel. (A) Normally, sensory neuron cell bodies within the dorsal root ganglia (DRG) are surrounded by several satellite cells that maintain neuronal homeostasis by regulating extracellular ion concentrations and nutrient levels. Resident macrophages survey the local environment for signs of tissue injury or infection. Peripheral nerve axons are surrounded by Schwann cells and are projected to the spinal cord and peripheral tissues. (B) 4 days post-treatment, a subset of DRG sensory neurons upregulate activating transcription factor 3 (ATF3, red nuclei) and exhibit displaced nuclei indicative of an injured phenotype. (C) 6 days post-treatment, there is activation of DRG satellite cells and an increase in the number of activated macrophages in the DRG. Both microglial and astrocyte activation is present in the spinal cord from 6 days post-treatment. (D) 10 days post-treatment, nodules of Nageotte form in the DRG and the number of macrophages and the ATF3 expression in Schwann cells are increased in the sciatic nerve (Figure taken from Peters et al., 2007b).

C) Loss of intraepidermal nerve fibers

The findings summarized in points A and B do not rule out the possibility of a dying-back process in which the pathology starts in the region near the peripheral terminal arbors of sensory neurons. Bennett´s group also examined this possibility by quantifying intraepidermal nerve fibers (IENFs) in the plantar hind paw skin. IENFs, along with their branches and receptor boutons, constitute the terminal arbor of the sensory axon. They found a decrease in IENFs associated with an increased activation of a type of epidermal resident immune cell known as the Langerhans cell (Siau et al., 2006; Jin et al., 2008; Xiao et al., 2009). They also inspected the small bundles of axons located a few microns below the basal lamina that separates the epidermis and dermis (the most distal level prior to the emergence of IENFs) in the plantar hind paw skin (Bennett et al., 2011). However, they found no sign of axonal degeneration below the epidermal basal lamina, even at higher doses of paclitaxel (total doses of 24 and 32 mg/kg) than used in this and previous articles, which produced a significant dose-dependent loss of IENFs (Bennett et al., 2011). They also found no expression of the injury marker ATF3 in DRG neurons (Flatters and Bennett, 2006), even at the highest dose (Bennett et al., 2011). They concluded that, within this dose range, paclitaxel-evoked degeneration is confined to the IENFs, which are the intraepidermal terminal arbors of the sensory fibers, and they proposed the designation "terminal arbor degeneration" (TAD) (Bennett et al., 2011). These nerve fibers innervating the skin are probably Aδ- and C-fiber nociceptors. However, they likely have a broader range of functions than simple sensory transduction, as suggested by their close association with immunocompetent dendritic Langerhans cells (Lauria et al., 2005). A detailed review of

the possible role of IENFs and Langerhans cells in paclitaxel-induced neuropathic pain is given below in section 2.3.3.4.A.

Based on these data, Bennett and colleagues suggested that paclitaxel-induced neuropathy is distal and symmetrical, but they expressed doubts about its progressive nature (like diabetes neuropathy). They argued that the TAD associated with paclitaxel painful neuropathy is not the initial manifestation of a progressive dying-back process, because no signs of peripheral nerve axon degeneration were detected at just a few tens of microns from the end of axons, even at the highest dose. In addition, observations of the simultaneous emergence of symptoms in the hands and feet of patients are not consistent with a progressive dying-back event. They also suggested that TAD might be a common lesion in toxic neuropathies, given that other authors have described similar findings (Bennett et al., 2011).

Supporting the hypothesis that the degeneration induced by paclitaxel is exclusively confined to IENFs, two very recent reports also found a gradual partial loss of IENFs corresponding to the development of paclitaxel-induced neuropathic pain (Lui et al., 2010; Boyette-Davis et al., 2011). However, other authors obtained results that contradict the TAD hypothesis, using higher (Jimenez-Andrade et al., 2006; Peters et al., 2007a, b), similar (Nishida et al., 2008), or even lower (Lui et al., 2010) cumulative doses of paclitaxel than the highest dose used by Bennett´s group (Bennett et al., 2011) and finding prominent ATF3 immunoreactivities in DRG neurons. Moreover, one of these studies showed that ATF3 staining was localized in DRG neurons expressing an A-fiber marker (NF200) but not in those expressing a C-fiber marker (IB4) or in satellite cells (Lui et al., 2010). Differences in the frequency of treatment or in the methodology used to evaluate ATF3 expression may possibly explain this contradiction.

Additional experiments would be helpful to address this discrepancy, although Peters et al. (2007b), who found increased ATF3 expression, detected no changes in other markers of cell injury or apoptosis at any time point examined.

2.3.3.3. Paclitaxel-induced atypical mitochondria and dysregulation of calcium homeostasis

Mitochondria are a dynamic reticular network of structures that fuse, branch, and divide within cells and are among the most prominent organelles, occupying around 20-25% of the total cellular volume. Their main function is to produce energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation, but they are also critical for redox control, fatty acid oxidation, Ca^{2+} homeostasis, amino acid metabolism, regulation of metabolic pathways, and physiological cell death mechanisms. They can also be affected by a variety of insults and are implicated in a number of human diseases, including cancer, diabetes, and neurodegenerative disorders (reviewed by Rismanchi and Blackstone, 2007).

Mitochondria have a typical double membrane structure that gives rise to four functional compartments: the outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and matrix. The inner mitochondrial membrane is highly invaginated, forming the cristae that considerably increase its overall surface area. Although all mitochondria share this basic structure, they can exhibit important cell- and tissue-specific differences. In fact, the number of mitochondria can vary widely with cell type, depending on the energy requirements. Thus, skin fibroblasts normally contain several hundred mitochondria, neurons may contain thousands, and cardiomyocytes tens of thousands. In addition, mitochondria from diverse tissues can

show differences in size, crista structure, and protein/lipid composition of their membranes, with major functional consequences. The distribution of mitochondria are also adapted to the physiological neeeds in each cellular area, and neurons are likely to exhibit prominent differential distributions because they are the most polarized cells in the body, with their long, highly specialized processes: the axons and dendrites (reviewed by Rismanchi and Blackstone, 2007).

In axons, mitochondria are more concentrated in particular regions with high demand for energy and ion flux (Fig. 6), such as the distal region of the initial segment and the nodes of Ranvier in myelinated fibers, where the mitochondria probably provide the ATP needed to maintain ionic gradients via Na^+-K^+ ATPase. Neuromuscular junction and sensory organs of the skin, such as corpuscles and free nerve endings, are also sites with abundant mitochondria, because they need ATP to support synaptic vesicle loading, mobilization, and recycling, maintaining Ca^{2+} homeostasis by direct Ca^{2+} sequestration and sustaining plasma membrane Ca^{2+}-ATPases. Moreover, they become differentially localized in pathologic states, accumulating in demyelinated axonal segments and in regions of disrupted axo-glial junctions (Baloh, 2011).

It is well-known that mitochondria play a significant role in Ca^{2+} handling in all cell systems and that this process has enormous functional ramifications for both cell physiology and pathophysiology. Thus, the uptake and release of mitochondrial Ca^{2+} control the amount of Ca^{2+} entering the cell, the Ca^{2+} concentration in cytoplasmic microdomains, the frequency of oscillatory Ca^{2+} signals, the rate of propagation of a Ca^{2+} signal across larger cells, and the spatial distribution of a Ca^{2+} signal throughout the volume of a cell. In particular, mitochondria have a huge capacity to accumulate Ca^{2+} and, in turn, Ca^{2+} accumulation modulates mitochondrial function in a variety of

ways, increasing fuel processing by enhancing substrate uptake and subsequent tricarboxylic acid cycle activity while simultaneously enhancing ATP synthase activity. These processes work together to improve the synthesis of ATP (reviewed by Duchen et al., 2008).

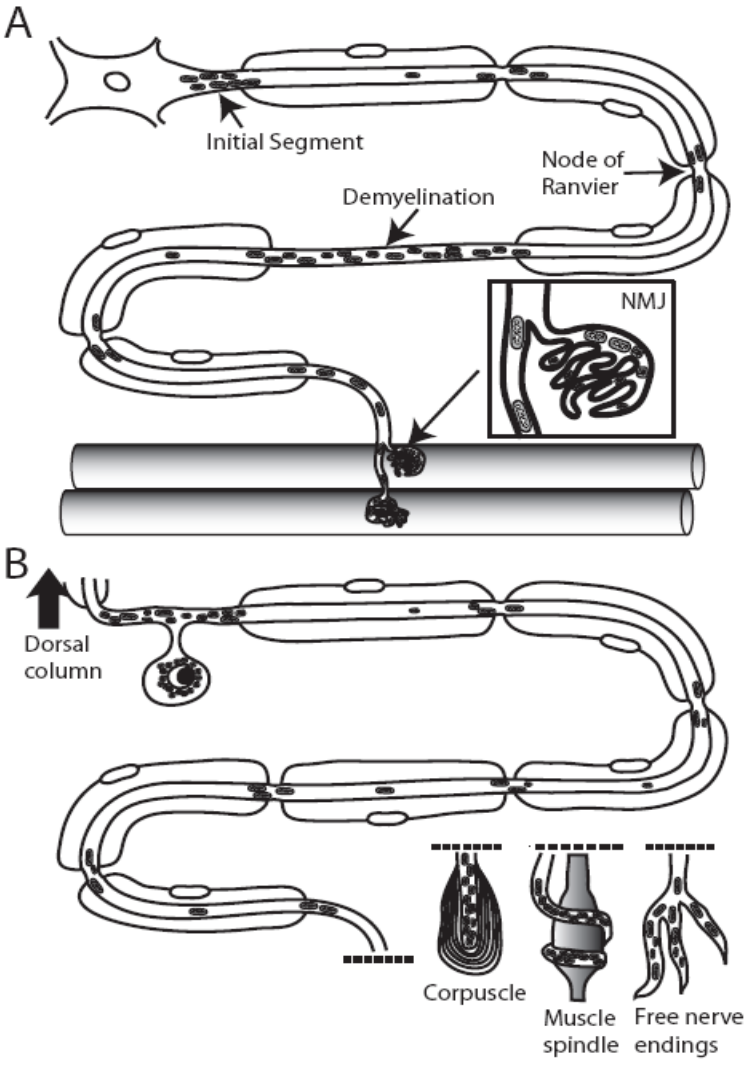

Fig. 6. Regions of mitochondrial localization in peripheral neurons. A, Scheme of a motor neuron, highlighting axonal regions in which mitochondria are preferentially located. B, Distribution of mitochondria in myelinated peripheral sensory neurons (Figure taken from Baloh, 2011).

Bennett´s group, using their normal paclitaxel schedule (4 injections of 2 mg/kg), found that paclitaxel-induced neuropathic pain was associated with increased atypical (swollen and vacuolated) mitochondria in C-fibers and myelinated axons without producing degeneration of peripheral nerves (Flatters and Bennett, 2006; Jin et al., 2008). They attributed this finding to a direct effect of paclitaxel on the mitochondrial permeability transition pore (mPTP), because paclitaxel causes mPTP opening, releasing Ca^{2+} from mitochondria and inducing swelling and vacuolization that finally result in their functional impairment (Flatters and Bennett, 2006; Jin et al., 2008). It is not clear whether the mPTP has a role in normal mitochondrial physiology, but under situations of mitochondrial Ca^{2+} overload (especially in oxidative stress situation), high phosphate concentrations, and adenine nucleotide depletion, the mPTP, a non-specific pore, opens in the inner mitochondrial membrane allowing free entrance of molecules of <1.5 kDa, including protons. The consequent uncoupling of oxidative phosphorylation leads to ATP depletion and necrotic cell death. Another result of mPTP opening is that all small molecular weight molecules equilibrate across the inner membrane, including cofactors and ions. This not only leads to the disruption of metabolic gradients between the mitochondria and cytosol, including the release of accumulated Ca^{2+}, but also increases mitochondrial swelling, because the greater permeability of the inner mitochondrial membrane to small molecules mediates the equilibration of all low molecular weight osmolytes while retaining proteins within their respective compartments. Given that the protein concentration is higher in the matrix than in the cytosol and intermembrane space, it exerts a colloidal osmotic pressure leading to swelling of the matrix compartment and consequent pressure on the outer membrane, which is eventually ruptured. It is important to note that one of the results of mPTP

opening is the release of accumulated Ca^{2+} from mitochondria (reviewed by Halestrap, 2009).

Although the exact composition of the mPTP remains uncertain, several proteins have been implicated in its structure or regulation (reviewed by Halestrap, 2009). In particular, a potential action mechanism for the effect of paclitaxel effect on the mPTP was suggested by the finding of an association between the voltage-dependent anion channel (VDAC), then thought to be a physical component of the mPTP, and tubulin (Carre et al., 2002). Based on this, Bennett´s group hypothesized that paclitaxel may bind to the tubulin associated with VDAC and open the mPTP, which would allow the release of Ca^{2+} from the mitochondria and might also cause mitochondria to swell and become vacuolated. This increase in intracellular Ca^{2+} may promote neuronal hyperexcitability, resulting in pain behavior (Flatters and Bennett, 2006; Jin et al., 2008). In accordance with this hypothesis, paclitaxel-evoked Ca^{2+} release from mitochondria was blocked by the mPTP inhibitor cyclosporin A in mouse brain neurons (Mironov et al., 2005) and in mouse pancreatic acinar cells (Kidd et al., 2002). In addition, paclitaxel produced a cyclosporin A-reversible mitochondrial swelling in rat liver, kidney, heart, and brain mitochondria (Varbiro et al., 2001) and in human neuroblastoma cells (Andre et al., 2000). However, emerging evidence rules out a direct involvement of VDAC in the mPTP (Halestrap, 2009). Furthermore, VDAC is localized in the outer mitochondrial membrane, whereas the mPTP is considered to form in the inner mitochondrial membrane. Nevertheless, VDAC is still considered to be an mPTP modulator.

It has been suggested that VDAC, the most abundant protein in the mitochondrial outer membrane, serves as a global regulator of mitochondrial function (Rostovtseva

and Bezrukov, 2008). Indeed, it is the major permeability pathway in the mitochondrial outer membrane controlling the flow of metabolites and ions, including Ca^{2+} (Tan and Colombini, 2007). It is known that VDAC can adopt a single "open state" and multiple "closed states" of significant smaller conductance. In "closed states", the VDAC is impermeable to ATP but remains permeable to small ions, including Ca^{2+} (Rostovtseva and Bezrukov, 2008). VDAC shows a higher permeability to Ca^{2+} in "closed states" than in the "open state". Therefore, VDAC closure promotes Ca^{2+} flux into mitochondria, which can also lead to mPTP opening (Tan and Colombini, 2007). It has been reported that tubulin can bind to VDAC, inducing reversible closure of the channel (Rostovtseva et al., 2008). Hence it is likely that paclitaxel and other microtubule-active drugs can modulate the interaction of microtubules and/or tubulin with VDAC, thereby transmitting a signal for mitochondria permeabilization (Rostovtseva and Bezrukov, 2008).

Moreover, VDAC is able to interact with other proteins located in the outer membrane, such as the translocator protein (TSPO; previously known as peripheral benzodiazepine receptor) and regulatory proteins of the Bcl-2 family (reviewed by Halestrap, 2009; Azarashvili et al., 2010). The Bcl-2 protein (a member of the antiapoptotic group of the Bcl-2 family) acts physiologically as a proton-efflux pump that prevents conductivity of the mPTP, participating in regulation of the mPTP and acting as a gatekeeper to decrease the sensitivity to apoptotic stimuli. However, Bcl-2 changes its function from a protector to a cell killer through direct interaction with Nur77, an orphan nuclear receptor induced by various stimuli, whose translocation from the nucleus to the mitochondrial membrane represents a potent cell death signal (Ferlini et al., 2009). It has been reported that paclitaxel directly binds to bcl-2 and suppresses

and reverses its function as an mPTP opening blocker, thereby facilitating the opening of the pore (Ferlini et al., 2009). Molecular modeling revealed an extraordinary similarity between paclitaxel binding sites in Bcl-2 and β-tubulin. This led authors to propose that paclitaxel may be a mimetic of the endogenous peptide Nur77, and they confirmed that Nur77 interacts with both paclitaxel targets (Bcl-2 and β-tubulin). They concluded that paclitaxel is a peptidomimetic compound that bears the same death signal as is physiologically delivered by Nur77, i.e., the disruption of microtubule dynamics and bcl-2-mediated mPTP opening (Ferlini et al., 2009).

Some pharmacological experiments have also implicated mitochondrial impairment in paclitaxel-induced neuropathic pain. Thus, the compound acetyl-L-carnitine, which appears to prevent mPTP opening (Furuno et al., 2001; Zanelli et al., 2005), not only prevented neuropathic pain after paclitaxel administration (Flatters et al., 2006; Jin et al., 2008) but also the appearance of atypical mitochondria in C-fibers (Jin et al., 2008) and the abnormal spontaneous discharge in A-fiber and C-fiber primary afferent neurons (Xiao and Bennett, 2008). An Italian group reported that acetyl-L-carnitine significantly reduced the neurotoxicity induced by high doses of paclitaxel and cisplatin (Pisano et al., 2003). In a later study, they found that acetyl-L-carnitine treatment prevented the development and inhibited the expression of mechanical hypersensitivity evoked by paclitaxel, cisplatin, and vincristine in rats (Ghirardi et al., 2005), suggesting a common pathophysiological mechanism.

Bennett and coworkers tested a new neuroprotective compound, olesoxime, which was reported to bind to the mitochondrial proteins TSPO and VDAC and to inhibit mPTP opening (Bordet et al., 2007), and found that it prevented and inhibited paclitaxel-evoked neuropathic pain. However, olesoxime only partially prevented

paclitaxel-induced IENF neurodegeneration and did not modify paclitaxel-evoked spontaneous discharges (Xiao et al., 2009).

Hence, although further research is needed to elucidate whether mPTP, VDAC, bcl-2, or another mitochondrial protein takes part in paclitaxel-induced neuropathy, the above findings may indicate that mitochondrial proteins play a role and that the increased atypical axonal mitochondria in peripheral nerves associated with paclitaxel plays some role in the neuropathic pain induced by this drug (Flatters and Bennett, 2006; Jin et al., 2008). In fact, it was recently reported that peripheral nerve mitochondria from paclitaxel-treated rats are indeed functionally impaired, with reduced capacity for oxidative phosphorylation, resulting in decreased energy production and signs of high oxidative stress (Zheng et al., 2011).

Bennett´s group suggested that energy deficiency produces neurodegeneration and that the threshold for this process is lowest in the neuronal compartment, which has the highest energy requirement. They proposed that the sensory axon's terminal receptor arbor, which has abundant mitochondria (see fig. 5), is the compartment with the highest energy requirement. They speculated that the high-energy needs of the intraepidermal terminal arbor derive from constantly alternating degeneration and regeneration in the constantly changing microenvironment produced by the continuous self-renewal of the epidermis. This may explain reports of IENF losses associated with paclitaxel neuropathy (Bennett et al., 2011).

Finally, Professor Bennett proposed an interesting theory in relation to the paclitaxel-induced neuropathic pain model. He suggested that paclitaxel interferes with mitochondrial energetics, producing an energy deficiency and consequently a dysfunction of the sodium-potassium pump that maintains the normal resting potential.

As a result, the axons depolarize to the threshold necessary for spontaneous discharge. This type of mechanism may also operate in other forms of toxic neuropathy (Bennett, 2010).

It was recently reported that a free radical scavenger (phenyl N-tert-butylnitrone) reduced paclitaxel-induced mechanical hypersensitivity (Kim et al., 2010). One of the consequences of mitochondrial dysfunction is the production and accumulation of free radicals within cells, and nerve cells are known to be especially vulnerable to the harmful actions of these molecules (Rismanchi and Blackstone, 2007). Other authors reported similar results in different models of neuropathic pain (Siniscalco et al., 2007; Yowtak et al., 2011) and in capsaicin-induced hyperalgesia (Lee et al., 2007). Moreover, an increase in reactive oxygen species (ROS) was observed in mitochondria from spinal dorsal horn neurons in models of neuropathic pain (Park et al., 2006) and capsaicin-evoked hypersensitivity (Schwartz et al., 2008). These findings suggest that ROS may be involved in the pathophysiology of neuropathic pain, including that induced by paclitaxel.

2.3.3.4. Peripheral and central sensitization mechanisms

Neuropathic pain develops as a result of lesions or disease involving the somatosensory nervous system and can be initiated or maintained at peripheral and/or central loci (Basbaum et al., 2009). Given that paclitaxel poorly penetrates to the CNS, its neurotoxic effects are normally located in the PNS (see section 2.2.1). Nevertheless, several findings suggest that paclitaxel-induced neuropathic pain is mediated by both peripheral and/or central mechanisms.

A) Role of peripheral sensitization

Tissue damage and inflammation lead to a reduced threshold and increased sensitivity of the peripheral terminals of the nociceptors, resulting in pain hypersensitivity – a process known as peripheral sensitization (Woolf and Salter, 2000). After paclitaxel treatment, several findings associated with paclitaxel-evoked painful neuropathy have been described in peripheral nerves in dorsal root ganglia (see section 2.3.3.2.), axons (see sections 2.3.3.1., 2.3.3.2 and 2.3.3.3.), and peripheral nerve endings in the skin (see section 2.3.3.2.). In particular, various authors found partial IENF loss in hind paw glabrous skin that corresponded to the development of neuropathic pain in paclitaxel-treated rats (Siau et al., 2006; Jin et al., 2008; Liu et al., 2010; Boyette-Davis et al., 2011). The role of this IENF loss in neuropathic pain is not fully understood, but it must play some part, because a similar loss to that found in paclitaxel-induced neuropathic pain has been reported in several painful sensory neuropathies in animals and humans. In fact, the density of epidermal axons is now used to diagnose different neuropathies, especially small fiber neuropathies, and it has been proposed as an early sign of peripheral neuropathy (reviewed by Sommer and Lauria, 2007 and Lauria et al., 2009).

IENFs are naked axons that lose the Schwann cell sheath when crossing the dermal-epidermal junction, and they are thought to have an exclusively somatic function. The most commonly used markers for nerve fibers are antibodies against PGP9.5, a carboxy-terminal ubiquitin hydrolase, which has proved particularly sensitive to identify small-diameter afferents in the skin. Some of these IENFs contain substance P or CGRP and intensely express the capsaicin receptor (TRPV-1), showing them to be nociceptors (reviewed by Lauria et al., 2009). In fact, most capsaicin-sensitive

nociceptors are C-fibers and, in a lesser proportion, Aδ fibers (Caterina and Julius, 2001). Therefore, the parent axons of these unmyelinated terminals are probably both myelinated and unmyelinated and, as suggested by Siau et al. (2006), it is likely that fibers lost from the epidermis are mainly Aδ and C fibers, including nociceptors and warming-specific and cooling-specific fibers.

The functional consequences of the IENF loss have not been fully elucidated. However, it is well-known that spontaneous activity is generated at multiple sites after peripheral nerve injury, including the site of injury (neuroma), neighboring intact afferents, and the cell body of injured DRG neurons (Costigan et al., 2009). The same schedule and dose of paclitaxel that produced IENF loss in the hind paw glabrous skin also produced abnormal spontaneous discharge in A-fiber and C-fiber primary afferent neurons at the time of peak pain severity (Xiao and Bennett, 2008). However, it did not produce degeneration of the saphenous nerve at mid-level (Flatters and Bennett, 2006) or in the parent axons of IENFs localized just below the epidermal basal lamina (Bennett et al., 2011), and it did not produce damage to nuclei of the afferent neurons (Flatters and Bennett, 2006; Bennett et al., 2011). Therefore, it is possible to hypothesize that the degeneration of these IENFs, which are the peripheral terminals of nociceptor neurons, might be the origin of these spontaneous discharges and therefore induce a process of peripheral sensitization.

In addition to this missing IENF, Bennett´s group also found activation of epidermal Langerhans cells (Siau et al., 2006; Jin et al., 2008), which are skin resident antigen-presenting cells belonging to the leukocyte system (Boulais and Misery, 2008). The origin of the activation of Langerhans cells is unknown. It may be produced by an immunological effect of paclitaxel itself or by the appearance of self-antigens due to

IENF degeneration. On the other hand, due to the nociceptor´s efferent function, activated nociceptors (and some nonneural cells) may release endogenous factors under certain conditions such as tissue damage, including the peptides substance P and CGRP, which are reported to stimulate Langerhans cells and other epidermal cells (Meyer et al., 2008).

The whole skin is an organ of communication in which sensory nerve endings and epidermal cells (keratinocytes, melanocytes, Langerhans cells, and Merkel cells), which express sensory proteins and neuropeptides, connect the skin to the nervous system through a complex communication network closely related to the neuroendocrine and immune systems. Langerhans cells are key cells in this process (reviewed by Boulais and Misery, 2008). However, the functional consequences for neuropathic pain of the activation of epidermal Langerhans cells remain unknown, although they are known to be close to IENF terminals (Gaudillere et al., 1996), suggesting a functional interaction. In fact, activated Langerhans cells express inducible nitric oxide synthase and produce large quantities of nitric oxide, at least *in vitro* (Qureshi et al., 1996; Iuga et al., 2004). Following partial sciatic nerve ligation, the enzyme cyclooxygenase-1 (COX-1) was up-regulated in Langerhans cells (Ma and Eisenach, 2002), which suggests that they can produce prostaglandins. Moreover, they are an important source of cytokines and pro-inflammatory molecules (Johnson-Huang et al., 2009). Therefore, molecules released by Langerhans cells may contribute to changes in the local chemical environment of the peripheral terminal nerve, sensitizing non-injured nociceptors and initiating a peripheral sensitization process.

Some authors have suggested that the disappearance of IENF reflects a Wallerian-like degeneration (Ko et al., 2002; Hsieh et al., 2009; Paleshok and Ribeiro-da-silva,

2011), one of the most elementary reactions of the peripheral nervous system, as a result of nerve fiber interruption from insults, including toxins. Wallerian degeneration normally leads to neuropathic pain (reviewed by Dubovy, 2011). Interestingly, Bennett and coworkers recently reported a correlation between the magnitude of this paclitaxel-induced insult and the severity of paclitaxel-induced pain, finding greater IENF loss and faster neuropathic pain with higher doses of paclitaxel (Bennett et al., 2011). A similar correlation between IENF density and the severity of sciatic nerve mechanical injury was also reported in rats (Hsieh et al., 2009).

A recent study using the low-dose Bennett protocol found that paclitaxel reduces the vasodilatation in rat dorsal hindpaw caused by sensory neuron activation by either intradermal capsaicin or electrical stimulation of the sciatic nerve. However, paclitaxel did not modify the release of CGRP from the terminals of sensory neurons in the dorsal spinal cord. The authors suggested that paclitaxel affects the peripheral endings of sensory neurons and alters the release of neurotransmitters (Gracias et al., 2011). These findings may be explained by a similar IENF loss to that found in paclitaxel-induced neuropathic pain. If there are fewer fibers expressing TRPV-1 receptors (paclitaxel-induced IENF loss), TRPV-1 agonists such as capsaicin are expected to have a lesser activation effect than in undamaged tissue (vehicle-treated animals). Moreover, this hypothesis is not contradicted by the correlation between IENF loss and spontaneous discharge in A-fiber and C-fiber primary afferent neurons. This is because, as suggested by Xiao and Bennett (2008), paclitaxel-induced spontaneous discharges may originate from intact axons with partially or completely degenerated sensory terminal endings or

from intact fibers adjacent to fibers undergoing terminal arbor degeneration, and/or may be produced by a chemotherapy-evoked inflammation-like condition.

Behavioral abnormalities associated with paclitaxel-induced neuropathic pain also support involvement of the peripheral sensitization process. Thus, paclitaxel induced heat hypersensitivity (although less prevalent, less prominent, and shorter-lasting than paclitaxel-induced mechanical hypersensitivity, see section 2.4.2.1), which is considered to be a feature of peripheral sensitization (Latremoliere and Woolf, 2009).

Finally, it is important to note that the loss of epidermal innervation is considered to be characterized by the exclusive or predominant impairment of Aδ and C-fibers, but studies of paclitaxel-induced human (see section 2.2.1) and animal (see section 2.4.1) neuropathies suggest that the damage largely involves large myelinated Aβ fibers. Hence, both small-diameter and large-diameter sensory nerves may be affected by paclitaxel treatment.

B) Role of central sensitization

Central sensitization occurs in pathological pain (e.g. inflammatory and neuropathic pain). It represents an enhanced function of neurons and circuits in nociceptive pathways, caused by increased membrane excitability and synaptic efficacy and by reduced inhibition, and it is a manifestation of the remarkable plasticity of the somatosensory nervous system in response to activity, inflammation, and neural injury (Latremoliere and Woolf, 2009).

The role of central sensitization in chemotherapy-induced neuropathic pain has not been studied, but there is some evidence that this pain may be at least partially mediated by this process. First, it is important to take into account that a peripheral sensitization

in response to peripheral nerve injury, such as that described for paclitaxel-induced neuropathic pain (see previous section), implies an increased primary afferent drive that probably contributes to changes in the spinal cord (Latremoliere and Woolf, 2009).

As reported in the previous section, a high incidence of spontaneous ectopic discharges have been described in A- and C-fiber of sural nerves of rats with paclitaxel-evoked neuropathic pain (Xiao and Bennett, 2008). This spinal cord input from C-fibers and A-fibers after chemical and structural changes can initiate and then maintain activity-dependent central sensitization in the dorsal horn, thereby producing and amplifying neuropathic pain abnormalities (Latremoliere and Woolf, 2009).

When neurons in the dorsal horn spinal cord are under the influence of central sensitization, they exhibit some or all of the following events: development of or increase in spontaneous activity, decrease in their threshold for activation by peripheral stimuli, increased responses to suprathreshold stimulation, and enlargement of their receptive fields. In addition, characteristics that may be particular to this phenomenon include: conversion of nociceptive-specific neurons to wide dynamic range (WDR) neurons that respond to both innocuous and noxious stimuli, progressive increases in the responses elicited by repeated innocuous stimuli (windup), expansion of the spatial extent of their input, and changes that outlast an initiating trigger (Latremoliere and Woolf, 2009).

Some of these changes in sensory processing of spinal cord neurons were observed by Cata et al. (2006) in paclitaxel-induced mechanical and thermal hyperalgesia in rats. Thus, paclitaxel-treated rats showed: increased rates of spontaneous discharge that could be due to a rise in the population of WDR neurons; prolonged afterdischarges to natural mechanical and thermal stimuli, which the authors correlated

with sustained paw lifting after stimulation of the hindpaw in neuropathic rats; increased responses to both skin heating and cooling; and increased windup to cutaneous electrical stimuli. In addition, the authors in this (Cata et al., 2006) and another study (Weng et al., 2005) found a downregulation of glutamate transporters in the spinal dorsal horn after paclitaxel treatment at the same time point as the physiological changes.

Neuronal glutamate transporters that are present presynaptically, postsynaptically, perisynaptically, and in glial cells are the main mechanism for clearance and maintenance of homeostasis in extracellular glutamate concentrations at excitatory synapses, and they therefore control the termination of signaling at these sites. Therefore, this reduction in glutamate transporters may result in a reduced glutamate clearance, which could produce in the spinal dorsal horn neurons an excessive and prolonged stimulation of glutamate receptors (NMDA, AMPA/kainate and metabotropic glutamate receptors) and their downstairs molecular mechanisms, which trigger the development of central sensitization that generates and maintains neuropathic pain (Tao et al., 2005). In fact, both glutamate uptake and the expression of neuronal and glial glutamate transporters in the spinal cord have been found to be changed under pathological (Sung et al., 2003; Binns et al., 2005) and inflammatory (Cheng et al., 2010) pain conditions. In addition, inhibition of glutamate uptake in the spinal cord induced hyperalgesia and increased the responses of spinal dorsal horn neurons to peripheral afferent stimulation (Weng et al., 2006). Therefore, this downregulation of glutamate transporters in the spinal cord dorsal horn is a reduction of one of the transmission inhibitory mechanisms. Disinhibition, mediated by whatever means, leaves

dorsal horn neurons more susceptible to activation by excitatory inputs (Latremoliere and Woolf, 2009).

Peripheral nerve injury also causes a reaction from the immune system. Both infiltration of inflammatory cells and activation of resident immune cells have been observed at various anatomical locations, including the injured nerve, DRG, spinal cord, and even the terminal receptor arbor of the sensory axon associated with paclitaxel-induced neuropathic pain (see sections 2.3.3.2. and 2.3.3.5). In addition to its role in regulating glutamate homeostasis, activation of these immune cells may result in the release of both pro- and anti-inflammatory cytokines as well as algesic and analgesic mediators, which may contribute to the development of central sensitization by enhancing excitatory and reducing inhibitory currents (Latremoliere and Woolf, 2009; Austin and Moalem-Taylor, 2010).

Section 2.3.3.3. describes paclitaxel-induced changes in axonal mitochondria of the peripheral nerves and their involvement in intracellular calcium homeostasis, which is expected to be increased. It has also been reported that paclitaxel induced up-regulation of the $\alpha_2\delta$-1 subunit of voltage-dependent calcium channel ($Ca\alpha_2\delta$-1) in the DRG (Matsumoto et al., 2006) and spinal cord (Xiao et al., 2007; Gauchan et al., 2009), which could result in an increase in intracellular calcium. In fact, elevation in intracellular calcium, by whatever means, is one of the main triggers of central sensitization by activating multiple calcium-dependent kinases that act on receptors and ion channels to increase synaptic efficacy (Latremoliere and Woolf, 2009). In addition, several recent findings suggested that increases in the number of $Ca\alpha_2\delta$-1 proteins at synapses in the dorsal horn are important for the production of central sensitization in animal models (Tauchman et al., 2010). Finally, the administration of antagonists of

both Ca$\alpha_2\delta$-1 (Matsumoto et al., 2006; Xiao et al., 2007; Gauchan et al., 2009) and T-type calcium channels (Flatters and Bennett, 2004; Xiao et al., 2008) and of calcium chelating agents (Siau and Bennett, 2006), which should reduce intracellular calcium, inhibited and/or prevented the neuropathic pain induced by paclitaxel.

With respect to paclitaxel-induced behavioral abnormalities, we are not aware of any report that paclitaxel does not induce mechanical hypersensitivity. This pain behavior, either mechanical allodynia or hyperalgesia, is always one of the behavioral manifestations induced by paclitaxel (see section 2.4.2.1), and mechanical sensitivity is considered to be a major feature of central sensitization (Latremoliere and Woolf., 2009).

