UNIVERSITAT POMPEU FABRA

PARTICIPACIÓN DEL SISTEMA CANNABINOIDE ENDÓGENO EN LOS FENÓMENOS DE ADICCIÓN. INTERACCIÓN CON OTROS SISTEMAS DE NEUROTRANSMISIÓN

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CERTIFICAN:

Que la presente Tesis Doctoral titulada “Participación del sistema cannabinoide endógeno en los fenómenos de adicción. Interacción con otros sistemas de neurotransmisión” presentada por Anna Castañé Forn, Licenciada en Farmacia, para optar al grado de Doctor por la Universitat Pompeu Fabra, ha sido realizada bajo su dirección y reúne todos los requisitos necesarios para ser juzgada.

Y para que conste, y a efectos oportunos, firman el presente certificado a 22 de abril de 2005.

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A la memoria de mi padre

A mi madre

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Quisiera expresar mi más sincero agradecimiento...

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ABREVIATURAS

Δ^8-THC	Delta8-tetrahidrocannabinol
Ach	Acetilcolina
AMPc	Adenosina-5'-monofosfato cíclico
ARNm	ARN mensajero
ATV	Área tegmental ventral
Ca^{+2}	Calcio
CCK	Colecistoquinina
CPF	Corteza prefrontal
CRF	Factor liberador de corticotrofina
DA	Dopamina
DOR	Receptor opioide delta
DSE	Supresión de la excitación inducida por despolarización
DSI	Supresión de la inhibición inducida por despolarización
Eje HPA	Eje adrenal hipotálamo-hipofisiario
GABA	Ácido γ-aminobutírico
K^+	Potasio
KOR	Receptor opioide kappa
LTD	Depresión a largo plazo
LTP	Potenciación a largo plazo
MOR	Receptor opioide mu
NAc	Núcleo accumbens
NAChRs	Receptores nicotínicos de acetilcolina
PDIN	Prodinorfina
PENC	Proencefalina
POMC	Proopiomelanocortina
SNC	Sistema nervioso central
THC	Delta9-tetrahidrocannabinol

ÍNDICE

INTRODUCCIÓN

1 SISTEMA CANNABINOIDE

Hace relativamente pocos años que se conoce la existencia de un sistema cannabinoide endógeno, teniendo en cuenta que los preparados derivados de la planta *Cannabis sativa* se han utilizado desde hace miles de años en las diferentes culturas con fines recreativos y terapéuticos (Mechoulam, 1986). El descubrimiento en los años noventa de receptores de membrana a los cuales se unían los compuestos cannabinomiméticos (Matsuda y cols., 1990; Munro y cols., 1993), abrió el camino hacia la comprensión de la farmacología de los cannabinoides, tales como el delta9-tetrahidrocannabinol (THC) (Gaoni y Mechoulam, 1964). Actualmente, los receptores cannabinoides junto con sus ligandos endógenos, enzimas involucradas en la biosíntesis y degradación de estos ligandos y proteínas transportadoras de membrana comprenden un nuevo e importante sistema de neuromodulación en el cerebro, el SISTEMA ENDOCANNABINOIDE (Piomelli, 2003).

1.1 CANNABINOIDES NATURALES Y SINTÉTICOS

En 1964, Gaoni y Mechoulam aislaron e identificaron a partir de la planta *Cannabis sativa* el THC, ingrediente psicoactivo y principal responsable de la actividad farmacológica de los extractos de la marihuana (Gaoni y Mechoulam, 1964). Desde entonces se han descrito más de sesenta compuestos activos en dicha planta (cannabinoides naturales), algunos de los cuales presentan propiedades psicoactivas como el delta8-tetrahidrocannabinol (Δ^8-THC) y el cannabinol y otros no, como es el caso del cannabidiol (Dewey, 1986; Mechoulam y cols., 1992) (figura 1).

Los estudios que relacionan la estructura de los compuestos cannabinoides con su actividad farmacológica han permitido diseñar moléculas con una conformación capaz de fijarse de manera selectiva a los receptores cannabinoides (ver apartado 1.2). Esto ha supuesto el punto de partida para el desarrollo de cannabinoides sintéticos con mejor potencial terapéutico y posible disminución de sus efectos psicoactivos. Actualmente, los cannabinoides sintéticos con actividad agonista incluyen dos grandes grupos de compuestos: (1) los derivados estructurales del THC tales como HU-210, CP-55,940 y nabilona y (2) los aminoalquilindoles, entre los que cabe destacar el WIN 55,212-2 (figura 2). Estos compuestos exhiben diferencias en cuanto a su afinidad y actividad intrínseca para los receptores cannabinoides (Breivogel y cols., 1998; Griffin y cols., 1998; Pertwee, 2001). Comparados con el THC, los compuestos CP-55,940 y WIN 55,212-2 tienen una mayor afinidad para los receptores cannabinoides CB1 y CB2, y su

eficacia para activar ambos receptores es mayor. El CP-55,940 presenta igual afinidad para el receptor CB1 que para el receptor CB2, mientras que el WIN 55,212-2 presenta una afinidad ligeramente mayor para el receptor CB2 (Pertwee, 2001a).

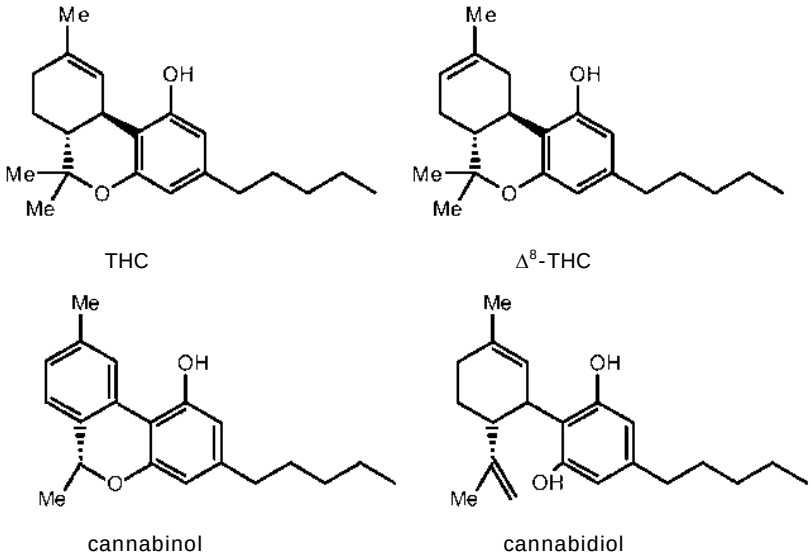

Figura 1. Estructura química de los cannabinoides naturales más importantes. THC, Δ^8-THC y cannabinol (con propiedades psicoactivas) y cannabidiol (sin propiedades psicoactivas).

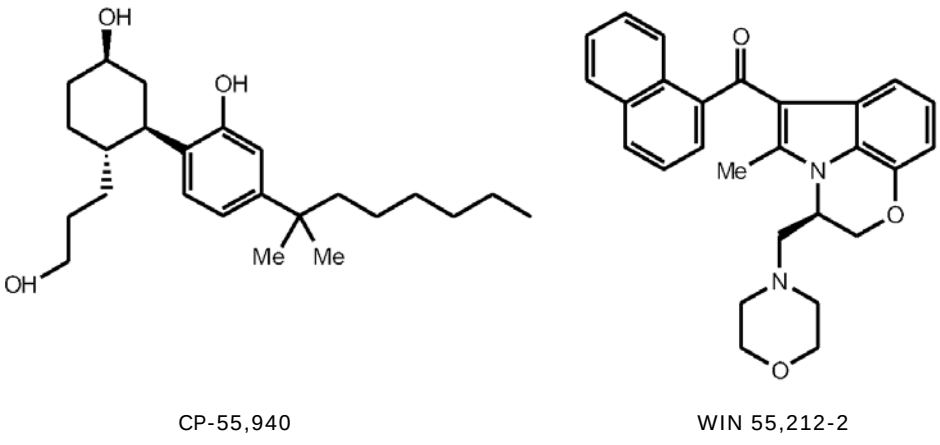

Figura 2. Estructura química de dos compuestos cannabinoides sintéticos. CP-55,940 (derivado estructural del THC) y WIN 55,212-2 (aminoalquilindol).

Los estudios de estructura-actividad también han permitido el desarrollo de antagonistas selectivos para los receptores cannabinoides. Así, el SR141716A (rimonabant) (Rinaldi-Carmona y cols., 1994) y el SR144528 (Rinaldi-Carmona y

cols., 1998) son dos compuestos antagonistas selectivos para los receptores CB1 y CB2 respectivamente.

1.2 RECEPTORES CANNABINOIDES

La primera hipótesis que se barajó para explicar de qué manera los cannabinoides producían sus efectos farmacológicos estuvo basada en la naturaleza química de dichas sustancias. Los cannabinoides son compuestos muy lipofílicos y se pensó que se disolvían en la membrana celular alterando así su función de una manera inespecífica (Hillard y cols., 1985). El desarrollo del ligando sintético CP-55,940 permitió demostrar su capacidad de unión a receptores específicos en membranas de cerebro de rata (Devane y cols., 1988). En 1990, Matsuda y colaboradores clonaron el primer receptor cannabinoide que hoy se conoce como receptor cannabinoide CB1 (CAN1) (Matsuda y cols., 1990). Tres años más tarde, Munro y colaboradores clonaron el segundo subtipo de receptor cannabinoide, llamado receptor cannabinoide CB2 (CAN2) (Munro y cols., 1993). Diversas evidencias farmacológicas sugieren la existencia de un tercer tipo de receptor cannabinoide llamado CB3 o CBx que hasta la fecha no ha sido clonado. Concretamente, se ha demostrado que el efecto vasodilatador del endocannabinoide anandamida (ver apartado 1.3) persiste en ratones deficientes de los receptores CB1 y también en los dobles mutantes CB1/CB2 (Járai y cols., 1999). Además, la anandamida es capaz de modificar la actividad espontánea y el umbral nociceptivo en ratones que carecen del los receptores cannabinoides CB1 (Di Marzo y cols., 2000). Por otro lado, Haller y colaboradores (2002) han observado que el antagonista de los receptores CB1 rimonabant es capaz de producir un efecto ansiolítico tanto en ratones normales como en ratones deficientes de los receptores CB1. Estudios bioquímicos también apoyan la hipótesis de la existencia de un tercer tipo de receptor cannabinoide. Así, la anandamida es capaz de estimular la unión de ^{35}S-GTPγS en preparaciones de cerebro de ratones deficientes de los receptores CB1 sin que este efecto sea inhibido ni por el antagonista de los receptores CB1, rimonabant, ni por el antagonista de los receptores CB2, SR144528 (Di Marzo y cols., 2000). Más recientemente, Breivogel y colaboradores han demostrado que el agonista WIN 55,212-2 es capaz de estimular la unión de ^{35}S-GTPγS en preparaciones de tejido cerebral de animales deficientes de los receptores cannabinoides CB1 (Breivogel y cols., 2001). De acuerdo con estos resultados, este supuesto nuevo receptor cannabinoide sería sensible a WIN 55,212-2, anandamida y rimonabant.

Los receptores cannabinoides CB1 y su variante de “splicing” alternativo $CB1_A$ (Shire y cols., 1995) junto con el receptor CB2 pertenecen a la super familia de

receptores acoplados a proteínas G (metabotrópico), con siete dominios transmembranales (figuras 3 y 4). En humanos, los receptores CB1 y CB2 comparten aproximadamente un 44% de homología en su secuencia de aminoácidos, porcentaje que aumenta hasta el 68% cuando se comparan únicamente los segmentos transmembranales (Lutz, 2002).

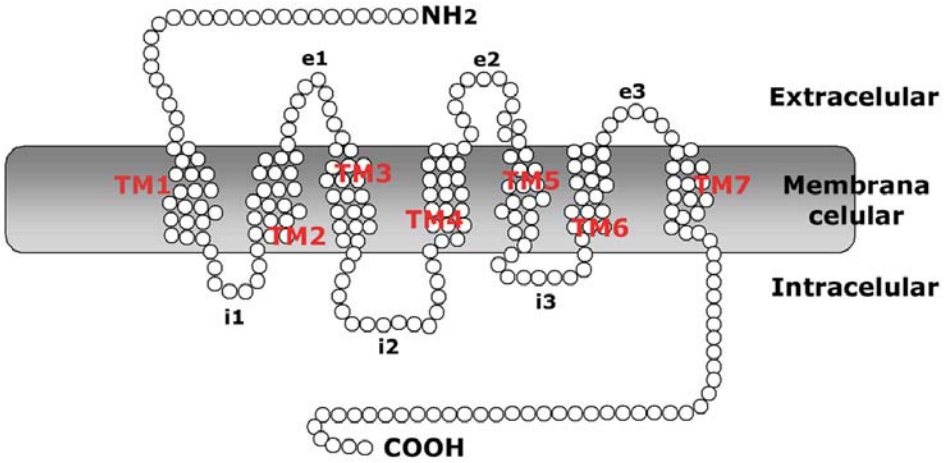

Figura 3. Representación de la estructura peptídica de los receptores acoplados a proteínas G. Abreviaturas: TM: segmento transmenbranal; e: giro extracelular; i: giro intracelular.

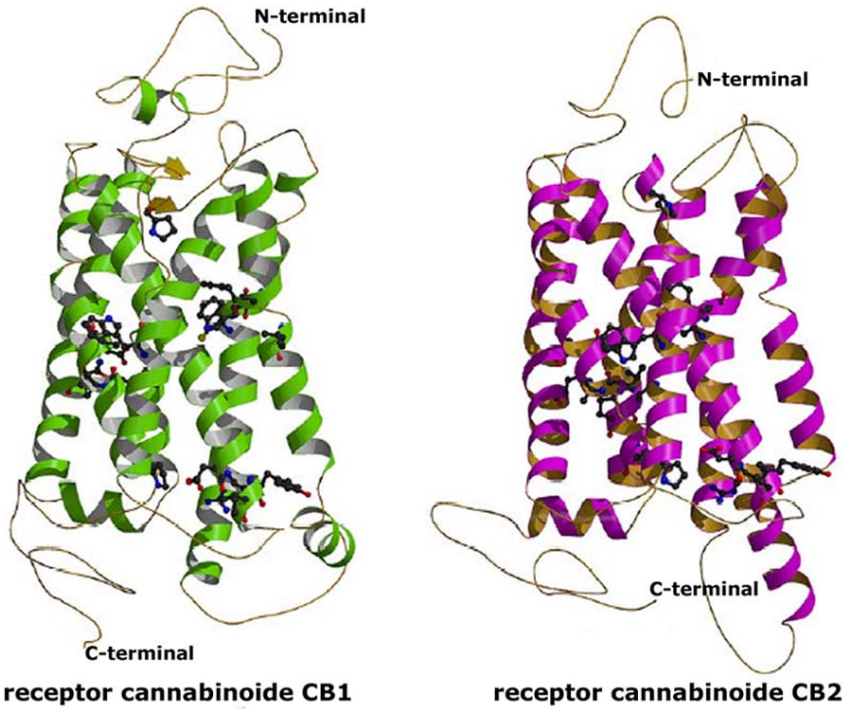

Figura 4. Representación tridimensional de la estructura de los receptores cannabinoides CB1 y CB2 (Modificado de Montero y cols., 2004).

1.2.1 DISTRIBUCIÓN ANATÓMICA DE LOS RECEPTORES CANNABINOIDES

El receptor cannabinoide CB1 se localiza fundamentalmente en el sistema nervioso central (SNC) (Howlett y cols., 2002). Se trata del receptor metabotrópico más abundante en el cerebro (Herkenham y cols., 1991). Mediante estudios autoradiográficos e inmunohistoquímicos se ha podido observar una alta densidad de receptores CB1 en la capa molecular del cerebelo, en el hipocampo y en los ganglios basales, tales como la sustancia negra *pars reticulata*, el núcleo entopeduncular, el globo pálido y el caudado-putamen lateral (figura 5; tabla 1). Otras áreas que presentan niveles más moderados de receptores CB1, pero que destacan por su importancia funcional son el núcleo accumbens (NAc), la corteza cerebral y el asta dorsal de la médula espinal (Herkenham y cols., 1991; Mailleux y Vanderhaeghen, 1992; Pettit y cols., 1998) (tabla 1).

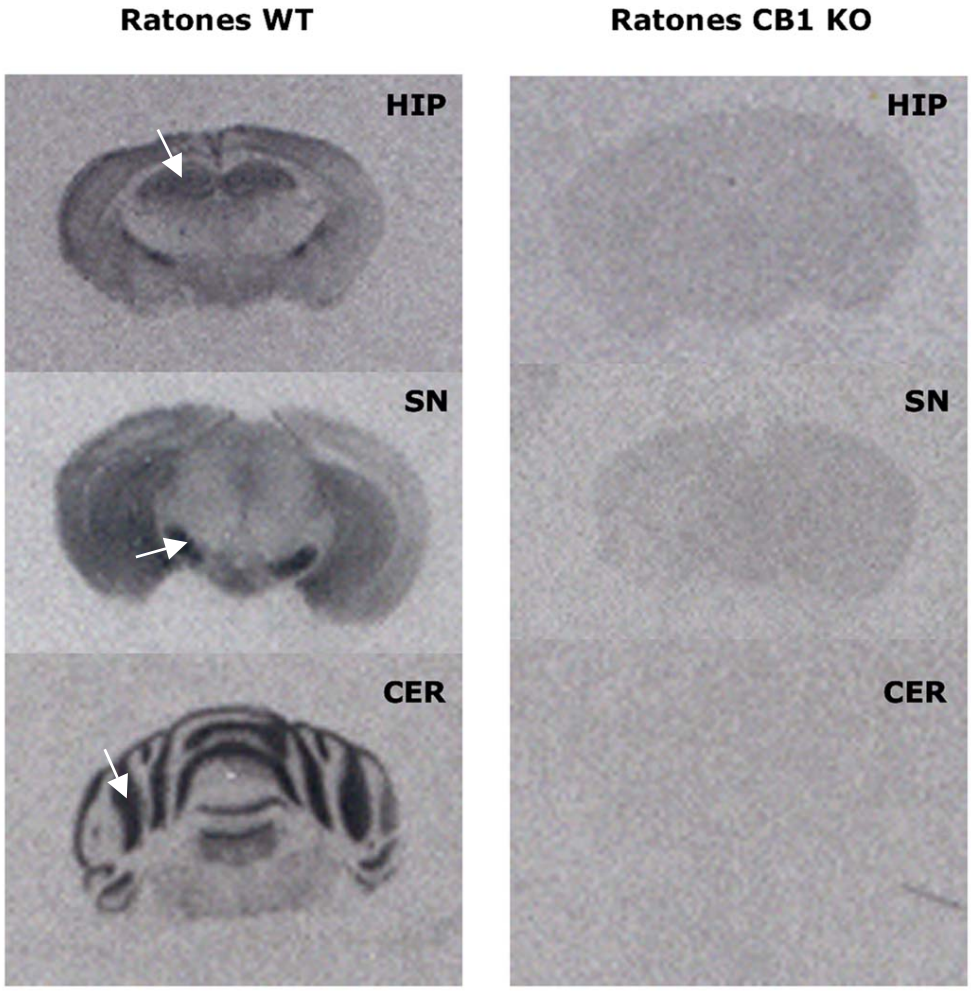

Figura 5. Distribución de los receptores cannabinoides CB1. Estudio de fijación del receptor cannabinoide CB1 con el radioligando [^{3}H]CP-55,940 en cortes coronales de cerebro de ratones normales (WT) y de ratones deficientes del receptor cannabinoide CB1 (CB1 KO), en el hipocampo (HIP), sustancia negra (SN) y cerebelo (CER) (Castañé, datos no publicados).

Tabla 1. Distribución del receptor cannabinoide CB1 en el cerebro de rata.

ESTRUCTURA	FIJACIÓN [^{3}H]CP-55,940
CORTEZA CEREBRAL	
Corteza cingular	++
Corteza frontal	++
Corteza parietal	++
HIPOCAMPO	
Capa molecular giro dentado	+++
Área CA3 del asta de Ammon	+++
Área CA1 del asta de Ammon	++
GANGLIOS BASALES	
Caudado-putamen medial	++
Caudado-putamen lateral	+++
Globo pálido	++++
Núcleo entopeduncular	+++
Sustancia negra *pars reticulata*	++++
Área tegmental ventral	+
Núcleo accumbens	++
AMÍGDALA	
Núcleo basolateral	+
Núcleo central	+
Núcleo medial	+
Núcleo lateral del tracto olfactorio	++
DIENCÉFALO	
Núcleo hipotalámico paraventricular	+
Hipotálamo lateral	+
Tálamo dorsal	+
TALLO CEREBRAL	
Núcleos del rafe	+
Locus coreuleus	+
Núcleo caudal del tracto solitario	++
CEREBELO	
Capa molecular	+++
Capa granular	+

Estudio de fijación del receptor cannabinoide CB1 con el radioligando [^{3}H]CP-55,940. Abreviaturas: +, 0-2; ++, 2-4; +++, 4-6; ++++, >6 pmol/mg de proteína. (Modificado de Herkenham y cols., 1991).

Además de su localización en el SNC, los receptores CB1 también están presentes en tejidos periféricos. De esta forma, se ha descrito su presencia en el bazo, pulmón, corazón, endotelio vascular, músculo liso y adipocitos, entre otras localizaciones (Galiegue y cols., 1995; Pertwee, 2001b).

A diferencia del receptor cannabinoide CB1, el receptor cannabinoide CB2 se localiza principalmente a nivel periférico, en células del sistema inmunitario tales como macrófagos, monocitos, neutrófilos, células "natural killer" y especialmente en los linfocitos B (Munro y cols., 1993; Galiegue y cols., 1995). No obstante, existen algunas evidencias de localización central de receptores cannabinoides CB2. Concretamente, se ha descrito su presencia en células de tumor glial (Sánchez y cols., 2001) y en la microglía (Walter y cols., 2003; Núñez y cols., 2004). Parece ser que el receptor CB2 no se localiza en neuronas, aunque un estudio ha descrito la presencia de ARN mensajero (ARNm) que codifica para este receptor en cultivos de células de Purkinje y células granulares de cerebelo de ratón (Skaper y cols., 1996).

Como se verá en el apartado 1.4, la ubicación de los receptores cannabinoides está estrechamente relacionada con los efectos farmacológicos de los compuestos cannabinoides.

1.2.2 MECANISMOS DE TRANSDUCCIÓN DE SEÑAL ASOCIADOS A LOS RECEPTORES CANNABINOIDES

Como consecuencia de la activación de los receptores cannabinoides, se produce un cambio funcional en la estructura de las proteínas G que se encuentran acopladas a dichos receptores. Los receptores cannabinoides se unen principalmente a proteínas G de tipo inhibitorio o $G_{i/o}$ y este fenómeno constituye el primer paso que dará lugar a un cambio en la actividad de diferentes rutas o vías de señalización intracelular (sistemas efectores o transductores de señal). Así, tras la activación de los receptores CB1 se produce una inhibición de la vía de la adenilato ciclasa, la regulación de diferentes canales iónicos y la activación de la ruta de las MAP quinasas (figura 6) (Howlett, 1984; Caulfield y Brown, 1992; Bouaboula y cols., 1995; Felder y cols., 1995; Henry y Chavkin, 1995; Mackie y cols., 1995; Derkinderen y cols., 2001). La inhibición de la vía de la adenilato ciclasa hace que los niveles intracelulares de adenosina-5'-monofosfato cíclico (AMPc) disminuyan. De esta forma se ve afectada la capacidad de fosforilación de proteínas quinasas dependientes de AMPc involucradas en procesos metabólicos y de expresión génica. La activación de los receptores CB1 también induce una inhibición de los canales de calcio (Ca^{+2}) voltaje dependiente tipo N y P/Q y un aumento de la conductancia de potasio (K^{+}). Este mecanismo podría ser el

responsable del efecto inhibitorio que tienen los cannabinoides sobre la liberación de neurotransmisores. Por otra parte, los cannabinoides son unos potentes activadores de la ruta de las MAP quinasas, vía implicada en la regulación de fenómenos de proliferación y diferenciación celular (Bouaboula y cols., 1995; Howlett, 1998).

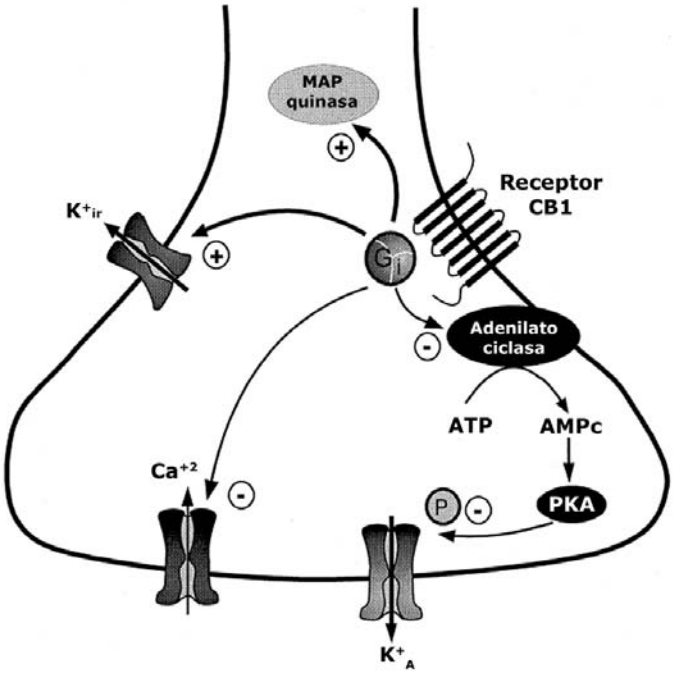

Figura 6. Mecanismos de transducción de señal estimulados por el receptor cannabinoide CB1. La activación de los receptores CB1 da lugar a una inhibición de la vía de la adenilato ciclasa, la regulación de diferentes canales iónicos y la activación de la ruta de las MAP quinasas (Modificado de Ameri, 1999).

La estimulación del receptor cannabinoide CB2 también es capaz de inhibir la vía de la adenilato ciclasa y activar la ruta de las MAP quinasas (Felder y cols., 1995).

1.3 ENDOCANNABINOIDES

La identificación y clonación del receptor cannabinoide CB1 sirvió como punto de partida en la búsqueda de sustancias endógenas capaces de activar dicho receptor. Así, la primera sustancia cannabinoide endógena que se aisló y se identificó fue la N-araquidonil etanolamina o también llamada anandamida (Devane y cols., 1992). El segundo endocannabinoide identificado fue el 2-araquidonil glicerol (2-AG) (Mechoulam y cols., 1995; Sugiura y cols., 1995) y ya recientemente se han descubierto otros compuestos endógenos con capacidad de fijarse a los receptores cannabinoides como el 2-araquidonil gliceril éter (noladín éter) (Hanus y cols., 2001), la virodamina (Porter y cols., 2002) y la N-

araquidonildopamina (Huang y cols., 2002). Todos ellos son compuestos de naturaleza lipídica que provienen de la degradación de fosfolípidos de membrana (Piomelli, 2003). Químicamente, se trata de derivados tipo amida, ester o éter, de acidos grasos poliinsaturados de cadena larga como el ácido araquidónico.

De manera similar a los neurotransmisores clásicos, los endocannabinoides son sintetizados en las neuronas, liberados en el espacio sináptico donde activan receptores de membrana y finalmente inactivados por recaptación y degradación enzimática. No obstante, los endocannabinoides presentan una clara diferencia respecto a los neurotransmisores clásicos y es que no se almacenan en vesículas sinápticas. Así, los endocannabinoides son mediadores que se sintetizan según demanda (Di Marzo y cols., 1994), actúan en las proximidades del lugar dónde se han liberado y una vez han actuado son rápidamente inactivados (Piomelli, 2003). En el cerebro, las concentraciones de anandamida son bajas. Los niveles más elevados se encuentran en el hipocampo, corteza cerebral y estriado (Felder y cols., 1996). Las concentraciones que se alcanzan de 2-AG en el cerebro son mucho mayores que las de anandamida, aproximadamente 200 veces superiores (Stella y cols., 1997; Bisogno y cols., 1999).

Otra característica muy particular de los endocannabinoides es que pueden actuar como mensajeros retrógrados, esto significa que la aplicación de estímulos concretos provoca su liberación desde las neuronas postsinápticas, siendo capaces de estimular receptores cannabinoides situados a nivel presináptico (Wilson y Nicoll, 2001, 2002) (figura 7). Mediante señalización retrógrada, los endocannabinoides son capaces de participar en la regulación a corto plazo de sinapsis inhibitorias (mediadas por la liberación de ácido γ-aminobutírico, GABA) y excitatorias (mediadas por la liberación de glutamato), dando lugar a fenómenos identificados como supresión de la inhibición inducida por despolarización, “depolarization-induced suppression of inhibition” (DSI) y supresión de la excitación inducida por despolarización, “depolarization-induced suppression of excitation” (DSE), respectivamente. De manera interasante, la señalización retrógrada mediada por endocannabinoides se ha relacionado con una regulación de las sinapsis a largo plazo dando lugar al fenómeno denominado depresión a largo plazo, “long-term depression” (LTD) (Robbe y cols., 2002). Claros ejemplos en los que los endocannabinoides actúan como mensajeros retrógrados han sido recientemente descritos en el cerebelo (Kreitzer y cols., 2002), hipocampo (Ohno-Shosaku y cols., 2001; Wilson y Nicoll, 2001), área tegmental ventral (ATV) (Melis y cols., 2004), NAc (Robbe y cols., 2002) y amígdala (Azad y cols., 2004), y contribuyen a explicar algunas de las funciones de los endocannabinoides en fenómenos tales como la motivación, la motricidad o los procesos cognitivos.

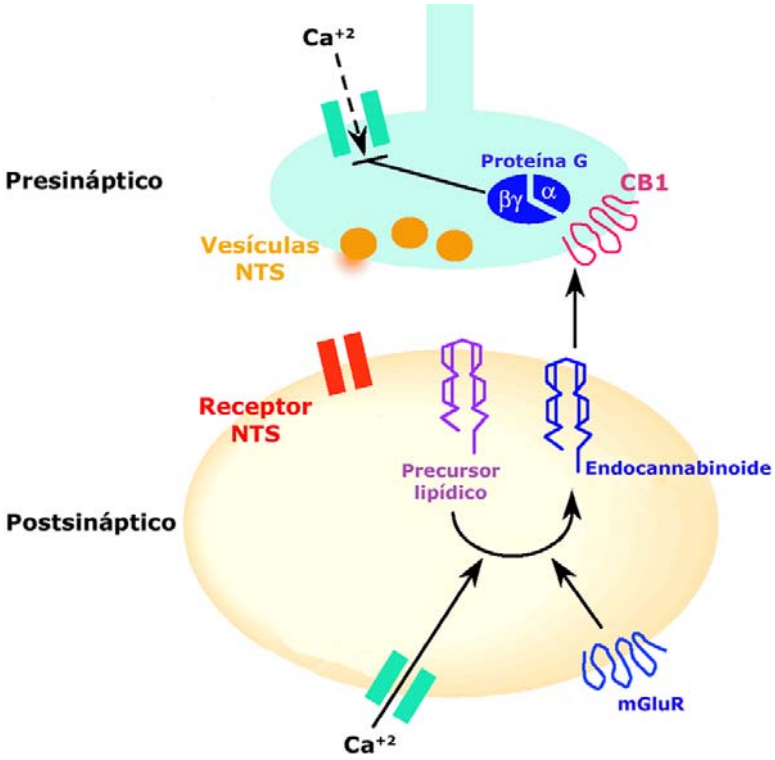

Figura 7. Esquema de actuación de los endocannabinoides como mensajeros retrógrados. El aumento de Ca^{+2} a nivel postsináptico secundario a la despolarización de las neuronas postsinápticas así como la estimulación de receptores metabotrópicos de glutamato (mGluR) en dichas neuronas actúan como señales independientes para la generación de endocannabinoides. Los endocannabinodes son liberados al espacio sináptico y activan los receptores CB1 situados a nivel presináptico. La subunidad βγ de la proteína G acoplada al receptor CB1 inhibe la entrada de Ca^{+2} en la neurona presináptica y esto disminuye la probabilidad de liberación de vesículas que contienen neurotransmisores (NTS) (Modificado de Wilson y Nicoll, 2002).

1.3.1 SÍNTESIS, LIBERACIÓN Y DEGRADACIÓN DE ENDOCANNABINOIDES

Como se ha comentado en el apartado anterior, los endocannabinoides son sintetizados en las neuronas bajo demanda y este proceso va inmediatamente seguido de su liberación al espacio sináptico. Una vez liberados, los endocannabinoides son rápidamente inactivados por recaptación y degradación enzimática. En estos procesos metabólicos participan diferentes enzimas y proteínas transportadoras. Hasta el momento, el endocannabinoide mejor caracterizado es la anandamida (figura 8).

En las neuronas, el proceso de formación de anandamida conlleva dos pasos sucesivos (figura 8a). En primer lugar, la enzima N-aciltransferasa, cuya actividad depende de Ca^{+2} y AMPc cataliza la formación del precursor llamado N-araquidonilfosfatidiletanolamina y en una segunda fase, el precursor N-

araquidonilfosfatidiletanolamina se hidroliza mediante la acción de una fosfolipasa D también regulada por Ca^{+2} (Di Marzo y cols., 1994; Okamoto y cols., 2004).

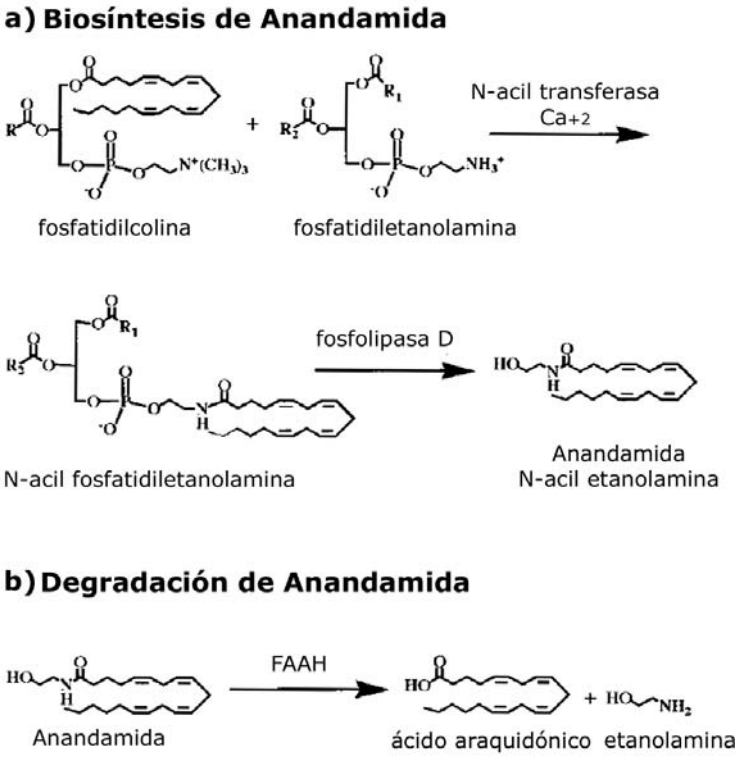

Figura 8. Enzimas involucradas en la síntesis (a) y degradación (b) de anandamida y otros lípidos endógenos de la familia de las N-acil etanolaminas. (Modificado de Cravatt y Lichtman, 2004).

Diversos estudios han demostrado que la entrada de Ca^{+2} en las neuronas, así como la activación de determinados receptores metabotrópicos como los receptores de dopamina (DA) D2, los receptores muscarínicos de acetilcolina (mAChR) y los receptores metabotrópicos de glutamato (mGluR) pueden estimular el proceso de formación y liberación de anandamida de una manera independiente (Giuffrida y cols., 1999; Varma y cols., 2001; Kim y cols., 2002). Una vez en el espacio sináptico, la anandamida interacciona con los receptores cannabinoides. Se ha descrito que presenta una cierta selectividad por el receptor cannabinoide CB1, y se comporta como un agonista parcial de ambos subtipos de receptores CB1 y CB2, siendo su eficacia mayor en la interacción con el receptor CB1 (Pertwee, 2001a). No obstante, no todos los efectos de la anandamida pueden explicarse a través de un mecanismo CB1 o CB2. Como hemos detallado anteriormente, diversos estudios farmacológicos y bioquímicos han demostrado que la anandamida es capaz de activar otros tipos de receptores acoplados a proteínas G diferentes de CB1 y CB2, sugiriendo así la existencia de un nuevo receptor cannabinoide CB3 o CBx (Di Marzo y cols., 2000; Breivogel y cols., 2001). También se ha demostrado

que la anandamida puede actuar sobre dianas moleculares no acopladas a proteínas G (Howlett y Mukhopadhyay, 2000) como por ejemplo los receptores vanilloides tipo I (Zygmunt y cols., 1999; Smart y Jerman, 2000). Una vez ha actuado, la anandamida es rápidamente recaptada del espacio sináptico hacia el interior de neuronas y astrocitos mediante un transportador que aún no ha sido clonado. Parece ser que este trasportador es también el responsable de la liberación de anandamida hacia el espacio extracelular. Una vez recaptada, la anandamida es degradada por la acción de la enzima amido hidrolasa de ácidos grasos (FAAH) dando lugar a la formación de ácido araquidónico y etanolamina (Cravatt y cols., 1996) (figura 8b). Actualmente, disponemos de compuestos inhibidores del transporte de anandamida como el AM404 (Beltramo y cols., 1997) e inhibidores de la FAAH como el URB532 y URB597 (Kathuria y cols., 2003) que nos han permitido profundizar en el estudio del sistema endocannabinoide.

El proceso de formación del 2-AG y otros 2-monoacilglicéridos parece estar mediado principalmente por la vía de la fosfolipasa C (Piomelli y cols., 2003) (figura 9a).

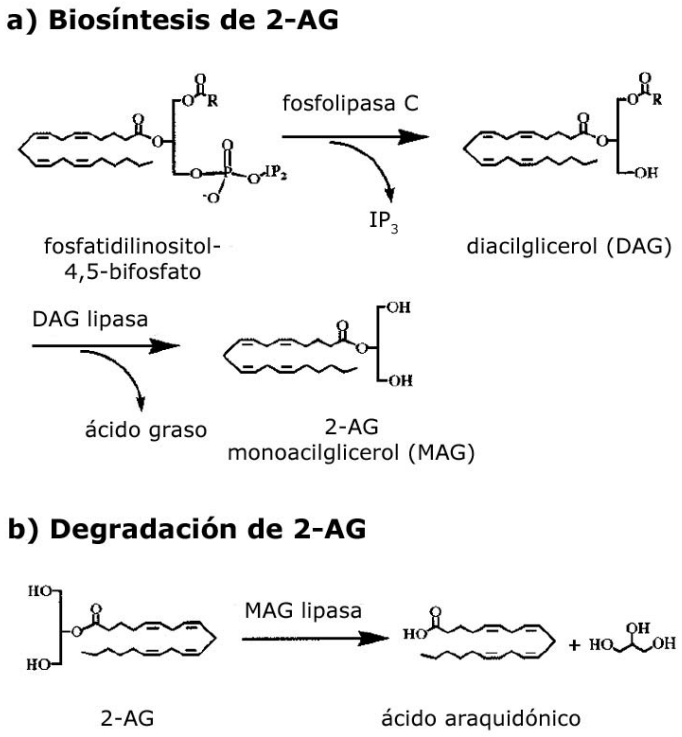

Figura 9. Enzimas involucradas en la síntesis (a) y degradación (b) de 2-AG y otros lípidos endógenos de la familia de los monoacilgliceroles (Modificado de Cravatt y Lichtman, 2004).

El 2-AG es eliminado, como la anandamida, a través de un proceso que consta de dos pasos sucesivos, recaptación y degradación enzimática. Parece ser que la recaptación del 2-AG tiene lugar a través del mismo sistema de transporte que la

anandamida (Piomelli y cols., 1999). Por otro lado, aunque el 2-AG es también sustrato de la FAAH (Goparaju y cols., 1998), se ha sugerido la existencia de otros enzimas implicados en su degradación tales como la monoacilglicerol lipasa (figura 9b) (Dinh y cols., 2002).

1.4 EFECTOS DE LOS COMPUESTOS CANNABINOIDES

El sistema endocannabinoide está implicado en la regulación de gran variedad de procesos fisiológicos, entre los que cabe destacar el aprendizaje y la memoria, la ingesta, la transmisión nociceptiva, la coordinación motora, el control de las emociones y el circuito del refuerzo, así como también en la mediación de diferentes procesos a nivel cardiovascular e inmunológico (Ameri, 1999). Los principales efectos de los cannabinoides sobre dichos procesos se encuentran resumidos en la tabla 2.

Tabla 2. Efectos más significativos de los compuestos cannabinoides.

PROCESOS FISIOLÓGIOS O FISIOPATOLÓGICOS	EFECTOS MÁS DESTACADOS	ARTÍCULO(s) de REFERENCIA
1. Aprendizaje y memoria	Déficits cognitivos	Sullivan, 2000
2. Transmisión nociceptiva	Antinocicepción	Pertwee, 2001b; Cravatt y Lichtman, 2004
3. Control motor	Hipolocomoción, Catalepsia, Ataxia	Fernández-Ruiz y cols., 2002
4. Temperatura corporal	Hipotermia	Pertwee, 1985
5. Sistema emocional	Ansiolisis, Ansiogénesis	Onaivi y cols., 1990
6. Motivación	Efectos gratificantes , efectos disfóricos/aversivos	Lupica y cols., 2004
7. Tolerancia y Dependencia	Tolerancia a respuestas agudas, síndrome de abstinencia	Tanda y Goldberg, 2003; Maldonado, 2002
8. Sistema cardiovascular	Vasodilatación, aumento de la frecuencia cardíaca, hipotensión	Randall y cols., 2004; Hiley y Ford, 2004
9. Sistema inmunitario	Inmunosupresión	Klein y cols., 2001
10. Ingesta	Aumento del apetito	Cooper, 2004
11. Supervivencia celular	Neuroprotección	Nagayama y cols., 1999; van der Stelt y cols., 2001

1.4.1 EFECTOS DE LOS CANNABINOIDES SOBRE EL APRENDIZAJE Y LA MEMORIA

Una de las propiedades comportamentales más comúnmente descrita para los compuestos cannabinoides es la de inducir alteraciones en los procesos de aprendizaje y memoria (Lichtman y cols., 1995; Sullivan, 2000). Este deterioro cognitivo inducido por los cannabinoides se ha definido a partir de estudios realizados en humanos (Chait y Pierri, 1992), primates no humanos y roedores (Hampson y Deadwyler, 1998) y se ha relacionado con una alteración en la funcionalidad del hipocampo. En humanos, la administración de THC induce una disrupción de la memoria a corto plazo así como efectos de desorientación (Miller y Branconnier, 1983; Chait y Perry, 1992). En el animal de experimentación, la administración de cannabinoides se ha relacionado con una alteración de la adquisición de ciertas tareas y con un deterioro de la memoria de trabajo (Molina-Holgado y cols., 1995; Lichtman y Martin, 1996; Winsauer y cols., 1999), especialmente la memoria de tipo espacial (Molina-Holgado y cols., 1995; Lichtman y Martin, 1996) y la memoria a corto plazo (Molina-Holgado y cols., 1995). No obstante, el papel del sistema cannabinoide endógeno en los procesos de aprendizaje y memoria no ha sido todavía del todo esclarecido. En roedores, los cannabinoides endógenos son capaces de prevenir la inducción del fenómeno de potenciación a largo plazo, "long-term potentiation (LTP) en el hipocampo (Stella y cols., 1997) y asimismo producen una alteración en la memoria para la realización de diversas tareas comportamentales, un efecto que se atenúa tras la administración del antagonista rimonabant (Mallet y Beninger, 1998). Por otro lado, el rimonabant es capaz de favorecer la memoria en ciertas condiciones experimentales (Hampson y Deadwyler, 2000). De acuerdo con estos datos farmacológicos, se ha observado que los ratones deficientes de los receptores cannabinoides CB1 presentan un incremento de la LTP en el hipocampo (Böhme y cols., 2000), una mejor retención de memoria en el paradigma de reconocimiento de objetos (Reibaud y cols., 1999; Maccarrone y cols., 2002, artículo 5 anexo) y un aumento de las respuestas condicionadas en el modelo de evitación activa (Martin y cols., 2002). Además, existen evidencias de que el sistema cannabinoide endógeno juega un papel facilitador de los procesos de extinción y/u olvido de tareas aprendidas (Varvel y Lichtman, 2002; Marsicano y cols., 2002). Mediante la utilización del paradigma de memoria espacial de la piscina de Morris, Varvel y Lichtman (2002) observaron que los ratones deficientes de los receptores cannabinoides CB1 presentaban dificultades para realizar una tarea de manera inversa a cómo había sido aprendida. Así, cuando la plataforma de la piscina de Morris se colocó en el lado opuesto al que estaba inicialmente, los ratones sin el receptor cannabinoide CB1 continuaban buscando la posición inicial de la

plataforma, siendo difícil encontrar su nuevo emplazamiento. Resultados similares han sido obtenidos por Marsicano y colaboradores (2002) mediante el paradigma del miedo condicionado. Así, los ratones deficientes de los receptores cannabinoides CB1 son incapaces de extinguir, a corto y largo plazo, un comportamiento de miedo condicionado a un estímulo sonoro, lo que demuestra que en ausencia del receptor CB1 la memoria de tipo aversivo está prolongada (Marsicano y cols., 2002).

El mecanismo concreto por el cual los cannabinoides modulan los procesos de aprendizaje y memoria no está claro. En el año 2000, Braida y Sala demostraron en ratas que el efecto de los cannabinoides de interferir la memoria estaba relacionado con una inhibición de la actividad colinérgica en el SNC (Braida y Sala, 2000). Recientemente, se ha evidenciado que los ratones deficientes de los receptores CB1 presentan niveles más elevados de acetilcolina (ACh) en el hipocampo (Kathmann y cols., 2001). El hipocampo y el neocórtex son dos estructuras que juegan un papel crucial en los procesos de aprendizaje y memoria. En ambas estructuras, los receptores cannabinoides CB1 se localizan presinápticamente en interneuronas GABAérgicas (Katona y cols., 1999; Marsicano y Lutz, 1999; Tsou y cols., 1999). Una propiedad interesante de las interneuronas CB1 positivas del hipocampo es que son capaces de establecer sinapsis inhibitorias de cinética rápida. Así, una única neurona inhibitoria es capaz de contactar con cientos de neuronas principales del hipocampo y facilitar que exista una descarga sincronizada de todas ellas. Esta sincronización parece jugar un papel importante en los procesos cognitivos (Buzsaki y Chrobak, 1995). Por otro lado, las interneuronas GABAérgicas parecen también controlar los cambios plásticos en las sinapsis excitatorias. Así, el bloqueo de una inhibición inducido por cannabinoides promueve la LTP en las sinapsis excitatorias (Wilson y Nicoll, 2002; Diana y Marty, 2004). Esta facilitación inducida por cannabinoides en las neuronas hipocampales parece sugerir que el sistema cannabinoide endógeno promueve el aprendizaje. No obstante, estudios farmacológicos y genéticos claramente demuestran que los cannabinoides inducen una alteración de la memoria. Para reconciliar estos datos discrepantes Wilson y Nicoll han elaborado una teoría. Según estos dos autores, a nivel fisiológico, los cannabinoides endógenos estarían modulando la actividad de las interneuronas GABAérgicas formando sinapsis rápidas en el hipocampo que orquestarían las oscilaciones sincrónicas en el rango gamma (Banks y cols., 2000). Según esta misma teoría, la administración de cannabinoides exógenos produciría cambios plásticos prematuros que suprimirían estas sinapsis inhibitorias hipocampales causando los déficits cognitivos y de memoria que hemos descrito (Wilson y Nicoll, 2002). Nuevos estudios deben ser realizados para demostrar esta

hipótesis y conciliar estos datos con los resultados obtenidos en el ratón deficiente en receptores CB1.

1.4.2 EFECTOS DE LOS CANNABINOIDES SOBRE LA TRANSMISIÓN NOCICEPTIVA

Una de las principales funciones fisiológicas atribuida al sistema endocannabinoide es la del control de la nocicepción (Pertwee, 2001b; Hohmann, 2002). La presencia de receptores cannabinoides en las diferentes vías de transmisión del dolor (Tsou y cols., 1998; Sañudo-Peña y cols., 1999; Ahluwalia y cols., 2000; Farquhar-Smith y cols., 2000), así como la liberación de endocannabinoides en ciertas áreas cerebrales tras la inducción de estímulos nociceptivos (Walker y cols., 1999) parece confirmar esta hipótesis. Además, la capacidad antinociceptiva de compuestos de naturaleza cannabinoide se ha puesto de manifiesto en numerosos estudios comportamentales de nocicepción y dolor (Martin y Lichtman, 1998). Entre los modelos nociceptivos agudos destacan aquellos que utilizan estímulos térmicos como el ensayo de retirada de la cola (Buxbaum, 1972) y el ensayo de la placa caliente (Buxbaum, 1972; Martin, 1985; Hutcheson y cols., 1998). Estos dos ensayos se caracterizan por evaluar respuestas comportamentales distintas. Mientras que las respuestas antinociceptivas observadas en el ensayo de la placa caliente están mediadas principalmente por la activación de mecanismos supraespinales, las respuestas comportamentales observadas en el ensayo de la sacudida de la cola son debidas sobre todo a reflejos espinales que presentan una baja influencia supraespinal. Otros modelos nociceptivos agudos donde los agonistas cannabinoides han mostrado su eficacia son aquellos modelos mecánicos que miden respuestas motoras (Smith y cols., 1998) o reflejas (Gilbert, 1981), modelos químicos como los de las constricciones abdominales inducidas por la administración de fenilbenzoquinona (Welch y cols., 1995), ácido acético o ácido fórmico (Bicher y Mechoulam, 1968), y los modelos de estimulación eléctrica de la pata (Weissman y cols., 1982), del nervio ciático (Bicher y Mechoulam, 1968) o de la pulpa dentaria (Kaymakcalan y cols., 1974). Los agonistas cannabinoides también inducen efectos antinociceptivos en modelos de dolor inflamatorio tales como el modelo de hiperalgesia producida por la administración de carragenina (Mazzari y cols., 1996), capsaicina (Li y cols., 1999), formalina (Moss y Johnson, 1980; Calignano y cols., 1998) y adyuvante de Freund (Martin y cols., 1999). Recientemente, los cannabinoides han mostrado su eficacia en modelos de dolor neuropático (Goya y cols., 2003; Lim y cols., 2003; Costa y cols., 2004), una dolencia que actualmente no posee un tratamiento adecuado en el humano.

Los receptores del dolor, constituidos por terminaciones nerviosas libres, se encuentran localizados en la piel, tejido subcutáneo y en diferentes tejidos internos. Las señales dolorosas son transmitidas por los nervios periféricos hasta el asta dorsal del la médula espinal a través de fibras de tipo Aδ (dolor rápido-punzante) y C (dolor lento-difuso). Desde la médula espinal, las fibras que transmiten las señales dolorosas se dirigen hacia el tálamo y de ahí a otras áreas del encéfalo y de la corteza cerebral sensitiva somática (vía ascendente). Existe un sistema descendente inhibitorio que se origina en el tronco del encéfalo el cual es activado por las proyecciones colaterales de la propia vía ascendente y frena la entrada de estímulos dolorosos en el asta dorsal de la médula espinal (Basbaum y Fields, 1984; Fields y cols., 1991). En este sistema inhibidor descendente participan diversos núcleos como la sustancia gris periacueductal y la médula rostroventromedial (Basbaum y Fields, 1984; Fields y cols., 1991).

El mecanismo de acción de los cannabinoides como inhibidores de la transmisión del dolor incluye acciones tanto a nivel central como periférico (Pertwee, 2001b; Hohmann, 2002) (figura 10). Igualmente, se ha sugerido que los cannabinoides pueden ejercer efectos antinociceptivos interaccionando no sólo con receptores cannabinoides sino también con receptores vanilloides (Pertwee, 2001b).

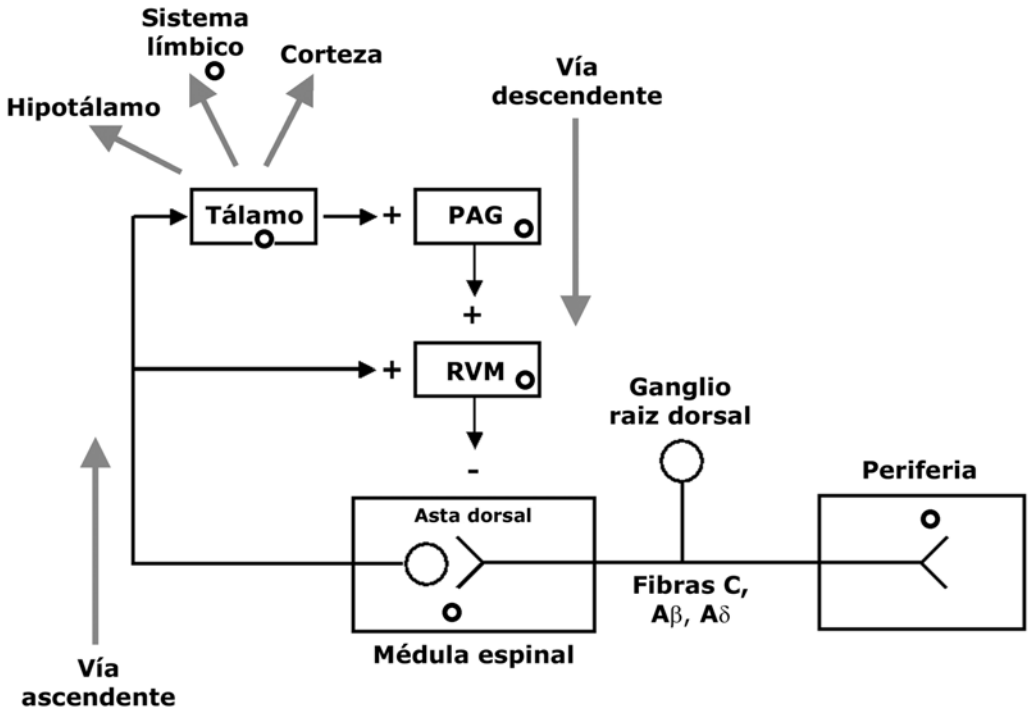

Figura 10. Esquema representativo de los lugares de acción de los cannabinoides para inducir efectos antinociceptivos en roedores (**O**). Otras áreas cerebrales importantes no representadas serían la amígdala y el colículo superior. Abreviaturas: PAG, área gris periacueductal; RVM: médula rostroventromedial (Modelo de Basbaum y Fields, 1984, Modificado de Pertwee, 2001b).

A nivel central, la actividad antinociceptiva de los cannabinoides se debe principalmente a la presencia de receptores CB1 en la médula espinal y en estructuras supraespinales (Meng y cols., 1998; Ledent y cols., 1999). Aunque los cannabinoides juegan un papel destacado en el control de las fibras primarias aferentes, otro mecanismo de acción central para la acción antinociceptiva de los cannabinoides parece ser el control de las vías inhibitorias descendentes. Diversos estudios han demostrado que a nivel de la sustancia gris periacueductal y la médula rostroventromedial existe un control bidireccional (facilitador o inhibidor) de la transmisión del dolor. Este control dual de la transmisión nociceptiva está conducido por dos subpoblaciones de neuronas diferentes que de manera teórica se denominan células On y células Off. Las células On facilitan la transmisión de la señal nociceptiva y las células Off la inhiben (Fields, 2004). Ambos tipos de células On y Off proyectan directamente en el asta dorsal de la médula espinal. La microinyección local de agonistas cannabinoides en la sustancia gris periacueductal (Martin y Lichtman, 1998; Martin y cols., 1999) y en la médula rostroventromedial (Martin y cols., 1998), así como la estimulación eléctrica de estas dos áreas (Fields y cols., 1991) produce efectos analgésicos. Esto sugiere que los cannabinoides desinhiben neuronas de ambas regiones y en consecuencia activan las vías inhibitorias descendentes. Se ha propuesto que este fenómeno ocurre a través de la reducción de la liberación de GABA en los botones presinápticos de las interneuronas localizadas en ambas áreas mediante un mecanismo similar al descrito para los opioides (figura 11).

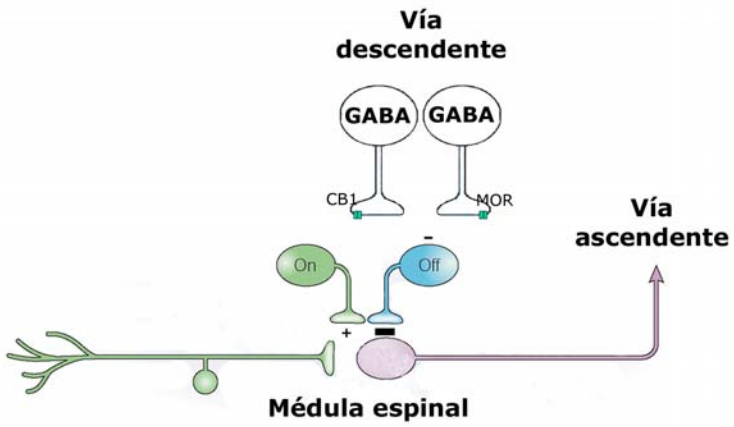

Figura 11. Mecanismo hipotético de actuación de los cannabinoide y los opioides para el control del sistema inhibidor descendente. Los cannabinoides a través del receptor CB1 y los opioides a través del receptor opioide mu (MOR) inhiben la liberación de GABA (neurotransmisor inhibidor). De esta manera se produce una desinhibición de la célula Off. Aunque el mecanismo para cannabinoides y opioides es parecido el lugar concreto de acción es diferente en cada caso.

A nivel supraespinal, los cannabinoides también son capaces de modificar la interpretación subjetiva del dolor mediante la modulación de la actividad neuronal en la amígdala (Manning y cols., 2001).

A nivel espinal, los receptores cannabinoides CB1 se localizan principalmente en el asta dorsal de la médula espinal. La mayoría de las neuronas aferentes primarias que expresan el ARNm para el receptor cannabinoide CB1 son aquellas compuestas por fibras de gran diámetro o Aβ que participan en la transmisión sensitiva de tipo no nociceptivo (Hohmann y Herkenham, 1998). No obstante, existen también receptores CB1 en las fibras nociceptivas de pequeño diámetro o fibras C capaces de inhibir la liberación de neurotransmisores mediadores del dolor (Drew y cols., 2000; Kelly y Chapman, 2001; Wilson y Nicoll, 2002) (figura 10). Además, el ARNm que codifica para los receptores CB1 también se encuentra altamente expresado en los ganglios de la raíz dorsal (Hohmann, 2002; Bridges y cols., 2003). A este nivel, la estimulación de los receptores CB1 parece producir una inhibición de los canales de Ca^{+2} presinápticos, atenuando así la liberación de neurotransmisores (Millns y cols., 2001) (figura 10).

Diversos estudios han demostrado que la anandamida tiene efectos analgésicos. Sin embargo, parte de estos efectos pueden ser debidos a la interacción con el receptor vanilloide VR1 (Di Marzo y cols., 2001). En este sentido, se ha observado que altas concentraciones de anandamida pueden excitar los terminales aferentes de los ganglios de la raíz dorsal incrementando la liberación de mediadores del dolor como la sustancia P, a través de la activación del receptor VR1 (Tognetto y cols., 2001).

A nivel periférico, los efectos antinociceptivos de los cannabinoides parecen estar mediados por la activación de ambos receptores cannabinoides CB1 y CB2. Existen estudios que demuestran un papel de los receptores periféricos CB2 en modelos animales de dolor crónico y se ha propuesto la existencia de un efecto sinérgico entre las respuestas mediadas por el receptor CB1 y CB2 a este nivel (Malan y cols., 2002).

Aunque las propiedades antinociceptivas de los cannabinoides exógenos son bien conocidas, el papel del sistema cannabinoide endógeno en el control del dolor no está claro. Estudios en los que se han utilizado antagonistas cannabinoides de los receptores CB1 como el rimonabant (Rinaldi-Carmona y cols., 1994; Richardson y cols., 1998; Costa y Colleoni, 1999; Cravatt y cols., 2001; Lichtman y cols., 2004) y ratones deficientes de los receptores CB1 (Zimmer y cols., 1999; Valverde y cols., 2000; Ledent y cols., 1999; Ibrahim y cols., 2003) han mostrado resultados contradictorios. Por ejemplo, estudios iniciales con el antagonista rimonabant mostraron que este compuesto no alteraba la sensibilidad dolorosa aguda en el

ensayo de retirada de la cola en ratas (Rinaldi-Carmona y cols., 1994). Trabajos posteriores realizados en ratones apoyaron este resultado. Así, el rimonabant no produjo ningún efecto sobre la sensibilidad dolorosa en el ensayo de la retirada de la cola y en el ensayo de la placa caliente (Cravatt y cols., 2001; Lichtman y cols., 2004). Contrariamente, otros autores han descrito la aparición de hiperalgesia tras la administración de rimonabant en el ensayo de la sacudida de la cola (Costa y Colleoni, 1999) y en el ensayo de la placa caliente (Richardson y cols., 1998) en ratas, lo que sugeriría la existencia de un tono cannabinoide endógeno en condiciones fisiológicas. En el caso de los estudios utilizando ratones deficientes de los receptores CB1 también se han podido observar resultados controvertidos. Así, mientras que los ratones mutantes generados por Ledent y colaboradores a partir de una cepa no consanguínea CD1 presentan umbrales nociceptivos similares a los de los ratones normales frente a estímulos térmicos en el ensayo de la placa caliente y de la retirada de la cola, mecánicos en el ensayo de presión de la cola y químicos en el ensayo de las constricciones abdominales inducidas por la administración de ácido acético (Ledent y cols., 1999), los ratones generados por Zimmer y colaboradores a partir de una cepa consanguínea C57BL/6J presentan una hiposensibilidad a estímulos térmicos en el ensayo de la placa caliente y químicos en el ensayo de la formalina (Zimmer y cols., 1999). Recientemente, Ibrahim y colaboradores han utilizado un nuevo modelo de ratón deficiente del receptor cannabinoide CB1 generado a partir de una cepa 129/SvJ que presenta una mayor sensibilidad a estímulos de tipo mecánico en el ensayo de los filamentos de von Frey (Ibrahim y cols., 2003). Los correspondientes autores de estos trabajos han sugerido que estas discrepancias podrían deberse al diferente fondo genético de estas tres líneas de ratones así como también a las diferentes condiciones experimentales utilizadas en cada trabajo.