2.3.3.5. Paclitaxel-induced neuropathic pain and immune system

Section 2.3.3.2. reviewed a series of articles that raise the possibility that the immune system is involved in paclitaxel-induced neuropathic pain. In fact, the implication of immune cells in neuropathic pain is well-known, and their involvement to some extent coincides with that described by authors in other types of neuropathic pain (Costigan et al., 2009; Austin and Moalem-Taylor, 2010 for reviews). In addition, it is known that paclitaxel exerts effects on the immune system and displays immunomodulatory traits (reviewed by Javeed et al., 2009).

Sections 2.3.3.2 and 2.3.3.4 reported that IENF degeneration in paclitaxel-treated animals is associated with the activation of cutaneous **Langerhans cells**, skin-resident antigen-presenting cells that belong to the leukocyte system (Boulais and Misery, 2008). The role of this type of immune cell in neuropathic pain is not clear, but they can release

several endogenous factors that may participate in a hypothetical paclitaxel-induced peripheral sensitization process (see section 2.3.3.4.A).

Painful neuropathy induced by paclitaxel may also involve injury or alterations in supporting cells such as **Schwann cells,** which are the glial cells of the peripheral nerves. In fact, morphological alterations of Schwann cells (see section 2.3.1.) and their increased expression of the injury marker ATF3 have been associated with paclitaxel treatment. Under physiological conditions, Schwann cells provide trophic support and maintain the local environment, as well as providing myelin sheath for large myelinated axons and enveloping small bundles of unmyelinated C-fiber axons (Remak bundles). In injured nerves however, Schwann cells undergo a phenotypic switch and recover the capacity to proliferate, migrate, and secrete numerous mediators that contribute to Wallerian degeneration and nerve regeneration. They may contribute to neuropathic pain by sensitizing or activating the axons of nociceptors through inflammatory cytokines and growth factors (Austin and Moalem-Taylor, 2010).

Paclitaxel appears to change other supporting cells into sensory ganglia. **Satellite cells**, which are peripheral glial cells that surround neuronal cell bodies, support normal sensory transmission and nociception by maintaining metabolic and ionic homeostasis. However, these satellite cells become activated and proliferate in neuropathic pain or under peripheral inflammation conditions, producing increased expression of glial fibrillary acidic protein (GFAP), as seen in paclitaxel neuropathy (Peters et al., 2007a; b), and releasing various cytokines and growth factors (Austin and Moalem-Taylor, 2010). As mentioned above (pages 92-67), paclitaxel-treatment induces hypertrophy of satellite cells and their expression of ATF3, especially in the satellite cells that constitute nodules of Nageotte (Jimenez-Andrade et al., 2006; Peters et al., 2007a, b). A

recent study further supported the role of DRG satellite cells in the development of paclitaxel-induced painful neuropathy. Thus, a decreased concentration of L-serine in the DRG but not in the sciatic nerve or spinal cord and a reduced expression of 3-phosphoglycerate dehydrogenase (3PGDH, a biosynthetic enzyme of L-serine) were associated with paclitaxel-induced neuropathic pain. 3PGDH staining was localized in satellite cells but not neurons in the DRG suggesting that satellite cell-derived L-serine in the DRG probably plays a major role in this painful neuropathy (Kiya et al., 2011). Given that glia-derived L-serine is a building block of proteins that maintain neuronal function and development, the authors proposed that a decrease in the supply of this amino acid from satellite cells may evoke abnormal sensations secondary to structural and/or morphological changes in peripheral sensory neurons rather than directly change neuronal signaling. In support of this hypothesis, they also showed that administration of L-serine normalized mechanical sensitivity and sensory nerve conduction velocity (Kiya et al., 2011).

Macrophages are large phagocytic leukocytes in tissue that are produced by the differentiation of monocytes and generally phagocytose foreign particles (e.g., microbes). However, in the context of peripheral or central nerve injury, macrophages play a central role in removing degenerating neuronal debris and myelin and contribute to subsequent regeneration (Austin and Moalem-Taylor, 2010). Besides, similar to satellite cells, paclitaxel-induced hypertrophy and hyperplasia of macrophages in the DRG and peripheral nerves (Jimenez-Andrade et al., 2006; Peters et al., 2007a, b; Nishida et al., 2008; Liu et al., 2010) may contribute to the development of painful symptoms associated with nerve injury, attributable to the release of several cytokines

and growth factors that can increase the excitability of sensory neurons (Austin and Moalem-Taylor, 2010).

Microglia are a population of normally quiescent resident macrophages that carry out immune vigilance in the CNS. Upon activation, they show hypertrophy, change their shape, express OX42, and can act as phagocytes, present antigen, and release mediators such as pro-inflammatory cytokines. In neuropathic pain conditions, microglia are one of the first spinal cord cell types to be activated after peripheral nerve injury, and their activation persists for at least several months in experimental neuropathies (Austin and Moalem-Taylor, 2010). **Astrocytes** represent the largest cell population in the CNS and express numerous transporter proteins to maintain homeostasis by regulating extracellular ions, protons, and neurotransmitter concentrations in their microenvironment. Astrocyte activation is characterized morphologically by hypertrophy and increased GFAP production and functionally by increased production of various pro-inflammatory substances (Austin and Moalem-Taylor, 2010). As reported above, paclitaxel-induced neuropathic pain is also associated with activation of microglia and astrocytes (Peters et al., 2007a, b), which are known to play a significant role in the creation and maintenance of exacerbated pain states with inflammatory and/or neuropathic etiologies (Milligan et al., 2008).

Paclitaxel-induced neuropathic pain therefore appears to be associated with the increase and hypertrophy of certain immune cells in the PNS and CNS that could release cytokines and other mediators, which may contribute to the induction and maintenance of neuropathic pain by altering neuronal function. Ledeboer et al. (2007) attempted to quantify some of these cytokines and found increased mRNA of proinflammatory cytokines IL-1β and TNF-α in lumbar DRG but not in lumbar

meninges or spinal cord of neuropathic paclitaxel-treated rats. Interestingly, mechanical hypersensitivity and the associated increase in proinflammatory cytokines were prevented by intrathecal (i.t.) IL-1 receptor antagonist and i.t. IL-10 (anti-inflammatory cytokine) gene therapy. The authors also showed that paclitaxel induced microglial but not astroglial activation in the lumbar DRG and that this microglial activation was inhibited by IL-10 gene therapy. Therefore, the data indicated that suppression of paclitaxel-induced allodynia by IL-10 is associated with decreased microglial and expression of TNF-α and IL-1β in lumbar DRG, suggesting that IL-10 inhibits activation and/or recruitment of microglia into the DRG and subsequent proinflammatory cytokine production.

In a recent report, Bennett´s group failed to detect any evidence of the activation of spinal microglia (Iba1 marker) in several painful peripheral neuropathies induced by paclitaxel, oxaliplatin, vincristine, and the anti-retroviral agent 2',3'-dideoxycytidine (ddC), whereas they detected a marked increase and hypertrophy of spinal microglia in nerve trauma models. The authors concluded that the response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is distinct from that evoked by traumatic nerve injuries (Zheng et al., 2011). However, these data are in disagreement with previous reports (Ledeboer et al., 2007; Peters et al., 2007a, b). Differences in the microglial marker and/or the dose/schedule of paclitaxel used may explain this discrepancy.

Finally, several pharmacological studies also support the hypothesis that the immune system is involved in paclitaxel-induced neuropathic pain. Thus, both mynocycline and thalidomide, which have profound immunomodulatory effects, prevented the development of neuropathic pain induced by paclitaxel in addition to their

originally intended pharmacological actions (Cata et al., 2008; Liu et al., 2010; Boyette-Davis et al., 2011). Interestingly, minocycline prevented not only paclitaxel-evoked pain but also the IENF loss, infiltration of macrophage infiltration, and ATF3 up-regulation associated with paclitaxel neuropathy (Liu et al., 2010; Boyette-Davis et al., 2011).

2.3.3.6. Other mechanisms involved in paclitaxel-induced neuropathic pain

Various laboratories have investigated the role of certain proteins in paclitaxel-induced neuropathic pain in rodents by testing changes in the expression of these proteins and/or their RNA, although few data are available on this issue. Thus, increased mRNA and protein levels of the $\alpha_2\delta$-1 subunit of voltage-dependent calcium channel ($Ca\alpha_2\delta$-1) were reported in medium/large-diameter DRG neurons of mice after a single low dose of paclitaxel. The time course of $Ca\alpha_2\delta$-1 protein expression was very close to that of paclitaxel-induced neuropathic pain (Matsumoto et al., 2006). However, other authors found increased $Ca\alpha_2\delta$-1 mRNA coinciding with peak pain severity in the dorsal spinal cord but not the DRG of paclitaxel-treated mice (Gauchan et al., 2009), although both studies used the same schedule and almost identical doses of paclitaxel. Bennett´s group also found that paclitaxel-induced neuropathic pain was associated with increased $Ca\alpha_2\delta$-1 protein in the dorsal spinal cord but not in the DRG (Xiao et al., 2007).

Other authors reported that transient receptor potential vanilloid 4 (TRPV4) is essential in paclitaxel-induced neuropathic pain, because rats administrated with TRPV4 antisense oligodeoxynucleotides (Alessandri-Haber et al., 2004) and TRPV4 knockout mice (Alessandri-Haber et al., 2008) had reduced paclitaxel-induced

mechanical hyperalgesia. According to these authors, the role of TRPV4 in paclitaxel-induced pain is not attributable to an increase in its protein expression but to specific interaction with second messenger integrin pathways (Dina et al., 2004; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2008). It was recently found that TRPV4 physically interacts with tubulin, actin, and neurofilament proteins as well as with second messengers such as PKCε in DRG neuronal culture. These authors proposed that microtubule dynamics regulate TRPV4 activity and that this channel forms a supra-molecular complex containing cytoskeletal proteins and regulatory kinases, which could integrate signaling of various intracellular second messengers and signaling cascades as well as cytoskeletal dynamics (Goswami et al., 2010). Moreover, paclitaxel-induced hyperalgesia was attenuated by the selective PKAε antagonist (Dina et al., 2001), one of the second messengers that may be part of the proposed supra-molecular complex. Therefore, the findings by Goswami and colleagues point to the presence of cross-talk between nonselective cation channels and the cytoskeleton at multiple levels, which may help us to understand the molecular basis of paclitaxel-induced neuropathic pain. However, more experiments are required to clarify this intriguing theory.

2.3.4 Experimental treatments to prevent and/or ameliorate paclitaxel-induced neuropathic pain in rodents

Nowadays, there are no clinically effective treatments to counteract or prevent paclitaxel-induced neuropathic pain, despite extensive preclinical and clinical efforts over recent years (Argyriou et al., 2008). Investigation of potential analgesic or neuroprotective drugs for chemotherapy-evoked neuropathic pain is particularly complex because cancer patients may have both inflammatory and neuropathic pain,

and the latter may arise from multiple causes (chemotherapy, tumor effects, radiotherapy, surgical trauma, etc.). However, experimental animal models of chemotherapy-induced neuropathic pain offer a simpler situation that allows researchers to test experimental treatments without interferences, although it does not ensure that this treatment is truly effective when the situation becomes more complex, as in patients with cancer. It is also important to note that a compound that has been shown to be effective in animal models of chemotherapy-induced neuropathic pain must not interfere with the effectiveness against cancer of the chemotherapeutic agent. This section describes the main experimental strategies to identify effective means of prevention and symptomatic treatments in these animal models.

2.3.4.1. Symptomatic treatment

Several authors have used animals with established paclitaxel-evoked neuropathic pain to test the efficacy of numerous compounds to ameliorate the pain behavior associated with paclitaxel treatment. Some of these compounds are commercially available drugs and others are experimental compounds.

A) Calcium channel blockers

Several calcium channel blockers have been tested in paclitaxel-induced neuropathic pain in animals. Thus, the anti-epileptic ethosuximide, which is a relatively selective T-type calcium channel blocker, elicited a nearly complete reversal of paclitaxel-induced mechanical allodynia/hyperalgesia and cold allodynia (Flatters and Bennett, 2004). In a subsequent article, Bennett´s group reported that NMED-126 (a mixed N- and T-type calcium channel blocker) produced significant reductions in mechanical allodynia/hyperalgesia that were more pronounced after the second, third, and fourth

injections (Xiao et al., 2008). Finally, a very recent report showed that intraplantar administration of other T-type calcium channel blockers, such as mibefradil and NNC 55-0396, also inhibited paclitaxel-induced hyperalgesia (Okubo et al., 2011).

The GABA analogues gabapentin and pregabalin, which were originally developed to treat epilepsy, are also considered first-line drugs for the treatment of neuropathic pain (Dworkin et al., 2007). They are not active at any known GABA-binding site but bind to the $\alpha_2\delta$-1 subunit of voltage-dependent calcium channels. The conformational changes produced by this binding inhibit abnormally intense neuronal activity by reducing the synaptic release of glutamate, norepinephrine, and substance P (Tuchman et al., 2010). Thus, systemic administration of gabapentin inhibited the mechanical allodynia and thermal hyperalgesia induced by a single paclitaxel treatment in mice. This effect of gabapentin was dose-dependent and lasted for more than 24 h (Matsumoto et al., 2006). In a more recent study that used a similar single dose of paclitaxel in mice, the authors found that oral and intrathecal administration of gabapentin dose-dependently reversed mechanical allodynia (Gauchan et al., 2009). Bennett´s group also tested gabapentin in its standard repeat-treatment paclitaxel model in rats and found that it was effective to reverse paclitaxel-induced mechanical allodynia/hyperalgesia but only after at least a second systemic injection of gabapentin (Xiao et al., 2007). In the case of this drug, there was a molecular correlation with the behavioral results, because the same authors reported a paclitaxel-induced up-regulation of the Ca$\alpha_2\delta$-1 (see sections 2.3.3.4. and 2.3.6.). Several findings suggest that Ca$\alpha_2\delta$-1 ligands such as pregabalin and gabapentin may be useful to treat numerous syndromes in which central sensitization plays a major role (Tuchman et al., 2010), as appears to be the case of paclitaxel-induced neuropathic pain. However, in patients, gabapentin was no better than placebo in

alleviating pain induced by chemotherapy, including paclitaxel (Rao et al., 2007), although further studies are required.

Therefore, several lines of evidence suggest that drugs that modulate calcium channels may have analgesic efficacy in paclitaxel-evoked pain. Another article by Bennett´s group explored the role of calcium in paclitaxel-induced neuropathy by using drugs that reduce intracellular or extracellular calcium ions (TMB-8, Quin-2, EGTA, EGTA-am), and they found that intrathecal administration of all of these drugs significantly inhibited the mechanical hypersensitivity produced by this antineoplastic (Siau and Bennett, 2006).

B) Cannabinoid receptors

Several studies suggested that cannabinoid receptors (CB) might be an important therapeutic target for the treatment of paclitaxel-induced neuropathic pain. First, it was demonstrated that systemic treatment with the cannabinoid agonist WIN55,212-2 significantly reduced mechanical allodynia and heat hyperalgesia. This effect was reversed by the CB1 antagonist SR 141716 A, suggesting the contribution of the CB1 receptor, although the participation of CB2 receptors could not be ruled out in this study. When WIN 55,212-2 was administered intraplantarly, no differences were observed between the injected paw and the contralateral paw, suggesting that systemic mechanisms are needed for efficacy to be achieved (Pascual et al., 2005). Two subsequent reports using CB2 agonists such as (R,S)-AM1241 and AM1714 (Rahn et al., 2008) and the new compound MDA7 (Naguib et al., 2008) demonstrated that the activation of CB2 receptors suppresses mechanical allodynia. In addition, these effects were selectively antagonized by a CB2 but not CB1 receptor antagonist. Therefore, it

appears that activation of both CB1 and CB2 receptors is able to inhibit signs of paclitaxel neuropathy.

C) Opioids and tramadol

Tests have also been conducted on opioid analgesics and tramadol, which are recommended as second-line treatments for neuropathic pain but can be considered for first-line use under certain clinical circumstances (Dworkin et al., 2007). One study showed that paclitaxel-induced neuropathic pain is relatively resistant to morphine treatment, because only elevated systemic doses of morphine elicited up to a 50% reversal of mechanical hypersensitivity (Flatters and Bennett, 2004). However, two subsequent reports demonstrated that morphine was effective at lower doses. Thus, Rahn et al., (2008) found that systemic morphine (4 mg/kg) suppressed paclitaxel-induced mechanical allodynia, and Pascual et al., (2010) showed that systemic administration of morphine abolished the mechanical and thermal hypersensitivy induced by paclitaxel in a dose-dependent and naloxone-sensitive manner, finding an antinociceptive effect at the highest doses. Interestingly, these authors demonstrated that morphine inhibited mechanical allodynia at 2.5 mg/kg (Pascual et al., 2010) and 4 mg/kg (Rahn et al., 2008), whereas Flatters and Bennett (2004) found no effect at a dose of 4 mg/kg. This discrepancy may be attributable to differences in the protocol and/or rat strain.

Pascual et al. (2010) also studied the effects of methadone, another potent μ-receptor agonist with affinity for the δ-opioid receptors and NMDA receptors. Methadone inhibited the mechanical and thermal hypersensitivity induced by paclitaxel and produced an analgesic effect at higher doses. These effects were completely antagonized by naloxone, suggesting that they are mediated by opioid receptor

activation. In addition, tramadol, a weak μ-opioid agonist that also inhibits reuptake of norepinephrine and serotonin (Dworkin et al., 2007), proved able to inhibit paclitaxel-induced mechanical hypersensitivity (Xiao et al., 2008).

However, despite the relative effectiveness of opioids drugs in animals and their frequent recommendation for treating chemotherapy-induced neuropathic pain in humans, there has been no clinical trial to date.

D) NMDA receptor antagonists

NMDA receptors are known to play a key role in the central sensitization process and in the development and maintenance of neuropathic pain (Latremoliere and Woolf, 2009). However, despite numerous findings suggesting the involvement of central sensitization phenomenon in paclitaxel-induced neuropathic pain (see section 2.3.3.4.B), NMDA receptor antagonists have had little effect in the treatment of this pathology. Thus, Flatters and Bennett, (2004) showed that the systemic administration of low doses of the NMDA receptor antagonist MK-801 did not significantly reverse paclitaxel-induced pain. In another study, the analgesic effect of ketamine (NMDA receptor antagonist) was only achieved at high doses, which were also associated with motor impairment. These authors also found that ketamine was more effective to inhibit paclitaxel-induced mechanical allodynia than thermal hyperalgesia (Pascual et al., 2010). In the same article, subanalgesic doses of morphine and ketamine produced an additive effect on heat hyperalgesia but not on mechanical allodynia.

E) Sodium channel blockers

Bennett´s group evaluated mexiletine (a class I cardiac antiarrhythmic drug) and various drugs that block the sodium channels, among other effects, including the tricyclic antidepressants (TCA) amitriptyline and the anticonvulsants topiramate and

oxcarbazepine, in paclitaxel-induced neuropathy in rats (Xiao et al., 2008). Mexiletine induced partial but statistically significant decreases in mechano-allodynia/hyperalgesia (Xiao et al., 2008). Amitriptyline also produced partial antiallodynic and antihyperalgesic effects, although only after repeated doses (Xiao et al., 2008). All of the TCAs, including amitriptyline, are known to exert a complex mixture of actions, which might contribute to their analgesic activity, but some authors attributed the efficacy of TCAs in neuropathic pain to their sodium channel blocking effect (Amir et al., 2006). In the clinical setting, TCAs such as nortriptyline or amitriptyline, which are recommended as first-line treatments for neuropathic pain (Dworkin et al., 2007), have been tested in patients for the treatment of neuropathic symptoms induced by chemotherapy, including paclitaxel, with contradictory results (Wolf et al., 2008; Argyriou et al., 2008 for references).

Topiramate has several mechanisms of action besides sodium channel blocking, and many of them may be relevant in treating neuropathic pain (Sang and Hayes, 2008). Moreover, topiramate may prevent or promote recovery of injured sensory axons and improve impaired mitochondrial function (see Xiao et al., 2008 for references), and both pathological conditions have been proposed to play a key role in the pathophysiology of paclitaxel-evoked painful neuropathy (see sections 2.3.3.2. and 2.3.3.3.). Based on this neuroprotective mechanism, the Bennett´s group administered topiramate for 12 consecutive days to rats with established paclitaxel-induced pain. No acute analgesic effect was observed, and antiallodynic and antihyperalgesic effects did not appear until days 6 and 8 of treatment, respectively (Xiao et al., 2008). In contrast, oral administration of oxcarbazepine to neuropathic paclitaxel-treated rats had no effect (Xiao et al., 2008). However, oxcarbazepine has other putative mechanisms of action,

including enhancement of potassium conductance and modulation of high voltage-activated calcium channels (Sang and Hayes, 2008).

F) Immunomodulatory agents

Given the immune system involvement in paclitaxel-induced neuropathic pain (see section 2.3.3.5.), it is not surprising that several authors have tested drugs with immunomodulatory properties in experimental treatments. In a study by Ledeboer et al. (2006), paclitaxel-induced mechanical allodynia was inhibited by the systemic administration of AV411 (ibudilast), which is a relatively nonselective phosphodiesterase inhibitor that also suppresses glial-cell activation. In addition, the highest concentrations of AV411 were found in the CNS, suggesting that it may block spinal cord glial activation. In a subsequent report, the same authors found that paclitaxel-induced mechanical allodynia is reversed by i.t. administration of an IL-1 (proinflammatory cytokine) receptor antagonist and by i.t. IL-10 (anti-inflammatory cytokine) gene therapy (Ledeboer et al., 2007).

Other authors later demonstrated that paclitaxel-induced neuropathic pain was attenuated by systemic treatment with minocycline (an antimicrobial agent) and thalidomide (introduced as a sedative-hypnotic), which are both glial activation inhibitors that decrease the release of multiple pro-inflammatory cytokines and cross the blood-brain barrier (Cata et al., 2008). These results support the hypothesis that this painful neuropathy has an important immunologic component.

G) Compounds that modulate mitochondrial functions

Because paclitaxel-evoked neuropathic pain is attributed to a toxic effect on axonal mitochondria (see section 2.3.3.3.), some authors have tested compounds (e.g., acetyl-L-carnitine and olesoxime) that have been found to improve mitochondrial

dysfunction. Thus, the paclitaxel-evoked neuropathic pain state was reversed by repeated treatment with acetyl-L-carnitine, an amino acid derivative that plays an essential role in transporting long chain free fatty acids into the mitochondria (Ghirardi et al., 2005; Flatters et al., 2006), and with olesoxime, a cholesterol-like compound with neuroprotective, pro-regenerative, and analgesic actions (Xiao et al., 2009).

H) Compound that regulate peripheral nerve blood flow

Given that paclitaxel also appears to have antiangiogenic properties (see section 1.3.), some authors have tested compounds that promote angiogenesis and/or increase the blood flow of the nerve. Thus, intramuscular gene transfer of VEGF to rats with established paclitaxel-induced neuropathy was found to ameliorate the associated diminution of the peripheral nerve vasculature, resulting in the recovery of peripheral nerve function (Kirchmair et al., 2007). Other authors subsequently reported that repeated treatment with the prostaglandin E_1 analog limaprost inhibited both the late phase of mechanical allodynia and the progressive decrease in peripheral blood flow induced by acute paclitaxel (Gauchan et al., 2009).

I) Other agents

Transient relief from paclitaxel-induced mechanical and cold hypersensitivity was obtained with the antiallergic agent pemirolast, which inhibits the release of chemical mediators such as histamine and leukotriene and of sensory nerve peptides such as substance P, neurokinin (NK) A, and calcitonin gene-related peptide (CGRP) (Tatsushima et al., 2011). The authors suggested that the ameliorative effect was probably caused in the PNS, because pemirolast has poor blood-brain barrier permeability and was orally administered in their study. They also demonstrated that pemirolast inhibited the release of substance P by cultured adult rat DRG neurons. In the same report, the authors showed that

paclitaxel-induced neuropathic pain was reversed by the i.t. administration of NK_1 and/or NK_2 receptor antagonists (binding sites of substance P). Taken together, these findings suggest that substance P is involved in paclitaxel-induced neuropathy (Tatsushima et al., 2011).

Dina et al., (2001) reported that the hyperalgesia produced by both acute and chronic paclitaxel treatment was attenuated by intraplantar injection of selective second messenger antagonists for PKCε and PKA, suggesting that both second messenger pathways contribute to paclitaxel-induced pain. In subsequent studies, the same authors reported that paclitaxel-induced neuropathic hypersensitivity was reversed by spinal administration of antisense oligodeoxynucleotides to integrins, which are transmembrane proteins that mediate cell adhesion and concomitant signaling and are important for a wide variety of cellular functions, including nociception (Dina et al., 2004; Alessandri-Haber et al., 2008). The same group also found that paclitaxel-induced neuropathy was abolished by spinal administration of TRPV4 antisense oligodeoxynucleotides (Alessandri-Haber et al., 2004; 2008).

Other authors tested two botulinum toxins (Dysport® and Botox®) in rats with paclitaxel-induced neuropathic pain. After intraplantar injection of the neurotoxins, the mechanical hyperalgesia was almost completely abolished during the 6-day observation period not only in the injected paw but also in the contralateral paw. The authors ruled out the possibility that this bilateral antinociceptive effect resulted from a systemic diffusion of the botulinum neurotoxins (Favre-Guilmard et al., 2009).

It has been reported that the repeated or single systemic administration of phenyl N-tert-butyl nitrone, a spin-trap reagent and potent free radical scavenger, counteracted paclitaxel-induced mechanical allodynia, suggesting an imbalance of free radical levels in

this pathologic condition (Kim et al., 2010). On the other hand, inconsistent outcomes were obtained from the treatment of paclitaxel-induced neuropathic pain with baclofen, a gamma amino butyric acid type-B (GABA-B) receptor agonist, which is primarily used to treat spasticity (Xiao et al., 2008). Finally, a single systemic dose of the neuroprotective peptide prosaposin (the intracellular precursor of lysosomal sphingolipid activator proteins called saposins) rapidly alleviated tactile allodynia in paclitaxel-treated rats (Jolivalt et al., 2006).

2.3.4.2. Preventive treatment

Authors have also evaluated the capacity of numerous compounds to prevent the neuropathic pain induced by paclitaxel in rodents. An effective prophylactic treatment is of enormous value in this type of neuropathy, because the exact timing of the toxic insult is known (start of anticancer treatment).

A) Neurotrophic factors

Some authors focused on the use of neurotrophic factors to prevent neuropathy induced by chemotherapy, including paclitaxel treatment. In fact, neurotrophic factors are able to target the most typical neurotoxicity sites of anticancer drugs, the DRG and peripheral nerves, where the presence of specific receptors for neurotrophic growth factors has been demonstrated (Cavaletti et al., 2011). Thus, two studies found that nerve growth factor (NGF) could prevent signs of paclitaxel-induced neurotoxicity in mice and rats (Apfel et al., 1991; Schmidt et al., 1995). Although there are no published clinical studies, these findings are in agreement with data on reduced circulating NGF levels in cancer patients with neuropathy treated with various neurotoxic chemotherapy schedules, including taxanes. In addition, the decrease in NGF levels appears to be

correlated with the severity of neurotoxicity (De Santis et al., 2000). Another article reported that 4-methylcatechol (4-MC), which induces NGF secretion, prevented the behavioral and electrophysiological abnormalities produced by paclitaxel (Callizot et al., 2008).

The neurotrophic factor prosaposin, the precursor of saposins A, B, C, and D, not only facilitated nerve regeneration *in vivo* but also prevented paclitaxel neurotoxicity (Campana et al., 1998). Some interleukins, such as leukemia inhibitory factor (Boyle et al., 2001; Kilpatrick et al., 2001) and IL-6 (Callizot et al., 2008), were also effective in preclinical models, but leukemia inhibitory factor did not prove efficacious in a clinical trial (Davis et al., 2005). Paclitaxel-induced mechanical hypersensitivity and reduction in sensory nerve conduction velocity were both improved by systemic treatment with the amino acid L-serine, which has important neurotrophic effects (Kiya et al., 2011).

B) Other agents

Several compounds that have been tested as "curative" treatments for established paclitaxel-induced neuropathy also proved able to avert it when administered before or simultaneously with paclitaxel treatment. Thus, paclitaxel-induced neuropathic pain was prevented by minocycline (Boyette-Davis et al., 2011; Lui et al., 2011), AV411 (Ledeboer et al., 2006), acetyl-L-carnitine (Pisano et al., 2003; Jin et al., 2008 Xiao and Bennett, 2008), olesoxime (Xiao et al., 2009), phenyl N-tert-butyl nitrone (Kim et al., 2010), and VEGF (Kirchmair et al., 2007).

Glutamine, a nonessential amino acid, was also able to prevent paclitaxel-induced neuropathy in a preclinical model (Boyle et al., 1999), but outcomes in patients have been controversial. Early clinical trials showed that glutamine and glutamate may be effective (Argyriou et al., 2008 for references), but it was recently reported that long-

term glutamate supplementation failed to protect against paclitaxel neurotoxicity (Loven et al., 2009).

BGP-15, a hydroxamic acid derivate, also protects against paclitaxel-induced peripheral neuropathy in rats. The authors suggested that its chemoprotective activity is related to the modulation of poly(ADP-ribose) polymerase activity and the protection of mitochondria from oxidative damage (Bárdos et al., 2003). Another study reported that the systemic inhibition of calpains with the peptide α-ketoamide calpain inhibitor AK295 also prevented paclitaxel-induced sensory neuropathy. Calpains are calcium-activated proteases that have been implicated in various neurological disorders, including peripheral neuropathies. The authors also showed that paclitaxel caused calpain activation in PC12 cells, suggesting that calpain activation was probably attributable to the sustained elevation of intracellular calcium (Wang et al., 2004).

Neurotropin, a non-protein extract from inflamed rabbit skin inoculated with vaccinia virus, which has been used to treat various chronic painful conditions, also proved effective to prevent paclitaxel-induced neuropathic pain in rats (Kawashiri et al., 2009). Other authors reported that treatment with the adrenocorticotropic hormone-(4-9) analog ORG 2766 (Hamers et al., 1993) or with ganglioside (Chentanez et al., 2003) could produce some beneficial effects in paclitaxel-induced neuropathy in rats. The herbal medicine Shakuyaku-kanzo-to also prevented paclitaxel-induced allodynia and hyperalgesia, whereas the non-steroidal anti-inflammatory drug loxoprofen sodium had no effect (Hidaka et al., 2009).

RATIONALE AND GOALS

Paclitaxel (Taxol®) is one of the most effective and widely used antineoplastic drugs for the treatment of solid tumors and is a first-line choice for the treatment of breast and ovarian cancer in women, among others (Balayssac et al., 2011; Argyriou et al., 2008). However, a large number of patients treated with paclitaxel frequently develop a clinically significant and dose-limiting painful peripheral neurotoxicity characterized by numbness, tingling, burning pain, and cold allodynia in a stocking-glove distribution (Dougherty et al., 2004; Jung et al., 2005; Argyriou et al., 2008). Unfortunately, there are no clinically validated treatments to prevent or ameliorate this painful neuropathy (Argyriou et al., 2008). Nevertheless, models of paclitaxel-induced neuropathic pain in rodents have recently been described that make possible to test new treatments and/or elucidate the pathogenesis of this pain (e.g.: Dina et al., 2001; Flatters and Bennett, 2004; Liu et al., 2010). These models have shown that repeated or acute administration of paclitaxel to rodents produces hyperalgesia and/or allodynia to mechanical, cold, and/or heat stimuli (listed in Authier et al., 2009). Most of the previously reported models of this neuropathy were performed in male rats; however, the clinical use of paclitaxel mainly involves female patients (Rowinsky, 2010), and most models of genetic engineering to eliminate or overexpress a given target are performed in mice. Therefore, the **general goal** of this Doctoral Thesis was to develop an experimental model of neuropathic pain induced by paclitaxel in female mice and to assess different potential therapeutic targets in this model, using diverse experimental approaches such as behavioral and morphological studies.

Voltage-gated sodium channels (VGSC) play a critical role in neuronal function under both physiological and pathological conditions (reviewed by Dib-Hajj et al., 2010; Liu and Wood, 2011). Nine isoforms of the sodium channel alpha-subunit

($Na_v1.1$-1.9) have been identified in mammals, which differ in their tissue localization, sensitivity to tetrodotoxin (TTX, a nonpeptidic guanidinium toxin), threshold of activation/inactivation, and frequency of activation/inactivation (Yu and Catterall, 2003). Low concentrations of TTX block $Na_v1.1$-1.4 and $Na_v1.6$-1.7 subtypes (TTX-sensitive channels), whereas markedly higher concentrations are necessary to block $Na_v1.5$ and $Na_v1.8$-1.9 subtypes (TTX-resistant channels) (Ogata and Ohishi, 2002).

Altered VGSC expression in sensory neurons contributes to neuropathic and inflammatory pain (Lai et al., 2004; Wood et al., 2004; Amir et al., 2006). In particular, TTX-sensitive channels are increased in nociceptive neurons localized in the dorsal root ganglion (DRG) (Dib-Hajj et al. 1999; Black et al., 1999; Kim et al., 2001; Hong et al., 2004), spinal cord dorsal horn (Hains et al., 2003, 2004), and thalamic nucleus (Zhao et al., 2006) in different neuropathic pain models. Upregulation of TTX-sensitive channels in these models leads to electrophysiological changes, e.g., an increase in background firing and hyperresponsiveness to mechanical and thermal stimuli in neurons, and to behavioral changes, e.g., an increase in pain in nociceptive tests (Rizzo et al., 1995; Cummins and Waxman, 1997; Black et al., 1999; Hains et al., 2004; Zhao et al., 2006). In order to control the neuropathic pain induced by this hyperexcitability, a drug that can selectively block these subtypes of VGSCs could therefore be of therapeutic interest, and TTX has this profile.

Administration of TTX reduces spontaneous ectopic discharges and pain behaviors in several models of mechanically-induced neuropathic pain (Omana-Zapata et al., 1997; Lyu et al., 2000; Liu et al., 2001; Xie et al., 2005; Marcil et al., 2006). However, the contribution of TTX-sensitive sodium channels to chemotherapy-induced neuropathic pain, such as that induced by paclitaxel, has not been investigated.