Otra estrategia que ha sido utilizada para estudiar el posible papel del sistema cannabinoide endógeno en el control del dolor ha consistido en aumentar el tono endocannabinoide mediante la inactivación genética o farmacológica de la FAAH. Estos estudios parecen apoyar la existencia de una señalización endocannabinoide en el control del dolor agudo y crónico (Cravatt y cols., 2001; Lichtman y cols., 2004).

Actualmente, los compuestos inhibidores de la FAAH así como también los agonistas de los receptores cannabinoides CB2 constituyen una potencial estrategia terapéutica para el tratamiento de diferentes tipos de dolor, con la ventaja de presentar menos efectos indeseables respecto a los agonistas de los receptores cannabinoides CB1.

1.4.3 EFECTOS DE LOS CANNABINOIDES SOBRE EL CONTROL MOTOR

En roedores, los principales efectos de los cannabinoides sobre el control del movimiento son hipoactividad, catalepsia y ataxia (Rodríguez de Fonseca y cols., 1998). No obstante, el efecto de los agonistas cannabinoides sobre la actividad locomotora parece ser bifásico. Así, a dosis bajas estos compuestos tienen un efecto estimulador sobre la actividad locomotora mientras que a dosis altas producen una inhibición de la misma (Davis y cols., 1972).

Los efectos locomotores de los cannabinoides están mediados principalmente por la activación de receptores cannabinoides CB1 a nivel de los ganglios basales, del sistema límbico y del cerebelo. Los ganglios basales están constituidos por un conjunto de núcleos cerebrales funcionalmente interconectados que incluyen el caudado y putamen, el globo pálido, el núcleo subtalámico, el globo pálido interno (o núcleo entopeduncular en roedores) y la sustancia negra (Albin y cols., 1989). Los núcleos caudado y putamen están conectados con la sustancia negra *pars reticulata* a través de dos vías, la directa y la indirecta. Estas dos vías de proyección del estriado ejercen acciones opuestas en cuanto al control del movimiento. Los efectos de los cannabinoides en los ganglios basales son complejos. Así, diversos estudios han demostrado que los agonistas cannabinoides como CP-55,940 activan el movimiento cuando son microinyectados en la vía directa de los ganglios basales (Sañudo-Peña y cols., 1996; Sañudo-Peña y cols., 1998), mientras que producen efectos inhibidores del movimiento cuando son microinyectados en la vía indirecta (Miller y cols., 1998; Sañudo-Peña y Walker, 1998). A nivel del cerebelo, los cannabinoides parecen modular aspectos de coordinación motora. Por otro lado, los efectos estimuladores observados tras la administración aguda de cannabinoides parecen estar originados en áreas del sistema límbico (Rodríguez de Fonseca y cols., 1998).

Estudios realizados en ratones deficientes de los receptores cannabinoides CB1 han descrito resultados diferentes dependiendo de la cepa de ratón y de las condiciones experimentales. Así, Zimmer y colaboradores han descrito una disminución en la actividad locomotora en los ratones sin el receptor CB1 (Zimmer y cols., 1999), mientras que Ledent y colaboradores han descrito una hiperactividad moderada en los ratones que carecen del receptor CB1 cuando estos son expuestos a un ambiente o entorno desconocido. Este efecto desaparece después de un periodo de adaptación (Ledent y cols., 1999).

1.4.4 EFECTOS DE LOS CANNABINOIDES SOBRE LA REGULACIÓN DE LA TEMPERATURA CORPORAL

Los compuestos cannabinoides son capaces producir hipotermia de manera dosis-dependiente a través de la activación de receptores cannabinoides CB1 (Fitton y Pertwee, 1982; Pertwee, 1985; Rawls y cols., 2002). Así, el efecto hipotérmico del agonista WIN 55,212-2 es revertido tras la administración del antagonista CB1 rimonabant, pero no tras la administración del antagonista CB2 SR144528 (Rawls y cols., 2002).

El efecto hipotérmico de los cannabinoides parece implicar la modulación de las vías serotonérgicas del área preóptica del hipotálamo. Diversos estudios han demostrado que la administración local de agonistas cannabinoides como THC (Fitton y Pertwee, 1982), HU-210 (Ovadia y cols., 1995) y WIN 55,212-2 (Rawls y cols., 2002) en el núcleo anterior preóptico del hipotálamo produce una respuesta hipotérmica dosis-dependiente. Además, este efecto es revertido tras la administración de rimonabant (Rawls y cols., 2002).

1.4.5 EFECTOS DE LOS CANNABINOIDES SOBRE EL CONTROL EMOCIONAL

Existen numerosas evidencias de que el sistema cannabinoide endógeno participa en la regulación de las respuestas de tipo emocional. Este hecho está apoyado en la elevada densidad de receptores cannabinoides CB1 en áreas del sistema límbico y corteza cerebral que participan en el control de dichos procesos. Además, los compuestos cannabinoides son capaces de producir una amplia variedad de respuestas psicotropas en el humano y también en el animal de experimentación en diferentes paradigmas comportamentales. Asimismo, los cannabinoides también ejercen un papel modulador del eje adrenal hipotálamo-hipofisiario (HPA) y son capaces de modificar la liberación de neurotransmisores importantes para el control de los estados emocionales, tales como GABA y colecistoquinina (CCK).

Los efectos de los cannabinoides sobre el control del estado de ansiedad son claramente bifásicos. A dosis bajas los cannabinodies suelen producir efectos ansiolíticos y por el contrario a dosis elevadas suelen tener un efecto ansiogénico (Onaivi y cols., 1990; Navarro y cols., 1993; Rodríguez de Fonseca y cols., 1996)

Recientemente, mediante la utilización de ratones deficientes de los receptores cannabinoides CB1 se ha podido demostrar que el sistema cannabinoide endógeno, a través de los receptores CB1, está implicado en el control de las respuestas emocionales. Los ratones sin el receptor CB1 exhiben un fenotipo ansiogénico en el paradigma del campo abierto, en el ensayo de la caja blanca y

negra y en el laberinto en cruz elevado (Haller y cols., 2002; Maccarrone y cols., 2002, artículo 5 anexo; Martin y cols., 2002; Urigüen y cols., 2004). Recientemente, Urigüen y colaboradores han demostrado que este fenotipo ansiogénico de los animales deficientes del receptor CB1 se acompaña de una alteración del eje HPA, así como también de una hipersensibilidad al estrés (Urigüen y cols., 2004). Concretamente, estos autores han demostrado que la concentración de corticosterona en plasma en condiciones basales es menor en los ratones que carecen del receptor CB1. Sin embargo, tras un estrés producido por inmovilización, los niveles de corticosterona aumentan de manera significativamente mayor en los ratones mutantes en comparación con los ratones normales (Urigüen y cols., 2004). En contraste con estos resultados, Barna y colaboradores han descrito un aumento de los niveles basales de corticosterona en plasma en los ratones mutantes sin el receptor CB1 (Barna y cols., 2004).

Además del fenotipo ansiogénico, los ratones deficientes del receptor CB1 presentan un comportamiento agresivo cuando se les somete al paradigma del intruso y una mayor predisposición a desarrollar un estado de anedonia tras la exposición al paradigma del estrés suave, crónico e impredecible (Martin y cols., 2002). Por otro lado, estudios realizados en estos mismos ratones han sugerido la existencia de un nuevo receptor cannabinoide que participaría en el control del estado anímico. Este receptor cannabinoide parece tener un efecto contrario al del receptor CB1. Así, Haller y colaboradores demostraron que el rimonabant era capaz de producir efectos ansiolíticos tanto en ratones normales como en ratones sin el receptor CB1 (Haller y cols., 2002).

1.4.6 EFECTOS GRATIFICANTES DE LOS CANNABINOIDES

Una de las propiedades de los derivados de la marihuana importante para el inicio de su consumo abusivo es sin duda alguna el efecto placentero que producen al ser consumidos. Actualmente, disponemos de modelos animales que nos permiten revelar los efectos gratificantes de los cannabinoides tales como el paradigma del condicionamiento espacial (Valjent y Maldonado, 2000), la auto-administración intravenosa (Martellotta y cols., 1998; Justinova y cols., 2003) y la auto-estimulación intracraneal (Gardner y Lowinson, 1991) (figura 12). No obstante, es importante destacar que la puesta a punto de estos modelos ha sido especialmente difícil en el caso del THC, debido principalmente a las caraterísticas farmacocinéticas (semivida larga) de este compuesto (Valjent y Maldonado, 2000; Justinova y cols., 2003).

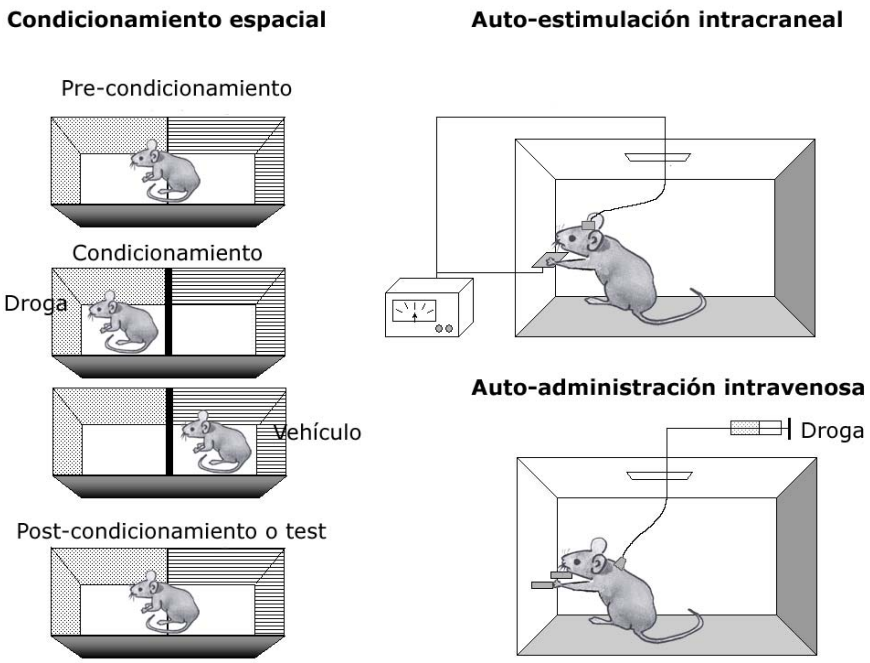

Figura 12. Representación esquemática de los paradigmas comportamentales utilizados para evaluar las propiedades reforzantes o gratificantes de las drogas de abuso. Paradigma del condicionamiento espacial, auto-estimulación intracraneal y auto-administración intravenosa.

Estudios farmacológicos en los que se ha utilizado el antagonista rimonabant y estudios realizados en ratones deficientes de los receptores cannabinoides CB1 han puesto de manifiesto que las respuestas de preferencia de plaza condicionada a la administración de cannabinoides y la auto-administración intravenosa de estos compuestos se originan a través de la activación de los receptores cannabinoides CB1 (Ledent y cols., 1999; Tanda y Golberg, 2003). Pero, ¿dónde y cómo actúan los cannabinoides para producir tales efectos?. Las propiedades gratificantes de los cannabinoides resultan de la interacción de estos compuestos con los circuitos centrales del refuerzo. El circuito más estudiado se origina en un grupo de neuronas dopaminérgicas del ATV cuyos axones proyectan hacia neuronas del sistema límbico, incluido el NAc y neuronas de la corteza prefrontal (CPF), de ahí que reciba también el nombre de sistema mesocorticolímbico (figura 13).

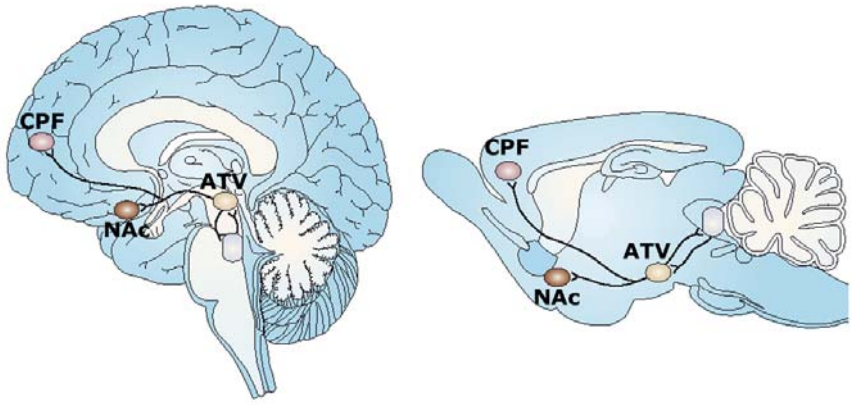

Figura 13. Sistema mesocorticolímbico. Localización del ATV, NAc y CPF en cerebro humano (izquierda) y de rata (derecha) (Modificado de Laviolette y van der Kooy, 2004).

De manera similar con otras drogas de abuso como cocaína, etanol, morfina anfetamina y nicotina, los cannabinoides son capaces de incrementar la actividad dopaminérgica en el circuito del refuerzo (Di Chiara y Imperato, 1988; Chen y cols., 1991), una propiedad que se ha relacionado directamente con los efectos gratificantes de las drogas de abuso (Wise, 2004). Mediante estudios de microdiálisis *in vivo* se ha podido observar que los cannabinoides son capaces de aumentar la concentración de DA en el NAc a través de un mecanismo que depende de potencial de acción, de Ca^{+2}, de la activación de los receptores CB1 y en el que los opioides endógenos juegan también un papel importante (ver apartado 2.1.4) (Chen y cols., 1990; Tanda y cols., 1997). No obstante, el mecanismo exacto por el cual se produce este aumento de DA en el NAc no se conoce con exactitud. A partir de registros electrofisiológicos de neuronas del ATV se ha comprobado que los cannabinoides aumentan la actividad de las neuronas dopaminérgicas a través de un mecanismo CB1-dependiente (French, 1997; Diana y cols., 1998a). Debido a la no presencia de receptores CB1 en las neuronas dopaminérgicas del ATV, se ha propuesto que los cannabinoides sean capaces de activar dichas neuronas a través de un mecanismo indirecto de desinhibición de las mismas, mecanismo parecido al descrito para los opioides. Así, los cannabinoides actuarían sobre receptores CB1 localizados en los terminales de neuronas GABAérgicas del ATV e inhibirían la liberación de neurotransmisor inhibidor GABA en esta área (figura 14). Esta hipótesis viene apoyada por estudios que muestran que el agonista cannabinoide WIN 55,212-2 es capaz de reducir los potenciales inhibitorios postsinápticos mediados por la activación de receptores $GABA_A$ en cortes de cerebro que incluyen el ATV, un efecto que se bloquea tras la administración de rimonabant (Szabo y cols., 2002).

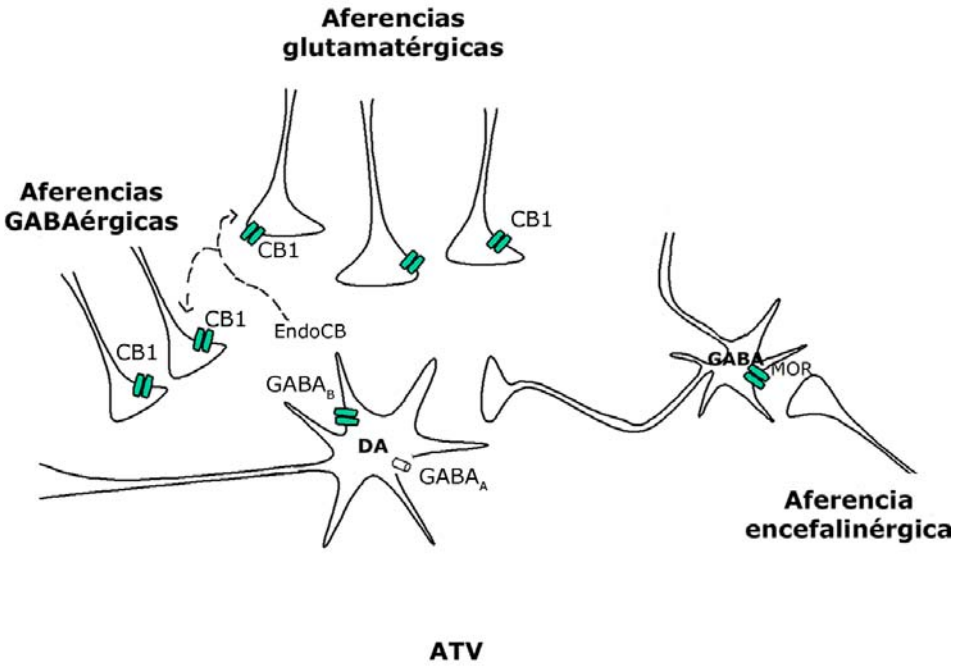

Figura 14. Esquema simplificado de los componentes celulares conocidos involucrados en los efectos de los cannabinoides en el ATV. Además de las neuronas dopaminérgicas, el ATV contiene neuronas GABAérgias que proyectan discretamente al NAc y CPF. Por otro lado, en el ATV proyectan neuronas glutamatérgicas que provienen de la CPF, amígdala, núcleo pedunculopontino y núcleo subtalámico y neuronas GABAérgicas que provienen del NAc. Abreviaturas: MOR, receptor opiode mu; EndoCB, endocannabinoides (Modificado de Lupica y cols., 2004).

Aunque el mecanismo de desinhibición de las neuronas dopaminérgicas del ATV está apoyado por múltiples estudios, existen evidencias de que la interacción entre los cannabinoides y dichas neuronas dopaminérgicas del ATV es mucho más compleja (figura 14). Por un lado, se ha descrito que la administración sistémica e intra-ATV de antagonistas opioides es capaz de revertir el aumento de dopamina que producen los cannabinoides en el NAc (Chen y cols., 1990; Tanda y cols., 1997) pero no el aumento de la velocidad de descarga de las neuronas dopaminérgicas que induce el THC (French, 1997). Además, la infusión directa de THC en el ATV no produce un aumento de la acumulación de DA en el NAc (Chen y cols., 1993a). Por otro lado, se ha descrito recientemente que los agonistas cannabinoides sintéticos así como los endocannabinoides, actuando de manera retrógrada, son capaces de inhibir la liberación de glutamato en el ATV *in vitro* (Melis y cols., 2004), lo que tendería a disminuir la aportación excitatoria hacia las neuronas dopaminérgicas del ATV y por tanto reduciría la probabilidad de descarga de las mismas (Johnson y cols., 1992; Kitai y cols., 1999). Finalmente, existen datos preliminares que indican que los receptores cannabinoides CB1 se localizan

también en los terminales de las neuronas GABAérgicas que provienen del NAc (Walaas y Fonnum, 1980; Heimer y cols., 1991), los cuales tienen como diana los receptores $GABA_B$ de las neuronas dopaminérgicas del ATV (Sugita y cols., 1992). Esto sugeriría la existencia de un posible segundo mecanismo de desinhibición importante para los efectos de los cannabinoides en el ATV (Riegel y cols., 2003) (figura 14). En conjunto, todos estos trabajos que examinan el efecto de los cannabinoides sobre las neuronas dopaminérgicas del ATV sugieren que, mientras que la activación de los receptores CB1 en dicha estructura puede contribuir a aspectos de la exposición a cannabinoides relevantes para el refuerzo, otros sitios adicionales dentro y fuera del ATV deben ser considerados.

Como hemos apuntado, parte de los efectos reforzantes de los cannabinoides podrían deberse a acciones directas de los cannabinoides en áreas diferentes del ATV como por ejemplo el NAc (figura 15). Así, estudios *in vitro* realizados en cortes de cerebro del NAc han demostrado que agonistas cannabinoides como WIN 55,212-2 (Hoffman y Lupica, 2001) y CP-55,940 (Manzoni y Bockaert, 2001) inhiben la liberación de GABA en el NAc a través de la activación de receptores CB1 localizados a nivel presináptico en terminales inhibitorias (Hoffman y Lupica, 2001; Manzoni y Bockaert, 2001). Hasta el momento, no se conoce con exactitud cuál es el mecanismo concreto que explica la inhibición de la liberación de GABA tras la administración de cannabinoides en el NAc. En esta área, las neuronas GABAérgicas de proyección hacia el ATV (neuronas espinosas de tamaño mediano) reciben influencias inhibitorias procedentes de interneuronas GABAérgicas del propio NAc (Koós y Tepper, 1999), así como también de interconexiones vía axones colaterales (Plenz, 2003), de manera que la inhibición de la liberación de GABA podría suceder a través de la activación de receptores CB1 en estos dos tipos celulares (figura 15). Por otro lado, los cannabinoides son también capaces de inhibir la liberación de glutamato en el NAc mediante la activación de receptores CB1 acoplados a canales de K^+ voltaje-dependientes situados en terminales glutamatérgicas que inervan dicha área (Robbe y cols., 2001) (figura 15). Por tanto, en el NAc nos encontramos de nuevo ante un mecanismo complejo en el que los cannabinoides son capaces de modular tanto un sistema inhibitorio como un sistema excitatorio, de manera que la actividad final de las neuronas GABAérgicas del NAc que proyectan hacia el ATV dependería del sistema que predomine en cada momento.

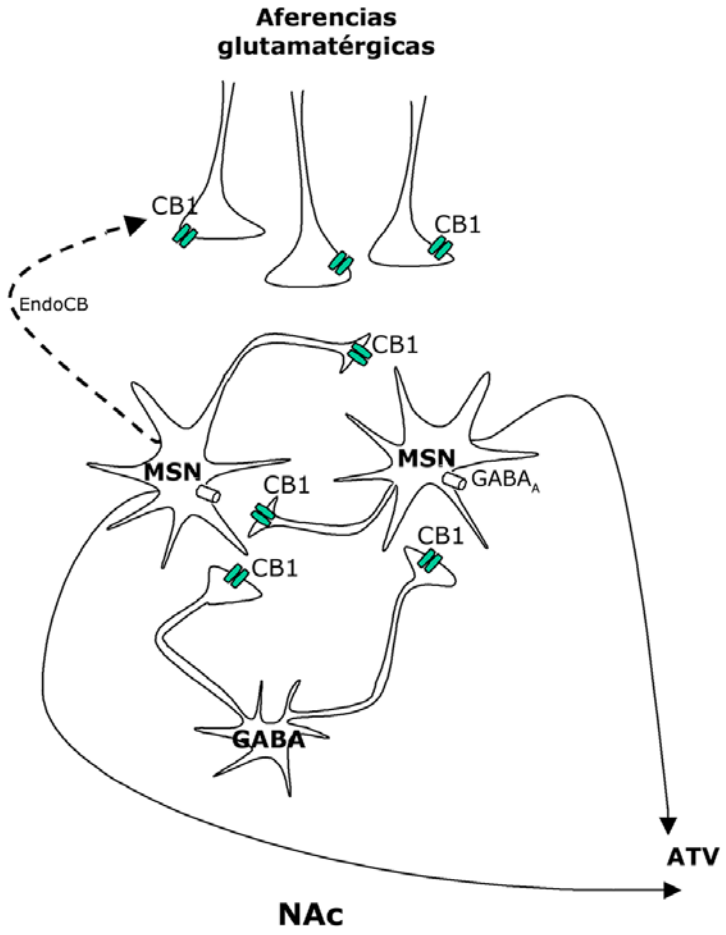

Figura 15. Esquema simplificado de los componentes celulares conocidos involucrados en los efectos de los cannabinodies en el NAc. Abreviaturas: MSN, neurona GABAérgica espinosa de tamaño mediano que proyecta hacia el ATV; EndoCB, endocannabinoides (Modificado de Lupica y cols., 2004).

1.4.7 TOLERANCIA Y DEPENDENCIA FÍSICA DE CANNABINOIDES

La administración crónica de cannabinoides induce cambios adaptativos en el sistema nervioso central que dan lugar a los fenómenos de tolerancia y dependencia física.

Tolerancia

La administración crónica de cannabinoides produce el desarrollo de tolerancia a la mayoría de sus efectos farmacológicos. Esto significa que la intensidad de los efectos agudos disminuye con la administración repetida de estos compuestos o que hace falta aumentar la dosis para conseguir el mismo efecto. En el caso particular de los cannabinoides, se ha descrito tolerancia a los efectos antinociceptivos (Hutcheson y cols., 1998), hipolocomotores (Davis y cols., 1972; Hutcheson y cols., 1998), hipotérmicos (Thompson y cols., 1974; Hutcheson y

cols., 1998), anticonvulsivantes (Colasanti y cols., 1982) y a los efectos sobre el peso corporal (Hutcheson y cols., 1998), entre otros. La aparición de tolerancia es especialmente rápida de manera que se puede observar un importante descenso de las respuestas agudas después de la segunda administración de un agonista cannabinoide (Abood y Martin, 1992; Hutcheson y cols., 1998). Aunque hay algunos estudios que muestran que mecanismos farmacocinéticos tales como cambios en la absorción, distribución, biotransformación y excreción de cannabinoides podrían participar en el desarrollo de tolerancia (Dewey y cols., 1972), los principales mecanismos involucrados son de tipo farmacodinámico. En concreto, nos referimos a modificaciones que afectan la expresión, características de fijación y actividad funcional del receptor cannabinoide CB1. En este sentido se ha demostrado que durante el tratamiento crónico con THC se produce una disminución de la densidad de receptores CB1 (Rodríguez de Fonseca y cols., 1994) así como del ARNm que codifica para este receptor en diversas áreas cerebrales (Romero y cols., 1998). También se ha observado un descenso generalizado de los niveles cerebrales de ARNm que codifica para diferentes subunidades tipo α de las proteínas G (Rubino y cols., 1997). Estos cambios en la expresión de los genes de las proteínas G están relacionados con una desensibilización de los receptores cannabinoides CB1, un hecho que se ha puesto de manifiesto con estudios que muestran una reducción de la fijación de [^{35}S]GTPγS estimulada por cannabinoides en el cerebro de ratas sometidas a un tratamiento crónico con cannabinoides (Sim y cols., 1996). Estos cambios adaptativos que acabamos de describir no son permanentes sino que revierten hasta valores basales tras el cese del tratamiento crónico, lo que concuerda perfectamente con la desaparición de la tolerancia a los efectos farmacológicos (Bass y Martin, 2000). Diversos estudios han descrito que existe una tolerancia cruzada entre compuestos exógenos agonistas de los receptores cannabinoides CB1. Concretamente, se ha observado tolerancia cruzada entre THC, CP-55,940 y WIN 55,212-2 para los efectos antinociceptivos, hipolocomotores, hipotérmicos y de catalepsia (Pertwee y cols., 1993). Resulta interesante comprobar la existencia de tolerancia cruzada entre compuestos cannabinoides y opioides lo que sugiere la existencia de una interacción funcional entre ambos sistemas (ver apartado 2.1.5).

Dependencia física

Numerosos estudios muestran que la administración crónica de dosis elevadas de cannabinoides como THC, WIN 55,212-2 y HU-210 produce un estado de dependencia física en el animal de experimentación (Maldonado y Rodríguez de Fonseca, 2002). Este estado de dependencia física se pone claramente de

manifiesto mediante la aparición de un síndrome de abstinencia tras la administración de un antagonista cannabinoide en animales tratados crónicamente con dichos agonistas.

En humanos, se ha descrito la aparición de un síndrome de abstinencia cannabinoide espontáneo en individuos expuestos a altas dosis de THC (210 mg/día) (Haney y cols., 1999). Este síndrome se caracteriza por la ausencia de signos somáticos y la presencia de síntomas subjetivos tales como ansiedad, irritabilidad y dolor hipogástrico, así como una disminución de la ingesta de comida (Haney y cols., 1999; 2004). Por el contrario, en el animal de experimentación no se ha podido observar la aparición de un síndrome de abstinencia espontáneo al finalizar un tratamiento crónico con THC (Aceto y cols., 2001). Únicamente en monos se ha observado una alteración en la ejecución de una conducta operante previamente adquirida tras la retirada del THC (Beardsley y cols., 1986). Esta alteración ha sido interpretada como la manifestación de una abstinencia espontánea. Recientemente, se ha descrito la aparición de un síndrome de abstinencia espontáneo al cesar un tratamiento crónico con el agonista cannabinoide WIN 55,212-2 en ratas (Aceto y cols., 2001). La corta semivida del WIN 55,212-2 en comparación con la del THC podría explicar las diferencias observadas entre estos dos compuestos.

El descubrimiento de antagonistas selectivos para los receptores cannabinoides ha permitido profundizar en el estudio de la dependencia física de cannabinoides. Así, diversos trabajos han descrito que la administración aguda del antagonista de los receptores cannabinoides CB1, rimonabant, es capaz de precipitar un síndrome de abstinencia en animales tratados crónicamente con THC (Lichtman y cols., 1998). En roedores, el síndrome de abstinencia de cannabinoides se caracteriza por la presencia de signos somáticos y la ausencia de signos vegetativos. Las manifestaciones somáticas principales incluyen sacudidas de perro mojado o “wet dog shakes”, temblor de patas, temblor corporal, ataxia o incoordinación motora, disminución de la actividad locomotora y masticación (tabla 3).

Mediante la utilización de animales modificados genéticamente se ha demostrado que los receptores cannabinoides CB1 son los principales responsables de las manifestaciones somáticas del síndrome de abstinencia de cannabinoides (Ledent y cols., 1999; Lichtman y cols., 2001). Así, la administración de rimonabant en ratones sin el receptor CB1 que habían recibido un tratamiento crónico con THC no desencadenó ningún signo de abstinencia (Ledent y cols., 1999; Lichtman y cols., 2001). No obstante y como veremos en el capítulo siguiente

parece ser que el sistema opioide es capaz de modular esta respuesta (ver apartado 2.1.5 *Dependencia*).

Tabla 3. Sintomatología más frecuente observada durante el síndrome de abstinencia de cannabinoides desencadenado por rimonabant en roedores

ESPECIE	AGONISTA	SIGNOS DE LA ABSTINENCIA CUANTIFICADOS	ARTÍCULO(s) de REFERENCIA
RATÓN	THC	"Wet dog shakes", frotamiento facial, ataxia, postura encorvada, temblor, ptosis, piloerección, masticación	Hutcheson y cols., 1998
		"Wet dog shakes", temblor de patas, piloerección, temblor corporal, "sniffing"	Ledent y cols., 1999
		Temblor de patas, sacudidas de cabeza	Cook y cols., 1998 Lichtman y cols., 2001
		Temblor, "wet dog shakes", ptosis, temblor de patas delanteras, ataxia, masticación, postura encorvada, "sniffing", piloerección, puntuación global de abstinencia	Valverde y cols., 2000b
		Temblor, ataxia, masticación, temblor de patas delanteras, piloerección, "wet dog shakes"	Tzavara y cols., 2000
	WIN 55,212-2	"Wet dog shakes", ptosis, temblor, temblor de patas, piloerección, postura encorvada, masticación, lameteo de genitales, ataxia, "sniffing", puntuación global de abstiencia	Castañé y cols., 2004 (artículo 1)
RATA	THC	Frotamiento facial, "wet dog shakes"	Aceto y cols., 1995, 1996
		Arañazos, frotamiento facial, "wet dog shakes", lameteo	Diana y cols., 1998b
		Supresión de la conducta operante	Beardsley y Martin, 2000
	WIN 55,212-2	"Wet dog shakes", frotamiento facial	Aceto y cols., 2001
	HU-210	Puntuación global de abstinencia	Rodríguez de Fonseca y cols., 1997
	CP-55,940	Giros, masticación, "digging"	Rubino y cols., 1998

(Modificado de Selley y cols., 2003).

El síndrome de abstinencia de cannabinoides se acompaña de cambios adaptativos a diferentes niveles. Por un lado, observamos un aumento en los niveles extracelulares del factor liberador de corticotrofina (CRF) en la amígdala y una marcada inhibición de la actividad dopaminérgica en el NAc (Diana y cols., 1998b), ambos eventos parecen ser comunes para el síndrome de abstinencia de otras drogas de abuso (Koob, 1996) y se relacionan con las consecuencias aversivas/disfóricas y el estrés de la abstinencia (Koob, 2003). De manera similar al síndrome de abstinencia de opioides, el síndrome de abstinencia de cannabinoides está asociado con una sobreregulación o “up-regulation” de la vía de la adenilato ciclasa. En el caso de los cannabinoides esta “up-regulation” aparece de manera específica en el cerebelo (Hutcheson y cols., 1998) y parece estar relacionada con la aparición de las manifestaciones somáticas de la abstinencia. Así, la microinyección del inhibidor de la actividad adenilato ciclasa, Rp-8Br-cAMPS en el cerebelo atenúa las manifestaciones somáticas de la abstinencia cannabinoide (Tzavara y cols., 2000). El síndrome de abstinencia de cannabinoides también ha sido relacionado con una alteración de la actividad de las redes neuronales del cerebelo. En este sentido, Ghozland y colaboradores demostraron que la administración crónica de THC en cortes de cerebelo disminuía la actividad y coordinación de las células granulares. Este efecto fue revertido tras incubar los cortes cerebelares con el antagonista rimonabant (Ghozland y cols., 2002a). Aunque el cerebelo parece jugar un papel crucial en la abstinencia cannabinoide (Tzavara y cols., 2000; Ghozland y cols., 2002a), no podemos descartar que otras estructuras cerebrales puedan participar en esta respuesta.

2 INTERACCIÓN DEL SISTEMA CANNABINOIDE CON OTROS SISTEMAS DE NEUROTRANSMISIÓN

En el SNC, el sistema cannabinoide interacciona funcionalmente con otros sistemas de neurotransmisión. A consecuencia de estas interacciones, los efectos farmacológicos de los compuestos cannabinoides pueden estar modulados por sistemas heterólogos y el propio sistema cannabinoide puede intervenir en las acciones de compuestos no cannabinoides. En nuestro estudio hemos investigado las interacciones entre el sistema cannabinoide y los sistemas opioide, purinérgico y nicotínico.

2.1 PARTICIPACIÓN DEL SISTEMA OPIOIDE EN LOS EFECTOS FARMACOLÓGICOS DE LOS CANNABINOIDES

2.1.1 SISTEMA OPIOIDE

El sistema opioide endógeno consiste en diferentes familias de péptidos opioides que actúan sobre tres tipos de receptores opioides denominados receptor opioide mu (MOR), delta (DOR) y kappa (KOR) (Kieffer, 1995; Kieffer y Gaveriaux-Ruff, 2002).

Receptores opioides

Los receptores opioides MOR, DOR y KOR fueron clonados a principios de los años noventa (Evans y cols., 1992; Kieffer y cols., 1992; Chen y cols., 1993b; Meng y cols., 1993; Yasuda y cols., 1993). El primer receptor opoide clonado fue el DOR (Evans y cols., 1992; Kieffer y cols., 1992). Posteriormente, el MOR y el KOR se identificaron gracias a la homología de secuencia que presentan con el DOR (Chen y cols., 1993b; Meng y cols., 1993; Yasuda y cols., 1993). Hasta la fecha, no se conoce la existencia de ningún otro receptor opioide. Sin embargo, se ha descrito la existencia de variantes de estos receptores debidas a cambios post-transcripcionales (Pan y cols., 2001; Cadet, 2004), así como también la formación de complejos heterodiméricos entre ellos. Concretamente, se ha descrito la existencia de heterodímeros de tipo DOR/KOR y MOR/DOR (Jordan y Devi, 1999; Gomes y cols., 2000). Estas variantes y asociaciones representan una nueva estructura funcional con unas propiedades diferentes a las de los receptores originales y podrían explicar algunas de las respuestas farmacológicas de los opioides que no se corresponden con la activación de MOR, DOR o KOR.

Los receptores opoides pertenecen a la super familia de receptores acoplados a proteínas G con siete dominios transmembranales. A través de la unión con proteínas G, principalmente G_i/G_o, los receptores opioides son capaces de inhibir la actividad de la adenilato ciclasa (Sharma y cols., 1977), modificar la conductividad de canales K^+ y Ca^{+2} (Surprenant y cols., 1990; Jin y cols., 1992) y activar las vías de señalización de la fosfolipasa C y de las MAP quinasas (Burt y cols., 1996; Fukuda y cols., 1996).

Los receptores opioides se encuentran ampliamente distribuidos en diversas estructuras del SNC pero también en diferentes tejidos periféricos. En el SNC, los tres receptores opoides presentan una localización diferencial (Mansour y cols., 1995). Los MORs son los receptores opioides que presentan una distribución más amplia en el cerebro, principalmente en estructuras relacionadas con el control de la transmisión nociceptiva, la motricidad y también la motivación. Así, se ha observado una elevada densidad de sitios de unión de tipo mu en el caudado-putamen, NAc, tálamo, hipocampo, sustancia gris periacueductal, sustancia negra y amígdala. En el estriado los MORs se distribuyen en forma de agrupaciones o parches. En comparación con los MORs, los DORs parecen tener una localización neuroanatómica más restringida en el SNC. Estos receptores se localizan en el estriado, tubérculo olfativo y en la corteza cerebral entre otras diversas estructuras. Parece ser que en el estriado, los DORs están distribuidos de manera densa y homogénea. Finalmente, los KORs también presentan una distribución central más circunscrita que los MORs. Así, destacaremos su presencia a nivel del NAc, tubérculo olfativo, área preóptica, amígdala e hipotálamo (Mansour y cols., 1995).

Péptidos opioides

En condiciones fisiológicas, los receptores opioides son activados por un conjunto de compuestos endógenos llamados péptidos opioides. Estos péptidos opioides provienen de la degradación enzimática de tres precursores peptídicos de gran tamaño, la proopiomelanocortina (POMC), la proencefalina (PENC) y la prodinorfina (PDIN). De la POMC se obtiene principalmente β-endorfina, de la PENC se obtienen diversos péptidos opioides entre los que cabe destacar la Met- y Leu-encefalina y de la PDIN se generan las dinorfinas y las neoendorfinas, entre otros. En 1997, Zadina y colaboradores propusieron las endomorfinas como otro grupo de péptidos opioides (Zadina y cols., 1997). No obstante, el precursor peptídico para dichos compuestos todavía no se conoce (tabla 4). Aunque los derivados peptídicos de POMC, PENC y PDYN presentan cierta preferencia hacia un tipo de receptor opioide, por ejemplo encefalinas para DOR y dinorfinas para KOR, la selectividad de

estos compuestos es tan solo relativa. Por el contrario, las endomorfinas son ligandos con una gran afinidad y selectividad para los MORs (tabla 4).

Tabla 4. Principales péptidos opioides, sus precursores y sitios de unión predominantes

PRECURSOR PEPTÍDICO	PÉPTIDOS OPIOIDES	RECEPTOR PREFERENCIAL	MOR	DOR	KOR
POMC	β-endorfina	MOR/DOR	+++	+++	
PENC	Met-encefalina	DOR	++	+++	
	Leu-encefalina	DOR	++	+++	
PDIN	Dinofina A	KOR	++		+++
	Dinorfina B	KOR	+	+	+++
	α-neoendorfina	KOR	+	+	+++
	β-neoendorfina	KOR	+	+	+++
?	Endomorfina 1	MOR	+++		
	Endomorfina 2	MOR	+++		

La potencia para activar cada receptor está indicada por el número de símbolos positivos (+).

Ligandos exógenos para los receptores opioides

Algunos de los principales compuestos exógenos que actúan sobre los receptores opioides se encuentran resumidos en la tabla 5.

Tabla 5. Ejemplos de ligandos exógenos de los receptores opioides.

LIGANDO	CATEGORIA	MOR	DOR	KOR
MORFINA	Agonista	+++		+
FENTANILO	Agonista	+++		
DPDPE	Agonista		+++	
DELTORFINA I	Agonista		+++	
DELTORFINA II	Agonista	+	+++	
U-50,488	Agonista			+++
ICI 199441	Agonista			+++
BUPRENORFINA	Agonista/Antagonista	+ parcial		--
NALOXONA	Antagonista	---	--	-
NALTREXONA	Antagonista	---	--	-
NALTRINDOL	Antagonista		---	
NOR-BINALTORFIMINA	Antagonista			---

La actividad de los compuestos agonistas está indicada con signos positivos (+) y la actividad de los compuestos antagonistas con símbolos negativos (-). La afinidad sobre cada receptor está indicada por el número de símbolos representados.

Procesos fisiológicos y fisiopatológicos en los que participa el sistema opioide

El sistema opioide endógeno ha sido implicado en el control de comportamientos que son esenciales para la supervivencia de las especies como las respuestas al dolor y al estrés, así como en el control de la motivación y el refuerzo y la motricidad. Los péptidos opioides y sus receptores también controlan funciones del sistema nervioso autónomo tales como la respiración, la termoregulación y la motilidad gastrointestinal. Además, el sistema opioide también parece modular las respuestas de tipo inmunitario. A consecuencia de la participación del sistema opioide endógeno en el control dichas funciones, las principales respuestas farmacológicas que inducen los ligandos opioides exógenos son antinocicepción, hipotermia, hipolocomoción, hipotensión, sedación, disminución de la motilidad gastrointestinal así como efectos gratificantes y dependencia física (Bodnar y Hadjimarkou, 2002).

2.1.2 BASES DE LA INTERACCIÓN CANNABINOIDE-OPIOIDE

Numerosos estudios han demostrado la existencia de interacciones funcionales entre los sistemas cannabinoide y opioide (Manzanares y cols., 1999; Maldonado y Valverde, 2003; Corchero y cols., 2004). Estas interacciones parecen estar fundamentadas en tres evidencias claras: (1) la co-localización de receptores cannabinoides y opioides en diversas estructuras del SNC, (2) el acoplamiento de los receptores cannabinoides y opioides a los mismos sistemas de señalización intracelular, y (3) el control, por parte de los sistemas cannabinoide y opioide, de procesos fisiológicos y fisiopatológicos comunes. La co-localización de receptores opioides y cannabinoides en estructuras similares y más concretamente la co-localización en las mismas neuronas abre la posibilidad de que dichos receptores compitan en el acoplamiento a sistemas de señalización intracelular comunes e incluso que interactúen físicamente para formar heterodímeros funcionales.

A parte de la posible interacción fisica de los receptores cannabinoides y opioides y de la modulación de mecanismos de señalización intracelular comunes, se ha descrito la aparición de compensaciones o adaptaciones bilaterales entre los dos sistemas que soportan aún más la existencia de una interacción. Algunos ejemplos de estas adaptaciones que comentamos podrían ser: la facilitación de la liberación y síntesis de péptidos opioides por parte de agonistas cannabinoides (Corchero y cols., 1997; Pugh y cols., 1997; Mason y cols., 1999; Houser y cols., 2000; Valverde y cols., 2001), la modificación de la capacidad funcional y densidad de los receptores cannabinoides CB1 tras un tratamiento crónico con opioides (González y cols., 2002b) y la aparición de cambios en la expresión génica de

péptidos opioides en animales deficientes de los receptores CB1 (Steiner y cols., 1999; Zimmer y cols., 1999).

En base a estas evidencias, diversos trabajos han abordado el estudio de la interacción entre el sistema cannabinoide y opioide a nivel del control de la transmisión del dolor, del control emocional, del refuerzo y de los procesos de tolerancia y dependencia física. La mayoría de estos estudios han descrito la existencia de una interacción bidireccional entre los sistemas cannabinoide y opioide, no obstante nosotros nos centraremos en la participación del sistema opioide endógeno en las respuestas inducidas por cannabinoides.

2.1.3 PARTICIPACIÓN DEL SISTEMA OPIOIDE EN LAS PROPIEDADES ANTINOCICEPTIVAS DE LOS CANNABINOIDES

Diversos estudios farmacológicos y moleculares han puesto de manifiesto la participación del sistema opioide endógeno en las respuestas antinociceptivas de los cannabinoides. Concretamente, estudios farmacológicos han demostrado que la administración de agonistas cannabinoides y opioides produce efectos sinérgicos en las respuestas antinociceptivas (Reche y cols., 1996a; Welch y Eads, 1999). Además, los antagonistas opioides son capaces de bloquear la antinocicepción inducida por cannabinoides en ciertas condiciones experimentales (Pertwee, 2001b). La administración de naloxona, un antagonista preferencial para los MORs, ha dado lugar a resultados controvertidos. Por un lado, se ha demostrado dicho antagonista es capaz de antagonizar los efectos antinociceptivos del THC en el ensayo de la placa caliente pero no en el ensayo de la sacudida de cola (Martin, 1985; Welch, 1993), lo que parece sugerir la participación de los MORs en las respuestas antinociceptivas del THC mediadas por la activación de mecanismos supraespinales. De acuerdo con esta hipótesis, la administración de naloxona no fue capaz de bloquear el efecto antinociceptivo del THC en el ensayo de la sacudida de cola cuando el THC se administró a nivel intratecal e intracerebroventricular (Martin, 1985; Welch y Stevens, 1992). No obstante, otros estudios en los que también se ha utilizado el antagonista naloxona han demostrado que este compuesto es capaz de atenuar las propiedades antinociceptivas del THC tanto en el ensayo de la placa caliente como en el ensayo de la sacudida de cola (Wilson y May, 1975; Reche y cols., 1996b). La utilización de antagonistas selectivos para los DORs ha demostrado que estos receptores no parecen estar implicados en la antinocicepción inducida por cannabinoides. En este sentido, ni el naltrindol ni el ICI-174,864 bloquearon las respuestas antinociceptivas inducidas por THC en el ensayo de la placa caliente y en el ensayo de la sacudida de cola (Welch, 1993; Reche y cols.,1996b). Finalmente, diversos estudios farmacológicos han evaluado la

posible implicación del KOR en las respuestas antinociceptivas de los cannabinoides. Así, la administración intratecal del antagonista específico de los KORs, nor-binaltorfimina (Welch, 1993; Welch y cols., 1995), la administración de oligonucleótidos antisentido contra los receptores kappa y la de anticuerpos contra las dinorfinas (Pugh y cols., 1997; Rowen y cols., 1998) diminuyen los efectos antinociceptivos inducidos tras la administración de THC (Welch, 1993; Welch y cols., 1995). Estos resultados parecen indicar que el KOR participa en las respuestas analgésicas de los cannabinoides que precisan la activación de mecanismos espinales (Reche y cols., 1996b).

La utilización de ratones modificados genéticamente deficientes de los diferentes receptores o péptidos opioides ha aportado nuevos datos para esclarecer el papel del sistema opioide endógeno en la antinocicepción inducida por cannabinoides. En este sentido, Ghozland y colaboradores demostraron que la supresión individual de los diferentes receptores opioides MOR, DOR y KOR no producía cambios en las respuestas antinociceptivas agudas del THC (Ghozland y cols., 2002b). De manera recíproca, el efecto antinociceptivo agudo inducido por la administración de agonistas selectivos de los receptores MOR, DOR y KOR no se vió modificado en el animal deficiente en el receptor cannabinoide CB1 (Valverde y cols., 2000a). Sin embargo, en concordancia con los estudios farmacológicos se ha observado una disminución de los efectos antinociceptivos del THC en la prueba de retirada de la cola en ratones deficientes en el gen que codifica para la prePENC (Valverde y cols., 2000b) y en los ratones deficientes del gen que codifica para la PDIN (Zimmer y cols., 2001), lo que sugiere que los derivados peptídicos de estas dos proteínas participan en la respuesta antinociceptiva del THC. Teniendo en cuenta que los derivados peptídicos de prePENC y PDIN no son selectivos para ninguno de los receptores opioides podríamos postular que la supresión individual de cada subtipo de receptor opioide no es suficiente para alterar la respuesta antinociceptiva aguda del THC.

Los mecanismos concretos que explican la participación del sistema opioide endógeno en los efectos antinociceptivos de los cannabinoides no han sido claramente elucidados. No obstante, existen evidencias de una co-localización de receptores opioides y cannabinoides en estructuras importantes para la transmisión nociceptiva. Concretamente, destacaremos la coexistencia de receptores cannabinoides CB1 y MOR en estructuras tales como el área gris periacueductal, la médula rostroventromedial y la médula espinal (Mansour y cols., 1988; Herkenham y cols., 1991; Lichtman y cols.,1996; Salio y cols., 2001). Además, se ha descrito que los cannabinoides son capaces de facilitar la liberación de péptidos opioides en áreas relacionadas con la transmisión del dolor. En este sentido, diversos estudios

bioquímicos han demostrado que la administración intratecal de THC y de otros agonistas exógenos, pero no de anandamida, es capaz de aumentar la liberación de dinorfinas en la médula espinal (Pugh y cols., 1997; Mason y cols., 1999; Houser y cols., 2000). Asimismo, estudios más recientes han demostrado que la administración sistémica de THC aumenta los niveles de met-encefalina (Valverde y cols., 2001) y β-endorfina (Solinas y cols., 2004) en áreas del sistema límbico.

2.1.4 PARTICIPACIÓN DEL SISTEMA OPIOIDE EN LAS PROPIEDADES GRATIFICANTES DE LOS CANNABINOIDES

Como se ha expuesto en el apartado 1.4.6, las propiedades gratificantes de los cannabinoides se ponen de manifiesto mediante técnicas de auto-estimulación intracraneal, condicionamiento espacial y auto-administración intravenosa. Estas mismas técnicas han sido utilizadas para estudiar el papel del sistema opioide endógeno en las propiedades gratificantes de los cannabinoides.

Diferentes estudios farmacológicos han investigado el papel del sistema opioide endógeno en las propiedades gratificantes de los cannabinoides. Así, mediante la técnica de auto-estimulación intracraneal se ha observado que la naloxona es capaz de bloquear la facilitación de la conducta de auto-estimulación eléctrica intracraneal inducida por THC (Gardner y Lowinson, 1991; Gardner y Vorel, 1998). Asimismo, la naloxona es capaz de bloquear la auto-administración intravenosa de agonistas cannabinoides como CP-55,940 (Braida y cols., 2001b), WIN 55,212-2 (Fattore y cols., 2001) o HU-210 (Navarro y cols., 2001) y la preferencia de plaza condicionada a CP-55,940 (Braida y cols., 2001a).

Estudios recientes realizados en ratones deficientes de MOR, DOR y KOR han utilizado el paradigma del condicionamiento espacial para evaluar el papel del sistema opioide endógeno en las propiedades reforzantes y aversivas de los cannabinoides (Ghozland y cols., 2002b). En concordancia con los estudios farmacológicos, Ghozland y colaboradores demostraron que la activación de MOR es necesaria para la manifestación de las propiedades reforzantes del THC. En este mismo estudio se observó que los ratones deficientes de KOR no exhibían aversión de plaza condicionada a THC y que en estos ratones la preferencia de plaza condicionada a THC se manifestaba sin la necesidad de administrar una inyección previa en la caja de estabulació (“priming”), lo que sugiriere un papel importante de KOR en mediar la disforia inducida por THC. De acuerdo con estos resultados los ratones deficientes en el gen de la PDIN tampoco muestran aversión de plaza condicionada de THC (Zimmer y cols., 2001).

Estudios bioquímicos y electrofisiológicos han demostrado que el sistema opioide endógeno está implicado en los efectos de los cannabinoides sobre la

actividad dopaminérgica mesolímbica. Así, antagonistas opioides son capaces de bloquear el aumento de DA en el NAc inducido tras la administración de agonistas cannabinoides (Gardner y Lowinson, 1991; Tanda y cols., 1997). Sin embargo, otros estudios han demostrado que el THC aumenta la actividad de las neuronas dopaminérgicas sin que dicho efecto sea modificado por la administración aguda de naloxona (French, 1997; Melis y cols., 2001). Esto indica la existencia de otros mecanismos reguladores de la acción de los cannabinoides en estas neuronas dopaminérgicas tal y como se ha descrito en el apartado 1.4.6.

2.1.5 PARTICIPACIÓN DEL SISTEMA OPIOIDE EN LA TOLERANCIA Y DEPENDENCIA FÍSICA DE CANNABINOIDES

Tolerancia

Numerosos estudios farmacológicos muestran la existencia de tolerancia cruzada entre compuestos cannabinoides y opioides a nivel de la respuesta antinociceptiva (Bloom y Dewey, 1978; Smith y cols., 1994; Thorat y Bhargava, 1994). Así, la administración de THC produce tolerancia para los efectos antinociceptivos de la morfina (Thorat y Bhargava, 1994) y viceversa; la morfina produce tolerancia a los efectos antinociceptivos del THC (Bloom y Dewey, 1978; Thorat y Bhargava, 1994). Estudios más recientes con animales modificados genéticamente han demostrado que ciertos componentes del sistema opioide endógeno, y en concreto los derivados peptídicos de la prePENC, podrían participar en el fenómeno de tolerancia para los efectos farmacológicos de los cannabinoides. Así, los ratones deficientes de la prePENC presentan una disminución en el desarrollo de tolerancia para el efecto antinociceptivo del THC y una leve atenuación de la tolerancia al efecto hipolocomotor de dicho compuesto (Valverde y cols., 2000b). Por el contrario, no se han observado cambios significativos en el desarrollo de tolerancia para los efectos farmacológicos que induce el THC en los ratones deficientes del precursor peptídico PDIN (Zimmer y cols., 2001). La invalidación de los diferentes subtipos de receptores opioides tampoco parece tener importantes consecuencias. La tolerancia para los efectos hipolocomotores, hipotérmicos y antinociceptivos del THC se desarrolla con normalidad en los ratones deficientes de MOR y DOR (Ghozland y cols., 2002b). No obstante, los ratones deficientes en KOR muestran una leve atenuación de la tolerancia para el efecto hipolocomotor (Ghozland y cols., 2002b).

Dependencia física

Como ya se había comentado, la administración aguda de un antagonista de los receptores cannabinoides CB1 es capaz de precipitar un síndrome de abstinencia en animales tratados crónicamente con THC (Lichtman y cols., 1998). De manera interesante el antagonista opioide naloxona es también capaz de inducir la aparición de signos de abstinencia en ratas que han recibido un tratamiento crónico con agonistas cannabinoides como HU-210 (Navarro y cols., 1998) o THC (Kaymakcalan y cols., 1977). Sin embargo, este antagonista opoide no precipitó un proceso de abstinencia en ratones dependientes de THC (Lichtman y cols., 2001). Trabajos en los que se han utilizado ratones modificados genéticamente han evaluado el papel de los diferentes componentes del sistema opioide en la dependencia física de cannabinoides. Así, los ratones deficientes del gen de la prePENC muestran una disminución de la severidad del síndrome de abstinencia a THC precipitado por rimonabant (Valverde y cols., 2000b). No se han observado cambios en la intensidad de la abstinencia cannabinoide en los ratones deficientes de DOR y KOR (Ghozland y cols., 2002b). No obstante, estudios realizados con ratones deficientes de MOR han mostrado resultados discordantes en función de la dosis de THC utilizada para inducir la dependencia. Así, ratones deficiente de MOR que recibieron dosis de 10 mg/kg una vez al día (Lichtman y cols., 2001) o de 20 mg/kg dos veces al día (Ghozland y cols., 2002b) durante 5 días no mostraron una modificación de la expresión de la abstinencia mientras que los ratones que recibieron dosis más altas de 30 o 100 mg/kg/día mostraron un síndrome de menor intensidad (Lichtman y cols., 2001).

En conjunto, los resultados que abordan la posible implicación del sistema opioide endógeno en la tolerancia y dependencia física de cannabinoides parecen indicar que los derivados peptídicos de la prePENC están implicados en dichos fenómenos. No obstante, quedaría por esclarecer cuál es el mecanismo y a través de qué receptores opioides sucede dicha modulación. Como hemos visto, la invalidación de un solo receptor opioide no parece modificar significativamente el desarrollo de los fenómenos de tolerancia y dependencia física de cannabinoides. En este sentido, la utilización de ratones deficientes de varios receptores opioides a la vez podría aportar datos interesantes al respecto. También resultaría interesante estudiar la posible existencia de dimeros entre los receptores CB1 y los distintos receptores opioides.

2.2 PARTICIPACIÓN DEL SISTEMA PURINÉRGICO EN LOS EFECTOS FARMACOLÓGICOS DE LOS CANNABINOIDES

2.2.1 SISTEMA PURINÉRGICO

Adenosina

La adenosina es un nucleótido purinérgico endógeno que participa en una gran variedad de procesos fisiológicos como la regulación del sueño, la transmisión nociceptiva, la motricidad, la neuroprotección y la atención. La adenosina también se ha relacionado con procesos patológicos tales como la epilepsia, enfermedad de Parkinson y más recientemente con los procesos de adicción (Ribeiro y cols., 2003). Se trata de un neuromodulador puesto que a diferencia de los neurotransmisores clásicos no se acumula en vesículas sinápticas y es liberado al espacio extracelular a través de transportadores de nucleósidos.

En el organismo, la adenosina se sintetiza a partir de AMPc y/o S-adenosil homocisteína tal y como se muestra en la figura 16.

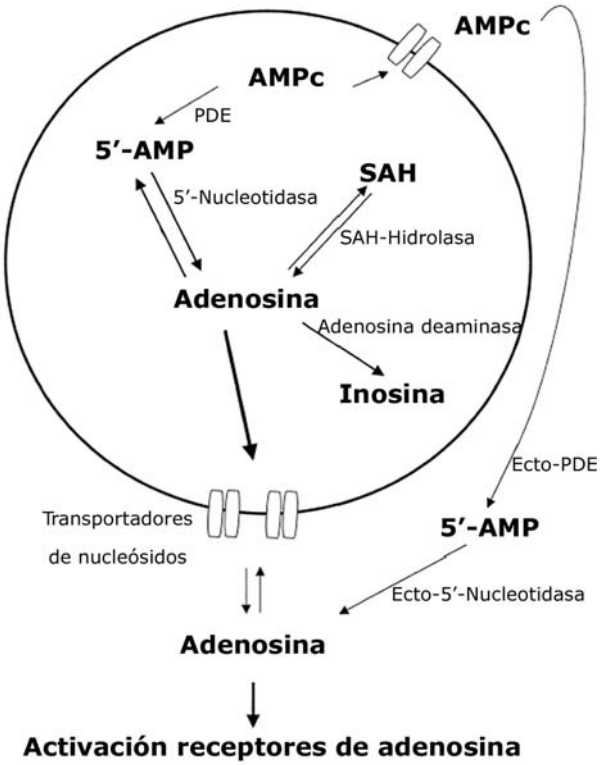

Figura 16. Vías para la producción, metabolismo y transporte de adenosina. Abreviaturas: SAH, S-adenosil homocisteína; PDE, fosfodiesterasa (Modificado de Latini y Pedata, 2001).

Receptores de adenosina

Los efectos fisiológicos de la adenosina tienen lugar a través de la activación de 4 subtipos de receptores llamados receptor de adenosina A_1, A_{2A}, A_{2B} y A_3 (Fredholm y cols., 1994). Estos receptores pertenecen a la familia de receptores acoplados a proteínas G con siete dominios transmembranales. Los receptores A_1 y A_{2A} son los principales mediadores de los efectos de la adenosina en el SNC. El receptor A_1 es el receptor de adenosina más abundante en el cerebro, con una alta concentración en estructuras tales como el hipocampo, cerebelo, corteza y ganglios basales (Weber y cols., 1988; Glass y cols., 1996). A nivel bioquímico, el receptor A_1 se encuentra acoplado negativamente a la vía de la adenilato ciclasa (tabla 6), de manera que su estimulación disminuye la acumulación de AMPc. Los receptores A_1 se localizan sobre todo a nivel presináptico, donde median el efecto inhibitorio de la adenosina sobre la liberación de neurotransmisores. En este sentido, se ha demostrado que la activación de los receptores A_1 es capaz de inhibir la liberación de GABA y glutamato en estructuras importantes para los fenómenos de adicción como son el hipocampo (Yoon y Rothman, 1991; Wu y Saggau, 1994), NAc (Uchimura y North, 1991), amígdala (Heinbockel y Pape, 1999), sustancia negra (Shen y Johnson, 1997) y sustancia gris periacueductal (Bagley y cols., 1999). El receptor de adenosina A_{2A} presenta una localización más circunscrita en el SNC. Así, los niveles más elevados de este receptor se encuentran en el caudado-putamen, NAc y tubérculo olfactorio (Moreau y Huber, 1999; DeMet y Chicz-DeMet, 2002). A diferencia del receptor A_1, la acción del receptor A_{2A} es generalmente excitatoria. Este receptor se encuentra acoplado positivamente a la vía de la adenilato ciclasa, de manera que tras su activación aumentan los niveles de AMPc. Los receptores de adenosina A_{2A} pueden actuar a través de otros mecanismos intracelulares como son la vía de las MAP quinasas y los canales de Ca^{+2} (tabla 6). Mediante estudios de hibridación *in situ* se ha demostrado que el receptor de adenosina A_1 co-localiza con el receptor de DA D1 en las neuronas del estriado que proyectan directamente hasta la sustancia negra. De manera similar, se ha observado que los receptores A_{2A} co-localizan con los D2 en las proyecciones indirectas del estriado a la sustancia negra. Ambas vías de proyección del estriado (directa e indirecta) ejercen acciones opuestas en el control de la actividad motora (Ferré y cols., 1997).

Algunas de las características de los receptores de adenosina A_{2B} y A_3 se encuentran esquematizadas en la tabla 6. A nivel de las vías de señalización intracelular destacaremos que el receptor A_{2B} es capaz de activar la enzima adenilato ciclasa mientras que el A_3 la inhibe.

Tabla 6. Características de los receptores de adenosina en el SNC.

RECEPTOR	MECANISMOS DE SEÑALIZACIÓN INTRACELULAR	PROTEÍNAS G	DISTRIBUCIÓN
A_1	(-) Adenilato ciclasa (+) GIRKs (+) Canales de Ca^{+2} (+) Fosfolipasa C	G_i/G_o	Ubicua y abundante
A_{2A}	(+) Adenilato ciclasa (+)/(-) canales de Ca^{+2} (+) MAP quinasas	G_s/G_{olf}	Tubérculo olfactorio, NAc, Caudado-putamen
A_{2B}	(+) Adenilato ciclasa (+) IP3 (+) MAP quinasas (+) Fosfolipasa C	G_s	Ubicua y baja
A_3	(-) Adenilato ciclasa (+) IP3 (+) Concentración intra-celular de Ca^{+2} (+) Fosfolipasa C	G_i/G_q	Ubicua y baja

Los signos positivos (+) representan una activación y los símbolos negativos (-) una inhibición. GIRK: canales rectificadores de K^+ dependientes de proteínas G; IP3: Inositoltrifosfato. (Modificado de Hack y Christie, 2003).