Accordingly, the **first specific goal** of this Doctoral Thesis was to examine the effect of the blockade of TTX-sensitive VGSC in paclitaxel-induced neuropathic pain. For this purpose, we evaluated the effects of acute systemic administration of low doses of TTX on the *expression* of different signs (allodynia and/or hyperalgesia) of paclitaxel-induced neuropathic pain in female mice once the neuropathy was fully established. In addition, because sodium channel blockade over an extended period in the early stages of nerve injury might constitute a mechanism of disease modification in mechanically-induced neuropathic pain models (Xie et al., 2005), we also tested the preventive effect of repeated systemic administrations of TTX on the *development* of paclitaxel-induced neuropathic pain.

Sigma-1 (σ_1) receptors are widely distributed in the central nervous system, including pain processing areas (Alonso et al., 2000; Kitaichi et al., 2000). Over recent years, there has been increasing evidence of the involvement of σ_1 receptors in pain modulation, especially in pain models that involve central sensitization (Drews and Zimmer, 2009). In fact, it was recently reported that both formalin-induced pain and capsaicin-induced mechanical hypersensitivity are reduced in σ_1 receptor knockout (σ_1-KO) mice (Cendán et al., 2005b; Entrena et al., 2009b) and in wild-type (WT) mice treated with σ_1 receptor antagonists (Cendán et al., 2005a; Kim et al., 2006; Entrena et al., 2009a). Moreover, sciatic nerve injury-induced neuropathic pain was diminished in σ_1-KO mice (De la Puente et al., 2009) and in WT mice treated with σ_1 receptor antagonists (Roh et al., 2008). These pain behavioral changes are accompanied by a reduction in the phosphorylation of both NMDA receptor subunit 1 (pNR1) (Roh et al., 2008) and extracellular signal-regulated kinase (pERK) (De la Puente et al., 2009) in the

ipsilateral spinal cord dorsal horn, which are important events in central sensitization (Latremoliere and Woolf, 2009).

Therefore, σ_1 receptors appear to play a key role in central sensitization and in models of mechanically-induced neuropathic pain. However, no studies have been published on the role of σ_1 receptors in highly prevalent painful neuropathies such as those induced by diabetes, herpes virus infection, or antineoplastic drugs. It is of interest to test this involvement, because the pathophysiological mechanisms and the response to drugs differ according to the origin of nerve injury (Aley and Levine, 2002; Baron et al., 2010; Bennett, 2010; Zheng et al., 2011). Hence, the **second specific goal** of this Doctoral Thesis was to determine the role of σ_1 receptors in the neuropathic pain induced by the antineoplastic drug paclitaxel. For this purpose, we evaluated whether acute systemic administration of σ_1 receptor antagonists inhibits the *expression* of the signs of neuropathic pain induced by paclitaxel, when this is fully developed. In addition, because the lack of σ_1 receptors or their blockade appears to prevent the development of mechanically-induced neuropathic pain and ERK activation, we also tested the preventive effect of repeated systemic administrations of σ_1 receptor antagonists on the *development* of paclitaxel-induced allodynia in WT mice. Finally, in order to use a non-pharmacological experimental approach, we examined whether the absence of σ_1 receptors impeded the development of neuropathic pain induced by paclitaxel by comparing the ability of the anticancer drug to induce neuropathic pain in σ_1-KO and WT mice.

Our understanding of the σ_1 receptor has made significant progress over the past few years. Thus, the σ_1 receptor was recently identified as a unique ligand-regulated molecular chaperone in the endoplasmic reticulum of cells (Hayashi and Su, 2007) and

was proposed to be a modulator of several receptors and ion channels (Maurice and Su, 2009). In particular, σ_1 receptors normally reside at the endoplasmic reticulum-mitochondrion interface known as the mitochondrion-associated endoplasmic reticulum membrane (MAM) where the σ_1 receptor chaperone modulates the intramitochondrial Ca^{2+} level, playing a key role in the control of intracellular Ca^{2+} homeostasis (Su et al., 2010).

An increased incidence of swollen and vacuolated axonal mitochondria has been reported in peripheral sensory fibers, which appears to be relevant to the pathogenesis of paclitaxel-induced neuropathic pain (Flatters and Bennett, 2006; Jin et al., 2008; Bennett, 2010). This increase of atypical mitochondria has been attributed to the binding of paclitaxel to mitochondrial β-tubulin, which may produce Ca^{2+} release from mitochondria and dysregulation of intracellular Ca^{2+} homeostasis (Flatters and Bennett, 2006; Siau and Bennett, 2006). Interestingly, disturbances of mitochondrial function and/or intracellular Ca^{2+} levels can affect membrane excitability, neurotransmitter release, and other cellular events that may contribute to diabetic neuropathy (Verkhratsky and Fernyhough, 2008) and other peripheral neuropathies (Fernyhough and Calcutt, 2010).

Given these facts, it is of interest to evaluate whether the beneficial effects of σ_1 receptor inactivation on paclitaxel-induced neuropathic pain observed in our behavioral studies are associated with a reduction of the mitochondrial abnormalities induced by the antineoplastic, and this question constitutes the **third specific goal** of this Doctoral Thesis. To this end, we performed transmission electronic microscopy analysis of the saphenous nerve ultrastructure of paclitaxel-treated animals and investigated whether the mitochondrial changes induced by this drug can be prevented by σ_1 receptor

blockade through administration of a selective σ_1 receptor antagonist or genetic inactivation (σ_1-KO mice).

PAPERS

1. Tetrodotoxin inhibits the development and expression of neuropathic pain induced by paclitaxel in mice

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1.1. ABSTRACT

We evaluated the effect of low doses of systemically administered tetrodotoxin (TTX) on the development and expression of neuropathic pain induced by paclitaxel in mice. Treatment with paclitaxel (2 mg/kg, i.p., once daily during 5 days) produced long-lasting (2-4 weeks) heat hyperalgesia (plantar test), mechanical allodynia (electronic Von Frey test) and cold allodynia (acetone drop method), with maximum effects observed on days 7, 10 and 10-14, respectively. Acute subcutaneous treatment with 1 or 3 µg/kg of TTX reduced the expression of mechanical allodynia, whereas higher doses (3 or 6 µg/kg) were required to reduce the expression of cold allodynia and heat hyperalgesia. In contrast, TTX (3 or 6 µg/kg, s.c.) did not affect the response to the same thermal and mechanical stimuli in control animals, which indicates that the antihyperalgesic and antiallodynic effects of TTX were not due to unspecific inhibition of the perception of these stimuli. Administration of TTX (6 µg/kg, s.c.) 30 min before each of the 5 doses of paclitaxel did not modify the development of heat hyperalgesia produced by the antineoplastic, but abolished the development of mechanical and cold allodynia. Coadministration of a lower dose of TTX (3 µg/kg) also prevented the development of mechanical allodynia. No signs of TTX-induced toxicity or motor incoordination were observed. These data suggest that low doses of TTX can be useful to prevent and treat paclitaxel-induced neuropathic pain, and that TTX-sensitive subtypes of sodium channels play a role in the pathogenesis of chemotherapy-induced neuropathic pain.

1.2. INTRODUCTION

Peripheral neurotoxicity induced by antineoplastic drugs (taxanes, vinca-alkaloids and platin-based compounds) is a clinically significant complication that can be dose-limiting and can substantially diminish quality of life (Kuroi and Shimozuma, 2004; Sul and Deangelis, 2006). In particular, paclitaxel produces sensory neuropathy with numbness, tingling, burning pain and cold allodynia in a stocking-glove distribution (Dougherty et al., 2004; Kuroi and Shimozuma, 2004). There are no well established treatments to prevent or minimize paclitaxel-induced nerve damage (Kuroi and Shimozuma, 2004), but models of this neuropathy in rodents (Polomano et al., 2001; Dina et al., 2001; Smith et al., 2004; Flatters and Bennett, 2004) have made it possible to test the effect of new treatments.

Voltage-gated sodium channels play a critical role in neuronal function under both physiological and pathological conditions (Wood et al., 2004; Amir et al., 2006). Nine isoforms of the sodium channel alpha-subunit ($Na_v1.1$-1.9) have been identified in mammals (Ogata and Ohishi, 2002). The isoforms differ in their sensitivity to tetrodotoxin (TTX), a nonpeptidic guanidinium toxin. Nanomolar concentrations of TTX block $Na_v1.1$-1.4, and $Na_v1.6$-1.7 subtypes (TTX-sensitive channels), whereas considerably higher (micromolar) concentrations are required to block $Na_v1.5$ and $Na_v1.8$-1.9 subtypes (TTX-resistant channels) (Ogata and Ohishi, 2002).

Altered sodium channel expression in sensory neurons contributes to neuropathic and inflammatory pain (Lai et al., 2004; Wood et al., 2004; Amir et al., 2006). In particular, TTX-sensitive channels (e.g., $Na_v1.3$ and $Na_v1.7$) are upregulated in nociceptive neurons located in dorsal root ganglion (DRG) (Dib-Hajj et al. 1999; Black et al., 1999; Kim et al., 2001; Hong et al., 2004), the spinal cord dorsal horn (Hains et

al., 2003, 2004) and thalamic nucleus (Zhao et al., 2006) in different models of neuropathic pain. The upregulation of TTX-sensitive channels (and downregulation of TTX-resistant ones) in these models leads to electrophysiological changes, e.g., an increase in background firing and hyperresponsiveness to mechanical and thermal stimuli in neurons, and to behavioral changes, e.g., an increase in pain in nociceptive tests (Rizzo et al., 1995; Cummins and Waxman, 1997; Black et al., 1999; Hains et al., 2004; Zhao et al., 2006).

Administration of TTX reduces spontaneous ectopic discharges and pain behaviors (mechanical allodynia and heat hyperalgesia) in several models of mechanically-induced neuropathic pain (Omana-Zapata et al., 1997; Lyu et al., 2000; Liu et al., 2001; Xie et al., 2005; Marcil et al., 2006). However, the contribution of TTX-sensitive sodium channels to chemotherapy-induced neuropathic pain has not been investigated. In addition, most of the previous studies applied TTX topically to DRGs or the injured nerves. Consequently, we evaluated the effect of the acute systemic (s.c.) administration of low, nontoxic, doses of TTX on the expression of different signs (heat hyperalgesia, mechanical- and cold-allodynia) of paclitaxel-induced neuropathic pain in mice. Because sodium channel blockade over an extended period in the early stages of nerve injury might constitute a mechanism of disease modification in neuropathic pain models (Xie et al., 2005), we also tested the preemptive effect of repeated systemic administrations of TTX on the development of paclitaxel-induced neuropathic pain.

1.3. METHODS

1.3.1. Animals

Experiments were performed in a total of 568 female CD-1 mice (Charles River, Spain) weighing 25-30 g each. We choose female animals because taxanes are frequently used in the treatment of breast and ovarian cancer in women (Crown and O'Learly, 2000; Bria et al., 2006), but no previous studies have evaluated the characteristics of and possible treatments for taxane-induced neuropathic pain in female animals. Treatments were randomly distributed across the phases of estrus, because we aimed to design our experiments in a way that reflects the clinical use of these drugs, and the phase of the menstrual cycle in treated women is not taken into account when taxane treatment is used, or in the management of taxane-induced peripheral neuropathy.

The animals were housed in colony cages with free access to food and water prior to the experiments. They were maintained in temperature- and light-controlled rooms (22 ± 1 ºC, lights on at 08.00 h and off at 20.00 h, air replacement every 20 min). Testing took place during the light phase (from 9.00 h to 15.00 h). Mice were handled in accordance with EEC Council Directive 86/609 and current guidelines for the investigation of experimental pain with conscious animals (Zimmermann, 1983). The experimental protocol was approved by the Research Ethics Committee of the University of Granada, Spain.

1.3.2. Drugs and drug administration

The drugs used (and their providers) were paclitaxel (Tocris Cookson Ltd., Bristol, United Kingdom), tetrodotoxin (Nanning Maple Leaf Pharmaceuticals; WEX

Pharmaceuticals Inc., Vancouver, BC, Canada), gabapentin and (±)-baclofen (both Sigma Aldrich, Madrid, Spain). Paclitaxel was dissolved in a solution made up of 50% Cremophor EL (Fluka Chemie, Buchs, Germany) and 50% absolute ethanol (Panreac Química S.A., Barcelona, Spain) to obtain a concentration of 6 mg/ml. This paclitaxel solution was stored at –20 ºC, during a maximum of 14 days, and was diluted in normal saline (NaCl 0.9%), just before administration, to a final concentration of 2 mg/10 ml. The vehicle for paclitaxel was diluted, at the time of injection, with saline at the same proportion as the paclitaxel solution.

Paclitaxel (2 mg/kg) was administered intraperitoneally (i.p.), in a volume of 10 ml/kg, once per day for 5 consecutive days. Therefore the cumulative dose was 10 mg/kg, a dose that has been previously shown to produce a painful neuropathy in rodents (Dina et al., 2001, 2004). In the control group the vehicle for paclitaxel was administered according to the same schedule.

The supplied TTX stock solution (concentration of 15 µg/ml) was diluted in normal saline, just before administration, to final concentrations of 1, 3 or 6 µg/5 ml. TTX (1, 3 or 6 µg/kg) or its solvent (normal saline) were administered subcutaneously (s.c.) in the interscapular area, using an injection volume of 5 ml/kg. Gabapentin and baclofen were dissolved in saline just before administration (at concentrations of 60 and 8 mg/5 ml, respectively) and were injected s.c. as described above.

1.3.3. General procedures for drug treatments and behavioral assays

The general procedure to identify the day of maximum hyper-responsiveness in each nociceptive test was as follows: pretreatment baseline responses were evaluated in each animal in a single nociceptive test 3 days before paclitaxel treatment was started,

then the animals received paclitaxel (or its vehicle) for 5 consecutive days, and the response of each animal to the nociceptive stimulus was tested again over the next 2-4 weeks (see 1.3.4. for details on the duration of each nociceptive test), without any additional treatment. When we evaluated the effect of TTX on the development of paclitaxel-induced pain, the only difference was that 30 min before each i.p. injection of paclitaxel, the animals received an s.c. injection of TTX (or saline). To investigate the effect of TTX on the expression of paclitaxel-induced pain, pretreatment baseline response was evaluated and treatment with paclitaxel was performed as in the general procedure described above. The procedures for evaluating posttreatment responses and the day of treatment with TTX were different depending on the nociceptive stimulus evaluated (see 1.3.4. for details).

In all cases the experiments in vehicle- and paclitaxel-treated groups were run in parallel, and saline- and TTX-treatment groups were also run in parallel. Each animal was tested in only one nociceptive test and received TTX (or saline) only once, because it is unknown whether initial treatment with TTX might enhance or reduce the effect of subsequent doses, and it is also unknown whether exposure to a pain test might affect the response to subsequent tests. The experimenter who evaluated the response to the nociceptive stimulus was blinded as to the treatment that the animal had received.

1.3.4. Behavioral assays

1.3.4.1. Procedure to measure heat hyperalgesia

Heat hyperalgesia was assessed with the method of Hargreaves et al. (1988), with slight modifications. Mice were habituated for 2 h in individual Plexiglas chambers (7 × 7 × 22 cm) placed on a glass floor. During this time the mice initially exhibited

exploratory behavior but subsequently stopped exploring and stood quietly with occasional bouts of grooming. After habituation, a beam of radiant heat was focused to the plantar surface of the hind paws with a plantar test apparatus (Ugo Basile, Comerio, Italy), until the mouse made a withdrawal response. The nocifensive withdrawal reflex interrupts the light reflected from the paw onto a photocell and automatically turns off the light and the timer. The latency of the withdrawal response (as an indirect measure of the heat-pain threshold) was thus recorded automatically.

Intensity of the light was adjusted at the start of the experiments such that average baseline latencies were about 8 s. This intensity was never changed. Each mouse was tested three times alternately on each hind paw, and the latencies for both paws were averaged for each measurement time. At least 1 min was allowed between consecutive measurements in the same paw. A cut-off latency time of 16 s was used in each measurement to avoid lesions to the skin and unnecessary suffering to the animals.

To elucidate the time-course of paclitaxel-induced heat hyperalgesia, pretreatment baseline latencies were obtained in each animal three days before paclitaxel administration was started. The animals were then treated with paclitaxel or its vehicle once daily for 5 days. Posttreatment latencies were assessed on days 7, 10, 14 and 17 after the first injection of paclitaxel. When the effect of TTX on the development of paclitaxel-induced heat hyperalgesia was evaluated, the procedure was the same except that TTX or its solvent was injected s.c. 30 min before each daily dose of paclitaxel (i.p.) during the 5 days of treatment.

The effect of TTX on the expression of paclitaxel-induced heat-hyperalgesia was tested on day 7 after the first injection of paclitaxel, because maximum heat hyperalgesia was observed that day (see Results, section 1.4.1.). In these experiments,

the pretreatment response to the heat stimulus was tested and the treatment with paclitaxel was performed as described above (see experiments to elucidate the time-course of heat hyperalgesia). On day 7, after habituation to the observation boxes, baseline latencies for hind paw withdrawal were recorded before drug treatment (time 0), then TTX or its solvent was injected s.c., and paw withdrawal latencies were measured again 30, 60, 90, 120 and 180 min after the injection. If the mean of the latency times recorded on day 7 at time 0 was not lower than the mean of the animal's own pretreatment value (obtained 3 days before paclitaxel treatment was started), the animal was considered a nonresponder (8.64% of the animals tested), and was not used in tests aimed to evaluate the effect of TTX on the expression of heat hyperalgesia.

1.3.4.2. Procedure to measure cold allodynia

Cold allodynia was tested as previously described by Smith et al. (2004), by gently touching the plantar skin of the hind paws with an acetone bubble formed with a syringe connected to a thin polyethylene tube. The mice were housed and habituated for 30 min in transparent plastic boxes (7 × 7 × 13 cm) with a floor made of wire mesh. After the adaptation period, acetone was applied alternately three times to each hind paw at intervals of 30 s, and the duration and frequency of licking or biting were recorded. A small mirror was placed behind the chambers to allow clear observation of the paws. The time spent licking or biting the paw was recorded with a stopwatch and reported as the cumulative time of licking/biting in all six measurements. Because animals rarely licked their hind paw for more than 10 s, a cut-off time of 10 s was used in each trial. The frequency of licking/biting was also measured and expressed as a

percentage, calculated with the formula: (number of trials in which licking or biting was observed/total number of trials) × 100.

To elucidate the time-course of paclitaxel-induced cold allodynia, the response to acetone was tested in each animal 3 days before paclitaxel administration was started (pretreatment value). Then, paclitaxel or its vehicle was injected once daily for 5 consecutive days, and posttreatment response to acetone was measured on different days (days 7, 10, 14, 17, 21 and 24) after the first paclitaxel or vehicle injection. The same procedure was used to test the effect of TTX on the development of cold allodynia, but in this case, TTX or its solvent was s.c. injected 30 min before each of the 5 i.p. paclitaxel injections.

To investigate the effect of TTX on the expression of paclitaxel-induced cold allodynia, the pretreatment response to acetone was tested and the treatment with paclitaxel was performed as described above (in the experiments to elucidate the time-course of cold-allodynia). We then tested the response of each animal to acetone on days 7 and 10 after the first dose of the antineoplastic. If the duration of acetone-induced licking or biting of the stimulated paws was less than 2 s each day, the animal was considered a nonresponder (33.48% of the animals tested), and was excluded from the study of the effect of TTX on the expression of cold allodynia. If the response to acetone on days 7 and 10 was ≥ 2 s each day, the animal was considered a responder and was tested again on day 14. On this day, once the baseline response to acetone was recorded before drug treatment (time 0), the animals received a single s.c. injection of TTX or its solvent, and the response to acetone was recorded again 60, 120 and 180 min after the injection.

1.3.4.3. Procedure to measure mechanical allodynia

To assess mechanical allodynia, mechanical thresholds of hind paw withdrawal were measured with a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) as previously described (Kondo et al., 2006), with slight modifications. The electronic Von Frey device uses a single nonflexible filament which applies increasing force (from 0 to 10 g) against the plantar surface of the hind paw over a 20-s period. The nocifensive paw withdrawal response automatically turns off the stimulus, and the mechanical pressure that evoked the response is recorded. On the day of the experiment, mice were placed individually in test compartments (9 × 9 × 14 cm) with a wire mesh bottom and allowed to habituate for 2 h. After habituation, each mouse was tested three times alternately in each hind paw, allowing at least 30 s between each measurement.

To elucidate the time-course of paclitaxel-induced mechanical allodynia, the response to the monofilament was tested in each animal 3 days before paclitaxel administration was started (pretreatment value). Treatment with paclitaxel (2 mg/kg, i.p.) or its vehicle was performed for 5 consecutive days, and posttreatment response to the monofilament was measured on days 7, 10, 14 and 17 after the first paclitaxel or vehicle injection. The same procedure was followed to test the effect of TTX on the development of paclitaxel-induced mechanical allodynia, except that TTX or its solvent was injected s.c. 30 min before each daily i.p. dose of paclitaxel or its vehicle during the 5 days of treatment.

We evaluated the effect of TTX on the expression of paclitaxel-induced mechanical allodynia on day 10 to coincide with the maximum reduction in the mechanical pressure threshold (see Results, section 1.4.1.). In these experiments, pretreatment response to the mechanical stimulus was measured and treatment with

paclitaxel was performed as described above (in the experiments to elucidate the time-course of mechanical allodynia). On day 10, after habituation to the observation boxes, baseline thresholds were recorded before drug treatment (time 0), then TTX or its solvent was injected s.c. and threshold pressures that evoked paw withdrawal were measured again 30, 60, 90, 120 and 180 min after the injection. If the mean of the threshold values recorded on day 10 at time 0 was not lower than the mean of the animal's pretreatment values (obtained 3 days before paclitaxel treatment was started), the animal was considered a nonresponder (4.65% of the animals), and was not used to test the effect of TTX on the expression of paclitaxel-induced mechanical allodynia.

1.3.4.4. Rotarod test

Changes in motor coordination were tested with an accelerating rotarod (Cibertec, Madrid, Spain) as previously described (Patel et al., 2001), with slight modifications. Briefly, mice were required to walk against the motion of an elevated rotating drum while the speed of rotation was increased from 4 to 40 revolutions/min over 5 min, and the latency to fall-down was recorded automatically. To accustom animals to the apparatus, 24 h before drug testing the animals were given 3 training sessions on the rotarod separated by 30-min intervals. On the day of the drug test rotarod latencies were measured immediately before the drug or saline was given (time 0) and several times (30, 60, 90, 120 and 180 min) after the s.c. injection. In all experiments we used a 300-s cut-off time.

1.3.5. Data analysis

The Kolmogorov-Smirnov test was used to verify normal distribution of the experimental data. When the data were not distributed normally, they were log-

transformed to obtain a normal distribution. The differences between values in all behavioral assays were analyzed with two-way repeated measures analysis of variance (ANOVA) followed by the Newman-Keuls test. All tests were performed with the SigmaStat 2.03 program (Systat Software Inc., San Jose, California, USA). The differences between means were considered statistically significant when the value of p was below 0.05.

1.4. RESULTS

1.4.1. Time-course of paclitaxel-induced heat hyperalgesia, cold allodynia and mechanical allodynia

The pretreatment values in the plantar (Fig. 1A), acetone (Fig. 1B) and Von Frey (Fig. 1C) tests were not significantly different in mice that were subsequently treated with paclitaxel or vehicle. Administration during 5 days of paclitaxel-vehicle did not significantly modify the posttreatment response in any test (Figs. 1A-C), whereas 5 days of treatment with paclitaxel (2 mg/kg, i.p.) significantly modified: a) paw withdrawal latency in the Hargreaves test [$F(1,38) = 18.20$, $p < 0.001$] (Fig. 1A), b) duration of paw licking/biting induced by acetone [$F(2,91) = 40.02$, $p < 0.001$] (Fig. 1B), and c) threshold force to evoke paw withdrawal in the electronic Von Frey test [$F(1,34) = 5.00$, $p = 0.029$] (Fig. 1C).

Paclitaxel treatment (2 mg/kg, i.p.) reduced paw withdrawal latencies to thermal stimulation of the hind-paws on all the posttreatment days compared to the pretreatment day value (Fig. 1A). Note that paclitaxel-induced heat hyperalgesia was maximal 7 days after the first injection of the antineoplastic (Fig. 1A), and this was thus the day chosen to evaluate the effect of TTX on the expression of heat hyperalgesia.

In the acetone test, the administration of paclitaxel (2 mg/kg, i.p.) once daily during 5 days enabled us to distinguish two groups of animals: responders and non-responders. Most animals (66.52%) treated with paclitaxel were considered responders according to the criteria explained in the Methods section (see 2.4.2). In these animals paclitaxel treatment significantly increased the time spent (Fig. 1B) and the frequency of (data not shown) licking or biting the stimulated paw on all posttreatment days when compared to the pretreatment day. On the other hand, 33.48% of the paclitaxel-treated animals did not show cold allodynia (nonresponders), and their response to acetone was indistinguishable from that of animals treated with paclitaxel-vehicle in both duration (Fig. 1B) and frequency (data not shown) of licking/biting. When we compared the values of these two variables among the different groups on the same day of evaluation, statistically significant differences were found between paclitaxel-responders and the other two groups (paclitaxel-nonresponders or paclitaxel-vehicle) in both duration (Fig. 1B) and frequency (data not shown) of licking/biting. Paclitaxel-induced cold allodynia was maximal 10-14 days after the first injection of the antineoplastic for both variables; we therefore evaluated the effect of TTX on the expression of cold allodynia on day 14.

Paclitaxel (2 mg/kg, i.p., during 5 days) induced mechanical allodynia in mice, significantly reducing the threshold force needed to evoke paw withdrawal in the Von Frey test on day 10 in comparison to both the pretreatment value and the value obtained the same day in paclitaxel-vehicle treated animals (Fig. 1C). We therefore tested the effect of TTX on the expression of mechanical allodynia on day 10.

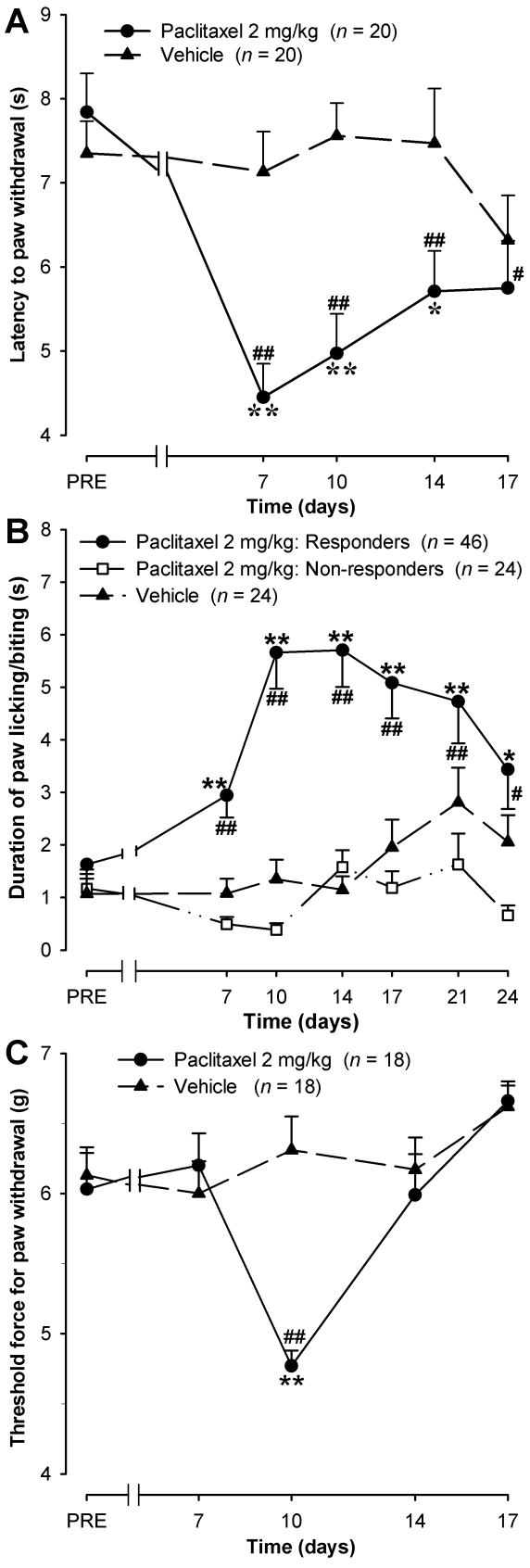

Fig. 1. Time-course of paclitaxel-induced heat hyperalgesia, cold allodynia and mechanical allodynia in mice. Animals were treated once daily from days 1 to 5 with paclitaxel (2 mg/kg) or its vehicle i.p. The latency to hind paw withdrawal in the plantar test (A), the duration of hind paw licking or biting in the acetone test (B), and the threshold force for hind paw withdrawal in the Von Frey test (C) were recorded 3 days before (PRE) and on several days after the first injection of paclitaxel or its vehicle. Each animal was tested in only one model of nociception. Each point and vertical line represent the mean ± S.E.M. of the values obtained in the number (n) of animals shown between brackets in the inset. Statistically significant differences between the paclitaxel- and vehicle-treated groups on the same day after treatment: * $p < 0.05$; ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: # $p < 0.05$; ## $p < 0.01$ (two-way repeated measures ANOVA followed by Newman-Keuls test). The original experimental data shown in A and B were transformed [ln(× + 1) function] to obtain a normal distribution before performing ANOVA analysis; data in C were distributed normally and therefore not transformed.

1.4.2. Effect of TTX on the expression of paclitaxel-induced heat hyperalgesia

Seven days after paclitaxel administration, before the injection of TTX or saline (time 0), paw withdrawal latencies in the plantar test were significantly lower than the pretreatment values in all groups of animals receiving antineoplastic treatment (Fig. 2A, time 0 versus PRE). In these animals, saline administration did not significantly modify paclitaxel-induced heat hyperalgesia, whereas the s.c. administration of TTX (3 or 6 μg/kg) inhibited this response [$F(2,53) = 7.580$, $p < 0.001$] (Fig. 2A). This effect of TTX was significantly different from that of saline from 30 to 120 min after injection of TTX 6 μg/kg, and from 60 to 120 min in the group of mice treated with TTX 3 μg/kg (Fig. 2A). Therefore, acute administration of TTX inhibited the expression of paclitaxel-induced heat hyperalgesia.

As expected, 5 days of treatment with paclitaxel-vehicle did not induce heat hyperalgesia (Fig. 2B, time 0 versus PRE). In these animals the s.c. administration of TTX (3 or 6 μg/kg) or saline did not significantly [$F(2,33) = 0.595$, $p = 0.557$] affect the latency of paw withdrawal responses to the thermal stimulus during the 3-h recording period (Fig. 2B). Hence TTX administration did not modify the normal response to a painful thermal stimulus in control animals.

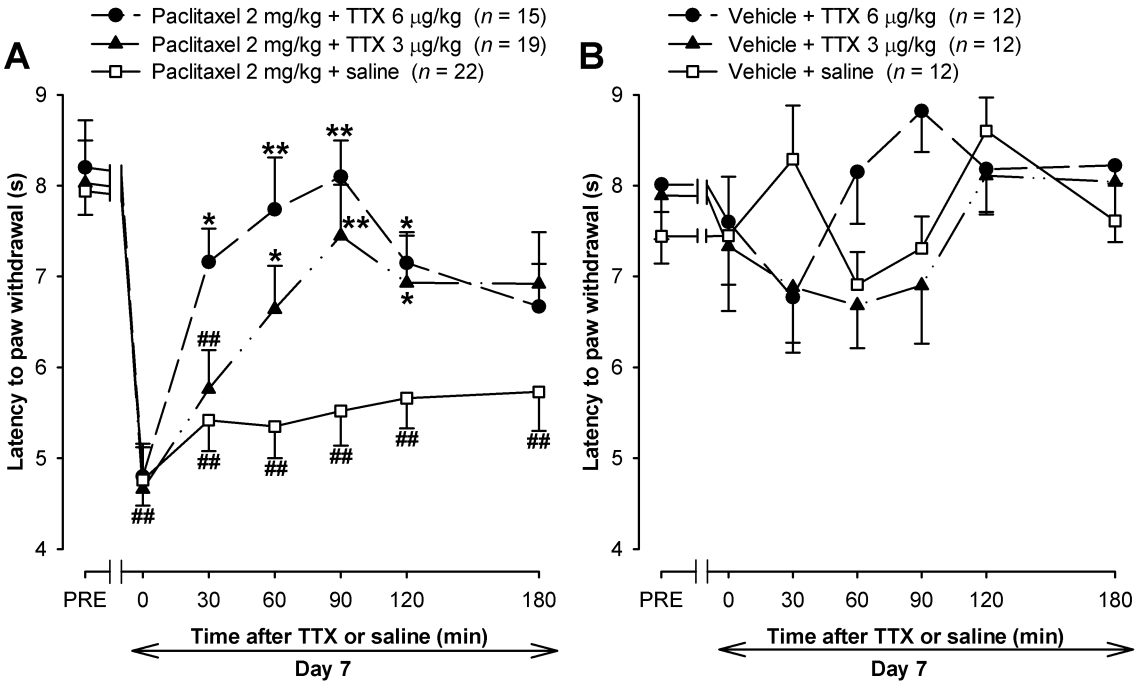

Fig. 2: Time-course of the effect on the latency to hind paw withdrawal (plantar test) of a single s.c. injection of tetrodotoxin (TTX; 3 or 6 μg/kg) or saline in mice pretreated with (A) paclitaxel or (B) paclitaxel-vehicle. Animals were treated once daily from days 1 to 5 with paclitaxel or its vehicle i.p., and on day 7 received a single s.c. injection of TTX or saline. The latency to hind paw withdrawal was recorded in each animal 3 days before (PRE) and 7 days after the first injection of paclitaxel or its vehicle. On that day, paw withdrawal latency was recorded immediately before (time 0) and at several times (30, 60, 90, 120 and 180 min) after the injection of TTX or saline. Each animal received either saline or one dose of TTX. Each point and vertical line represent the mean ± S.E.M. of the values obtained in the number (n) of animals shown between brackets in the inset. (A) Statistically significant differences among the values of TTX- and saline-treated groups on the same day after treatment: * $p < 0.05$; ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: # $p < 0.05$; ## $p < 0.01$ (two-way repeated measures ANOVA followed by Newman-Keuls test). The original experimental data were transformed [ln(× + 1) function] to obtain a normal distribution before performing ANOVA analysis. (B) No statistically significant differences among the three groups were observed at any observation time, or in comparison to their own pretreatment day values (two-way repeated measures ANOVA). Experimental data were distributed normally and therefore not transformed before performing ANOVA analysis.

1.4.3. Effect of TTX on the expression of paclitaxel-induced cold allodynia

In all groups of animals treated with paclitaxel the duration of acetone-induced paw licking/biting on day 14, before treatment with TTX or saline (time 0), was significantly different from that on the pretreatment day (Fig. 3A, time 0 versus PRE). In these animals, on day 14, a single s.c. injection of TTX (3 or 6 μg/kg; s.c.), but not of saline, reduced the duration of paw licking/biting induced by acetone [$F(2,39) = 3.186$, $p = 0.049$] (Fig. 3A), i.e. TTX inhibited the expression of paclitaxel-induced cold allodynia. This effect of TTX was significantly different from that of saline 1 h after the injection of TTX 3 or 6 μg/kg (Figs. 3A). Similar results were obtained when the frequency of paw licking/biting was evaluated, but in this case only the effect of TTX 6 μg/kg was significant (data not shown).

As expected, treatment with paclitaxel-vehicle during 5 days did not significantly change the duration (Fig. 3B; time 0 versus PRE) or frequency (data not shown) of paw licking/biting on day 14. In these mice the administration on day 14 of a single s.c. injection of saline or TTX (6 μg/kg) did not significantly change the duration [$F(1,47) = 1.959$, $p = 0.164$] (Fig. 3B) or frequency (data not shown) of paw licking/biting during the 3-h evaluation period. Therefore the administration of paclitaxel-vehicle did not induce cold allodynia in the acetone test on day 14, and a single injection of TTX did not affect the baseline response to acetone in this test.

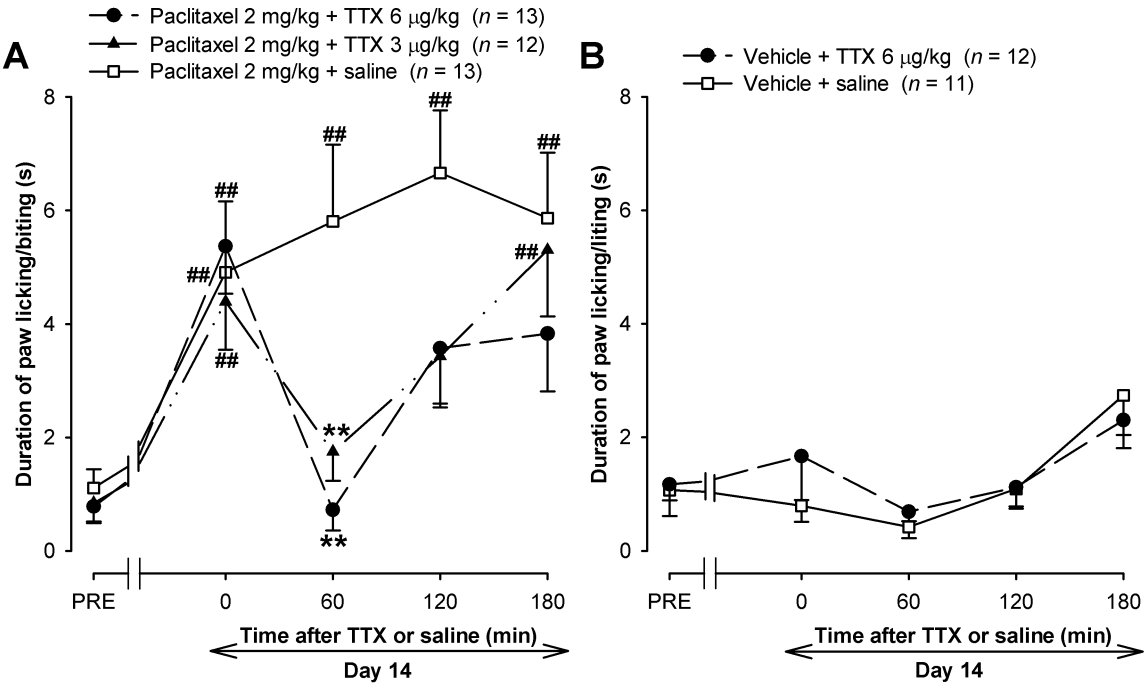

Fig. 3: Time-course of the effect on the duration of hind paw licking/biting (acetone test) of a single s.c. injection of tetrodotoxin (TTX; 3 or 6 μg/kg) or saline in mice pretreated with (A) paclitaxel or (B) paclitaxel-vehicle. Animals were treated once daily from days 1 to 5 with paclitaxel or its vehicle i.p., and on day 14 received a single s.c. injection of TTX or saline. The duration of hind paw licking/biting was recorded in each animal 3 days before (PRE) and 14 days after the first injection of paclitaxel or its vehicle. On that day, duration of hind paw licking/biting was recorded immediately before (time 0) and at several times (60, 120 and 180 min) after the injection of TTX or saline. Each animal received either saline or one dose of TTX. Each point and vertical line represent the mean ± S.E.M. of the values obtained in the number (n) of animals shown between brackets in the inset. (A) Statistically significant differences among the values of TTX- and saline-treated groups at the same time after treatment: ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Newman-Keuls test). Experimental data were distributed normally and therefore not transformed before performing ANOVA analysis. (B) No statistically significant differences between the two groups were observed at any observation time, or in comparison to their own pretreatment day values (two-way repeated measures ANOVA). Experimental data were distributed normally and therefore not transformed before performing ANOVA analysis.

1.4.4. Effect of TTX on the expression of paclitaxel-induced mechanical allodynia

On day 10, before the administration of TTX or saline (time 0), mechanical allodynia in the paclitaxel-treated mice was manifested as a significant reduction of the threshold force needed to evoke paw withdrawal (Fig. 4A; time 0 versus PRE). In these mice, saline (TTX-solvent) administration did not significantly modify paclitaxel-induced mechanical allodynia, but acute administration of TTX (1 and 3 µg/kg) inhibited mechanical allodynia induced by the antineoplastic [$F(2,37) = 17.118$, $p < 0.001$] (Fig. 4A). The effect of TTX was statistically significant from 30 to 90 min after administration in both groups of TTX-treated animals (1 and 3 µg/kg) compared to saline-treated animals (Fig. 4A).

As expected, 5 days of treatment with paclitaxel-vehicle did not significantly change the value of threshold force for hind paw withdrawal on day 10 (Fig. 4B, time 0 versus PRE), and the subcutaneous administration of TTX (3 or 6 µg/kg, s.c.) or saline did not significantly modify the threshold force to evoke paw withdrawal [$F(2,19) = 1.192$, $p = 0.325$] (Fig. 4B). Therefore TTX administration did not alter the response to a mechanical stimulus in control animals.

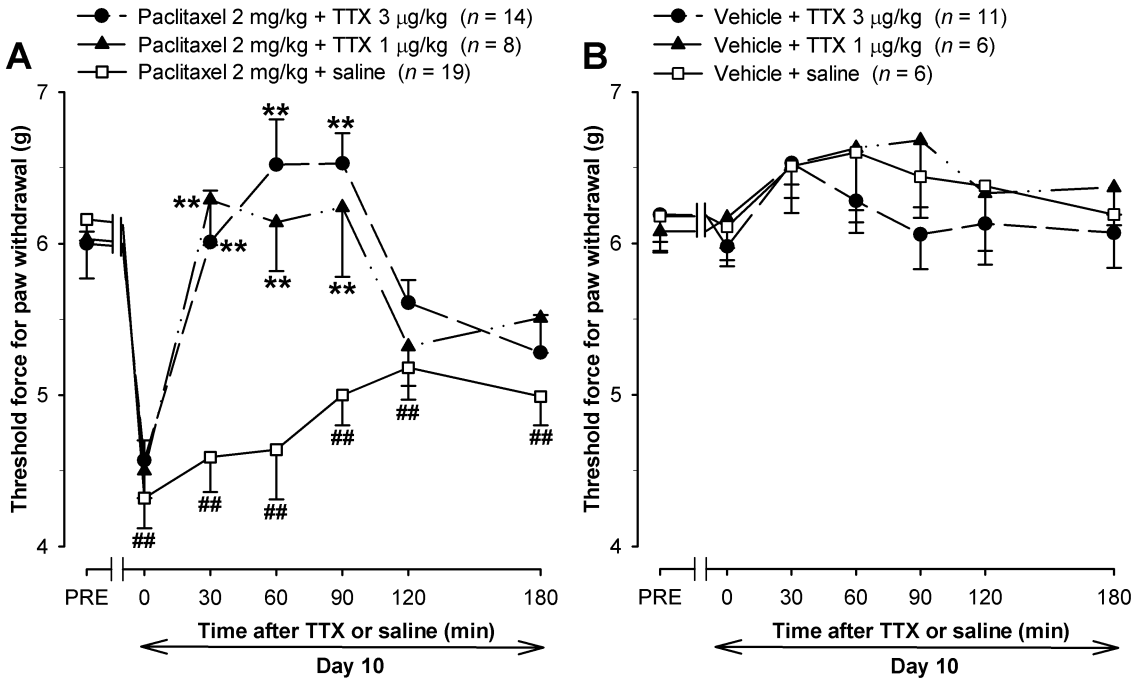

Fig. 4: Time-course of the effect on the threshold force for hind paw withdrawal (Von Frey test) of a single s.c. injection of tetrodotoxin (TTX; 1 or 3 μg/kg) or saline in mice pretreated with (A) paclitaxel or (B) paclitaxel-vehicle. Animals were treated once daily from days 1 to 5 with paclitaxel or its vehicle i.p., and on day 10 received a single s.c. injection of TTX or saline. The threshold force for hind paw withdrawal was recorded in each animal 3 days before (PRE) and 10 days after the first injection of paclitaxel or its vehicle. On that day, paw withdrawal latency was recorded immediately before (time 0) and at several times (30, 60, 90, 120 and 180 min) after the injection of TTX or saline. Each animal received either saline or one dose of TTX. Each point and vertical line represent the mean ± S.E.M. of the values obtained in the number (n) of animals shown between brackets in the inset. (A) Statistically significant differences among the TTX- and saline-treated groups at the same time after treatment: ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Newman-Keuls test). Experimental data were distributed normally and therefore not transformed before performing ANOVA analysis. (B) No statistically significant differences among the the three groups were observed at any observation time, or in comparison to their own pretreatment day values (two-way repeated measures ANOVA). Experimental data were distributed normally and therefore not transformed before performing ANOVA analysis.

1.4.5. Effect of TTX on the development of paclitaxel-induced heat hyperalgesia, cold allodynia and mechanical allodynia

The pretreatment values were similar in the two experimental groups (paclitaxel + saline and paclitaxel + TTX) in all the tests (plantar, acetone and Von Frey tests) (Figs. 5A, 5B and 5C).

Paw withdrawal latency values to thermal stimulation of the hind-paws were significantly lower than the pretreatment values in both experimental groups at all times from 7 to 17 days after the first injection of paclitaxel [$F(1,4) = 18.269$, $p < 0.001$] (Fig. 5A). However, we found no significant differences [$F(1,39) = 0.014$, $p = 0.905$] in paw withdrawal latencies (throughout the 17-day observation period) when we compared the effect of coadministration of paclitaxel + TTX 6 µg/kg with that of paclitaxel + saline (Fig. 5A). We therefore concluded that coadministration of TTX with paclitaxel did not affect the development of heat hyperalgesia induced by paclitaxel.

In the acetone test, the duration of paw licking/biting in the group of animals in which paclitaxel (i.p.) and saline (s.c.) were coadministered differed significantly from the duration in animals that received paclitaxel + TTX [$F(1,36) = 6.732$, $p = 0.013$] (Fig. 5B). The increases in the duration (Fig. 5B) and frequency (data not shown) of paw licking/biting were maximal on days 10-14 after the first injection of paclitaxel. However, in animals which received a s.c. injection of TTX (6 µg/kg) 30 min before each dose of i.p. paclitaxel, the duration (Fig. 5B) and frequency (data not shown) of paw licking/biting throughout the 24-day observation period after coadministration were not significantly different from those on the pretreatment day. In addition, statistically significant differences between the values in both groups (paclitaxel + saline and paclitaxel + TTX 6 µg/kg) were observed on days 10 and 14 for duration of

licking/biting (Fig. 5B), and on days 7, 10 and 14 for frequency of licking/biting (data not shown). Therefore coadministration of TTX 6 µg/kg and paclitaxel inhibited the development of cold allodynia induced by paclitaxel. We also evaluated the effect of coadministration of paclitaxel with a lower dose of TTX (3 µg/kg), but this dose of TTX did not modify the development of paclitaxel-induced cold allodynia (data not shown).

The threshold force values that evoked paw withdrawal (Von Frey test) in paclitaxel + saline-treated animals were significantly lower than those in animals in which TTX (3 or 6 µg/kg) was injected 30 min before each paclitaxel dose [$F(2,38) = 5.825$, $p = 0.006$] (Fig. 5C). A reduction in mechanical threshold values on days 10 and 14 was observed when paclitaxel and saline were coadministered; however, there were no significant differences between the pretreatment and posttreatment values on any day in animals treated with paclitaxel + TTX 3 or 6 µg/kg (Fig. 5C). We observed statistically significant differences in the threshold force values of paw withdrawal after the coadministration of paclitaxel with TTX (3 or 6 µg/kg) and paclitaxel with saline (Fig. 5C) on days 10 and 14. Therefore coadministration of TTX and paclitaxel inhibited the development of mechanical allodynia induced by paclitaxel in mice.

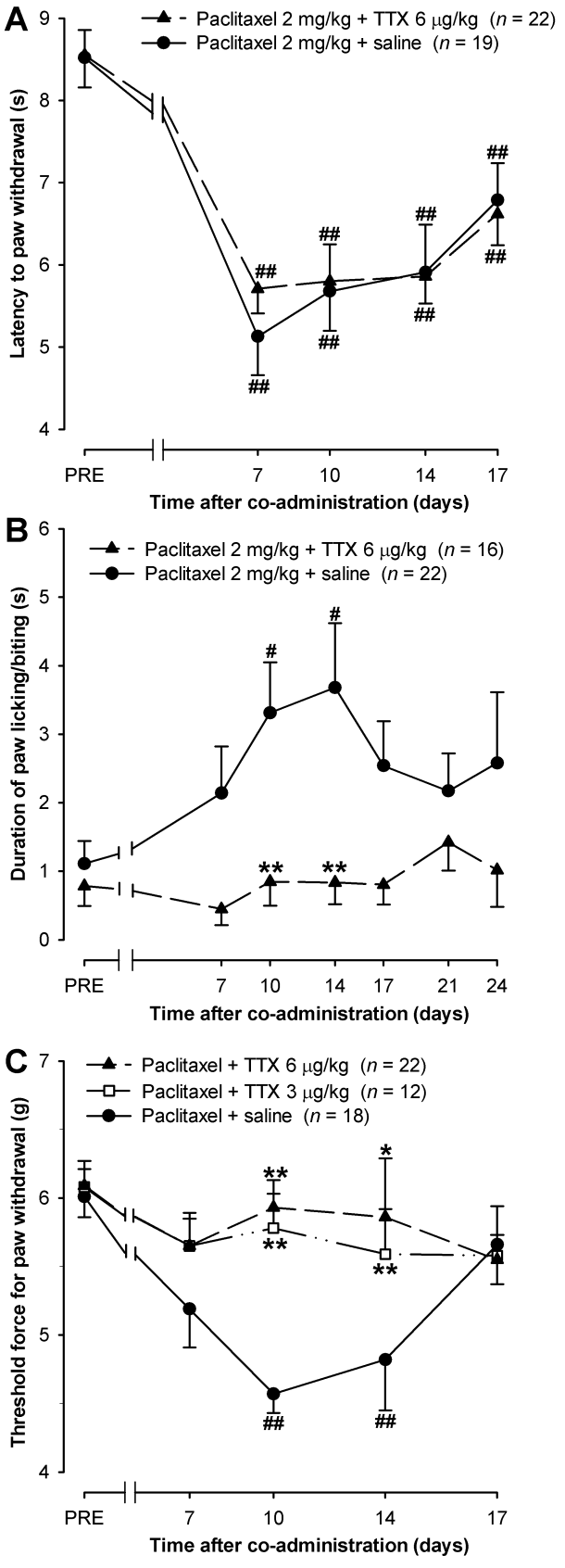

Fig. 5: Time-course of the effect of coadministration of paclitaxel + TTX (3 or 6 μg/kg) and paclitaxel + saline on latency to hind paw withdrawal in the plantar test (A), duration of hind paw licking/biting in the acetone test (B), and threshold force for hind paw withdrawal in the Von Frey test (C). Mice were treated once daily from days 1 to 5 with an s.c. injection of TTX (3 or 6 μg/kg) or saline 30 min before each i.p. injection of paclitaxel (2 mg/kg). The response was recorded in each animal 3 days before (PRE) and on different days after the first injection of paclitaxel + TTX or paclitaxel + saline. Each animal was tested in only one model of nociception. Each point and vertical line represent the mean ± S.E.M. of the values obtained in the number (n) of animals shown between brackets in the inset. No statistically significant differences between the two treatment groups were found in the plantar test (two-way repeated measures ANOVA). Statistically significant differences in comparison to paclitaxel + saline: * $p < 0.05$, ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: # $p < 0.05$, ## $p < 0.01$ (two-way repeated measures ANOVA followed by Newman-Keuls test). The original experimental data shown in A and B were transformed [ln(x + 1) function] to obtain a normal distribution before performing ANOVA analysis. Data in C were distributed normally and therefore not transformed before performing ANOVA analysis.

1.4.6. Effect of TTX on rotarod test

Animals treated with TTX (at the same doses used in nociception experiments) were tested with the accelerating rotarod test to rule out the involvement of a hypothetical locomotor disturbing effect of the drug on the results of the pain experiments. As comparison drugs we used baclofen and gabapentin at doses previously reported to impair rotarod performance (Patel et al., 2001; Hong-Jue et al., 2004).

The latency to fall-down from the rotarod before the injection of drugs or saline (time 0) was similar in all experimental groups, but significant differences between groups were found after drug administration [$F(4,45) = 4.248$, $p = 0.005$] (Fig. 6). During the 3-h evaluation period after the s.c. administration of saline or TTX (3 or 6 μg/kg), the rotarod latency values were nonsignificantly different from the values at time 0 (Fig. 6). Moreover, there were no differences in rotarod latencies between saline- and TTX-treated animals at any time after administration (Fig. 6). In contrast, animals treated with gabapentin (60 mg/kg, s.c.) or baclofen (8 mg/kg, s.c.) showed significantly reduced latencies in the rotarod test in comparison to both their own values at time 0 and the values obtained in saline-treated animals at different intervals after administration (Fig. 6).

1.5. DISCUSION

We found that acute systemic administration of TTX inhibited the expression of paclitaxel-induced heat hyperalgesia, mechanical and cold allodynia in mice, and that the repeated coadministration of TTX and paclitaxel prevented the development of both kinds of allodynia without affecting heat hyperalgesia. These findings suggest that TTX-sensitive sodium channels play an important role in generating and maintaining

neuropathic pain induced by paclitaxel. The effects were observed after the administration of low doses of TTX that did not affect the response to mechanical and thermal stimuli in control animals. The differential effects of TTX in neuropathic and control animals may be related to the finding that TTX inhibits abnormal low-threshold and ectopic discharges coming from injured nerves at doses much lower than those needed to interfere with the generation and conduction of action potentials in normal (noninjured) nerves (Omana-Zapata et al., 1997; Lyu et al., 2000; Liu et al., 2001).

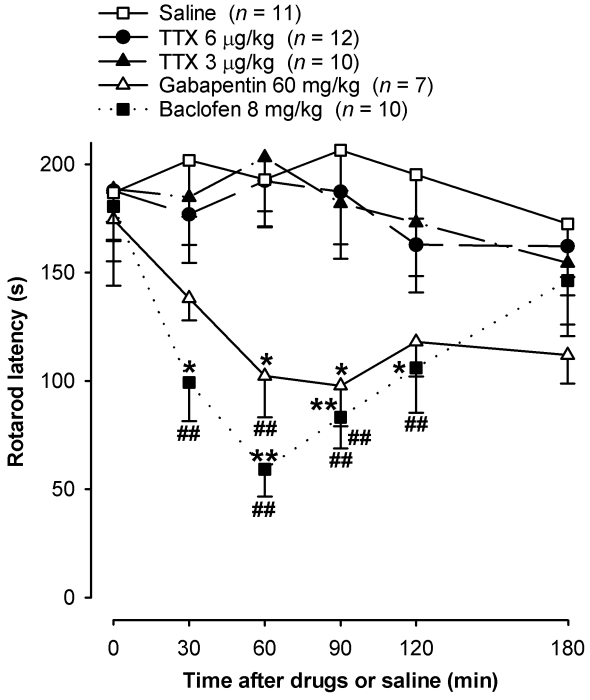

Fig. 6: Time-course of the effects on the rotarod test of saline, TTX and two comparison drugs (gabapentin and baclofen). Twenty-four hours before drug testing the animals were trained on the accelerating rotarod in three sessions, as described in Methods, to accustom them to the apparatus. On the day of the experiment the latency to fall-down from the rotarod was recorded in each mouse immediately before (time 0) and at several times (30, 60, 90, 120 and 180 min) after the s.c. injection of the drug. Each animal was treated with a single s.c. injection of TTX (3 or 6 μg/kg), baclofen (8 mg/kg), gabapentin (60 mg/kg) or saline. Each point and vertical line represent the mean ± S.E.M. of the values obtained in the number (n) of animals indicated between brackets in the inset. Statistically significant differences among the drug-treated and saline-treated groups at the same time after treatment: * $p < 0.05$; ** $p < 0.01$; and between the values at time 0 and at several times after the s.c. injection: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Newman-Keuls test; experimental data were not transformed before performing ANOVA analysis).

Although TTX is a potent neurotoxin, at the doses used in the present study we found no signs of toxicity or motor incoordination in the rotarod test. Interestingly, the two drugs used as positive controls (gabapentin and baclofen) altered the response in the rotarod test, as previously described (Patel et al., 2001; Hong-Jue et al., 2004), which indicates that the lack of effect of TTX in our study is not a false-negative result. A previous study that also used low doses of TTX (0.3-6 µg/kg, s.c.) likewise found no signs of toxicity (Marcil et al., 2006). Higher doses (>10 µg/kg) injected i.v. are necessary to affect cardiovascular parameters, whereas doses as high as 100-300 µg/kg i.v. produce cardiac arrest (Omana-Zapata et al., 1997). These data indicate that in rodents, TTX has a therapeutic window that allows the drug to be used to reduce pain. In humans, TTX injected intramuscularly reduced pain intensity (in an open-label, dose-escalation study) in patients with severe cancer pain refractory to standard treatments. The most frequent adverse effects were perioral hyperesthesia and paresthesia, although two patients experienced clinically significant ataxia (Hagen et al., 2007). New placebo-controlled studies with larger number of patients are necessary to better characterize the analgesic efficacy and toxicity of TTX in humans. Recently, another natural toxin, ziconotide (SNX-111), was marketed for the treatment of refractory chronic pain. This drug is efficacious but produces numerous adverse effects (Wallace, 2006).

Although the effects of TTX on chemotherapy-induced neuropathic pain were previously unknown, TTX has been tested before in mechanically-induced nerve injury models. These earlier findings reported that TTX inhibited the generation of ectopic discharges and the expression of mechanical allodynia produced by nerve transection or ligation (Omana-Zapata et al., 1997; Lyu et al., 2000; Liu et al., 2001; Marcil et al., 2006). Moreover, continuous local infusion (during 3-7 days) of TTX directly on the

injured nerve, at the beginning of nerve injury, prevented the appearance of spontaneous nerve activity and mechanical allodynia in two different models of neuropathic pain induced by nerve ligation (Xie et al., 2005). Taken together, these studies and the present results show that TTX inhibits the development and expression of neuropathic pain produced by mechanically- and chemically-induced nerve injury.

We found that the expression of mechanical allodynia was inhibited by lower doses of TTX than heat hyperalgesia. These results agree with those obtained in a model of nerve ligation by Marcil et al. (2006). Tetrodotoxin also inhibited the expression of paclitaxel-induced cold allodynia; this effect of TTX had not been previously described in any model of neuropathic pain. We also found differential effects of TTX on the development of different signs of paclitaxel-induced neuropathic pain: mechanical allodynia was prevented by coadministering paclitaxel and both doses of TTX (3 and 6 μg/kg), whereas only the higher dose of TTX significantly prevented cold allodynia, and heat hyperalgesia was not prevented by TTX even at the highest dose tested (6 μg/kg). The differential effects of TTX against heat hyperalgesia and mechanical or cold allodynia may be related with the differential involvement of myelinated and unmyelinated peripheral nerve fibers in the pathogenesis of each sign of pain. Mechanical allodynia is evoked by inputs from myelinated fibers (mainly Aβ-fibers, but also Aδ-fibers), whereas heat hyperalgesia is evoked mainly by unmyelinated C-fibers (Ossipov et al., 1999; Khan et al., 2002), and both Aδ- and C-fibers are recruited to mediate cold allodynia (Hao et al., 1996; Kim et al., 1999). This apparent functional preference of TTX for inhibiting A-fiber-mediated pain signs is consistent with electrophysiological studies reporting that low doses of TTX applied to the nerve preferentially blocked A-fiber (Aβ and Aδ) conduction without significantly affecting

C-fibers (Clarke et al., 2003). Interestingly, large- and medium-diameter DRG neurons (with Aβ and Aδ fibers) showed greater upregulation of TTX-sensitive sodium channels and sodium currents than small-diameter DRG neurons (with C-fibers) in several models of neuropathic pain (Everill et al., 2001; Kim et al., 2001; Craner et al., 2002; Hong and Wiley, 2006). Moreover, paclitaxel treatment induced hyperresponsiveness of Aβ- and Aδ-fibers, but not of C-fibers, to electrical stimulation (Matsumoto et al., 2006). It seems therefore that the greater efficacy of TTX against mechanical and cold allodynia appears to indicate a greater contribution of TTX-sensitive channels to the pathogenesis of these signs of neuropathic pain.

We can not determine with certainty whether the effect of TTX in our study was exerted peripherally or centrally, since we injected TTX subcutaneously. However, the ectopic discharges that occur after sciatic nerve transection were inhibited in dorsal horn neurons with higher doses of systemically (i.v.) injected TTX (ED50=36.2 μg/kg) than those necessary to inhibit ectopic discharges in neuroma and DRG neurons (ED50=0.8 and 4.3 μg/kg, respectively) (Omana-Zapata et al., 1997). In addition, when a very low dose of TTX (5 μl of 25 nM) was injected systemically (i.p.), epidurally, or applied directly on the DRG, the order of magnitude of antiallodynic efficacy was DRG > epidural > i.p. (Lyu et al., 2000). These results are not surprising since TTX is a hydrophilic charged molecule, so access to the spinal cord and brain through the blood-brain barrier is expected to be low. Accordingly, the contribution of central effects of TTX on the inhibition of neuropathic pain in the present study would be expected to be lower than the contribution of peripheral effects. Interestingly, the topical administration of TTX near the injured nerve or DRG neurons inhibited ectopic discharges and mechanical allodynia induced by ligation of the peripheral nerves (Lyu et al., 2000; Xie

et al., 2005). Therefore the antiallodynic and antihyperalgesic efficacy of TTX in the present study might be due to effects of TTX limited to peripheral nerves or DRG neurons injured by paclitaxel. The peripherally-mediated effects of TTX might be sufficient to inhibit neuropathic pain-related altered central processing, since this processing is initiated, and at least partially maintained, by peripheral inputs (Woolf and Salter, 2000; Xie et al., 2005).

The mechanism of action of TTX involves the selective blockade of some sodium channels acting on specific alpha-subunit isoforms. Among the isoforms that are sensitive to TTX, numerous studies have reported that $Na_v1.3$ and $Na_v1.7$ are upregulated in DRG neurons (and also in central second- and third-order nociceptive neurons) in several models of neuropathic pain (see the Introduction for references). Recently, an increase in the immunoreactivity of $Na_v1.3$ alpha-subunit in DRG neurons was also reported in mice with vincristine-induced neuropathic pain (Uceyler et al., 2006). If similar changes also occur in paclitaxel-induced neuropathic pain (which is unknown at present), the protective effect of TTX against this neuropathic pain may be explained by the blockade of $Na_v1.3$ and/or $Na_v1.7$ channels.

In fact, treatment with an antisense oligodeoxynucleotide against $Na_v1.3$ or with glial cell line-derived neurotrophic factor (GDNF) reduced the upregulation of $Na_v1.3$ channels, the hyper-responsiveness of dorsal horn neurons to mechanical stimuli, and the mechanical allodynia and heat hyperalgesia induced by several types of painful neuropathies (Boucher et al., 2000; Hains et al., 2003, 2004). However, the specificity of the oligonucleotides used in these studies was not absolute, and another study using a different $Na_v1.3$-specific antisense oligodeoxynucleotide failed to confirm the inhibition of mechanical allodynia (Lindia et al., 2005). Moreover, the ectopic discharges and

mechanical allodynia induced by spinal nerve ligation were fully expressed in $Na_v1.3$ knockout mice (Nasssar et al., 2006), as well as in $Na_v1.7$ nociceptor-specific knockout and double $Na_v1.7/Na_v1.8$ knockout mice (Nassar et al., 2005). Taken together, these results suggest that changes affecting the activity and expression of multiple sodium channel subunits, rather than a change in any particular channel, most likely contributed to the pathogenesis of neuropathic pain. This could explain why TTX was able to inhibit paclitaxel-induced neuropathic pain in our study, whereas knockdown or knockout of only one subtype of TTX-sensitive channel was less efficacious in other models of neuropathic pain.

In conclusion, we found that the systemic administration of low, nontoxic doses of TTX inhibited hyper-responsiveness to thermal and mechanical stimuli in paclitaxel-treated mice. These findings suggest that TTX-sensitive sodium channels play a role in the pathogenesis of chemotherapy-induced neuropathic pain.

1.6. REFERENCES

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

2. Role of sigma-1 receptors in paclitaxel-induced neuropathic pain

Francisco Rafael Nieto, Cruz Miguel Cendán, Cristina Sánchez-Fernández, Enrique José Cobos, José Manuel Entrena, Daniel Zamanillo, José Miguel Vela, José Manuel Baeyens

(SUBMITTED)

Note: the results shown in this paper have given rise to several international patents (see below). Consequently, the final publication of this article has been delayed due to the patenting process.

International patents:

-Baeyens Cabrera JM, Buschmann H, Vela Hernández JM, Zamanillo Castañedo D, Nieto López FR. Sigma ligands for the prevention or treatment of pain induced by chemotherapy. Patent numbers: WO 2011/018487; WO 2011/018488; EP 2292236; EP 2292237.

-Baeyens Cabrera JM, Buschmann H, Vela Hernández JM, Zamanillo Castañedo D, Nieto López FR. Use of compounds binding to the sigma receptor ligands for the treatment of neuropathic pain developing as a consequence of chemotherapy. Patent numbers: WO 2009/103487; US 2011/0052723; EP 2090311

2.1. ABSTRACT

Sigma-1 (σ_1) receptors play a role in different types of pain and in central sensitization mechanisms; however, it is unknown whether σ_1 receptors are involved in the neuropathic pain induced by antineoplastic drugs. We compared the ability of paclitaxel to induce cold (acetone test) and mechanical (electronic Von Frey test) allodynia in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. We also tested the effect on paclitaxel-induced painful neuropathy of BD-1063 (16-64 mg/kg, s.c.) and S1RA (32-128 mg/kg, s.c.), two selective σ_1 receptor antagonists that show high affinity for the σ_1 receptor labeled with [^{3}H](+)-pentazocine, and interact with it competitively. The responses to cold and mechanical stimuli were similar in WT and σ_1-KO mice not treated with paclitaxel; however, treatment with paclitaxel (2 mg/kg, i.p., once per day during 5 consecutive days) produced cold and mechanical allodynia in WT but not in σ_1-KO mice. The subcutaneous administration of BD-1063 or S1RA 30 min before each paclitaxel dose prevented the development of cold and mechanical allodynia in WT mice. Moreover, the acute administration of both σ_1 receptor antagonists dose-dependently inhibited the expression of both types of paclitaxel-induced allodynia after they had fully developed. These results suggest that σ_1 receptors play a key role in paclitaxel-induced painful neuropathy and that antagonists of these receptors may have therapeutic value for the treatment and/or prevention of this neuropathy. This latter possibility is especially interesting in the context of chemotherapy-induced neuropathy, where the onset of nerve damage is predictable and preventive treatment could be administered.

2.2. INTRODUCTION

Antineoplastic drugs frequently induce clinically significant and dose-limiting peripheral neurotoxicity (Sul and Deangelis, 2006; Park et al., 2008; Paice, 2010). Specifically, paclitaxel, a first-line chemotherapeutic agent for the treatment of several types of cancer (Woodward and Twelves, 2010), frequently produces painful peripheral neuropathies with numbness, tingling, burning pain and cold allodynia in a stocking-glove distribution (Dougherty et al., 2004; Jung et al., 2005; Argyriou et al., 2008). There are no well-established treatments to ameliorate this neuropathy (Argyriou et al., 2008), but models of paclitaxel-induced neuropathic pain in rodents make it possible to test new treatments for this (Dina et al., 2001; Smith et al., 2004; Nieto et al., 2008; Xiao et al., 2009).