Ligandos exógenos para los receptores de adenosina

Los principales compuestos exógenos que actúan sobre los receptores de adenosina se describen en la tabla 7.

Tabla 7. Principales ligandos exógenos de los receptores de adenosina.

LIGANDO	CATEGORIA	SELECTIVIDAD DE RECEPTOR
CPA	Agonista	A_1
CGS 21680	Agonista	A_2
NECA	Agonista	Equipotente A_1, A_{2A} y A_3
CAFEÍNA	Antagonista	Inespecífico, preferencial A_{2A}
TEOFILINA	Antagonista	No selectivo y de baja afinidad
DPCPX	Antagonista	A_1
CGS 15943	Antagonista	No selectivo A_1 = A_{2A}

(Modificado de Hack y Christie, 2003).

2.2.2 BASES Y EVIDENCIAS DE LA INTERACCIÓN CANNABINOIDE-ADENOSINA

La única evidencia descrita hasta el momento de interacción cannabinoide/adenosina proviene de estudios que examinan los mecanismos subyacentes a la descoordinación motora inducida por cannabinoides. En este sentido, se ha demostrado que la administración intracerebelar de un agonista selectivo de los receptores de adenosina A_1 acentúa la descoordinación causada por la activación de los receptores CB1 en el ratón (Dar, 2000; DeSanty y Dar, 2001) y la administración de antagonistas A_1 atenúa este comportamiento (Dar, 2000). No obstante, existen evidencias para sugerir un papel del sistema purinérgico en otras respuestas comportamentales de los cannabinoides, especialmente en aquellas que están relacionadas con sus propiedades adictivas. Diversos trabajos han demostrado la existencia de interacciones funcionales entre el sistema de adenosina y el sistema dopaminérgico en el SNC. Estas interacciones han sido estudiadas a diferentes niveles (Ferré y cols., 1998). Estudios de fijación con radioligandos han demostrado que los agonistas de los receptores A_1 afectan negativamente la afinidad de la dopamina hacia los receptores de DA D1 (Ferré y cols., 1998). Por el contrario, la presencia de agonistas A_{2A} afecta negativamente la afinidad de la dopamina hacia los receptores D2 (Ferré y cols., 1991; Dasgupta y cols., 1996; Kull y cols., 1999) (figura 17).

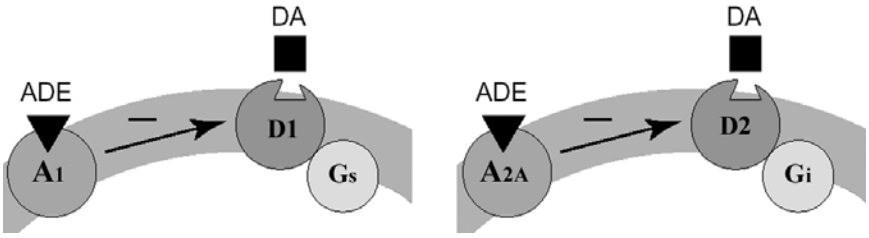

Figura 17. Representación esquemática de la interacción antagónica que existe entre los receptores de adenosina y los receptores de DA. ADE: adenosina. (Modificado de Ferré y cols., 1998).

Las interacciones antagónicas entre receptores A_1/D1 y A_{2A}/D2 han sido también evidenciadas a nivel de los mecanismos de señalización intracelular. Así, la presencia de agonistas adenosinérgicos produce una disminución de la producción de AMPc inducida por la activación de receptores D1 mientras que la presencia de antagonistas A1 es capaz de potenciar dicho efecto (Ferré y cols., 1998). Diversos trabajos han demostrado que la adenosina juega un papel opuesto al de la DA en el control de diversas respuestas comportamentales. Así, los antagonistas dopaminérgicos y los agonistas adenosinérgicos producen efectos similares sobre la

actividad locomotora (Hauber y Munkle, 1997). Como se ha detallado en el apartado 1.4.6, la transmisión dopaminérgica mesolímbica juega un papel importante en las propiedades reforzantes de los cannabinoides y en general de la mayoría de drogas de abuso. Teniendo en cuenta que existe una alta densidad de receptores de adenosina A_{2A} en el NAc (Ongini y Fredholm, 1996; Rosin y cols., 2003) sería interesante examinar el papel que puedan jugar estos receptores en las respuestas motivacionales de los cannabinoides.

Por otro lado, estudios recientes sugieren que la adenosina juega un papel crucial en los fenómenos de dependencia física y abstinencia de diferentes drogas de abuso. Este efecto parece estar estrechamente relacionado con la capacidad que tienen la mayoría de drogas de abuso para producir, tras un tratamiento crónico, cambios adaptativos en las vías de señalización mediadas por adenosina. Así, el síndrome de abstinencia de compuestos como los opioides, cannabinoides y nicotina y se ha asociado con un aumento de la actividad de la vía de la adenilato ciclasa en el locus coeruleus, cerebelo y amígdala respectivamente (Maldonado y cols., 1996; Hutcheson y cols., 1998; Tzavara y cols., 2004). Este aumento de la actividad de la enzima adenilato ciclasa se corresponde con un aumento de los niveles de AMPc, el sustrato endógeno para la formación de adenosina. Diversos estudios farmacológicos han demostrado que el bloqueo del metabolismo de adenosina es capaz de disminuir la severidad del síndrome de abstinencia de morfina (Kaplan y Coyle, 1998). Un efecto parecido se ha observado tras la administración del agonista purinérgico CGS 21680 (Kaplan y Sears, 1996). Por el contrario, se ha demostrado que los antagonistas de adenosina o la invalidación del gen que codifica el receptor de adenosina A_{2A} incrementan la expresión del síndrome de abstinencia de morfina (Salem y Hope, 1997; Berrendero y cols., 2003, artículo 6 anexo; Bailey y cols., 2004). No obstante, hasta el momento ningún estudio ha evaluado la posible implicación de la adenosina y de los diferentes receptores de adenosina en los efectos agudos y crónicos inducidos tras la administración de cannabinoides y en particular, en los fenómenos relacionados con la dependencia de THC.

2.3 PARTICIPACIÓN DEL SISTEMA CANNABINOIDE ENDÓGENO EN LOS EFECTOS FARMACOLÓGICOS DE LA NICOTINA

2.3.1 NICOTINA Y RECEPTORES NICOTÍNICOS DE ACETILCOLINA

La nicotina es un alcaloide que se encuentra principalmente en plantas de la familia de las solanáceas tales como la patata, el tomate y el tabaco (Doolittle y cols., 1995) (figura 18a). Este compuesto parece tener un papel primordial en la iniciación y mantenimiento de la adicción al tabaco (Buisson y Bertrand, 2002). No obstante, la nicotina ejerce otros efectos en el organismo. Así, se ha descrito que la nicotina es capaz de modular la neurotransmisión del dolor, el control del movimiento, los procesos cognitivos y también las respuestas de tipo emocional (Picciotto y cols., 1995; File y cols., 2002; Katner y cols., 2004; Schochet y cols., 2004; Decker y cols., 2004).

Los efectos farmacológicos de la nicotina tienen lugar a través de la activación de los receptores nicotínicos de acetilcolina (nAChRs) (figura 18b). Estos receptores se encuentran ampliamente distribuidos en el SNC y también en la periferia. A nivel periférico se localizan en la placa muscular, en las neuronas vegetativas postganglionares y en la médula adrenal. Los nAChRs pertenecen a la familia de canales iónicos que se activan mediante la unión de un ligando (Le Novère y Changeux, 1999) (tabla 8). El ligando endógeno para los nAChRs es la acetilcolina.

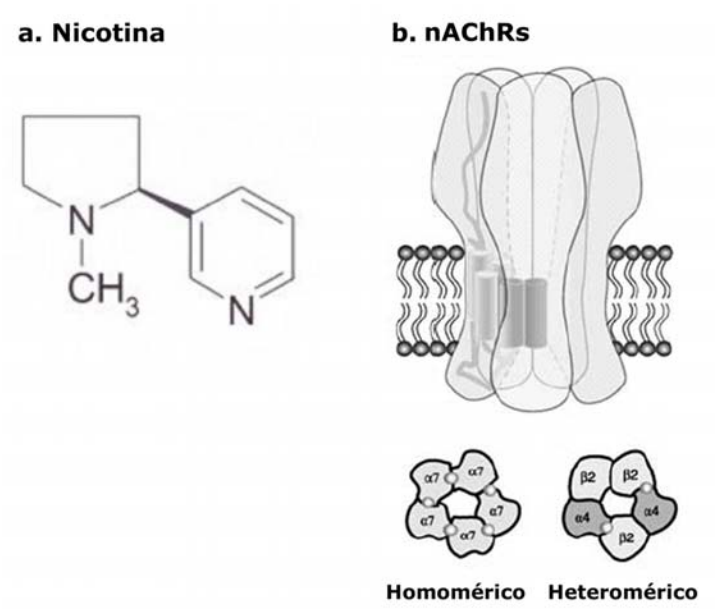

Figura 18. a. Estructura química de la nicotina. b. Esquema representativo de los nAChRs (Modificado de Hogg y cols., 2003).

Los nAChRs tienen una estructura pentamérica resultado de la combinación de cinco subunidades proteicas. En el sistema nervioso, estos receptores están formados por la combinación de subunidades tipo α y β. Diversos estudios han demostrado que las neuronas expresan nueve tipos de subunidades α (α2-α10) y tres tipos de subunidades β (β2-β4). Estas subunidades se pueden combinar de manera homomérica (p.e. α7) o heteromérica (p.e. α4/β2) para generar una gran diversidad de subtipos de nAChRs que presentan propiedades funcionales y características farmacológicas diferentes (Le Novère y cols., 2002; Itier y Bertrand, 2001; Hogg y cols., 2003). En base a su composicíon y características farmacológicas se han establecido 4 grupos de nAChRs (Marubio y Changeux, 2000) (tabla 8).

Tabla 8. Grupos de nAChRs.

nAChRs	COMPOSICIÓN	ALTA AFINIDAD PARA..	LOCALIZACIÓN PREDOMINANTE EN EL SNC
GRUPO 1	α7	α-bungarotoxina	Corteza Áreas límbicas
GRUPO 2	β2 α4 (α5?) β2 (α2?) β2 (α3?) β2 (α6 β3?)	Epibatadina > Nicotina = Citisina = Metilcarbamilcolina = Acetilcolina	Todo SNC Núcleo interpeduncular Hipocampo Núcleo catecolaminérgico Colículo superior
GRUPO 3	β4 α3 (α5?)	Epibatadina	Habénula medial Núcleo interpeduncular Médula dorsal
GRUPO 4	(β4 α4?) (β4 α2?)	Epibatadina > Citisina > Metilcarbamilcolina = Acetilcolina	Habénula medial lateral Núcleo interpeduncular dorsal

(Modificado de Marubio y Changeux, 2000).

El primer grupo de nAChRs incluye aquellos receptores que son sensibles a α-bungarotoxina y que están compuestos por subunidades de tipo α7. El segundo grupo incluye aquellos nAChRs insensibles a α-bungarotoxina y que contienen las subunidades α2/β2 o α4/β2. Estos receptores se caracterizan por presentar una alta afinidad para epibatadina y nicotina. El tercer grupo está compuesto por receptores que no contienen las subunidades α4, α7 o β2 y que presentan una alta afinidad para epibatadina pero no para nicotina y citisina. Se ha propuesto que la

composición de estos receptores sea de tipo α3/β4. Finalmente, existe un cuarto grupo de nAChRs que está formado por receptores que no contienen la subunidad β2, que tienen una alta afinidad para la epibatadina y una baja afinidad para la nicotina, pero que a diferencia de los receptores del tercer grupo presentan una alta afinidad para citisina. Las combinaciones que se han propuesto para este grupo son de tipo α4/β4 y α2/β4.

Un aspecto importante a destacar de los nAChRs es que pueden existir en cuatro estados funcionales: cerrado, abierto, desensibilizado e inactivo. En general, los nAChRs permanecen largo tiempo en estado cerrado antes de que se una el agonista. Estos receptores se encuentran en estado abierto durante un breve espacio de tiempo cuando el canal está conduciendo cationes y en estado de desensibilización e inactivo cuando no responden a agonistas (Dani y Heinemann, 1996). El porcentaje de tiempo que los nAChRs pasan en cada uno de estos estados depende, entre otras cosas, del tipo de subunidades que los formen. Así, se ha demostrado que en el ATV los nAChRs que contienen la subunidad α7 se desensibilizan menos ante la presencia de nicotina que aquellos que no contienen esta subunidad (Mansvelder y Mc Gehee, 2002).

Ligandos exógenos para los nAChRs

Algunos de los ligandos exógenos que actúan sobre los nAChRs se describen en la tabla 9.

Tabla 9. Ligandos exógenos de los nAChRs.

LIGANDO	CATEGORIA	SELECTIVIDAD DE RECEPTOR
NICOTINA	Agonista	no
EPIBATIDINA	Agonista	no
ABT-594	Agonista	α4β2
MECAMILAMINA	Antagonista	excepto α7
METILCACONITINA	Antagonista	α7
α-BUNGAROTOXINA	Antagonista	α7
DIHIDRO-β-ERITROIDINA	Antagonista	β2

2.3.2 EFECTOS FARMACOLÓGICOS DE LA NICOTINA A NIVEL DEL SNC

La nicotina es capaz de afectar múltiples funciones en el sistema nervioso central algunas de las cuales se detallan brevemente a continuación.

Efectos de la nicotina sobre la transmisión nociceptiva

Diversos estudios farmacológicos han demostrado que la nicotina es capaz de inducir respuestas antinociceptivas en el animal de experimentación (Aceto y cols., 1983; Decker y cols., 2004). Estudios neuroanatómicos han puesto de manifiesto que los nAChRs se encuentran ampliamente distribuidos en los núcleos y vías que participan en la transmisión del dolor, lo que sugiere que los efectos antinociceptivos de la nicotina pueden estar mediados por la acción de este compuesto en múltiples lugares. Uno de los mecanismos que se ha propuesto es la facilitación de la liberación de ciertos neurotrasmisores como adrenalina, acetilcolina y serotonina en áreas relacionadas con la transmisión nociceptiva. Así, la administración de antagonistas α2-adrenérgicos, muscarínicos y serotonérgicos directamente en la médula espinal atenúa las respuestas antinociceptivas de la nicotina (Iwamoto y Marion, 1993; Rogers y Iwamoto, 1993). Más recientemente, diversos estudios farmacológicos y moleculares han implicado los péptidos opioides en la analgesia inducida por nicotina. En este sentido, tanto la administración del antagonista opioide naloxona, como la supresión del MOR y del gen que codifica para la PENC, disminuye las propiedades antinociceptivas de la nicotina en el ratón (Zarrindast y cols., 1997; Berrendero y cols., 2002; 2005). Otro mecanismo importante para el efecto antinociceptivo de la nicotina parece ser la modulación de las vías inhibitorias descendentes reguladoras de la nocicepción. Así, la administración directa de nicotina en el núcleo tegmental pedunculopontino y en el núcleo del raphe fue capaz de inducir repuestas antinociceptivas en el ensayo de la placa caliente y en el ensayo de la sacudida de cola en la rata (Iwamoto, 1991).

El uso de ratones modificados genéticamente ha permitido avanzar en el conocimiento de las subunidades de los nAChRs más importantes para los efectos antinociceptivos de la nicotina. Así, los ratones deficientes de las subunidades α4 o β2 mostraron una disminución del efecto antinociceptivo de la nicotina en el ensayo de la placa caliente y una sensibilidad menor a la nicotina en el ensayo de la sacudida de cola (Marubio y cols., 1999).

Efectos centrales de la nicotina sobre el control motor

En roedores, la nicotina ejerce efectos complejos a nivel de la locomoción. En un ambiente conocido, la administración de nicotina puede resultar en una

activación locomotora (Clarke y cols., 1988). Además, la administración repetida de nicotina es capaz de inducir una sensibilización de estos efectos hiperlocomotores (Clarke y Kumar, 1983; Museo y Wise, 1990), una respuesta que ha sido asociada a la administración de sustancias psicoestimulantes (Pierce y Kalivas, 1997). Por el contrario, el tratamiento agudo con nicotina en un ambiente nuevo produce una significativa disminución de la motricidad en roedores (Marks y cols., 1989). Este efecto disminuye tras sucesivas administraciones del compuesto demostrando el desarrollo de una tolerancia (Marks y cols., 1991).

Efectos gratificantes de la nicotina

Los efectos reforzantes de la nicotina se han puesto de manifiesto en el animal de experimentación mediante la utilización de diversos modelos comportamentales tales como el paradigma del condicionamiento espacial, la auto-administración intravenosa y la auto-estimulación intracraneal (Laviolette y van der Kooy, 2004).

Mediante la utilización de ratones modificados genéticamente, Picciotto y colaboradores pusieron de manifiesto que la subunidad β2 es importante para la manifestación de los efectos reforzantes de la nicotina. Así, la auto-administración intravenosa de nicotina fue menor en los ratones mutantes para dicha subunidad (Picciotto y cols., 1998). Recientemente, Berrendero y colaboradores han demostrado que el MOR y los péptidos opioides derivados de PENC participan en la manifestación de las propiedades gratificantes de la nicotina mediante el paradigma de preferencia de plaza condicionada (Berrendero y cols., 2002; 2005).

Las propiedades gratificantes de la nicotina resultan de su interacción con el circuito central del refuerzo. En este sentido, diversos estudios han demostrado que la nicotina es capaz de estimular la transmisión dopaminérgica mesolímbica (Pontieri y cols., 1996; Dani y De Biasi, 2001), un efecto que parece depender de un balance funcional entre las aferencias excitatorias e inhibitorias que reciben las neuronas dopaminérgicas del ATV, así como también del efecto directo de la nicotina sobre las propias neuronas dopaminérgicas (Mansvelder y McGehee, 2002). Diferentes subtipos de nAChRs presentes en el ATV parecen regular este proceso (figura 19).

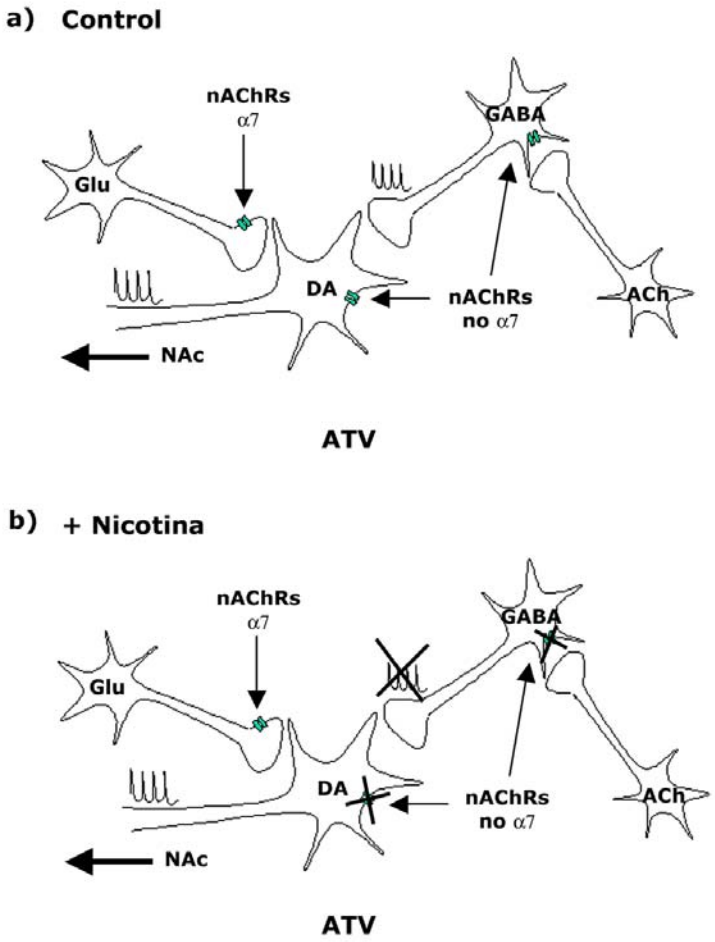

Figura 19. Papel de los nAChRs en el control de la excitabilidad de las neuronas dopaminérgicas del ATV: a) situación control; b) tras la administración de nicotina (Modificado de Mansvelder y McGehee, 2002).

Tal y como se esquematiza en la figura 19a, en condiciones normales los nAChRs que no contienen la subunidad α7 son capaces de activar las neuronas dopaminérgicas y GABAérgicas directamente (Mansvelder y cols., 2002), mientras que los nAChRs que contienen la subunidad α7 favorecen la liberación de glutamato en las terminales glutamatérgicas que provienen de la corteza prefrontal (Mansvelder y McGehee, 2000). En este contexto, la acetilcolina endógena es capaz de favorecer la liberación de GABA en el ATV. En presencia de nicotina (figura 19b), los nAChRs que no contienen la subunidad α7 se desensibilizan rápidamente, deprimiendo la liberación de GABA. Por el contrario, los nAChRs que contienen la subunidad α7 se desensibilizan menos y esto hace que se siga liberando glutamato. La inhibición de la liberación de GABA junto con la estimulación de la liberación de glutamato hace que exista un incremento neto de la excitación de las neuronas dopaminérgicas del ATV. Por tanto, según Mansvelder y McGehee (2002), la diferente velocidad de desensibilización de estos dos grupos de receptores, los que

contienen la subunidad α7 y los que no, hace que el efecto de la nicotina sobre las neuronas dopaminérgicas del ATV se decante hacia la excitación y no hacia la inhibición. Este efecto parece contribuir a la liberación sostenida de DA que se produce tras la administración de nicotina.

Dependencia física y síndrome de abstinencia de nicotina

Existen claras evidencias de que el consumo repetido de nicotina induce un estado de dependencia física en el hombre. Este hecho viene abalado por la aparición de un síndrome de abstinencia en aquellas personas que interrumpen el consumo crónico de nicotina cuando abandonan la adicción al tabaco (Kenny y Markou, 2001). En humanos, el síndrome de abstinencia de nicotina comprende tanto síntomas de tipo físico como afectivos. La sintomatología física más representativa incluye bradicardia, malestar gastrointestinal y un aumento considerable del apetito. Dichos síntomas se acompañan de un estado de ansiedad, irritabilidad, disforia y dificultad en la concentración (Glassman y cols., 1990; West y cols., 1991; Parrott, 1993). En el animal de experimentación, el síndrome de abstinencia de nicotina puede aparecer espontáneamente después de finalizar un tratamiento crónico con nicotina o puede precipitarse de forma brusca mediante la administración de antagonistas nicotínicos tales como la mecamilamina y la dihidro-β-eritroidina (Damaj y cols., 2003). Análogamente al síndrome de abstinencia en humanos, el descrito en roedores se caracteriza por la aparición de signos físicos y signos afectivos. En la actualidad, disponemos de modelos que reproducen tanto el aspecto físico como el aspecto emocional o afectivo del síndrome de abstinencia de nicotina. De manera general podemos decir que los signos físicos más representativos de la abstinencia de nicotina en roedores incluyen constricciones abdominales, contracciones de los músculos faciales, arañazos, intentos de escape, lameteo de los genitales, “wet dog shakes” y temblores (Isola y cols., 1999; Watkins y cols., 2000). Por otro lado, la sintomatología afectiva de tipo disfórico asociada a la abstinencia de nicotina se ha puesto de manifiesto mediante modelos de auto-estimulación intracraneal y aversión de plaza condicionada. Así, mediante técnicas de estimulación intracraneal se ha podido observar que el síndrome de abstinencia de nicotina, tanto espontáneo (Epping-Jordan y cols., 1998) como precipitado por antagonistas nicotínicos (Epping-Jordan y cols., 1998; Watkins y cols., 2000), produce un aumento de los umbrales del refuerzo cerebral en ratas. Se ha demostrado la aparición de aversión de plaza condicionada inducida por la administración del antagonista nicotínico dihidro-β-eritroidina en ratas dependientes de nicotina (Watkins y cols., 2000). Recientemente, Balerio y colaboradores han

descrito la aparición de una aversión de plaza condicionada tras la administración de naloxona en ratones nicotina-dependientes (Balerio y cols., 2004).

A nivel bioquímico, el síndrome de abstinencia de nicotina en ratas se ha asociado con un aumento de la actividad adenilato ciclasa en la amígdala (Tzavara y cols., 2002), una disminución de la liberación de DA en la amígdala y en el NAc y un aumento de la expresión de c-fos en la amígdala (Hildebrand y cols., 1998; Panagis y cols., 2000).

Existen pocos estudios que hayan evaluado la participación de las diferentes subunidades de los nAChRs en el síndrome de abstinencia de nicotina. En este sentido, un estudio reciente ha demostrado que la subunidad β4 parece jugar un papel importante. Así, los ratones deficientes de la subunidad β4 mostraron un síndrome de abstinencia nicotínico de menor severidad (Salas y cols., 2004).

2.3.3 BASES Y EVIDENCIAS DE LA INTERACCIÓN CANNABINOIDE-NICOTINA

Aunque el cannabis y el tabaco se asocian habitualmente para el consumo en humanos, se conoce poco acerca de las interacciones que pueden ocurrir entre estos dos compuestos y especialmente en el desarrollo de los procesos adictivos. Ambos compuestos contienen sustancias psicoactivas, el THC y la nicotina respectivamente, los cuales son capaces de afectar (aunque en ocasiones de forma divergente) funciones similares en el SNC tales como la locomoción, la antinocicepción, la ansiedad, el aprendizaje y la memoria. Además, tanto el THC como la nicotina son capaces de inducir efectos gratificantes y dependencia física. Por otro lado, existe una amplia distribución de los receptores cannabinoides CB1 y nAChRs en el SNC. Esto hace que existan áreas donde la expresión de ambos receptores está solapada, lo cual parece facilitar la posible existencia de una interacción funcional entre estos dos sistemas.

Hasta el momento dos estudios farmacológicos han investigado la posible interacción entre la nicotina y los agonistas cannabinoides a nivel de las respuestas agudas. Estos dos estudios han demostrado que la nicotina es capaz de facilitar, en el animal de experimentación, diversas respuestas comportamentales inducidas tras la administración aguda de cannabinoides tales como hipolocomoción, ansiolisis, hipotermia, incoordinación motora y bradicardia (Pryor y cols., 1978; Valjent y cols., 2002). Asimismo, la administración conjunta de dosis sub-efectivas de THC y nicotina produce una respuesta de tipo ansiolítico y efectos reforzantes evaluados en el paradigma del condicionamiento espacial (Valjent y cols., 2002). En este mismo trabajo, Valjent y colaboradores observaron que el efecto facilitador de la nicotina sobre las respuestas del THC se manifestaba también a nivel bioquímico. Así, la administración conjunta de THC y nicotina incrementó los niveles de

expresión de c-fos en el NAc, núcleo central y basolateral de la amígdala, núcleo del lecho de la estría terminal y núcleo paraventricular del hipotálamo. A nivel crónico, estos mismos autores observaron que el co-tratamiento de nicotina y THC producía una disminución de la tolerancia a los efectos farmacológicos del THC y un incremento del síndrome de abstinencia de este compuesto (Valjent y cols., 2002). Por otro lado, se ha descrito que el THC atenúa las manifestaciones somáticas y motivacionales de la abstinencia de nicotina en ratones (Balerio y cols., 2004). Estudios bioquímicos han demostrado que el tratamiento crónico con nicotina modifica los niveles de endocannabinoides en determinadas áreas cerebrales. Concretamente, se ha observado un incremento de los niveles de anandamida en áreas del sistema límbico y una disminución de dicho cannabinode endógeno en el hipocampo, estriado y corteza cerebral (González y cols., 2002a). Este resultado junto con los datos farmacológicos anteriormente descritos sugiere que los efectos de la nicotina podrían estar mediados o modulados por la acción de cannabinoides endógenos. En este sentido, resulta de especial interés investigar las respuestas de la nicotina en animales deficientes de los receptores cannabinoides CB1.

OBJETIVOS

OBJETIVO 1

Evaluar la posible implicación de diversas estructuras cerebrales con alta densidad de receptores cannabinoides CB1 como cerebelo, estriado, hipocampo y amígdala en el síndrome de abstinencia de cannabinoides.

Artículo 1:

Castañé A, Maldonado R, Valverde O (2004). "Role of different brain structures in the behavioural expression of WIN 55,212-2 withdrawal in mice". *British Journal of Pharmacology* 142:1309–1317.

OBJETIVO 2

Examinar la posible existencia de una acción cooperativa entre los receptores MOR y DOR para mediar los efectos comportamentales de los cannabinoides mediante el uso de ratones doble mutantes deficientes de MOR y DOR.

Artículo 2:

Castañé A, Robledo P, Matifas A, Kieffer BL, Maldonado R (2003). "Cannabinoid withdrawal syndrome is reduced in double mu and delta opioid receptor knockout mice". *European Journal of Neuroscience* 17:155–159.

OBJETIVO 3

Estudiar la participación de los receptores de adenosina A_{2A} en la regulación de los efectos farmacológicos de los cannabinoides, especialmente en aquellas respuestas comportamentales relacionadas con las propiedades adictivas de estos compuestos, mediante el uso de ratones deficientes de los receptores A_{2A}.

Artículo 3:

Soria G*, Castañé A*, Berrendero F, Ledent C, Parmentier M, Maldonado R, Valverde O (2004). "Adenosine A_{2A} receptors are involved in physical dependence and place conditioning induced by THC". *European Journal of Neuroscience* 20:2203-2213.

* igual contribución

OBJETIVO 4

Investigar la implicación de los receptores cannabinoides CB1 en las respuestas farmacológicas de la nicotina, mediante el uso de ratones deficientes de los receptores cannabinoides CB1.

Artículo 4:

Castañé A, Valjent E, Ledent C, Parmentier M, Maldonado R, Valverde O (2002). Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence. *Neuropharmacology* 43:857-867.

RESULTADOS

ARTÍCULOS

Artículo 1:
Castañé A, Maldonado R, Valverde O. “Role of different brain structures in the behavioural expression of WIN 55,212-2 withdrawal in mice”. *British Journal of Pharmacology.* 2004 Aug;142(8):1309–17.

Artículo 2:
Castañé A, Robledo P, Matifas A, Kieffer BL, Maldonado R. “Cannabinoid withdrawal syndrome is reduced in double mu and delta opioid receptor knockout mice”. *European Journal of Neuroscience.* 2003 Jan;17(1):155–9. Blackwell Publishing

Erratum in: *European Journal of Neuroscience.* Jan;17(2):427.

Artículo 3:
Soria G, Castañé A, Berrendero F, Ledent C, Parmentier M, Maldonado R, Valverde O. “Adenosine A_{2A} receptors are involved in physical dependence and place conditioning induced by THC”. *European Journal of Neuroscience.* 2004 Oct;20(8):2203-13. Blackwell Publishing

Artículo 4:
Castañé A, Valjent E, Ledent C, Parmentier M, Maldonado R, Valverde O. “Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence”. *Neuropharmacology.* 2002 Oct;43(5):857-67.

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Role of different brain structures in the behavioural expression of WIN 55,212-2 withdrawal in mice

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1 We have evaluated several responses induced by the cannabinoid agonist WIN 55,212-2 related to its addictive properties, including rewarding effects and the development of physical dependence in mice. Moreover, we have studied the specific involvement of several brain regions with high density of CB1 cannabinoid receptors, such as striatum, hippocampus, amygdala and cerebellum, in the behavioural expression of SR 141716A-precipitated WIN 55,212-2 withdrawal.

2 The systemic administration of the CB1 receptor antagonist SR 141716A (10 mg kg^{-1}, s.c.) precipitated behavioural signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 and 2 mg kg^{-1}, intraperitoneal (i.p.)), revealing the development of physical dependence.

3 The microinjection of SR 141716A (1.5 and 3 μg) into the cerebellum induced severe manifestations of abstinence in mice dependent on WIN 55,212-2 (1 mg kg^{-1}, i.p.). Out of 10 signs evaluated, seven were statistically significant: wet dog shakes, body tremor, paw tremor, piloerection, mastication, genital licks and sniffing. When the cannabinoid antagonist was administered into the hippocampus and the amygdala, a moderate but significant withdrawal syndrome was also observed. However, no signs of abstinence were induced when SR 141716A was microinjected into the striatum.

4 WIN 55,212-2 produced rewarding effects in the place-conditioning paradigm in mice pre-exposed to a priming injection of the drug.

5 These results show a reliable behavioural model to reveal rewarding effects and physical dependence induced by the repeated administration of WIN 55,212-2 in mice. The cerebellum and to a lesser extent the hippocampus and the amygdala participate in the behavioural expression of cannabinoid withdrawal.

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Keywords: Amygdala; cannabinoids; cerebellum; hippocampus; physical dependence; WIN 55,212-2; withdrawal

Abbreviations: AC, adenylyl cyclase; CNS, central nervous system; CP 55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol; DMSO, dimethylsulphoxide; HU 210, *R*(−)-7-hydroxy-delta(6)-tetra-hydrocannabinol-dimethylheptyl; IP3, inositol triphosphate; MAPKs, mitogen-activated protein kinases; SR 141716A, (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4,-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxyamidehydrochloride); THC, delta9-tetrahydrocannabinol; WIN 55,212-2, ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]1-naphthyl)methanone)

Introduction

Molecular cloning of membrane receptors for *Cannabis Sativa* derivatives was a key step in the understanding of cannabinoid pharmacology. Two types of cannabinoid receptors have been identified so far: the CB1 receptor, mainly located in the central nervous system (CNS) (Matsuda *et al*., 1990), and the CB2 receptor, which has a predominant peripheral distribution in immune cells (Munro *et al*., 1993). Both receptor subtypes are coupled to $G_{i/o}$ proteins and their activation induces intracellular signalling events including inhibition of adenylyl cyclase (AC) activity, activation of mitogen-activated protein kinases (MAPKs), and changes in Ca^{2+} and K^+ currents (Wilson & Nicoll, 2002; Piomelli, 2003). Several natural (delta9-tetrahydrocannabinol, THC) and synthetic agonists (CP 55,940, HU 210, WIN 55,212-2) as well as endogenous ligands (anandamide, 2-arachidonyl glycerol, noladin ether and palmitoylethanolamide) that bind to cannabinoid receptors have been identified (Fride & Mechoulam, 2003). These compounds exhibit differences in their affinities and intrinsic activities for CB1 and CB2 receptors (Breivogel *et al*., 1998; Griffin *et al*., 1998). In this sense, WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzo xazin-6-yl]1-naphthyl)methanone), the most typical aminoalkylindole cannabinoid agonist, reveals a higher intrinsic activity at CB1 receptors than the natural agonist THC (Breivogel *et al*., 1998; Griffin *et al*., 1998; Howlett *et al*., 2002). Previous studies have shown that CB1 receptors mediate the main effects of cannabinoids in the CNS (Ledent *et al*., 1999). Thus, CB1 receptors are responsible for the effects of cannabinoids on nociceptive transmission, motor function, learning and memory processes, and drug addiction (Ameri, 1999; Ledent *et al*., 1999; Zimmer *et al*., 1999; Maldonado & Rodríguez de Fonseca, 2002; Martin *et al*., 2002). Behavioural responses related to the addictive properties of cannabinoids,

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including rewarding effects and the development of physical dependence, have been previously investigated. Rewarding effects of cannabinoids have been revealed in several behavioural paradigms such as conditioned place preference and self-administration procedures, although different results have been reported mainly depending on the experimental designs used (Takahashi & Singer, 1979; Sañudo-Peña *et al*., 1997; Martellotta *et al*., 1998; Cheer *et al*., 2000; Valjent & Maldonado, 2000; Braida *et al*., 2001; Fattore *et al*., 2001; Navarro *et al*., 2001; Justinova *et al*., 2003). Several studies have shown that the administration of SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4,-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxyamidehydrochloride), a selective CB1 receptor antagonist, precipitated a withdrawal syndrome in animals chronically treated with cannabinoids (Tsou *et al*., 1995; Hutcheson *et al*., 1998; Rubino *et al*., 1998). In rodents, this withdrawal syndrome is characterized by the presence of somatic signs such as wet dog shakes, head shakes, facial rubbing, front paw tremor, body tremor, ataxia, hunched posture, ptosis, piloerection, mastication, hypolocomotion and scratching, and the absence of vegetative manifestations. Additionally, several biochemical changes were also observed during cannabinoid withdrawal such as a compensatory upregulation in the AC pathway that selectively occurs in the cerebellum (Hutcheson *et al*., 1998; Tzavara *et al*., 2000), an enhancement in the release of corticotrophin-releasing factor (CRF) and c-Fos inmunoreactivity in the amygdala (Rodríguez de Fonseca *et al*., 1997), and a reduced dopaminergic transmission in the ventral striatum (Diana *et al*., 1998). Although these previous studies pointed out a role of several brain structures in cannabinoid withdrawal, the anatomical sites that mediate the somatic manifestations of physical dependence have not been identified.

Here, we have further investigated several pharmacological responses of WIN 55,212-2 related to its addictive properties. We have evaluated the rewarding properties of WIN 55,212-2 in the place-conditioning paradigm. We have developed a model of WIN 55,212-2 physical dependence in mice in order to investigate the neuroanatomical regions involved in the manifestation of somatic signs of cannabinoid withdrawal, with particular interest in those regions with high CB1 receptor density. For this purpose, the CB1 antagonist SR 141716A was directly microinjected into the striatum, hippocampus, amygdala and cerebellum of mice chronically treated with the cannabinoid agonist WIN 55,212-2.

Methods

Animals

Male CD1 mice (Charles River, France) weighing 26–30 g at the start of the study were housed grouped (five per cage) in a temperature ($21 \pm 1^{\circ}C$) and humidity ($55 \pm 10\%$) controlled room with a 12 h light–dark cycle (light between 0800 and 2000). Food and water were available *ad libitum*. Mice were habituated to their new environment for 1 week after arrival, before starting the experimental procedure. Behavioural tests and animal care were conducted in accordance with the Guidelines of The European Communities Council Directive 86/609/EEC regulating animal research and were approved by the Local Ethical Committee (CEEA-IMAS-UPF). The observer was blind to the treatment in all the experiments.

Place preference paradigm

An unbiased place-conditioning procedure was used to evaluate the rewarding properties of WIN 55,212-2, as previously reported (Matthes *et al*., 1996; Maldonado *et al*., 1997). During the preconditioning phase, drug-naive mice had free access to explore both compartments of the conditioning apparatus during 20 min. No initial place preference or aversion for the different compartments was observed. The conditioning phase consisted of five pairings with WIN 55,212-2 (0.1 and 1 mg kg^{-1}, intraperitoneal (i.p.)) (days 1, 3, 5, 7 and 9) and five pairings with vehicle (days 2, 4, 6, 8 and 10) during a 45 min conditioning time. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The test phase was conducted exactly as the preconditioning one, that is, free access to both compartments during 20 min. In order to exclude the possible dysphoric effect of the first drug administration, two groups of mice received a single injection (priming) of WIN 55,212-2 (0.1 and 1 mg kg^{-1} respectively, i.p.) in their home cages 24 h before starting the conditioning procedure, as previously reported for THC place-conditioning experiments (Valjent & Maldonado, 2000). The time in the central area was proportionally shared and added to the time value of each compartment, as previously reported (Matthes *et al*., 1996; Valverde *et al*., 1996). A place-conditioning score was calculated for each mouse as the difference between the time spent in the drug-paired compartment during the testing and preconditioning phases.

Physical dependence induced by WIN 55,212-2 and evaluation of withdrawal syndrome after systemic administration of SR 141716A

The CB1 cannabinoid receptor agonist WIN 55,212-2 (1 and 2 mg kg^{-1}, i.p.) or its vehicle was administered twice daily during 5 days (0900 and 2000) in order to induce physical dependence in mice ($n = 10$–12 per group). On day six, mice only received the morning injection and 2 h later mice were placed in a circular plastic observation area for a 15 min period. At the end of this period, the CB1 cannabinoid receptor antagonist SR 141716A (10 mg kg^{-1}, s.c.) was administered to precipitate the withdrawal syndrome. The following abstinence signs were evaluated, 15 min before and 30 min after SR 141716A injection: wet dog shake, ptosis, body tremor, ataxia, front paw tremor, piloerection, hunched posture, mastication, genital lick, cramp and sniffing. The number of wet dog shakes, front paw tremors, sniffings and cramps was counted. Ptosis, body tremor, ataxia, piloerection, hunched posture, mastication and genital licks were scored 1 for appearance and 0 for nonappearance within each 5 min time. A quantitative value was calculated in each animal for the different checked signs by adding the scores obtained in each 5 min period. A global withdrawal score, ranging from 0 to 100, was calculated for each animal by giving to each individual sign a relative weight, as previously reported (Valverde *et al*., 2000).

Physical dependence induced by WIN 55,212-2 and evaluation of withdrawal syndrome after intracerebral administration of SR 141716A

In this set of experiments, the participation of several brain structures in the somatic manifestations of WIN 55,212-2 withdrawal was examined. For this purpose, the CB1 cannabinoid receptor antagonist SR 141716A (0.75, 1.5 and 3 μg per mouse) was directly administered into the brain of WIN 55,212-2-dependent mice (1 mg kg^{-1}, i.p., twice daily, 5 days). Mice were first anaesthetized with a ketamine/xylazine mixture and subsequently mounted in a stereotaxic frame (KOPF Instruments, Tujunga, CA, U.S.A.). They were implanted with guide cannuli (7 mm long, 30 gauge) into different brain regions: third ventricle (i.c.v.), striatum, hippocampus, amygdala and cerebellum. The coordinates (expressed in mm) were taken from bregma and the skull surface according to the stereotaxic atlas (Paxinos & Franklin, 1997). Mice were implanted unilaterally into the third ventricle (AP: −0.5; L: 0; H: −3) ($n=17$) and cerebellum (AP: −7.0; L: 0; H: −1.7) ($n=19$), and bilaterally into the striatum (AP: +0.38; L: ±2.0; H: −4.0) ($n=19$), hippocampus (AP: −1.7; ML: ±1.75, DV: −2.1) ($n=10$) and amygdala (AP: −1.46; ML: ±2.80, DV: −4.80) ($n=13$). Cannuli were subsequently fixed to the skull with dental cement. After surgery, mice were individually housed for recovering during 4 days. After this period, mice were chronically treated with WIN 55,212-2 (1 mg kg^{-1}, i.p., twice daily, 5 days), as described above. Following the induction of WIN 55,212-2 dependence, a withdrawal syndrome was precipitated by the administration of different doses of SR (0.75, 1.5 and 3 μg). At 2 h after each morning injection of WIN 55,212-2, the withdrawal syndrome was precipitated by the administration of SR 141716A or its vehicle (first) through the cannuli in an injection volume of 2 μl (animals implanted bilaterally received 1 μl through each cannula). Each animal received the different doses of SR 141716A in consecutive days by using a Latin Square design. In order to verify that the withdrawal syndrome was not conditioned to the experimental context, the last day, mice received the morning injection of WIN 55,212-2, and 2 h later they were observed for withdrawal signs after the intracerebral administration of vehicle (second) (Figure 4, Table 1). During the different days of withdrawal syndrome evaluation, mice received the chronic WIN 55,212-2 treatment (1 mg kg^{-1}, i.p., twice daily).

After completion of the experimental sequence, the histological verification of the cannuli was performed. Mice were killed, and the brains were removed and frozen. Coronal sections (40 μm) of the brain were cut on a cryostat at −26°C and the site of injection was compared to standard stereotaxic plates (Paxinos & Franklin, 1997) (Figure 3). Data from mice with wrong location of the cannuli were removed from the study.

Table 1 WIN 55,212-2 withdrawal precipitated by intracerebral administration of SR141716A

	Wet dog shakes	*Ptosis*	*Body tremor*	*Paw tremor*	*Piloerection*	*Hundred posture*	*Mastication*	*Genital licks*	*Sniffing*	*GWS*
i.c.v.										
Veh (first)	1.88±0.38	0.00±0.00	0.00±0.00	4.29±0.67	0.00±0.00	0.00±0.00	0.18±0.10	0.29±0.14	0.00±0.00	2.89±0.42
SR 1.5	2.47±0.67	1.06±0.33[a]	0.53±0.23	5.00±1.65	1.82±0.44[b]	0.00±0.00	1.00±0.28[a]	0.88±0.24	0.00±0.00	7.78±0.91[b]
SR 3	2.35±0.59	1.41±0.51[b]	2.65±0.59[b]	6.18±0.98	3.06±0.56[b]	0.12±0.12	1.41±0.33[b]	0.47±0.15	0.00±0.00	11.62±1.20[b]
Veh (second)	1.41±0.33	0.00±0.00	0.00±0.00	4.18±0.90	0.24±0.18	0.00±0.00	0.06±0.06	0.47±0.17	0.00±0.00	2.92±0.46
Cerebellum										
Veh (first)	2.06±0.42	0.00±0.00	0.12±0.12	7.00±1.06	0.18±0.18	0.06±0.06	0.18±0.13	0.53±0.12	0.12±0.08	5.12±0.77
SR 0.75	2.68±0.45	0.05±0.05	0.00±0.00	3.68±0.58	0.00±0.00	0.00±0.00	0.00±0.00	0.21±0.10	0.00±0.00	2.85±0.37
SR 1.5	6.25±0.80[b]	0.00±0.00	0.50±0.33	17.25±2.54[b]	0.00±0.00	0.13±0.13	1.63±0.42[b]	0.75±0.16	1.75±0.73[a]	12.80±1.52[b]
SR 3	6.13±1.61[b]	0.00±0.00	0.13±0.52[b]	24.25±3.71[b]	0.88±0.44[b]	0.13±0.13	3.63±0.53[b]	1.38±0.32[b]	4.50±1.27[b]	20.36±2.40[b]
Veh (second)	2.47±0.36	0.00±0.00	0.05±0.05	3.16±0.74	0.00±0.00	0.00±0.00	0.11±0.07	0.47±0.12	0.26±0.13	2.93±0.41
Striatum										
Veh (first)	1.42±0.32	0.26±0.15	0.00±0.00	3.21±0.94	0.00±0.00	0.00±0.00	0.11±0.07	0.42±0.14	0.05±0.05	2.67±0.54
SR 1.5	1.20±0.30	0.00±0.00[a]	0.00±0.00	1.80±0.39	0.00±0.00	0.00±0.00	0.00±0.00	0.30±0.11	0.00±0.00	1.43±0.29
SR 3	1.05±0.43	0.00±0.00[a]	0.00±0.00	2.70±0.76	0.00±0.00	0.00±0.00	0.10±0.07	0.25±0.12	0.00±0.00	2.06±0.52
Veh (second)	1.17±0.42	0.00±0.00[a]	0.00±0.00	2.33±0.65	0.00±0.00	0.00±0.00	0.00±0.00	0.44±0.15	0.00±0.00	1.80±0.41
Hippocampus										
Veh (first)	1.50±0.60	1.92±0.47	2.25±0.37	3.92±0.82	3.50±0.62	0.00±0.00	0.75±0.35	0.50±0.15	0.08±0.08	10.26±1.08
SR 1.5	2.83±0.97	2.58±0.48	3.83±0.60	7.17±2.40	3.83±0.69	0.00±0.00	1.83±0.44	1.00±0.25	0.17±0.11	15.84±1.92[a]
SR 3	5.00±1.33	2.60±0.56	3.30±0.60	10.00±1.64	5.50±0.22[a]	0.00±0.00	2.00±0.33[a]	1.00±0.30	1.20±0.00	19.61±1.31[b]
Veh (second)	2.33±0.62	1.22±0.52	0.89±0.39	6.11±1.64	2.11±0.56	0.00±0.00	0.33±0.17	0.44±0.18	0.00±0.00	7.92±1.25
Amygdala										
Veh (first)	2.46±0.68	1.31±0.41	2.00±0.52	5.15±1.30	2.69±0.61	0.08±0.08	0.31±0.17	0.15±0.10	0.15±0.10	8.99±1.18
SR 1.5	3.62±0.98	1.23±0.38	2.46±0.61	7.92±2.18	3.46±0.63	0.00±0.00	0.92±0.31	0.31±0.13	0.15±0.10	12.22±1.99
SR 3	5.54±1.29	2.00±0.57	3.15±0.63	9.85±2.40	3.85±0.55	0.31±0.13	1.54±0.30[a]	0.54±0.14	0.46±0.31	16.24±1.58[b]
Veh (second)	2.54±0.64	0.92±0.54	1.85±0.59	5.85±1.48	2.77±0.61	0.00±0.00	0.38±0.14	0.15±0.10	0.00±0.00	8.92±1.43

Individual signs of withdrawal and global withdrawal score (GWS) in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}, i.p.) after the microinjection of SR 141716A (0.75, 1.5 and 3 μg 2 μl^{-1}) into the third ventricle (i.c.v.), cerebellum, striatum, hippocampus and amygdala (see Methods). Data are expressed as mean±s.e.m.
[a]$P<0.05$ vs Veh (first) group (*post hoc* Dunnett).
[b]$P<0.01$ vs Veh (first) group (*post hoc* Dunnett).

Drugs

R-(+)-WIN 55,212-2 mesylate salt (Sigma Chemical Co., Madrid, Spain) was dissolved in a solution consisting of ethanol/cremophor EL (Sigma Chemical Co., Madrid, Spain)/distilled water (1 : 1 : 8) and was administered by i.p. route in a volume of 10 ml kg^{-1} body weight. The selective CB1 cannabinoid receptor antagonist SR 141716A was kindly provided by Sanofi (Montpellier, France). SR 141716A was dissolved in a solution consisting of ethanol/cremophor EL/distilled water (1 : 1 : 8) and was injected in a volume of 20 ml kg^{-1} body weight (s.c.). For intracerebral administration, SR 141716A was dissolved in 5% dimethylsulphoxide (DMSO) (Scharlau Chemie S.A., Barcelona, Spain) and the injection volume was 2 μl. Ketamine chlorhydrate (100 mg kg^{-1}) (Imalgène 1000®, Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20 mg kg^{-1}) (Sigma Chemical Co., Madrid, Spain) were mixed and dissolved in ethanol and distilled water (1 : 9). The anaesthetic mixture was administered in an injection volume of 20 ml kg^{-1} body weight (i.p.).

Statistical analysis

Results from the conditioned place preference paradigm were analysed using a two-way ANOVA with priming and treatment as factors between subjects followed by one-way ANOVAs and the Dunnett test as *post hoc* analysis. Data from physical dependence studies were analysed by using a one-way ANOVA between subjects followed by the Dunnett test after significant main effects. The level of significance was $P<0.05$ in all experiments.

Results

WIN 55,212-2-induced conditioned place preference

The motivational responses induced by WIN 55,212-2 (0.1 and 1 mg kg^{-1}) administration were explored by using the place-conditioning paradigm. As shown in Figure 1, WIN 55,212-2 (0.1 mg kg^{-1}) induced rewarding effects only when animals were pretreated with a priming injection of the drug. Such a priming injection (WIN 55,212-2 at the dose of 0.1 mg kg^{-1}, i.p.) was given 24 h before the first conditioning session. In contrast, animals not receiving the WIN 55,212-2 pre-exposure did not exhibit any rewarding response in the place-conditioning paradigm. Thus, two-way ANOVA showed no treatment effect ($F(2,47)=3.031$; $P=0.058$), no priming effect ($F(1,47)=2.193$; NS), but a significant interaction between these two factors ($F(1,47)=4.888$; $P<0.05$). Subsequent, one-way ANOVA and *post hoc* analysis showed a significant preference for the compartment associated with WIN 55,212-2 in the group of mice receiving the dose of 0.1 mg kg^{-1} and preinjected with the same dose of this CB1 agonist, when compared to control mice ($P<0.01$) (Figure 1).

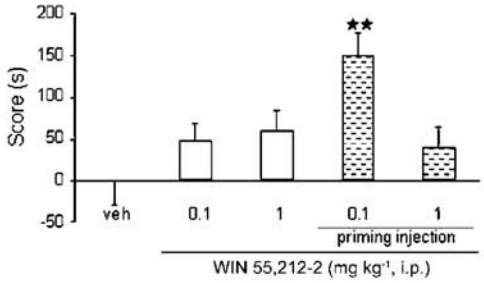

Figure 1 Rewarding properties of WIN 55,212-2 (0.1 and 1 mg kg^{-1}, i.p.) in the place-conditioning paradigm. Vertical axis represents the place preference score, calculated as the time spent in the conditioned compartment on the testing day minus the time in the same compartment on the preconditioning day. Data are expressed as mean ± s.e.m. ($n=10$–11 per group). ** $P<0.01$, WIN 55,212-2-treated vs vehicle group (*post hoc* Dunnett).

WIN 55,212-2 withdrawal precipitated by systemic administration of SR 141716A

Mice were chronically treated with WIN 55,212-2 (1 and 2 mg kg^{-1}) twice daily during 5 days to induce physical dependence. On day six, a withdrawal syndrome was precipitated by the systemic administration of SR 141716A (10 mg kg^{-1}). Thus, SR 141716A challenge precipitated a variety of somatic signs of withdrawal in mice chronically treated with WIN 55,212-2 (Figure 2). One-way ANOVA revealed a significant effect of WIN 55,212-2 treatment for ataxia ($F(2,33)=18.066$; $P<0.01$), hunched posture ($F(2,33)=33.577$; $P<0.01$), mastication ($F(2,33)=36.175$; $P<0.01$), paw tremor ($F(2,33)=16.146$; $P<0.01$), piloerection ($F(2,33)=5.299$; $P<0.05$), body tremor ($F(2,33)=7.054$; $P<0.01$) and wet dog shakes ($F(2,33)=10.285$; $P<0.01$). The global withdrawal score calculated for each experimental group revealed a severe degree of abstinence in WIN 55,212-2-dependent mice ($F(2,33)=58.711$; $P<0.01$). Taking into account that the same severity of abstinence was obtained after the chronic treatment with both doses of WIN 55,212-2 (1 and 2 mg kg^{-1}), the lowest dose, 1 mg kg^{-1}, was chosen for the following experiments.

WIN 55,212-2 withdrawal following intracerebral administration of the CB1 receptor antagonist SR 141716A

Neuroanatomical distribution of the final injection site in each brain structure in the different animals is shown in Figure 3. The use of a within-subject design for the evaluation of cannabinoid withdrawal was validated since there were no significant differences between the symptoms observed after the first and second vehicle administrations (Figure 4, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the third ventricle

Intracerebroventricular (i.c.v.) administration of the CB1 receptor antagonist SR 141716A (1.5 and 3 μg) precipitated the expression of behavioural signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}). One-way ANOVA showed a significant effect for ptosis ($F(3,67)=5.700$; $P<0.01$), body tremor ($F(3,67)=15.692$; $P<0.01$), piloerection ($F(3,67)=15.325$; $P<0.01$) and mastication ($F(3,67)=8.333$; $P<0.01$). For the global withdrawal score, one-way ANOVA exhibited a significant response ($F(3,67)=26.780$; $P<0.01$) and *post hoc* analysis revealed a

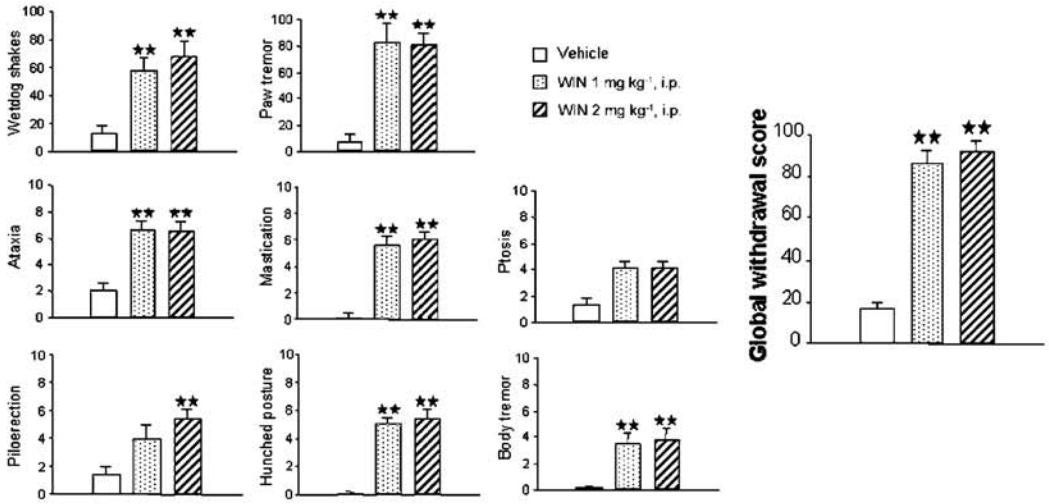

Figure 2 WIN 55,212-2 withdrawal in mice precipitated by systemic SR 141716A administration. Abstinence was precipitated by the administration of SR 141716A (10 mg kg^{-1}, s.c.) in mice receiving a chronic administration of WIN 55,212-2 (1 and 2 mg kg^{-1} i.p., twice daily, 5 days). Counted (wet dog shakes and paw tremor) and checked (ptosis, ataxia, mastication, body tremor, piloerection and hunched posture) signs of withdrawal were observed during 30 min after SR 141716A administration. The global withdrawal score was calculated by giving a relative weight for each individual sign (see Methods for details). Data are expressed as mean ± s.e.m. ($n = 10$–12 per group). $^{**}P<0.01$, WIN 55,212-2-treated vs vehicle group (*post hoc* Dunnett).

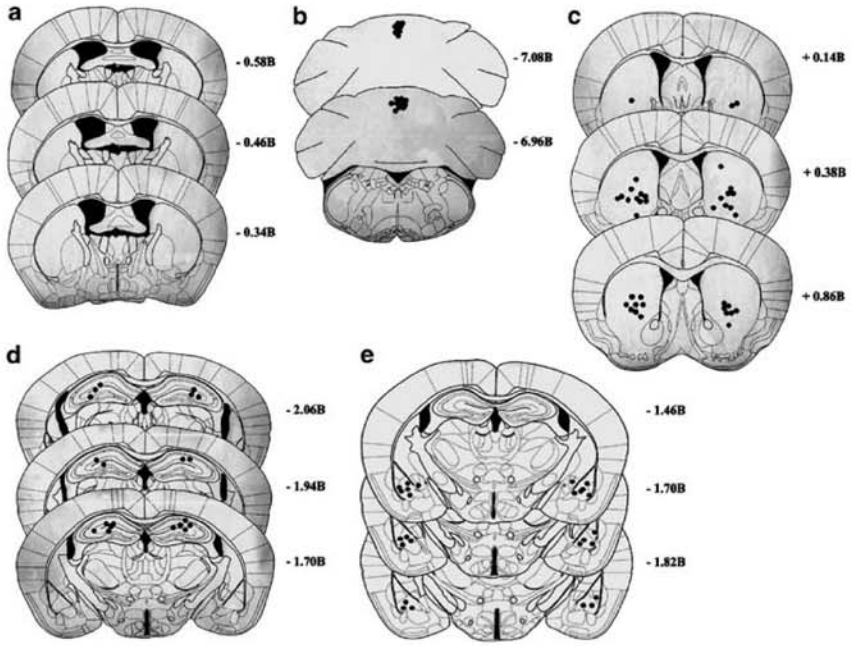

Figure 3 Representative coronal sections of the final site of injection of SR 141716A in WIN 55,212-2-dependent mice based on the atlas (Paxinos & Franklin, 1997). Grey points correspond to the localization of the sites of injection in (a) the third ventricle, (b) cerebellum, (c) striatum, (d) hippocampus and (e) amygdala. Values on the right of each section refer to the distance from Bregma.

significant effect of SR 141716A administration at both doses used, 1.5 and 3 μg ($P<0.01$) (Figure 4a, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the cerebellum

Cerebellar administration of the CB1 receptor antagonist SR 141716A (0.75, 1.5 and 3 μg) precipitated several somatic signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}). One-way ANOVA demonstrated a significant effect for seven out of 10 signs analysed: wet dog shakes ($F(4,70) = 8.618$; $P<0.01$), body tremor ($F(4,70) = 5.679$; $P<0.01$), paw tremor ($F(4,70) = 34.249$; $P<0.01$), piloerection ($F(4,70) = 4.474$; $P<0.01$), mastication ($F(4,70) = 49.421$; $P<0.01$), genital licks ($F(4,70) = 6.829$; $P<0.01$) and sniffing ($F(4,70) = 18.494$; $P<0.01$). One-way ANOVA for the global

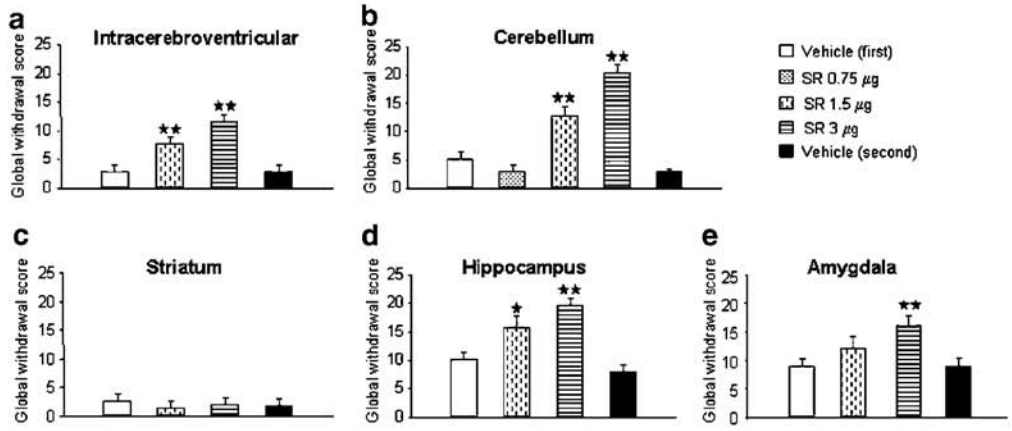

Figure 4 Global withdrawal score after microinjection of SR 141716A into several brain areas of mice chronically treated with WIN 55,212-2. SR 141716A (0.75, 1.5 and 3 μg) or its vehicle was microinjected into (a) the third ventricle, (b) cerebellum, (c) striatum, (d) hippocampus and (e) amygdala of WIN 55,212-2-dependent mice (1 mg kg^{-1}, twice daily, 5 days) by using a Latin Square design. Data are expressed as mean $\pm$ s.e.m. ($n = 10$–19). $^{\star}P<0.05$; $^{\star\star}P<0.01$, SR 141716A-administered vs vehicle (first) group (*post hoc* Dunnett).

withdrawal score also revealed a significant response ($F(4,70) = 54.985$; $P<0.01$), and subsequent *post hoc* analysis showed a significant effect of SR 141716A administration at the doses of 1.5 and 3 μg ($P<0.01$) (Figure 4b, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the striatum

Microinjection of SR 141716A (1.5 and 3 μg) in the striatum did not induce any behavioural manifestation of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}) (Figure 4c, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the hippocampus

Microinjection of SR 141716A (1.5 and 3 μg) in the hippocampus precipitated a significant expression of several somatic signs of withdrawal in mice chronically treated with WIN 55,212-2 (1 mg kg^{-1}). One-way ANOVA revealed a significant effect for body tremor ($F(3,42) = 6.071$; $P<0.01$), piloerection ($F(3,42) = 5.090$; $P<0.01$) and mastication ($F(3,42) = 4.887$; $P<0.01$). For the global withdrawal score, one-way ANOVA revealed a significant effect of SR 141716A ($F(3,42) = 12.130$; $P<0.01$), and subsequent *post hoc* analysis demonstrated a significant effect for both doses used, 1.5 μg ($P<0.05$) and 3 μg ($P<0.01$) (Figure 4d, Table 1).

WIN 55,212-2 withdrawal following the administration of SR 141716A in the amygdala

SR 141716A (1.5 and 3 μg) injection into the amygdala elicited a withdrawal syndrome with a significant expression of mastication ($F(3,51) = 2.903$; $P<0.05$). Although other signs of withdrawal were not significantly observed, one-way ANOVA for the global withdrawal score also revealed a significant effect of SR 141716A ($F(3,51) = 4.855$; $P<0.01$), and subsequent *post hoc* analysis demonstrated a significant effect with the highest dose used, 3 μg ($P<0.01$) (Figure 4e, Table 1).

Discussion

In this study we have evaluated the ability of the cannabinoid agonist WIN 55,212-2 to produce rewarding effects in the place-conditioning paradigm and the somatic expression of WIN 55,212-2 withdrawal. Furthermore, we have investigated the participation of different brain structures in the behavioural expression of WIN 55,212-2 withdrawal. We show that WIN 55,212-2 induced rewarding effects in the place-conditioning paradigm. This response was only observed with the lowest dose tested and when mice received a priming injection of the drug. In agreement with our results, THC induced a conditioned place preference only when mice received a previous priming THC exposure (Valjent & Maldonado, 2000). In the present study and in the previous report, mice received the first cannabinoid administration in their home cages, and thus the negative consequences of the first exposure to the drug cannot be associated with the contextual cues of the conditioning apparatus.

SR 141716A challenge precipitated several somatic signs of withdrawal in mice chronically treated with WIN 55,212-2. These signs included wet dog shakes, paw tremor, ataxia, mastication, body tremor, piloerection and hunched posture, and are similar to those reported during the withdrawal syndrome to other cannabinoid agonists in rodents such as THC (Hutcheson *et al*., 1998; Ledent *et al*., 1999; Lichtman *et al*., 2001; Ghozland *et al*., 2002b; Maldonado, 2002; Castañé *et al*., 2003). The administration of SR 141716A in vehicle-treated mice precipitated a slight presence of some somatic signs similar to those observed during cannabinoid withdrawal, suggesting an intrinsic activity of this compound as previously reported (Hutcheson *et al*., 1998; Rubino *et al*., 1998). The main focus of our study was to investigate the involvement of several brain structures containing a high

density of CB1 cannabinoid receptors and related to cannabinoid behavioural effects, such as the striatum, hippocampus, amygdala and cerebellum (Herkenham *et al*., 1990), in the behavioural expression of cannabinoid withdrawal. We also investigated the consequences of i.c.v. SR 141716A administration in WIN 55,212-2-dependent mice as a positive control for the other intracerebral administrations, considering that the ventricle allows the diffusion of the drug in the whole brain. We show that the microinjection of the CB1 cannabinoid receptor antagonist SR 141716A into the third ventricle, cerebellum, hippocampus and amygdala but not into the striatum precipitated somatic signs of cannabinoid withdrawal in mice chronically treated with WIN 55,212-2. The severity of WIN 55,212-2 abstinence was dependent on the site of injection of the cannabinoid antagonist. The microinjection of SR 141716A in the third ventricle precipitated a withdrawal syndrome in WIN 55,212-2-dependent mice, which was qualitatively and quantitatively different from the one observed after systemic administration of the antagonist. Several signs reported during systemic SR 141716A administration such as wet dog shakes, paw tremor and hunched posture did not appear statistically significant after i.c.v. SR 141716A administration and the intensity of the withdrawal was lower. These differences may be due to the use of higher doses of antagonist when administered systemically. After the microinjection of SR 141716A into the several brain structures evaluated, the highest severity of cannabinoid withdrawal was observed when SR 141716A was microinjected in the cerebellum. A total of seven out of 10 signs evaluated (wet dog shakes, body tremor, paw tremor, piloerection, mastication, genital licks and sniffing) were statistically significant and the global withdrawal score reached the highest value. After the microinjection of SR 141716A in the hippocampus and amygdala, the global withdrawal score achieved significant values but in these brain areas the basal withdrawal score obtained after vehicle administration was also elevated, suggesting that the involvement of the hippocampus and amygdala in the control of emotional responses could induce a higher behavioural reactivity to the microinjection procedure. However, no signs of cannabinoid withdrawal were observed when the CB1 antagonist was administered in the striatum.

In agreement with the high severity of cannabinoid withdrawal after SR 141716A microinjection in the cerebellum, previous studies have also suggested a crucial role of this structure in the somatic expression of cannabinoid withdrawal (Hutcheson *et al*., 1998; Tzavara *et al*., 2000; Ghozland *et al*., 2002a). Thus, cannabinoid withdrawal syndrome was associated to a compensatory increase of AC activity (Hutcheson *et al*., 1998) and a downstream activation of the protein kinase A (PKA) (Tzavara *et al*., 2000) in this brain area, which was not observed in other brain structures also containing CB1 receptors. The cerebellar granule neurons have been reported to be involved in the adaptive changes induced in this structure by chronic cannabinoid administration and withdrawal (Ghozland *et al*., 2002a). All these previous studies used the natural cannabinoid agonist THC, whereas no data have been yet published about the adaptive changes produced by the synthetic agonist WIN 55,212-2.

Our study indicates that the striatum is not involved in the somatic expression of cannabinoid abstinence. This is in agreement with previous results showing that AC activity in the striatum was unaffected during SR 141716A-precipitated withdrawal (Hutcheson *et al*., 1998). However, chronic CP-55,940 administration induced a desensitization of the CB1 cannabinoid receptors and the transduction system ($G_{\alpha s}$ and $G_{\alpha i}$) coupled to CB1 receptors in the rat striatum, which recovered during withdrawal, and might be part of the molecular mechanisms underlying cannabinoid dependence (Rubino *et al*., 1998). Interestingly, we also show in our study that the hippocampus, a brain structure mainly related to learning and memory processes, participates in the behavioural expression of cannabinoid withdrawal syndrome since a moderate withdrawal was observed after microinjection of the cannabinoid antagonist (significant manifestation of three withdrawal signs: body tremor, piloerection and mastication). Learning and memory processes have been suggested to play an important role in opiate addiction (Fan *et al*., 1999; Lu *et al*., 2000). However, no compensatory changes in the cAMP pathway into the hippocampus has been previously reported after cannabinoid withdrawal (Hutcheson *et al*., 1998; Tzavara *et al*., 2000). Therefore, the role of this brain area in cannabinoid abstinence might be mediated through adaptive changes in other intracellular signalling mediators. Indeed, the stimulation of cannabinoid receptors modulates several intracellular signalling systems including MAP kinases, IP3 and cationic channels (Piomelli, 2003), and it has been recently reported that cannabinoids induce long-term alterations in the hippocampus through the activation of the ERK pathway (Derkinderen *et al*., 2003). These long-term ERK changes may be important for the effects of cannabinoids in the context of drug abuse. Furthermore, a recent study showed significant alterations in the hippocampal cannabinoid receptors associated to the behavioural withdrawal syndrome precipitated by SR 141716A in THC-dependent rats, which could be related to cannabinoid tolerance and dependence (Breivogel *et al*., 2003).

Finally, a mild withdrawal syndrome was induced when the cannabinoid antagonist was administered in the amygdala, and only one withdrawal sign, mastication, significantly appeared. Interestingly, the administration of the opiate antagonist methylnaloxonium in the amygdala of morphine-dependent rats also resulted in a mild withdrawal syndrome, being mastication one of the most sensitive signs (Maldonado *et al*., 1992). Recent studies have related the amygdala and other limbic structures to the aversive/dysphoric stimulus associated to withdrawal from drugs of abuse (Koob, 2003). Thus, cannabinoid withdrawal is accompanied by a marked increase in extracellular CRF and enhanced c-Fos immunoreactivity in the central nucleus of the amygdala, which could be related to the dysphoric aspects of cannabinoid abstinence (Rodriguez de Fonseca *et al*., 1997). Nevertheless, further studies are needed to clarify which are the mechanisms responsible for the implication of the amygdala in the somatic expression of cannabinoid withdrawal.

Taken together, our findings show that WIN 55,212-2 induces rewarding effects and physical dependence in mice. Moreover, the cerebellum plays a crucial role in the behavioural expression of cannabinoid abstinence, but other brain structures such as the hippocampus and the amygdala also play a significant role. Therefore, the neuroanatomical substrate of cannabinoid somatic abstinence is not restricted to a single brain structure, and several brain areas seem to be required for the complete expression of cannabinoid withdrawal.

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Cannabinoid withdrawal syndrome is reduced in double mu and delta opioid receptor knockout mice

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Abstract

Several studies have shown a functional relationship between the endogenous cannabinoid and opioid systems. However, acute effects of Δ^9-tetrahydrocannabinol (THC) and physical dependence were not modified in knockout mice with single deletion of mu (MOR), delta (DOR) or kappa (KOR) opioid receptors. To further investigate the neurobiological basis of cannabinoid dependence, we have evaluated acute pharmacological responses, rewarding effects, tolerance and dependence to THC in double MOR/DOR knockout mice. Antinociception and hypolocomotion induced by acute THC administration remained unaffected, whereas the hypothermic effect was slightly attenuated in these double knockout mice. During chronic THC treatment, knockout mice developed slower tolerance to the hypothermic effect, but the development of tolerance to antinociceptive and hypolocomotor effects was unchanged. The rewarding properties of THC, measured in the conditioned place preference paradigm, were reduced in knockout mice. Interestingly, the somatic manifestations of THC withdrawal were also significantly attenuated in mutant mice, suggesting that a cooperative action of MOR and DOR is required for the entire expression of THC dependence.

Introduction

Cannabinoids such as Δ9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, produce their pharmacological effects by stimulating two types of cannabinoid receptors: the CB_1 cannabinoid receptor, localized mainly in the CNS (Devane *et al*., 1988) and the CB_2 cannabinoid receptor found primarily in the immune system (Munro *et al*., 1993). Activation of the CB_1 cannabinoid receptors is thought to mediate the central pharmacological effects of cannabinoids including hypolocomotion, hypothermia and antinociception (review in Ameri, 1999). Opioid compounds through activation of three types of receptors, namely mu (MOR), delta (DOR) and kappa (KOR), also produce hypothermia and antinociception, although unlike cannabinoids, they mostly increase locomotor activity (reviewed in Kieffer, 1999). Bidirectional interactions between the cannabinoid and opioid systems have been reported with respect to the development of tolerance (Thorat & Bhargava, 1994; Rowen *et al*., 1998) and dependence (Navarro *et al*., 1998; Ledent *et al*., 1999; Valverde *et al*., 2001), as well as for their rewarding properties (Gardner & Lowinson, 1991; Tanda *et al*., 1997; Ledent *et al*., 1999; Martin *et al*., 2000; Ghozland *et al*., 2002). Recently, it has been demonstrated that the somatic expression of the cannabinoid withdrawal is attenuated in preproenkephalin knockout mice suggesting that peptide derivatives from this protein precursor participate in the development of physical THC dependence (Valverde *et al*., 2000). However, the manifestations of THC withdrawal were not modified in any of the single knockout mice deficient in either MOR, DOR or KOR (Ghozland *et al*., 2002). One hypothesis that can be put forward to reconcile these results is that the simultaneous activation of different opioid receptors is needed for the development of THC withdrawal (Ghozland *et al*., 2002). Indeed, peptide derivatives from preproenkephalin are not selective agonists of any opioid receptor, and activate both DOR and MOR. Furthermore, cooperative actions between MOR and DOR have been postulated based on anatomical and pharmacological studies. Thus, MOR and DOR colocalize in dendrites and spines of striatal patches (Wang & Pickel, 2001), where they may interact in reward-related motor functions (White & Hiroi, 1998). In addition, there is new evidence demonstrating that MOR and DOR associate to form a heterodimer exhibiting functional activity (Gomes *et al*., 2000). Pharmacological data also point to a possible collaboration between these two receptors in opioid antinociceptive and dependence processes (Abdelhamid *et al*., 1991; Porreca *et al*., 1992; Traynor & Elliott, 1993).

Considering the different results obtained on THC dependence in the preproenkephalin (Valverde *et al*., 2000) and the single opioid receptor knockout mice (Ghozland *et al*., 2002), we sought to examine whether a cooperative role exists between MOR and DOR in the behavioural actions produced by acute and chronic administration of THC. For this purpose, we have investigated THC acute effects, tolerance, physical dependence and motivational responses in double MOR/DOR knockout mice.

Materials and methods

Animals

Mice with double opioid receptor gene deletion (MOR/DOR) as well as their corresponding wild-type littermates were used. Animals lacking both MOR and DOR were F2 from heterozygous breeding pairs and maintained on a hybrid 50% 129/SV 50% C57B1/6 genetic background, as in our earlier reports (Filliol *et al*., 2000; Ghozland *et al*., 2002). Mice weighing from 25 to 30 g at the start of the study

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were housed five per cage and acclimatized to the laboratory conditions (12 h light : 12 h dark cycle, 21 ± 1 °C room temperature, 55 ± 10% humidity) 1 week before the experiment with *ad libitum* access to food and water. In each experimental group, mice were matched for age and sex. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health, 1995; Council of Europe, 1996) and approved by the local ethical committee. The observer was blind to the genotype and treatment in all experiments.

Drugs

THC (THC Pharm, Frankfurt) was dissolved in a solution of 5% ethanol, 5% cremophor El, and 90% distilled water, and injected in a volume of 0.1 mL per 10 g body weight. The selective CB1 cannabinoid receptor antagonist SR141716A was dissolved in a solution of 10% ethanol, 10% cremophor El, and 80% distilled water, and injected by intraperitoneal route in a volume of 0.2 mL per 10 g body weight.

Tolerance and withdrawal

Animals were injected by intraperitoneal route twice daily at 09.00 h and 19.00 h for 5 days with THC (20 mg/kg) or its vehicle. On day 6, mice only received the morning injection. Three different responses were measured once a day during the chronic THC treatment using a test battery schedule: locomotor activity, nociception and rectal temperature. Locomotor measurements for each mouse were taken 20 min after morning injections by placing animals in individual actimeters (9 × 20 × 11 cm) (Imetronic, Bordeaux, France) equipped with two lines of six infrared beams, and recording both horizontal and vertical activity for 10 min under a dim light (15 lux). Antinociceptive measurements for each mouse were taken 30 min after morning injection by using the tail-immersion assay as described previously (Janssen *et al.*, 1963). The time to withdraw the tail from the bath (50 ± 0.5 °C) was registered with a cut-off latency of 15 s in order to prevent tissue damage. Rectal temperature was measured in each mouse by placing an electronic thermocouple flexible rectal probe for 3 cm into the rectum of the mice for 20 s (Panlab, Madrid, Spain). Measures were taken immediately before and 40 min after morning injection.

On the sixth day, 4 h after the last THC or vehicle injection, mice were placed in a circular clear plastic observation area (25 cm diameter, 40 cm height) for a 15-min period. At the end of this period, body weight and rectal temperature were consecutively measured, and animals received administration of SR141716A (10 mg/kg, i.p.). Mice were then immediately replaced in the observation area, and observed for 45 min. Measurement of somatic signs before and after SR141716A challenge were divided into 5 min time intervals, as previously described (Hutcheson *et al.*, 1998). The number of bouts of sniffing, writhing, wet dog shakes, and front paw tremor were counted. Genital licking or erection, tremor, ptosis, and piloerection were scored 1 for appearance and 0 for nonappearance within each 5 min time period. Scores for the level of activity were made by giving in each 5-min period a value of 0 for low activity (less than five complete crossings of the observation area), 1 for normal activity (between five and 20 complete crossings of the observation area), or 2 for increased activity (more than 20 complete crossings of the observation area). A quantitative value was calculated in each animal for the different checked signs by adding the scores obtained in each 5 min time period. A global withdrawal score was calculated for each animal by giving to each individual sign a relative weight, as previously described (Ghozland *et al.*, 2002): 0.9 for the appearance of each checked sign in each 5 min time period and 0.4 for each bout of counted sign.

Place conditioning

The rewarding properties of THC were measured as previously described (Valjent & Maldonado, 2000). The apparatus consisted of two main square conditioning compartments (15 × 15 × 15 cm) separated by a triangular central area (Maldonado *et al.*, 1997). The light intensity within the conditioning chambers was 30 ± 5 lux. During the preconditioning phase, drug-naive mice were placed in the middle of the central area and had free access to both compartments (striped and dotted) of the apparatus for 20 min. The time spent in each compartment was recorded by computerized monitoring software (Videotrack; View Point, Lyon, France). During the conditioning phase, THC-treated mice were injected with THC (1 mg/kg, i.p.) or vehicle on alternate days, and then immediately confined into one of the two conditioning compartments. Five pairings were carried out with THC and five pairings with vehicle. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The test phase was conducted exactly as the preconditioning phase, i.e. free access to each compartment for 20 min. In order to avoid the dysphoric effects of the first drug exposure, THC-treated mice received a single injection of THC (1 mg/kg, i.p.) in their home cages 24 hours before starting the conditioning procedures, and control mice received a vehicle injection (Valjent & Maldonado, 2000). A preference score was calculated for each group as the difference between the time spent in the drug-paired compartment during the test and preconditioning phases.

Statistics

Acute responses, somatic signs of withdrawal as well as conditioned place preference scores were analyzed using a two-way ANOVA between subjects (genotype and treatment) followed by the Tukey *post-hoc* test when significant interactions were observed. Tolerance data were evaluated using a two-way ANOVA with repeated measures: genotype (between subjects) and day (within subjects) followed by the Tukey *post-hoc* when significant interactions were observed.

Results

Acute THC effects in double MOR/DOR knockout mice

The first administration of THC (20 mg/kg) produced similar antinociceptive responses in the tail-immersion test in wild-type and knockout mice. Two-way ANOVA revealed a significant main effect of treatment ($F_{1,57} = 104.173$; $P < 0.01$), no effect of genotype, and no interaction between these two factors. In addition, no significant differences in the hypolocomotor responses were observed between genotypes. Two-way ANOVA revealed a significant effect of treatment ($F_{1,57} = 23.441$; $P < 0.01$), no effect of genotype, and no interaction between these two factors. However, the hypothermia induced by acute THC administration was attenuated in mutant mice. Two-way ANOVA revealed a significant effect of treatment ($F_{1,37} = 93.725$; $P < 0.001$), genotype ($F_{1,37} = 5.759$; $P < 0.05$), and a significant interaction between the two factors ($F_{1,37} = 4.101$; $P < 0.05$) (Fig. 1A).

Tolerance to the THC hypothermic effects and THC withdrawal syndrome were attenuated in double MOR/DOR knockout mice

During repeated THC administration (20 mg/kg, twice daily during 6 days), a progressive decrease in the antinociceptive, hypolocomotor, and hypothermic effects of the drug was observed. Two-way ANOVA revealed a significant effect of day in all these responses ($F_{4,112} = 11.901$; $P < 0.001$); ($F_{4,112} = 16.878$; $P < 0.001$); ($F_{4,72} = 23.309$;

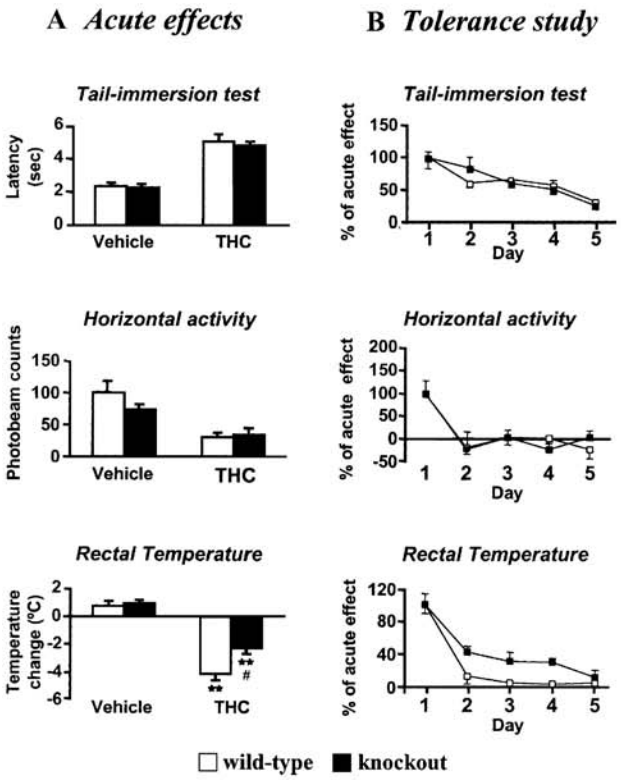

FIG. 1. Acute effects and development of tolerance to THC in double MOR/DOR knockout mice. (A) Acute effects of THC (20 mg/kg) on nociceptive responses, locomotor activity and rectal temperature. Data are expressed as mean ± SEM in wild-type (white bars, $n = 6$–13) and knockout (black bars, $n = 13$–18) mice. ★★ $P < 0.01$, THC-treated vs. respective vehicle control group. # $P < 0.05$, knockout vs. wild-type group (ANOVA followed by *post-hoc* Tukey's test). (B) Tolerance to the antinociceptive, hypolocomotor and hypothermic effects of THC (20 mg/kg twice daily for 5 days). Data are expressed as percentage of acute effect (mean ± SEM), in wild-type (white squares, $n = 6$–13) and knockout (black squares, $n = 13$–18) mice.

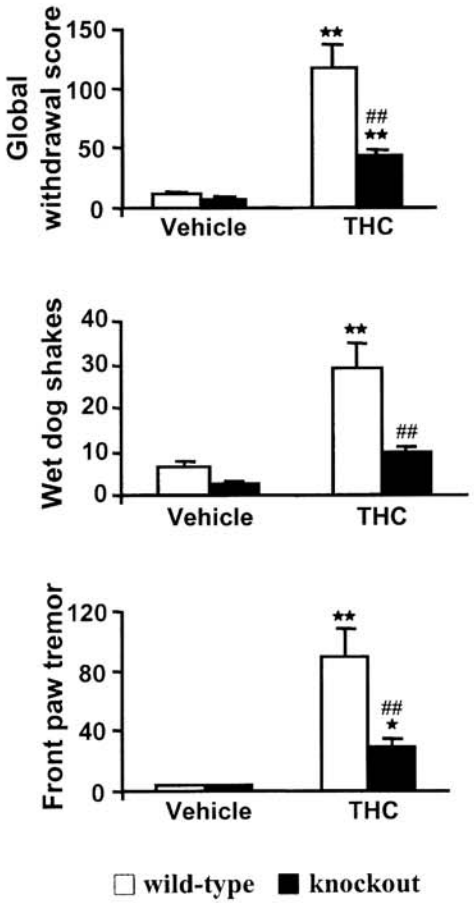

FIG. 2. SR141716A-precipitated THC withdrawal is reduced in double MOR/DOR knockout mice. Two of the main signs of cannabinoid withdrawal (wet dog shakes and front paw tremor) were reduced in double knockout mice, as well as the global withdrawal score. Data are expressed as the mean ± SEM in wild-type (white bars) and knockout (black bars) mice ($n = 12$–19 in each group). ★ $P < 0.05$; ★★ $P < 0.01$, THC-treated vs. respective vehicle control group. ## $P < 0.01$, knockout vs. wild-type group (ANOVA followed by *post-hoc* Tukey's test).

$P < 0.001$), respectively. Although, both wild-type and mutant mice achieved the same degree of tolerance to the antinociceptive, hypolocomotor and hypothermic effects of THC on day 5 (Fig. 1B), mutant mice showed a slower development of tolerance to the hypothermic effect of this drug. Two-way ANOVA revealed a significant effect of genotype ($F_{1,18} = 5.109$; $P < 0.05$), without interaction between factors (Fig. 1B). For the antinociceptive and hypolocomotor responses, no effect of genotype and no interaction between factors were observed.

The administration of the CB_1 receptor antagonist SR141716A (10 mg/kg) induced a significant expression of somatic signs of withdrawal in wild-type and mutant mice chronically treated with THC, but not in vehicle control groups (Fig. 2). The expression of piloerection, body tremor and ptosis was comparable in THC-dependent mutant and wild-type groups. Other signs of withdrawal such as writhing and genital licks were slightly reduced in knockout mice as compared to wild-type littermates, while the episodes of sniffing were increased in the mutant animals. Interestingly, two of the main signs of THC withdrawal (wet dog shakes and front paw tremor) were strongly reduced in knockout mice. Thus, for wet dog shakes statistical analysis revealed a significant main effect of treatment ($F_{1,57} = 38.736$, $P < 0.001$), a significant main effect of genotype ($F_{1,57} = 23.494$, $P < 0.001$), and a significant interaction between the two factors ($F_{1,57} = 10.409$, $P < 0.01$). For paw tremor, a significant main effect of treatment ($F_{1,57} = 47.339$, $P < 0.001$), a significant main effect of genotype [$F_{1,57} = 12.929$, $P < 0.01$], and a significant interaction between the two factors ($F_{1,57} = 12.978$, $P < 0.01$) was observed. For the global withdrawal score, a significant main effect of treatment ($F_{1,57} = 70.542$, $P < 0.001$), a significant main effect of genotype ($F_{1,57} = 19.607$, $P < 0.001$), and a significant interaction between the two factors ($F_{1,57} = 16.068$, $P < 0.001$) was observed.

THC-induced place preference was reduced in double MOR/DOR knockout mice

The motivational responses to THC were explored by using the place conditioning paradigm as previously reported (Valjent & Maldonado, 2000). Mice received a single injection of THC (1 mg/kg) in their home cages, and were subsequently conditioned to the same dose of the drug. Figure 3 shows the preference scores for the different groups. Two-way ANOVA showed a significant main effect of treatment ($F_{1,47} = 8.96$, $P < 0.01$), no significant effect of genotype, and a significant interaction between the two factors ($F_{1,47} = 4.43$, $P < 0.05$).

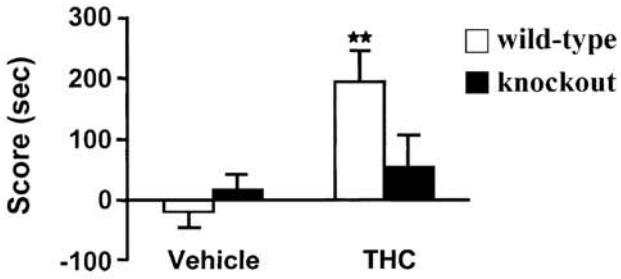

FIG. 3. The rewarding properties of THC are reduced in double MOR/DOR knockout mice. Data represent preference scores (mean ± SEM) in wild-type (white bars) and knockout (black bars) mice treated with THC or vehicle ($n = 12$–19 in each group). ** $P < 0.01$, THC-treated vs. respective vehicle control group (ANOVA followed by *post-hoc* Tukey's test).

Discussion

In this study we show that the acute hypothermic response induced by THC, the development of tolerance to this hypothermic effect, as well as THC withdrawal syndrome precipitated by SR141717A are reduced in double MOR/DOR knockout mice. In addition, the rewarding properties of THC are reduced in these double mutant mice. Our recent study using single knockout mice lacking either MOR, DOR or KOR has shown an abolishment of THC-induced conditioned place preference in MOR, but not DOR or KOR knockout mice. In addition, THC-induced conditioned place aversion was suppressed in KOR knockout mice. Together these data showed an opposite role for MOR and KOR in the motivational responses of THC (Ghozland *et al*., 2002). Our results corroborate the previously reported essential role of MOR in mediating the rewarding effects of THC.

Deletion of both MOR and DOR did not modify the acute antinociceptive responses induced by THC. In accordance with these findings, we have recently found no changes in the acute responses to THC in mice lacking either MOR or DOR (Ghozland *et al*., 2002). These results obtained with knockout mice are in apparent contradiction with those previously reported by using pharmacological tools. Thus, high doses of the nonselective opioid antagonist, naloxone (Smith *et al*., 1998) or the irreversible mu opioid antagonist betafunaltrexamine (Reche *et al*., 1996) blocked some of the antinociceptive effects of cannabinoids. However, this was mainly observed in the hot-plate test and, in fact, the involvement of MOR on THC antinociception seems to implicate supraspinal but not spinal mechanisms (Manzanares *et al*., 1999). In this study as in those previously performed with single knockout mice we used the tail-immersion test, which preferentially involves spinal mechanisms. The spinal nociceptive responses recorded in this test could explain the maintenance of the THC effects in double MOR/DOR knockout mice (present study), as well as in single mutant mice deficient in MOR or DOR (Ghozland *et al*., 2002). On the contrary, we found that THC-induced hypothermia was significantly diminished in double knockout mice. In the same way, a previous pharmacological study showed that pretreatment with the nonselective opioid antagonist naloxone (1 mg/kg) partially antagonized the hypothermia produced by THC in mice (Bloom & Dewey, 1978). These findings, together with our previous data in single knockout mice showing no genotype effect in the hypothermic response induced by THC, suggest a possible cooperative action between MOR and DOR in this acute cannabinoid response.

Concerning tolerance to THC-induced antinociception, there are several pharmacological studies demonstrating reciprocal cannabinoid–opioid interactions (Manzanares *et al*., 1999). In our experimental conditions, mutant mice lacking both MOR and DOR developed the same degree of tolerance to the antinociceptive actions of THC as wild-type controls. Similarly, single deletion of MOR or DOR genes had no major consequences on the development of THC antinociceptive tolerance (Ghozland *et al*., 2002). However, in contrast with the present data, THC-induced antinociceptive tolerance was reduced in knockout mice lacking the preproenkephalin gene (Valverde *et al*., 2000). The discrepancies between these findings could be due to the different genetic background used to generate the preproenkephalin knockout mice (100% C57Bl/6), and the single and double opioid receptor knockout mice (50% 129/SV, 50% C57Bl/6). Interestingly, tolerance to the hypothermic effects of THC developed slower in double opioid receptor knockout mice than in wild-type animals. Tolerance to this cannabinoid response developed similarly in single knockout mice lacking MOR, DOR or KOR (Ghozland *et al*., 2002). Considering these results, our findings suggest that MOR and DOR, in cooperation, could take part in the development of tolerance to THC-induced hypothermia.

We have previously evaluated the involvement of opioid receptors in THC-induced physical dependence in knockout mice lacking the different opioid receptors (Ghozland *et al*., 2002) or preproenkephalin (Valverde *et al*., 2000). Thus, cannabinoid withdrawal was not altered in MOR, DOR or KOR mutant mice chronically treated with 20 mg/kg (twice daily) of THC (Ghozland *et al*., 2002), but was attenuated in THC-dependent preproenkephalin knockout mice (Valverde *et al*., 2000). Another study has shown that the cannabinoid withdrawal syndrome was attenuated in MOR knockout mice chronically treated once daily with 30 and 100 mg/kg of THC, but not in those treated with 10 mg/kg of THC (Lichtman *et al*., 2001). In the present study, when the cannabinoid antagonist SR141716A was administered to double MOR/DOR knockout mice chronically treated with 20 mg/kg of THC, a decrease in the expression of cannabinoid withdrawal was observed. The present findings in double knockout mice could resolve the apparent discrepancies between knockout mice deficient in opioid receptor and opioid peptide precursor by postulating a cooperative action of MOR and DOR in the expression of THC withdrawal.

Pharmacological data indicate that MOR and DOR are capable of collaborating in mediating several effects of opioids (Abdelhamid *et al*., 1991; Traynor & Elliott, 1993). Cooperation between MOR and DOR has been demonstrated in neurons of striatal patches where they modulate postsynaptic excitability of spines (Wang & Pickel, 2001). A similar colocalization of MOR and DOR has been reported in other central areas, such as the spinal cord (Arvidsson *et al*., 1995). The possible formation of MOR–DOR complexes suggested from these studies has been recently confirmed in a report showing that MOR and DOR associate to form heterodimers in transfected cells with distinct binding and signalling properties (Gomes *et al*., 2000). Nonetheless, other types of interactions between these two opioid receptors cannot be excluded. For instance, MOR and DOR localized separately in the same neuron could interact with similar second messenger systems. Interactions between these two receptors could also take place in separate neurons belonging to a similar neuronal circuit. Finally, these findings provide further evidence that the endogenous opioids play a major role in the expression of behavioural signs related to THC-withdrawal.

In conclusion, the present study shows that MOR and DOR cooperate in mediating the acute hypothermic effects of THC, the development of tolerance to these hypothermic effects and the somatic expression of THC withdrawal syndrome. For the rewarding properties of THC, our results confirm the preferential involvement of MOR. These results provide new information to further our understanding of the involvement of each opioid receptor in acute and chronic cannabinoid effects.

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Abbreviations

DOR, delta opioid receptor; G, genotype; GWS, global withdrawal score; i.p., intraperitoneal; KO, knockout; KOR, kappa opioid receptor; MOR, mu opioid receptor; T, treatment; THC, Δ9-tetrahydrocannabinol; Veh, vehicle; WT, wild-type.

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Adenosine A_{2A} receptors are involved in physical dependence and place conditioning induced by THC

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Abstract

A_{2A} adenosine and CB1 cannabinoid receptors are highly expressed in the central nervous system, where they modulate numerous physiological processes including adaptive responses to drugs of abuse. Both purinergic and cannabinoid systems interact with dopamine neurotransmission (through A_{2A} and CB1 receptors, respectively). Changes in dopamine neurotransmission play an important role in addictive-related behaviours. In this study, we investigated the contribution of A_{2A} adenosine receptors in several behavioural responses of Δ^9-tetrahydrocannabinol (THC) related to its addictive properties, including tolerance, physical dependence and motivational effects. For this purpose, we first investigated acute THC responses in mice lacking A_{2A} adenosine receptors. Antinociception, hypolocomotion and hypothermia induced by acute THC administration remained unaffected in mutant mice. Chronic THC treatment developed similar tolerance to these acute effects in wild-type and A_{2A}-knockout mice. However, differences in the body weight pattern were found between genotypes during such chronic treatment. Interestingly, the somatic manifestations of SR141716A-precipitated THC withdrawal were significantly attenuated in mutant mice. The motivational responses of THC were also evaluated by using the place-conditioning paradigm. A significant reduction of THC-induced rewarding and aversive effects was found in mice lacking A_{2A} adenosine receptors in comparison with wild-type littermates. Binding studies revealed that these behavioural changes were not associated with any modification in the distribution and/or functional activity of CB1 receptors in knockout mice. Therefore, this study shows, for the first time, a specific involvement of A_{2A} receptors in the addictive-related properties of cannabinoids.

Introduction

Adenosine is an endogenous nucleoside acting as a neuromodulator in the CNS. Four receptor subtypes that specifically bind to adenosine have been identified, namely A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 1994). A_{2A} and A_{2B} receptors are positively coupled to adenylate cyclase activity, whereas A_1 and A_3 receptors are negatively coupled to this enzyme. Adenosine modulates numerous physiological processes and participates in mediating different responses induced by several drugs of abuse, such as psychostimulants and opioids (Sweeney *et al.*, 1991; Chen *et al.*, 2000; Berrendero *et al.*, 2003). Thus, amphetamine-mediated hyperlocomotion is potentiated by adenosine antagonists and attenuated by adenosine agonists (Turgeon *et al.*, 1996; Ferré, 1997). In addition, genetic inactivation of A_{2A} receptors attenuates amphetamine and cocaine-induced locomotion (Chen *et al.*, 2000). Pharmacological studies have also suggested a role of adenosine in opioid dependence. Indeed, the blockade of adenosine metabolism by adenosine kinase inhibitors decreases the severity of morphine withdrawal (Kaplan & Coyle, 1998). Furthermore, the adenosine agonist CGS 21680 inhibits the expression of morphine withdrawal, whereas adenosine antagonists or the deletion of A_{2A} gene increase the severity of morphine abstinence (Kaplan & Sears, 1996; Salem & Hope, 1997; Berrendero *et al.*, 2003; Bailey *et al.*, 2004).

Endocannabinoids have been described as important neuromodulators in the brain (Di Marzo *et al.*, 1998) that regulate several behavioural and physiological responses (Maldonado & Rodríguez de Fonseca, 2002; Piomelli, 2003). Cannabinoid effects in the CNS are mediated through the activation of CB1 cannabinoid receptors (Ledent *et al.*, 1999), which are negatively coupled to the adenylate cyclase activity. A_{2A} and CB1 receptors have been found in common areas in the CNS including the striatum, cerebellum, hippocampus and cerebral cortex (Herkenham *et al.*, 1991; Svenningsson *et al.*, 1999). Both adenosine and cannabinoids participate in the modulation of different physiopathological processes such as nociception, anxiety, aggressiveness, motor activity and drug addiction (Fredholm & Svenningsson, 2003; Piomelli, 2003).

There is evidence that dopamine transmission in the mesolimbic system plays a crucial role in the modulation of the rewarding processes (Koob, 1996; Di Chiara, 2002) and therefore in the addictive properties of drugs of abuse. Adenosine regulates dopamine transmission through antagonistic interactions of adenosine A_1/dopamine D_1 receptors and adenosine A_{2A}/dopamine D_2 receptors (Franco *et al.*, 2000). A close relationship between the cannabinoid and dopamine systems has also been described (Nava *et al.*, 2000; Hermann *et al.*, 2002). Thus, THC and other cannabinoid agonists

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increase dopamine levels in limbic areas related to reward (Tanda *et al*., 1997), and the D_2 agonist quinpirole is able to decrease some behavioural responses induced by the cannabinoid agonist CP 55 940 (Sañudo-Peña *et al*., 1998). Furthermore, chronic treatment with D2 receptor antagonists results in an up-regulation of CB1 receptor mRNA in the striatum (Mailleux & Vanderhaeghen, 1993). However, the role of adenosine receptors in the behavioural effects of cannabinoids and in cannabinoid addiction has not yet been investigated. Therefore, the aim of the present study was to investigate the specific role of A_{2A} adenosine receptors in the behavioural responses of THC that are related to its addictive properties. For this purpose, we have investigated the acute pharmacological responses of THC, the development of tolerance and physical dependence after chronic THC administration, and the different motivational effects induced by this drug in mice lacking A_{2A} adenosine receptors. In addition, the possible existence of compensatory changes in CB1 cannabinoid receptors was investigated in these knockout mice by using autoradiography of cannabinoid receptor binding and cannabinoid-agonist-stimulated [^{35}S]-GTPγS binding.

Materials and methods

Animals

Mice lacking A_{2A} adenosine receptors were generated as previously reported (Ledent *et al*., 1997), and were bred on a CD1 background. Fourteen-weeks-old A_{2A} knockout mice and wild-type littermates (30–35 g) were housed five per cage in temperature- (21 ± 1 °C) and humidity- (55 ± 10%) controlled rooms, with a 12-h light : 12-h dark cycle (light between 8 : 00 a.m. and 8 : 00 p.m). Food and water were available *ad libitum* during all experiments except for the food self-administration study. Mice were handled for 1 week before starting the experiments.

Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-IMAS-UPF). All experiments were performed under blind conditions.

Drugs

THC was purchased from THC Pharm (Frankfurt, Germany), Cremophor EL was provided by Sigma Chemical Co. (Madrid, Spain). THC was dissolved in vehicle as follows: 5% ethanol, 5% Cremophor EL, 90% distilled water. The volume of injection was 0.1 mL per 10 g body weight and the administration route was intraperitoneal (i.p.). SR141716A, kindly provided by SANOFI, was dissolved in vehicle based on: 10% ethanol, 10% Cremophor EL, 80% distilled water. The volume of injection was 0.2 mL per 10 g body weight and the administration route was subcutaneous (s.c.).

Acute THC effects after the first administration

Locomotor activity responses induced by THC (20 mg/kg, i.p.) or vehicle were evaluated by using locomotor activity boxes (9 × 20 × 11 cm) (Imetronic, Bordeaux, France). The boxes were provided with two lines of photocells, one 2 cm above the floor to measure horizontal activity, and the other located 6 cm above the floor to measure vertical activity (rears), in a low-luminosity environment (5 lux). Mice were habituated to the locomotor cages for 10 min each day for three consecutive days. On the fourth day, 20 min after THC or vehicle injection, mice were placed in the locomotor activity boxes for 10 min.

Antinociceptive effects induced by an acute administration of THC (20 mg/kg, i.p.) or vehicle were evaluated 30 min after injection by using the tail-immersion test, as previously described (Simonin *et al*., 1998). The latency to a rapid tail-flick in the bath (50 ± 0.5 °C) was registered with a cut-off at 15 s in order to prevent tissue damage. Subsequently, the hot-plate test was performed 35 min after THC or vehicle injection in the same experimental sequence, as previously reported (Simonin *et al*., 1998). A glass cylinder was used to maintain the heated surface of the plate, which was kept at a temperature of 52 ± 0.5 °C (Columbus Instruments, Columbus Ohio, USA). The nociceptive threshold evaluated was the jumping and the licking response, and a 240-s cut-off was used to prevent tissue damage.

Rectal temperature was measured before and 1 h after the injections by using a digital thermometer (TMP 812, Letica, Barcelona, Spain) with an electronic thermocouple flexible rectal probe. The probe was lubricated and introduced 3 cm into the rectum of the mice for 20 s (Valverde *et al*., 2000).

THC tolerance and withdrawal

Mice were injected twice daily at 9 : 00 a.m and 7 : 00 p.m. for 5 days with THC (20 mg/kg, i.p.) or vehicle. On day 6, mice received only the morning injection. For the tolerance study, three different parameters were measured each day during THC chronic treatment: nociception, rectal temperature and body weight. Antinociceptive responses were evaluated on the tail-immersion test (50 °C), 30 min after morning and evening injections from day 1 to day 5. Rectal temperature was measured before, and 1 h after, the injection on days 1, 2 and 3, after both morning and evening injections of THC, and on days 4 and 5 only after the morning injection. Body weight was recorded for each animal twice a day using an electronic balance (Mettler PM 4800, sensitive to 0.01 g), before morning and evening injections. Body weight changes were calculated by subtracting each weight value from the preceding value.

On the 6th day of THC chronic treatment, the THC withdrawal syndrome was precipitated by injecting the CB1 cannabinoid receptor antagonist SR141716A (10 mg/kg, s.c.) or its corresponding vehicle, 4 h after the THC or the corresponding vehicle morning injection. Each mouse was placed in an identical test chamber consisting of a round box (30 cm diameter × 35 cm high) with white floor and moderate lighting (30 lux), and was evaluated for any somatic manifestation of abstinence during the 15 min before the antagonist injection. After SR141716A administration, the somatic signs of abstinence were measured for 45 min, as described previously (Hutcheson *et al*., 1998). The number of bouts of writhing, wet dog shakes and forepaw tremor were counted. Penile licking or erection, hunched posture, tremor, ptosis and piloerection were scored 1 for appearance and 0 for nonappearance within each 5 min during the observation time period. A quantitative value was calculated in each animal for the different checked signs by adding the scores obtained in each 5 min time period. A global withdrawal score, ranging from 0 to 100, was calculated for each animal by giving to each individual sign a relative weight, as previously described (Valverde *et al*., 2000).

Rewarding and aversive effects of THC in the place-conditioning paradigm

The rewarding and the aversive properties of THC (1 and 5 mg/kg, i.p., respectively) were measured using a place-conditioning paradigm,

as previously described (Valjent & Maldonado, 2000). The apparatus consisted of two main square conditioning compartments (15 × 15 × 15 cm), with differences in texture and colours, separated by a triangular central area (Matthes *et al*., 1996). The light intensity within the conditioning chambers was 30 lux. During the preconditioning phase, drug-naive mice were placed in the middle of the central area and had free access to both compartments of the apparatus for 20 min. The time spent in each compartment was recorded by computerized monitoring software (Videotrack; View Point, Lyon, France). During the conditioning phase, mice received alternating injections of THC (1 or 5 mg/kg) or vehicle, and were immediately confined to one of the two conditioning compartments for 45 min. Five pairings were carried out with THC and five pairings with vehicle on alternate days. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The postconditioning phase was conducted exactly as the preconditioning phase, i.e. free access to each compartment for 20 min. When evaluating the rewarding properties of THC (1 mg/kg, i.p.), the possible negative motivational effects of the first drug exposure were avoided by administering mice a single injection of THC (1 mg/kg, i.p.) in their home cage 24 h before starting the conditioning phase, as previously reported (Valjent & Maldonado, 2000). This THC pre-exposure was not administered when aversive properties of THC (5 mg/kg, i.p.) were evaluated.

Operant paradigm for food self-administration

The food self-administration experiment was conducted in mouse operant chambers (Model ENV-307 A-CT, Medical Associates, Georgia, VT, USA) equipped with two holes, one was selected as the active hole for delivering the reinforcer and the other as the inactive hole. Nose-poking on the active hole resulted in a reinforcer (food pellet) while nose-poking on the inactive hole had no consequences. The chambers were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and ambient noise. A removable food dispenser equidistant between the two nose-pokes permitted delivery of food pellets when required. A stimulus light, located above each hole, signalled the delivery of the reinforcer. Mice were deprived of food (3.5 g of food were provided daily) for four days, in order to obtain 95% of their initial weight. The same food deprivation regime was maintained during the whole evaluation of food-maintained operant behaviour. Water was available *ad libitum* during the whole study. Four days after starting food deprivation, mice were trained in the operant chambers to nose-poke for food pellets (Noyes Precision Pellets, Research Diets. Inc., New Brunswick, NJ, USA). One-hour daily self-administration sessions were conducted 6 days per week. The house light was on at the beginning of the session for 3 s and then remained off for the whole session. First, mice were trained under a FR1 schedule of reinforcement. A 10-s time-out period was established after each reinforcement. During this 10-s period, the cue light was off and no reward was provided on the active hole. Responses on the inactive hole and all the responses during the 10 s time-out period were also recorded. The session was terminated after 100 reinforcers were delivered or after 1 h, whichever occurred first. The criteria for the acquisition of FR1 was achieved when mice maintained a stable responding with $<20\%$ deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability). Then, the response requirement to earn a reward was changed to a FR3 schedule. When the 80% stability criteria was reached in FR3, the experiment was finished. After each session, mice were returned to their home-cages.

Brain slicing

Mice were decapitated and their brains were quickly removed and frozen by immersion in 2-methyl-butane surrounded by dry ice. Coronal sections 20 μm-thick were cut in a cryostat according to the Paxinos & Franklin atlas (1997). Sections were thaw-mounted on gelatin/chrome-coated slides, dried briefly at 30 °C and stored at −80 °C until used. For the identification of the different brain nuclei, adjacent sections to those used for autoradiographic analysis were stained with cresyl-violet and analysed according to the Paxinos & Franklin atlas (1997).

Autoradiography of cannabinoid receptor binding

The protocol used was basically the method described by Herkenham *et al*. (1991). Slide-mounted brain sections were incubated for 2.5 h at 37 °C in a buffer containing 50 mM TRIS with 5% bovine serum albumin (fatty acid-free), pH 7.4, and 10 nM [^{3}H]CP-55, 940 (Perkin Elmer Life Sciences, Madrid, Spain) prepared in the same buffer, in the absence or presence of 10 μM unlabelled CP-55 940 (Tocris, Madrid, Spain) to determine total and nonspecific binding, respectively. Following this incubation, slides were washed in 50 mM TRIS buffer with 1% bovine serum albumin (fatty acid-free), pH 7.4, for 4 h (2 × 2 h) at 0 °C, dipped in ice-cold distilled water, then dried under a stream of cool dried air. Autoradiograms were generated by apposing the labelled tissues, together with autoradiographic standards ([^{3}H]microscales, Amersham, Barcelona, Spain), to tritium-sensitive film (Biomax MS, Amersham, Barcelona, Spain) for a period of 4 weeks and developed for 4 min at 20 °C.

Analysis of WIN 55,212-2-stimulated [^{35}S]-GTPγS binding

The protocol used was the method described by Sim *et al*. (1995). Briefly, slide-mounted brain sections were rinsed in assay buffer (50 mM TRIS, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 0.5% bovine serum albumin fatty acid-free, pH 7.4) at 25 °C for 10 min, then pretreated for 15 min with an excess concentration (2 mM) of GDP (Sigma Chemical Co., Madrid, Spain) in assay buffer. Afterwards, sections were incubated at 25 °C for 2 h in assay buffer containing 0.04 nM [^{35}S]-GTPγS (Amersham, Barcelona, Spain), 2 mM GDP, and 5 μM WIN-55,212-2 (Sigma Chemical Co., Spain). Basal activity was assessed in the absence of agonist, whereas nonspecific binding was measured in the presence of 10 μM unlabelled GTPγS. In pilot experiments, additional brain sections were incubated in the presence of the cannabinoid receptor antagonist SR141716A (1.5 μM) (Rinaldi-Carmona *et al*., 1994) in addition to 0.04 nM [^{35}S]-GTPγS, 2 mM GDP, and 5 μM WIN-55,212-2. SR141716A significantly antagonized the increase in WIN-55,212-2-stimulated [^{35}S]-GTPγS binding, thus supporting that this increase was specifically caused through activation of CB1 cannabinoid receptors (data not shown). Slices were rinsed twice in 50 mM TRIS buffer, pH 7.4, at 4 °C and deionized once in water, then dried under a stream of cool dry air. Autoradiograms were generated by apposing the labelled tissues to film (Biomax MR, Amersham, Barcelona, Spain) for a period of 3 days and developed for 4 min at 20 °C. For all autoradiographic studies, developed film was analysed and quantified in a computerized image analysis system (MCID, St. Catharines, Ontario, Canada).

Statistical analysis

Acute effects of THC administration, body weight loss and withdrawal data were compared by using two-way ANOVA (genotype and treatment as factors of variation) between subjects, followed by one-way ANOVA and Scheffé *posthoc* comparisons when required. Data from the tolerance study were compared using three-way ANOVA (genotype and treatment as between-group factors and day as the within-group factor of variation). Subsequent two-way and one-way ANOVA were made when required. For the conditioned place preference and place aversion experiments, paired two-tailed Student's *t*-tests were made between the postconditioning and preconditioning time spent in the drug paired compartment. For food-maintained operant responding, two-way ANOVA was calculated on the mean of nose-pokes performed during the last 3 days required to reach the stability criteria, with hole (active vs. inactive) and genotype (knockout vs. wild-type) as factors of variation. This statistical analysis was performed for FR1 and FR3 schedules of reinforcement. One-way ANOVA was made when required. Biochemical studies were analysed by using one-way ANOVA. Differences were considered significant if the probability of error was $<5\%$. The level of significance was corrected by Bonferroni when consecutive analysis were made.

Results

Acute THC effects after the first administration

The acute administration of THC (20 mg/kg) induced antinociceptive responses in the hot-plate and tail-immersion tests, a decrease of body temperature and a decrease of locomotor activity in both wild-type and knockout animals. No differences between genotypes were found for any of the acute responses evaluated. Antinociceptive responses in the jumping threshold of the hot-plate test (time to jump in seconds) were similar in wild-type (vehicle-treated, 73.48 ± 11.57; THC-treated, 176.99 ± 18.59) and knockout mice (vehicle-treated, 60.58 ± 5.46; THC-treated, 154.73 ± 10.89). Indeed, two-way ANOVA showed a significant effect of THC treatment ($F_{1,46} = 55.699$, $P < 0.01$), without an effect of genotype ($F_{1,46} = 1.763$, n.s.) and without interaction between these two factors ($F_{1,49} = 0.125$, n.s.). Antinociceptive effects on the licking threshold of the hot-plate test were also similar in wild-type (vehicle-treated, 11.30 ± 1.52; THC-treated, 21.14 ± 2.04) and knockout mice (vehicle-treated, 9.57 ± 0.75; THC-treated, 25.18 ± 1.53). Thus, two-way ANOVA revealed a significant effect of THC treatment ($F_{1,47} = 63.764$, $P < 0.01$), but no genotype effect ($F_{1,47} = 0.527$, n.s.), and no interaction between genotype and treatment ($F_{1,47} = 3.255$, n.s.). In the tail-immersion test, THC also induced similar antinociceptive effects in both wild-type and knockout mice (Fig. 1a, D1, a.m.). Two-way ANOVA revealed a significant effect of the treatment ($F_{1,47} = 70.936$, $P < 0.01$), without a genotype effect ($F_{1,47} = 0.481$, n.s.), and with no interaction between genotype and treatment ($F_{1,47} = 1.507$, n.s.). The body temperature was also similarly decreased by THC (20 mg/kg) treatment in both genotypes (Fig. 1b, D1, a.m.). Two-way ANOVA indicated a significant effect of the THC treatment ($F_{1,47} = 237.336$, $P < 0.01$), without a genotype effect ($F_{1,47} = 0.008$, n.s.) and without interaction between these two factors ($F_{1,47} = 0.024$, n.s.). Locomotor activity was reduced in a similar way by the acute injection of THC (20 mg/kg) in both genotypes (photobeam counts per 10 min, mean values ±SEM in: vehicle-treated wild-type, 61.38 ± 10.52; vehicle-treated knockout, 57.4 ± 6.24; THC-treated wild-type, 11.5 ± 3.28; THC-treated knockout, 15.30 ± 5.29). Two-way ANOVA revealed a significant effect of treatment ($F_{1,47} = 52.769$, $P < 0.01$) but no effect of genotype ($F_{1,47} = 0.103$, n.s.) and no interaction between these two factors ($F_{1,47} = 36.310$, n.s.). The level of significance was $P < 0.025$ for all the cases after applying Bonferroni's correction.

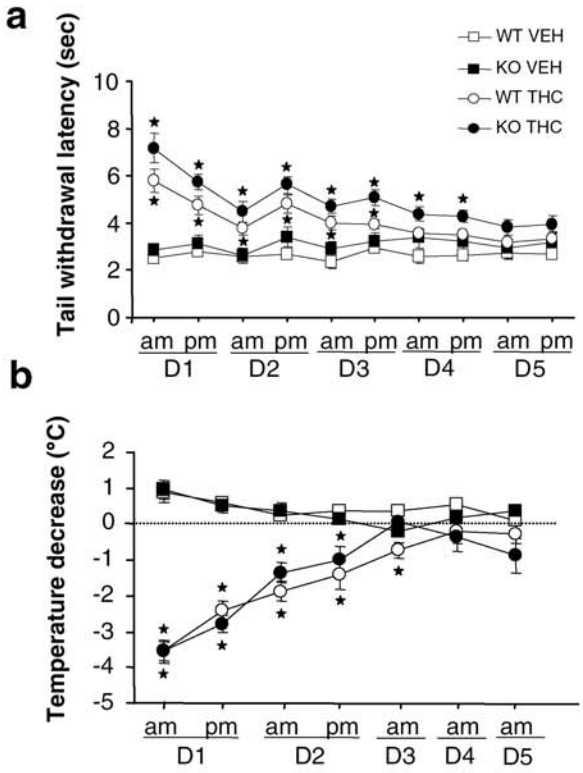

FIG. 1. Development of THC tolerance in A_{2A} knockout (KO) and wild-type (WT) mice. Development of tolerance during chronic THC treatment (20 mg/kg, i.p., twice daily for 5 days) was evaluated by measuring antinociceptive responses in the tail-immersion test (a), and changes in body temperature (b). Data are expressed as mean ±SEM in WT vehicle (white squares, $n = 11$), WT THC (white circles, $n = 14$), KO vehicle (black squares, $n = 12$) and KO THC (black circles, $n = 13$) mice. *$P < 0.01$, comparing treatments (one-way ANOVA). D1–D5, day 1 to day 5.

Tolerance to the effects of THC after repeated administration

Repeated administration of THC (20 mg/kg) developed tolerance as revealed by a progressive decrease in the antinociceptive, and hypothermic effects of the drug during the treatment (Fig. 1). Development of tolerance to THC antinociceptive effects was observed in the tail-immersion test in both genotypes (Fig. 1a). Three-way ANOVA revealed a significant effect of day, treatment and genotype, and an interaction between day and treatment (Table 1). Subsequent two-way ANOVA calculated for each genotype showed, for wild-type animals, a significant effect of day ($F_{9,225} = 7.159$, $P < 0.01$), treatment ($F_{1,25} = 38.511$, $P < 0.01$), and an interaction between these two factors ($F_{9,225} = 8.029$, $P < 0.01$). Two-way ANOVA for knockout mice also revealed a significant effect of day ($F_{9,198} = 6.237$, $P < 0.01$), treatment ($F_{1,22} = 24.845$, $P < 0.01$) and interaction between these two factors ($F_{9,198} = 6.841$, $P < 0.01$). One-way ANOVA to compare treatment effects on each day showed significant differences

TABLE 1. Three-way ANOVA of antinociception, hypothermia, hypolocomotion and weight loss induced during chronic THC treatment in mice lacking A_{2A} adenosine receptor

	Antinociception (TI)		Hypothermia		Loss of body weight	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Day	$F_{9,47} = 13.14$	0.001	$F_{6,47} = 15.66$	0.001	$F_{3,14} = 2.74$	n.s.
Treatment	$F_{1,47} = 59.22$	0.001	$F_{1,47} = 169.68$	0.001	$F_{1,47} = 19.78$	0.001
Genotype	$F_{1,47} = 9.48$	0.01	$F_{1,47} = 0.01$	n.s.	$F_{1,47} = 0.42$	n.s.
Day × treatment	$F_{9,47} = 14.40$	0.001	$F_{6,47} = 35.03$	0.001	$F_{4,47} = 1.55$	n.s.
D × genotype	$F_{9,47} = 0.38$	n.s.	$F_{6,47} = 0.71$	n.s.	$F_{4,47} = 0.44$	n.s.
Treatment × genotype	$F_{1,47} = 0.97$	n.s.	$F_{1,47} = 0.48$	n.s.	$F_{1,47} = 0.78$	n.s.
Day × treatment × genotype	$F_{9,47} = 0.55$	n.s.	$F_{6,47} = 1.94$	n.s.	$F_{4,47} = 0.75$	n.s.

Three-way ANOVA repeated measures with treatment and genotype as between-subject factors, and day as within-subject factor. See Materials and methods for details. TI, tail-immersion test.

between vehicle- and THC-treated mice, until the 4th day in wild-type mice and until the 3rd day in knockout animals. The level of significance was $P < 0.016$ for all the cases after applying Bonferroni's correction.

Tolerance to the hypothermic effects developed similarly in both genotypes (Fig. 1b) reaching the initial body temperature baseline at the end of the chronic treatment. Three-way ANOVA revealed a significant effect of day, treatment and interaction between these two factors (Table 1). Subsequent two-way ANOVA on each genotype showed for wild-type animals a significant effect of the day ($F_{6.150} = 9.868$, $P < 0.01$), treatment ($F_{1.25} = 97.317$, $P < 0.01$) and interaction between these two factors ($F_{6.150} = 17.737$, $P < 0.01$). Two-way ANOVA for knockout mice revealed a significant effect of the day ($F_{6.132} = 6.753$, $P < 0.01$), treatment ($F_{1.22} = 74.214$, $P < 0.01$) and interaction between these two factors ($F_{6.132} = 18.853$, $P < 0.01$). One-way ANOVA to compare treatment effects on each day indicated significant differences between vehicle- and THC-treated mice until the 3rd day in wild-type mice and until the 2nd day in knockout animals. The level of significance was $P < 0.016$ for all the cases after applying Bonferroni's correction.

Effects of THC on body weight after repeated administration

A decrease of body weight during chronic treatment was observed in the THC-treated groups (Fig. 2a). Thus, three-way ANOVA showed a significant effect of treatment (Table 1).

We have also analysed the loss of body weight observed between each injection of the chronic treatment. The chronic administration of THC in wild-type mice revealed a different pattern of food intake compared to A_{2A} adenosine receptor knockout mice (Fig. 2b). Indeed, THC-treated wild-type animals presented an homogeneous and progressive loss of weight during day and night periods. This pattern was not observed in the other groups of animals, including THC-treated knockout mice, whose body weight changes were dependent on the circadian activity and the loss of weight was mainly observed during the light period. Three-way ANOVA revealed a significant effect of day ($F_{3.47} = 28.708$, $P < 0.01$), no effect of treatment ($F_{1.47} = 2.389$, n.s.) but the interaction between these two factors ($F_{3.47} = 7.246$, $P < 0.01$). However, no effect of genotype ($F_{1.47} = 3.659$, n.s.), and no interactions between treatment and genotype ($F_{1.47} = 0.733$, n.s.), day and genotype ($F_{3.47} = 2.155$, n.s.) or day, genotype and treatment ($F_{3.47} = 1.196$, n.s.) were observed. Subsequent two-way ANOVA performed for each genotype, showed for wild-type animals, a significant effect of the day ($F_{8.200} = 12.030$, $P < 0.01$), no effect of the treatment ($F_{1.25} = 0.163$, n.s.) and an interaction between these two factors ($F_{8.200} = 6.571$, $P < 0.01$). Two-way ANOVA for knockout mice revealed an effect of day ($F_{8.176} = 18.232$, $P < 0.01$), of treatment ($F_{1.22} = 7.353$, $P < 0.01$) but no interaction between the two factors ($F_{8.176} = 2.210$, n.s.). One-way ANOVA calculated for wild-type animals to compare treatment effects on each day indicated significant differences between vehicle- and THC-treated mice until the 4th day. The level of significance was $P < 0.016$ for all the cases after applying Bonferroni's correction.