Two subtypes of sigma receptors have been characterized, although only the sigma-1 (σ_1) receptor has been cloned (Cobos et al., 2008; Maurice and Su, 2009 for reviews). The σ_1 receptors are highly expressed in the central nervous system, including important areas for pain control (Alonso et al., 2000; Kitaichi et al., 2000). Evidence that σ_1 receptors are involved in pain modulation has accumulated steadily, and their role as modulators of opioid analgesia has been extensively documented (see Cobos et al., 2008; Maurice and Su, 2009 for references). However, σ_1 receptors are also involved in pain control *per se* (i. e. without modulating the effect of opioids), especially when central sensitization occurs (Drews and Zimmer, 2009). In fact, both formalin-induced pain and capsaicin-induced mechanical hypersensitivity, models of pain which involve central sensitization (South et al, 2003; Woolf, 2010), are reduced in σ_1 receptor knockout

(σ_1-KO) mice (Cendán et al., 2005b; Entrena et al., 2009b) and in wild-type (WT) mice treated with σ_1 receptor antagonists (Cendán et al., 2005a; Kim et al., 2006; Entrena et al., 2009a). In addition, the allodynia induced by sciatic nerve injury is attenuated in σ_1-KO mice (De la Puente et al., 2009) and in rats treated with a σ_1 receptor antagonist (Roh et al., 2008). These behavioral changes are accompanied by a reduction in the phosphorylation of both NMDA receptor subunit 1 (pNR1) (Roh et al., 2008) and extracellular signal-regulated kinase (pERK) (De la Puente et al., 2009) in the ipsilateral spinal cord dorsal horn, and both of these events are important in central sensitization (Latremoliere and Woolf, 2009).

Consequently, σ_1 receptors appear to play a key role in central sensitization and in models of mechanically induced neuropathic pain. However, it is not known whether σ_1 receptors are also involved in antineoplastic drug-induced neuropathy and other types of highly prevalent painful neuropathies such as those induced by diabetes or herpes virus infection. Determining their possible role in these types of neuropathies is of interest since the pathophysiological mechanisms and response to drugs differ in part depending on the origin of nerve injury (Aley and Levine, 2002; Baron et al., 2010; Bennett, 2010; Dworkin et al., 2010; Zheng et al., 2011). Therefore, we evaluated the preventive effect of the repeated systemic (s.c.) administration of σ_1 receptor antagonists on the development of paclitaxel-induced cold and mechanical allodynia in WT mice. We also tested whether the acute administration of σ_1 receptor antagonists inhibited the expression of both types of paclitaxel-induced allodynia after they had fully developed. Finally, to use a nonpharmacological experimental approach, we compared the ability of paclitaxel to induce neuropathic pain in σ_1-KO and WT mice.

2.3. METHODS

2.3.1. Animals

Experiments were performed in female wild-type (Charles River, Barcelona, Spain) and σ_1-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25-30 g. The σ_1-KO mice were generated on a CD-1 background as described previously (Entrena et al., 2009a). The animals were acclimated in our animal facilities for at least 1 week before testing, and were housed in colony cages. They were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water *ad libitum* until the beginning of the experiments, and maintained in temperature- and light-controlled rooms (22 ± 1 ºC, lights on at 08.00 h and off at 20.00 h, air replacement every 20 min). Testing took place during the light phase (from 9.00 h to 15.00 h) at random times throughout the estrous cycle. Mice were always handled in accordance with the European guidelines regarding protection of animals used for experimental and other scientific purposes (Council Directive of 24 November 1986, 86/609/ECC). The experimental protocol was approved by the University of Granada Research Ethics Committee.

2.3.2. Drugs and drug administration

The drug used to induce neuropathic pain was paclitaxel (Tocris Cookson Ltd., Bristol, UK). It was dissolved in a solution made up of 50% cremophor EL (Fluka Chemie, Buchs, Germany) and 50% absolute ethanol (Panreac Química S.A., Barcelona, Spain) to obtain a concentration of 6 mg/ml. This paclitaxel solution was stored at –20 ºC during a maximum of 14 days and was diluted in sterile physiological

saline (NaCl 0.9%) to a final concentration of 2 mg/10 ml just before administration. The vehicle for paclitaxel was diluted at the time of injection with saline at the same proportion as the paclitaxel solution. Paclitaxel (2 mg/kg) was administered intraperitoneally (i.p.) in a volume of 10 ml/kg once per day for 5 consecutive days (cumulative dose 10 mg/kg). We previously reported that this schedule of paclitaxel treatment produces a painful neuropathy in mice (Nieto et al., 2008). In the control group the vehicle for paclitaxel was administered according to the same schedule.

For behavioral studies we used the well-known σ_1 receptor antagonist BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) (Tocris Cookson) and the newly synthesized σ_1 antagonist S1RA (E-52862.HCl; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine, provided by Laboratorios Esteve) (Cobos et al., 2008; Romero et al., 2011). Both drugs were dissolved to their final concentrations in sterile physiological saline just before administration, and were administered s.c. in the interscapular area in an injection volume of 5 ml/kg. The control animals received the same volume of the drug solvent (saline) s.c..

For σ_1 binding assays, the radioligand used was [^{3}H](+)-pentazocine, with a specific activity of 33.6 Ci/mmol (PerkinElmer Life Sciences, Boston, MA, USA). Dilutions from the stock [^{3}H](+)-pentazocine solution were prepared with ice-cold incubation buffer (50 mM HCl-Tris buffer pH 8 at 30 ºC). The cold drugs used were: haloperidol (Sigma-Aldrich Química S.A., Madrid, Spain), BD-1063 and S1RA. Haloperidol was dissolved in absolute ethanol to make up a stock solution of 1 mM, from which further dilutions were prepared with incubation buffer to yield a final 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 (Cobos et al., 2005 and 2007). BD-1063 and S1RA were dissolved at a concentration of 1 mM in ultrapure water, and further diluted in incubation buffer at the concentrations required for the experiments.

2.3.3. Brain membrane preparations and [^{3}H](+) pentazocine binding assays

Crude synaptosomal membranes (P_2 fraction) were prepared strictly according to a previously described method (Entrena et al., 2009 a and b). Each pellet was resuspended in 1 ml 10 mM Tris-HCl, pH 7.4, and frozen in aliquots (protein concentration 10.5-12 mg/ml) at –80 °C. The binding characteristics were stable for at least 1 month when the tissues were stored at –80 °C (Cobos et al., 2006).

Aliquots of mouse brain membranes were slowly thawed and resuspended in fresh incubation buffer, and [^{3}H](+)-pentazocine binding assays were done as described previously (Entrena et al., 2009 a and b). Resuspended membrane preparations at a final protein concentration of 0.79-1.10 mg/ml were incubated at 30 °C, pH 8, for 240 min, with [^{3}H](+)-pentazocine and with the cold ligand of interest or its solvent. To measure the affinity of BD-1063 and S1RA for σ_1 receptor, we performed radioligand competition binding assays. In these experiments several concentrations (from 10^{-10} to 10^{-5} M) of the cold ligands or their solvent were tested against [^{3}H](+)-pentazocine (5 nM) binding. To determine whether BD-1063 and S1RA has competitive or noncompetitive interactions at the σ_1 receptor we used a previously described procedure (Bowen et al., 1989; Brammer et al., 2006) with slight modifications. Briefly, saturation binding studies for [^{3}H](+)-pentazocine (0.06-33 nM) were conducted in the absence or presence of fixed concentrations of the unlabeled ligands, which were 12.5 nM for BD-1063 and 75 nM for S1RA. These concentrations were selected to obtain approximately

the same displacement of [^{3}H](+)-pentazocine (taking into account the results of the competition experiments described above). Nonspecific binding was determined in the presence of 10 µM haloperidol.

To stop [^{3}H](+)-pentazocine binding to the mouse brain membranes, 5 ml ice-cold filtration buffer (Tris 10 mM pH 7.4) was added to the tubes. The bound and free radioligand were separated by rapid filtration under a vacuum using a Brandel cell harvester (Model M-12 T, Brandel Instruments, SEMAT Technical Ltd., St. Albans, Hertfordshire, UK) over Whatman GF/B glass fiber filters (SEMAT Technical Ltd.), presoaked with 0.5% polyethylenimine in filtration buffer for at least 1 h prior to use, to reduce nonspecific binding. The filters were washed under a vacuum twice with 5 ml-volumes of the ice-cold filtration buffer and transferred to scintillation counting vials. After the addition of 4 ml liquid scintillation cocktail (CytoScint Scintillation Counting Solution, MP Biomedicals, Irvine, CA), the samples were allowed to equilibrate overnight. The radioactivity retained in the filter was measured with a liquid scintillation spectrometer (Beckman Coulter España S.A., Spain), with an efficiency of 52%. Each assay was done in triplicate.

2.3.4. General procedures for drug treatments and behavioral assays in pain models

The general procedures were performed as described previously (Nieto et al., 2008), with slight modifications. To elucidate the time-course of paclitaxel-induced cold or mechanical allodynia in WT and σ_1-KO mice, the behavioral responses were tested in each animal 3 days before paclitaxel administration was started (pretreatment value). Then paclitaxel or its vehicle was injected once daily for 5 consecutive days, and

posttreatment responses were measured on different days (7, 10, 14, 17, 21 and 24 for cold allodynia; 7, 10, 14 and 17 for mechanical allodynia) after the first paclitaxel or vehicle injection. The same procedure was used to test the effects of BD-1063 and S1RA on the development of cold and mechanical allodynia, but in this case, one of the σ_1 receptor antagonists or their solvent (saline) was injected s.c. 30 min before each of the 5 i.p. injections of paclitaxel or its vehicle

To investigate the effect of the drugs of interest on the expression of paclitaxel-induced pain, pretreatment response was evaluated and paclitaxel treatment was done as described above. Then 7 and 10 days after the first injection of the antineoplastic drug or its vehicle, the response of the animals in the pain test was recorded and nonresponder animals were excluded from further study as previously described (Nieto et al., 2008). On day 10, the response in the pain test was evaluated immediately before drug treatment (time 0), then the drug or its solvent was injected s.c., and the response to the stimulus was measured again (60, 120 and 180 min after the injection for cold allodynia; 30, 60, 90, 120 and 180 min after the injection for mechanical allodynia). We studied this effect on day 10 because in this day we observed evident hyperresponsiveness in both pain tests (see section 2.4).

The experimenter who evaluated the behavioral responses was blinded to the treatment and genotype of each experimental animal. In all cases the experiments in the σ_1-KO or WT groups, vehicle- or paclitaxel-treated groups, and saline- or drug-treatment groups were run in parallel. Each animal was tested in only one pain test and received a drug (or saline) only once.

2.3.5. Procedure to measure cold allodynia

Cold allodynia was tested by gently touching the plantar skin of the hind paws with an acetone bubble formed with a syringe connected to a thin polyethylene tube as previously described (Nieto et al., 2008). On each day of evaluation the mice were housed and habituated for 30 min in individual transparent plastic boxes (7 × 7 × 13 cm) with a floor made of wire mesh (a small mirror was placed behind the chambers to allow clear observation). After the adaptation period, acetone was applied alternately three times to each hind paw at intervals of 30 s, and the duration of licking or biting was recorded. The time spent licking or biting the paw was recorded with a stopwatch and reported as the cumulative time of licking/biting in all six measurements. A cut-off time of 10 s was used in each of the six trials, because animals rarely licked their hind paw for more than 10 s.

2.3.6. Procedure to measure mechanical allodynia

To assess mechanical allodynia, threshold force for hind paw withdrawal was measured with an electronic Von Frey apparatus (Dynamic Plantar Aesthesiometer, Ugo Basile, Comerio, Italy) as previously described (Nieto et al., 2008). This electronic device uses a single nonflexible filament which applies increasing force (from 0 to 10 g) against the plantar surface of the hind paw over a 20-s period. The nocifensive paw withdrawal response automatically turns off the stimulus, and the mechanical pressure that evoked the response is recorded. On each day of the experiment, the mice were habituated for 2 h in individual transparent plastic boxes (9 × 9 × 14 cm) with a wire mesh bottom. After the adaptation period, each mouse was tested three times alternately

in each hind paw, allowing at least 30 s between each measurement. The mean of the six trial values was considered the response of the animal.

2.3.7. Rotarod test

Possible disturbances in motor coordination in σ_1-KO mice or disturbances produced by the σ_1 receptor antagonists in WT mice were tested with an accelerating rotarod (Cibertec, Madrid, Spain). As a comparison drug we used pregabalin, which has been reported to impair rotarod performance (Yokoyama et al., 2007). The method used was the same as described previously by Nieto and coworkers (2008), with slight modifications. Briefly, the mice were required to walk against the motion of an elevated rotating drum while the speed of rotation was increased from 4 to 40 revolutions/min over 5 min, and the latency to fall-down was recorded automatically. To accustom animals to the apparatus, 24 h before drug testing the animals were given 3 training sessions on the rotarod separated by 30-min intervals. On the day of the drug test, rotarod latencies were measured immediately before the drug or saline was administrated (time 0) and several times (60, 120 and 180 min) after the s.c. injection. In all experiments we used a 300-s cut-off time.

2.3.8. Data analysis

For behavioral studies, the differences between values obtained in several experimental groups were analyzed with two-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni test and a Student's *t*-test was used for comparison between two means. All tests were done with the SigmaStat 2.03 program (Systat Software Inc., San Jose, CA, USA). The differences between means were considered statistically significant when the value of *p* was below 0.05.

For *in vitro* assays, the data were analyzed with the SigmaPlot 2002 v.8.0 program (Systat Software Inc). The IC_{50} (concentration of unlabeled drug that inhibited 50% of [^{3}H](+)-pentazocine specific binding) was estimated from the inhibition curves with nonlinear regression analysis of the equation for a sigmoid plot, assuming one-site competition. Pseudo-Hill coefficients (n'_H) were calculated as the slope of the linear regression obtained by plotting the data from competition assays as log [% B/(100 - % B)] *vs.* log [cold drug (M)], where B is the degree of [^{3}H](+)-pentazocine-specific binding displacement by several concentrations of the cold drug tested.

The equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}) from saturation assays were calculated by nonlinear regression analysis of the results fitted to a rectangular hyperbola equation. We used the GraphPad Prism 5.00 program (GraphPad Software Inc., San Diego, CA, USA) to compare the parameters obtained from saturation experiments with the sum-of-squares *F* test to check the goodness of fit of different models that shared one or more parameters. The differences between values were considered significant when the *p* value was below 0.05. Saturation assays were also analyzed by linear regression from the Scatchard analysis as [B/F] *vs.* B, assuming B to be specific binding and F to be the free concentration of radioligand. The K_i values for unlabeled ligands (which indicate the affinity of the inhibitor for the receptor) were calculated with the Cheng–Prussoff equation: $K_i = IC_{50} /(1 + [L]/K_D)$, where [*L*] is the concentration of radioligand used and K_D is the value obtained with nonlinear regression analysis from the control saturation experiments.

2.4. RESULTS

2.4.1. Characteristics of the interaction of S1RA and BD-1063 with σ_1 receptor in mouse brain

We used competition binding assays to measure the affinity of the drugs for the σ_1 receptor, labeled with [^{3}H](+)-pentazocine, in WT mouse brain membranes (P_2 fraction). As shown in Figure 1, both BD-1063 and S1RA inhibited the specific binding of [^{3}H](+)-pentazocine (which always represented more than 80 % of the total binding) in a concentration-dependent manner. The inhibition constants (K_i values) of BD-1063 and S1RA were 4.43 ± 0.86 and 29.99 ± 6.85 nM, respectively, indicating a high affinity of both unlabeled drugs for the σ_1 receptor. The pseudo-Hill analysis of these competition assays yielded straight lines whose slopes or pseudo-Hill coefficients (n'_H) were close to unity for both BD-1063 (n'_H= 1.02 ± 0.03) and S1RA (n'_H= 1.21 ± 0.07). This suggests the existence of a single population of binding sites.

We performed saturation binding studies with [^{3}H](+)-pentazocine in the presence of BD-1063 and S1RA to test whether the interaction of the drugs with WT mice brain σ_1 receptors is competitive. Control saturation binding assays showed that [^{3}H](+)-pentazocine bound in a saturable manner to its specific binding sites in mice brain membranes. Scatchard analysis of these results yielded straight lines, consistent with the existence of a single class of σ_1 binding sites (Fig. 2). The values of the equilibrium dissociation constant (K_D) and the maximum number of receptors (B_{max}) from the control saturation assays were 1.82 ± 0.12 nM and 0.41 ± 0.01 pmol/mg protein, respectively.

Saturation binding assays in the presence of the prototypical σ_1 ligand BD-1063 (12.5 nM) showed that there was no significant change ($p > 0.05$) in B_{max} (0.43 ± 0.02 pmol/mg protein) in comparison to the control results. However, there was a patent increase ($p < 0.01$) in [^{3}H](+)-pentazocine K_D (5.22 ± 0.84 nM), as reflected by the large decrease in the slope of the Scatchard analysis, which suggests that BD-1063 competitively interacts with the [^{3}H](+)-pentazocine binding site (Fig. 2). The results for the newly synthesized σ_1 ligand S1RA (75 nM) were similar to the competitive antagonist BD-1063, exhibiting a clear decrease ($p < 0.01$) in [^{3}H](+)-pentazocine affinity (K_D = 5.68 ± 0.75 nM), with no statistically significant change in B_{max} (0.45 ± 0.02 pmol/mg prot) (Fig. 2). Therefore, both BD-1063 and S1RA appear to interact with the mouse σ_1 receptor in a competitive manner.

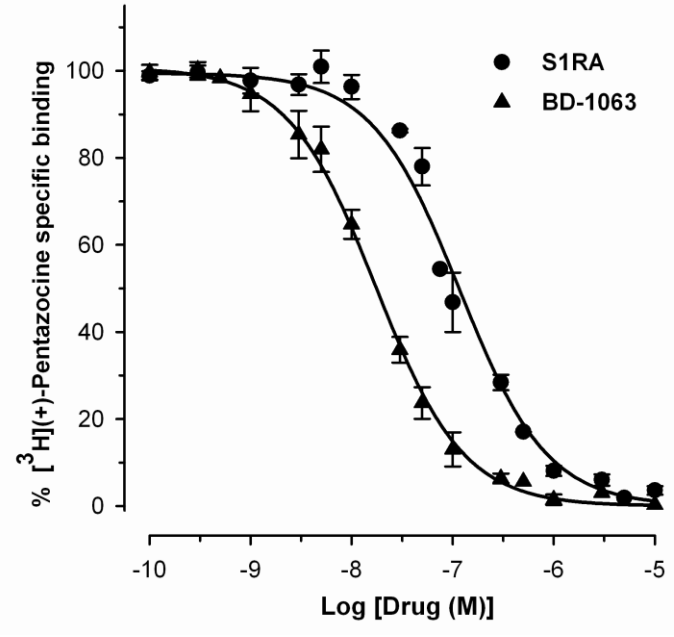

Fig. 1. Inhibition by unlabeled drugs of [^{3}H](+)-pentazocine specific binding to WT mouse brain membranes (P_2 fraction). Mouse brain membranes (0.79-1.10 mg of protein/ml) were incubated at 30 ºC, pH 8, for 240 min with [^{3}H](+)-pentazocine (5 nM) and increasing concentrations of BD-1063 or S1RA (cold ligands). Each point and vertical line represents the mean ± SEM of the values obtained in 3 experiments carried out in triplicate.

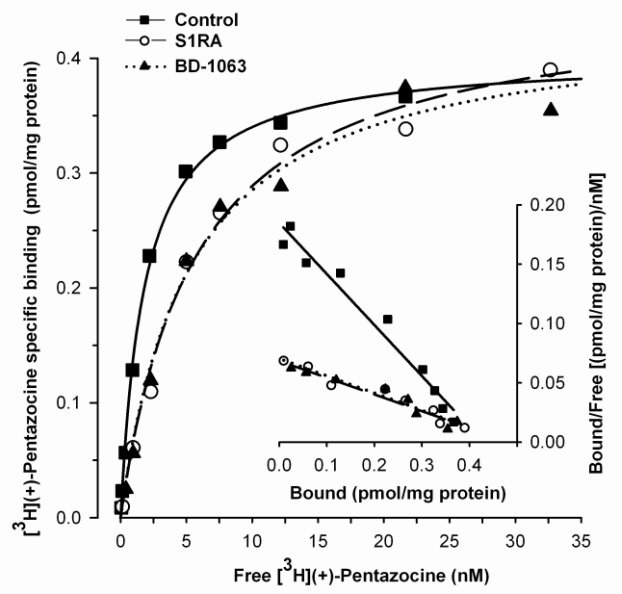

Fig. 2. Representative saturation binding assays for the selective σ_1 receptor radioligand [^{3}H](+)-pentazocine in the absence (control) or presence of 12.5 nM BD-1063 and 75 nM S1RA. Membranes (0.79-1.10 mg/ml protein) were incubated at 30 °C, pH 8, for 240 min, with several concentrations of [^{3}H](+)-pentazocine (0.06–33 nM) in the presence of haloperidol 10 μM (for nonspecific binding) or its solvent. The corresponding Scatchard plots are inserted. There was an apparent change in K_D ($p < 0.01$), but not in B_{max} ($p = 0.07$) when BD-1063 or S1RA was present (F test). The data shown are representative of three experiments carried out in triplicate.

2.4.2. Effect of σ_1 receptor antagonists on the expression of paclitaxel-induced cold and mechanical allodynia in wild-type mice

To test the role of σ_1 receptor on the expression of paclitaxel-induced neuropathy, BD-1063 or S1RA were tested after the neuropathy was fully expressed (10 days after the first injection of paclitaxel). In all groups of WT mice treated with paclitaxel, the duration of paw licking/biting produced by acetone on day 10, before treatment with the drugs or saline (time 0), was significantly longer than on the pre-treatment day (Figs. 3A and 3C, time 0 versus PRE). In these mice, on day 10 a single s.c. injection of saline (drug solvent) did not modify the response to acetone at any of the time-points (Figs. 3A and 3C). However, a single s.c. injection of BD-1063 (16-64 mg/kg) or S1RA (32-128 mg/kg) dose-dependently reduced the duration of acetone-induced paw licking/biting (Figs. 3A and 3C).

We also found that treatment with paclitaxel-vehicle during 5 days did not significantly change the response to acetone on day 10 (Figs. 3B and 3D, time 0 versus PRE). The administration on day 10 of a single s.c. injection of BD-1063 (32 mg/kg) or

S1RA (64 mg/kg), doses that markedly inhibited paclitaxel-induced cold allodynia (see Figs. 3A and 3C), did not change the duration of paw licking/biting induced by acetone during the 3-h test period (Figs. 3B and 3D).

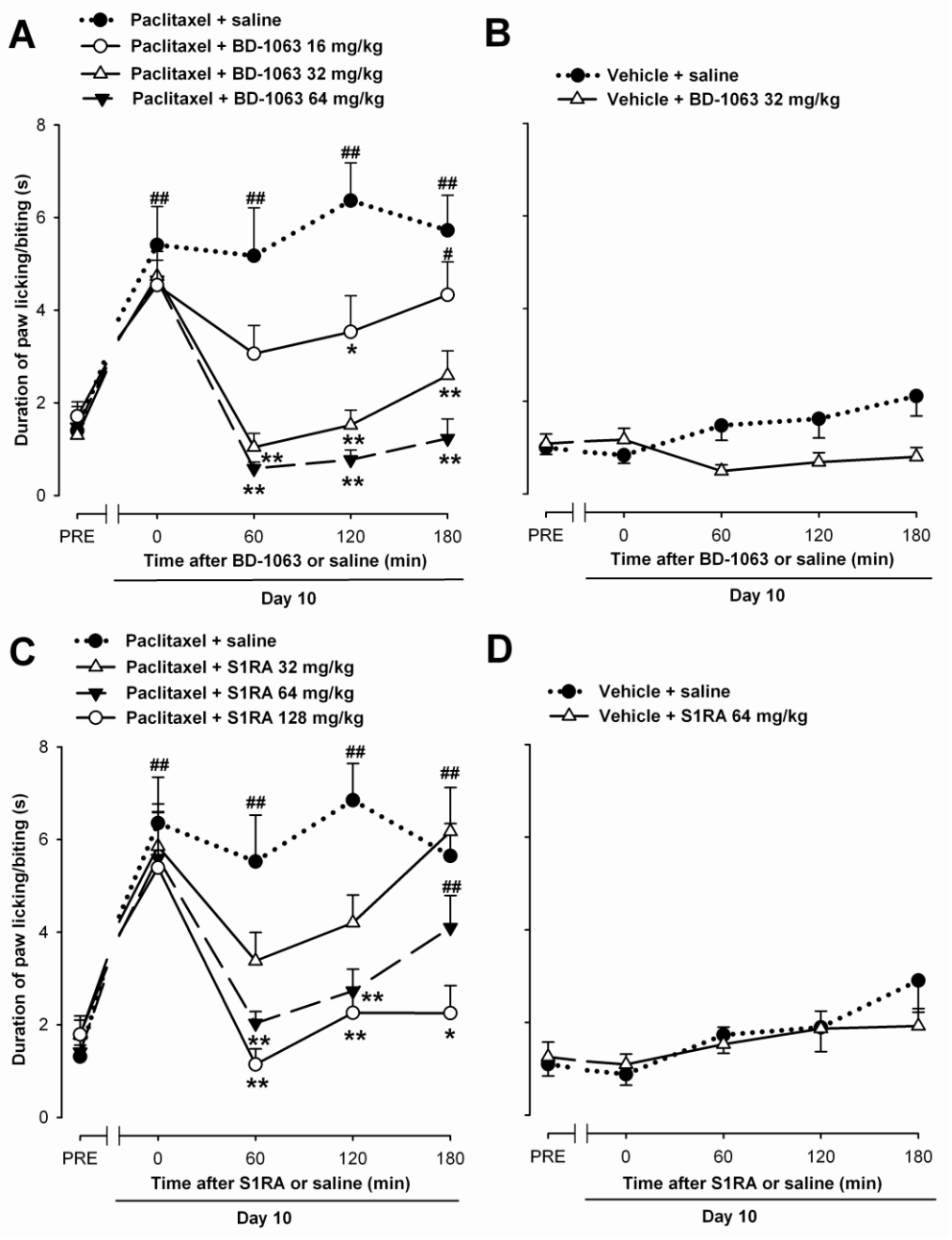

Fig. 3. Time-course of the effect of a single s.c. injection of BD-1063 (16, 32 or 64 mg/kg) **(A** and **B)**, S1RA (32, 64 or 128 mg/kg) **(C** and **D)** or saline **(A-D)** in the acetone test, in mice pretreated with paclitaxel (**A** and **C**) or its vehicle (**B** and **D**). The animals were treated once daily from days 1 to 5 with paclitaxel or paclitaxel-vehicle i.p., and on day 10 they received a single s.c. injection of one of the drugs or saline. The duration of hind paw licking/biting was recorded in each animal 3 days before (PRE) and 10 days after the first injection of paclitaxel or its vehicle. On day 10, the duration of hind paw licking/biting was recorded immediately before (time 0) and at several times (60, 120 and 180 min) after the injection of the drug or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 14-22 animals. Statistically significant differences between drug- and saline-treated groups on the same day after treatment: * p <0.05; ** p <0.01; and between the values on the pretreatment day and the days after treatment: ## p <0.01 (two-way repeated measures ANOVA followed by Bonferroni test).

Mechanical allodynia was also observed 10 days after paclitaxel administration in WT mice. This allodynia was manifested as a statistically significant reduction in the threshold force needed to evoke paw withdrawal (Figs. 4A and 4C, time 0 versus PRE). In these animals, saline administration did not significantly modify paclitaxel-induced mechanical allodynia during the 3-h test period (Figs. 4A and 4C). In contrast, the s.c. administration of BD-1063 (32 and 64 mg/kg) significantly inhibited the allodynia in comparison to saline, in a dose-dependent way (Fig. 4A). Both doses of S1RA (32 and 64 mg/kg) completely reversed the mechanical hypersensitivity induced by paclitaxel (Fig. 4C).

Treatment of mice with paclitaxel-vehicle did not induce mechanical hyperresponsiveness on the day 10 (Figs. 4B and 4D, time 0 versus PRE). In these mice the administration of 64 mg/kg of BD-1063 or S1RA (a dose that evoked the maximum decrease in paclitaxel-induced mechanical allodynia, Figs. 4A and 4C) did not significantly change the mechanical threshold that evoked paw withdrawal in comparison to saline-treated mice (Figs. 4B and 4D).

Therefore, the acute administration of both σ_1 receptor antagonists inhibited the expression of paclitaxel-induced cold and mechanical allodynia, whereas neither of them altered the response to these stimuli in paclitaxel-vehicle treated animals.

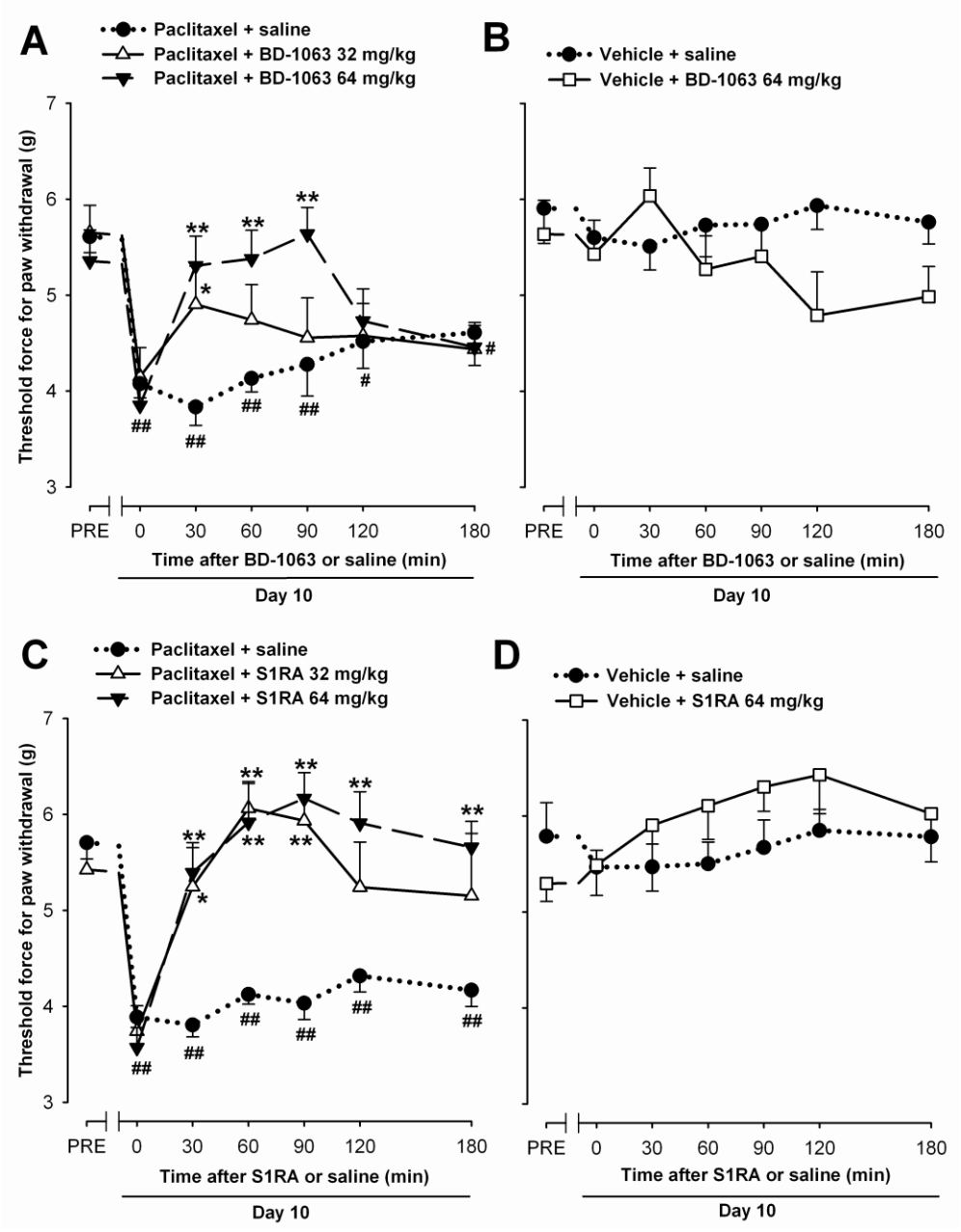

Fig. 4. Time-course of the effect of a single s.c. injection of BD-1063 (32 or 64 mg/kg) **(A** and **B)**, S1RA (32 or 64 mg/kg) **(C** and **D)** or saline **(A-D)** on the threshold force for paw withdrawal (electronic Von Frey test) in mice pretreated with paclitaxel (**A** and **C**) or its vehicle (**B** and **D**). The animals were treated once daily from days 1 to 5 with paclitaxel or paclitaxel-vehicle i.p., and on day 10 they received a single s.c. injection of one of the drugs or saline. The mechanical threshold was recorded in each animal 3 days before (PRE) and 10 days after the first injection of paclitaxel or its vehicle. On day 10, the threshold force was recorded immediately before (time 0) and at several times (30, 60, 90, 120 and 180 min) after the injection of the drug or saline. Each point and vertical line represent the mean ± SEM of the values obtained in 10-14 animals. Statistically significant differences between the drug- and saline-treated groups on the same day after treatment: * $p < 0.05$; ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: # $p < 0.05$; ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

2.4.3. Effect of σ_1 receptor antagonists on the development of paclitaxel-induced cold and mechanical allodynia in wild-type mice

To study the effect of pharmacological antagonism of the σ_1 receptor on the development of paclitaxel-induced neuropathy, we gave the animals an s.c. injection of the drug (or saline in the control group) 30 min before each dose of paclitaxel and tested allodynia on several posttreatment days with the acetone or the electronic Von Frey test.

The posttreatment values of acetone-induced paw licking/biting in the groups of animals treated with saline + paclitaxel were significantly higher than the pretreatment values on several days (Figs. 5A and 5C). However, in animals that received BD-1063 (32 mg/kg) or S1RA (64 mg/kg) before each dose of paclitaxel, the effect of acetone throughout the 24-day observation period after cotreatment was not significantly different from the pretreatment day (Figs. 5A and 5C). Statistically significant differences in the duration of licking/biting between the control group (paclitaxel + saline) and the group treated with paclitaxel + BD-1063 were observed on days 7, 10 and 14 (Fig. 5A), and on every posttreatment day in the group treated with S1RA + paclitaxel (Fig. 5C).