SR141716A-precipitated THC withdrawal

The severity of THC withdrawal was evaluated in A_{2A} knockout and wild-type mice. Thus, mice chronically treated with THC (20 mg/kg, i.p., twice daily) or the corresponding vehicle for 6 consecutive days were challenged with the cannabinoid antagonist SR141716A (10 mg/kg, s.c.) or its corresponding vehicle. No signs of withdrawal were observed in any group of mice during the behavioural observation period 15 min before the antagonist injection (data not shown). A significant expression of different behavioural signs of withdrawal was observed in both genotypes after the antagonist injection during the 45 min period of observation (Fig. 3). Two-way ANOVA was performed for each individual sign of withdrawal. A significant effect of treatment was observed in wet dog shakes ($F_{3.90} = 19.314$, $P < 0.01$), paw tremor ($F_{3.90} = 33.622$, $P < 0.01$), tremor ($F_{3.90} = 19.371$, $P < 0.01$), ptosis ($F_{3.90} = 23.489$, $P < 0.01$), piloerection ($F_{3.90} = 40.515$, $P < 0.01$), genital licks ($F_{3.90} = 5.413$, $P < 0.01$) and mastication ($F_{3.90} = 9.138$, $P < 0.01$). A significant effect of genotype was observed in wet dog shakes ($F_{1.90} = 14.659$, $P < 0.01$), paw tremor ($F_{1.90} = 7.626$, $P < 0.01$) and ptosis ($F_{1.90} = 7.240$, $P < 0.01$). A significant interaction between genotype and treatment was observed for paw tremor ($F_{3.90} = 4.340$, $P < 0.01$) and piloerection ($F_{3.90} = 4.318$, $P < 0.01$). For the global withdrawal score, two-way ANOVA revealed a significant effect of treatment ($F_{3.90} = 92.346$, $P < 0.01$), a significant effect of genotype ($F_{1.90} = 13.815$, $P < 0.01$) and an interaction between these two factors ($F_{3.90} = 4.126$, $P < 0.01$). One-way ANOVA for wild-type animals revealed a significant effect of treatment in paw tremor ($F_{3.48} = 18.830$, $P < 0.01$), piloerection ($F_{3.48} = 14.007$, $P < 0.01$) and the global withdrawal score ($F_{3.48} = 49.881$, $P < 0.01$). Subsequent *posthoc* comparisons revealed a significant difference between the THC-SR group and VEH-VEH group in paw tremor,

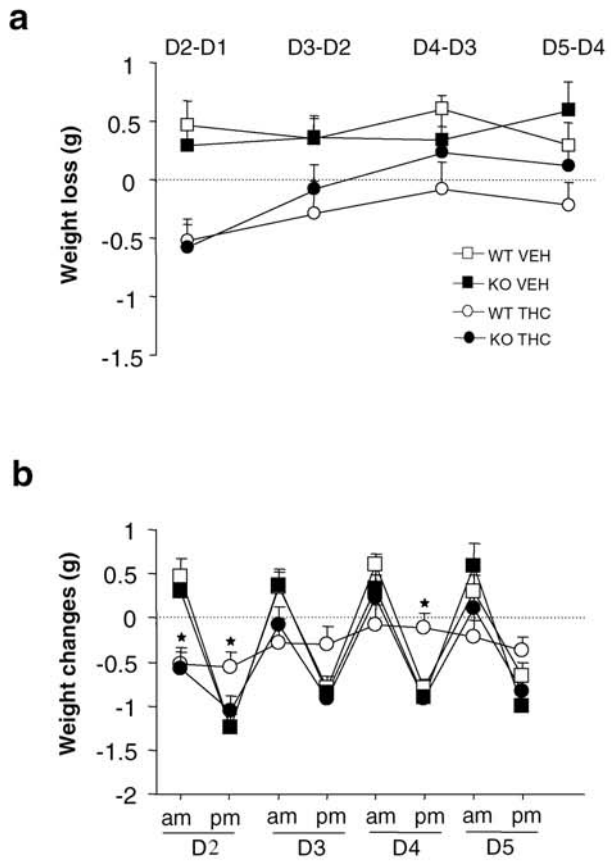

FIG. 2. Loss of weight in A_{2A} knockout (KO) and wild-type (WT) mice during chronic treatment with THC. (a) Daily loss of body weight (g) during 5 days of THC or vehicle treatment. Data are expressed as mean ± SEM of the difference between daily a.m. measurement of body weight and each previous p.m. determination in WT vehicle (white squares, $n = 11$), WT THC (white circles, $n = 14$), KO vehicle (black squares, $n = 12$) and KO THC (black circles, $n = 13$) mice. Three-way ANOVA showed a significant effect of treatment ($P < 0.01$). (b) Body weight differences (g) measured after each injection of THC (20 mg/kg, i.p., twice daily for 5 days). Data are expressed as mean ± SEM in the different groups. *$P < 0.01$; comparing treatments (one-way ANOVA).

piloerection and the global withdrawal score. One-way ANOVA for knockout animals revealed a significant effect of treatment in paw tremor ($F_{3.48} = 19.574$, $P < 0.01$), piloerection ($F_{3.48} = 32.903$, $P < 0.01$) and the global withdrawal score ($F_{3.48} = 45.078$, $P < 0.01$). Subsequent *posthoc* comparisons revealed a significant difference between the THC-SR group and VEH-VEH group in paw tremor, piloerection and the global withdrawal score. The level of significance was $P < 0.016$ for all the cases after applying Bonferroni's correction. No significant differences were observed between VEH-VEH, VEH-SR and THC-VEH groups in either wild-type or mutant mice. One-way ANOVA calculated for THC-VEH-treated mice revealed a significant difference between genotypes in piloerection and global withdrawal score. One-way ANOVA calculated for THC-SR-treated mice revealed a significant difference between genotypes in paw tremor and global withdrawal score. The level of significance was $P < 0.025$ for all the cases after applying Bonferroni's correction.

Rewarding and aversive effects induced by different doses of THC in the place-conditioning paradigm

Motivational responses induced by THC were investigated in A_{2A} knockout and wild-type mice using the place-conditioning paradigm. We first investigated the rewarding properties of THC (1 mg/kg, i.p.), by using a protocol that minimizes the dysphoric consequences of the first THC exposure (Valjent & Maldonado, 2000). Under these experimental conditions, a significant conditioned place preference was observed in wild-type mice treated with THC, as revealed by a significant increase in the time spent in the drug-paired compartment in the postconditioning vs. the preconditioning phase ($t_{1.12} = -2.89$, $P < 0.05$). In contrast, A_{2A} knockout mice did not show any significant difference in the time spent in the drug-paired compartment in the postconditioning vs. the preconditioning phase, demonstrating the absence of THC rewarding effects of THC in these mutant mice ($t_{1.16} = 1.21$, n.s.) (Fig. 4a). To evaluate the aversive effects of THC (5 mg/kg i.p.), we used a similar place-conditioning protocol without pre-exposing mice to THC, as previously reported (Valjent & Maldonado, 2000). In these experimental conditions, THC induced a significant decrease in the time spent in the drug-paired compartment in the postconditioning vs. the preconditioning phase in wild-type mice, revealing an aversive effect of THC in this genotype ($t_{1.12} = 2.40$, $P < 0.05$) (Fig. 4b). However, this aversive response was not observed in A_{2A} knockout mice, as revealed by the similar time spent in the drug-paired compartment during postconditioning and preconditioning phases ($t_{1.16} = -0.14$, n.s.) (Fig. 4b).

Food-maintained operant behaviour

As shown in Fig. 5, nose-poke behaviour maintained by food pellets was similarly acquired by both wild-type and A_{2A} knockout mice. All the wild-type and A_{2A} knockout mice reached the stability criteria in FR1 and FR3, and both genotypes maintained active nose-poke discrimination during the whole experiment. Thus, two-way ANOVA showed in FR1 a significant effect of nose-poke discrimination ($F_{1.26} = 122.903$, $P < 0.01$), no effect of genotype ($F_{1.26} = 2.991$, n.s.), and no interaction between genotype and nose-poke ($F_{1.26} = 0.880$, n.s.). The maintenance of nose-poke responding by food in FR3 schedule of reinforcement was also similar in both genotypes, with a clear discrimination between the active and the inactive nose-pokes. Two-way ANOVA showed a significant effect of nose-poke ($F_{1.26} = 60.598$, $P < 0.01$), no effect of genotype ($F_{1.26} = 0.000$, n.s.), and no interaction between genotype and nose-poke ($F_{1.26} = 0.342$, n.s.). Subsequent one-way ANOVAs revealed significant discrimination between the active and the inactive holes in wild-type and A_{2A} knockout mice during FR1 and FR3 schedules of reinforcement. The level of significance was $P < 0.025$ for all the cases after applying Bonferroni's correction.

Autoradiography of cannabinoid receptor binding

The distribution of CB1 cannabinoid receptors labelled with [^{3}H]CP-55 940 in coronal sections of brain of control mice was similar to those previously reported (Herkenham *et al.*, 1991). Thus, a high density of CB1 cannabinoid receptors was found in several brain structures including basal ganglia, cerebellum, hippocampus and cerebral cortex. There were no significant differences between wild-type and A_{2A} knockout mice in the levels of CB1 cannabinoid receptor in any of the brain structures analysed (Table 2).

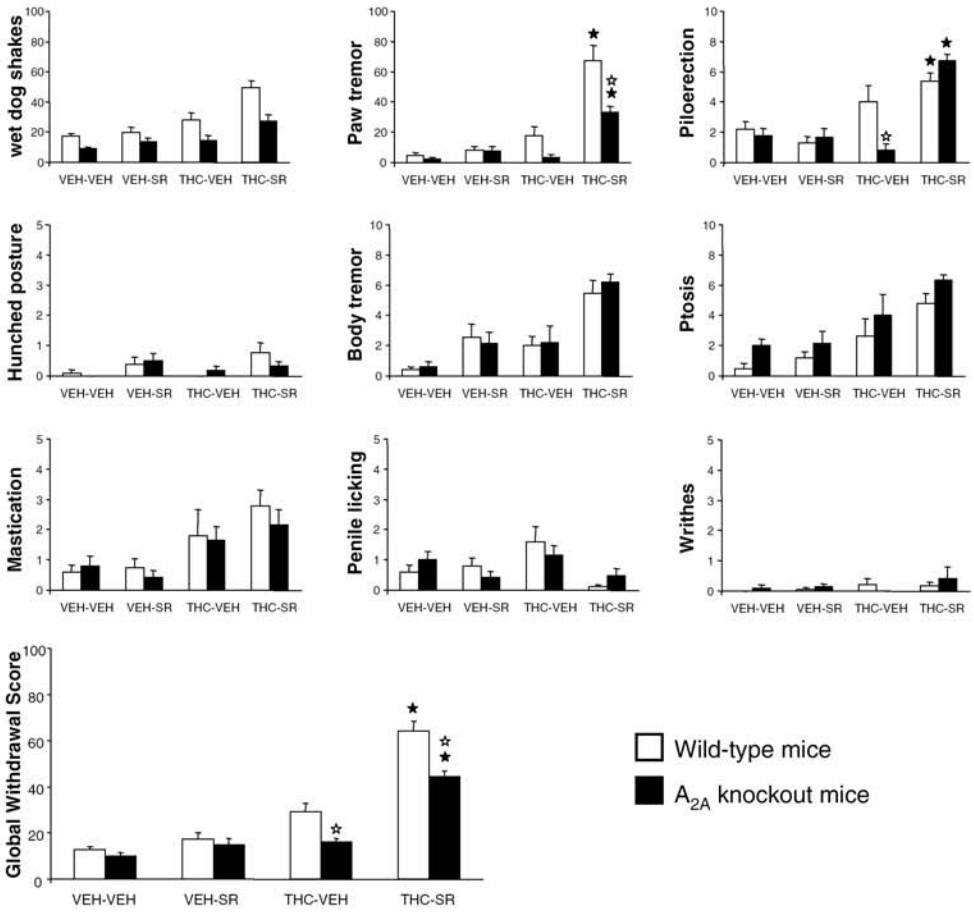

Fig. 3. SR141716A-precipitated THC withdrawal signs in A_{2A} knockout (KO) and wild-type (WT) mice. Withdrawal syndrome was precipitated by the administration of SR141716A (10 mg/kg, s.c.) or the corresponding vehicle (VEH) in mice receiving chronic treatment with either vehicle or THC (20 mg/kg, i.p.). Counted (wet dog shakes, paw tremor and writhes) and checked (ptosis, body tremor, hunched posture, piloerection, mastication and penile licking) signs of withdrawal were observed for the 45 min immediately after SR141716A administration. The global withdrawal score was calculated for each animal by giving each individual sign a relative weight. Data are expressed as mean ± SEM in WT (white bars, VEH-VEH $n = 10$, VEH-SR $n = 16$, THC-VEH $n = 5$, THC-SR $n = 18$) and KO (black bars, VEH-VEH $n = 10$, VEH-SR $n = 14$, THC-VEH $n = 6$, THC-SR $n = 19$) mice. *$P < 0.01$ (posthoc comparison vs. VEH-VEH group). ☆$P < 0.025$ comparing genotypes (one-way ANOVA).

Autoradiography of WIN 55,212-2-stimulated [^{35}S]-GTPγS binding

The anatomical distribution of WIN 55,212-2-stimulated [^{35}S]-GTPγS binding qualitatively paralleled receptor distribution as determined in the previous experiment by receptor binding autoradiography. The highest functional activity of CB1 cannabinoid receptor was revealed in several nuclei of the basal ganglia such as globus pallidus, entopeduncular nucleus and substantia nigra, as previously reported (Sim *et al*., 1995). As shown in Table 3, no differences between A_{2A} adenosine receptor knockout mice and wild-type animals were detected in the stimulation of [^{35}S]-GTPγS binding in any of the brain areas investigated.

Discussion

The aim of the present study was to investigate the behavioural responses related to THC addictive properties in A_{2A} adenosine receptor knockout mice. The deletion of A_{2A} adenosine receptors abolished both rewarding and aversive responses produced by THC in the place-conditioning paradigm and attenuated the severity of THC withdrawal. These effects were produced without any compensatory change in the distribution and functional activity of CB1 cannabinoid receptors in the brain.

Deletion of A_{2A} adenosine receptors did not modify several acute effects induced by THC. Thus, THC (20 mg/kg) acute antinociceptive responses in both the tail-immersion and hot-plate tests were similar in wild-type and knockout mice. A hypoalgesic phenotype has been previously reported, but the thermal stimulus was stronger than that used in the present study (Ledent *et al*., 1997; Bailey *et al*., 2002). Changes in basal nociceptive responses in A_{2A} knockout mice do not seem reliable when using the tail-immersion test at 50 °C. THC administration induced a similar decrease in both locomotor activity and rectal temperature in wild-type and knockout animals. Lower doses of THC (5 and 10 mg/kg) also induced the same effects on nociceptive thresholds, locomotion and rectal temperature in both genotypes (data not shown). These data demonstrate that A_{2A} receptors do not participate in the acute effects induced by THC.

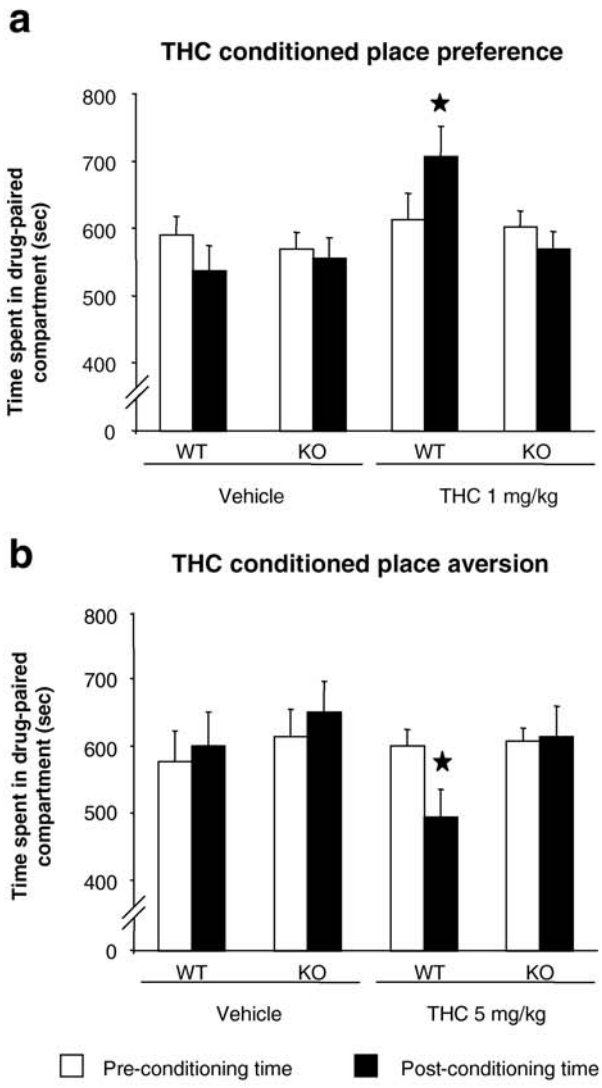

FIG. 4. THC conditioned place preference and aversion in A_{2A} knockout (KO) and wild-type (WT) mice. Results are expressed as means ± SEM of the times spent in the drug-paired compartment (s) during the preconditioning phase (white bars) and postconditioning phase (black bars). Conditioned place preference experiment: WT vehicle ($n = 15$), KO vehicle ($n = 17$), WT THC ($n = 13$), KO THC ($n = 17$). Conditioned place aversion: WT vehicle ($n = 12$), KO vehicle ($n = 12$), WT THC ($n = 13$), KO THC ($n = 17$). *$P < 0.05$; comparing the times spent in the drug-paired compartment during the preconditioning and postconditioning phase (two tailed paired Student's *t*-test).

Chronic treatment with THC produced a similar degree of tolerance to antinociceptive, hypothermic and body weight loss effects of the drug in wild-type and A_{2A} knockout mice, although tolerance developed slightly faster in the knockout group. Interestingly, the analysis of body weight changes for the different experimental groups revealed that THC treatment modified the pattern of daily weight oscillation in wild-type but not in knockout mice. Both groups of vehicle-treated mice showed the expected weight pattern based on the light/dark cycle, and exhibited an increase of body weight during the dark phase, corresponding to the normal eating period in rodents, and a decrease during the light phase. THC-treatment modified this pattern in wild-type mice; they exhibited a stable line of body weight loss without the rhythmic oscillations observed in vehicle-treated mice. Interestingly, chronic treatment with THC did not modify the daily oscillation pattern of body weight in A_{2A} knockout mice. Cannabinoid receptors and their endogenous ligands have been implicated in the control of feeding and body weight regulation (Di Marzo *et al.*, 2001; Kirkham *et al.*, 2002; Williams & Kirkham, 2002). Thus, THC treatment interfered with the satiety threshold in rodents (Williams *et al.*, 1998). It is important to note that the dose of THC used in this study was high in order to induce a reliable degree of tolerance and dependence, and does not correspond to the doses currently used to reveal THC hyperphagic effects (Williams & Kirkham, 2002). Several studies have also proposed a role for A_{2A} adenosine receptors in the regulation of feeding behaviour (Nagel *et al.*, 2003; Kittner *et al.*, 2004). Thus, our findings provide evidence that A_{2A} adenosine receptors could regulate the effects of THC on feeding behaviour.

We have evaluated the involvement of A_{2A} adenosine receptors in THC-induced physical dependence. SR141716A precipitated a withdrawal syndrome in THC-dependent wild-type and A_{2A} adenosine knockout mice. However, in mice lacking A_{2A} adenosine receptors the withdrawal syndrome was attenuated as revealed by the significant decrease of the global withdrawal score. Both A_{2A} adenosine and CB1 cannabinoid receptors are present in common brain areas related to the control of motor activity such as the cerebellum and the striatum (Herkenham *et al.*, 1991; Svenningsson *et al.*, 1999). The cannabinoid withdrawal syndrome is accompanied by a strong motor impairment (Hutcheson *et al.*, 1998; Tzavara *et al.*, 2000) and the cerebellum has been reported to play an important role in the somatic manifestations of THC withdrawal (Tzavara *et al.*, 2000). A_{2A} adenosine receptors are positively coupled to adenylyl cyclase, and the activity of this enzyme was selectively increased in the cerebellum during SR141716A-precipitated cannabinoid withdrawal. Therefore, we postulate that A_{2A} receptors may contribute to the increased cAMP levels during THC withdrawal and the deletion of these purinergic receptors would then result in an attenuation in the severity of cannabinoid withdrawal. However, other second messenger systems coupled to CB1 receptors, such as the MAP-kinase pathway, could also be implicated in the somatic manifestations of cannabinoid withdrawal, which would justify the moderate magnitude of the behavioural effect in A_{2A} knockout mice.

Motivational responses to THC were explored by using the place-conditioning procedure. Rewarding and aversive effects of THC were revealed in wild-type mice, as previously reported (Valjent & Maldonado, 2000), but these effects were suppressed in mice lacking A_{2A} receptors. To exclude the possibility of a general learning impairment in knockout mice lacking A_{2A} receptors, food-maintained operant behaviour was investigated in a self-administration paradigm. Both wild-type and A_{2A} knockout mice acquired and maintained operant self-administration for food in a similar way in FR1 and FR3 schedules of reinforcement, which suggests that learning processes were not impaired in mutant mice. Previous pharmacological studies using adenosine agonists and antagonists have provided confusing data about the contribution of A_{2A} receptors in reward-related processes (Brockwell & Beninger, 1996; Baldo *et al.*, 1999; Sahraei *et al.*, 1999; Poleszak & Malec, 2003). The use of different behavioural paradigms and compounds makes the interpretation of these pharmacological studies difficult. Our study represents the first direct evidence showing an interaction between A_{2A} adenosine receptors and THC motivational effects in the place-conditioning paradigm. A_{2A} receptors are mainly located in striatal neurons where they interact with multiple neurotransmitter systems. Thus, A_{2A}

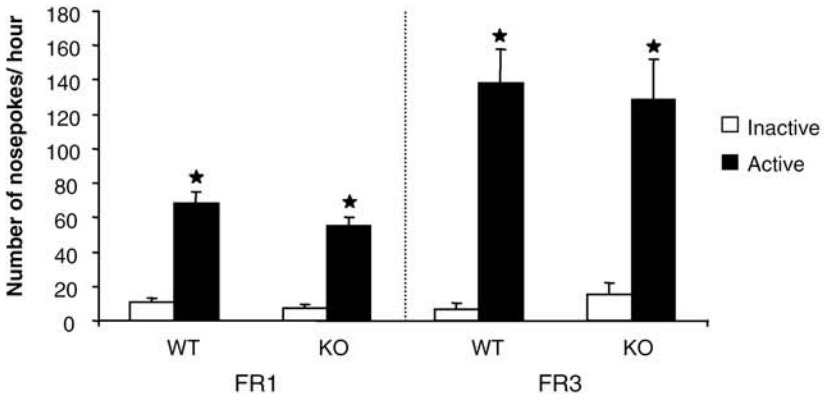

FIG. 5. Food-maintained operant behaviour in A_{2A} knockout (KO) and wild-type (WT) mice Average of the number of nose-pokes in both the active and the inactive holes, made in the three consecutive sessions (1 h each) required to achieve the stability criteria in FR1 and FR3 schedule of reinforcement $*P < 0.025$ comparing holes (one-way ANOVA).

TABLE 2. CB1 receptor binding of [^{3}H]CP-55 940 in several brain regions of A_{2A} knockout mice and wild-type littermates

	[^{3}H]CP-55 940 (fmol/mg of tissue)	
Brain regions	Wild-type	A_{2A} knockout
Cerebral cortex		
Superficial layer (I)	94.28 ± 14.54	93.39 ± 4.58
Deep layer (VI)	93.09 ± 15.47	86.22 ± 5.33
Basal ganglia		
Lateral caudate-putamen	97.38 ± 18.10	95.23 ± 15.38
Medial caudate-putamen	73.52 ± 12.97	74.00 ± 7.99
Globus pallidus	254.47 ± 40.57	289.02 ± 38.74
Entopeduncular nucleus	199.39 ± 43.90	270.65 ± 45.29
Substantia nigra	216.52 ± 8.12	200.38 ± 15.78
Cerebellum molecular layer	193.97 ± 25.70	182.08 ± 29.39
Hippocampus	83.13 ± 14.58	81.44 ± 9.35
Periaqueductal grey matter	67.68 ± 9.50	71.62 ± 19.27

Values are mean ± SEM of six determinations per group. No significant differences were found between genotypes (one-way ANOVA with genotype as between factor).

TABLE 3. WIN-55,212-2-stimulated [^{35}S] GTPγS binding in several brain regions of A_{2A} knockout mice and wild-type littermates

	[^{35}S] GTPγS binding (%)*	
Brain regions	Wild-type	A_{2A} knockout
Cerebral cortex		
Superficial layer (I)	20.70 ± 4.26	24.30 ± 4.51
Deep layer (VI)	30.39 ± 5.06	28.55 ± 3.54
Basal ganglia		
Lateral caudate-putamen	22.00 ± 3.56	19.65 ± 4.80
Medial caudate-putamen	16.80 ± 2.92	14.16 ± 3.68
Globus pallidus	86.32 ± 8.55	103.05 ± 11.96
Entopeduncular nucleus	102.52 ± 19.54	76.22 ± 6.73
Substantia nigra	64.21 ± 12.20	64.42 ± 9.31
Cerebellum molecular layer	52.33 ± 7.62	50.30 ± 9.25
Hippocampus	23.56 ± 5.10	24.61 ± 2.67

Values are mean ± SEM of seven determinations per group. *Percentage of stimulation of basal binding. No significant differences were found between genotypes (one-way ANOVA with genotype as between factor).

receptors are coexpressed with postsynaptic D2 receptors in GABAergic neurons in this brain structure (Fink *et al*., 1992). CB1 cannabinoid receptors are also highly expressed in the striatum where they interact with A_{2A} adenosine receptors in the control of different behavioural responses (Berrendero *et al*., 2003). Taking into account the participation of the opioid system in the responses induced by cannabinoids (for review see Manzanares *et al*., 1999; Valverde *et al*., 2000; Ghozland *et al*., 2002) and the regulation of proenkephalin gene expression by A_{2A} adenosine receptors (Ledent *et al*., 1997), we cannot exclude a possible involvement of the endogenous opioid system in the modification of THC responses in A_{2A} knockout mice. Indeed, THC place preference was abolished in mice lacking mu-opioid receptors (Ghozland *et al*., 2002), whereas THC-induced place aversion was suppressed in both mice deficient in kappa-opioid receptors (Ghozland *et al*., 2002) and deficient in the dynorphin gene (Zimmer *et al*., 2001). However, no changes in the binding properties of the different opioid receptors were detected in the brains of A_{2A} knockout mice (Bailey *et al*., 2002). On the other hand, the suppression of A_{2A} adenosine receptors leads to a functional hypodopaminergic state in the striatum and cerebral cortex (Dassesse *et al*., 2001), and dopamine neurotransmission plays a crucial role in the regulation of the rewarding effects of drugs of abuse (Koob, 1996; Di Chiara, 2002). The activation of CB1 cannabinoid receptors has been suggested to disinhibit dopaminergic neurons producing an increase of dopamine release (Van der Stelt & Di Marzo, 2003), and it could be suggested that A_{2A} receptors have a modulatory role on cannabinoid-induced dopamine release. Thus, DARPP-32, a potent endogenous inhibitor of Protein Phosphatase 1 which plays an obligatory role in dopaminergic transmission, is altered in mice lacking A_{2A} adenosine receptors (Svenningsson *et al*., 2000). Interestingly, a deficit in DARPP-32 is sufficient to decrease the conditioned place preference for cocaine, indicating that modulation of downstream processes of dopamine release appears to be critical for cocaine rewarding properties (Zachariou *et al*., 2002). A recent study reported the existence of an important synergy in protein kinase A signalling between CB1 cannabinoid and D_2 dopamine receptors in the nucleus accumbens. Adenosine A_2 receptor activation is required for this synergy (Yao *et al*., 2003). These authors proposed that colocalization of D_2 and CB1 receptors on the same neurons confers hypersensitivity to cannabinoids. All these complex interactions between A_{2A} adenosine receptors and the dopamine system may explain that the effects of THC on dopamine transmission could be

impaired in the absence of A_{2A} receptors, thereby producing a suppression of both THC-induced rewarding and aversive responses.

Finally, the distribution and the functional activity of CB1 cannabinoid receptors was measured in several brain structures related to cannabinoid behavioural effects, such as cerebellum, hippocampus, cortex, and different nuclei of the basal ganglia (Ameri, 1999). The distribution of CP 55 940 binding sites was similar in A_{2A} knockout mice and wild-type littermates. Similarly, WIN 55,212-2 produced a similar stimulation of [^{35}S]-GTPγS binding in both genotypes in all the brain areas evaluated, indicating that the functional activity of CB1 cannabinoid receptors was not modified in A_{2A} knockout mice. These results indicate the absence of compensatory changes in CB1 cannabinoid receptors in A_{2A} knockout mice that could have been of relevance to explain the modification of THC behavioural responses.

In summary, the present study shows that A_{2A} adenosine receptors do not participate in the acute effects induced by THC, or in the development of THC tolerance to these effects. However, A_{2A} adenosine receptors seem to play a role in THC control of feeding behaviour. Furthermore, A_{2A} adenosine receptors are involved in the motivational properties of THC and the somatic expression of THC withdrawal. These behavioural changes were not due to a learning impairment, or to compensatory changes in the distribution or functional activity of CB1 cannabinoid receptors in A_{2A} knockout mice. Therefore, these results demonstrate for the first time the participation of A_{2A} adenosine receptors in the behavioural manifestations of THC addictive properties and provide a further advance in understanding the complex neurobiological processes underlying cannabinoid addiction.

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Abbreviation

THC, Δ^9-tetrahydrocannabinol.

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Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence

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Abstract

Cannabis is the most widely consumed illicit drug and its consumption is currently associated with tobacco, which contains another psychoactive compound, namely nicotine. Interactions between cannabinoids and other drugs of abuse, such as opioids, have been previously reported. The aim of the present study was to evaluate the possible role of CB1 cannabinoid receptor in responses induced by acute and repeated nicotine administration by using knockout mice lacking the CB1 cannabinoid receptor and their wild-type littermates. Acute nicotine (0.5, 1, 3 and 6 mg/kg, sc) administration decreased locomotor activity and induced antinociceptive responses in the tail-immersion and the hot-plate test, in wild-type animals. The antinociceptive effects in the tail-immersion test were significantly enhanced in CB1 knockout mice. In wild-type mice nicotine (0.5 mg/kg, sc) produced a significant rewarding effect, as measured by a conditioned place preference paradigm. This response was absent in CB1 knockout mice. Finally, a model of mecamylamine-induced abstinence in chronic nicotine-treated mice (10 mg/kg/day, sc) was developed. Mecamylamine (1 and 2 mg/kg, sc) precipitated several somatic signs of nicotine withdrawal in wild-type dependent mice. However, no difference in the severity of nicotine withdrawal was observed in CB1 knockout mice. These results demonstrate that some acute effects and motivational responses elicited by nicotine can be modulated by the endogenous cannabinoid system and support the existence of a physiological interaction between these two systems.

Keywords: Cannabinoid; CB1 cannabinoid receptor; Nicotine; Nociception; Conditioning place preference; Withdrawal; Mice

1. Introduction

Marijuana is the most widely consumed illicit drug in humans (Adams and Martin, 1996; Watson et al., 2000), frequently used in association with tobacco (Degenhardt et al., 2001; Schorling et al., 1994). The endogenous cannabinoid system has been reported to be involved in the modulation of many functions within the central nervous system. For example, previous studies in rodents have shown that administration of cannabinoid agonists modifies locomotion, anxiety, memory, nociception and processing of reward signals (Dewey, 1986; Hernandez-Tristan et al., 2000; Ledent et al., 1999; Walker et al., 1999; Zimmer et al., 1999). Similarly, previous studies in rodents have shown that nicotine also modifies locomotion, anxiety, learning and memory, nociception, and its repeated administration produces physical dependence (Clarke and Kumar, 1983; Hildebrand et al., 1999; Marubio et al., 1999; Picciotto et al., 1995). Therefore, a number of common central nervous system functions are affected by cannabinoid agonists and by nicotine, although often in opposite directions. Recent studies carried out with mutant mice lacking the CB1 cannabinoid receptor gene have demonstrated that this receptor mediates the pharmacological effects of cannabinoids in the central nervous system (Ledent et al., 1999; Zimmer et al., 1999). Neuroanatomical studies have shown a high density of CB1 receptors in neurons of the cerebellum, basal ganglia, limbic cortices, hippocampus, hypothalamus and different nuclei of the extended amygdala (Tsou et al., 1998). Interestingly, an overlapping distribution

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of CB1 cannabinoid receptors and nACh receptors has been reported in several brain structures such as the hippocampus and the amygdala (Picciotto et al., 2000), which suggests the possibility of functional interactions between these two systems.

The aim of the present study was to investigate the involvement of the endogenous cannabinoid system on nicotine pharmacological effects. For this purpose, several well-known behavioural responses induced by acute and repeated nicotine administration (Watkins et al., 2000) were evaluated in CB1 cannabinoid receptor knockout mice and their wild-type littermates. Locomotor effects induced by acute nicotine administration were measured in activity boxes. Acute nicotine antinociceptive responses were assayed using two models where different neural pathways are involved in processing the nociceptive signals, the tail-immersion and the hot-plate test (Grossman et al., 1982; Morgan et al., 1989). The conditioned place preference paradigm was used to evaluate the rewarding properties of nicotine. Finally, the behavioural expression of mecamylamine–precipitated withdrawal was evaluated in chronic nicotine-treated mice.

2. Materials and Methods

2.1. Animals

Male CB1 knockout mice and wild-type littermates, weighing 26–30 g at the beginning of the experiments, were used. The generation of mice lacking CB1 cannabinoid receptor has been previously described (Ledent et al., 1999). In order to homogenise the genetic background of mice, the first generation heterozygotes were bred for 15 generations on a CD1 background, with selection for the mutant CB1 gene at each generation. All animals used in a given experiment originated from the same breeding series and were matched for age and weight. In preliminary experiments, male CD1 mice (Charles River, France), weighing 26–30 g at the beginning of the study, were used in order to find the optimal range of nicotine doses and to validate the experimental protocol for the evaluation of nicotine abstinence.

Mice were housed five per cage in a temperature-controlled room (21 ± 1 °C) with a 12 h light–dark cycle (light between 08:00 and 20:00h). Food and water were available ad libitum. Mice were habituated to their new environment for 1 week after arrival before starting the experimental procedure. The observer was blind to the mouse genotype and treatment in all the experiments. All the procedures met the guidelines of The European Communities Council Directive 86/609/EEC regulating animal research and were approved by the Local Ethical Committee within our institute.

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridyl]pyrrolidine) and mecamylamine hydrochloride (Sigma Chemical Co., Madrid Spain) were dissolved in physiological saline (0.9%), and administered in a volume of 10 ml/kg.

2.3. Acute nicotine responses

2.3.1. Experimental procedure

In the first experiment, wild-type CD1 mice were used in order to find the optimal range of nicotine doses. Mice (n = 12 per group) were injected with nicotine (0.5, 1, 2, and 3 mg/kg, sc) or saline. Locomotor activity and the antinociceptiception (tail-immersion and hot-plate) were evaluated along a test battery scheme (see Fig. 1). Preliminary experiments with subsequent exposure to the tail-immersion and the hot-plate test showed that the previous tail-immersion exposure did not influence the results obtained in the hot-plate test. Each mouse received only one treatment.

Subsequently, the pharmacological effects of nicotine were evaluated in CB1 knockout mice. CB1 knockout mice and wild-type littermates (n = 10 per group) were injected with nicotine (0.5, 1, 3 and 6 mg/kg, sc) or saline and locomotor activity and antinociceptive responses were measured using the same protocols as described above.

2.3.2. Locomotor activity

The locomotor responses induced by nicotine were evaluated by using small locomotor activity boxes (9 × 20 × 11 cm) (Imetronic, Lyon France) in a low luminosity room (5 lux), and with white noise. Each box contained a line of photocells 2 cm above the floor to measure horizontal movements, and another line located 6 cm above the floor to measure vertical activity (rearing). Mice were individually placed in the boxes 5 min after nicotine or saline injection and the number of activity counts was recorded for a period of 10 min.

2.3.3. Antinociceptive responses

Two different nociceptive models, the tail-immersion and the hot-plate test were used to evaluate the antinociceptive responses elicited by nicotine.

2.3.3.1. Tail-immersion test The antinociceptive responses were determined as previously reported (Simonin et al., 1998), 15 min after nicotine or saline injection. The water temperature was maintained at 50 ± 0.5 °C using a thermo regulated water circulating pump (Clifton, North Somerset UK). The time to withdraw the tail was determined and a cut-off was set-up at 15 s in order to prevent tissue damage.

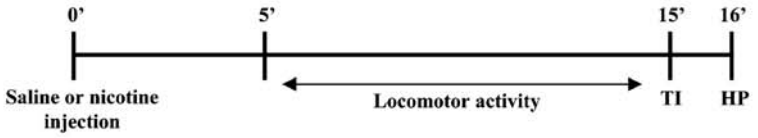

Fig. 1. Schematic representation of the protocol used for locomotor activity and nociceptive tests. TI: tail-immersion test, HP: hot-plate test. Numbers express time in minutes after nicotine or saline injection

2.3.3.2. Hot-plate test This test was performed as previously described (Simonin et al., 1998), 16 min after nicotine or saline injection. The temperature of the plate (Columbus instruments, Columbus OH USA) was kept at 52 ± 0.5 °C. The time to elicit the first jump was determined and a cut-off was set-up at 240 s in order to prevent tissue damage.

2.4. Place preference paradigm

The rewarding effects of nicotine were evaluated by using the conditioned place preference paradigm, as previously described (Maldonado et al., 1997; Matthes et al., 1996). The conditioning apparatus used was a Plexiglas box with two compartments and a central neutral area (Maldonado et al., 1997; Matthes et al., 1996). In a preliminary experiment, nicotine (0.12, 0.25, 0.5 and 1 mg/kg, sc) effects were evaluated in wild-type CD1 mice. In a second experiment, nicotine (0.1, 0.25, and 0.5 mg/kg, sc) was tested in CB1 knockout mice and wild-type littermates. The place preference protocol was performed in three different phases.

2.4.1. Pre-conditioning phase

The first day, each mouse (n = 15 per group) was placed in the middle of the neutral area of the conditioning box and was allowed to explore both compartments. The time spent in each compartment was recorded during 18 min by a camera connected to a computer (Videotrak, View Point, Lyon France). Treatments were counterbalanced between compartments in order to use an unbiased procedure. No initial place preference or aversion for the different compartments was observed in any of the experiments.

2.4.2. Conditioning phase

Animals were treated over 8 days with alternate injections of nicotine or saline. Mice were confined to the corresponding compartment immediately after injection by using guillotine doors matching walls for 20 min. Nicotine was administered on days 1, 3, 5 and 7, and saline on days 2, 4, 6 and 8. Control animals received saline every day.

2.4.3. Testing phase

This phase was conducted exactly as for the pre-conditioning phase, i.e. free access to both compartments during 18 min, and the time spent in each compartment recorded. The time in the central area was proportionally shared and added to the time value of each compartment as previously described (Maldonado et al., 1997; Valverde et al., 1996). A score value was calculated for each mouse as the difference between the time spent in the drug-paired compartment during the testing and pre-conditioning phases.

2.5. Nicotine dependence and withdrawal

2.5.1. Induction of nicotine dependence

Mice were divided in two groups and were implanted subcutaneously with Alzet osmotic minipumps (Model, 2001) (Alzet®, Cupertino, CA), under brief diethyl ether anaesthesia. The minipumps contained saline or nicotine solutions and delivered a constant subcutaneous flow in a rate of 1μl/h. The concentration of nicotine was adjusted to compensate for differences in the body weight of the subjects. Thus, average-weight mouse received a dose of approximately 10 mg/kg/day nicotine hydrogen tartrate salt over 6 days.

2.5.2. Mecamylamine-precipitated nicotine withdrawal syndrome

Six days after minipump implantation, mice were placed inside a circular clear plastic observation area and the withdrawal syndrome was precipitated by injection of the nicotinic receptor antagonist, mecamylamine. In a preliminary experiment, withdrawal syndrome was precipitated in nicotine dependent wild-type CD1 mice by using different doses of mecamylamine (1 and 2 mg/kg, sc). Taking into account these preliminary results, the dose of mecamylamine chosen to test the nicotine withdrawal syndrome in CB1 knockout mice and wild-type littermates was 1 mg/kg (sc). The following abstinence signs were evaluated during a period of 10 min before and 30 min after mecamylamine injection: locomotor activity, body tremor, ptosis, wet dog shakes, teeth chattering, paw tremor, scratching, genital licks, sniffing and piloerection. The number of wet dog shakes, front paw tremors, sniffing and scratches was counted. Ptosis, genital licks, tremor, piloerection and teeth chattering were scored 1 for appearance or 0 for non-appearance within each 5 min time. The locomotor activity over 5 min periods was rated 0, 1 or 2 (0 for inactivity, 1 for low activity and 2 for normal activity). A global withdrawal score was calculated for each animal by giving each individual sign a relative weight: 0.5 for each epi-

sode of wet dog shake, front paw tremor, sniffing and scratching; and 1 for the presence of ptosis, genital lick, tremor, piloerection and teeth chattering during each observation period of 5 min. The relative weight of locomotor activity for each 5 min period was 0 normal activity, 0.5 low activity and 1 inactivity.

2.6. Statistical analysis

Data from preliminary experiments performed in wild-type CD1 mice were compared by using a one-way ANOVA between subjects and post-hoc (Dunnett test) after significant main effects. All experiments performed using CB1 knockout mice and their wild-type littermates were compared by using the two-way ANOVA (genotype and treatment) between subjects followed by a corresponding one-way ANOVA and post-hoc (Dunnett test) after significant main effects. The level of significance was $p < 0.05$ in all experiments.

3. Results

Previous studies have characterized the spontaneous behavioural phenotype of CB1 cannabinoid receptor knockout mice (Ledent et al., 1999; Martin et al., 2002; Valverde et al., 2000). Thus, CB1 knockout mice appear fertile and healthy and exhibit a similar nociceptive threshold to wild-type littermates. However, CB1 cannabinoid receptor knockout mice have been reported to show changes in emotional behaviour, a decrease in the motivational responses induced by morphine as well as a reduction in the severity of morphine withdrawal syndrome.

3.1. Nicotine decreased locomotor activity in wild-type and CB1 knockout mice

In a preliminary set of experiments, we evaluated the locomotor effects of nicotine in wild-type CD1 mice. Nicotine (0.5, 1, 2 and 3 mg/kg) induced a significant decrease in horizontal [$F(4,59) = 13.296$, $p < 0.001$] and vertical [$F(4,59) = 15.335$, $p < 0.001$] locomotor activity. Post-hoc analysis for horizontal locomotor activity showed a significant effect when nicotine was given at the doses of 1 ($p < 0.05$), 2 ($p < 0.01$) and 3 ($p < 0.01$) mg/kg. Vertical locomotor activity was significantly decreased when nicotine was injected at the doses of 2 and 3 mg/kg ($p < 0.01$); (data not shown).

In a second experiment, the locomotor effects of nicotine (0.5, 1, 3 and 6 mg/kg) were evaluated in CB1 knockout mice and wild-type littermates. Nicotine decreased locomotion in both genotypes (Fig. 2). Two-way ANOVA calculated for horizontal locomotor activity, showed a significant effect following treatment [$F(4,100) = 65.961$, $p < 0.001$], without genotype effect and without interaction between treatment and genotype. Subsequent one-way ANOVA (treatment) for wild-type [$F(4,54) = 39.416$, $p < 0.001$] and knockout [$F(4,54) = 29.910$, $p < 0.001$] mice exhibited a significant effect. Post-hoc analysis revealed a reduction of horizontal locomotor activity when nicotine was given at the doses of 1, 3 and 6 mg/kg ($p < 0.001$) in both genotypes. Two-way ANOVA calculated for vertical locomotor activity, showed a significant effect following treatment [$F(4,100) = 62.932$, $p < 0.001$], without genotype effect, and without interaction between treatment and genotype. One-way ANOVA (treatment) for wild-type [$F(4,54) = 33.083$, $p < 0.001$] and knockout [$F(4,54) = 30.853$, $p < 0.001$] mice as also significant. Post-hoc analysis revealed a significant reduction of ver-

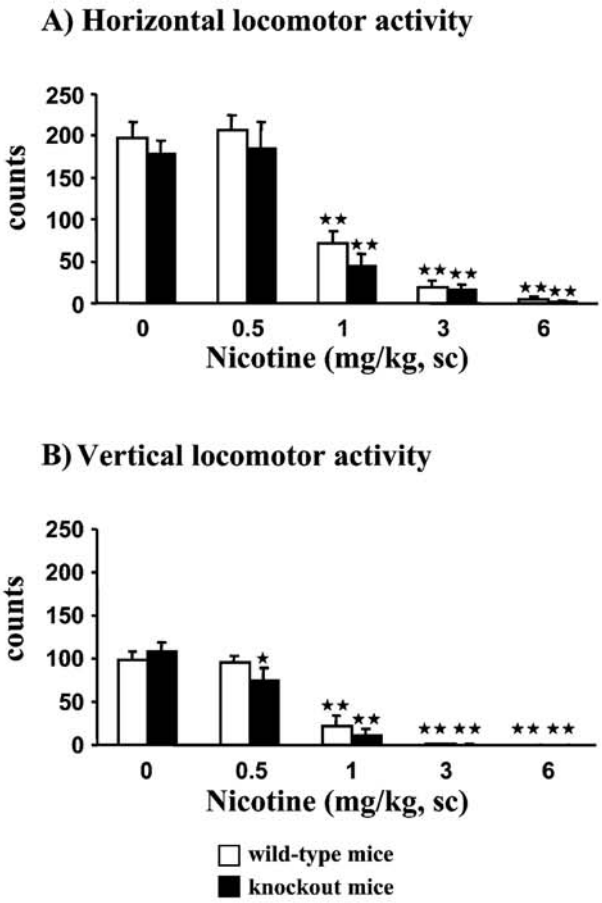

Fig. 2. Nicotine decreased locomotor activity in CB1 knockout mice and wild-type littermates. Horizontal (A) and vertical (B) locomotor activity was measured 5 min after acute nicotine administration (0.5, 1, 3 and 6 mg/kg, sc). Results are expressed as mean ± S.E.M of photocell counts during a 10 min period in wild-type (white bars) and knockout (black bars) mice (n = 10–15 mice for each group). Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post-hoc comparisons using the Dunnett test. ★★$p < 0.01$ when comparing with vehicle group of the same genotype

tical locomotor activity at the dose of 0.5 mg/kg in knockout mice ($p < 0.05$) and 1, 3 and 6 mg/kg ($p < 0.001$) in both genotypes.

3.2. Antinociceptive responses induced by nicotine in wild-type and CB1 knockout mice

We have performed a preliminary experiment in order to verify whether the response to the hot-plate test was influenced by previous exposure to the tail-immersion test. For this purpose, wild-type CD1 mice were divided into six different groups. In the first three groups, mice were tested using both antinociceptive models (tail-immersion and hot-plate) along the test battery scheme. As described above, the tail-immersion and the hot-plate tests were performed 15 and 16 min respectively, after nicotine (1 and 3 mg/kg, sc) or saline injection. In the last three groups, mice were only tested using the hot-plate test after saline or nicotine (1 and 3 mg/kg, sc) administration. Two-way ANOVA calculated for the jumping response in the hot-plate test showed a significant effect of nicotine treatment [$F(2, 54) = 7.942$; $p < 0.01$] without effect of tail-immersion pre-exposure [$F(1, 54) = 1{,}994$; n.s] and without interaction between these two factors [$F(2, 54) = 1.994$; n.s]. These data demonstrate that the behavioural response to the hot-plate test was not influenced by the previous tail-immersion test exposure.

In a preliminary experiment, the antinociceptive effects of nicotine (0.5, 1, 2, 3 and 6 mg/kg) were evaluated in wild-type CD1 mice using the tail-immersion and the hot-plate tests. In the tail-immersion test, one-way ANOVA revealed a global significant effect induced by nicotine administration [$F(5,80) = 4.939$, $p < 0.01$]. Post-hoc analysis indicated a significant effect of nicotine at the doses of 3 and 6 mg/kg ($p < 0.01$) in this test (data not shown). In the hot-plate test, one-way ANOVA revealed a global significant effect of nicotine administration [$F(5,80) = 5.937$, $p < 0.001$] and post-hoc comparisons showed significant nicotine effects at the doses of 2 ($p < 0.01$), 3 ($p < 0.01$) and 6 ($p < 0.05$) mg/kg (data not shown).

In a second experiment, the antinociceptive effects of nicotine (0.5, 1, 3 and 6 mg/kg) were evaluated in CB1 knockout mice and their wild-type littermates (Fig. 3). Nicotine-induced antinociception was enhanced in CB1 knockout mice in the tail-immersion but not in the hot-plate test. Indeed, two-way ANOVA revealed a significant effect of treatment [$F(4,100) = 31.283$ $p < 0.001$], genotype [$F(1,100) = 36.072$, $p < 0.001$], and interaction between treatment and genotype [$F(4,100) = 8.897$, $p < 0.001$]. Subsequent one-way ANOVA (treatment) revealed significant effects in wild-type [$F(4,54) = 8.832$, $p < 0.001$] and knockout [$F(4,54) = 24.066$, $p < 0.001$] mice. Post-hoc comparisons revealed a significant effect of nicotine treatment

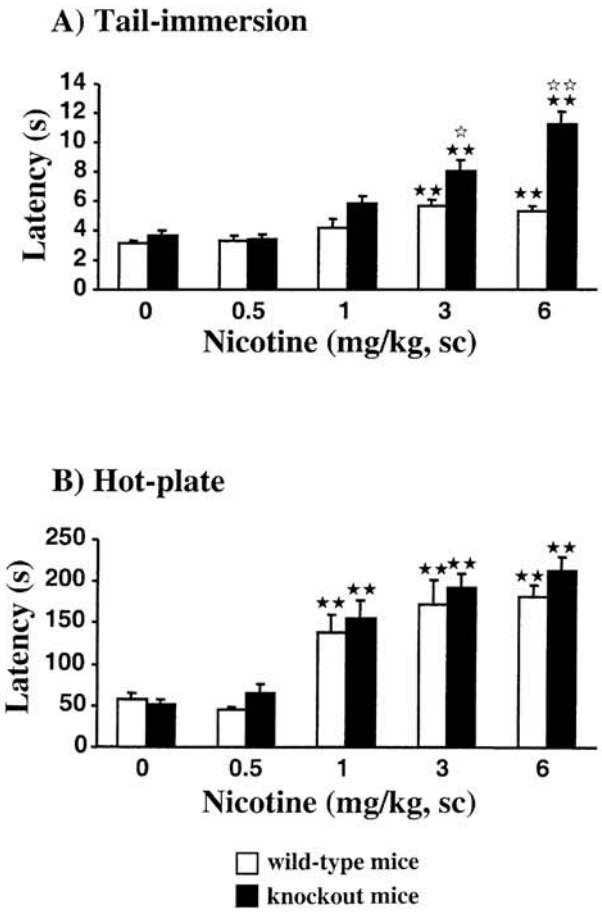

Fig. 3. Antinociceptive effects of nicotine were enhanced in CB1 knockout mice in the tail immersion but not in the hot plate test. Antinociceptive responses in the tail-immersion (A) and hot-plate (B) test were measured 15 and 16 min respectively after nicotine administration (0.5, 1, 3, 6 mg/kg, sc). Results are expressed as mean ± S.E.M of latency time in wild-type (white bars) and knockout (black bars) mice ($n = 10$–15 mice for each group). Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post-hoc comparisons using the Dunnett test. ★$p < 0.05$; ★★$p < 0.01$ when compared to the vehicle group of the same genotype. ☆$p < 0.05$; ☆☆$p < 0.01$ genotype comparisons.

at doses of 3 and 6 mg/kg ($p < 0.01$) when compared to the saline group in both wild-type and knockout mice. Significant differences between genotypes were observed using doses of 3 ($p < 0.05$) and 6 ($p < 0.01$) mg/kg of nicotine (Fig. 3A). In the hot-plate test (jumping response), two-way ANOVA revealed a significant effect of treatment [$F(4,100) = 39.808$, $p < 0.001$], but no significant effect of genotype nor interaction between these two factors. Subsequent one-way ANOVA for wild-type [$F(4,54) = 10.009$, $p < 0.001$] and knockout [$F(4,54) = 26.810$, $p < 0.001$] mice showed a significant effect of nicotine treatment. Post-hoc comparisons revealed similar antinociceptive

responses to nicotine at 1, 3 and 6 mg/kg ($p < 0.01$) in both genotypes (Fig. 3B).

3.3. Nicotine induced rewarding effects in the conditioned place preference in wild-type but not in CB1 knockout mice

In a preliminary study, the rewarding effects of nicotine (0.25 and 0.5 mg/kg) were evaluated in wild-type CD1 mice. Nicotine administered at the dose of 0.5 mg/kg produced rewarding effects in the conditioned place preference. Indeed, one-way ANOVA showed a global significant effect of nicotine treatment [$F(2,43) = 11.874$, $p < 0.001$]. Post-hoc analysis revealed a significant place preference score of nicotine following a dose of 0.5 mg/kg ($p < 0.01$) (data not shown). The dose of 1 mg/kg of nicotine was also evaluated in this paradigm. This dose of nicotine did not produce any conditioned place preference in wild-type mice (one-way ANOVA [$F(1,23) = 0.89$; n.s] (Fig. 4, Table 1).

We then evaluated the effects of nicotine in the conditioned place preference paradigm in CB1 knockout mice and their wild-type littermates. Nicotine (0.5 mg/kg) produced rewarding effects in wild-type animals but failed to produce any motivational response in CB1 knockout mice at all the doses used (0.1, 0.25, 0.5 mg/kg). Two-way ANOVA did not reveal either a significant effect for treatment or for genotype, but the interaction between treatment and genotype was significant [$F(3,114) = 2.994$, $p < 0.05$]. Subsequent one-way anova (treatment) showed a significant effect in wild-type mice [$F(3,56) = 5.650$, $p < 0.01$] but not in knockout groups. Post hoc analysis revealed a significant effect of 0.5 mg/kg nicotine in wild-type mice ($p < 0.01$), in agreement with the previous experiment (Fig. 4, Table 1).

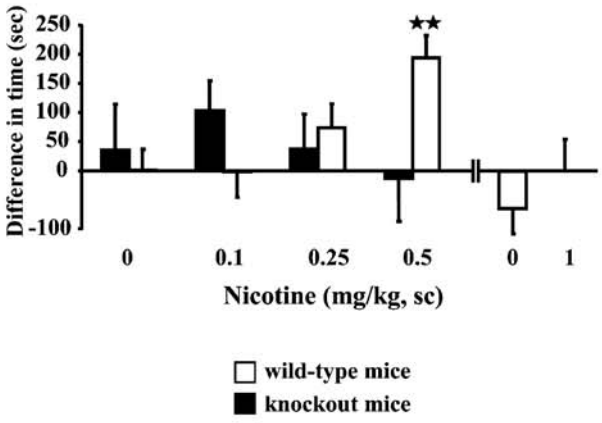

Fig. 4. Nicotine induced place preference in wild-type but not in CB1 knockout mice. The place preference score was calculated as the difference between post-conditioning (testing day) and pre-conditioning time (sec) spent in the compartment associated with the drug. Results are expressed as mean ± S.E.M of values in wild-type (white bars) and knockout (black bars) mice ($n = 15$ mice for each group). Nicotine (0.1, 0.25, 0.5 and 1 mg/kg, sc) was administered immediately before each conditioning session. The dose of 1 mg/kg of nicotine was only evaluated in wild-type mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation followed by corresponding one-way ANOVA and post-hoc comparisons using the Dunnett test. ★★$p < 0.01$ when compared to the vehicle group of the same genotype.

3.4. Mecamylamine precipitated similar nicotine withdrawal in wild-type and CB1 knockout mice

In the first experiment, two different doses of mecamylamine were used (1 and 2 mg/kg, sc) to precipitate abstinence in nicotine-dependent wild-type CD1 mice. No behavioural signs related to nicotine withdrawal were observed in any group of animals before mecamylamine injections. The severity of nicotine withdrawal syndrome was similar when using these two doses of mecamylamine, as revealed by the different individual signs observed after mecamylamine injection and the global withdrawal score. Indeed, two-way ANOVA calculated for the global withdrawal score revealed a significant effect of nicotine treatment [$F(1,52) = 25.726$, $p < 0.001$], but no effect of the mecamylamine dose or interaction between these two factors was observed. Two-way ANOVA calculated for the individual signs of withdrawal revealed a significant effect of nicotine treatment for body tremor [$F(1,49) = 12.861$, $p < 0.01$], ptosis [$F(1,49) = 14.234$, $p < 0.001$], wet dog shakes [$F(1,49) = 8.468$, $p < 0.01$], teeth chattering [$F(1,49) = 17.302$, $p < 0.001$] and piloerection [$F(1,49) = 20.382$, $p < 0.001$] but no effect of mecamylamine dose or interaction between treatment and mecamylamine dose (Table 2).

Taking into account the results obtained in this preliminary experiment, we used the dose of 1 mg/kg of mecamylamine to precipitate nicotine withdrawal syndrome in CB1 knockout mice and wild-type littermates. No behavioural signs related to nicotine withdrawal were observed in any group of mice before the precipitation of the withdrawal syndrome. After mecamylamine injection, chronic nicotine-treated wild-type and CB1 knockout mice displayed a similar expression of the withdrawal syndrome that was characterized by the somatic signs previously described in the preliminary experiment. Two-way ANOVA revealed a significant effect of nicotine treatment in the case of ptosis [$F(1,51) = 20.416$, $p < 0.001$], teeth chattering [$F(1,51) = 11.420$, $p < 0.01$], paw tremor [$F(1,51) = 7.695$, $p < 0.01$], piloerection [$F(1,51) = 16.727$, $p < 0.001$], locomotor activity [$F(1,51) = 4.502$, $p < 0.05$] and body tremor [$F(1,51) = 10.066$, $p < 0.01$], and a significant effect of genotype in the case of body tremor [$F(1,51) = 4.365$, $p < 0.05$]. No significant interaction

Table 1
Time spent in the three compartments of the conditioning apparatus[a]

	Pre-conditioning Drug-paired compartment	Vehicle-paired compartment	Central area	Post-conditioning Drug-paired compartment	Vehicle-paired compartment	Central area
Wild-type mice						
Saline	452.77±27	447.00±25	180.23±22	461.05±21	450.35±15	168.60±11
Nicotine (mg/kg)						
0.10	451.27±26	450.50±27	178.24±17	432.55±30	441.61±37	205.85±18
0.25	409.44±23	476.27±28	194.29±12	489.07±36	389.21±32	201.72±24
0.5	401.69±15	482.10±26	196.20±25	558.24±35	312.76±23	208.99±23
CB1 Knockout mice						
Saline	467.51±29	445.01±32	167.48±13	478.39±66	390.95±61	210.66±40
Nicotine (mg/kg)						
0.10	425.86±43	460.09±42	194.05±31	526.72±53	379.82±45	173.46±29
0.25	402.59±41	509.46±48	167.96±33	407.50±57	433.50±54	238.99±36
0.5	409.77±33	468.95±45	201.29±26	410.25±63	484.06±66	185.69±26
Wild-type mice						
Saline	442.64±17	446.86±17	190.50±20	417.93±42	537.14±42	124.93±15
Nicotine (mg/kg)						
1	415.30 ± 26	410.80 ± 21	253.90 ± 37	432.90 ± 90	430.20 ± 49	216.90 ± 28

[a] Data are expressed as mean ± S.E.M of time spent in the drug-paired compartment, the vehicle-paired compartment and the central area of the conditioning apparatus during the pre-conditioning and the post-conditioning phases.

Table 2
Mecamylamine-precipitated nicotine withdrawal in wild-type mice[a]

	Piloerection	Ptosis	Paw tremor	Wet dog shakes	Body tremor	Teeth chat	Scratches	GWS [b]
Mecamylamine 1 mg/kg								
Control mice	0.70±0.30	2.70±0.63	4.50±2.20	2.80±0.87	0.80±0.33	0.40±0.22	5.20±1.94	15.15±1.16
Nicotine-treated mice	3.47±0.67	4.67±0.42	9.20±2.21	5.26±1.13	3.67±0.65	3.07±0.60	5.67±1.94	33.55±2.87
Mecamylamine 2 mg/kg								
Control mice	0.90±0.43	2.90±0.77	4.50±1.91	1.80±0.42	1.60±0.65	0.50±0.34	2.50±1.28	15.02±2.39
Nicotine-treated mice	3.60±0.62	5.00±0.40	8.06±2.42	4.93±0.84	3.00±0.52	2.27±0.56	11.73±4.04	33.10±4.16

[a] Data are expressed as mean ± S.E.M. Two-way ANOVA revealed a significant effect of nicotine treatment for piloerection, ptosis, wet dog shakes, body tremor, teeth chattering and global withdrawal score (GWS) ($p < 0.01$ in all cases) (see results section). Abstinence was precipitated by acute administration of the nicotine antagonist mecamylamine (1 and 2 mg/kg, sc) after six days of nicotine infusion (10 mg/kg/day) by using subcutaneous minipumps. Counted (wet dog shakes, paw tremor and scratches) and checked (body tremor, ptosis, teeth chattering and piloerection) somatic signs of withdrawal were observed for 30 min immediately after mecamylamine administration.