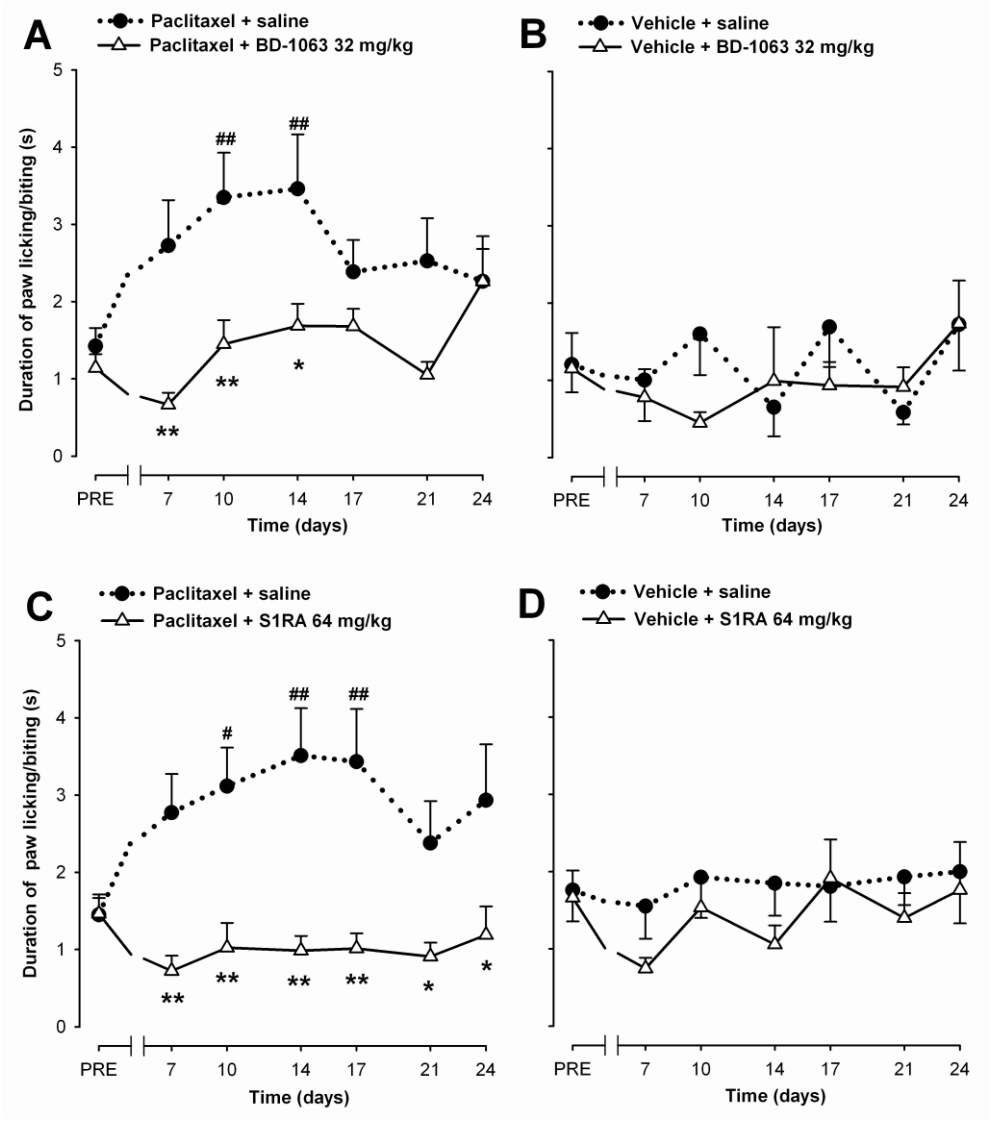

Fig. 5. Time-course of the effect of cotreatment with paclitaxel + saline, + BD-1063 (32 mg/kg) or + S1RA (64 mg/kg) (**A** and **C**) and paclitaxel-vehicle + saline, + BD-1063 (32 mg/kg) or + S1RA (64 mg/kg), (**B** and **D**) on the duration of hind paw licking/biting in the acetone test. The mice were treated once daily from days 1 to 5 with an s.c. injection of one of the drugs or saline 30 min before each i.p. injection of paclitaxel or its vehicle. The response was recorded in each animal 3 days before (PRE) and on different days after the start of cotreatment. Each point and vertical line represent the mean ± SEM of the values obtained in 12-28 animals. Statistically significant differences between drug- and saline-treated groups on the same day after treatment: * $p < 0.05$; ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: # $p < 0.05$; ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

As expected, mice treated with paclitaxel-vehicle and cotreated with saline, BD-1063 or S1RA did not show any change on the response to acetone (Figs. 5B and 5D).

In the electronic Von Frey test, the basal threshold forces for paw withdrawal (pretreatment values) were almost identical in all experimental groups (Figs. 6A-D). When pre- and posttreatment values were compared in the paclitaxel + saline treated groups, a statistically significant reduction in mechanical threshold values was observed on days 7, 10 and 14 (Figs. 6A and 6C). However, there were no significant differences between the pre- and posttreatment values at any time-point in animals treated with BD-1063 (64 mg/kg; Fig. 6A) or S1RA (64 mg/kg; Fig. 6C), before each dose of paclitaxel. Moreover, on days 10 and 14 the threshold force values that produced paw withdrawal in paclitaxel + saline-treated animals were significantly lower than in animals in which BD-1063 (Fig. 6A) or S1RA (Fig. 6C) was injected before paclitaxel.

Treatment with paclitaxel-vehicle 30 min after each dose of saline, BD-1063 (64 mg/kg) or S1RA (64 mg/kg) did not have any effect on the response to the mechanical stimulus (Figs. 6B and 6D)

Therefore, treatment with σ_1 receptor antagonists before each dose of paclitaxel inhibited the development of cold and mechanical allodynia induced by this antineoplastic, without modifying the mechanical and cold response in mice treated with paclitaxel-vehicle.

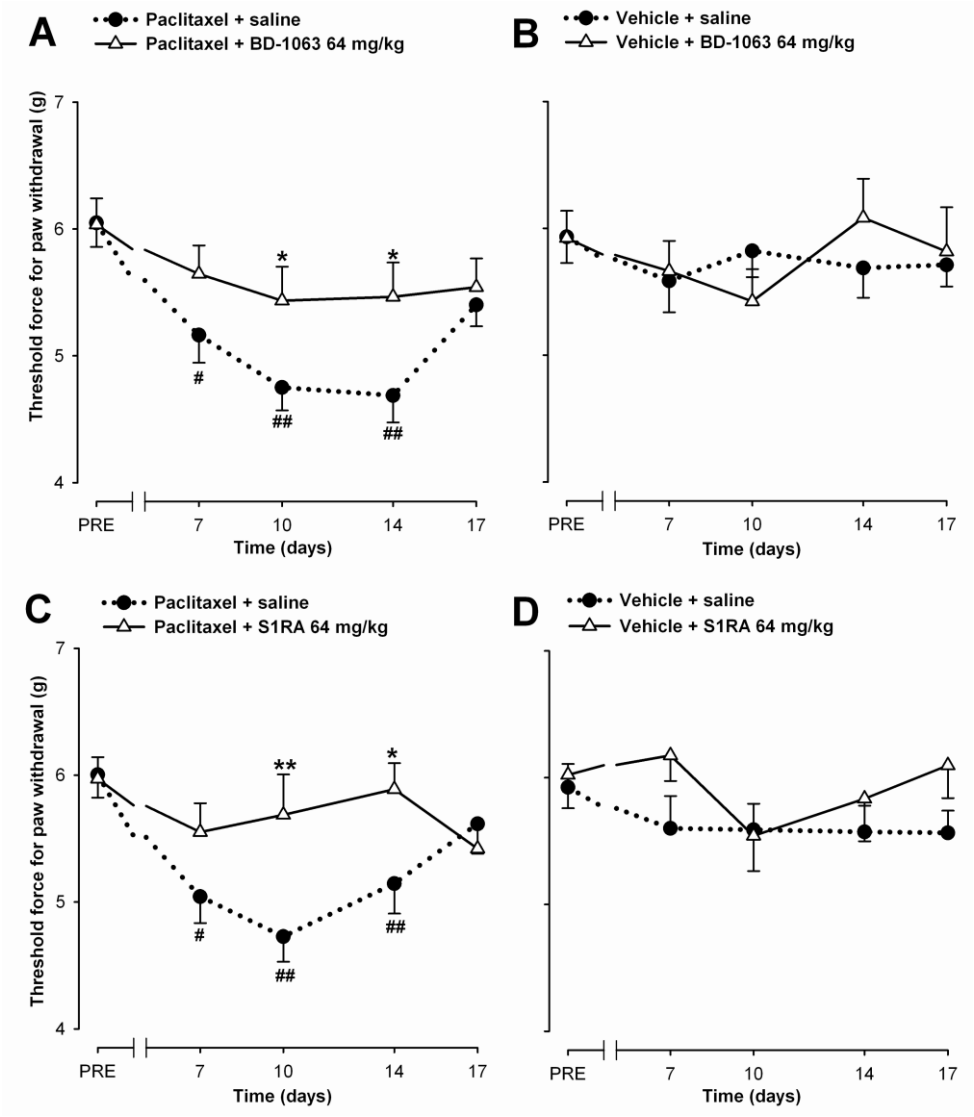

Fig. 6. Time-course of the effect of cotreatment with paclitaxel + saline, + BD-1063 (64 mg/kg) or + S1RA (64 mg/kg) (**A** and **C**) and paclitaxel-vehicle + saline, + BD-1063 (64 mg/kg) or + S1RA (64 mg/kg) (**B** and **D**) on the threshold force for paw withdrawal (electronic Von Frey test). The mice were treated once daily from days 1 to 5 with an s.c. injection of one of the drugs or saline 30 min before each i.p. injection of paclitaxel or its vehicle. The response was recorded in each animal 3 days before (PRE) and on different days after the start of cotreatment. Each point and vertical line represent the mean ± SEM of the values obtained in 12-22 animals. Statistically significant differences between a drug- and saline-treated groups on the same day after treatment: * $p < 0.05$; ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: # $p < 0.05$; ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

2.4.4. Comparison of paclitaxel-induced cold and mechanical allodynia in σ_1 receptor knockout and wild-type mice

To further test the involvement of σ_1 receptor in paclitaxel-induced neuropathic pain, we compared the response to cold and mechanical stimuli of WT and σ_1-KO mice. The baseline responses to the acetone and the electronic Von Frey tests were not significantly different between σ_1-KO and WT mice that were subsequently treated with paclitaxel or its vehicle (Figs. 7A-D). The administration of paclitaxel-vehicle during 5 days did not significantly modify the posttreatment responses in both tests in σ_1-KO or WT animals (Figs. 7B and 7D). However, 5 days of treatment with paclitaxel (2 mg/kg, i.p.) in WT mice significantly increased the duration of paw licking/biting induced by acetone (Fig. 7A) and reduced the threshold force to evoke paw withdrawal in the mechanical test (Fig. 7C). The enhanced responsiveness was maximal 10 days after the first paclitaxel injection in both behavioral tests (Figs. 7A and 7C). In contrast, the same treatment with the antineoplastic had no significant effect on the posttreatment responses in σ_1-KO mice (Figs. 7A and 7C). When we compared the posttreatment values in WT and σ_1-KO mice on the same day, we found statistically significant differences in both pain tests at several time-points (Figs. 7A and 7C).

Therefore, treatment with the antineoplastic induced cold and mechanical allodynia in WT but not in σ_1-KO mice.

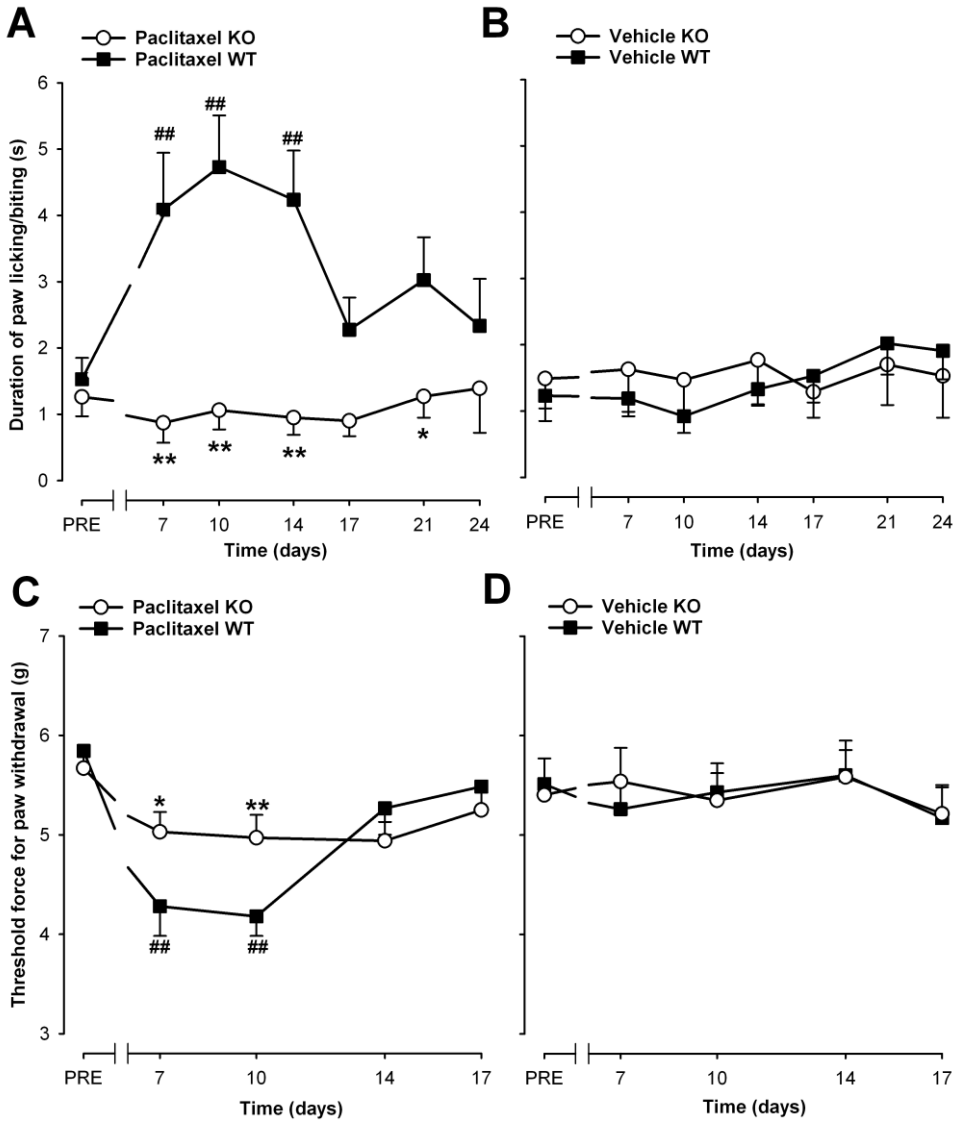

Fig. 7. Time-course of the effects of paclitaxel or its vehicle on the duration of hind paw licking/biting in the acetone test (**A** and **B**), and threshold force for hind paw withdrawal in the electronic Von Frey test (**C** and **D**), in σ_1 receptor knockout (KO) and wild-type (WT) mice. The animals were treated i.p. once daily from days 1 to 5 with paclitaxel (2 mg/kg) or paclitaxel vehicle. The response was recorded in each animal 3 days before (PRE) and on different days after the first injection of the antineoplastic. Each mouse was tested in only one model of nociception. Each point and vertical line represent the mean ± SEM of the values obtained in 16-32 (**A** and **B**) or 12-20 (**C** and **D**) animals. Statistically significant differences between the σ_1 receptor KO and WT mice on the same day after paclitaxel treatment: ** $p < 0.01$, * $p < 0.05$; and between the values on the pretreatment day and the days after treatment: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test). In animals treated with paclitaxel vehicle there were no statistically significant differences between the two groups (KO and WT mice) at any time point, or between each groups' respective pretreatment day value and the values obtained several days after treatment with paclitaxel vehicle (two-way repeated measures ANOVA).

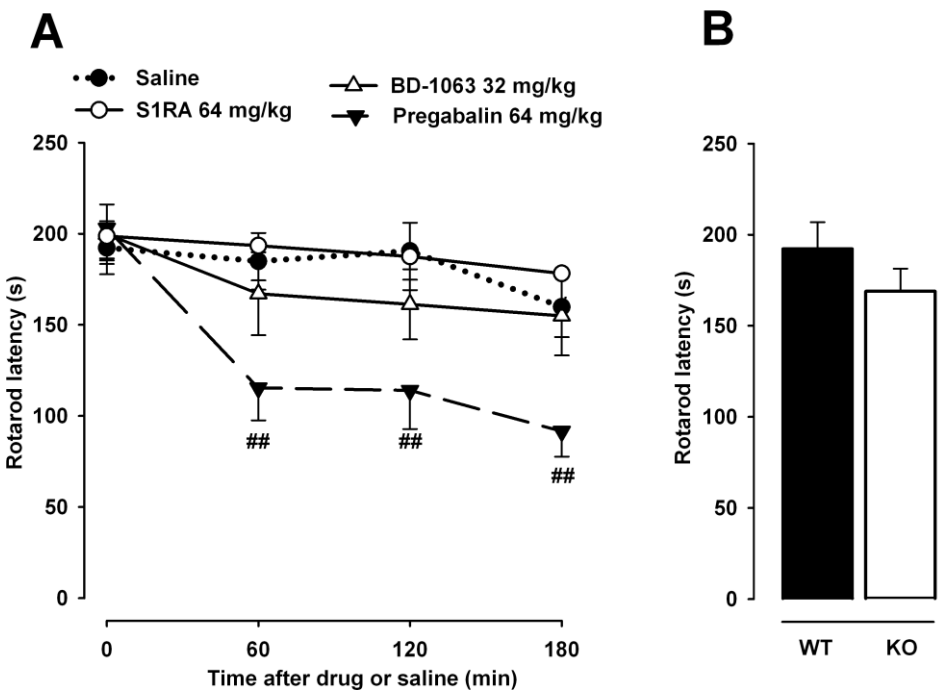

Fig. 8. (**A**) Time-course of the effect on the rotarod test of s.c. treatment with saline, BD-1063 (32 mg/kg), S1RA (64 mg/kg) or pregabalin (32 mg/kg) in wild-type (WT) mice. Twenty-four hours before the drug or saline was administered, the animals were trained on the accelerating rotarod in three sessions to accustom them to the apparatus. On the day of the experiment the latency to fall-down from the rotarod was recorded in each mouse immediately before (time 0) and at several times after the administration of saline or drugs. Each point and vertical line represent the mean ± SEM of the values obtained in 15-20 animals. Statistically significant differences between the values at time 0 and the times after the administration of saline or drugs: $^{\#\#}p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test). (**B**) Comparison of latencies to fall down in the rotarod test in WT and σ_1 receptor knockout (KO) mice. Twenty-four hours before the testing day, the animals were trained on the accelerating rotarod in three sessions to accustom them to the apparatus. Each bar and vertical line represent the mean ± SEM of the values obtained in 15-20 animals No significant differences were found between WT and σ_1-KO mice (Student´s *t*-test).

2.4.5. Effect of σ_1 receptor antagonism and σ_1 genetic inactivation on the rotarod test

Mice treated with both σ_1 receptor antagonists (at doses used in pain experiments) were tested with the accelerating rotarod test to rule out the involvement of a hypothetical locomotor disturbing effect of the drugs on the results of the pain experiments. As a positive control, we used pregabalin, which has been shown to impair

rotarod performance (Yokoyama et al., 2007). Wild-type mice treated s.c. with BD-1063 (32 mg/kg) or S1RA (64 mg/kg) show latencies to fall-down similar to those of animals treated with saline at all times, and none of the posttreatment latencies were significantly different from the pretreatment values obtained in the same animals in each experimental group (Fig. 8A). In contrast, WT mice treated with pregabalin (32 mg/kg, s.c.) showed a significant reduction of the latency to fall-down 60, 120 and 180 min after administration in comparison to their basal values (Fig. 8A). In σ_1-KO animals the latencies to fall-down in the rotarod test were slightly shorter but were not significantly different from those observed in WT mice (Fig. 8B).

2.5. DISCUSSION

The main finding of this study is that both pharmacological blockade and genetic knockout of σ_1 receptors inhibit neuropathic pain induced by paclitaxel in mice. We thus provide the first available evidence that σ_1 receptors are involved in chemotherapy-induced neuropathic pain. We also show that the prototypic σ_1 receptor antagonist BD-1063 and the new drug S1RA interact with the σ_1 receptor in a similar way.

In the binding studies we found that BD-1063 and S1RA had a marked affinity (K_i in the low nM range) for the σ_1 receptor labeled with [^{3}H](+)-pentazocine in mouse brain membranes. The pseudo-Hill analysis for both drugs yielded n'_H values close to unity, which suggest that they interact with a single population of binding sites. The K_i and n'_H values of BD-1063 in our experiments agree with those of previous studies in rat and guinea pig brain membranes (Matsumoto et al., 1995; Cobos et al., 2005, 2006), and in human neuroblastoma cells (Cobos et al., 2007). Together, these findings suggest that the BD-1063-σ_1 receptor interaction is similar in mice and other species including

humans. Saturation binding studies with [^{3}H](+)-pentazocine in the presence of BD-1063 and S1RA revealed a significant change in K_D, but not in B_{max}, which suggests that both drugs interact competitively with the σ_1 receptors. A study in rat brain membranes had previously described a competitive type of interaction between BD-1063 and [^{3}H](+)-pentazocine (Brammer et al., 2006).

In our behavioral studies we found that without paclitaxel treatment, the values in the cold and mechanical tests in σ_1-KO mice were indistinguishable from those in WT mice. These results are in agreement with those of previous studies that found normal mechanical, heat and cold perception in naive σ_1-KO mice (Entrena et al., 2009b; De la Puente et al., 2009). However, after paclitaxel treatment the results were clearly different. Wild-type mice developed cold- and mechanical-allodynia whereas the responses in σ_1-KO mice changed very little compared to their own baseline values, and therefore these animals did not develop neuropathic pain. Interestingly, we obtained similar results in a model of neuropathy induced by mechanical nerve injury where both mechanical- and cold-allodynia were also attenuated in σ_1-KO mice (De la Puente et al., 2009). These data suggest that the σ_1 receptor must be present for, and may play a key functional role in, the development or expression of these neuropathies.

We also found that the s.c. administration of the σ_1 receptor antagonists BD-1063 and S1RA before each paclitaxel dose in WT mice (to block σ_1 receptors during the development of neuropathy), totally prevented paclitaxel-evoked cold- and mechanical-allodynia. In agreement with these data, Roh et al. (2008) demonstrated that intrathecal treatment with another σ_1 antagonist (BD-1047) during the induction phase of sciatic nerve mechanical injury-induced neuropathy also impeded the development of allodynia in rats. Together, these data suggest that σ_1 receptors must be activated during the

development of a painful neuropathy for its full progression. This possibility is particularly interesting in the context of chemotherapy-induced neuropathy, where the moment of onset of the nerve insult is readily identifiable and preventive treatment can be given.

Another finding of the present study was that the systemic treatment of WT mice with either σ_1 receptor antagonist after paclitaxel treatment had ended also inhibited cold- and mechanical-allodynia. This suggests that σ_1 receptors also play a role in the expression of the neuropathy after it has fully developed. In contrast with our results, Roh and collaborators (2008) found that the intrathecal injection of BD-1047 when mechanically-induced neuropathy was already established failed to inhibit the pain behavior. This lack of effectiveness of BD-1047 may be due to the greater severity of or the different pathophysiological mechanisms involved in nerve trauma- and chemotherapy-induced neuropathic pain. In fact, it has been proposed that the pathophysiological mechanisms responsible for neuropathic pain are at least partly different depending on the source of nerve damage, and that these mechanistic differences might also be reflected in different responses to antineuropathic drugs (Aley and Levine, 2002; Baron et al., 2010; Bennett, 2010; Dworkin et al., 2010; Zheng et al., 2011). Another possible explanation for the differences is the route of drug administration. We injected the σ_1 receptor antagonists s.c., so they may act not only at spinal cord but also at supraspinal structures and peripheral nerves, where σ_1 receptors are also present (Palacios et al., 2004; Cobos et al., 2008).

The doses of BD-1063 (32 mg/kg) and S1RA (64 mg/kg) that inhibited cold- and mechanical-allodynia expression in WT mice were inactive in the rotarod test. This lack of effect was not due to any methodological pitfall since pregabalin (32 mg/kg)

significantly reduced the latency to fall-down, as previously described (Yokoyama et al., 2007). These data suggest that the inhibition of the expression of paclitaxel-induced pain by the σ_1 receptor antagonists in our experiments was not due to any motor interference. Similarly, the inhibition of the pain responses observed when σ_1 receptor antagonists and paclitaxel were injected during the development phase of neuropathy could not be due to any motor interference induced by the drugs, since they were injected days or weeks before the pain experiments were done. We also found that the response of WT and σ_1-KO mice in the rotarod test did not differ, which also suggest that no motor deficit could account for the inhibition of cold- and mechanical-allodynia in σ_1-KO mice. The experiments in animals treated with paclitaxel-vehicle further support the lack of involvement of any unspecific effect on the inhibition of paclitaxel-induced pain by σ_1 receptor blockade, since acute or repeated treatment with the σ_1 antagonists tested did not change the responses to cold or mechanical stimuli, and σ_1-KO mice showed virtually identical responses to WT mice treated with paclitaxel-vehicle.

Paclitaxel-induced neuropathy is associated with several electrophysiological abnormalities in peripheral nerves and the spinal cord, such as (1) spontaneous discharges in A- and C-fiber nociceptors and in spinal wide-dynamic-range neurons (Cata et al., 2006, Xiao and Bennett, 2008), and (2) prolonged afterdischarges to mechanical stimuli and increased wind-up to cutaneous stimuli (Cata et al., 2006). These abnormal discharges as well as wind-up and long-term potentiation (LTP) are considered important aspects of central sensitization in the production and amplification of neuropathic pain abnormalities (Latremoliere and Woolf, 2009). Remarkably, σ_1-KO mice showed reduced wind-up responses compared to WT mice (De la Puente et al.,

2009), and σ_1 receptors are known to modulate LTP (Martina et al., 2007). In addition, neuropathic pain following paclitaxel treatment is associated with downregulation of glutamate transporters in the spinal dorsal horn (Weng et al., 2005; Cata et al., 2006). This can produce excessive and prolonged stimulation of glutamate receptors, including NMDA receptor, and its downstream molecular mechanisms (such as phosphorylation of ERK) in the spinal dorsal horn neurons, which trigger the development of central sensitization that generates and maintains neuropathic pain (Ji et al., 2009, Chen et al., 2010). Interestingly, the upregulation of both the NMDA receptor NR1 subunit and its phosphorylated form (pNR1) induced by mechanical nerve injury were prevented by BD-1047 (Roh et al., 2008), and the phosphorylation of ERK following sciatic nerve injury did not occur in σ_1-KO mice (De la Puente et al., 2009). Consequently, our results might be explained by the inhibition of these paclitaxel-induced electrophysiological and biochemical processes by σ_1 receptor pharmacological blockade or genetic knockout.

Low concentrations of paclitaxel, by stabilizing microtubules within the dendritic spines, increase dendritic spine formation and produce more mature (mushroom-shaped) spines (Gu et al., 2008), which have been proposed to play a key role in paclitaxel-induced neuropahtic pain (Goswami and Goswami, 2010). Then, another possible explanation for our results is related to the modulation of dendritic spine plasticity-dependent neuropathic pain phenomena by σ_1 receptors. This possibility is supported by two recent findings. Firstly, inhibition of the small GTP-binding protein Rac1 (with intrathecal NSC23766) attenuated spinal cord injury-induced dendritic spine remodeling, as well as the subsequent neuronal hyperexcitability and neuropathic pain signs (Tan et al., 2008). Secondly, the knockdown of neuronal σ_1 receptors by

transfection with a siRNA inhibits dendritic spine formation by reducing the neuronal signaling through the Tiam1/Rac1 pathway (Tsai et al., 2009). Taken together, these findings suggest the hypothesis that the pharmacological or genetic blockade of σ_1 receptors might inhibit paclitaxel-dependent dendritic spine remodeling and the resulting neuronal hyperexcitability that underlies mechanical and cold allodynia. Further studies should be done to test this hypothesis.

In summary, we found that paclitaxel induced cold and mechanical allodynia in WT but not in σ_1-KO mice, and that two competitive σ_1 receptor antagonists not only inhibited the expression of both kinds of allodynia, but also prevented the development of the painful neuropathy. These results raise the possibility of using the blockade of σ_1 receptor-dependent mechanisms as an approach to the preventive treatment of chemotherapy-induced neuropathic pain.

2.6. REFERENCES

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

3. Genetic inactivation and pharmacological blockade of sigma-1 receptors prevent paclitaxel-induced sensory-nerve mitochondrial abnormalities and neuropathic pain in mice

Francisco Rafael Nieto, Eduardo Fernández Segura; Francisco Javier Cañizares, Cruz Miguel Cendán, María Angustias Cubero, Daniel Zamanillo, José Manuel Baeyens

(PENDING PUBLICATION)

3.1. ABSTRACT

Background

Paclitaxel, a widely-used antineoplastic drug, frequently produces a dose-limiting painful peripheral neuropathy that has no clinically validated treatment. Paclitaxel administration to rodents induces mitochondrial alterations that are relevant to neuropathic pain. The sigma-1 (σ_1) receptor is a ligand-regulated molecular chaperone involved in mitochondrial calcium homeostasis and pain hypersensitivity. We recently reported that σ_1 receptors play a key role in paclitaxel-induced neuropathic pain, but it is not known whether they also modulate mitochondrial abnormalities.

Results

Paclitaxel administration (2 mg/kg, i.p. once per day for 5 consecutive days) to wild type (WT) mice produced cold-allodynia (acetone test), mechanical-allodynia (electronic Von Frey test) and an increase in the frequency of swollen and vacuolated mitochondria in myelinated A-fibers (Aβ>Aδ) but not in C-fibers of the saphenous nerve (transmission electronic microscopy analysis). Behavioral and morphological alterations were marked at 10 days after paclitaxel-administration and had resolved by day 28. In contrast, the same schedule of paclitaxel treatment did not induce allodynia or mitochondrial abnormalities in σ_1 receptor knockout mice at any time. Moreover, treatment of WT mice with the selective σ_1 receptor antagonist BD-1063 (32 mg/kg, s.c. 30 min before each paclitaxel injection) prevented the neuropathic pain (cold and mechanical allodynia) and saphenous nerve mitochondrial abnormalities induced by the antineoplastic.

Conclusions

These results suggest that activation of the σ_1 receptor is necessary for development of the sensory nerve mitochondrial damage and neuropathic pain produced by paclitaxel. Therefore, σ_1 receptor antagonists might have therapeutic value for the prevention of paclitaxel-induced neuropathy.

3.2. INTRODUCTION

Peripheral neuropathy induced by antineoplastic drugs is a clinically significant complication that can be dose-limiting (Park et al., 2008; Paice, 2010; Farquhar-Smith, 2011). Paclitaxel, a first-line chemotherapeutic agent used for the treatment of several classes of cancer (Balayssac et al., 2011), frequently produces neuropathic pain characterized by numbness, tingling, burning pain, and cold allodynia in a stocking-glove distribution (Dougherty et al., 2004; Jung et al., 2005; Argyriou et al., 2008). Unfortunately, there are no clinically validated treatments to prevent or ameliorate this painful neuropathy (Argyriou et al., 2008). However, models of paclitaxel-induced neuropathy in rodents have allowed the testing of novel treatments and/or elucidation of pathophysiological mechanisms (Flatters and Bennett, 2004; Matsumoto et al., 2006; Nieto et al., 2008; Liu et al., 2010).

Paclitaxel was assumed to exert its neurotoxicity by binding to β-tubulin of microtubules and disturbing axonal transport, leading to a "dying back" axonal degeneration (Park et al., 2008). However, evidence has been published that questions this assumption (Polomano et al., 2001; Flatters and Bennett, 2006; Jin et al., 2008) and alternative mechanisms for paclitaxel-induced neuropathy have been proposed (reviewed in Balayssac et al., 2011). In particular, an increased incidence of swollen and vacuolated axonal mitochondria in peripheral sensory fibers appears to be relevant (Flatters and Bennett, 2006; Jin et al., 2008). This increase in atypical mitochondria has been attributed to the binding of paclitaxel to mitochondrial β-tubulin, which may produce Ca^{2+} release from mitochondria and dysregulated intracellular Ca^{2+} homeostasis (Flatters and Bennett, 2006; Siau and Bennett, 2006). In support of this proposition, paclitaxel-induced neuropathic pain in experimental animals is prevented and/or

reversed by agents that either enhance mitochondrial function (Ghirardi et al., 2005; Flatters et al., 2006; Jin et al., 2008; Xiao and Bennett, 2008; Xiao et al., 2009) or reduce intracellular Ca^{2+} (Flatters and Bennett, 2004; Matsumoto et al., 2006; Siau and Bennett, 2006; Xiao et al., 2007; 2008; Gauchan et al., 2009; Okubo et al., 2011). Alterations of mitochondrial function and/or intracellular Ca^{2+} levels, affecting membrane excitability, neurotransmitter release, and other cellular events, may also contribute to diabetic neuropathy (Verkhratsky and Fernyhough, 2008) and other peripheral neuropathies (Fernyhough and Calcutt, 2010).

Two sigma receptor subtypes have been described, although only the sigma-1 (σ_1) receptor has been cloned (Cobos et al., 2008; Maurice and Su, 2009 for reviews). The σ_1 receptor has been identified as a ligand-regulated molecular chaperone (Hayashi and Su, 2007) and proposed as a modulator of several receptors and ion channels (Maurice and Su, 2009). Specifically, at mitochondrion-associated endoplasmic reticulum membrane (MAM) level, the σ_1 receptor chaperone modulates the intramitochondrial Ca^{2+} level and plays a key role in the control of intracellular Ca^{2+} homeostasis (Su et al., 2010). σ_1 receptors are highly expressed in the central nervous system, including areas important for pain control (Alonso et al. 2000; Kitaichi et al. 2000), and their involvement in pain modulation is well documented (Cendán et al., 2005a; 2005b; Kim et al., 2006; Roh et al., 2008; Entrena et al., 2009a; 2009b; De la Puente et al., 2009; Romero et al., 2011). Accordingly, σ_1 receptors have been proposed as an emerging target for the treatment of neuropathic pain (Diaz et al., 2009). In favor of this proposal, our group recently reported that paclitaxel-induced pain is reduced in σ_1 receptor knockout (σ_1-KO) mice and in wild-type (WT) mice treated with σ_1 receptor antagonists (Nieto et al., 2011). However, it is not known whether the beneficial effects of σ_1 receptor blockade on

paclitaxel-induced neuropathic pain are associated with a reduction of the mitochondrial abnormalities induced by the antineoplastic.