[b] A global withdrawal score (GWS) was calculated for each animal as described in the methods.

between treatment and genotype was observed in any case. Two-way ANOVA calculated for global withdrawal score showed a significant effect of nicotine treatment [$F(1, 52) = 24.884$, $p < 0.001$] without genotype effect nor interaction between treatment and genotype. Therefore, the severity of nicotine withdrawal was not modified by the deletion of CB1 cannabinoid receptors (Fig. 5, Table 3).

4. Discussion

The present results support the existence of a functional interaction between the endogenous cannabinoid system and nicotine, mediated by the CB1 cannabinoid receptor. Indeed, some behavioural responses induced by nicotine (antinociception in the tail-immersion test and conditioned place preference) were modified in animals lacking CB1 cannabinoid receptors, whereas others (hypolocomotion, antinociception in the hot-plate test and physical dependence) remained unaffected.

Nicotine-induced antinociception was evaluated in the tail-immersion and the hot-plate test, two models with different neural pathways involved in processing the nociceptive signals. Whereas a spinal reflex mainly mediates the response in the tail-immersion test, hot-plate responses require a supraspinal integration of the nociceptive stimuli. No changes in nicotine responses in

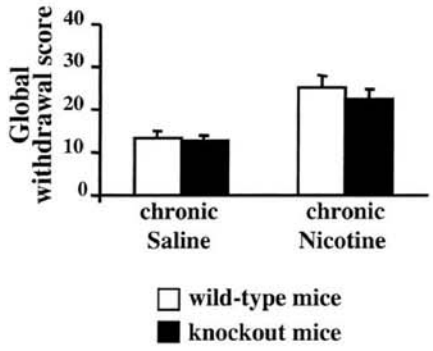

Fig. 5. The severity of nicotine withdrawal was not modified in CB1 knockout mice. Abstinence was precipitated by acute administration of the nicotine antagonist mecamylamine (1 mg/kg, sc) after a 6-day period of nicotine infusion (10 mg/kg/day) by using subcutaneous minipumps. Mice were observed for 30 min immediately after mecamylamine administration and a global withdrawal score was calculated for each animal as described previously in the text. Results are expressed as mean ± S.E.M in chronic saline-treated and chronic nicotine-treated wild-type (white bars) and knockout (black bars) mice ($n = 13$ mice for each group). Two-way ANOVA revealed a significant main effect of nicotine treatment in global withdrawal score ($p < 0.001$).

the hot-plate test were observed in knockout animals, while nicotine-induced antinociception in the tail-immersion test was enhanced in CB1 knockout mice. Cannabinoids produce antinociception through multiple spinal and supraspinal mechanisms (Lichtman and Martin, 1991; Martin et al., 1993, 1995), supporting the existence of distinct cannabinoid antinociceptive pathways, which could represent sites for interaction with other neurotransmitters involved in the control of pain. The spinal cord is an important site where cannabinoids act to produce antinociception (Lichtman and Martin, 1991). Spinal CB1 cannabinoid receptors are thought to be located on intrinsic spinal neurons and at terminals that project from the brain. Immunocytochemical experiments have revealed the presence of CB1 cannabinoid receptors in rat dorsal root ganglia and dorsal roots (Sañudo-Peña et al., 1999). Additional evidence for the presence of CB1 cannabinoid receptors on primary afferent neurons has also been obtained (Ahluwalia et al., 2000).

Nicotinic receptors have also been reported to modulate nociceptive transmission at the level of the primary sensory neuron (Puttfarcken et al., 1997), as well as in the spinal cord (Khan et al., 1994). Therefore, the enhancement of nicotine responses observed in CB1 knockout mice in the tail-immersion test can be explained by an action of nicotine at similar levels as cannabinoids (Mailleux and Vanderhaeghen, 1992; Quik et al., 2000). However, the involvement of other supraspinal neural structures that control the spinal reflex, such as the cerebellum, cortex, basal ganglia and/or brain steam cannot be excluded.

Acute nicotine administration decreased locomotor activity in both wild-type and knockout mice, but no significant differences were observed between genotypes. In the present study, the spontaneous locomotor activity of CB1 knockout mice was similar to that observed in wild-type littermates, in agreement with previous studies using the same line of knockout mice (Ledent et al., 1999; Valverde et al., 2000). The specific behavioural consequences of an interaction between nicotine and cannabinoids have been poorly studied in animal models (Jones et al., 1976; Pryor et al., 1978). Recently, it has been reported that acute nicotine administration enhanced the hypolocomotion produced by Δ^9-tetrahydrocannabinol (Δ^9-THC) (Valjent et al., 2002). However, we found that the absence of CB1 cannabinoid receptors does not seem to significantly affect the nicotine-induced hypolocomotion.

Given that the consumption of cannabis is highly associated with tobacco (which contains the psychoactive

Table 3
Mecamylamine-precipitated nicotine withdrawal in CB1 knockout mice[a]

	Piloerection	Ptosis	Paw tremor	Wet dog shakes	Body tremor	Teeth chat	Scratches	GWS[b]
Wild-type mice								
Control mice	0.08±0.08	1.85±0.50	2.92±0.95	2.00±0.47	1.38±0.42	0.46±0.22	6.00±2.92	13.38±1.52
Nicotine-treated mice	2.69±0.59	3.62±0.51	8.23±1.98	4.08±1.25	2.31±0.57	1.77±0.46	7.92±3.47	25.00±2.96
CB1 Knockout mice								
Control mice	0.31±0.24	1.69±0.63	2.62±0.81	4.50±1.81	1.77±0.47	0.08±0.08	2.31±2.14	12.60±1.42
Nicotine-treated mice	1.23±0.58	4.54±0.35	5.15±1.59	4.62±1.75	4.00±0.52	1.23±0.52	1.38±1.30	22.33±2.28

[a] Data are expressed as mean ± S.E.M. Two-way ANOVA revealed a significant effect of nicotine treatment for piloerection, ptosis, body tremor, teeth chattering, paw tremor and global withdrawal score (GWS) ($p < 0.01$ in all cases), and a significant effect of genotype for body tremor ($p < 0.05$) (see results section). Abstinence was precipitated by acute administration of the nicotine antagonist mecamylamine (1 mg/kg, sc) after six days of nicotine infusion (10 mg/kg/day) by using subcutaneous minipumps. Counted (wet dog shakes, paw tremor and scratches) and checked (body tremor, ptosis, teeth chattering and piloerection) somatic signs of withdrawal were observed for 30 min immediately after mecamylamine administration.

[b] A global withdrawal score (GWS) was calculated for each animal as described in the methods.

compound nicotine) we have investigated, in CB1 knockout mice, several responses induced by repeated nicotine administration and related to addictive behaviour. Nicotine administration has been previously reported to produce motivational responses in several behavioural models including the conditioned place preference paradigm (Piccioto et al., 1998; Pontieri et al., 1996; Risinger and Oakes, 1995; Watkins et al., 1999). Furthermore, chronic nicotine administration has been shown to induce physical dependence revealed by the incidence of several somatic signs after nicotine withdrawal in rodents (Epping-Jordan et al., 1998; Hildebrand et al., 1998; Isola et al., 1999), and irritability and stress in humans (Hughes et al., 1991; West et al., 1989). In our experimental conditions, nicotine induced a significant place preference in wild-type animals when administered at the dose of 0.5 mg/kg. However, 0.5 mg/kg nicotine did not produce any significant positive place preference in CB1 knockout mice. Higher and lower doses of nicotine were also evaluated in wild-type mice, using this paradigm, but also produced no place conditioning (data not shown). These results are in agreement with previous studies in mice showing that higher doses of nicotine (1 and 2 mg/kg) did not produce place preference or even induced place aversion (Risinger and Oakes, 1995). In our experimental conditions, different hypotheses could explain the lack of place preference in CB1 knockout mice. One possible explanation is the existence of an interaction between cannabinoids and nicotine neurotransmitter systems and/or a modulation in the activity of heterologous systems such as the dopaminergic and the opioidergic system. Indeed, nicotine and cannabinoids have been reported to modulate the neural circuitry related to motivation and reward in the limbic system. The CB1 cannabinoid receptor is involved in the motivational properties induced by other drugs of abuse such as opiates (Ledent et al., 1999; Martin et al., 2000; Navarro et al., 2001). In this sense, morphine-induced reward (Ledent et al., 1999; Martin et al., 2000) and increased release of dopamine in limbic areas (Mascia et al., 1999) were suppressed in animals lacking CB1 cannabinoid receptors. Mesolimbic dopaminergic neurons are thought to be a final common neuronal pathway for mediating rewarding processes, and most drugs of abuse have been reported to increase dopamine transmission in the mesolimbic system (Koob and Nestler, 1997). Nicotine and cannabinoids are both able to increase the release of dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988; Gardner et al., 1988; Szabo et al., 1999;), and to produce rewarding responses in different animal models (Fattore et al., 1999; Valjent and Maldonado, 2000). Thus, CB1 cannabinoid receptors could modulate dopamine release induced by nicotine in the limbic areas, however, this interpretation is limited to the particular experimental conditions used in our study. Neurochemical studies are necessary to elucidate a possible role of the CB1 cannabinoid receptor in the motivational responses to nicotine. Another hypothesis to explain this result could be the existence of a shift to the left of the dose–response curve of nicotine in these knockout animals. Indeed, rewarding effects of nicotine are difficult to assess using a place preference paradigm, since effects are generally biphasic, with low doses ineffective, an intermediate doses producing a positive place preference and higher doses either not effective or producing place aversion (Risinger and Oakes, 1995). In order to clarify this possible shift of the curve in CB1 knockout mice, we tested lower doses of nicotine (0.05 and 0.1 mg/kg) in the conditioned place preference paradigm. These lower doses did not produce any rewarding response either in wild-type or in knockout mice, suggesting that the dose–response curve of nicotine was not shifted to the left. Only a mild tendency to produce place preference was observed in CB1 knockout mice at the dose of 0.1 mg/kg of nicotine.

In contrast with the present place preference data, Cossu et al. (2001) have recently reported that the absence of CB1 cannabinoid receptors did not modify self-administration induced by nicotine. The effective doses to find a reliable response in these two models are different and do not allow comparisons to be drawn between the two studies (Bardo and Bevins, 2000 for review). In agreement with our results, the co-administration of THC and nicotine produced a facilitation of conditioned place preference, as well as an enhancement of c-Fos expression in limbic areas (Valjent et al., 2002). Taken together, these data suggest a specific interaction between nicotine and cannabinods in brain areas involved in reward related processes.

Finally, nicotine dependence was induced by using minipumps delivering a constant rate of nicotine solution over a 6-day period. In wild-type mice chronically treated with nicotine, the nicotine antagonist mecamylamine at 1 or 2 mg/kg precipitated a withdrawal syndrome with a similar severity index. Behavioural manifestations of the nicotine abstinence syndrome have been previously reported in rats by using similar experimental conditions (Epping-Jordan et al., 1998; Hildebrand et al., 1997). In the present experiment, using mice, most of the somatic signs of nicotine withdrawal reported in rats have been observed, including ptosis, shakes, teeth chattering, piloerection, escapes, genital licks, and changes in locomotor activity. However, nicotine abstinent rats displayed other behavioural signs such as gasps, abdominal constrictions and yawns that were not observed in mice (Epping-Jordan et al., 1998; Hildebrand et al., 1997). In the present study, nicotine-dependent CB1 knockout mice also exhibited a significant degree of abstinence after injection of mecamylamine and there was no difference in the severity of the withdrawal syndrome compared to wild-type mice. This finding sug-

gests that the endogenous cannabinoid system is not involved in the expression of the somatic component of nicotine withdrawal. Interestingly, CB1 cannabinoid receptors have been reported to participate in the development of physical dependence to opioids. A strong attenuation in the severity of morphine abstinence has been observed in CB1 knockout mice (Ledent et al., 1999).

In conclusion, our results provide evidence supporting the involvement of the endogenous cannabinoid system, through the CB1 cannabinoid receptor, in two pharmacological responses induced by nicotine, acute antinociception in the tail-immersion test and place preference conditioning, and advances the understanding of the interactions that may occur when tobacco and cannabis consumption overlap in humans.

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DISCUSIÓN

El sistema cannabinoide está implicado en el control de una gran variedad de respuestas fisiológicas. Este sistema también está estrechamente relacionado con el desarrollo de los procesos adictivos. Así, los cannabinoides son capaces de inducir efectos reforzantes y dependencia física en diversos modelos experimentales. El sistema cannabinoide interacciona con otros sistemas de neurotransmisión y participa en la manifestación de las propiedades adictivas de otras drogas de abuso como los opiáceos, el etanol y los psicoestimulantes. En este trabajo, hemos pretendido esclarecer ciertos aspectos referentes a las bases neurobiológicas de la adicción de cannabinoides. Por una parte hemos evaluado la capacidad de una agonista sintético más potente que el THC para inducir efectos reforzantes y dependencia física en el ratón. La puesta a punto de estos modelos nos ha permitido estudiar el sustrato neuroanatómico de la dependencia física de cannabinoides. También hemos evaluado la posible implicación de los sistemas opioide y purinérgico en determinadas acciones farmacológicas del THC, en especial aquellas relacionadas con los fenómenos de adicción. Los sistemas cannabinoide, opioide y purinérgico participan en el control de respuestas fisiológicas comunes. Finalmente, teniendo en cuenta que el sistema cannabinoide parece estar implicado en la modulación de las propiedades adictivas de otras drogas de abuso hemos investigado la posible implicación del receptor CB1 en las propiedades adictivas de la nicotina.

Efectos gratificantes del agonista sintético WIN 55,212-2 en el ratón

Las propiedades gratificantes de los cannabinoides han sido difíciles de revelar en el animal de experimentación. Recientemente, se ha descrito el aprendizaje de un comportamiento de auto-administración de THC en monos (Justinova y cols., 2003). Sin embargo, no existen evidencias de auto-administración de THC en otras especies animales. En el caso particular del ratón, únicamente dos estudios han demostrado auto-administración de agonistas sintéticos como WIN 55,212-2 o HU-210 mediante modelos experimentales de auto-administración aguda (Martellotta y cols., 1998; Navarro y cols., 2001). Las respuestas gratificantes de los cannabinoides también han sido difíciles de evidenciar en el paradigma del condicionamiento espacial en roedores. En el año 2000, Valjent y Maldonado demostraron que el THC, a la dosis de 1 mg/kg, era capaz de inducir una respuesta de preferencia de plaza. No obstante, este efecto tenía lugar bajo unas condiciones experimentales particulares (Valjent y Maldonado, 2000). Por una parte, era necesario alargar los tiempos de condicionamiento hasta 45 minutos y por otra,

evitar las posibles consecuencias disfóricas de la primera administración de THC. Para ello, los ratones recibieron una inyección previa de THC "priming" en sus jaulas de estabulación 24 horas antes de ser expuestos al primer condicionamiento. Hasta la fecha, ningún otro estudio ha evaluado la capacidad de los agonistas cannabinoides sintéticos para inducir efectos gratificantes en el ratón mediante el paradigma del condicionamiento espacial. En este trabajo, hemos descrito la aparición de una preferencia de plaza condicionada a la administración de WIN 55,212-2 en el ratón (artículo 1). De acuerdo con el estudio de Valjent y Maldonado, este efecto aparece únicamente cuando los animales reciben un "priming" de WIN 55,212-2, 24 horas antes del primer condicionamiento, lo que demuestra que también en el caso de utilizar agonistas cannabinoides sintéticos es necesario contrarrestar los efectos disfóricos asociados a la primera administración. Recientemente, mediante la aplicación de la estrategia experimental del "priming", nuestro grupo de investigación ha sido capaz de poner a punto un modelo de auto-administración intravenosa de WIN 55,212-2 en el ratón (datos no publicados).

Dependencia física de WIN 55,212-2 en el ratón. Sustrato neuroanatómico de la abstinencia cannabinoide

Además de establecer un modelo de preferencia de plaza condicionada a la administración de WIN 55,212-2 también hemos desarrollado un modelo de dependencia física y abstinencia de este compuesto en el ratón. En nuestras condiciones experimentales, el antagonista cannabinoide rimonabant precipitó la aparición de un síndrome de abstinencia en animales dependientes de WIN 55,212-2 (1 y 2 mg/kg) (artículo 1). Los principales signos de abstinencia observados fueron similares a los descritos durante el síndrome de abstinencia de otros agonistas cannabinoides (Cook y cols., 1998; Hutcheson y cols., 1998; Ledent y cols., 1999; Lichtman y cols., 2001) e incluyen ataxia, masticación, postura encorvada, temblor de patas, piloerección, temblor corporal y "wet dog shakes". En un paso posterior, hemos estudiado el sustrato neuroanatómico de la abstinencia de WIN 55,212-2. Para ello, hemos evaluado la participación de diferentes estructuras cerebrales como el cerebelo, el hipocampo, el estriado y la amígdala en la expresión del síndrome de abstinencia de dicho compuesto. Estas estructuras se caracterizan por presentar una elevada densidad de receptores CB1 (Herkenham y cols., 1991) y por estar estrechamente relacionadas con los efectos comportamentales de los cannabinoides. La microinyección de rimonabant en el cerebelo, el hipocampo y la amígdala, pero no en el estriado precipitó signos de

abstinencia en ratones tratados crónicamente con WIN 55,212-2 (artículo 1). Además, tras la microinyección de rimonabant en el cerebelo pudimos observar el síndrome de abstinencia de mayor severidad. En efecto, trabajos previos de nuestro grupo de investigación habían atribuido al cerebelo un papel principal en el fenómeno de dependencia física de cannabinoides. Hutcheson y colaboradores (1998) observaron un aumento en la actividad adenilato ciclasa específicamente en el cerebelo durante la abstinencia precipitada por rimonabant en ratones dependientes de THC. En esta misma línea, Tzavara y colaboradores observaron, dos años más tarde, que el perfil temporal de dicho aumento se correlacionaba con el curso de las manifestaciones somáticas de la abstinencia cannabinoide (Tzavara y cols., 2000). Además, la administración local en el cerebelo del inhibidor de la proteína quinasa A (PKA), el Rp-8Br-cAMPS, disminuyó la expresión de la abstinencia cannabinoide (Tzavara y cols., 2000). En estos dos trabajos, la administración de rimonabant se realizó por vía sistémica, de manera que no se podía descartar que las modificaciones observadas en la actividad adenilato ciclasa fueran debidas a modificaciones inducidas por el antagonista en otras regiones del cerebro con altos niveles de receptores CB1 y que proyectan al cerebelo. Posteriormente, en un estudio *in vitro*, Ghozland y colaboradores (2002a) analizaron la actividad de las neuronas del cerebelo en cortes de cerebro obtenidos a partir de ratones dependientes de THC. Se observó que el tratamiento crónico con THC provocaba una disminución de la actividad espontánea de las neuronas granulares del cerebelo y que la administración directa de rimonabant en el líquido de perfusión que baña los cortes cerebrales de los ratones THC-dependientes inducía una estimulación de la actividad de dichas neuronas. Sobre la base de estas observaciones, resultaba interesante estudiar las consecuencias de la administración *in vivo* del rimonabant en el cerebelo de animales dependientes de cannabinoides. Como hemos descrito, la microinyección de rimonabant en el cerebelo precipitó la aparición de signos de abstinencia en ratones tratados crónicamente con WIN 55,212-2, poniendo en evidencia que el cerebelo está directamente implicado en los fenómenos de dependencia de cannabinoides. Además, resulta interesante resaltar que nuestro estudio demuestra que el sustrato neuroanatómico de la abstinencia cannabinoide no se restringe a una única área cerebral. Hemos demostrado que el hipocampo y también la amígdala parecen participar en la manifestación física de la abstinencia cannabinoide. No obstante, quedaría por esclarecer cuáles son los mecanismos subyacentes a dicha participación. El hipocampo es una estructura cerebral principalmente relacionada con los procesos de aprendizaje y memoria. Existen evidencias de que los mecanismos moleculares y celulares relacionados con la formación de la memoria

también participan en el desarrollo de la adicción (Fan y cols., 1999; Lu y cols., 2000; Jones y Bonci, 2005). En este sentido, se ha propuesto que los fenómenos de plasticidad sináptica inducidos por el consumo de una droga en áreas que forman parte o se relacionan funcionalmente con los circuitos del refuerzo como es el caso del hipocampo, podrían contribuir al aprendizaje de ciertos comportamientos adictivos (Jones y Bonci, 2005). A diferencia de lo que ocurre en el cerebelo, no existen cambios adaptativos en la actividad adenilato ciclasa en el hipocampo durante la abstinencia de THC (Hutcheson y cols., 1998; Tzavara y cols., 2000), lo que sugiere la participación de otras vías de señalización intracelular en dicho fenómeno. Como se ha expuesto en la parte introductoria, la actividad del receptor cannabinoide CB1 es capaz de afectar otras rutas de señalización intracelular entre las que se incluyen la ruta de las MAP quinasas y la actividad de diferentes canales iónicos (Piomelli, 2003). Un estudio reciente llevado a cabo por Derkinderen y colaboradores (2003) ha demostrado que la administración de cannabinoides induce cambios adaptativos en la ruta de la ERK MAP quinasa en el hipocampo. Estos cambios adaptativos podrían ser, en parte, responsables de la participación del hipocampo en la expresión de la abstinencia de cannabinoides.

Finalmente, hemos observado que la administración local de rimonabant en la amígdala de ratones dependientes de WIN 55,212-2 es capaz de precipitar un síndrome de abstinencia de muy baja severidad. La limitada participación de la amígdala en la manifestación física de la abstinencia cannabinoide contrasta con el papel que parece ejercer sobre el componente motivacional de la abstinencia. En efecto, la amígdala es una estructura que forma parte del sistema límbico y que se ha relacionado principalmente con el estado emocional de disforia que acompaña la abstinencia de diferentes drogas de abuso incluido los cannabinoides (Rodríguez de Fonseca y cols., 1997; Koob, 2003).

Participación de los sistemas opioide y purinérgico en los efectos motivacionales y de dependencia física de los cannabinoides

Uno de los objetivos de nuestro trabajo ha sido investigar la posible participación de otros sistemas de neurotransmisión, como el sistema opioide y el sistema purinérgico, en los efectos que inducen los cannabinoides, en particular en las respuestas motivacionales y la dependencia física de estos compuestos. Para ello hemos utilizado ratones modificados genéticamente deficientes de los receptores opioides MOR/DOR (cedidos por Brigitte L. Kieffer. Illkirch, Francia) y de los receptores de adenosina A_{2A} (cedidos por Catherine Ledent. Bruselas, Bélgica).

En este trabajo hemos demostrado que la supresión conjunta de ambos receptores opioides MOR y DOR modifica las propiedades gratificantes de los cannabinoides, así como también la expresión del síndrome de abstinencia de dichos compuestos. Concretamente, en los ratones doble mutantes MOR/DOR los efectos gratificantes del THC están abolidos y observamos un síndrome de abstinencia de THC de menor severidad (artículo 2). En un estudio previo en el que se utilizaron ratones genéticamente modificados deficientes de los diferentes receptores opioides (MOR, DOR y KOR), Ghozland y colaboradores (2002b) demostraron un papel crucial del MOR en las propiedades gratificantes de los cannabinoides y del KOR en sus propiedades aversivas. Este mismo estudio, propone que el DOR no parece estar involucrado en los aspectos motivacionales de los cannabinoides. De acuerdo con estos resultados, en los ratones doble mutantes MOR/DOR observamos una abolición de los efectos gratificantes del THC en el paradigma del condicionamiento espacial, lo que corrobora el papel importante del MOR en la mediación de los efectos gratificantes de los cannabinoides. El estudio de Ghozland y colaboradores (2002b) describe también que la supresión individual de los diferentes receptores opioides no altera la expresión del síndrome de abstinencia de THC. Este resultado era totalmente inesperado teniendo en cuenta que se había demostrado que la naloxona, un antagonista opioide preferencial MOR pero no selectivo, era capaz de precipitar la aparición de signos de abstinencia en ratas que habían recibido un tratamiento crónico con agonistas cannabinoides como HU-210 y THC (Kaymakcalan y cols., 1977; Navarro y cols., 1998). Además, un estudio previo de nuestro laboratorio demostró que los ratones deficientes del gen de la prePENC (Valverde y cols., 2000b) presentan una reducción importante de la severidad del síndrome de abstinencia de THC. Estos resultados parecían sugerir una implicación de los péptidos opioides derivados de la prePENC en la dependencia física de cannabinoides. Estos péptidos tienen una afinidad preferencial por los receptores DOR aunque también son capaces de activar de manera importante los receptores MOR. Para verificar esta hipótesis, en este trabajo evaluamos el síndrome de abstinencia cannabinoide en los ratones doble mutantes MOR/DOR. Estos ratones presentaron una reducción significativa de la expresión del síndrome de abstinencia de THC (artículo 2), de manera que podemos concluir que la transmisión encefalinérgica participa en los fenómenos de dependencia física de cannabinoides mediante la acción simultanea de ambos receptores MOR y DOR. No obstante, quedaría por esclarecer que tipo que cooperación se establece entre estos dos tipos de receptores. En efecto, podría tratarse de una interacción de proximidad entre MOR y DOR debida a la localización de ambos receptores en neuronas comunes o de una interacción más lejana debido a la localización de estos

receptores en neuronas diferentes que forman parte de una mismo circuito neuronal. En relación con la primera hipótesis, diversos estudios han demostrado la co-localización de MOR y DOR en neuronas ubicadas en diversas áreas del SNC (Arvidsson y cols., 1995a; 1995b; Wang y Pickel, 2001). De esta manera, estos dos receptores podrían interaccionar físicamente para formar complejos funcionales y/o participar en la modulación de mecanismos de señalización intracelular comunes. Efectivamente, diversos estudios han demostrado que los receptores MOR y DOR son capaces de formar heterodímeros funcionales tanto en células transfectadas como en células que expresan dichos receptores (Gomes y cols., 2004; Law y cols., 2005). También se ha demostrado que la estimulación de MOR y DOR modifica vías de señalización intracelular comunes. Puesto que hemos demostrado que el cerebelo juega un papel crucial en la abstinencia cannabinoide sería interesante evaluar si existe algún tipo de interacción entre los receptores MOR y DOR en neuronas dicha estructura. Recientemente, se ha demostrado que ambos tipos de receptores opioides se expresan en la capa granular y en células de Purkinje del cerebelo de rata (Mrkusich y cols., 2004).

La supresión conjunta de los receptores MOR y DOR también modificó el efecto hipotérmico agudo del THC (artículo 2). Son pocos los trabajos que han evaluado la posible implicación de los receptores opioides en los efectos hipotérmicos de los cannabinoides. De acuerdo con nuestros resultados, un estudio del año 1978 demostró que la naloxona era capaz de antagonizar parcialmente la respuesta hipotérmica inducida tras la administración aguda de THC en ratones (Bloom y Dewey, 1978). Teniendo en cuenta que la supresión individual de los receptores MOR o DOR no produce ninguna consecuencia sobre los efectos hipotérmicos agudos del THC (Ghozland y cols., 2002b), también podríamos postular la existencia de una acción cooperativa entre MOR y MOR en la modulación de esta respuesta cannabinoide aguda.

Otro sistema de neurotransmisión que parece estar modulando las propiedades adictivas de los cannabinoides es el sistema purinérgico. En el presente trabajo, la utilización de ratones deficientes de los receptores de adenosina A_{2A} nos ha permitido observar que dichos receptores modulan los aspectos motivacionales y la dependencia física de cannabinoides (artículo 3). Así, en ausencia del receptor A_{2A} tanto las propiedades gratificantes como las propiedades aversivas de los cannabinoides estaban inhibidas. Estos efectos no parecen ser debidos a un déficit de aprendizaje en los ratones mutantes, ya que estos ratones son capaces de adquirir y mantener un comportamiento operante de auto-administración de comida (artículo 3). Además, los ratones sin el receptor A_{2A} presentaron un síndrome de abstinencia de THC de menor severidad (artículo 3).

Hasta la fecha, pocos estudios han evaluado la posible implicación de los receptores A_{2A} en los procesos relacionados con la adicción. Sin embargo, existen datos que nos pueden ayudar a formular algunas hipótesis. En el estriado, los receptores de adenosina A_{2A} están co-expresados con los receptores de dopamina D2 en las neuronas GABAérgicas estriatopalidades y regulan la expresión del gen de la PENC (Ledent y cols., 1997). Mediante estudios de hibridación *in situ*, Ledent y colaboradores (1997) demostraron que el producto de trascripción del gen de la PENC está disminuido en los ratones sin el receptor $A_{2A.}$ Teniendo en cuenta la demostrada participación del sistema opioide en las propiedades gratificantes de los cannabinoides (Ghozland y cols., 2002b; artículo 2) y la regulación de la expresión del gen de la PENC por los receptores de adenosina A_{2A} (Ledent y cols., 1997), no podemos excluir que participación de los receptores de adenosina A_{2A} en los efectos gratificantes del THC sea a través de una modulación del sistema opioide endógeno.

Por otro lado, se conoce que el sistema dopaminérgico mesolímbico juega un papel crucial en la regulación de los efectos gratificantes de las drogas de abuso (Koob, 1996; Di Chiara, 2002; Di Chiara y cols., 2004). Numerosos trabajos han demostrado la existencia de una interacción funcional y antagónica entre el sistema dopaminérgico y purinérgico de adenosina. Así, la estimulación de los receptores de adenosina A_{2A} disminuye la afinidad de los receptores de DA D2 y la estimulación de los receptores de adenosina A_1 disminuye la afinidad de los receptores de DA D1 (Ferré y cols., 1997; Ferré y cols., 1998; Franco y cols., 2000). Sobre la base de esta interacción antagónica entre los sistemas dopaminérgico y purinérgico cabría esperar un aumento de las propiedades reforzantes de los cannabinoides en ausencia del receptor A_{2A}, sin embargo nos encontramos con un resultado claramente opuesto. Diversos estudios han demostrado que la invalidación del receptor A_{2A} es capaz de alterar ciertos aspectos de la transmisión dopaminérgica. Así, los ratones sin el receptor A_{2A} presentan un estado funcional hipodopaminérgico en el estriado y córtex cerebral (Dassesse y cols., 2001). También se ha demostrado que estos ratones mutantes presentan un déficit de la proteína DARPP-32, un elemento intracelular clave para la transmisión dopaminérgica (Svenningsson y cols., 2000). Recientemente, se ha demostrado que la proteína DARPP-32 participa en la mediación de los efectos farmacológicos de una gran variedad de drogas de abuso (Nairn y cols., 2004). Además, los receptores A_{2A} podrían regular directamente la fosforilación de DARPP-32 (Svenningsson y cols., 2004). Teniendo en cuenta estos hallazgos hemos querido estudiar la posible implicación de los receptores A_{2A} en los efectos motivacionales de otras drogas de abuso. Así, un estudio reciente de nuestro laboratorio ha

demostrado que los receptores A_{2A} modulan las respuestas motivacionales de la cocaína mediante el paradigma de auto-administración (Soria y cols., en revisión). Actualmente, estamos evaluando la posible implicación de los receptores de adenosina A_{2A} en las propiedades motivacionales de los opiáceos. Mediante el paradigma del condicionamiento espacial hemos observado que los efectos gratificantes de la morfina están inhibidos en los ratones sin el receptor A_{2A} (Castañé y cols., en preparación). De forma conjunta estos resultados podrían sugerir la existencia de un mecanismo purinérgico común, a través de la activación de los receptores de adenosina A_{2A}, para los efectos motivacionales de las drogas de abuso. De manera interesante, Yao y colaboradores (2003) han demostrado que los receptores A_2 son indispensables para que exista una señalización sinérgica entre los receptores de DA D2 y los receptores CB1 o DOR. Según estos autores la adenosina a través de la activación del receptor A_2 sería capaz de potenciar la señalización mediada por el receptor de DA D2 cuando los receptores CB1 o DOR, D2 y A2 se expresan en una misma célula.

Los receptores de adenosina A_{2A} no parecen estar involucrados en los fenómenos de tolerancia a los efectos farmacológicos del THC pero sí participan en la expresión del síndrome de abstinencia de cannabinoides. Así, en ausencia del receptor A_{2A} los ratones presentan un síndrome de abstinencia de THC de menor severidad. Curiosamente, se ha descrito que los receptores A_{2A} también modulan la expresión del síndrome de abstinencia de opiáceos, aunque en sentido opuesto. Así, en los ratones deficientes de los receptores de adenosina A_{2A} el síndrome de abstinencia de la morfina está aumentado (Berrendero y cols., 2003: artículo 6 anexo; Bailey y cols., 2004). Estos dos resultados sugieren que no existe un mecanismo purinérgico adaptativo común para los fenómenos de dependencia física *per se*, sino que la adenosina modula estos fenómenos mediante mecanismos específicos para cada tipo de droga.

En este trabajo hemos observado que los receptores A_{2A} podrían estar involucrados en el control que hacen los cannabinoides del comportamiento alimentario. Durante el estudio de tolerancia observamos que en condiciones normales el peso de los ratones seguía un patrón cíclico. Así, los ratones ganaban peso durante la noche (fase de oscuridad/actividad) y perdían peso durante el día (fase de luz/reposo). La administración de THC fue capaz de interferir estos cambios oscilantes de peso en los animales normales, sin embargo no tuvo ningún efecto en los animales sin el receptor A_{2A} los cuales seguían presentando cambios cíclicos en el peso corporal. Futuros estudios deben ser realizados para esclarecer el papel de los receptores A_{2A} en dicho fenómeno.

Para concluir esta parte de nuestro estudio, hemos demostrado que las respuestas comportamentales que induce el THC en los ratones mutantes sin el receptor A_{2A} no son debidas a un cambio en la densidad y funcionalidad del receptor CB1 como consecuencia de la invalidación del receptor A_{2A} (artículo 3). Mediante el estudio de fijación con el radioligando [H^3]CP-55,940 hemos observado una distribución similar de los receptores CB1 en el cerebelo, el hipocampo, la corteza y los ganglios basales de ratones normales y de ratones que carecen del receptor A_{2A}. Además, el agonista WIN 55,212-2 produce una estimulación similar de la fijación de [S^{35}]GTPγS en dichas áreas cerebrales en los ratones de ambos genotipos.

Participación del receptor cannabinoide CB1 en los efectos motivacionales y dependencia física de nicotina

Estudios previos han demostrado que el sistema cannabinoide participa en las propiedades adictivas de otras drogas de abuso como los opiáceos (Ledent y cols., 1999; Martin y cols., 2000), la cocaína (De Vries y cols., 2001; Soria y cols., 2005), el etanol (Houchi y cols., 2005) y la MDMA (Braida y cols., 2005). En este trabajo hemos estudiado la posible participación del sistema cannabinoide en las propiedades adictivas de la nicotina. El estudio de esta posible interacción presenta un especial interés puesto que el cannabis y el tabaco se asocian para el consumo en humanos (Aung y cols., 2004).

Mediante la utilización de ratones deficientes de los receptores CB1 hemos demostrado un papel crucial de dichos receptores en las propiedades gratificantes de la nicotina. Sin embargo, los receptores CB1 no parecen estar implicados en el desarrollo y expresión de la dependencia física de dicho compuesto (artículo 4).

Un estudio farmacológico previo de Valjent y colaboradores (2002) había mostrado la existencia de una interacción farmacológica entre el THC y la nicotina. Estos autores evaluaron las consecuencias de la administración de nicotina en ciertas respuestas comportamentales y bioquímicas inducidas por THC. Así, la nicotina facilita la hipotermia, la antinocicepción y la hipolocomoción inducida tras la administración aguda de dosis efectivas de THC (5 y 10 mg/kg). En dicho trabajo observamos también que los animales co-tratados crónicamente con nicotina y THC presentan un síndrome de abstinencia precipitado por rimonabant de mayor severidad. Además, la co-administración de dosis sub-efectivas de THC y nicotina produce efectos gratificantes en el paradigma del condicionamiento espacial, lo que claramente indica la existencia de una interacción entre el sistema cannabinoide y la nicotina. En el presente trabajo hemos pretendido esclarecer el papel del

receptor cannabinoide CB1 en los efectos farmacológicos de la nicotina mediante la utilización de ratones que carecen del receptor cannabinoide CB1. Uno de los principales hallazgos de nuestro trabajo ha sido que la nicotina no produce efectos gratificantes en el paradigma del condicionamiento espacial en los ratones mutantes (artículo 4), lo que indica que el receptor CB1 es esencial para la manifestación dichos efectos. En contraste con nuestros resultados, Cossu y colaboradores (2001) publicaron que los ratones deficientes del receptor CB1 son capaces de auto-administrarse nicotina mediante un paradigma de auto-administración aguda. No obstante, este estudio no demuestra si estos ratones mutantes son capaces de adquirir y mantener una respuesta estable de auto-administración de nicotina. Posteriores estudios farmacológicos han apoyado nuestros resultados. Así, se ha descrito que el bloqueo farmacológico del receptor CB1 por rimonabant inhibe la expresión de la respuesta de condicionamiento espacial de nicotina (Le Foll y Goldberg, 2004), además de disminuir la auto-administración de nicotina en la rata (Cohen y cols., 2002). En esta misma línea, estudios neuroquímicos de microdiálisis *in vivo* en el roedor han demostrado que el rimonabant bloquea la liberación de DA en el NAc inducida por nicotina en ratas (Cohen y cols., 2002), lo que podría explicar la disminución de los efectos gratificantes de la nicotina por el antagonista cannabinoide. La liberación de DA en áreas límbicas ha sido relacionada, en estudios clásicos, con las respuestas condicionadas asociadas al consumo de las drogas de abuso (Everitt y cols., 2001; Shaham y cols., 2003). En este sentido, Cohen y colaboradores han demostrado recientemente, que el bloqueo farmacológico del receptor CB1 con rimonabant es capaz de inhibir el re-establecimiento de un comportamiento de auto-administración de nicotina precipitado por la presentación de estímulos previamente asociados con la obtención de la droga en ratas (Cohen y cols., 2005). Esto sugiere la participación de los receptores cannabinoides CB1 en los fenómenos de recaída al consumo de nicotina.

En humanos, datos preliminares procedentes del estudio clínico en fase III STRATUS-US (“smoking cessation in smokers motivated to quit”) han demostrado que el tratamiento crónico con rimonabant (Acomplia®) aumenta la probabilidad de dejar de fumar así como también reduce el aumento de peso asociado al abandono de la adicción al tabaco (Anthenelli y Despres, 2004). En resumen, todos estos datos preclínicos y clínicos, sugieren que los antagonistas de los receptores CB1 podrían ser de gran utilidad para el tratamiento de la adicción a nicotina.

Los ratones sin el receptor cannabinoide CB1 presentaron un síndrome de abstinencia de nicotina similar a los ratones normales (artículo 4). De acuerdo estos resultados estudios farmacológicos posteriores han mostrado que el bloqueo

farmacológico del receptor CB1 mediante rimonabant no es capaz de precipitar la aparición de un síndrome de abstinencia en animales tratados crónicamente con nicotina (Balerio y cols., 2004), lo que sugiere que los receptores CB1 no están directamente implicados en la manifestación somática de la abstinencia nicotínica. Estos mismos autores han observado que no existen cambios adaptativos que afecten la densidad y funcionalidad de los receptores CB1 después de un tratamiento crónico con nicotina en ratones (Balerio y cols., 2004). Resultados similares han sido descritos en estudios de densidad de receptores en ratas (González y cols., 2002a).

Aunque la invalidación del receptor CB1 no parecen alterar el desarrollo y expresión de la dependencia de nicotina, la activación farmacológica de los receptores CB1 puede modificar la severidad de dicha abstinencia. Así, Balerio y colaboradores (2004) observaron que la administración aguda de THC atenúa la severidad de la abstinencia de nicotina en ratones (Balerio y cols., 2004), así como también los aspectos motivacionales aversivos asociados a dicha abstinencia. En conjunto estos trabajos sugieren que el fenómeno de dependencia de nicotina está asociado con una baja actividad tónica del sistema cannabinoide, sin embargo la activación de los receptores cannabinoides mediante agonistas exógenos modifica la expresión de dicho fenómeno. Futuros estudios son necesarios para clarificar el sustrato neurobiológico de la dependencia de nicotina y el posible papel del sistema cannabinoide en dicho fenómeno.

CONCLUSIONES

El trabajo desarrollado en la presente Tesis Doctoral permite extraer las siguientes conclusiones:

1. El agonista sintético WIN 55,212-2 induce efectos reforzantes y un estado de dependencia física en el ratón tras su administración repetida. Por ello, la administración repetida del agonista WIN 55,212-2 representa un modelo adecuado de dependencia de cannabinoides.

2. El estudio neuroanatómico de microinyección cerebral de rimonabant en ratones dependientes de WIN 55,212-2 confirma la participación principal del cerebelo en la expresión de la dependencia física de cannabinoides. Por primera vez queda demostrada la función del hipocampo en dicho fenómeno y revela un papel menor de la amígdala, probablemente más involucrada en el componente motivacional de la dependencia de cannabinoides.

3. El sistema opioide participa en la modulación de diversas respuestas farmacológicas de los cannabinoides. Además, se requiere una acción cooperativa entre los receptores opioides MOR y DOR para la completa expresión de la abstinencia cannabinoide.

4. El sistema purinérgico, a través de los receptores A_{2A}, también regula las propiedades adictivas de los cannabinoides. La presencia de dicho receptor es necesaria para la manifestación de los efectos motivacionales de los cannabinoides y su ausencia disminuye la severidad de la abstinencia cannabinoide. El sistema purinérgico se perfila como un sistema capaz de afectar la capacidad de diferentes drogas de abuso para producir dependencia.

5. Los receptores cannabinoides CB1 son imprescindibles para que se manifiesten las propiedades gratificantes de la nicotina. Estos receptores modulan las propiedades adictivas de otras drogas de abuso, lo que ha sugerido la utilización de antagonistas de los receptores CB1 para el tratamiento de los fenómenos de dependencia de diversas drogas de abuso, en particular de la nicotina.

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ANEXO

ARTÍCULOS

Artículo 5:
Macarrone M, Valverde O, Barbaccia ML, Castañé A, Maldonado R, Ledent C, Parmentier M, Finazzi-Agro A. "Age-related changes of anandamide metabolism in CB1 cannabinoid receptor knockout mice: correlation with behaviour". *European Journal of Neuroscience.* 2002 Apr;15(7):1178–86. Blackwell Publishing

Artículo 6:
Berrendero F, Castañé A, Ledent C, Parmentier M, Maldonado R, Valverde O. "Increase of morphine withdrawal in mice lacking A_{2A} receptors and no changes in CB1/A_{2A} double knockout mice". *European Journal of Neuroscience.* 2003, Jan;17(2):315–24. Blackwell Publishing

Artículo 7:
Celerier E, Yazdi MT, Castañé A, Ghozland S, Nyberg F, Maldonado R. "Effects of nandrolone on acute morphine responses, tolerance and dependence in mice". *European Journal of Pharmacology. 2003 Mar* 28:465(1-2):69-81.

Artículo 8:
Castañé A, Berrendero F, Maldonado R. "The role of cannabinoid system in nicotine addiction". *Pharmacology; Biochemistry and Behavior.* 2005 Jun;81(2):381-6.

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Age-related changes of anandamide metabolism in CB_1 cannabinoid receptor knockout mice: correlation with behaviour

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Abstract

Anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are the most active endocannabinoids at brain (CB_1) cannabinoid receptors. CD1 mice lacking the CB_1 receptors ('knockout' [KO] mutants) were compared with wildtype (WT) littermates for their ability to degrade AEA through an AEA membrane transporter (AMT) and an AEA hydrolase (fatty acid amide hydrolase, FAAH). The age dependence of AMT and FAAH activity were investigated in 1- or 4-month-old WT and KO animals, and found to increase with age in KO, but not WT, mice and to be higher in the hippocampus than in the cortex of all animals. AEA and 2-AG were detected in nmol/mg protein (μM) concentrations in both regions, though the hippocampus showed approximately twice the amount found in the cortex. In the same regions, 2-AG failed to change across groups, while AEA was significantly decreased (≈ 30%) in hippocampus, but not in cortex, of old KO mice, when compared with young KO or age-matched WT animals. In the open-field test under bright light and in the lit-dark exploration model of anxiety, young KO mice, compared with old KO, exhibited *a* mild anxiety-related behaviour. In contrast, neither the increase in memory performance assessed by the object recognition test, nor the reduction of morphine withdrawal symptoms, showed age dependence in CB_1 KO mice. These results suggest that invalidation of the CB_1 receptor gene is associated with age-dependent adaptive changes of endocannabinoid metabolism which appear to correlate with the waning of the anxiety-like behaviour exhibited by young CB_1 KO mice.

Introduction

Endocannabinoids, an emerging class of lipid mediators isolated from brain and peripheral tissues (Devane *et al*., 1992; Pop, 1999), include amides and esters of long chain polyunsaturated fatty acids. Anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG), produced in the brain through the action of phospholipases D and C, respectively (Piomelli *et al*., 2000), are the main endogenous agonists of cannabinoid receptors described to date (for reviews, see Pop, 1999; Salzet *et al*., 2000). They bind to both peripheral (CB_2) and brain (CB_1) cannabinoid receptors, thus mimicking some of the peripheral and central effects of Δ^9-tetrahydrocannabinol, the (psycho)active principle of hashish and marijuana. CB_1 receptor-mediated actions have been suggested to play a role in the hypothermia, analgesia, reduction of motor behaviour and catalepsis induced by cannabinoids (Chaperon & Thiebot, 1999). Moreover, cannabinoids impair working memory (Lichtman *et al*., 1995) and, consistent with this effect, enhance mesoprefrontal dopaminergic activity (Diana *et al*., 1998) and inhibit hippocampal GABA-mediated network oscillations (Hajos *et al*., 2000) in a CB_1-receptor-dependent manner. This evidence alludes to a physiological role for the endocannabinoid system in the modulation of cognition and motor activity. Accordingly, an increased performance in an object recognition test for memory and an enhanced hippocampal long-term potentiation have been reported in CB_1 receptor knockout mice (Reibaud *et al*., 1999; Bohme *et al*., 2000). Endocannabinoids, however, appear to interfere with neuronal networks by inhibiting gap junction communication (Venance *et al*., 1995) or by interacting with GABAergic (Tsou *et al*., 1998; Katona *et al*., 1999), serotonergic (Cheer *et al*., 1999), glutamatergic (Hampson *et al*., 1998) and dopaminergic (Beltramo *et al*., 2000) neurotransmission in CB_1 receptor-dependent and -independent manners. The biological activity of AEA depends on its life-span in the extracellular space, which is regulated by: (i) cellular uptake by the AEA membrane transporter (AMT) (Hillard *et al*., 1997; Maccarrone *et al*., 2000a), and (ii) intracellular degradation by the enzyme anandamide hydrolase (fatty acid amide hydrolase, FAAH) (Deutsch & Chin, 1993; Ueda *et al*., 1995; Maccarrone *et al*., 2000b). Actually, a coupling between CB_1 receptor activation and increased AMT activity, and thereby AEA

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degradation, has been recently demonstrated *in vitro* (Maccarrone *et al.*, 2000a). Therefore, mutant mice lacking a functional CB_1 receptor gene represent a powerful tool to investigate the presence of a CB_1–AMT coupling *in vivo*, and to assess whether the impairment of such a coupling would result in altered brain endocannabinoid concentrations that may interfere with brain function in a CB_1-dependent and/or -independent manner. Here, 1- and 4-month-old homozygous CB_1 knockout (KO) and wildtype (WT) mice (Ledent *et al.*, 1999) were used to ascertain the influence of CB_1 receptor invalidation on AMT and FAAH activity in cortex and hippocampus, two brain regions primarily involved in the psychotropic actions of exogenous and, perhaps, endogenous cannabinoids. The concentration of AEA and 2-AG in these areas was also measured. Moreover, CB_1 KO and WT mice of both ages were subjected to behavioural tests aimed at evaluating: (i) anxiety-related responses, (ii) memory in the object recognition task, (iii) nociception and (iv) naloxone-precipitated morphine withdrawal syndrome.

Materials and methods

Materials and animals

All chemicals were of the purest analytical grade. AEA and naloxone were from Sigma Chemical (St Louis, MO, USA). [^{3}H]AEA (223 Ci/mmol) was purchased from NEN Dupont de Nemours (Köln, Germany). Deuterated anandamide was synthesized from d4-ethanolamine (Isotec, Miamisburg, OH, USA) according to Schmid *et al.* (1995). 2-AG was from Research Biochemicals International (Natick, MA, USA). Identity and chemical purity of AEA and 2-AG standards were determined by ^{1}H NMR and mass spectrometric analysis under electron impact mode (Maccarrone *et al.*, 2001a).

The generation of CD1 mice lacking the cannabinoid CB_1 receptors ('knockout' mutants) was described earlier (Ledent *et al.*, 1999). In order to homogenize the genetic background of the mice, first-generation heterozygotes (CB_1+/–) were bred for five generations on a CD1 background, with selection for the mutant CB_1 gene at each generation. Fifth-generation heterozygotes were bred together, to generate the homozygous mice, either wildtype (CB_1+/+, WT) or knockout (CB_1–/–, KO), used in this study. Mice were maintained for the indicated periods of time under standard animal housing conditions in a 12-h dark : 12-h light cycle with free access to food and water. For the biochemical and behavioural tests, two groups of male CB_1 knockout mice and wildtype littermates animals were used: 1-month-old ('young'), ranging 20–22 g at the start of the experiments, and 4-month-old ('old'), ranging 28–30 g at the start of the experiments. Animal care was in accordance with ethical guidelines (European Communities Council Directive 86/609/EEC). All experimental procedures were approved by local animal care committees.

Determination of anandamide uptake and hydrolysis

Synaptosomes were prepared from the different areas of mouse brain, mice were killed by decapitation and processed immediatley as described previously (Barbaccia *et al.*, 1983). Tissues were resuspended in ice-cold 0.32 M sucrose, 5 mM Tris.HCl buffer (pH 7.4) and were gently disrupted by 10 up-and-down strokes in a Teflon-glass homogenizer (weight : volume ratio, 1 : 20). The homogenates were centrifuged at 1000 *g* for 5 min, at 4 °C, then supernatants were centrifuged again at 17 000 *g* for 15 min, at 4 °C. The final pellets were resuspended in 136 mM NaCl, 5 mM KCl, 0.16 mM $CaCl_2$, 0.1 mM EGTA, 1.3 mM $MgCl_2$, 10 mM glucose, 10 mM Tris.HCl buffer (pH 7.4), at a protein concentration of 3 mg/mL. The activity of the AMT was measured as described (Maccarrone *et al.*, 2001b), using 100 µL synaptosomes and 300 nM [^{3}H]AEA per test. Q_{10} value was calculated as the ratio of AEA uptake at 30 °C and 20 °C (Hillard *et al.*, 1997).

Anandamide hydrolase (arachidonoylethanolamide amidohydrolase, EC 3.5.1.4; FAAH) activity was assayed in mouse brain homogenates by reversed-phase high-performance liquid chromatography, using 5 µM [^{3}H]AEA as substrate (Maccarrone *et al.*, 1998). FAAH activity was expressed as pmol arachidonate released per min per mg protein.

GC/MS analysis

Immediately after decapitation, mouse brains were washed in phosphate-buffered saline, precooled at 4 °C, dissected and frozen in liquid nitrogen, and kept at –70 °C until processed. A maximum of 8 min elapsed between mouse decapitation and freezing of dissected tissues, a time not sufficient to cause artefactual rises in endocannabinoid levels (Schmid *et al.*, 1995; Maccarrone *et al.*, 2001a). Lipids were extracted from frozen tissues and analysed by gas chromatography-electron impact mass spectrometry (GC/MS), as described previously (Maccarrone *et al.*, 2001a). Lipid extracts were injected into a Carlo Erba model HRGC5160 gas chromatograph (Rome, Italy), equipped with a BP5 silica capillary column (30 m × 0.25 mm inner diameter) from SGE (Milan, Italy), and interfaced with a VG Micromass model QUATTRO spectrometer (Manchester, UK). Analyses were performed in 'splitless' mode at temperatures rising from 70 to 250 °C, at a rate of 30 °C/min. The identity of AEA and 2-AG was assessed by comparison of the retention times and the mass spectra recorded at 70 eV with those of authentic standards. Quantification of AEA was achieved by isotope dilution with deuterated anandamide, whereas 2-AG was quantified by the internal standard method with deuterated anandamide. Calibration solutions and calibration curves were obtained as described previously (Maccarrone *et al.*, 2001a).

Behavioural tests

Open-field

Each animal was placed in an open-field apparatus (Simonin *et al.*, 1998) consisting of a rectangular area (70 cm wide × 90 cm long × 60 cm high) under bright illumination (500 lux). We drew 63 squares (10 × 10 cm^2) with black lines on the white floor of the apparatus. The parameters measured during a 5-min observation were: (i) the number of rearings, (ii) the number of squares crossed in the peripheral and central areas of the field, and (iii) the time spent in the central area of the field.

Lit-dark box

We used a box (Timpl *et al.*, 1998) consisting of a small (15 cm wide × 20 cm long × 25 cm high) compartment with black walls and black floor dimly lit (5 lux), connected by a 4-cm long tunnel to a large compartment (30 cm wide × 20 cm long × 25 cm high) with white walls and a white floor, intensely lit (500 lux). We drew lines on the floor of both compartments to permit measurement of locomotor activity by counting the number of squares crossed. Each animal was placed in the dark compartment facing the tunnel at the beginning of the observation session. Locomotor activity (number of squares crossed), time spent in each compartment, as well as latency of time to go to the lit compartment for the first time, were recorded for 5 min.

Object recognition task

This test was performed as reported previously (Meziane *et al.*, 1998) in a Plexiglas open-field box (51 cm wide × 51 cm long × 58 cm

high) with white vertical walls and a white floor divided into 25 equal squares. The light intensity in the middle of the field was 30 lux. The objects to be discriminated were a marble (5.5 cm high, object A) and a plastic (4.5 cm high, object B) figure. First, mice were individually habituated to the open-field for 50 min. The next day, they were submitted to a 10-min acquisition trial (first trial) during which they were placed in the open-field in the presence of the object A. Locomotor activity (number of squares crossed), rearings and time that animal took to explore object A (animal's snout direct toward the object at a distance < 1 cm) were recorded. A 10-min retention trial (second trial) occurred 3 h or 24 h later. During this second trial, objects A and B were placed in the open-field, and locomotor activity, rearings and time that animal took to explore object A (t_A) and object B (t_B) were recorded. A recognition index was defined as $[t_B/(t_A + t_B)] \times 100$.

Formalin test

The test was performed in a glass cylinder chamber (16 cm high × 16 cm diameter) (Hunskaar *et al.*, 1985). Each mouse was placed in the test chamber for 30 min habituation. After this period, 20 µL of 5% formalin was injected subcutaneously into the dorsal surface of the right hindpaw of the mouse, using a 26-gauge needle connected to a microsyringe. Each mouse was immediately returned to the observation chamber after injection and its nociceptive response was recorded. Recording of the early response (early phase) started immediately and lasted for 5 min (0–5 min), whereas recording of the late response (late phase) started 15 min after formalin injection and lasted for 15 min (15–30 min). In both phases only licking or biting of the injected hindpaw was defined as a nociceptive response and the total duration of the response was registered by means of a stopwatch.

Morphine withdrawal syndrome

Opiate dependence was induced in mice by repeated intraperitoneal injection of morphine, a kind gift of the 'Ministerio de Sanidad y Consumo' (Madrid, Spain), at an interval of 12 h, for 6 days (Simonin *et al.*, 1998; Ledent *et al.*, 1999). The morphine dose was progressively increased as follows: day 1, 20 mg/kg bodyweight; day 2, 40 mg/kg; day 3, 60 mg/kg; day 4, 80 mg/kg; days 5 and 6, 100 mg/kg (only one injection in the morning). Control mice were treated with saline under the same conditions. Withdrawal was precipitated only once in each animal by injecting subcutaneously 1 mg/kg naloxone 2 h after the last morphine administration. Mice were placed individually into test chambers, consisting of transparent round plastic boxes (50 cm high × 30 cm diameter) with a white floor, 15 min after naloxone injection for habituation. Different somatic signs of withdrawal were evaluated immediately after the injection of the opiate antagonist during a period of 30 min: the number of wet-dog-shakes, jumping, paw tremor and sniffing, and the presence of teeth chattering, diarrhoea, tremor, piloerection and ptosis. Taking into account all the individual signs, a global withdrawal score was calculated for each animal, as reported previously (Maldonado *et al.*, 1992).

Statistical analysis

Data reported in this paper are the mean (± SD) of at least four independent determinations, each performed in duplicate. Statistical analysis of biochemical data was performed by the nonparametric Mann–Whitney *U*-test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science, San Diego, CA, USA). The results of the behavioural tests were expressed as mean (± SEM) and were analysed by two-way ANOVA (genotype and age) between subjects. Subsequent one-way ANOVA values were calculated for genotype and age effects. In the case of the object recognition task, recognition index of WT and KO groups, and retention from 3 h vs. 24 h trials, were compared using one-way ANOVA. In all cases, differences with $P < 0.05$ were considered significant.

Results

AEA degradation in CB_1+/+ and CB_1–/– mice

Biochemical analyses were performed on 1-month-old (young) or 4-month-old (old) mice in order to correlate the biochemical background with the behavioural performances of the same animals. Mice older than 4 months could not be used because of the general impairment of the behavioural performance occurring in these animals (Ledent *et al.*, 1999; Valverde *et al.*, 2000).

In pilot experiments, synaptosomes prepared from the cortex of WT mice were found to take up [^{3}H]AEA in a temperature- ($Q_{10} \approx 1.5$) and time- ($t_{1/2} \approx 5$ min) dependent manner, suggesting that an AMT was responsible for [^{3}H]AEA uptake (Bisogno *et al.*, 1997; Hillard *et al.*, 1997; Maccarrone *et al.*, 1998, 2000a; Jarrahian *et al.*, 2000). To our knowledge, only one report has yet shown AMT activity *ex vivo*, i.e. in synaptosomes prepared from brain areas (Maccarrone *et al.*, 2001b). In all groups of WT and KO animals, AMT activity was higher in the hippocampus than in the cortex (Table 1). Moreover, in both brain areas of WT mice AMT failed to change with age, whereas in KO animals it increased up to 290% (cortex) or 260% (hippocampus) in old vs. young mice (Table 1). The kinetic parameters, i.e. apparent Michaelis–Menten constant (Km) and maximum velocity (Vmax) of AMT in the cortex of young and old WT mice were 280 ± 30 nM and 150 ± 15 pmol/min/mg protein, respectively, whereas the same Km value of 280 ± 30 nM but higher Vmax

TABLE 1. Activity of AEA membrane transporter (AMT) and AEA hydrolase (FAAH) in the cortex and the hippocampus of wildtype (WT) or knockout (KO) male mice

	AMT activity (pmol/min/mg protein)		FAAH activity (pmol/min/mg protein)	
Mouse brain	Cortex	Hippocampus	Cortex	Hippocampus
WT (young)	75 ± 8 (100%)	100 ± 10 (100%)	650 ± 70 (100%)	1000 ± 100 (100%)
KO (young)	70 ± 7 (93%)*	120 ± 12 (120%)*	630 ± 65 (97%)*	1000 ± 100 (100%)*
WT (old)	70 ± 7 (93%)*	100 ± 10 (100%)*	680 ± 70 (105%)*	1000 ± 100 (100%)*
KO (old)	220 ± 22 (293%)†§	260 ± 26 (260%)†§	1100 ± 110 (169%)‡¶	2000 ± 200 (200%)†§

Values in brackets represent percentage of the WT (young) animals. *$P > 0.05$ vs. WT (young); †$P < 0.01$ vs. WT (young); ‡$P < 0.05$ vs. WT (young); §$P < 0.01$ vs. WT (old); ¶$P < 0.05$ vs. WT (old) ($n = 4$ in all cases).

TABLE 2. Levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the cortex and the hippocampus of wildtype (WT) or knockout (KO) male mice

	AEA concentration (nmol/mg protein)		2-AG concentration (nmol/mg protein)	
Mouse brain	Cortex	Hippocampus	Cortex	Hippocampus
WT (young)	0.23 ± 0.06 (100%)	0.50 ± 0.14 (100%)	0.14 ± 0.02 (100%)	0.31 ± 0.08 (100%)
KO (young)	0.24 ± 0.05 (104%)*	0.43 ± 0.03 (86%)*	0.14 ± 0.02 (100%)*	0.27 ± 0.07 (87%)*
WT (old)	0.22 ± 0.04 (96%)*	0.43 ± 0.03 (86%)*	0.13 ± 0.02 (93%)*	0.30 ± 0.08 (97%)*
KO (old)	0.19 ± 0.02 (83%)*‡	0.31 ± 0.04 (62%)†§	0.14 ± 0.02 (100%)*‡	0.28 ± 0.07 (90%)*‡

Values in brackets represent percentage of the WT (young) animals. *$P > 0.05$ vs. WT (young); †$P < 0.05$ vs. WT (young); ‡$P > 0.05$ vs. WT (old); §$P < 0.05$ vs. WT (old) ($n = 4$ in all cases).

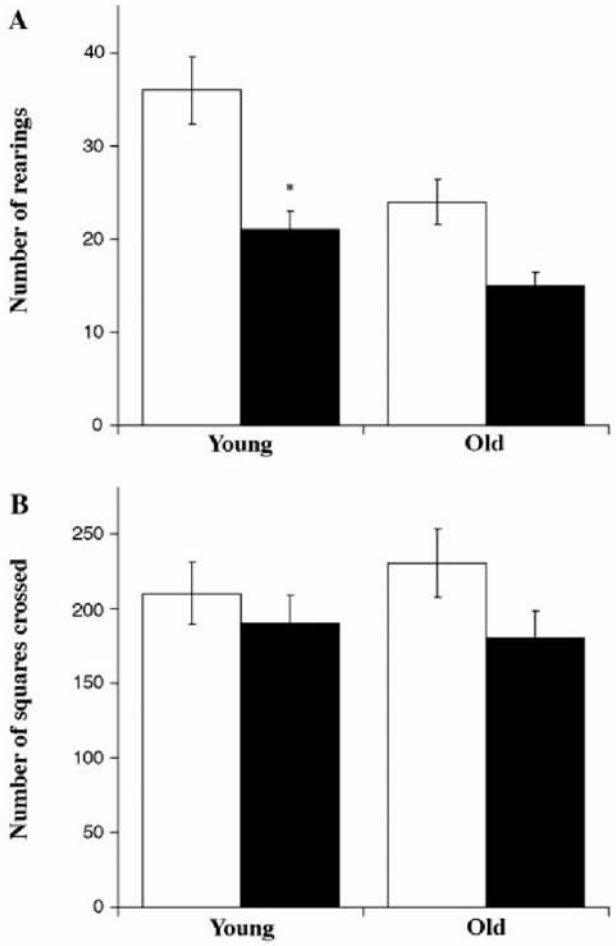

FIG. 1. Effects observed in the open-field test in wildtype (white bars) and CB_1 receptor knockout (black bars) mice of two different ages, during the first day of observation. The number of rearings and the number of squares crossed are reported in A and B, respectively. Vertical bars represent SEM values. *$P < 0.05$, KO vs. WT (one-way ANOVA) ($n = 9$–10 for each group).

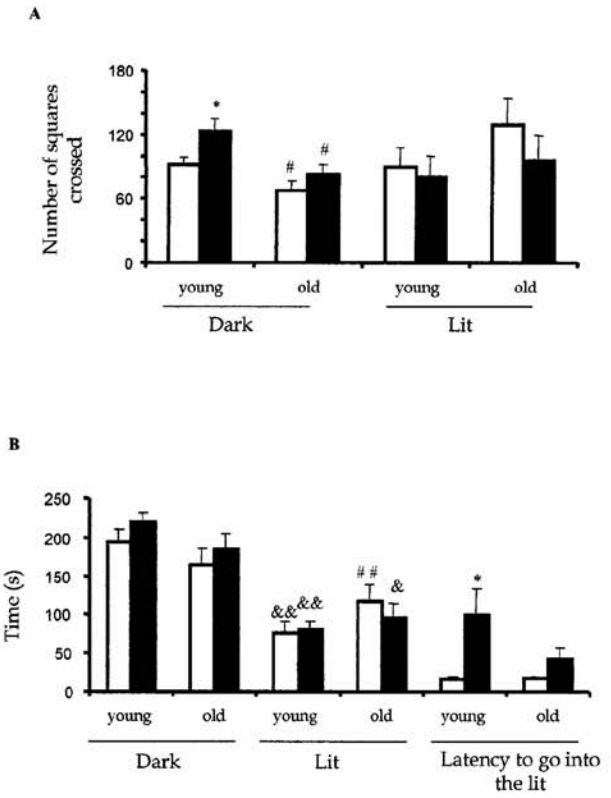

FIG. 2. Effects observed in the lit-dark box in wildtype (white bars) and CB_1 receptor knockout (black bars) mice of two different ages. The number of squares crossed is reported in A, whereas the time spent in each part of the box (left side) and the latency time to go to the white box (right side) are reported in B.Vertical bars represent SEM values. *$P < 0.05$, KO vs. WT (one-way ANOVA); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, old vs. young (one-way ANOVA); $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$, lit vs. dark (one-way ANOVA) ($n = 10$ for each group).

values were found in old vs. young KO animals (350 ± 40 vs. 150 ± 15 pmol/min/mg protein, respectively).

As for AMT, FAAH activity of WT or KO mice was higher in the hippocampus than in the cortex. However, while in both brain regions of WT animals FAAH did not change with age, it was significantly higher in old vs. young KO animals in both brain areas (Table 1). Kinetic analysis of [^{3}H]AEA hydrolysis by cortex FAAH showed the same Vmax values in young and old WT mice (1700 ± 170 pmol/min/mg protein), whereas higher Vmax values were found in old vs. young KO animals (2200 ± 200 vs. 1600 ± 170 pmol/min/mg protein, respectively) and Km values of FAAH were 4.0 ± 0.5 μM in all groups.

Levels of AEA and 2-AG in CB_1+/+ and CB_1–/– mice

GC/MS analysis of rapidly frozen samples showed that the concentrations of AEA and 2-AG in mouse cortex and hippocampus are in

the nmol/mg protein range (Table 2). No traces of 1(3)-AG were detected in either standard 2-AG solutions or brain samples, suggesting that no appreciable isomerization occurred under our experimental conditions, as observed recently in human and rat brains (Maccarrone *et al.*, 2001a). Hippocampus contained approximately twice the amount of AEA and 2-AG than the cortex in both groups of WT and in the young KO animals. While age failed to affect the AEA and 2-AG content in cortex or hippocampus of WT mice, a significant decrease of AEA, but not of 2-AG, was observed in the hippocampus of old CB_1 KO mice (Table 2).

Behavioural responses of CB_1+/+ and CB_1–/– mice of different ages

The behaviour of WT and KO mice at both ages was studied using several paradigms. In the open-field test, performed under bright light, i.e. under stressful conditions, two-way ANOVA calculated for the number of rearings indicated significant effect of age ($F_{1,39} = 5.352$, $P < 0.05$), and mutation ($F_{1,39} = 7.273$, $P < 0.05$), without interaction between these two factors. When we analysed the effect of the mutation, we observed a decrease in the number of rearings in young KO vs. young WT mice ($P < 0.05$) (Fig. 1A). The number of squares crossed in the open-field (an index of the locomotor activity) was similar for the two genotypes and for the two groups of ages (Fig. 1B).

In the lit-dark box, two-way ANOVA for the number of the squares crossed in the dark compartment indicated significant effects of age ($F_{1,39} = 10.812$, $P < 0.01$), and mutation ($F_{1,39} = 5.427$, $P < 0.05$), without interaction between these two factors. One-way ANOVA showed that the number of squares crossed in the dark compartment was significantly increased ($P < 0.05$) in young KO vs. young WT mice (Fig. 2A). This response was not observed in the group of old mice. Indeed, when we compared the genotypes in the two groups of ages, we observed a significant decrease ($P < 0.05$) in the activity in the old group of mice, similar for the two genotypes (Fig. 2A). When we analysed the time spent in each part of the box, two-way ANOVA for the lit compartment revealed an effect of age ($F_{1,40} = 5.398$, $P < 0.05$), without effect of mutation, nor interaction between these two factors. One-way ANOVA showed a significant difference between old WT and young WT groups ($P < 0.01$) (Fig. 2B). We have also calculated one-way ANOVA for the effect observed in the two compartments of the box. In this case, we observed that young WT mice spent less time in lit vs. dark compartments ($P < 0.01$). Such an effect was not observed in old WT mice. Two-way ANOVA for KO mice indicated an effect of compartment ($F_{1,40} = 60.910$, $P < 0.01$), without effect of age and with interaction between age and compartment ($F_{1,40} = 5.048$, $P < 0.05$). One-way ANOVA showed a more significant effect of compartment for young KO mice ($P < 0.01$) than for the old KO group ($P < 0.05$) (Fig. 2B). We also analysed the latency to go into the lit compartment. Two-way ANOVA indicated an effect of mutation ($F_{1,40} = 8.134$, $P < 0.01$) without effect of age. One-way ANOVA showed an increase in the latency to go to the lit compartment in the young KO vs. the young WT group ($P < 0.05$). This effect was not observed in old KO vs. old WT mice. No significant differences between groups of ages were observed in WT mice for this parameter (Fig. 2B).

In the object recognition task, two-way ANOVA did not reveal any significant effect for the two factors investigated (age and genotype). Young CB_1 KO and WT animals exhibited a similar recognition index 3 h after the exploration training of object A, suggesting a similar ability for recognition in both groups of young mice (Fig. 3). When the test was performed 24 h after the training session, the recognition index declined significantly ($P < 0.05$) in the young WT

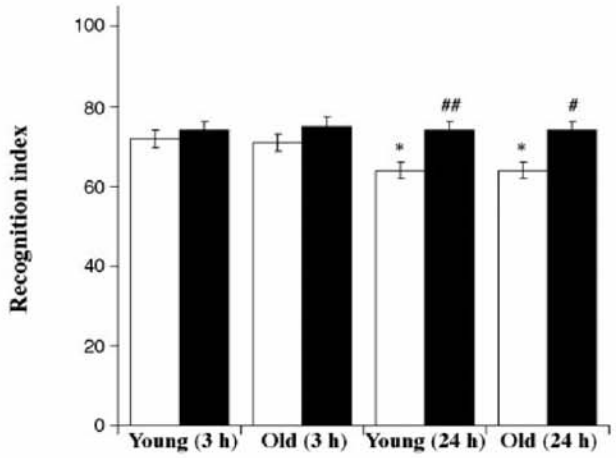

FIG. 3. Recognition index measured in wildtype (white bars) and CB_1 receptor knockout (black bars) mice of two different ages. Vertical bars represent SEM values. $^{*}P < 0.05$, 24 h vs. 3 h trials (one-way ANOVA); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, KO vs. WT (one-way ANOVA) ($n = 9$–10 for each group).

but not KO mice, leading to a significant difference ($P < 0.01$) between the two groups of animals at this time-point (Fig. 3). Old CB_1 KO and WT mice also showed a similar recognition index 3 h after the exploration training of object A, and again the recognition index significantly ($P < 0.05$) decreased in the old WT but not KO mice 24 h after the training session, leading to a significant difference ($P < 0.05$) between the two groups of animals at this time-point (Fig. 3).

The severity of irritative/inflammatory pain was measured in the formalin test. Typically, two phases of nociceptive responses can be observed in this test. An early phase (5–10 min) and a late phase, which begins about 15 min after the injection and may last up to 45 min. It is thought that the early phase is triggered through the direct activation of nociceptors by formalin, while the late phase involves peripheral inflammatory responses, as well as plastic changes in the spinal cord (Tjolsen *et al.*, 1992; Noble *et al.*, 1997). A similar nociceptive threshold was observed during the early phase in the different groups of mice (not shown). In contrast, a decrease of the nociceptive response in the late phase (inflammatory pain) was observed in both groups of old animals. Two-way ANOVA in the late phase indicated an effect of the age ($F_{1,39} = 21.525$, $P < 0.01$), without mutation effect and without interaction between these two factors. One-way ANOVA for age groups showed a significant response when comparing old and young WT ($P < 0.05$) and KO ($P < 0.01$) mice (Fig. 4A).

Finally, the expression of naloxone-precipitated morphine withdrawal syndrome was investigated, and a decrease in the severity of morphine withdrawal was observed in both groups of CB_1 KO mice. Indeed, two-way ANOVA for the global withdrawal scores (age and genotype factors), revealed an effect of the mutation ($F_{1,37} = 17.307$, $P < 0.01$), without effect of the age and without interaction between these two factors. Accordingly, one-way ANOVA showed a decrease in the severity of the withdrawal syndrome in knockout mice in both groups of ages ($P < 0.01$ for young, and $P < 0.05$ for old KO mice) (Fig. 4B).

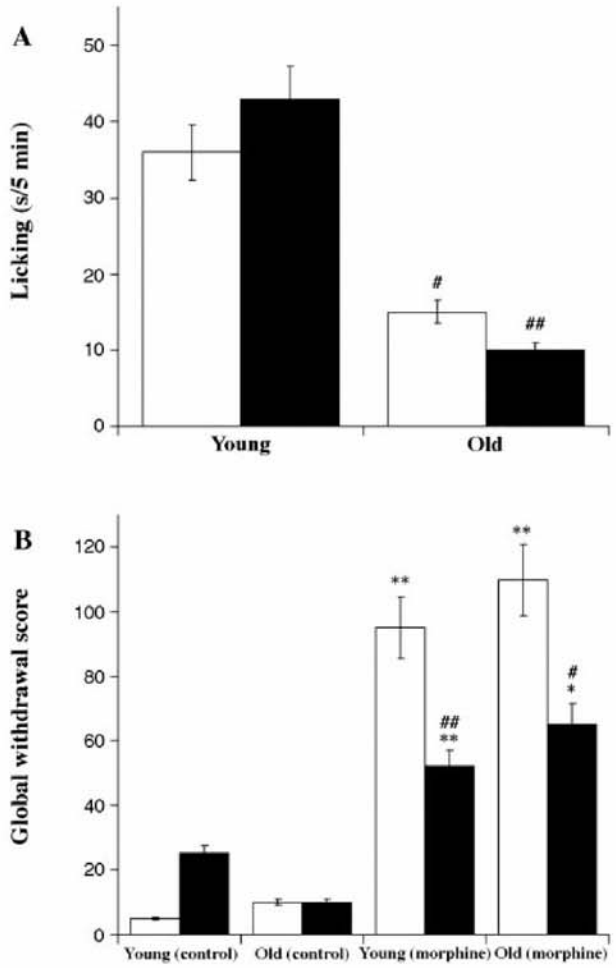

FIG. 4. Nociceptive responses in the late (15–30 min) phase after 5% formalin injection (A) were measured as the amount of time spent licking or biting the injected paw. Naloxone-precipitated morphine withdrawal syndrome and spontaneous nociceptive threshold in wildtype (white bars) and CB_1 receptor knockout (black bars) mice of two different ages. Naloxone-precipitated morphine withdrawal syndrome was expressed as a global withdrawal score (B). Vertical bars represent SEM values. In (A), $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, old vs. young (one-way ANOVA) ($n = 10$ for each group). In (B), $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control groups (one-way ANOVA), whereas $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, KO vs. WT (one-way ANOVA) ($n = 9$–10 for each group).

Discussion

The main findings of this report are that: (i) the endogenous cannabinoid system appears to adapt with age in mice carrying a constitutive knockout of the CB_1 gene; indeed, no changes in endogenous AEA and 2-AG brain content as well as in AMT or FAAH activity were observed in 1-month-old CB_1 KO mice, with respect to their age-matched WT controls; in contrast, 4-month-old CB_1 KO mice, when compared with age-matched WT controls or to young KO mice, showed a significant decrease in hippocampal AEA content associated with significant increases in hippocampal and cortical AMT and FAAH activity; (ii) these age-dependent adaptive changes in endocannabinoid metabolism are associated with a mild decrease in anxiety-like behaviour in old vs. young KO mice; (iii) the age-dependent changes in endocannabinoid metabolism, however, failed to impact on the increased memory performance in the object recognition test as well as on the reduction of naloxone-precipitated morphine withdrawal symptoms, previously described in CB_1 KO mice (Ledent *et al.*, 1999; Reibaud *et al.*, 1999); (iv) the age-dependent modifications observed in the response to the formalin test were similar for both genotypes.

Quantitative analysis of AEA and 2-AG in the cortex and hippocampus of young and old WT and KO mice yielded the results shown in Table 2. We could not extend GC/MS analysis to other brain areas because of the limited number of animals available. We chose to normalize the amounts of AEA and 2-AG to the protein content of the samples, in order to better compare different tissues, and also in consideration of the fact that determination of proteins is much more accurate than that of fresh weight. Remarkably, AEA and 2-AG were detected in similar nmol/mg protein amounts, extending to the mouse previous observations on human and rat brains (Maccarrone *et al.*, 2001a). These concentrations, which cannot be due to an artefactual accumulation by the killing procedure (Schmid *et al.*, 1995; Maccarrone *et al.*, 2001a), are in the micromolar range, which is compatible with a role for AEA and 2-AG in the central nervous system as modulatory substances (Cadas *et al.*, 1997) and/or true neurotransmitters (Self, 1999). A major finding of this investigation is that AEA, but not 2-AG, significantly decreased only in the hippocampus of old KO vs. young KO or WT mice, while these endocannabinoids showed minor changes in the cortex of old KO mice (Table 2). These adaptive changes in AEA hippocampal concentrations are not only in keeping with the increased AMT and FAAH activity, measured contextually in the same brain region of these animals (Table 1), but also further suggest a physiological role for AEA as a neurotransmitter/neuromodulator. A functional coupling between CB_1 and AMT has been recently shown in human endothelial cells, where AEA binding to CB_1 induces the release of nitric oxide, which in turn activates AEA uptake by AMT and subsequent intracellular degradation by FAAH (Maccarrone *et al.*, 2000a). In this line, AMT in mouse brain synaptosomes has also been shown to be activated by nitric oxide (Maccarrone *et al.*, 2001b). The fact that 2-AG, which is also a good substrate for FAAH (Goparaju *et al.*, 1998), failed to change in hippocampus and that AEA concentration did not change in cortex of old KO mice, where instead AMT and FAAH activities increased, suggests that whatever the mechanism responsible for endocannabinoid steady state levels, it should be brain region-specific and, particularly for 2-AG, may involve other metabolic enzymes in addition to FAAH (Goparaju *et al.*, 1999). In keeping with this hypothesis, a recent report showed specific brain regional changes in endocannabinoid concentrations in rats tolerant to Δ^9-tetrahydrocannabinol (Di Marzo *et al.*, 2000). Moreover, the steady state levels of each endocannabinoid, besides depending on the degradation rate, might also depend on an alternative synthetic pathway activated in KO mice, possibly as a consequence of CB_1 receptor invalidation, which would account for the reduced levels of AEA and the stable 2-AG concentrations. These findings suggest that CB_1 receptors might play a critical role in modulating endogenous AEA levels, with a beneficial effect on its potential neurotoxicity. Indeed, AEA has been shown to induce programmed cell death of neuronal cells *in vitro* (Maccarrone *et al.*, 2000c) and *in vivo* (Galve-Roperh *et al.*, 2000), and to increase during glutamate-induced neurotoxicity (Hansen *et al.*, 1999). In this line, it is noteworthy that the hippocampus expresses

considerably more CB_1 receptors than cortex in WT mice (Pertwee, 1997), suggesting that the different effects on AEA levels of KO and WT animals might reflect a differential reduction in receptor population in the two brain areas of these mice. Recent time-course experiments on 2-, 6- and 12-month-old mice have shown that AMT and FAAH increase with age in KO animals, whereas they show minor changes in WT animals (Maccarrone *et al.*, 2001b). In the same study, kinetic analysis showed that the enhancement of AMT and FAAH is due to increased amounts of the same proteins rather than to different isoforms of them, strengthening the concept that a different regulation of AMT and FAAH expression is taking place in KO animals. Moreover, in 6- and 12-month-old KO mice not only AEA, but also *N*-palmitoylethanolamine, *N*-oleoylethanolamine and *N*-stearoylethanolamine, though not 2-AG, were considerably decreased in the hippocampus (Maccarrone *et al.*, 2001b). *N*-palmitoylethanolamine, in particular, has been recently shown to inhibit the expression of FAAH in peripheral cell lines (Di Marzo *et al.*, 2001). Should this mechanism also be operative in the brain, the decrease of *N*-palmitoylethanolamine in CB_1 KO mice may relate to the increased FAAH expression and activity.

In order to establish the physiological relevance of the reported biochemical differences among young and old KO mice, we also investigated the possible behavioural differences between these animals. Locomotor activity was not different between the two genotypes, as shown by the similar number of squares crossed in the open field test. In the lit-dark box paradigm, young WT and young KO mice similarly spent a lower amount of time in the light vs. the dark compartment. This 'anxiety-related' response to light was decreased in old WT mice and, though to a lesser degree, in KO mice. Furthermore, while young KO mice showed a longer latency to enter the light compartment compared with the young WT mice, this parameter (an index of higher basal anxiety-related behaviour) was not different in old KO mice compared with the old WT group. Animal studies reveal that cannabinoids can induce both anxiolytic- and anxiogenic-like responses, depending on the doses and the familiarity of the environment (Rodríguez de Fonseca *et al.*, 1996). In agreement with the present results, a recent study demonstrated that CB_1 KO mice presented an anxiogenic-like behaviour in the lit-dark box and increased behavioural responses in a chronic model of depression (Martin *et al.*, 2002). The higher anxiety-related behaviour exhibited by young CB_1 KO mice, and the temporal relationship between behavioural responses, lower hippocampal AEA levels and increased AMT and FAAH activities in old KO mice, suggest a non-CB_1 receptor-mediated anxiogenic effect of AEA. Accordingly, a novel non-CB_1 and non-CB_2 AEA receptor has been recently characterized in the mouse brain (Breivogel *et al.*, 2001). In this line, AEA, apparently in a CB_1 receptor-independent manner, potentiates serotonergic transmission at $5HT_{1a}$ receptors (Boger *et al.*, 1998), and serotonin in the hippocampus is known to modulate the expression of anxiety-related behaviours (File *et al.*, 2000). The presence of a normal repertoire of CB_1 receptors, shown to reduce serotonin release in brain (Nakazi *et al.*, 2000), may overcome or attenuate these non-CB_1-mediated actions of endocannabinoids in young WT mice, which show endocannabinoid levels similar to those of young KO animals.

On the other hand, the hippocampus-specific memory test showed a better performance of young and old KO animals compared with their WT littermates 24 h after training (Fig. 3). These data support the hypothesis that the enhancement of memory performance might depend on the absence of CB_1 receptors (Reibaud *et al.*, 1999). Similarly, in a previous study (Ledent *et al.*, 1999), we have reported that CB_1 knockout mice exhibited a significant decrease in the severity of morphine withdrawal syndrome. Here, we evaluated the age dependence of this response (Fig. 4B). In agreement with previous experiments, the somatic signs of naloxone-precipitated morphine withdrawal syndrome were significantly attenuated in CB_1 knockout mice and no differences were observed in relation with age, suggesting that the changes of AEA metabolism in hippocampus and cortex of the older KO mice did not participate in the reduced expression of morphine abstinence. CB_1 receptor expression and age-related changes in endocannabinoid levels also failed to interfere with the nociceptive threshold, which was similar in the early (irritative) and late (inflammatory) phases of the formalin test in WT and KO animals (Fig. 4A and data not shown).

Altogether, the present report shows plasticity of the endocannabinoid system in mice carrying a constitutive knockout of the CB_1 receptor gene. These adaptive changes appear to be confined to the hippocampus and to mildly affect anxiety-related behaviour, which may be independent of the interaction of endocannabinoids with CB_1 receptors. However, they do not affect memory performance nor the sensitivity of the CB_1 KO animals to naloxone-precipitated morphine withdrawal syndrome, implying a role for CB_1 receptor-mediated events in these behavioural responses.

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Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonoylethanolamine (anandamide); AMT, AEA membrane transporter; FAAH, fatty acid amide hydrolase; GC/MS, gas chromatography-mass spectrometry; KO, knockout; WT, wild-type.

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Increase of morphine withdrawal in mice lacking A_{2a} receptors and no changes in CB_1/A_{2a} double knockout mice

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Abstract

CB_1 cannabinoid and A_{2a} adenosine receptors are highly expressed in the central nervous system where they modulate numerous physiological processes including emotional behaviour and the responses of several drugs of abuse. To investigate the contribution of these receptors in emotional-like responses and opioid dependence we have generated CB_1/A_{2a} double deficient mice ($CB_1^{-/-}/A_{2a}^{-/-}$). The spontaneous locomotor activity was reduced in double knockout as compared to wild-type animals. Emotional-like responses of $CB_1^{-/-}/A_{2a}^{-/-}$ mice were investigated using the elevated plus-maze and the lit-dark box. Mutant mice exhibited an increased level of anxiety in both behavioural models. The specific involvement of CB_1 and A_{2a} receptors in morphine dependence was evaluated by using A_{2a} knockout mice and CB_1/A_{2a} double mutant mice. The severity of naloxone-precipitated morphine withdrawal syndrome was significantly increased in the absence of A_{2a} adenosine receptors whereas no modifications were observed in the double knockout mice. These results suggest that both receptors participate in the control of emotional behaviour and seem to play an opposite role in the expression of opioid physical dependence.

Introduction

Adenosine is an important inhibitory neuromodulator in the central nervous system which induces its physiological effects by acting on four different adenosine receptor subtypes, A_1, A_{2a}, A_{2b} and A_3 (Olah & Stiles, 1995). A_1 and A_3 receptor activation inhibits adenylate cyclase activity whereas A_{2a} and A_{2b} receptors are positively coupled to this enzyme. The highest levels of A_{2a} receptors are found in the striatum where they are coexpressed with postsynaptic D_2 dopamine receptors in GABAergic striatopallidal neurons, and regulate proenkephalin gene expression (Fink *et al.*, 1992; Schiffmann & Vanderhaeghen, 1993). The specific function of A_{2a} receptors has been investigated by using A_{2a} knockout mice (Ledent *et al.*, 1997; Chen *et al.*, 1999), and an important increase in basal levels of anxiety was observed in these mutant mice. Adenosine has been suggested to regulate different responses induced by opioids. Thus, the antinociceptive effects of morphine in the spinal cord seem to be mediated at least in part by release of endogenous adenosine and subsequent activation of A_1 and A_2 receptors (Sweeney *et al.*, 1987; Sweeney *et al.*, 1991). Adenosine also participates in opioid dependence as the blockade of adenosine metabolism by adenosine kinase inhibitors decreases the severity of morphine withdrawal (Kaplan & Coyle, 1998). In agreement, adenosine agonists inhibit the expression of morphine withdrawal while adenosine antagonists increase the incidence of withdrawal signs (Kaplan & Sears, 1996; Salem & Hope, 1997). However, the low specificity of adenosine antagonists makes it difficult to clarify the participation of the different adenosine receptor subtypes in opioid dependence.

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On the other hand, the cannabinoid system represents another important inhibitory neuromodulator acting in the central nervous system through the stimulation of CB_1 cannabinoid receptors (Matsuda *et al.*, 1990). Cannabinoid receptor stimulation reduces adenylyl cyclase activity also through activation of G proteins. The CB_1 receptor has been reported to be responsible for the addictive properties of cannabinoids, including rewarding effects and physical dependence (Ledent *et al.*, 1999), and bi-directional interactions between cannabinoid and opioid systems in these processes have been recently confirmed (reviews in Manzanares *et al.*, 1999; Maldonado & Rodríguez de Fonseca, 2002). Thus, the severity of opioid withdrawal is reduced in mice lacking the CB_1 cannabinoid receptor (Ledent *et al.*, 1999), whereas cannabinoid withdrawal syndrome is attenuated in preproenkephalin-deficient mice (Valverde *et al.*, 2000a). CB_1 cannabinoid receptors seem to be also involved in the regulation of emotional behaviour as an increase in the basal level of anxiety has been reported in CB_1 knockout mice (Martin *et al.*, 2002).

The aim of the present study was to investigate the specific role of CB_1 cannabinoid receptors and A_{2a} adenosine receptors in emotional-like responses and opioid dependence by disrupting these genes in mice. For this purpose, basal levels of locomotor activity and anxiety-like behaviour were analyzed in $CB_1^{-/-}/A_{2a}^{-/-}$ mice and wild-type animals. In addition, the contribution of CB_1 and A_{2a} receptors on the expression of the opioid withdrawal syndrome was evaluated following chronic morphine treatment in mice lacking A_{2a} receptors (Ledent *et al.*, 1997) and $CB_1^{-/-}/A_{2a}^{-/-}$ mice. These behavioural responses were compared with the results obtained in A_{2a} and CB_1 single mutant mice in the same paradigms. All the studies were performed under identical experimental conditions in order to make possible a direct comparison of double vs. single knockout mice.

Materials and methods

Animals

Mice lacking A_{2a} adenosine receptors or CB_1 cannabinoid receptors were generated as previously reported (Ledent *et al.*, 1997, 1999). $CB_1^{-/-}/A_{2a}^{-/-}$ mice were generated by interbreeding CB_1 and A_{2a} knockout mice, that were in a CD_1 outbred background. Animals (25–30 g at the beginning of the study) were housed five per cage in a temperature (21 ± 1 °C) and humidity ($55 \pm 10\%$) controlled-room with a 12-h light : 12-h dark cycle (light between 08.00 h and 20.00 h). Food and water were available *ad libitum*. Mice were habituated to their new environment and handled for one week before starting the experiments. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEFA-IMAS-UPF). All experiments were performed under blind conditions. Several spontaneous behavioural responses were evaluated in the same group of mice (locomotor activity, elevated plus-maze and lit-dark box). The spontaneous nociceptive threshold was then evaluated after thermal stimuli (tail-immersion and hot-plate tests). One week later, morphine dependence experiments were started in these mice.

Drugs

Morphine was provided by the Ministerio de Sanidad y Consumo (Spain). Naloxone was purchased from Sigma Chemical Co. (Madrid, Spain). These compounds were dissolved in physiological saline (0.9% NaCl).

Locomotor activity

The spontaneous locomotor responses were measured by using individual locomotor activity boxes ($9 \times 20 \times 11$ cm) (Imetronic, Bordeaux, France). Each box contained a line of photocells 2 cm above the floor to measure horizontal activity, and another line located 6 cm above the floor to measure vertical activity (rears), in a low luminosity environment (5 lux). Mice were individually placed in the boxes and locomotion was recorded during 15 min for three consecutive days ($n = 14$–15 animals per group in the experiment using $CB_1^{-/-}/A_{2a}^{-/-}$ mice; $n = 15$ animals per group in the experiments using CB_1 and A_{2a} single knockout mice).

Elevated plus-maze

The elevated plus-maze consisted of a black plastic apparatus with four arms (16×5 cm) set in a cross from a neutral central square (5×5 cm). Two opposite arms were delimited by vertical walls (closed arms), whereas the two other opposite arms had unprotected edges (open arms). The maze was elevated 30 cm above the ground and illuminated from the top (100 lux). At the beginning of the 5-min observation session, each mouse was placed in the central neutral area, facing one of the open arms. The total number of visits to the closed and open arms, and the cumulative time spent in the open and closed arms were then observed on a monitor through a videocamera system (ViewPoint, Lyon, France). Measurements in the open and closed arms were recorded when the mouse moved two forepaws and head into the arm. Mice were exposed to the test for three consecutive days ($n = 14$–15 animals per group in the experiment using $CB_1^{-/-}/A_{2a}^{-/-}$ mice; $n = 15$ animals per group in the experiments using CB_1 and A_{2a} single knockout mice).

Lit-dark box

Mice were individually exposed for 5 min to a box consisting of a small compartment ($15 \times 20 \times 25$ cm) with black walls and black floor dimly lit (5 lux) connected by a 4-cm long tunnel to a large compartment ($30 \times 20 \times 25$ cm) with white walls and floor, under intense illumination (500 lux). Lines were drawn on the floor of both compartments to allow measurement of locomotor activity by counting the number of squares (5×5 cm) crossed. Floor lines separated the lit compartment into three equal zones, from the tunnel to the opposite wall, designated as proximal, median and distal zone. Locomotor activity, time spent in and number of entries into each compartment as well as number of visits into each zone of the lit compartment, was recorded ($n = 14$ animals per group in the experiment using $CB_1^{-/-}/A_{2a}^{-/-}$ mice; $n = 19$–20 animals per group in the experiment using CB_1 knockout mice; $n = 15$ animals per group in the experiment using A_{2a} knockout mice).

Tail-immersion test

The tail-immersion test was measured as previously described (Simonin *et al.*, 1998). The water temperature was maintained at 48 °C, 50 °C or 52 ± 0.5 °C using a thermo regulated water circulating pump (Clifton, North Somerset, England). The mice were maintained in a cylinder and their tails were immersed in the heated water. The latency to a rapid flick of the tail was taken as the endpoint, and the cut-off latency was 10 s. Mice were exposed to the test for three consecutive days and a different water temperature was used each day ($n = 9$ animals per group in the experiment using $CB_1^{-/-}/A_{2a}^{-/-}$ mice; $n = 10$ animals per group in the experiment using CB_1 knockout mice; $n = 15$ animals per group in the experiment using A_{2a} knockout mice).

Hot-plate test

The hot plate test was performed as previously described (Simonin *et al.*, 1998). A glass cylinder (16×16 cm) was used to maintain the mice on the heated surface of the plate, which was kept at a temperature of 52 ± 0.1 °C (Columbus instruments, Columbus Ohio USA). The nociceptive threshold evaluated was the jumping response, and a 240-s cut-off was used to prevent tissue damage ($n = 9$ animals per group in the experiment using $CB_1^{-/-}/A_{2a}^{-/-}$ mice).

Induction of morphine dependence and withdrawal

Opioid dependence was induced by repeated injection (i.p.) of morphine HCl, as previously described (Maldonado *et al.*, 1997). Doses of morphine (twice a day) were progressively increased from 20 to 100 mg/kg over a period of 5 days. Behavioural observations were performed on day 6. On this day, mice were injected in the morning with morphine (100 mg/kg, i.p.), and the withdrawal syndrome was precipitated 2 h later by a naloxone injection (1 mg/kg, s.c.). Mice were observed during a period of 30 min and the manifestation of the different withdrawal signs was evaluated. Test chambers consisted of round boxes (30 cm diameter $\times$ 35 cm high) with white floor and moderate lighting. Mice were habituated for 15 min to the chambers before naloxone administration. Two types of signs were measured during abstinence. The number of jumps, wet dog shakes, paw tremors and sniffing were counted. Ptosis, diarrhoea, teeth chattering, body tremor and piloerection were evaluated over 5-min periods with a point being given for the presence of each sign during each period. The number of periods showing the sign were then counted (maximum score, 6). In order to summarize the results obtained from the different observations, a global withdrawal score was calculated individually for each mouse. To obtain this global value and to give all the signs a proportional weighting, the score obtained from each sign was multiplied by a constant as previously reported (Maldonado *et al.*, 1996) and as follows: jumping $\times$ 0.8; wet dog shakes $\times$ 1; diarrhoea $\times$ 1.5; paw tremor $\times$ 0.35; sniffing $\times$ 0.5; ptosis $\times$ 1.5; teeth chattering $\times$ 1.5; body tremor $\times$ 1.5 and piloerection $\times$ 1.5 ($n = 10$–13 animals per group in

the experiment using $CB_1^{-/-}/A_{2a}^{-/-}$ mice; $n = 9$–10 animals per group in the experiment using CB_1 knockout mice; $n = 16$–20 animals per group in the experiment using A_{2a} knockout mice).

Statistical analysis

The values obtained from locomotor activity and elevated-plus maze experiments were analyzed using a within subjects two-way ANOVA (time as within group factor and genotype as between group factor), followed by a one-way ANOVA when required. Somatic signs of morphine withdrawal were compared by using a between subjects two-way ANOVA (genotype and treatment as factors of variation), followed by a one-way ANOVA when applicable. For the lit-dark box, tail-immersion test and hot-plate test experiments, data were compared using the Student's *t*-test. Differences were considered significant if the probability of error was less than 5%.

Results

Spontaneous behavioural responses in $CB_1^{-/-}/A_{2a}^{-/-}$ mice

Locomotor activity

We have compared the responses of $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice on spontaneous locomotion during three consecutive days. Double mutant mice showed a reduction of locomotor activity on the second and third day (Fig. 1). Two-way ANOVA revealed a significant effect of genotype on the horizontal locomotor activity ($F_{1,81} = 17.32$, $P < 0.0001$), without time effect ($F_{2,81} = 2.14$; ns) and a significant interaction between these two factors ($F_{2,81} = 3.30$, $P < 0.05$). The comparison between genotypes indicated a significant reduction of horizontal locomotor activity in $CB_1^{-/-}/A_{2a}^{-/-}$ mice on the second and third day ($P < 0.01$; Fig. 1A).

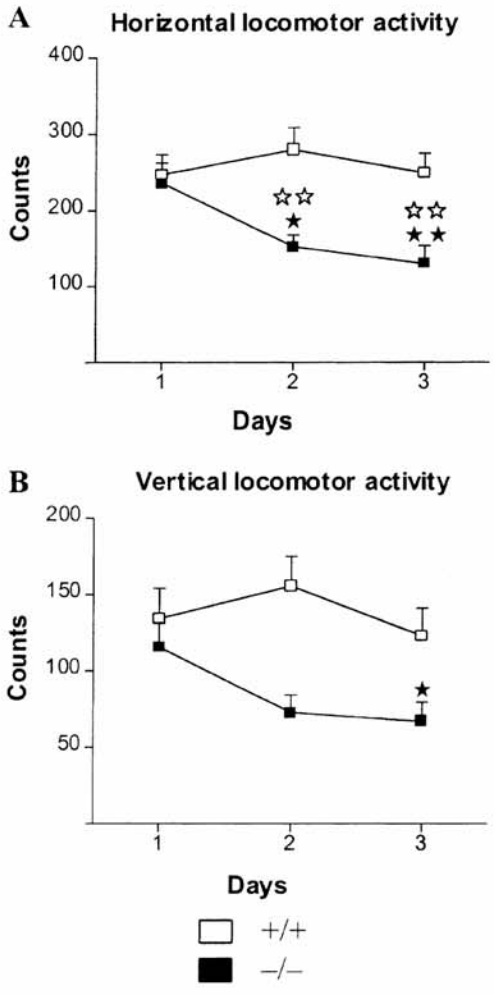

FIG. 1. Spontaneous locomotor responses in $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice.(A) Horizontal locomotor activity; (B) vertical locomotor activity. Data are expressed as mean ± SEM of photocell counts during a 15-min period in wild-type (white squares, $n = 15$) and double knockout (black squares, $n = 14$) mice. ★$P < 0.05$; ★★$P < 0.01$ when comparing to the first day of the same genotype; ☆☆$P < 0.01$ comparison between genotypes (Dunett's test) when a significant interaction between day and genotype was revealed by two-way ANOVA.

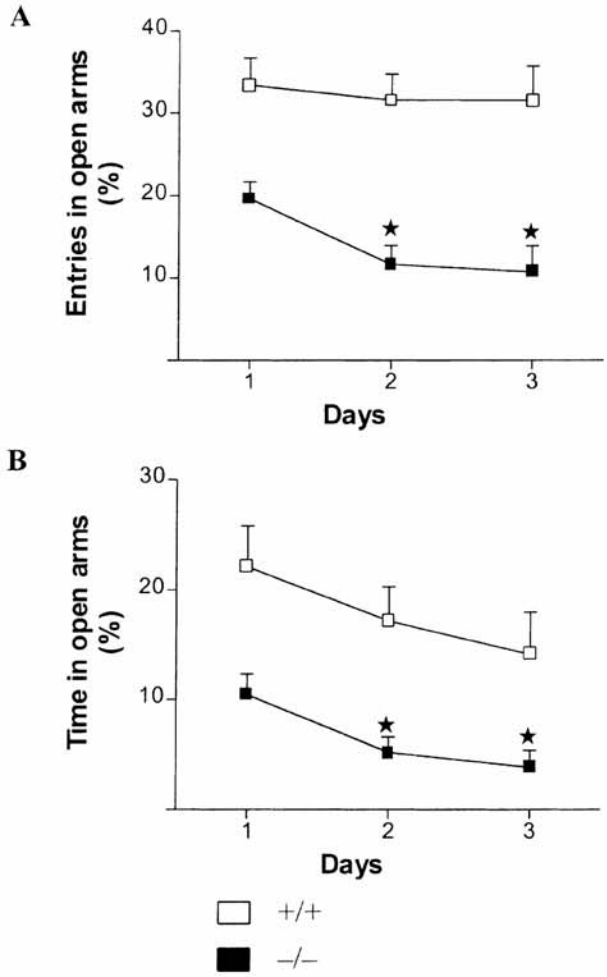

FIG. 2. Emotional-like responses exhibited by $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice in the elevated-plus maze. (A) Percentage of entries in open arms; (B) Percentage of time spent in open arms. Data are expressed as mean ± SEM in wild-type (white squares, $n = 15$) and double knockout (black squares, $n = 14$) mice. ★$P < 0.05$ when comparing to the first day of the same genotype. Two way ANOVA revealed a significant effect of genotype for the number of visits ($P < 0.0001$) and time spent ($P < 0.0001$) in open arms, although no significant interaction between day and genotype was revealed in any case.

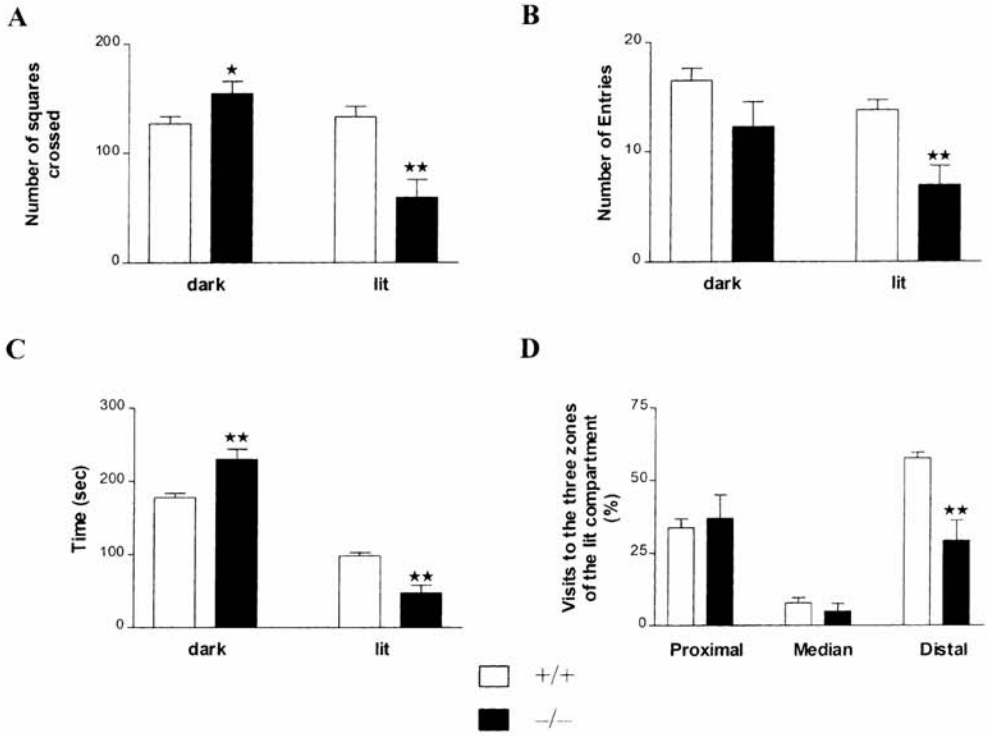

FIG. 3. Emotional-like responses exhibited by $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice in the lit-dark box. (A) Number of squares crossed in each compartment; (B) number of entries in each compartment; (C) time (s) spent in each compartment; (D) percentage of visits for each zone of the lit compartment. Data are expressed as mean ± SEM in wild-type (white bars) and double knockout (black bars) mice ($n = 14$ mice for each group). ★$P < 0.05$; ★★$P < 0.01$ comparison between genotypes (Student's *t*-test).

Two-way ANOVA calculated for vertical locomotor activity also showed a genotype effect ($F_{1,81} = 14.73$, $P < 0.001$) but not time effect ($F_{2,81} = 1.64$, ns) nor interaction between genotype and time ($F_{2,81} = 1.82$, ns). Double knockout mice exhibited a decrease of vertical locomotion as compared to wild-type mice on the second (53.28% of decrease) and third day (45.68% of decrease) (Fig. 1B).

Emotional-related responses

Emotional-related responses of $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice were investigated using two models of anxiety: the elevated plus-maze and the lit-dark box. Double mutant mice showed increased anxiety-like responses in both behavioural models (Figs 2 and 3) (Table 3).

In the elevated plus-maze, double knockout mice showed an increase in anxiety state during the three test days (Fig. 2). Two-way ANOVA calculated for the percentage of entries in open arms showed a significant effect of genotype ($F_{1,81} = 50.43$, $P < 0.0001$) no effect of time ($F_{2,81} = 1.82$, ns) and no interaction between these two factors ($F_{2,81} = 0.76$, ns). $CB_1^{-/-}/A_{2a}^{-/-}$ mice showed a decreased percentage of entries in the open arms compared with wild-type mice on day 1 (42.4% of decrease), day 2 (63.21% of decrease) and day 3 (66.03% of decrease) (Fig. 2A). Two-way ANOVA calculated for the percentage of time spent in open arms also showed a significant effect of genotype ($F_{1,81} = 24.21$, $P < 0.0001$), time ($F_{2,81} = 3.52$, $P < 0.05$) without interaction between these two factors ($F_{2,81} = 0.05$, ns). $CB_1^{-/-}/A_{2a}^{-/-}$ mice also showed a reduction in the percentage of time spent in the open arms compared with wild-type mice on day 1 (52.8% of decrease), day 2 (69,96% of decrease) and day 3 (72.82% of decrease) (Fig. 2B).

In the lit-dark box $CB_1^{-/-}/A_{2a}^{-/-}$ mice also exhibited an enhancement of anxiety in all parameters evaluated (Fig. 3) (Table 3). Thus, the number of squares crossed and entries in the lit compartment were lower in double mutants as compared to wild-type mice ($t = 3.936$, $P < 0.01$; $t = 3.518$, $P < 0.01$) (Fig. 3A and B). $CB_1^{-/-}/A_{2a}^{-/-}$ mice spent less time in the lit compartment ($t = 4.348$, $P < 0.01$) and showed fewer visits to the distal zone of the lit compartment ($t = 3.962$, $P < 0.01$) (Fig. 3C and D).

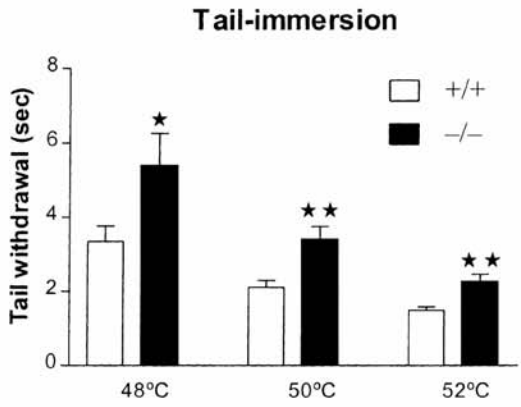

FIG. 4. Spontaneous nociceptive thresholds of $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice in the tail-immersion test. Data are expressed as mean ± SEM of tail withdrawal latency (s) in wild-type (white bars) and double knockout (black bars) mice ($n = 9$ mice for each group). ★$P < 0.05$; ★★$P < 0.01$ comparison between genotypes (Student's *t*-test).

TABLE 1. Two-way analysis of variance (ANOVA) of behavioural signs of morphine withdrawal after naloxone administration in A_{2a} knockout and wild-type mice

	Treatment		Genotype		Interaction	
Withdrawal signs	$F_{(1,65)}$	P	$F_{(1,65)}$	P	$F_{(1,65)}$	P
Jumping	583.90	0.0001	17.39	0.0001	14.86	0.001
Wet Dog Shakes	424.71	0.0001	31.57	0.0001	46.59	0.0001
Paw tremor	476.47	0.0001	27.20	0.0001	27.86	0.0001
Sniffing	487.95	0.0001	79.78	0.0001	82.16	0.0001
Tremor	2547.86	0.0001	17.67	0.0001	10.58	0.01
Ptosis	3401.25	0.0001	3.26	ns	31.97	0.0001
Diarrhoea	49.43	0.0001	221.11	0.0001	18.85	0.0001
Teeth chattering	2407.96	0.0001	41.22	0.0001	50.19	0.0001
Piloerection	7878.88	0.0001	8.95	0.01	0.63	ns
Global withdrawal score	2651.00	0.0001	87.32	0.0001	75.83	0.0001

The factors of variation were treatment and genotype (between subjects). ns, not significant.

TABLE 2. Two-way analysis of variance (ANOVA) of behavioural signs of morphine withdrawal after naloxone administration in CB_1/A_{2a} double knockout and wild-type mice

	Treatment		Genotype		Interaction	
Withdrawal signs	$F_{(1,39)}$	P	$F_{(1,39)}$	P	$F_{(1,39)}$	P
Jumping	36.95	0.0001	7.88	0.01	6.36	0.05
Wet Dog Shakes	7.32	0.01	0.64	ns	0.003	ns
Paw tremor	11.67	0.01	0.67	ns	1.93	ns
Sniffing	3.06	ns	0.06	ns	0.77	ns
Tremor	40.88	0.0001	9.00	0.01	0.45	ns
Ptosis	97.73	0.0001	2.05	ns	0.10	ns
Diarrhoea	9.15	0.01	0.46	ns	0.46	ns
Teeth chattering	57.43	0.0001	0.94	ns	0.005	ns
Piloerection	62.96	0.0001	9.13	0.01	3.32	ns
Global withdrawal score	130.60	0.0001	5.03	0.05	0.05	ns

The factors of variation were treatment and genotype (between subjects). ns, not significant.

TABLE 3. Comparative responses observed in A_2a and CB_1 single, as well as double CB_1/A_2a knockout mice in different behavioural paradigms

	A_2a ko mice		CB_1 ko mice		CB_1/A_{2a} ko mice	
Tail-immersion	+/+	−/−	+/+	−/−	+/+	−/−
48 °C	3.16 ± 0.35	4.55 ± 0.48*	–	–	3.34 ± 0.42	5.38 ± 0.86*
50 °C	2.60 ± 0.19	3.81 ± 0.25**	0.97 ± 0.08	1.04 ± 0.06	2.11 ± 0.18	3.39 ± 0.35**
52 °C	1.74 ± 0.13	2.58 ± 0.20**	0.85 ± 0.05	0.96 ± 0.06	1.48 ± 0.09	2.25 ± 0.21**
Locomotor activity						
Vertical	189.13 ± 12.33	164.66 ± 7.47	–	–	134.33 ± 19.64	115.35 ± 15.52
Horizontal	475.71 ± 36.30	388.26 ± 24.70*	151.40 ± 5.00	180.70 ± 4.60**	246.93 ± 26.64	236.00 ± 26.52
Plus maze						
Entries day 1 (%)	57.04 ± 1.55	47.71 ± 2.24**	56.70 ± 2.30	55.10 ± 1.80	33.39 ± 3.28	19.64 ± 2.08**
Entries day 2 (%)	55.52 ± 2.55	43.64 ± 3.99*	40.50 ± 3.10	41.20 ± 2.20	31.56 ± 3.12	11.61 ± 2.33**
Entries day 3 (%)	54.40 ± 2.92	41.02 ± 6.82	38.10 ± 1.50	33.20 ± 2.20	31.53 ± 4.13	10.71 ± 3.20**
Time day 1 (%)	50.58 ± 4.43	35.10 ± 3.79*	9.50 ± 1.00	12.70 ± 1.50	22.14 ± 3.67	10.43 ± 1.87**
Time day 2 (%)	41.49 ± 5.38	21.77 ± 3.96**	8.01 ± 0.70	10.20 ± 1.10	17.18 ± 3.13	5.16 ± 1.40**
Time day 3 (%)	35.39 ± 6.75	30.67 ± 7.85	7.80 ± 1.20	9.1 ± 0.90	14.13 ± 3.82	3.84 ± 1.49**
Lit-dark box						
Time black	139.05 ± 8.69	149.68 ± 10.20	144.47 ± 6.08	188.20 ± 6.82**	177.64 ± 5.68	229.85 ± 13.80**
Time white	111.89 ± 7.59	99.53 ± 8.79	116.47 ± 5.39	73.05 ± 7.31**	98.07 ± 4.57	47.07 ± 10.80**
Squares black	96.64 ± 4.87	100.80 ± 5.51	89.36 ± 2.72	122.75 ± 6.86**	126.85 ± 6.76	154.35 ± 11.45*
Squares white	138.00 ± 11.52	94.20 ± 9.64**	124.89 ± 11.24	59.65 ± 9.61**	133.14 ± 9.68	59.28 ± 16.06**
Entries black	17.64 ± 0.76	15.67 ± 1.00	14.26 ± 0.47	13.70 ± 0.95	16.50 ± 1.10	12.28 ± 2.27
Entries white	19.36 ± 1.23	15.00 ± 1.25*	14.10 ± 0.61	9.55 ± 1.07**	13.78 ± 0.88	6.92 ± 1.73**
GWS						
Saline	5.20 ± 0.96	6.15 ± 0.96	4.50 ± 1.00	6.50 ± 1.10	13.38 ± 4.23	2.50 ± 1.15*
Morphine	69.06 ± 6.38†	96.00 ± 10.57*†	99.50 ± 2.60†	53.50 ± 5.90**†	62.59 ± 4.18†	53.74 ± 5.96†

Mean values ± SEM are shown for tail-immersion test (tail withdrawal latency (sec)), locomotor activity (photo beam counts), plus-maze (percentage of entries and percentage of time in the open arms), lit-dark box [time (sec), squares (number), entries (number)] and the global withdrawal score (GWS). The results in each behavioural paradigm were obtained under identical experimental conditions (see material and methods section for details). *$P < 0.05$; **$P < 0.01$ (+/+ vs -/-); †$P < 0.01$ (saline vs morphine) (unpaired Student's t-test).

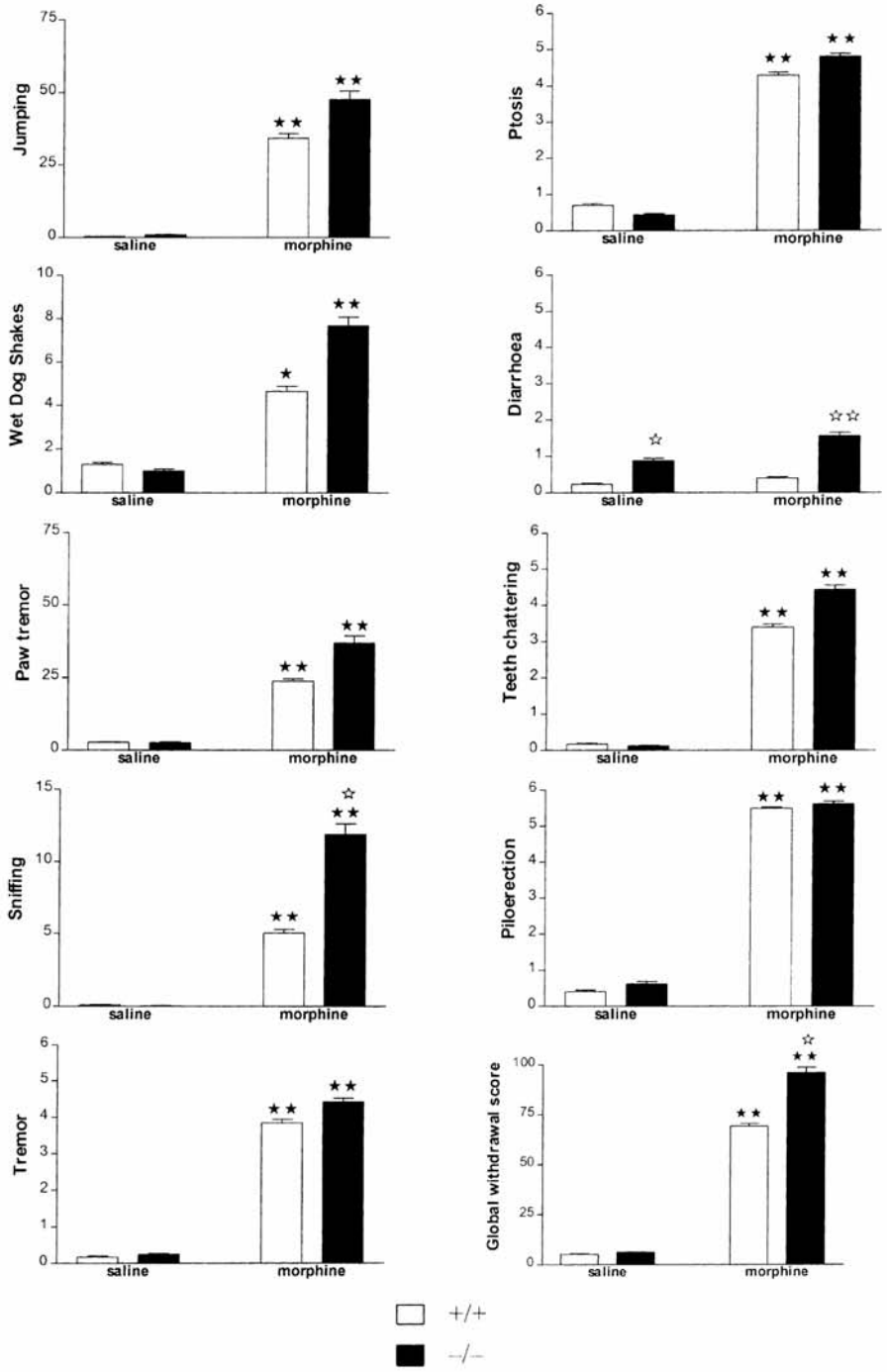

FIG. 5. Naloxone-precipitated morphine withdrawal signs in A_{2a} knockout and wild-type mice. Abstinence was precipitated by the administration of naloxone (1 mg/kg, s.c.) in mice receiving a chronic administration of morphine for 6 days. Counted (jumping, wet dog shakes, paw tremor and sniffing) and checked (ptosis, diarrhoea, teeth chattering and piloerection) signs of withdrawal were observed 30 min immediately after the naloxone administration. The global withdrawal score was calculated for each animal by giving each individual sign a relative weight. Data are expressed as mean ± SEM in wild-type (white bars, $n = 17$ saline group; $n = 20$ morphine group) and knockout (black bars, $n = 16$, saline and morphine groups). ★$P < 0.05$; ★★$P < 0.01$ when compared to the saline group of the same genotype. ☆$P < 0.05$; ☆☆$P < 0.01$ comparison between genotypes (one-way ANOVA) when a significant interaction between treatment and genotype was revealed by two-way ANOVA.

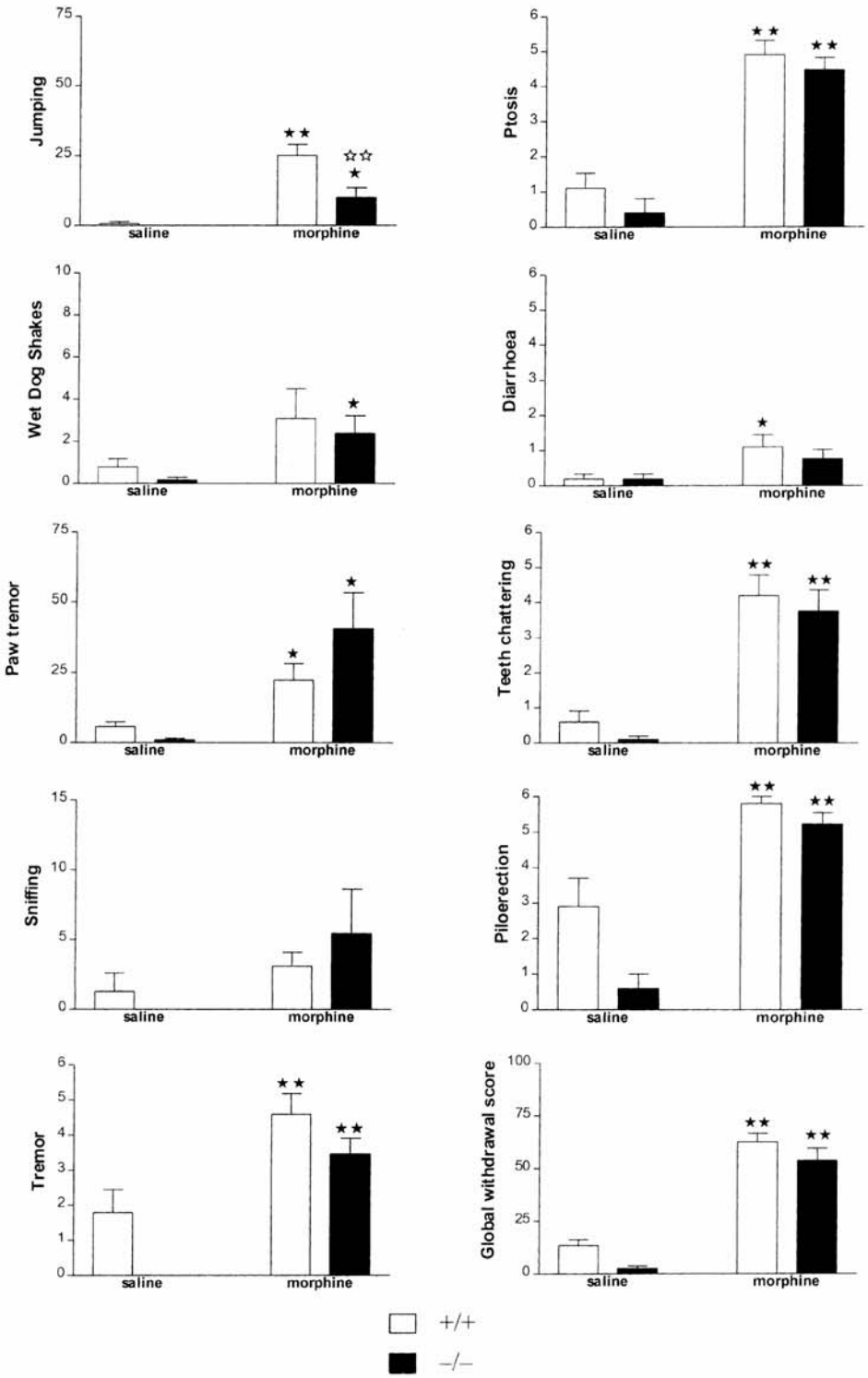

FIG. 6. Naloxone-precipitated morphine withdrawal signs in $CB_1^{-/-}/A_{2a}^{-/-}$ and wild-type mice. Abstinence was precipitated by the administration of naloxone (1 mg/kg, s.c.) in mice receiving a chronic administration of morphine for 6 days. Counted (jumping, wet dog shakes, paw tremor and sniffing) and checked (ptosis, diarrhoea, teeth chattering and piloerection) signs of withdrawal were observed 30 min immediately after the naloxone administration. The global withdrawal score was calculated for each animal by giving each individual sign a relative weight. Data are expressed as mean ± SEM in wild-type (white bars, $n = 10$, saline and morphine groups) and double knockout (black bars, $n = 10$, saline group; $n = 13$, morphine group). ★$P < 0.05$; ★★$P < 0.01$ when compared to the saline group of the same genotype. ☆$P < 0.05$; ☆☆$P < 0.01$ comparison between genotypes (one-way ANOVA), when a significant interaction between treatment and genotype was revealed by two-way ANOVA.