In the present study, we first compared the time-course of paclitaxel-induced changes in hind paw pain perception (acetone and electronic Von Frey tests) and in saphenous nerve mitochondrial characteristics (electronic microscopy analysis) in WT mice. We then evaluated whether σ_1 receptor blockade by treatment with a selective σ_1 receptor antagonist (BD-1063) or genetic inactivation (σ_1-KO mice) prevents the neuropathic pain behaviors and mitochondrial changes induced by paclitaxel administration. Our results show that paclitaxel-induced behavioral and mitochondrial abnormalities followed a similar time course in WT mice, and that pharmacologically- or genetically-induced σ_1 receptor blockade prevents both types of abnormality.

3.3. METHODS

3.3.1. Animals

Experiments were performed in female WT (Charles River, Barcelona, Spain) and σ_1-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25-30 g. The σ_1-KO mice were generated on a CD-1 background as previously described (Entrena et al., 2009a). The animals were acclimated in our animal facilities for at least 1 week before testing and were housed in colony cages. They were fed a standard laboratory diet (Harlan Teklad Research diet, Madison, WI, USA) and tap water *ad libitum* until the beginning of the experiments and kept in temperature- and light-controlled rooms (22 ± 1 ºC, lights on at 08.00 h and off at 20.00 h, air replacement every 20 min). Testing took place during the light phase (from 9.00 h to 15.00 h) on random days throughout the estrous cycle. Mice were handled in accordance with the European Communities Council

Directive of 24 November 1986 (86/609/ECC). The experimental protocol was approved by the University of Granada Research Ethics Committee.

3.3.2. Drugs and drug administration

The drugs used (and their suppliers) were paclitaxel and the σ_1 receptor antagonist BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) (both from Tocris Cookson Ltd., Bristol, United Kingdom). Paclitaxel was dissolved in a solution of 50% Cremophor EL (Fluka Chemie, Buchs, Germany) and 50% absolute ethanol (Panreac Química S.A., Barcelona, Spain) to obtain a concentration of 6 mg/ml. This paclitaxel solution was stored at –20ºC for a maximum of 14 days and was diluted in sterile physiological saline (NaCl 0.9%) to a final concentration of 2 mg/10 ml just before its administration. For control treatments, the paclitaxel-vehicle solution was also diluted just before its administration in saline at the same proportion as the paclitaxel solution. The paclitaxel (2 mg/kg) was administered intraperitoneally (i.p.) in a volume of 10 ml/kg once per day for 5 consecutive days (cumulative dose of 10 mg/kg). We previously reported that this schedule of paclitaxel treatment produces a painful neuropathy in mice (Nieto et al., 2008 and 2011). The control group was administered with the vehicle for paclitaxel according to the same schedule.

BD-1063 (32 mg/kg) was dissolved in sterile physiological saline just before the s.c. administration of a volume of 5 ml/kg in the interscapular area. This dose of BD-1063 produces a significant anti-allodynic effect in several models of pain (Entrena et al., 2009b; Nieto et al., 2011). The control animals received s.c. the same volume of the drug solvent (saline).

3.3.3. General procedures for drug treatments and behavioral assays in pain models

The general procedures were performed as previously described (Nieto et al., 2008 and 2011) with slight modifications. First, behavioral responses were tested in each animal at 3 days before the start of paclitaxel administration (pretreatment value). Then, animals were treated with drugs once daily for 5 consecutive days. On each treatment day, animals received a s.c. injection of saline or BD-1063 and then, after a 30-min interval, an i.p. injection of paclitaxel or its vehicle. Post-treatment responses were measured on days 10 and 28 after the first paclitaxel or vehicle injection. Selection of these days was based on previous findings by our group that the maximum expression of paclitaxel-induced cold and mechanical allodynia is on day 10 and that there is no pain behavior on day 28 (Nieto et al., 2008 and 2011). Each animal was tested alternately in both pain tests, with an interval of 24 h between evaluations, and was sacrificed after the final measurement in order to obtain the saphenous nerve, as described below (see section 2.7). Only paclitaxel-treated animals that showed both cold and mechanical allodynia on day 10 were selected for the study of saphenous nerve ultrastructure.

The experimenter who evaluated the behavioral responses was blinded to the treatment and genotype of experimental subjects. In all cases, experiments in the σ_1-KO or WT groups, vehicle- or paclitaxel-treated groups, and saline- or BD-1063-treated groups were run in parallel.

3.3.4. Procedure to measure cold allodynia

Cold allodynia was tested by gently touching the plantar skin of the hind paws with an acetone bubble formed with a syringe connected to a thin polyethylene tube as previously described (Nieto et al., 2008). On each evaluation day, the mice were housed and habituated for 30 min in individual transparent plastic boxes (7 × 7 × 13 cm) with a floor made of wire mesh (a small mirror was placed behind the chambers to allow clear observation). After the adaptation period, acetone was applied alternately three times to each hind paw at intervals of 30 s, and the duration of licking or biting was recorded. The time spent licking or biting the paw was recorded with a stopwatch and reported as the cumulative time of licking/biting at all six measurements. A cut-off time of 10 s was used in each of the six trials, because animals rarely licked their hind paw for more than 10 s.

3.3.5. Procedure to measure mechanical allodynia

Mechanical allodynia was assessed by measuring the threshold force for hind paw withdrawal with an electronic Voy Frey apparatus (Dynamic Plantar Aesthesiometer, Ugo Basile, Comerio, Italy) as previously described (Nieto et al., 2008). This electronic device uses a single nonflexible filament that applies increasing force (from 0 to 10 g) against the plantar surface of the hind paw over a 20-s period. The nocifensive paw withdrawal response automatically turns off the stimulus, and the mechanical pressure that evokes the response is recorded. On each day of the experiment, the mice were habituated for 2 h in individual transparent plastic boxes (9 × 9 × 14 cm) with a wire mesh bottom and then tested three times alternately in each hind paw, allowing at least

30 s between each measurement. The mean of the six trial values was considered the response of the animal.

3.3.6. Procedure to obtain saphenous nerves and electron microscopy analysis

In order to evaluate the ultrastructural morphology of the saphenous nerve in σ_1-KO and WT groups, mice were anesthetized with isofluorane (IsoVet®, B. Braun, Barcelona, Spain) and perfused intracardially with 20 ml saline followed by 30 ml of freshly prepared 2% glutaraldehyde/1% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 15 min. After perfusion, saphenous nerves were dissected and processed as previously described by Flatters and Bennett (2006) with slight modifications. Briefly, 5 mm of saphenous nerves were dissected at mid-thigh level and fixed with 2% glutaraldehyde/1% paraformaldehyde in 0.1 M PB, pH 7.4, overnight at 4º C. After fixation, samples were transferred to 10% sucrose in 0.1M PB for 24 h at 4º C and then postfixed with 0.1% osmium tetroxide in 0.1 M PB, pH 7.4, containing 1% potassium ferrocyanure for 1 h at 4º C, dehydrated in a graded series of alcohols, and embedded in Epoxy resin. After embedding, transverse sections (1 μm) were cut and stained with toluidine blue for examination under light microscopy to assess specimen quality. Samples were sectioned with an Ultracut E Reichert-Jung ultramicrotome (Leica, Barcelona Spain) to obtain ultrathin sections (70 nm) and then stained with uranyl acetate and lead citrate.

Ultrathin sections were viewed in a Zeiss EM 902 (Zeiss, Oberckochem, Germany) transmission electron microscope equipped with a monochrome CCD TV camera. Micrographs were taken with the camera connected to a video frame grabber (Snappy Video Snapshot, Play Inc., Rancho Cordova, CA) plugged into a personal

computer (1500 x 1125 resolution). To perform ultrastructural and morphometric analyses, microphotographs were taken of myelinated (n=30) and unmyelinated (n=30) axons of the saphenous nerves from each mouse. ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used to measure the area (A), perimeter (P), circularity ($4\ \pi[A/P^2]$; 1.0 = perfect circle), and Feret's diameter (longest distance between any two points along the selection boundary). Micrographs were taken at 4400x, 7000x, and 20000x. At 4400x, myelinated fibers (n=2100) were classified according to their diameter as Aβ fibers (6-12 μm) or Aδ fibers (< 6 μm) (Gardner et al., 2000). C-fibers (n=2100) were identified at 7000x. Morphometric measurements were conducted at 20000x for the area, perimeter, circularity, and Feret´s diameter in mitochondria from Aβ-, Aδ-, and C-fibers. Area values alone are given in the Results section for the mitochondria, because the other morphometric data were closely related to the area and were similarly affected by treatments (data not shown). Analysis of mitochondrial area (n=10679) in the different fiber types in both WT and σ_1-KO control mice allowed the differentiation of three mitochondrial populations according to area size (see Results section and figure 4). Morphometric analyses were performed by observers who were blind to the genotype or treatment group of the mice.

3.3.7. Data analysis

Differences between values in the behavioral assays were analyzed with two-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni test. Differences between the frequencies of atypical mitochondria were analyzed with two-way analysis of variance (ANOVA) followed by the Bonferroni test. Differences between the values of mitochondrial area distribution or mean mitochondrial area were

analyzed with one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test. All tests were done with the SigmaStat 2.03 program (Systat Software Inc., San Jose, CA). Differences between means were considered statistically significant when the value of *p* was below 0.05.

3.4. RESULTS

3.4.1. Behavioral pain studies

On the pretreatment day, WT and σ_1-KO mice showed a similar duration of acetone-induced paw licking/biting (Fig. 1A) and a similar threshold force for paw withdrawal in the electronic Von Frey test (Fig. 1B). Treatment with paclitaxel vehicle did not significantly modify the response of WT or σ_1-KO mice in any test at any measurement time point (Figs. 1A and 1B). However, on day 10 after the first paclitaxel injection, WT mice showed a statistically significant increase in acetone-induced paw licking/biting duration (cold-allodynia) and a reduction in threshold force for paw withdrawal in the electronic Von Frey test (mechanical allodynia) (Figs. 1A and 1B). On day 28 after the first paclitaxel dose, the response of WT animals had returned to normal values in both tests (Figs. 1A and 1B). In contrast, the paclitaxel-treated σ_1-KO mice showed no sign of cold or mechanical allodynia at any time point (Figs.1A and 1B). Likewise, the s.c administration of the selective σ_1 receptor antagonist BD-1063 (32 mg/kg) to WT animals at 30 min before each paclitaxel dose totally prevented the development of cold and mechanical allodynia (Figs. 1C and 1D). Therefore, σ_1 receptor blockade induced by genetic inactivation (σ_1-KO mice) or treatment with a selective σ_1 receptor antagonist (BD-1063) completely averted the neuropathic pain behavioral manifestations induced by paclitaxel administration.

All treated animals (i.p. paclitaxel/vehicle and s.c. saline/BD-1063 groups) were in good general health at the end of the treatment and all gained weight normally (data not shown).

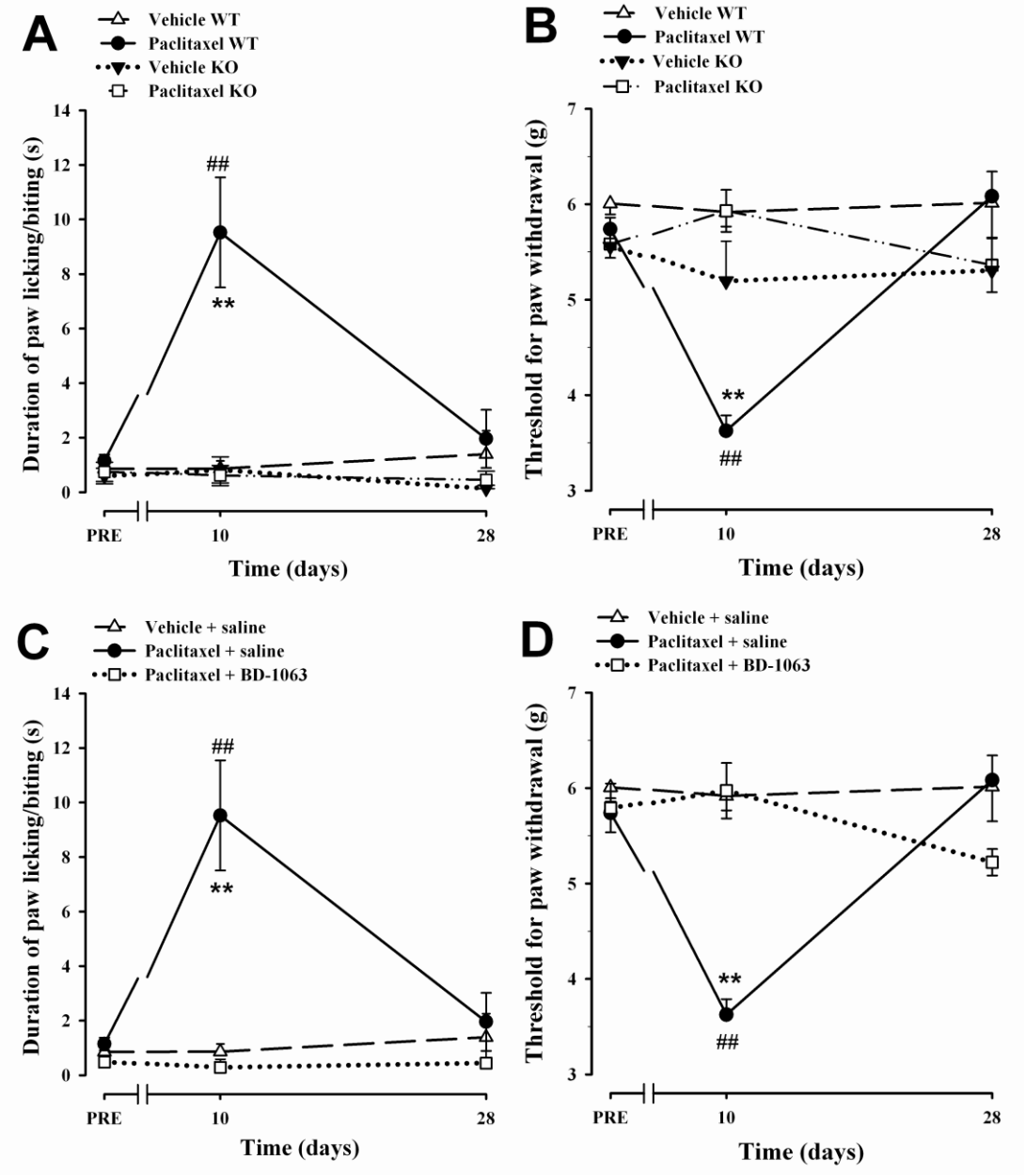

Fig. 1: Time-courses of the effect of paclitaxel and paclitaxel-vehicle on the duration of hind paw licking/biting in the acetone test (**A**) and on the threshold force for hind paw withdrawal in the electronic Von Frey test (**B**) in wild-type (WT) and σ_1 receptor knockout (KO) mice. Time-courses of the effect of paclitaxel + saline, + BD-1063 (32 mg/kg) and paclitaxel-vehicle + saline in the acetone test (**C**) and in the electronic Von Frey test (**D**) in WT mice. The animals were treated once daily from days 1 to 5 with an i.p. injection of paclitaxel (2 mg/kg) or its vehicle (A–B) and with an s.c. injection of BD-1063 or saline 30 min before each paclitaxel dose (C–D). The response was recorded in each animal 3 days before (PRE) and on days 10 and 28 after the start of treatment. Each point and vertical line represents the mean ± SEM of the values obtained in 6-12 animals. Statistically significant differences between WT mice treated with paclitaxel + saline and the rest of the groups on the same day after treatment: ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

3.4.2. Electronic microscopy analysis of saphenous nerve fibers in control WT and σ_1-KO mice

Figure 2 illustrates the ultrastructural characteristics of myelinated and unmyelinated fibers in WT control mice. Normal axoplasmic structures (neurotubules and neurofilaments) can be identified in both myelinated and unmyelinated fibers. Mitochondria within fibers are typically characterized as circular or oval structures containing cristae and amorphous electron dense material enveloped by double membranes (Fig. 2). The ultrastructural characteristics of myelinated and unmyelinated fibers in σ_1-KO control mice were similar to those of WT control mice (Fig 3). Hence, the results show an absence of significant histological differences between the different types of fibers in both groups of animals.

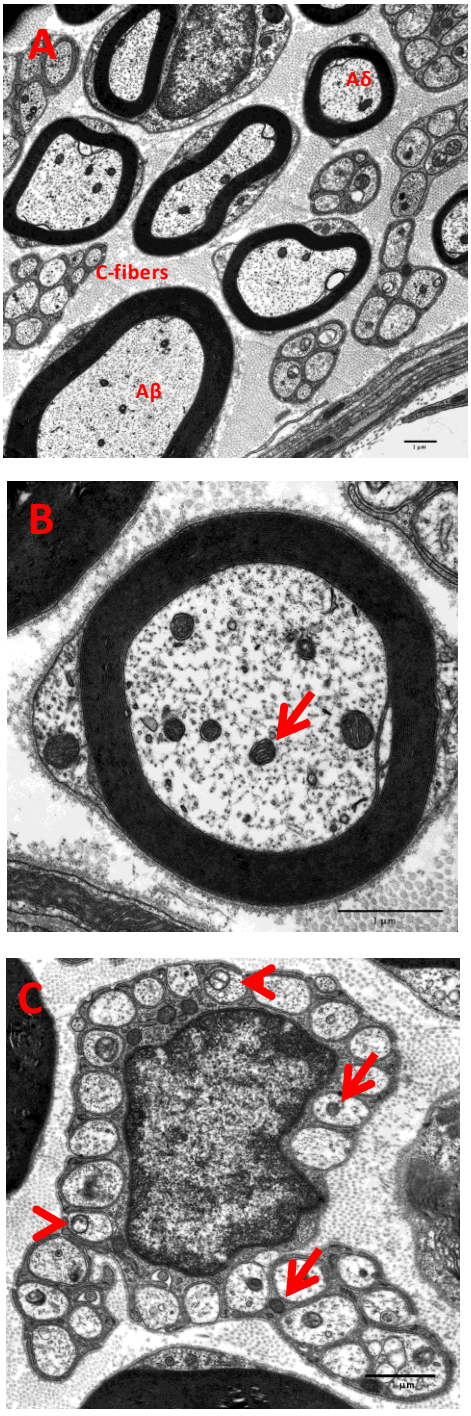

Fig. 2. Transmission electron micrographs of myelinated and unmyelinated fibers of the saphenous nerve from wild-type (WT) control mice. **(A)** Cross-section of myelinated Aβ-, Aδ-, and C-fibers in saphenous nerve from control WT mice observed at 4400x. (**B)** Cross section of a myelinated fiber observed at 20000x. Normal mitochondria (*arrow*) show a circular morphology containing cristae and amorphous electron-dense material enveloped by double membrane. Note the circular microtubule profile in the myelinated axon. **(C)** Remak bundle with Schwann cell nucleus from a control WT mouse observed at 7000x. Normal (arrows) and atypical (*arrowheads*) mitochondria in unmyelinated axons. Atypical mitochondria were characterized by a large area with a double membrane, pronounced swelling, and/or vacuolization. Scale bar: 1 µm.

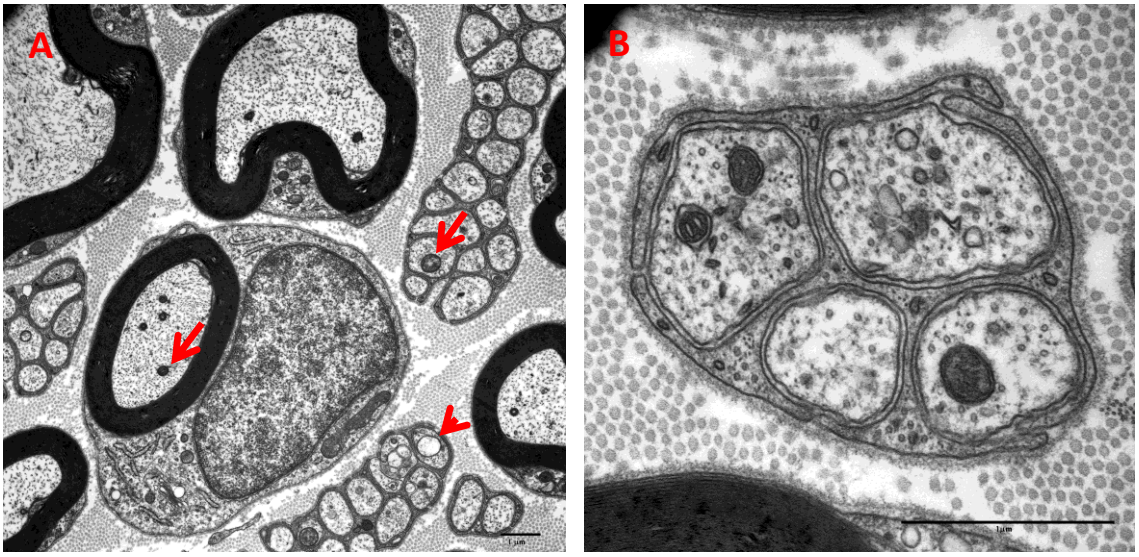

Fig. 3. Transmission electron micrographs of myelinated and unmyelinated fibers from σ_1 receptor knockout (σ_1-KO) control mice. **(A)** Cross-section of the saphenous nerve from σ_1-KO control mice observed at 7000x. Myelinated fibers show normal (*arrow*) mitochondria and Schwann cells. Axons from unmyelinated fibers present normal (*arrow*) and atypical (*arrowhead*) mitochondria similar to control wild-type mice. **(B)** High magnification of unmyelinated C-fiber from control σ_1-KO mice. Note the normal appearance of mitochondria and circular microtubule profile in axons. Scale bar: 1 µm.

Morphometric measurement of the mitochondrial area showed an ample range of values (2 to > 60 x 10^{-2} µm^2) in all types of saphenous nerve fiber, both in WT and σ_1-KO control mice. The mean areas of the mitochondria of each fiber type were virtually identical in both WT mice (10.06 ± 0.2; 10.4 ± 0.3 and 13.02 ± 0.4 x 10^{-2} µm^2, for Aβ, Aδ and C fibers, respectively) and σ_1-KO controls (10.64 ± 0.2; 10.81 ± 0.4 and 13.36 ± 0.5 x 10^{-2} µm^2, for Aβ, Aδ and C fibers, respectively). However, in both WT and σ_1-KO control mice, the mean mitochondrial area was significantly larger ($p < 0.001$) in unmyelinated C-fibers than in either type of myelinated fiber.

Detailed analysis of the mitochondrial area distribution allowed three different populations to be distinguished: small (area ≤ 9 x 10^{-2} µm^2), medium (9.1-18 x 10^{-2} µm^2) and large (> 18 x 10^{-2} µm^2) in both WT and σ_1-KO control mice (Figs. 4A and

4B). In Aβ fibers, most mitochondria were small or medium (96.8 ± 1.2% of total mitochondria in WT mice and 91.7 ± 1.9% in σ_1-KO mice, $P > 0.05$), with a slightly higher percentage of medium- than small-area mitochondria (Figs. 4A and 4B). Likewise, most mitochondria in Aδ fibers were small or medium (96.1 ± 2.9% in WT mice and 95.6 ± 2.5% in σ_1-KO mice, $P > 0.05$), with a higher percentage of medium- than small-area mitochondria in both groups (Figs. 4A and 4B). Small and medium mitochondria were less predominant in unmyelinated C-fibers, (78.9 ± 5.7% in WT mice and 70.9 ± 5.1% in σ_1-KO mice, $P > 0.05$) (Figs. 4A and 4B). The percentage of large mitochondria was 5- to 6-fold higher in C-fibers than in myelinated fibers in both WT and σ_1-KO (Figs. 4A and 4B). All mitochondria included in the large-area group ($> 18 \times 10^{-2}$ μm^2) were characterized by a double membrane, pronounced swelling (2- to 6-fold enlargement of mean area, with diameter > 480 nm), and/or vacuolization and can be considered atypical mitochondria.

In summary, the results of the morphometric analyses show: A) no significant differences between mitochondrial populations in WT and σ_1-KO control mice in any of the three fiber types, and B) a higher percentage of large mitochondria in unmyelinated than in myelinated fibers in both types of animal.

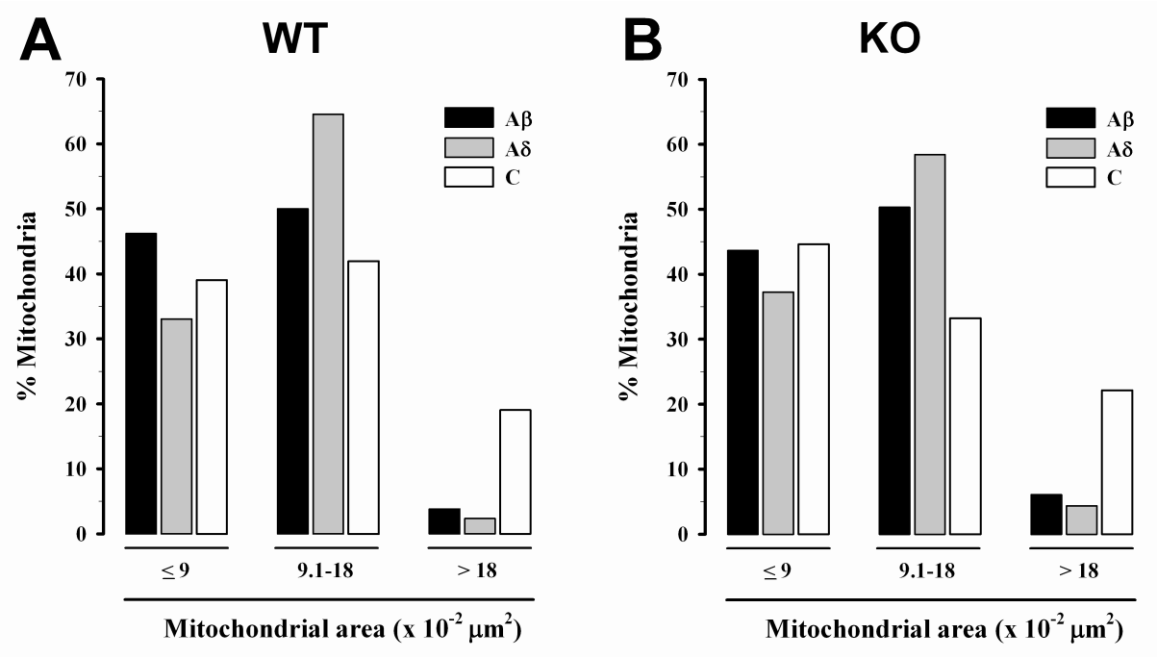

Fig. 4. Comparison of the mitochondrial area distribution of all types of saphenous nerve fibers in control wild-type **(A)** and σ_1 receptor knockout (KO) **(B)** mice. Morphometric analysis of the mitochondrial area showed a wide range of values (2 to >60) in all types of nerve fiber and allowed classification of the mitochondria in 3 groups: small ($\leq 9 \times 10^{-2}$ μm^2), medium (9.1-18 x 10^{-2} μm^2) and large (>18 x 10^{-2} μm^2). Bars represent the percentage of each type of mitochondria in Aβ-, Aδ- and C-fibers.

3.4.3. Electronic microscopy analysis of saphenous nerve fibers in paclitaxel-treated WT and σ_1-KO mice

Ultrastructural study of saphenous nerves from paclitaxel-treated WT and σ_1-KO mice showed an absence of significant morphological alterations in Schwann cells in both myelinated and unmyelinated fibers. In addition, there were no myelin degeneration phenomena or aggregates of neurotubules. However, mitochondrial alterations were observed especially in WT mice, at day 10 from the start of paclitaxel treatment. These alterations were associated with an increased size and vacuolization of mitochondria, resulting in an increase in mitochondrial area (Fig. 5). Analysis of the distribution of mitochondrial area in mice at 10 and 28 days of paclitaxel treatment showed that the only change that was statistically significant was in the large-area (> 18

x 10^{-2} μm^2) mitochondrial subset. Consequently, we analyzed in detail this population of mitochondria as the target population for comparisons among the experimental groups.

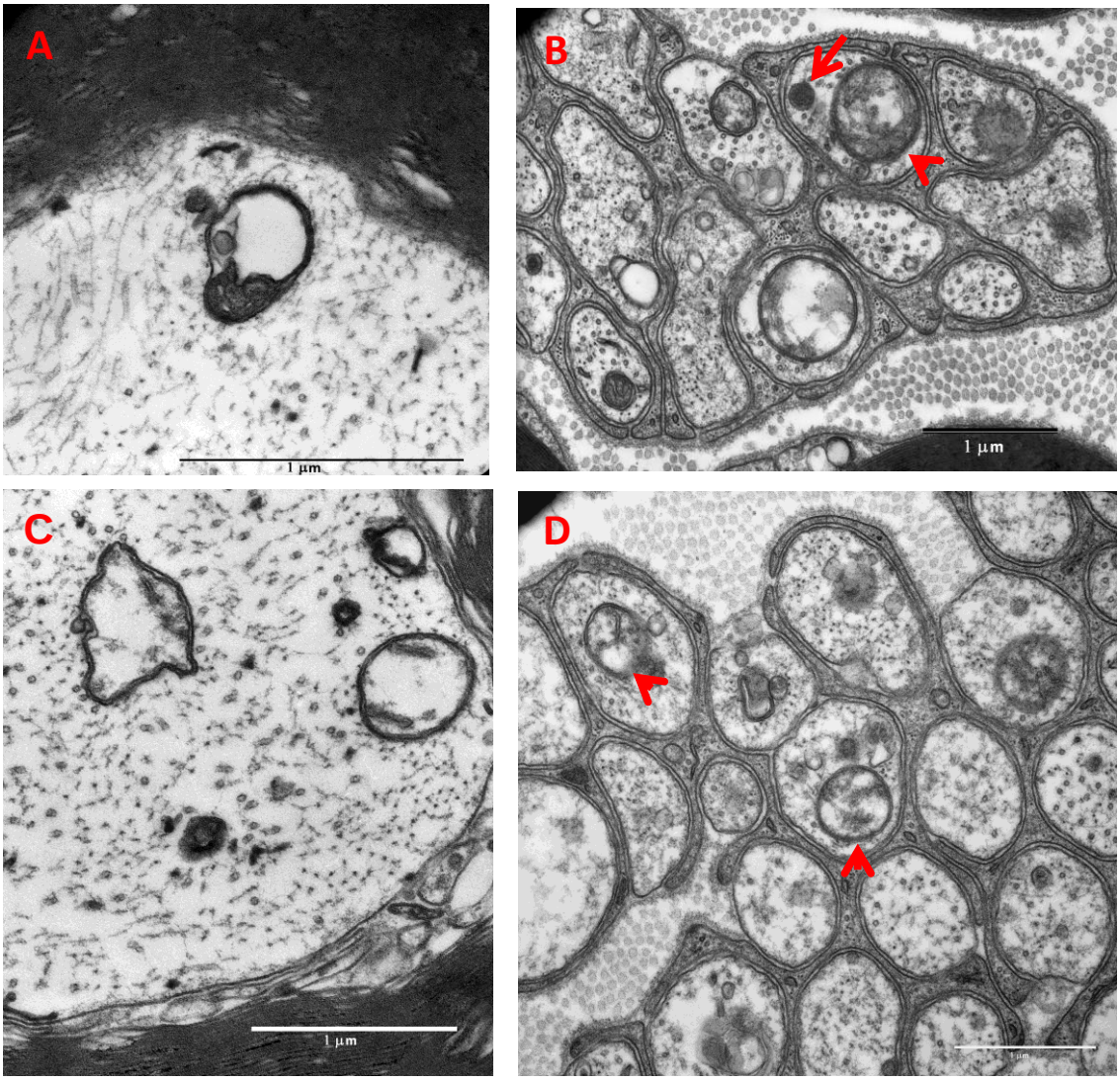

Fig. 5. Transmission electron micrographs of atypical mitochondria in myelinated and unmyelinated fibers of the saphenous nerve from WT **(A, B)** and σ_1-KO **(C, D)** mice treated with paclitaxel + saline. **(A)** Atypical mitochondria, i.e., with a double membrane, pronounced swelling (2- to 6-fold enlargement of mean area) and/or vacuolization, in Aβ fiber from WT mice. Note the collapsed cristae at one pole of the swollen mitochondria. **(B)** Atypical mitochondria in C-fiber. Some axons show simultaneously normal (*arrow*) and swollen (*arrowhead*) mitochondria. **(C)** Atypical mitochondria (swollen and vacuolated) in myelinated axon from σ_1-KO mice. Note the circular microtubule profile in axon. **(D)** Unmyelinated C-fibers from paclitaxel-treated σ_1-KO mice showing swollen and vacuolated mitochondria (*arrowheads*). Scale Bar: 1 µm.

Figure 6 depicts the percentage of large mitochondria in the Aβ, Aδ, and C-fibers of WT and σ_1-KO mice before any treatment and at 10 and 28 days after the first dose of paclitaxel or its vehicle. Treatment with the paclitaxel vehicle produced no statistically significant change in the percentage of large mitochondria in any of the three fiber types in either WT or σ_1-KO mice (Figs. 6A, 6B and 6C). However, at 10 days after the first administration of paclitaxel, a statistically significant and substantial increase in the percentage of large mitochondria was observed in the Aβ fibers of WT mice (from 3.15 ± 1.19% in pretreatment group to 20.26 ± 3.57% at day 10 posttreatment, a posttreatment:pretreatment ratio of 6.43) (Fig. 6A). In contrast, the percentage of large mitochondria in the Aβ fibers of σ_1-KO mice showed only a small and non-significant increase after 10 days of paclitaxel treatment (posttreatment/pretreatment ratio = 1.81) (Fig. 6A). Paclitaxel treatment also produced an increase in the percentage of large mitochondria in the Aδ fibers of WT mice (posttreatment/pretreatment ratio = 3.80) and a very small increase in σ_1-KO mice (posttreatment/pretreatment ratio = 1.33) (Fig. 6B), although statistical significance was not reached in either case. After 10 days of paclitaxel treatment, only small and non-significant increases were observed in the percentage of large mitochondria in the unmyelinated C-fibers of WT (posttreatment/pretreatment ratio = 1.63) and σ_1-KO mice (posttreatment/pretreatment ratio = 1.65) (Fig. 6C).

At 28 days of paclitaxel treatment, no statistically significant changes were observed in the percentage of large mitochondria in myelinated or unmyelinated fibers in either WT or σ_1-KO mice (Figs. 6A, 6B and 6C).

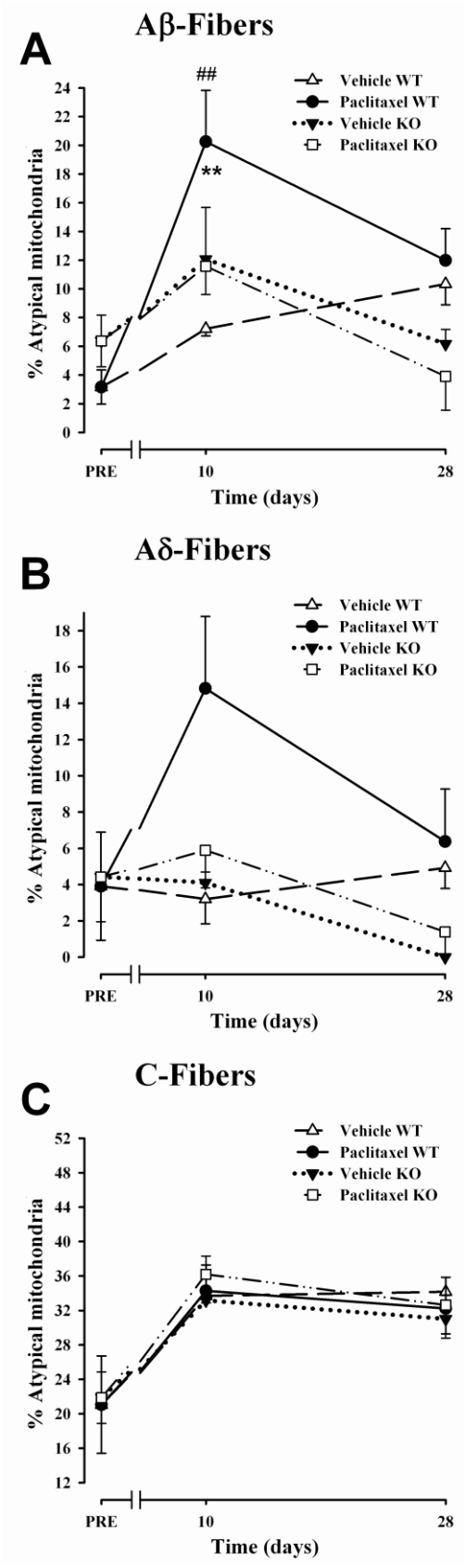

Fig. 6. Time-course of the effect on the percentage of atypical mitochondria of the treatment with paclitaxel or its vehicle on Aβ-, Aδ- and C-fibers (**A**, **B** and **C**, respectively) of saphenous nerves from wild-type (WT) and σ_1 receptor knockout (KO) mice. Each point and vertical line represent the mean ± SEM of the percentage of atypical mitochondria relative to the total number of mitochondria in each type of fiber at day PRE (before treatment) and at days 10 and 28 posttreatment (n=3-5 animals per day). Statistically significant differences between WT and σ_1-KO mice on the same day after paclitaxel treatment: ** $p < 0.01$; and between the values on the pretreatment day and the days after treatment: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

3.4.4. Electronic microscopy analysis of mitochondria in WT mice treated with paclitaxel and the σ_1 receptor antagonist BD-1063

Figure 7 depicts the effects of treatment with paclitaxel and BD-1063 on the large (> 18 x 10^{-2} μm^2) mitochondria subset in myelinated and unmyelinated fibers of WT mice. Administration of BD-1063 before each paclitaxel dose completely prevented the paclitaxel-induced increase in percentage of large mitochondria in myelinated fibers of WT mice (Figs. 7A and 7B) but produced a statistically significant increase in the percentage of large mitochondria in the C-fibers of these mice (Fig. 7C).

3.5. DISCUSSION

The main finding of this study was that the pharmacological blockade or genetic knockout of σ_1 receptor prevented the increased incidence of atypical axonal mitochondria in saphenous nerve Aβ- and Aδ-fibers and the neuropathic pain signs associated with the administration of paclitaxel in mice. These findings suggest, for the first time, an involvement of the σ_1 receptor in the paclitaxel-evoked mitochondrial abnormalities that appear to be important in the physiopathology of paclitaxel-induced neuropathy.

We confirm here that paclitaxel induces cold and mechanical allodynia in WT mice (Nieto et al., 2008, 2011). However, when activation of σ_1 receptor was hindered through a genetic or pharmacologic approach, the development of paclitaxel-induced allodynia was completely prevented, suggesting a key role for the σ_1 receptor in this type of neuropathic pain. These results are in agreement with those of previous behavioral studies demonstrating that σ_1-KO mice (Cendán et al., 2005b; Entrena et al.,

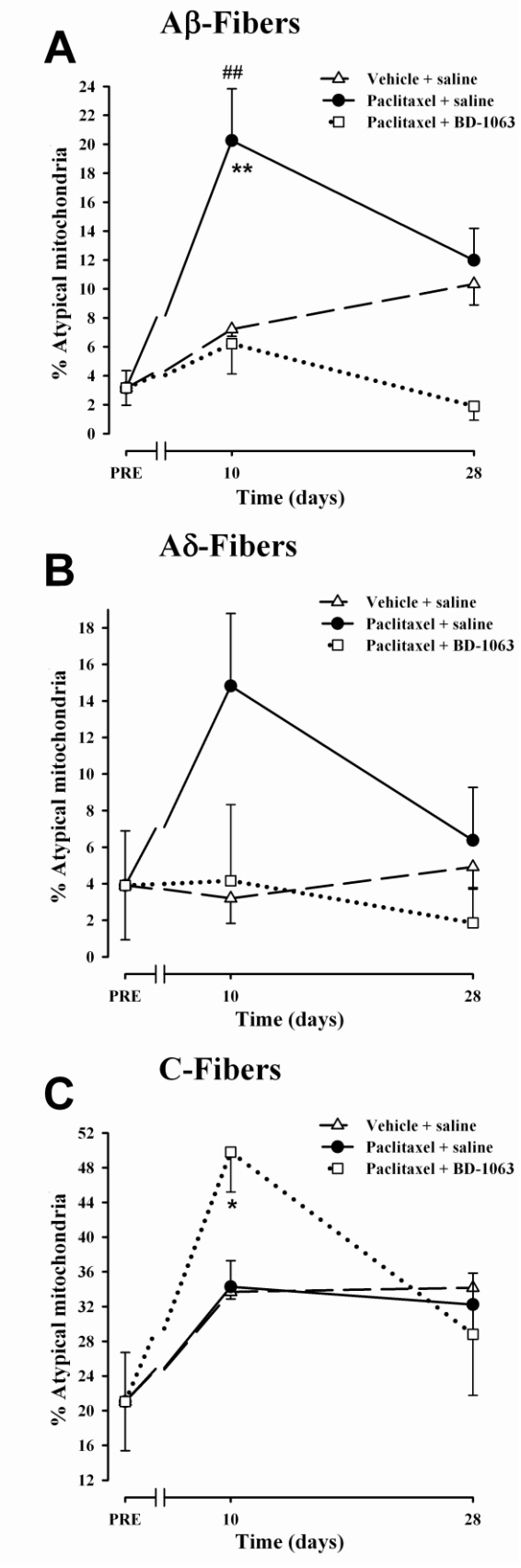

Fig. 7. Time-course of the effect of treatment with paclitaxel + saline, paclitaxel-vehicle + saline, or paclitaxel + BD-1063 on the percentage of atypical mitochondria in Aβ-, Aδ- and C-fibers (**A**, **B** and **C**, respectively) of saphenous nerves from WT mice. Each point and vertical line represent the mean ± SEM of the percentage of atypical mitochondria relative to the total number of mitochondria in each type of fiber before treatment (PRE) and at days 10 and 28 posttreatment (n=3-5 animals per day). Statistically significant differences between WT mice treated with paclitaxel + saline and the rest of the groups on the same day after treatment: ** $p < 0.01$, * $p < 0.05$; and between the values on the pretreatment day and the days after treatment: ## $p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

2009b; De la Puente et al., 2009) and WT animals pretreated with σ_1 receptor antagonists (Cendán et al., 2005a; Kim et al., 2006; Roh et al., 2008; Entrena et al., 2009a; Son et al., 2010; Romero et al., 2011; Nieto et al., 2011) showed a marked reduction of pain in different models that activate central sensitization mechanisms. Therefore, the behavioral data presented here and previously by our group and others strongly support the involvement of σ_1 receptor in modulating pain, especially neuropathic pain.

Our paclitaxel treatment schedule induced an increase in the frequency of large mitochondria (area > 18 x 10^{-2} μm^2) in mice saphenous nerve. We considered large mitochondria to be atypical because they were always swollen (diameter >480 nm) and/or vacuolated. These criteria are very similar to those used in previous reports that also found an increased incidence of atypical axonal mitochondria in peripheral nerves of rats with paclitaxel- (Flatters and Bennett, 2006; Jin et al., 2008) and oxaliplatin-induced neuropathy (Xiao and Bennett, 2010). An augmentation of swollen and/or vacuolated mitochondria has also been reported in the peripheral nerves (Carson et al., 1980) and DRGs (Rusell et al., 1999) of animals with diabetic neuropathy and in the sural nerves of patients with painful peripheral neuropathy induced by 2´3´-dideoxycitidine (ddC) and HIV infection (Dalakas et al., 2001). Hence, these mitochondrial structural alterations may be a common characteristic of these kinds of peripheral neuropathy.

We found a low incidence of axonal atypical mitochondria in naive mice, which appears to be a common observation in normal animals fixed with aldehydes (Rusell et al., 1999; Flatters and Bennett, 2006). It is highly unlikely that the paclitaxel-induced increase in the percentage of large mitochondria was due to an unsuitable fixation, given

that these mitochondria conserved their double membrane, their neighboring microtubules were well preserved, and the mitochondria from other cells (Schwann cells, fibroblasts, endothelial cells) were normal. In addition, we processed all nerves using the same methodology; consequently, if there was a fixation problem, the percentage of atypical mitochondria would be similar in all groups, regardless of treatment, and this was not the case. Therefore, the abnormal incidence of atypical mitochondria was a neurotoxic effect of paclitaxel and not a fixation artifact.

We also found an increase, although not statistically significant, in atypical mitochondria in the saphenous nerves of mice treated with paclitaxel vehicle. This is not surprising because one of its main components, Cremophor EL, is not totally innocuous (Gelderblom et al., 2001). Flatters and Bennett (2006) found a similar increase in vehicle groups, and it has been reported that Cremophor EL can directly damage mitochondria (Sanchez et al., 2001).

We found that paclitaxel induced a significant increase in atypical axonal mitochondria in A-fibers but not C-fibers in WT mouse saphenous nerves. The time-course of the mitochondrial and behavioral alterations were similar, with both being evident on day 10 and resolved by day 28. An increase in atypical mitochondria (also in parallel with the time-course of behavioral changes) was previously reported in paclitaxel-treated rats, in which both myelinated and unmyelinated fibers were affected; furthermore, the behavioral and mitochondrial changes were more delayed than in mice, with both peaking at 27 days (Flatters and Bennett, 2006; Jin et al., 2008). Differences in mitochondria counting methods, paclitaxel schedules, and/or between species may explain this discrepancy. Interestingly, the greatest mitochondrial damage in our mice was in large myelinated (Aβ) fibers and, although paclitaxel can affect all sensory

neurons, it mainly impairs Aβ fibers in humans (Dougherty et al., 2004) and mice (Matsumoto et al., 2006). Therefore, our results are in at least partial agreement with the findings by Bennett´s group in rats and suggest that paclitaxel-induced neuropathic pain may result from an impairment of axonal mitochondria. In fact, functional impairment of mitochondria was very recently reported in peripheral nerves from paclitaxel- and oxaliplatin-treated rats (Zheng et al., 2011).

Previous studies in mice (Mimura et al., 2000; Ogawa et al., 2001; Carozzi et al., 2010) and rats (Cavaletti et al., 1995; Cavaletti et al., 1997; Kilpatrick et al., 2001; Persohn et al., 2005) found evidence of axonal degeneration or alterations in Schwann cells or microtubules after paclitaxel administration. None of these structural irregularities were observed in the present study, probably because the single and cumulative doses used by us were markedly below those administered in the above studies (single dose, 2 vs. 5-50 mg/kg; cumulative dose, 10 vs. 20-280 mg/kg). This explanation is supported by the absence of these structural anomalies in other studies using similarly low doses (single dose, 2 mg/kg; cumulative dose 8 mg/kg) (Flatters and Bennett, 2006; Xiao et al., 2011).

The genetic inactivation (σ_1-KO mice) or pharmacological blockade (σ_1 receptor antagonist) of the σ_1 receptor prevented paclitaxel-induced mitochondrial abnormalities and neuropathic pain signs in a parallel manner. However, paclitaxel-treated WT mice (with a normal σ_1 receptor function) developed a prominent allodynia in parallel with the mitochondrial abnormalities. This suggests that σ_1 receptor must be present and play a key functional role in the development of paclitaxel-induced painful neuropathy and atypical mitochondria. Therefore, the prophylactic effect of σ_1 receptor antagonists such as BD-1063 (present work; Nieto et al., 2011) and S1RA (Nieto et al., 2011) on the

development of paclitaxel-induced cold and mechanical allodynia may be related to the prevention of these mitochondrial abnormalities. These data support the proposal of selective σ_1 receptor antagonists as a novel approach to the treatment of neuropathic pain (Diaz et al., 2009).

The mechanisms by which paclitaxel cause the mitochondrial abnormalities are unclear. It has been suggested that the mitotoxic effect of paclitaxel may derive from its binding to the β-tubulin associated with the voltage-dependent anion channel (VDAC) (Zheng et al., 2011). VDAC is the most abundant protein in the mitochondrial outer membrane (Tan and Colombini, 2007) and may open the mitochondrial permeability transition pore (mPTP). This would eventually produce alterations in mitochondrial function and structure, including the release of accumulated Ca^{2+} and mitochondrial swelling (reviewed by Halestrap, 2009). Several *in vitro* studies found that paclitaxel induced swollen and vacuolated mitochondria (Varbiro et al., 2001; Andre et al., 2000; Jiang et al., 2011) and Ca^{2+} release from mitochondria (Mironov et al., 2005; Kidd et al., 2002), which were blocked by the mPTP inhibitor cyclosporin A. A further possible explanation of paclitaxel-induced mitochondrial abnormalities is the indirect regulation of mPTP opening through the drug's action on bcl-2. It has been reported that paclitaxel directly binds to bcl-2, reversing its function as an mPTP opening blocker and thereby facilitating the opening of the mPTP (Ferlini et al., 2009). Taken together, these data suggest that paclitaxel may induce mPTP opening by binding to the β-tubulin associated with VDAC and/or to bcl-2, which would augment the mitochondrial size and increase the release of Ca^{2+} to the cytoplasm from the mitochondria. This abnormal intracellular Ca^{2+} increase may activate multiple calcium-dependent kinases and trigger a central sensitization process, resulting in pain behavior (Latremoliere and Woolf, 2009).

The σ_1 receptor chaperone is highly concentrated at the mitochondrion-associated endoplasmic reticulum membrane (MAM) and participates in the modulation of Ca^{2+} homeostasis (Su et al., 2010). In particular, σ_1 receptor can chaperone inositol 1,4,5-triphosphate receptors IP_3 (IP_3R) and increase the Ca^{2+} flux into mitochondria (Hayashi and Su, 2007). Consequently, the absence or blockade of σ_1 receptor may prevent the mitochondrial Ca^{2+} overload and impede the resulting mPTP opening. In addition, bcl-2 is also highly enriched at the MAM and interacts with IP_3R to inhibit the channel opening of IP_3R as well as the Ca^{2+} overloading (Rong et al., 2008). In this context, the σ_1 receptor is known to tonically regulate the expression of bcl-2 proteins, because bcl-2 mRNA is downregulated by knockdown of this receptor and upregulated by its overexpression (Meunier and Hayashi, 2010). Hence, σ_1 receptor may also indirectly regulate mPTP opening. In addition, σ_1 receptor may translocate from MAM to the endoplasmic reticulum network and plasma membrane and regulate several functional proteins, including ion channels, receptors, and kinases (Su et al., 2010). It has been proposed that activation of σ_1 receptor increases the intracellular Ca^{2+} concentration by enhancing the entry of Ca^{2+} at plasma membrane level (NMDA-induced Ca^{2+} influx) and its mobilization from endoplasmic stores (IP_3-induced Ca^{2+} mobilization) (De la Puente et al., 2009). Therefore, the absence or blockade of σ_1 receptor may compensate for the enhancement of intracellular Ca^{2+} derived from paclitaxel-induced mPTP opening and prevent its concentration from reaching a threshold level that would trigger the central sensitization process and neuropathic pain.

In conclusion, we found that the pharmacological blockade (σ_1 receptor antagonists) or genetic inactivation of σ_1 receptor (knockout mice) prevents the sensory nerve mitochondrial abnormalities induced by paclitaxel in parallel with the prevention

of neuropathic pain development. These findings suggest that σ_1 receptor antagonists might have therapeutic value for the prevention of paclitaxel-induced neuropathic pain.

3.6. REFERENCES

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

CONCLUSIONS

SPECIFIC CONCLUSIONS

1. The repeated administration of paclitaxel induces long-lasting neuropathic pain in female mice, which manifests as cold allodynia, mechanical allodynia, and heat hyperalgesia.

2. Acute treatment with the voltage-gated sodium channel blocker tetrodotoxin (TTX) inhibits the expression of heat hyperalgesia and cold and mechanical allodynia induced by paclitaxel. However, TTX does not affect the response to heat, cold, and mechanical stimuli in control animals (not treated with paclitaxel).

3. Preventive treatment with TTX inhibits the paclitaxel-induced development of cold and mechanical allodynia but not that of heat hyperalgesia.

4. The selective σ_1 receptor antagonists BD-1063 and S1RA dose-dependently inhibit the expression of paclitaxel-induced cold and mechanical allodynia in wild-type mice. However, neither antagonist affects the response to cold and mechanical stimuli in control animals (not treated with paclitaxel).

5. The prophylactic administration of either BD-1063 or S1RA prevents the paclitaxel-induced development of cold and mechanical allodynia in wild-type mice. Likewise, mice lacking σ_1 receptor do not develop neuropathic pain after paclitaxel administration.

6. Paclitaxel-induced neuropathic pain is associated with an increase in the percentage of swollen and/or vacuolated mitochondria in saphenous nerve myelinated fibers in wild-type mice.

7. The pharmacological blockade (σ_1 receptor antagonists) or genetic inactivation of σ_1 receptor (knockout mice) prevents the sensory nerve mitochondrial abnormalities induced by paclitaxel in parallel with the prevention of neuropathic pain development.

GENERAL CONCLUSIONS

1. The repeated administration of paclitaxel to mice mimics some signs of paclitaxel-induced neuropathy in humans and represents a suitable model to explore potential therapeutic targets against this type of neuropathic pain.

2. The opening of TTX-sensitive voltage-gated sodium channels appears to play an important role in the generation and maintenance of neuropathic pain signs induced by paclitaxel (especially signs mediated by myelinated sensitive fibers).

3. An endogenous σ_1 receptor-activating system appears to play a pivotal facilitatory role in the mechanisms underlying the development and expression of paclitaxel-induced neuropathic pain signs and mitochondrial abnormalities in sensitive myelinated fibers.

4. TTX-sensitive voltage-gated sodium channels and σ_1 receptors do not appear to play a relevant role in nociceptive/physiologic pain, which (together with the above conclusions) suggest that these targets are activated during paclitaxel-induced neuropathy but not in a physiological situation.

5. Blockers of the two pharmacological targets explored in this Doctoral Thesis, the TTX-sensitive voltage-gated sodium channels and the σ_1 receptors, might be of therapeutic interest against paclitaxel-induced neuropathic pain, and their study in humans appears to be warranted.

CONCLUSIONES

CONCLUSIONES ESPECÍFICAS

1. La administración repetida de paclitaxel induce dolor neuropático de larga duración en ratones hembra, que se manifiesta por alodinia al frío, alodinia mecánica e hiperalgesia al calor.

2. El tratamiento agudo con el bloqueante de los canales de sodio dependientes de voltaje tetrodotoxina (TTX) inhibe la expresión de la hiperalgesia al calor, de la alodinia al frío y de la alodinia mecánica inducidas por paclitaxel. Sin embargo, la TTX no afecta a la respuesta al calor, al frío o a estímulos mecánicos de los animales controles (no tratados con paclitaxel).

3. El tratamiento preventivo con TTX inhibe el desarrollo de la alodinia al frío y de la alodinia mecánica, pero no el de la hiperalgesia al calor.

4. Los antagonistas selectivos del receptor σ_1 BD-1063 y S1RA inhiben, de manera dosis-dependiente, la expresión de la alodinia al frío y de la alodinia mecánica inducidas por paclitaxel en ratones salvajes. Sin embargo, ninguno de estos antagonistas afecta la respuesta a estímulos fríos y mecánicos en animales controles (no tratados con paclitaxel).

5. La administración profiláctica tanto de BD-1063 como de S1RA previene el desarrollo de la alodinia al frío y de la alodinia mecánica inducidas por paclitaxel en ratones salvajes. Asimismo, los ratones desprovistos del receptor σ_1 no desarrollan dolor neuropático tras la administración de paclitaxel.

6. El dolor neuropático inducido por paclitaxel está asociado con un incremento en el porcentaje de mitocondrias hinchadas y/o vacuolizadas en las fibras mielínicas del nervio safeno en animales salvajes.

7. El bloqueo farmacológico (con antagonistas del receptor σ_1) o la inactivación genética del receptor σ_1 (con ratones "knockout") previene las anomalias mitocondriales del nervio sensorial inducidas por paclitaxel, en paralelo con la prevención del desarrollo del dolor neuropático.

CONCLUSIONES GENERALES

1. La administración repetida de paclitaxel a ratones reproduce algunos signos característicos de la neuropatía inducida por paclitaxel en humanos y representa un modelo adecuado para explorar dianas terapéuticas potenciales contra este tipo de dolor neuropático.

2. La apertura de los canales de sodio dependientes de voltaje sensibles a TTX parece jugar un papel importante en la generación y el mantenimiento de los signos del dolor neuropático inducidos por paclitaxel (especialmente aquellos mediados por fibras sensitivas mielínicas).

3. Un sistema endógeno activado por el receptor σ_1 parece jugar un papel facilitador esencial de los mecanismos responsables del desarrollo y expresión de los signos del dolor neuropático y de las anomalías mitocondriales de las fibras mielínicas sensitivas inducidos por paclitaxel.

4. Los canales de sodio dependientes de voltaje y los receptores σ_1 no parecen jugar un papel relevante en el dolor nociceptivo/fisiológico, lo cual (junto con las conclusiones anteriores) sugiere que estas dianas son activadas durante la neuropatía inducida por paclitaxel, pero no en una situación fisiológica.

5. Los bloqueantes de las dos dianas farmacológicas exploradas en esta Tesis Doctoral, los canales de sodio dependientes de voltaje y los receptores σ_1,

podrían ser de interés terapéutico contra el dolor neuropático inducido por paclitaxel, y su estudio en humanos parece estar justificado.

LIST OF ABBREVIATIONS

Aβ fiber: A-beta fiber

ABC transporters: ATP-binding cassette transporters

Aδ fiber: A-delta fiber

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

ANOVA: analysis of variance

ATF3: activating yranscription factor 3

ATP: adenosine triphosphate

Bcl-2 family: (B-cell lymphoma 2) family of apoptosis regulator proteins

Bcl-2 protein: member of the antiapoptotic group of the Bcl-2 family

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

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

Ca^{2+}: calcium

$Ca\alpha_2\delta$-1: $\alpha_2\delta$-1 subunit of voltage-dependent calcium channel

CB1: cannabinoid receptor 1

CB2: cannabinoid receptor 2

CGRP: calcitonin gene-related peptide

CNS: central nervous system

Cremophor EL®: polyoxyethyleneglycerol triricinoleate 35 (polyoxyethylated castor oil)

CYP450: cytochrome P450

ddc: 2',3'-dideoxycytidine (ddC

DMSO: dimethylsulfoxide

DRG: dorsal root ganglion

E-52862: 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine

ERK: extracellular signal-regulated kinase

FDA: Food and Drug Administration of the United States

G2 phase: the final subphase of interphase in the cell cycle

GABA: gamma-amino butyric acid

GDNF: glial cell line-derived neurotrophic factor

GFAP: glial fibrillary acidic protein

GTP: Guanosine triphosphate

H-1 antagonists: histamine antagonist of the H-1 receptor

HIV: human immunodeficiency virus

IASP: International Association for the Study of Pain

IENF: intraepidermal nerve fibers

IL: interleukin

i.p.: intraperitoneal

IP_3: inositol 1,4,5-trisphosphate

IP_3R: IP_3 receptors

i.t.: intrathecal

i.v.: intravenously

K^+: potassium

KO: knockout

LTP: long-term potentiation

M phase: mitosis phase

MAM: mitochondrion-associated endoplasmic reticulum membrane

MDR proteins: multidrug resistance proteins

MK-801: dizocilpine

MMP: matrix metalloproteinase-3

mPTP: mitochondrial permeability transition pore

mRNA: messenger ribonucleic acid

Na^+: sodium

Na_v: sodium channel alpha-subunit

NCI: National Cancer Institute of the United States

NGF: nerve growth factor

NK: neurokinin

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

NR1: NMDA receptor 1 subunit

Nur77: member of the Nur nuclear receptor family of intracellular transcription factors

P_2 fraction: crude synaptosomal/mitochondrial fraction

P53: tumor suppressor protein

Paclitaxel: (Taxol®) $C_{47}H_{51}NO_{14}$; 5β,20-epoxy-1,2β,4,7β,-10β,13α-hexahydroxy-tax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester

PB: phosphate buffer

pERK: phosphorylated extracellular signal-regulated kinase

PKAε: protein kinase A epsilón

PKCε: protein kinase C epsilón

pNR1: phosphorylated NMDA receptor 1 subunit

PNS: peripheral nervous system

PRE: pretreatment

ROS: reactive oxygen species

s.c.: subcutaneuous

σ_1 receptors: sigma-1 receptors

σ_1-KO: σ_1 receptor knockout

SR1A: 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl]

TAD: terminal arbor degeneration

Taxol®: paclitaxel

TCA: tricyclic antidepressants

TNF-α: Tumor necrosis factor-alpha

TRPV-1: transient receptor potential vanilloid type 1

TRPV-4: transient-receptor potential vanilloid type 4

TSPO: translocator protein (previously known as peripheral benzodiazepine receptor)

TTX: tetrodotoxin

Tween-80: polyoxyethylene (20) sorbitan monooleate

VDAC: voltage-dependent anion channel

VGSC: voltage-gated sodium channels

WDR neurons: wide dynamic range neurons

VEGF: vascular endothelial growth factor

WHO: World Health Organization

WT: wild-type

PARAMETERS

B: specific radioligand binding

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

F: free concentration of radioligand.

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

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

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

L: concentration of radioligand used in competition binding assays

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

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APENDIX

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Tetrodotoxin inhibits the development and expression of neuropathic pain induced by paclitaxel in mice

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Abstract

We evaluated the effect of low doses of systemically administered tetrodotoxin (TTX) on the development and expression of neuropathic pain induced by paclitaxel in mice. Treatment with paclitaxel (2 mg/kg, i.p., once daily during 5 days) produced long-lasting (2–4 weeks) heat hyperalgesia (plantar test), mechanical allodynia (electronic Von Frey test) and cold allodynia (acetone drop method), with maximum effects observed on days 7, 10 and 10–14, respectively. Acute subcutaneous treatment with 1 or 3 μg/kg of TTX reduced the expression of mechanical allodynia, whereas higher doses (3 or 6 μg/kg) were required to reduce the expression of cold allodynia and heat hyperalgesia. In contrast, TTX (3 or 6 μg/kg, s.c.) did not affect the response to the same thermal and mechanical stimuli in control animals, which indicates that the antihyperalgesic and antiallodynic effects of TTX were not due to unspecific inhibition of the perception of these stimuli. Administration of TTX (6 μg/kg, s.c.) 30 min before each of the 5 doses of paclitaxel did not modify the development of heat hyperalgesia produced by the antineoplastic, but abolished the development of mechanical and cold allodynia. Coadministration of a lower dose of TTX (3 μg/kg) also prevented the development of mechanical allodynia. No signs of TTX-induced toxicity or motor incoordination were observed. These data suggest that low doses of TTX can be useful to prevent and treat paclitaxel-induced neuropathic pain, and that TTX-sensitive subtypes of sodium channels play a role in the pathogenesis of chemotherapy-induced neuropathic pain.

Keywords: Tetrodotoxin; Paclitaxel; Neuropathic pain; Heat hyperalgesia; Mechanical allodynia; Cold allodynia

1. Introduction

Peripheral neurotoxicity induced by antineoplastic drugs (taxanes, vinca-alkaloids and platin-based compounds) is a clinically significant complication that can be dose-limiting and can substantially diminish quality of life [27,43]. In particular, paclitaxel produces sensory neuropathy with numbness, tingling, burning pain and cold allodynia in a stocking-glove distribution [12,27]. There are no well-established treatments to prevent or minimize paclitaxel-induced nerve damage [27], but models of this neuropathy in rodents [10,14,40,42] have made it possible to test the effect of new treatments.

Voltage-gated sodium channels play a critical role in neuronal function under both physiological and pathological conditions [1,47]. Nine isoforms of the sodium channel alpha-subunit ($Na_v1.1$–1.9) have been identified in mammals [36]. The isoforms differ in their sensitivity to tetrodotoxin (TTX), a nonpeptidic guanidinium toxin. Nanomolar concentrations of TTX block $Na_v1.1$–1.4, and $Na_v1.6$–1.7 subtypes (TTX-sensitive channels), whereas considerably higher (micromolar)

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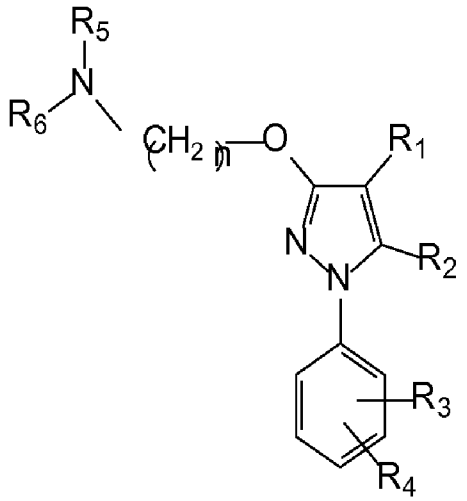

(I)

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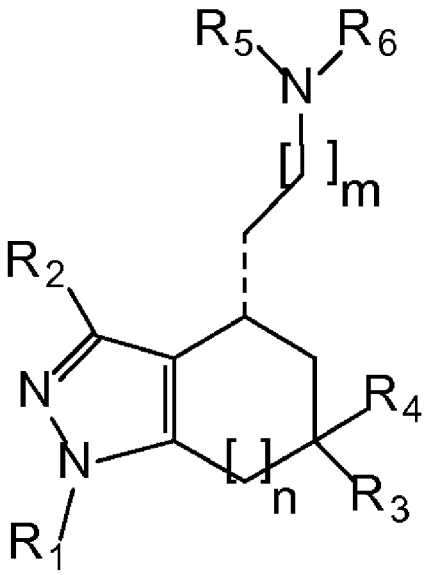

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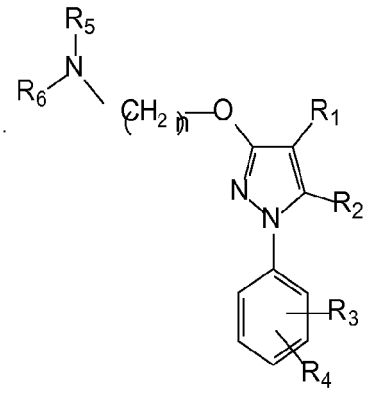
(54) **Sigma ligands for the prevention or treatment of pain induced by chemotherapy**

(57) The invention refers to the use of a sigma ligand of formula (I) to prevent or treat pain induced by a chemotherapeutic agent, especially pain induced by taxanes, vinca alkaloids or platin chemotherapeutic drugs.



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(54) **Sigma ligands for the prevention or treatment of pain induced by chemotherapy**

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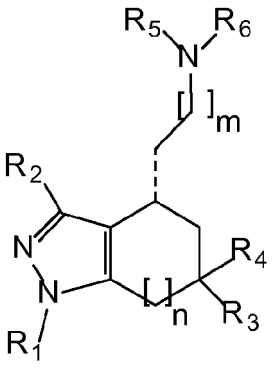

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(54) **Title**: USE OF COMPOUNDS BINDING TO THE SIGMA RECEPTOR LIGANDS FOR THE TREATMENT OF NEUROPATHIC PAIN DEVELOPING AS A CONSEQUENCE OF CHEMOTHERAPY

(57) **Abstract**: The present invention refers to the use of compounds binding to the sigma receptor for the treatment or prevention of neuropathic pain resulting from chemotherapy.

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(54) **USE OF COMPOUNDS BINDING TO THE SIGMA RECEPTOR LIGANDS FOR THE TREATMENT OF NEUROPATHIC PAIN DEVELOPING AS A CONSEQUENCE OF CHEMOTHERAPY**

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(57) **ABSTRACT**

The present invention refers to the use of compounds binding to the sigma receptor for the treatment or prevention of neuropathic pain resulting from chemotherapy.

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Remarks:
The references to the drawing(s) no. 6 are deemed to be deleted (Rule 56(4) EPC).

(54) **Use of compounds binding to the sigma receptor ligands for the treatment of neuropathic pain developing as a consequence of chemotherapy**

(57) The present invention refers to the use of compounds binding to the sigma receptor for the treatment or prevention of neuropathic pain resulting from chemotherapy.

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