Nociceptive responses

Reactivity to pain was investigated in double knockout and control mice by using a tail-immersion test that involved thermal stimuli. $CB_1^{-/-}/A_{2a}^{-/-}$ mice showed a decreased response to acute pain stimulus at all temperatures evaluated (48 °C, $P < 0.05$; 50 °C and 52 °C, $P < 0.01$) (Fig. 4) (Table 3), indicating the presence of a higher nociceptive threshold in double deficient mice. The same tendency was observed in the hot-plate test as the latency of jumping response was higher in double mutant as compared to wild-type animals (25.77% of increase). However, no significant differences between genotypes were observed in the hot-plate test (data not shown).

Evaluation of morphine withdrawal in A_{2a} knockout mice and $CB_1^{-/-}/A_{2a}^{-/-}$ mice

Opioid withdrawal syndrome was precipitated by the administration of naloxone (1 mg/kg, s.c.) in mice receiving a chronic treatment of increasing doses of morphine from 20 to 100 mg/kg for six consecutive days. No signs of withdrawal were observed in any group of mice during the behavioural observation performed before the administration of naloxone (data not shown). A significant incidence of several behavioural signs of abstinence was observed after naloxone administration in all the groups of morphine-dependent mice. The severity of morphine withdrawal was increased in the absence of A_{2a} adenosine receptors (Table 1; Fig. 5). Indeed, we found a significant increase of diarrhoea ($P < 0.01$), sniffing ($P < 0.05$) and the global withdrawal score ($P < 0.05$) in A_{2a} knockout mice in comparison with wild-type animals (Fig. 5). In contrast, the manifestations of morphine withdrawal syndrome were not modified in $CB_1^{-/-}/A_{2a}^{-/-}$ mice (Table 2; Fig. 6). Although double mutant mice showed a lower incidence of jumping as compared to wild-type mice ($P < 0.01$), the other signs and the global withdrawal score were similar in both genotypes (Fig. 6) (Table 3).

Discussion

This study evaluates the spontaneous behavioural and nociceptive responses, and the expression of morphine withdrawal syndrome in mice with a double mutation lacking the CB_1 cannabinoid receptor and the A_{2a} adenosine receptor. Spontaneous locomotion, anxiety-like and nociceptive responses were modified as a consequence of this mutation. Furthermore, CB_1 and A_{2a} receptors seem to play an opposite role in opioid physical dependence as revealed by a decreased morphine withdrawal syndrome in mice lacking CB_1 receptors (Ledent *et al.*, 1999), an increase in the manifestation of withdrawal in mice lacking A_{2a} receptors, and the absence of changes in $CB_1^{-/-}/A_{2a}^{-/-}$ mice. All these behavioural studies were compared with the results obtained in A_{2a} and CB_1 single knockout mice in the same paradigms. In order to make a direct comparison between double and single knockout mice all studies were performed under identical experimental conditions (Table 3).

An important decrease in spontaneous locomotor activity was observed in double mutants in comparison with wild-type mice. Both CB_1 and A_{2a} receptors are highly expressed in the striatum (Tsou *et al.*, 1997; Svenningsson *et al.*, 1998), where they could be involved in the physiological control of motor activity. CB_1 knockout mice have been reported to display hypoactivity in the open field test (Zimmer *et al.*, 1999) or an increased locomotion in a novel nonstressful environment (Ledent *et al.*, 1999; Valverde *et al.*, 2000b). Furthermore, CB_1 agonists are able to strongly decrease the locomotor activity in several animal species, including rodents (Crawley *et al.*, 1993; Romero *et al.*, 1995; Chaperon & Thiebot, 1999). An important reduction in spontaneous exploratory behaviour was also observed in A_{2a} knockout mice (Table 3) (Ledent *et al.*, 1997), although specific agonists of these receptors, such as CGS 21680, induce a similar decrease in locomotion in wild-type mice (Ledent *et al.*, 1997; Chen *et al.* 2001). In this sense, several studies suggest a potential therapeutic benefit of drugs acting on CB_1 and A_{2a} receptors as treatment for motor pathologies. Thus, the administration of A_{2a} receptor antagonists has been shown to improve symptoms of Parkinson's disease by enhancing D_2 receptor function (Ongini & Fredholm, 1996; Ikeda *et al.*, 2002), whereas cannabinoid receptor antagonists might also be useful to treat some of the symptoms of this disease (Porter & Felder, 2001; Silverdale *et al.*, 2001). Our results confirm the physiological relevance of A_{2a} and CB_1 receptors in the control of locomotor behaviour, and support their possible potential interest as therapeutic targets for motor disorders.

$CB_1^{-/-}/A_{2a}^{-/-}$ mice revealed higher spontaneous anxiety-like responses in two different anxiety-like behavioural models, the elevated plus maze and the lit-dark box. This response is not related to the decreased locomotor activity observed in these animals as no difference was observed between wild-type and double knockout mice when the number of squares crossed in the dark compartment of the lit-dark box was measured. In agreement with our data, both CB_1 and A_{2a} knockout mice have been reported to be more anxious as compared to wild-type controls (Table 3) (Ledent *et al.*, 1997; Martin *et al.*, 2002). In our experimental conditions, the severity of this anxiogenic-like response in double mutant mice was higher compared with the responses reported in each single knockout mouse line as significant effects were observed in these double mutant mice for all the parameters evaluated (Table 3). This result suggests that both CB_1 and A_{2a} receptors control anxiety-like responses in a similar manner, and a facilitation of this response is produced by the deletion of both receptors. The physiological involvement of the endogenous cannabinoid system in regulating emotional-like responses has been suggested by several pharmacological studies showing an anxiogenic-like effect of the CB_1 antagonist SR141716A (Navarro *et al.*, 1997; Berrendero & Maldonado, 2002). On the other hand, the nonselective adenosine antagonist caffeine has also been shown to induce anxiogenic-like effects in several animal models (File *et al.*, 1988; El Yacoubi *et al.*, 2000).

We found a higher nociceptive threshold in $CB_1^{-/-}/A_{2a}^{-/-}$ mice by using the tail-immersion test, and a similar tendency was observed in the hot-plate test. Controversial results have been reported on the spontaneous nociceptive threshold of knockout mice lacking CB_1 cannabinoid receptors. Thus, no differences in the nociceptive threshold between knockout and wild-type mice (Table 3) (Ledent *et al.*, 1999; Valverde *et al.*, 2000b), or hypoalgesia in mutant mice in the hot-plate and formalin tests (Zimmer *et al.*, 1999) have been described. These latter results are surprising as cannabinoid agonists elicit antinociception by both spinal and supraspinal routes (Martin & Lichtman, 1998), and have a potential usefulness in the alleviation of pain (Porter & Felder, 2001). The discrepancies between these findings could be due to the different genetic background used to generate each line of CB_1 knockout mice. Concerning the role of adenosine in nociception, it is known that this neurotransmitter acts through A_{2a} receptors located on sensory pain fibres to mediate pain perception. Indeed, the disruption of A_{2a} adenosine receptors revealed reduced pain responses in the hot-plate and the tail-flick tests (Table 3) (Ledent *et al.*, 1997). The results obtained in these knockout mice support the possible therapeutic indication of A_{2a} antagonists as analgesic drugs (Snyder, 1997). In addition, the absence of CB_1 receptors does not seem to influence the hypoalgesia revealed by the disruption of A_{2a} receptors as we obtained a similar increase of nociceptive threshold in double mutant mice (Table 3).

We also investigated the role of CB_1 and A_{2a} receptors in opioid physical dependence by using mice lacking A_{2a} adenosine receptors and double mutant $CB_1^{-/-}/A_{2a}^{-/-}$ mice. Several studies have suggested that endogenous adenosine receptors play a role in the expression of morphine withdrawal. Thus, the enhancement of extracellular adenosine levels induced by adenosine kinase inhibitors reduced several opioid withdrawal signs and this attenuation was blocked by the nonselective adenosine antagonist caffeine, suggesting a selective involvement of adenosine receptors (Kaplan & Coyle, 1998). Higher concentrations of the adenosine metabolites, hypoxanthine and inosine were also detected in the nucleus accumbens during naloxone-precipitated morphine withdrawal (Salem & Hope, 1999). Furthermore, an electrophysiological study has recently reported that caffeine increases the activity of the paragigantocellularis nucleus, a structure known to participate in the somatic expression of opioid abstinence, more pronouncedly in morphine-dependent rats than in control animals (Khalili *et al*., 2001). Pharmacological studies showed that both A_1 and A_{2a} selective adenosine receptor agonists decreased the incidence of some morphine withdrawal signs, such as wet-dog shakes and diarrhoea, whereas the administration of adenosine antagonists produced the opposite responses (Kaplan & Sears, 1996; Salem & Hope, 1997; Zarrindast *et al*., 1999). In agreement with these findings, we found an enhancement in the expression of morphine withdrawal in the absence of A_{2a} receptors as revealed by a higher global abstinence score in knockout morphine-dependent mice. While an inhibitory effect on up-regulated cAMP pathway associated with opioid withdrawal has been proposed to explain the effects of A_1 adenosine agonists (Kaplan & Coyle, 1998), further experiments will be necessary to elucidate the mechanisms by which A_{2a} receptors mediate this response. On the other hand, CB_1 knockout mice presented a decrease in the expression of naloxone-precipitated morphine withdrawal (Table 3) (Ledent *et al*., 1999), demonstrating that CB_1 receptors are required to obtain a complete manifestation of the somatic signs of opioid abstinence. The severity of morphine physical dependence was unaltered in mice lacking both CB_1 and A_{2a} receptors, confirming that these receptors act in an opposite manner to regulate opioid withdrawal response. The lack of changes in $CB_1^{-/-}/A_{2a}^{-/-}$ mice obtained in the present study could indicate a balance of the opposite responses observed in each single knockout mouse line.

In summary, the present results show that both CB_1 and A_{2a} receptors have similar effects on anxiety-like responses and opposite effects on opioid withdrawal. Furthermore, the phenotype previously reported for A_{2a} knockout mice on spontaneous locomotion and nociceptive responses was not modified in double mutants also lacking CB_1 cannabinoid receptors.

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Effects of nandrolone on acute morphine responses, tolerance and dependence in mice

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Abstract

Anabolic–androgenic steroid exposure has been proposed to present a risk factor for the misuse of other drugs of abuse. We now examined whether the exposure to the anabolic–androgenic steroid, nandrolone, would affect the acute morphine responses, tolerance and dependence in rodents. For this purpose, mice received nandrolone using pre-exposure (for 14 days before morphine experiments) or co-administration (1 h before each morphine injection) procedures. Nandrolone treatments increased the acute hypothermic effects of morphine without modifying its acute antinociceptive and locomotor effects. Nandrolone also attenuated the development of tolerance to morphine antinociception in the hot plate test, but did not affect tolerance to its hypothermic effects, nor the sensitisation to morphine locomotor responses. After nandrolone pre-exposure, we observed an attenuation of morphine-induced place preference and an increase in the somatic manifestations of naloxone-precipitated morphine withdrawal. These results indicate that anabolic–androgenic steroid consumption may induce adaptations in neurobiological systems implicated in the development of morphine dependence.

Keywords: Anabolic–androgenic steroid; Nandrolone; Hypothermia; Morphine tolerance; Reward; Dependence; (Mouse)

1. Introduction

Anabolic–androgenic steroids are synthetic derivates of testosterone widely used in the clinic as androgen replacement therapy and as chemotherapy for certain types of cancer (Kamel et al., 2001; Wilson and Griffin, 1980). However, there is also a wide nonmedical use of these steroids by athletes and others that has evoked considerable concern (Wilson, 1988). Indeed, during the last five decades, anabolic–androgenic steroids have been used at doses 10–100 times the therapeutic dose by many athletes and bodybuilders to enhance their physical performance, increase muscle mass and intensify training regimens (Kuhn, 2002; Lukas, 1993; Wilson, 1988; Yesalis and Bahrke, 1995). The original goal in making these drugs was to promote the anabolic effects of testosterone without its androgenic qualities. Although the androgenic effects were reduced, they were not eliminated, and this remains one of the main problems with anabolic steroids today (Marshall, 1988). Androgen action is linked to its ability to bind and activate specific androgen receptors (Falkenstein et al., 2000) throughout various regions of the brain, suggesting that anabolic–androgenic steroids may be involved in a wide variety of neural functions (Simerly et al., 1990). Effects of anabolic–androgenic steroids on physical and mental health have been widely reported (Pope and Katz, 1994). Thus, the development of psychological side-effects such as psychosis, increased irritability, hostility, aggression, loss of inhibition, lack of judgement, mood swings and increased self-esteem have been described in association with anabolic–androgenic steroid intake (Bahrke et al., 1996; Williamson and Young, 1992).

Interestingly, a concurrent abuse of anabolic–androgenic steroids has recently been reported among addicts not connected to sports (DuRant et al., 1993; Kindlundh et al., 1999; Lukas, 1993; Yesalis and Bahrke, 1995). Several studies have suggested the association between use of

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anabolic–androgenic steroids and consumption of alcohol, tobacco and psychotropic substances such as cannabis, opiates, amphetamine and ecstasy (DuRant et al., 1993; Kindlundh et al., 1999; Yesalis and Bahrke, 1995). Moreover, results of animal studies have indicated that anabolic–androgenic steroids evoke neurobiochemical alterations related to behavioural responses and reward in rats (Clark et al., 1996; Hallberg et al., 2000; Le Grevès et al., 1997; Schlussman et al., 2000; Thiblin et al, 1999), particularly by affecting the endogenous opioid and dopamine systems (Johansson et al., 1997, 2000a,b; Lukas, 1993; Menard et al., 1995). Based on these data, it has been proposed that anabolic–androgenic steroid may serve as "gateway" drugs to opioid dependence (Arvary and Pope, 2000).

The aim of this study was to use different behavioural models in mice in order to evaluate the effects of the anabolic–androgenic steroid, nandrolone, on acute morphine effects and on several behavioural responses induced by repeated morphine administration and related to its addictive properties. For this purpose, we first evaluated the influence of nandrolone on the changes in nociception, body temperature and locomotor activity induced by acute morphine administration. In a second step, we evaluated the development of tolerance to morphine antinociception, sensitisation to its locomotor effects and conditioned place preference after repeated morphine administration. We also investigated whether nandrolone exposure affects expression of the naloxone-precipitated morphine withdrawal syndrome.

2. Material and methods

2.1. Animals

Albino male CD-1 mice (CRIFFA, France) weighing 20–22 g after arrival were housed in cages of 10 and maintained at a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%). The mice were given access to food and water ad libitum. Lighting was maintained at 12-h cycles (on at 8 a.m. and off at 8 p.m.). All the experiments were performed during the light phase of the dark/light cycle. The animals were habituated to the experimental room and handled for 1 week before the start of the experiments. All animal procedures met the guidelines of the National Institute of Health detailed in the "Guide for the Care and Use of Laboratory Animals", the European Communities directive 86/609/EEC regulating animal research and of the Local Ethical Committees.

2.2. Drugs

Morphine was provided by the Ministerio de Sanidad y Consumo (Spain). The anabolic–androgenic steroid, nandrolone decanoate, and naloxone were purchased from Sigma (Spain). Nandrolone was diluted in vehicle preparation (10% ethanol/10% cremophor EL/80% distilled water). All other compounds were dissolved in saline (0.9%). All the compounds were administered in a volume of 10 ml/kg.

2.3. Experimental procedure

Four series of experiments were performed in order to evaluate the effects of nandrolone treatment on acute and chronic morphine effects. Two different protocols (co-administration or pre-exposure) were used for nandrolone administrations in an attempt to represent conditions similar to its use by drug addicts (drug intake concomitant to nandrolone use or drug intake with a past of long-term consumption of nandrolone). In the first protocol (pre-exposure), nandrolone (15 mg/kg, i.m.) was administered chronically once daily for 14 days before starting morphine treatment. In the second protocol (co-administration), nandrolone (15 mg/kg, i.m.) was administered 1 h before each morphine injection. In the pre-exposure protocol, the intramuscular injections were given in the left and right hind legs, alternatively. A supratherapeutic dose of nandrolone was used (i) because this mimics the dose self-administered by heavy nandrolone abusers (Williamson and Young, 1992) and (ii) because it has previously been shown to induce biochemical changes in the endogenous opioid and dopamine systems in rodents (see Johansson et al., 1997).

All the experiments included four groups, receiving vehicle and morphine, nandrolone and morphine, vehicle and saline, or nandrolone and saline.

2.3.1. Acute pharmacological effects of morphine

The acute effects induced by different doses of morphine (1, 3 or 9 mg/kg, s.c.) on nociception, locomotion and body temperature were evaluated in each experimental group.

Two nociceptive tests were used, the tail immersion and the hot plate. In the tail immersion test, mice were gently placed in a restrainer cylinder. The nociceptive threshold was assessed as described previously (Janssen et al., 1963), by measuring the time to withdraw the tail immersed in a thermostated water bath (50 ± 0.1 °C) (Clifton, Scientific Instruments, England), with a cutoff latency of 15 s to prevent tissue damage. Nociceptive responses were also measured using a hot plate analgesia meter (Columbus, OH, USA). A glass cylinder (19 cm high, 19 cm diameter) was used to keep the mice on the heated surface of the plate, which was maintained at a temperature of 52 ± 0.1 °C. Two nociceptive thresholds, licking of the paws and jumping, were evaluated. The cutoff time was 30 and 240 s, respectively.

Locomotor responses were evaluated using locomotor activity boxes (9 × 20 × 11 cm; Imetronic, Lyon, France). The boxes contained a line of photocells 2 cm above the floor to measure horizontal movements and another line located 6 cm above the floor to measure vertical activity (rearing). On the experimental day, the mice were individually placed in the boxes and the ambulatory, horizontal

(ambulatory movements plus small movements) and vertical activities were recorded for 10 min in a low luminosity environment (5–15 lx).

Rectal temperature was measured in each mouse using an electronic thermocouple flexible probe (Panlab, Barcelona, Spain). The probe was placed 3 cm into the rectum of the mice for 20 s before the temperature was recorded.

In order to habituate the animals to the test environment and to obtain a stable baseline, tail immersion response and locomotor activity were measured for 2 days before the experiment. On the experimental day, locomotor activity was evaluated 10 min after morphine injection (1, 3 or 9 mg/kg, s.c.) during 10 min. Then (i.e. 20 min after morphine), the tail immersion test was performed, immediately followed by the hot plate test. Rectal temperature was measured just before and 40 min after the morphine injection.

2.3.2. Tolerance to the morphine antinociceptive effects and sensitisation to its locomotor responses

As in the previous experiment, the basal tail immersion response and locomotor activity were evaluated for 2 days before the beginning of the chronic morphine treatment on day 1. Morphine (12 mg/kg, s.c.) was administered chronically twice a day (12-h interval) for 14 days (days 1–14). Locomotor activity was recorded 10 min after the morning morphine injection on days 1, 2, 4, 6, 8, 10 and 12. Changes in nociceptive threshold were assessed 20 min after morning injection of 3 and 9 mg/kg (s.c.) of morphine using the tail immersion test (days 0, 7 and 14) and the hot plate test (day 14). The morning of day 7, animals received only this morphine injection. Morphine-induced changes in body temperature were also evaluated on day 14. Data for antinociceptive tolerance were expressed as the percentage of maximal possible effect (%MPE). The calculation of the %MPE was performed using the following formula: (test latency − control mean latency)/(cutoff − control mean latency) × 100.

2.3.3. Rewarding effects of morphine

The rewarding effects of morphine were evaluated using the conditioned place preference paradigm, as previously described (Maldonado et al., 1997). The place preference apparatus consisted of two different cubic compartments (15 × 15 × 15 cm) separated by a triangular central neutral area (15 cm per side). The place preference conditioning schedule consisted of three phases. During the preconditioning phase, the mouse was placed in the middle of the neutral area and allowed to explore both compartments, and the time spent in each compartment was measured for 18 min. After the session, animals were randomised for pairing to drug or vehicle administration and for assignment to a compartment. Care was taken to ensure that treatments were counterbalanced as closely as possible between compartments. During the conditioning phase, the animals were treated for 6 consecutive days with an injection of morphine (5 mg/kg, s.c.) or saline. Doors matching the walls of the compartment allowed confinement of the mice for 20 min immediately after morphine or saline injections. Mice received morphine on days 1, 3 and 5 and vehicle on days 2, 4 and 6. Control animals received vehicle every day. Finally, the test phase was conducted 24 h after the last conditioning session exactly as was the preconditioning phase, i.e. free access to both compartments and the time spent in each compartment measured for 18 min. A score was calculated for each mouse as the difference between the post-conditioning and the pre-conditioning time spent in the drug-paired compartment.

2.3.4. Naloxone-precipitated morphine withdrawal

The naloxone-precipitated withdrawal syndrome in morphine-dependent mice was evaluated as previously described (Maldonado et al., 1997). Morphine was injected i.p. twice a day (9 a.m. and 9 p.m.) for 6 days. The morphine dose was progressively increased as follows: 1st day, 20 mg/kg; 2nd day, 40 mg/kg; 3rd day, 60 mg/kg; 4th day, 80 mg/kg; 5th day, 100 mg/kg; 6th day (only morning injection), 100 mg/kg. Control mice were treated with saline under the same conditions. Withdrawal was precipitated in each animal by injecting naloxone (1 mg/kg, s.c.) 2 h after the last morphine administration. The animals were placed individually into test chambers to evaluate the behavioural signs of withdrawal. The chambers consisted of transparent round plastic boxes (30 cm in diameter and 50 cm in height) with a white floor. Behaviour was observed in two sessions: the first session was during the 15 min preceding naloxone injection and the second session was for 30 min immediately after this injection. The wet dog shakes, jumping, paw tremor and sniffing were counted. Teeth chattering, piloerection and ptosis were evaluated over 5-min periods, 1 point being given for the presence of each sign during each period. The periods with a sign were then counted (maximum score: 6). Body weight was determined before and 30 min after naloxone injection. Taking into account all the individual signs, a global withdrawal score was calculated for each animal by using a range of possible scores from 0 to 100, as previously reported (Maldonado et al., 1992a).

2.4. Statistical analysis

For the analysis of the data obtained for acute morphine pharmacological responses, a one-way analysis of variance (ANOVA) was first used to determine the doses of morphine that produced significant effects. A two-way ANOVA was then performed to evaluate the influence of the nandrolone treatment in the groups of mice where significant morphine effects were observed. Consecutive post hoc comparisons, using Dunnett's test, were performed when appropriate. The development of tolerance to morphine antinociception in the hot plate test and the effects on the naloxone-precipitated morphine withdrawal syndrome were analysed by using a two-way ANOVA with nandrolone/vehicle and morphine/saline treatments as factors of variation. One-way ANOVAs

were used to reveal main morphine or nandrolone effect. Data from the study of development of tolerance to morphine antinociception in the tail immersion test and sensitisation to locomotor activities were analysed using a three-way ANOVA with one within-subjects (time), and two between-subjects (nandrolone/vehicle and morphine/saline treatments) factors of variation. When required, the three-way ANOVA was followed by two-way and one-way ANOVAs, as well as by post hoc comparisons (Dunnett's test). In conditioning place preference experiments, individual comparisons of time spent in the drug-paired compartment during preconditioning and test phases were made in each experimental group by using the paired two-tailed Student's *t*-test. Additionally, one-way ANOVA was used to compare the score values, followed by consecutive post hoc comparisons (Dunnett's test). The statistical significance criterion was $P<0.05$.

3. Results

3.1. Effects of nandrolone treatments on acute morphine responses

The influence of nandrolone pre-exposure and co-administration on the acute pharmacological effects of morphine (1, 3 and 9 mg/kg, s.c.) was evaluated by measuring the changes induced in nociception, locomotor activity and body temperature. Control experiments showed that nandrolone had no intrinsic effect when injected in combination with saline on any of the parameters studied (data not shown).

Chronic nandrolone pre-exposure for 14 days did not influence acute morphine effects on nociception as evaluated in tail immersion (Fig. 1A) and hot plate (Fig. 1B and C) tests. In the tail immersion test, significant antinociceptive effects of 3 and 9 mg/kg of morphine were revealed in vehicle-pre-exposed mice (one-way ANOVA, $F(3,39)=18.14$, $P<0.001$). Two-way ANOVA revealed a significant morphine effect ($F(2,59)=25.46$, $P<0.001$) but no nandrolone effect ($F(1,59)=1.56$, nonsignificant, NS) and no morphine/nandrolone interaction ($F(2,59)=1.18$, NS). For the licking threshold in the hot plate test, significant antinociceptive effects of 3 and 9 mg/kg of morphine were revealed in vehicle pre-exposed mice (one-way ANOVA, $F(3,38)=14.25$, $P<0.001$). Two-way ANOVA revealed a significant morphine effect ($F(2,59)=51.70$, $P<0.001$), no nandrolone effect ($F(1,59)=0.04$, NS) and a significant morphine/nandrolone interaction ($F(2,59)=4.24$, $P<0.05$), but subsequent one-way ANOVA revealed no significant differences between the different vehicle and nandrolone pre-exposed groups (morphine 3 mg/kg: $F(1,19)=3.06$, NS; morphine 9 mg/kg: $F(1,18)=3.83$, NS). For the jumping threshold in the hot plate test, significant antinociceptive effects of 3 and 9 mg/kg of morphine were revealed in vehicle-co-treated mice (one-way ANOVA, $F(3,37)=26.66$, $P<0.001$). Two-way ANOVA revealed a significant morphine effect ($F(2,51)=154.21$, $P<0.001$) but no nandrolone effect ($F(1,51)=0.01$, NS) and no morphine/nandrolone interaction ($F(2,51)=0.76$, NS). Similar morphine dose-dependent antinociceptive responses were obtained in both vehicle and nandrolone-co-treated mice as shown by the nociceptive threshold in the tail immersion (Fig. 1E) and hot plate (Fig. 1F and G) tests. In the tail immersion test, significant antinociceptive effects of 3 and 9 mg/kg of morphine were revealed in vehicle-co-treated mice (one-way ANOVA, $F(3,68)=24.59$, $P<0.001$). Two-way ANOVA revealed a significant morphine effect ($F(2,96)=42.57$, $P<0.001$) but no nandrolone effect ($F(1,96)=2.56$,

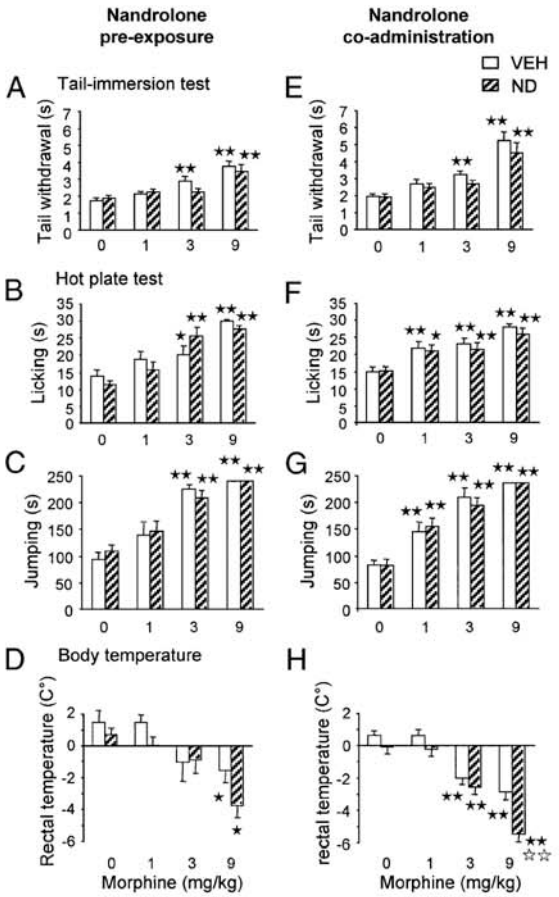

Fig. 1. Acute effects of morphine (1, 3 and 9 mg/kg, s.c.) on nociception (A–C and E–G) and body temperature (D and H) in mice pre-exposed to or co-treated with nandrolone (ND) or vehicle (VEH). Two behavioural tests were used to evaluate changes in nociception: the tail immersion (A and E) and hot plate (licking threshold: B and C; jumping threshold: F and G) tests. Antinociceptive effects were evaluated 20 min after morphine injection. The rectal temperature was measured just before and 40 min after morphine injection. Number of mice per group in the nandrolone co-treatment experiments = 19–20. Number of mice per group in the nandrolone pre-exposure experiments = 9–10. Data are expressed as means ± S.E.M. $^{\star}P<0.05$, $^{\star\star}P<0.01$ (Dunnett's test, comparison with the respective saline control group) and $^{\star\star}P<0.01$ (one-way ANOVA, VEH- vs. ND-treated mice).

NS) and no morphine/nandrolone interaction ($F(2,96) = 0.65$, NS). In the licking threshold in the hot plate test, significant antinociceptive effects of 1, 3 and 9 mg/kg of morphine were revealed in vehicle co-treated mice (one-way ANOVA, $F(3,68) = 15.90$, $P < 0.001$). Two-way ANOVA revealed a significant morphine effect ($F(3,128) = 22.28$, $P < 0.001$) but no nandrolone effect ($F(1,128) = 1.38$, NS) and no morphine/nandrolone interaction ($F(3,128) = 0.17$, NS). For the jumping threshold in the hot plate test, significant antinociceptive effects of 1, 3 and 9 mg/kg of morphine were revealed in vehicle-co-treated mice (one-way ANOVA, $F(3,69) = 44.95$, $P < 0.001$). Two-way ANOVA revealed a significant morphine effect ($F(3,136) = 79.71$, $P < 0.001$) but no nandrolone effect ($F(1,136) = 0.07$, NS) and no morphine/nandrolone interaction ($F(3,136) = 0.43$, NS).

Nandrolone pre-exposure and co-administration had different effects on the hypothermic responses to morphine (Fig. 1D and H). In mice chronically pretreated with vehicle (Fig. 1D), significant hypothermia was found after treatment with the dose of 9 mg/kg of morphine (one-way ANOVA, $F(3,38) = 4.10$, $P < 0.05$). Two-way ANOVA revealed a significant hypothermic effect of 9 mg/kg of morphine in nandrolone pre-exposed mice, but no significant difference was found from the vehicle pre-exposed control group (morphine effect: $F(1,38) = 35.57$, $P < 0.001$; nandrolone effect: $F(1,38) = 5.59$, $P < 0.05$; and no morphine/nandrolone interaction: $F(2,38) = 1.29$, NS).

In the nandrolone co-administration groups, significant hypothermia was revealed after 3 and 9 mg/kg of morphine in vehicle co-treated mice (Fig. 1H, one-way ANOVA, $F(3,48) = 20.41$, $P < 0.001$). Two-way ANOVA revealed a significant morphine effect ($F(2,73) = 53.14$, $P < 0.001$), a nandrolone effect ($F(1,73) = 12.78$, $P < 0.01$) and a morphine/nandrolone interaction ($F(2,73) = 7.77$, $P < 0.05$). Subsequent one-way ANOVA revealed a significantly increased hypothermic effect of 9 mg/kg of morphine in nandrolone-co-treated mice as compared to the vehicle-co-treated group ($F(1,23) = 11.92$, $P < 0.01$).

Nandrolone pre-exposure and co-administration did not produce major changes in the effects of morphine on ambulatory, horizontal and vertical activities (data not shown). In nandrolone pre-exposed mice, two-way ANOVA revealed a significant morphine effect on ambulatory ($F(3,79) = 5.92$, $P < 0.01$), horizontal ($F(3,76) = 3.77$, $P < 0.05$) and vertical ($F(2,56) = 22.02$, $P < 0.001$) activities. However, no significant effect of nandrolone (ambulatory: $F(1,79) = 0.26$, NS; horizontal: $F(1,76) = 1.14$, NS; vertical: $F(1,56) = 1.79$, NS) and no morphine/nandrolone interaction (ambulatory: $F(3,79) = 0.35$, NS; horizontal: $F(3,76) = 1.15$, NS; vertical: $F(2,56) = 1.50$, NS) was observed in any case. In nandrolone co-treated mice, one-way ANOVA ($F(3,69) = 10.67$, $P < 0.01$) and Dunnett's test post hoc comparisons indicated that 9 mg/kg of morphine significantly ($P < 0.05$) increased ambulatory activity in vehicle co-treated mice. Two-way ANOVA revealed a significant morphine effect ($F(1,70) = 8.06$, $P < 0.01$) but no nandrolone effect ($F(1,66) = 2.87$, NS) and no morphine/nandrolone interaction ($F(1,66) = 1.12$, NS). One-way ANOVA ($F(3,68) = 3.55$, $P < 0.05$) and Dunnett's test post hoc comparisons revealed an inhibitory effect of 3 mg/kg of morphine ($P < 0.05$) on horizontal activity of vehicle-co-treated mice. Two-way ANOVA revealed a significant morphine effect ($F(1,66) = 11.46$, $P < 0.01$) but no nandrolone effect ($F(1,66) = 0.02$, NS) and no morphine/nandrolone interaction ($F(1,66) = 0.31$, NS). One-way ANOVA ($F(3,64) = 15.88$, $P < 0.001$) and Dunnett's test post hoc comparisons indicated that 1, 3 and 9 mg/kg of morphine significantly ($P < 0.001$) decreased vertical activity in vehicle co-treated mice. Two-way ANOVA revealed a significant morphine effect ($F(3,129) = 22.23$, $P < 0.001$) but no nandrolone effect ($F(1,129) = 0.48$, NS) and no morphine/nandrolone interaction ($F(3,129) = 1.10$, NS).

3.2. Effects of nandrolone treatments on the development of tolerance to morphine antinociception

Nandrolone pre-exposure and co-administration did not produce any change in the development of tolerance to antinociception produced by chronic morphine administration (12 mg/kg, s.c., twice a day for 14 days) as evaluated by the tail immersion test (Fig. 2). The changes in nociceptive threshold were evaluated on days 0, 7 and 14 of treatment. Nandrolone treatments did not modify the tail-withdrawal latencies in saline-treated control mice (Fig. 2). In morphine chronically treated mice, tolerance to the antinociceptive effects of morphine developed similarly in both vehicle and nandrolone-pre-exposed mice (Fig. 2A–C). Three-way ANOVA revealed a morphine effect ($F(2,79) = 43.61$, $P < 0.001$), a time effect ($F(2,158) = 41.89$, $P < 0.001$) and a morphine/time interaction ($F(4,158) = 14.95$, $P < 0.001$), but no nandrolone effect ($F(1,79) = 1.90$, NS) and no morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(2,79) = 1.70$, $F(2,158) = 0.53$ and $F(4,158) = 0.96$, respectively, NS). Similar results were obtained in morphine mice co-treated with nandrolone (Fig. 2D–F). Three-way ANOVA revealed a morphine effect ($F(2,87) = 39.50$, $P < 0.001$), a time effect ($F(2,174) = 12.15$, $P < 0.001$) and a morphine/time interaction ($F(4,174) = 6.59$, $P < 0.001$), but no nandrolone effect ($F(1,87) = 0.23$, NS) and no morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(2,87) = 0.29$, $F(2,174) = 1.26$ and $F(4,174) = 0.13$, respectively, NS).

On the contrary, a nandrolone effect was found on the development of tolerance to morphine antinociception as evaluated by the hot plate test on day 14 of opioid treatment (jumping threshold; Fig. 3). Tolerance to the antinociceptive effects of 9 mg/kg of morphine was observed in vehicle-pre-exposed mice (-47%), but not in nandrolone-pre-exposed animals (-6%). Two-way ANOVA revealed a significant time effect ($F(1,47) = 12.75$, $P < 0.01$), a nandrolone effect ($F(1,47) = 17.34$, $P < 0.01$) and a nandrolone/time interaction ($F(1,47) = 7.34$, $P < 0.01$). Significant differences be-

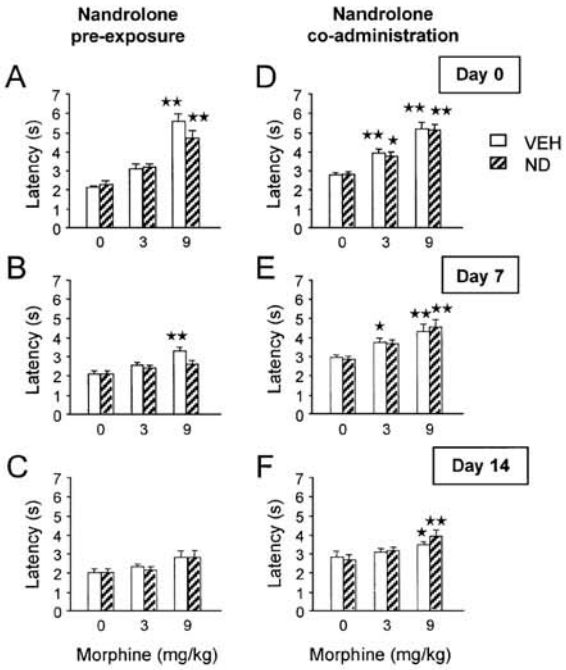

Fig. 2. Tolerance to morphine-induced antinociception in the tail immersion test in mice pre-exposed to (A–C) or co-treated with (D–F) nandrolone (ND) or vehicle (VEH). Mice received repeated morphine administration (12 mg/kg, s.c., twice a day for 12 days). Nociceptive threshold was evaluated 20 min after the injection of 3 and 9 mg/kg (s.c.) of morphine on the morning of days 0, 7 and 14. Number of mice per group in the nandrolone co-treatment experiments = 15. Number of mice per group in the nandrolone pre-exposure experiments = 10–16. Data are expressed as means ± S.E.M. $^{\star}P<0.05$, $^{\star\star}P<0.01$ (Dunnett's test, comparison with the respective saline control group) and $^{\star\star}P<0.01$ (one-way ANOVA, VEH- vs. ND-treated mice).

tween nandrolone- and vehicle-co-treated mice appeared on day 14 ($F(1,25)=6.83$, $P<0.01$). This effect of nandrolone pre-exposure was not observed with the lower dose of morphine (3 mg/kg). In this case, tolerance to antinociception developed to a similar degree in vehicle- (−86%) and nandrolone-pre-exposed mice (−74%). Two-way ANOVA revealed a significant time effect ($F(1,46)=62.59$, $P<0.001$), no nandrolone effect ($F(1,46)=0.01$, NS) and no nandrolone/time interaction ($F(1,46)=3.05$, NS).

In the co-administration experiments, nandrolone attenuated the development of tolerance to morphine antinociception for both 3 and 9 mg/kg doses of morphine (Fig. 3C and D). Tolerance to the antinociceptive effects of 3 mg/kg of morphine developed to a lower degree in nandrolone-co-treated mice (−74%) as compared to the vehicle-treated group (−100%). Two-way ANOVA revealed a significant time effect ($F(1,60)=79.39$, $P<0.001$), no nandrolone effect ($F(1,60)=0.78$, NS) and a significant nandrolone/time interaction ($F(1,60)=4,46$, $P<0.05$). Significant differences between the nandrolone- and vehicle-co-treated groups appeared on day 14 ($F(1,30)=7.17$, $P<0.05$). Moreover, tolerance to the antinociceptive effects of 9 mg/kg of morphine developed in vehicle-co-treated mice (−42%), but not in nandrolone-treated animals (−4%). Two-way ANOVA revealed a significant time effect ($F(1,61)=18.08$, $P<0.001$), a nandrolone effect ($F(1,61)=10.75$, $P<0.01$) and a nandrolone/time interaction ($F(1,61)=10.75$, $P<0.01$). Significant differences between nandrolone and vehicle co-treated mice also appeared on day 14 ($F(1,31)=10.42$, $P<0.01$).

3.3. Effects of nandrolone treatments on the development of tolerance to morphine hypothermia

The influence of nandrolone on the development of tolerance to morphine-induced hypothermia was also evaluated (data not shown).

Nandrolone pre-exposure did not modify the development of tolerance to the hypothermic effects of 3 mg/kg of morphine (two-way ANOVA: time effect, $F(1,47)=48.00$, $P<0.01$; nandrolone effect, $F(1,47)=0.04$, NS; nandrolone/time interaction, $F(1,47)=0.14$, NS). A similar development of tolerance to the hypothermic effects of 9 mg/kg morphine was also observed in both groups (two-way ANOVA: time effect, $F(1,48)=30.54$, $P<0.001$; nandrolone effect, $F(1,48)=8.60$, $P<0.01$; nandrolone/time interaction, $F(1,48)=1.92$, NS).

The development of tolerance to the hypothermic effects of 3 mg/kg of morphine on day 14 was similar in both vehicle- and nandrolone-co-treated mice (two-way ANOVA: time effect, $F(1,50)=23.11$, $P<0.001$; nandrolone effect, $F(1,50)=0.57$, NS; nandrolone/time interaction, $F(1,50)=0.65$,

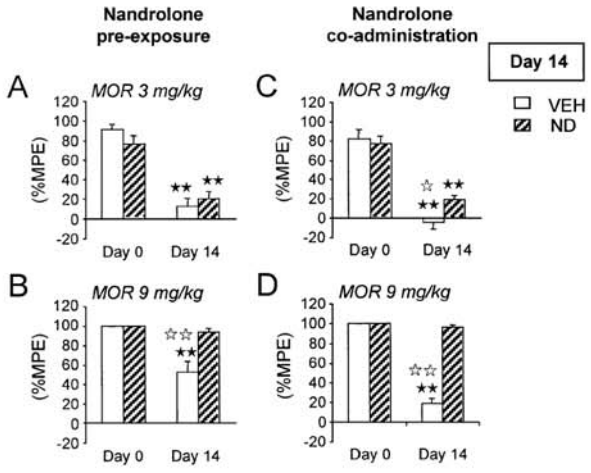

Fig. 3. Tolerance to morphine-induced antinociception in the hot plate (jumping) test in mice (A and B) pre-exposed to or co-treated with (C and D) nandrolone (ND) or vehicle (VEH). Mice received repeated morphine administration (MOR, 12 mg/kg, s.c., twice a day for 14 days). Jumping threshold was evaluated 20 min after the injection of 3 and 9 mg/kg (s.c.) of morphine on the morning of day 14. Number of mice per group in the nandrolone pre-exposure experiments = 10–16. Number of mice per group in the nandrolone co-treatment experiments = 15. Data are expressed as means ± S.E.M. $^{\star\star}P<0.01$ (one-way ANOVA, Day 0 vs. Day 14); $^{\star}P<0.05$, $^{\star\star}P<0.01$ (one-way ANOVA, VEH- vs. ND-treated mice).

NS). Tolerance to the hypothermic effects of 9 mg/kg morphine also developed in a similar way in both vehicle- and nandrolone-co-treated mice (two-way ANOVA: time effect, $F(1,52)=43.19$, $P<0.001$; nandrolone co-treatment effect, $F(1,52)=0.89$, NS; nandrolone/time interaction, $F(1,52)=2.95$, NS).

3.4. Effects of nandrolone treatments on morphine-induced locomotor sensitisation

Nandrolone pre-exposure did not modify the ability of morphine to elicit sensitisation to ambulatory movements (Fig. 4A). Three-way ANOVA revealed a significant morphine effect ($F(1,81)=53.33$, $P<0.001$), a time effect ($F(7,567)=14.13$, $P<0.001$) and a morphine/time interaction ($F(7,567)=16.22$, $P<0.001$), but no nandrolone effect ($F(1,81)=0.04$, NS) and no morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(1,81)=0.07$, $F(7,567)=0.29$ and $F(7,567)=0.51$, respectively, NS). Nandrolone pre-exposure also did not modify morphine-induced sensitisation of horizontal activity (Fig. 4B). Three-way ANOVA revealed a significant morphine effect ($F(1,81)=30.37$, $P<0.001$), a time effect ($F(7,567)=12.36$, $P<0.001$) and a morphine/time interaction ($F(7,567)=12.08$, $P<0.001$), but no nandrolone effect ($F(1,81)=0.58$, NS) and no morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(1,81)=0.01$, $F(7,567)=0.74$ and $F(7,567)=0.48$, respectively, NS). We also found no influence of nandrolone pre-exposure on the morphine-induced decrease in vertical locomotion (data not shown). Three-way ANOVA revealed a significant morphine effect ($F(1,81)=8.41$, $P<0.001$), a time effect ($F(7,567)=7.74$, $P<0.001$) and a morphine/time interaction ($F(7,567)=4.19$, $P<0.001$), but no nandrolone effect ($F(1,81)=0.86$, NS) and no morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(1,81)=0.01$, $F(7,567)=0.32$ and $F(7,567)=0.51$, respectively, NS).

Similarly, nandrolone co-treatment did not modify the sensitisation to morphine effects on ambulatory movements (Fig. 4C). Three-way ANOVA revealed a morphine effect ($F(1,71)=115.62$, $P<0.001$), a time effect ($F(7,497)=28.44$, $P<0.001$) and a morphine/time interaction ($F(7,497)=27.35$, $P<0.001$), but no nandrolone effect ($F(1,71)=0.04$, NS) and no morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(1,71)=0.42$, $F(7,497)=0.10$ and $F(7,497)=0.16$, respectively, NS). Nandrolone co-treatment also did not modify morphine-induced sensitisation of horizontal activity (Fig. 4D). Three-way ANOVA revealed a morphine effect ($F(1,72)=73.00$, $P<0.001$), a time effect ($F(7,504)=20.25$, $P<0.001$) and a morphine/time interaction ($F(7,504)=22.92$, $P<0.001$), but no nandrolone effect ($F(1,72)=0.11$, NS) and no significant morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(1,72)=2.64$, $F(7,504)=0.43$ and $F(7,504)=0.24$, respectively, NS). We also found no influence of nandrolone

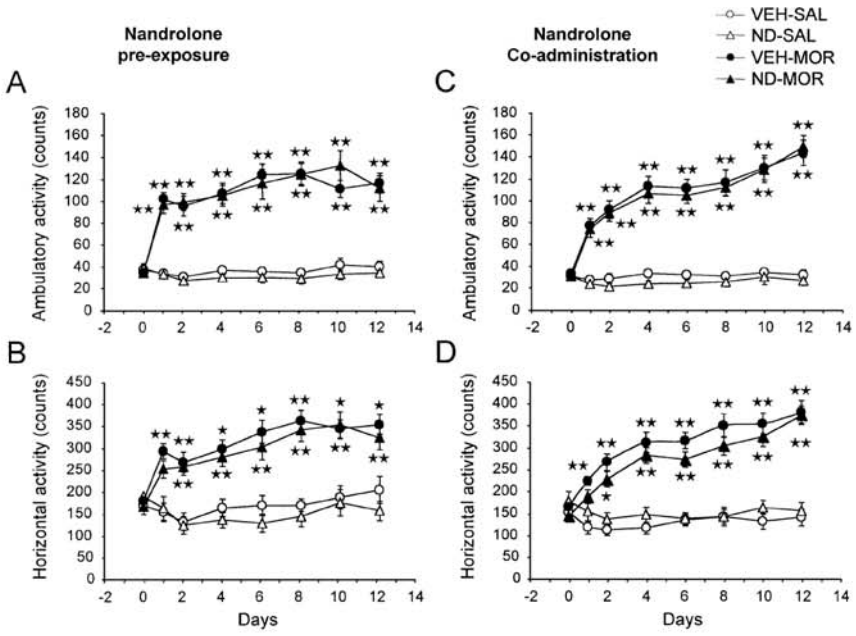

Fig. 4. Sensitisation to locomotor effects induced by repeated morphine administration in mice pre-exposed to (A and B) or co-treated with (C and D) nandrolone (ND) or vehicle (VEH). Mice received repeated morphine (12 mg/kg, s.c., twice a day for 12 days) or saline administration. Ambulatory (AC) and horizontal (BD) activities were recorded 10 min after the daily morning injection, for a period of 10 min. Number of mice per group in the nandrolone co-treatment experiments = 15. Number of mice per group in the nandrolone pre-exposure experiments = 10–16. Data are expressed as means ± S.E.M. $^{\star}P<0.05$, $^{\star\star}P<0.01$ (Dunnett's test, comparison with the respective control basal value on day 0).

co-treatment on the effects of chronic morphine on vertical locomotion (data not shown). Three-way ANOVA revealed a significant time effect ($F(7,504)=6.52$, $P<0.001$), but no morphine effect ($F(1,72)=2.30$, NS), nandrolone effect ($F(1,72)=0.09$, NS), morphine/time, morphine/nandrolone, nandrolone/time and morphine/nandrolone/time interactions ($F(7,504)=1.01$, $F(1,72)=1.52$, $F(7,504)=0.73$ and $F(7,504)=0.71$, respectively, NS).

3.5. Effects of nandrolone treatments on morphine rewarding effects

We explored the influence of nandrolone on morphine rewarding effects by using the conditioned place preference paradigm (Fig. 5). No initial preference or aversion for any compartment was observed in any of the experiments.

The effects of nandrolone pre-exposure were first evaluated (Fig. 5A and B). A significant rewarding effect of morphine (5 mg/kg, s.c.) was observed in the place conditioning paradigm in mice previously receiving intramuscular vehicle for 14 days. This was revealed by a significant increase in the time spent in the drug-paired compartment from the preconditioning to the test phase in the morphine-treated mice (Fig. 5A, Student's t-test, $t(1,12)=-3.28$, $P<0.01$), as well as by significant differences in the score values when those for the morphine-treated mice were

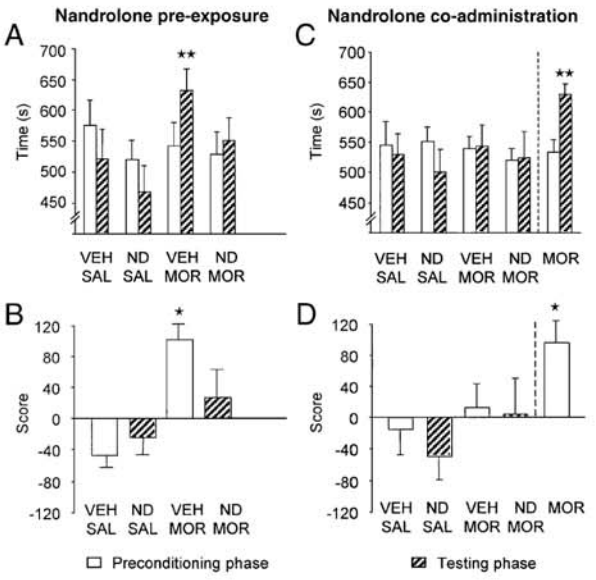

Fig. 5. Rewarding effects of morphine (MOR, 5 mg/kg, s.c.) in the place preference test in mice pre-exposed to (A and B) or co-treated with (C and D) nandrolone (ND) or vehicle (VEH). Control saline (SAL) animals did not receive morphine. The time spent in the morphine-associated compartment during the preconditioning (white bars) and testing (black bars) phases (A and C) and the scores values (B and D) are presented. Number of mice per group in the nandrolone pre-exposure experiments = 12–14. Number of mice per group in the nandrolone co-treatment experiments = 11–14. Values are expressed as means ± S.E.M. $^{**}P<0.01$ (one-way ANOVA, comparison with the respective saline group).

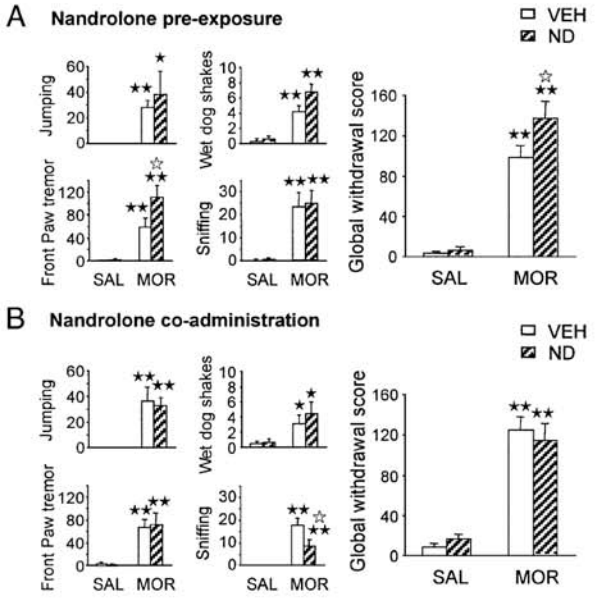

Fig. 6. Number of signs of withdrawal (jumping, wet dog shakes, front paw tremor and sniffing) and global withdrawal score after naloxone administration in morphine (MOR)-dependent mice pre-exposed to (A) or co-treated with (B) nandrolone (ND) or vehicle (VEH). Control saline (SAL) animals did not receive morphine. Abstinence was precipitated by the administration of the opioid receptor antagonist, naloxone (1 mg/kg, s.c.), in mice receiving a chronic treatment of increasing doses of morphine for 6 days. Somatic signs of withdrawal were observed for 30 min immediately after naloxone administration. The global withdrawal score was calculated for each animal by giving each sign a relative weight. Number of mice per group in pre-exposure and co-administration experiments = 9–10. Data are expressed as means ± S.E.M. $^{\star}P<0.05$, $^{\star\star}P<0.01$ (Dunnett's test, comparison with the respective saline control group); $^{\star}P<0.05$ (one-way ANOVA, VEH- vs. ND-treated mice).

compared to those for the respective vehicle group (Fig. 5B, one-way ANOVA, $F(3,49)=3.68$, $P<0.05$, followed by Dunnett's test, $P<0.01$). On the contrary, morphine did not produce any place preference in mice pre-exposed to nandrolone. No difference in the time spent in the drug-paired compartment from the preconditioning to the test phase was found in the morphine-treated mice previously exposed to nandrolone (Student's t-test, $t(1,11)=-0.57$, NS). Moreover, no differences in the scores were observed when those for the morphine-treated mice were compared to those for the respective vehicle control mice (one-way ANOVA, $F(3,49)=3.68$, $P<0.05$, followed by Dunnett's test, NS).

The influence of chronic nandrolone co-administration on morphine rewarding effects was also evaluated (Fig. 5C and D). In our study, the influence of nandrolone co-administration on morphine responses could not be assessed due to the interference of the previous intramuscular injection. Indeed, morphine (5 mg/kg, s.c.) did not produce conditioned place preference when mice received an intramuscular injection of vehicle or nandrolone 1 h before the conditioning session. There were no significant differences in the time spent in the drug-paired compartment from the

preconditioning to the test phase in the morphine-treated mice (Fig. 5C, Student's *t*-test, $t(1,13) = -0.17$, NS, in vehicle group and $t(1,11) = -0.07$, NS, in nandrolone group). Moreover, no differences in the scores were observed when those for the morphine-treated mice were compared to those for the respective vehicle control mice (Fig. 5D, one-way ANOVA, $F(4,57) = 2.86$, $P < 0.05$, followed by Dunnett's test, NS). On the contrary, a significant place preference was observed in a group of mice receiving morphine alone, without any intramuscular co-treatment with vehicle or nandrolone, studied concurrently with the co-administration groups. Thus, a significant increase in the time spent in the drug-paired compartment during the test phase was observed on comparison to the preconditioning value (Student's *t*-test, $t(1,10) = -3.46$, $P < 0.01$), and significant differences in the scores were found when comparing the morphine-treated mice with the respective vehicle control mice (one-way ANOVA, $F(4,57) = 2.86$, $P < 0.05$, followed by Dunnett's test, $P < 0.05$).

3.6. Effects of nandrolone treatments on the naloxone-precipitated morphine withdrawal syndrome

The opioid withdrawal syndrome was precipitated by the administration of naloxone (1 mg/kg, s.c.) in mice receiving chronic increasing doses of morphine for 6 days (Fig. 6). No signs of withdrawal were observed in any group of mice during behavioural observation before the administration of naloxone. Moreover, no behavioural manifestations of withdrawal were observed after the injection of naloxone in saline control mice pre-exposed to or co-treated with nandrolone. Naloxone injection in chronic morphine-treated mice precipitated a withdrawal syndrome manifested by the presence of several somatic signs. The intensity of the syndrome was significantly increased in nandrolone pre-exposed mice (Fig. 6A). Indeed, two-way ANOVA revealed a significant increase of front paw tremor and global withdrawal score (Table 1) in the chronic morphine group pre-exposed to nandrolone in comparison with the group pre-exposed to vehicle. In contrast, the intensity of morphine withdrawal was not modified by the co-administration of nandrolone (Fig. 6B). Indeed, two-way ANOVA (see Table 1) revealed a similar manifestation of jumping, wet dog shakes, front paw tremor, ptosis, teeth chattering, piloerection and body weight loss in chronic morphine-treated mice co-administered with vehicle vs. nandrolone. Only sniffing was significantly reduced in nandrolone co-treated mice. The analysis of the global withdrawal scores also indicated no effect of nandrolone co-treatment on the severity of the morphine withdrawal syndrome (Table 1).

4. Discussion

Pre-exposure to high doses of the anabolic–androgenic steroid nandrolone has been previously reported to induce biochemical changes in the endogenous opioid system in various regions of the brain in rodents (Johansson et al., 1997, 2000a,b; Lukas, 1993; Menard et al., 1995). However, the consequences of such a treatment on morphine-induced behavioural responses related to its addictive properties remained to be examined. In this study, mice were exposed to high doses of nandrolone, comparable to those used by competitive bodybuilders, and the effects on different be-

Table 1
Nandrolone effect on naloxone-precipitated morphine withdrawal

	Two-way ANOVA					
	Morphine	*P*-value	Nandrolone	*P*-value	Interaction	*P*-value
Pre-exposure						
Jumping	$F(1,48) = 13.97$	< 0.001	$F(1,48) = 0.32$	n.s.	$F(1,48) = 0.32$	n.s.
Wet dog shakes	$F(1,48) = 56.32$	< 0.001	$F(1,48) = 4.68$	< 0.05	$F(1,48) = 3.32$	n.s.
Front paw tremor	$F(1,48) = 48.04$	< 0.001	$F(1,48) = 4.71$	< 0.05	$F(1,48) = 4.33$	< 0.05
Sniffing	$F(1,48) = 33.00$	< 0.001	$F(1,48) = 0.06$	n.s.	$F(1,48) = 0.02$	n.s.
Teeth chattering	$F(1,48) = 60.82$	< 0.001	$F(1,48) = 4.04$	n.s.	$F(1,48) = 3.09$	n.s.
Piloerection	$F(1,48) = 133.33$	< 0.001	$F(1,48) = 1.01$	n.s.	$F(1,48) = 0.01$	n.s.
Weight loss	$F(1,48) = 0.15$	n.s.	$F(1,48) = 0.01$	n.s.	$F(1,48) = 0.05$	n.s.
Global withdrawal score	$F(1,48) = 142.69$	< 0.001	$F(1,48) = 5$	< 0.05	$F(1,48) = 7.18$	< 0.05
Co-administration						
Jumping	$F(1,35) = 32.78$	< 0.001	$F(1,35) = 0.07$	n.s.	$F(1,35) = 0.07$	n.s.
Wet dog shakes	$F(1,35) = 11.67$	< 0.01	$F(1,35) = 0.69$	n.s.	$F(1,35) = 0.38$	n.s.
Front paw tremor	$F(1,35) = 31.70$	< 0.001	$F(1,35) = 0.02$	n.s.	$F(1,35) = 0.08$	n.s.
Sniffing	$F(1,35) = 44.87$	< 0.001	$F(1,35) = 5.44$	< 0.05	$F(1,35) = 5.44$	< 0.05
Teeth chattering	$F(1,35) = 32.04$	< 0.001	$F(1,35) = 0.12$	n.s.	$F(1,35) = 0.01$	n.s.
Piloerection	$F(1,35) = 15.62$	< 0.001	$F(1,35) = 0.56$	n.s.	$F(1,35) = 2.44$	n.s.
Weight loss	$F(1,35) = 9.31$	< 0.01	$F(1,35) = 41.82$	n.s.	$F(1,35) = 0.76$	n.s.
Global withdrawal score	$F(1,35) = 124.49$	< 0.001	$F(1,35) = 0.02$	n.s.	$F(1,35) = 0.94$	n.s.

Two-way ANOVA with morphine and nandrolone (between subjects) as factor of variations.
n.s., not significant.

havioural and somatic responses induced by acute and chronic morphine administration were evaluated. Two different protocols of nandrolone administration were used, pre-exposure (repeated administration for two weeks before opioid treatment) and co-administration (concomitant with opioid treatment).

We found no modification of acute morphine-induced antinociception and locomotor responses after both nandrolone pre-exposure and co-administration. On the contrary, nandrolone co-administration, which did not produce any intrinsic effect on body temperature, increased the hypothermia induced by the highest dose of morphine used in this study. In agreement with our results, nandrolone has been described to act on the systems implicated in the hypothermic effects of morphine. Morphine has complex effects on body temperature in rodents and induces hyperthermia at low doses by acting on mu-opioid receptors whereas high doses of morphine produce hypothermia by acting on hypothalamic kappa-opioid receptors (Benamar et al., 2001; Geller et al., 1983; Xin et al., 1997). High doses of morphine increase plasma levels of antipyretic substances, such as the adrenocorticotropin hormone (ACTH) and corticosterone (Nikolarakis et al., 1989). A role of the *N*-methyl-D-aspartate (NMDA)/nitric oxide (NO) pathway in the hypothermic effects of morphine has also been proposed (Ulugol et al., 2000). It is noteworthy in this context that nandrolone administration has been reported to modify endogenous opioid systems (Johansson et al., 2000a), as well as hypothalamic expression of the NMDA receptor NR1 subunit (Le Grevès et al., 1997) and the circulating levels of corticosterone and ACTH (Schlussman et al., 2000). Our results showing an acute pharmacological interaction between morphine and nandrolone in the co-administration, but not in the pre-exposure protocol, suggest a rapid non-genomic action of nandrolone. Rapid androgen actions have been already described in the brain. Thus, testosterone has been shown to increase the spike frequency of neurons within seconds in the lateral hypothalamus of male rats (Orsini et al., 1985). This kind of rapid effect is likely to be mediated through steroid intracellular receptors, nonclassic steroid receptors (ion-gated neurotransmitter receptors) or through direct action on physicochemical membrane properties (Falkenstein et al., 2000). However, we found that nandrolone was more effective to influence morphine rewarding effects and the somatic expression of morphine withdrawal when it was chronically administered before the start of opioid treatment (see below). This is in line with the traditional model of steroid action on intracellular androgen receptors that subsequently modulate transcription and protein synthesis, thus triggering genomic events finally responsible for delayed effects (Falkenstein et al., 2000).

Tolerance to the antinociceptive effects of morphine is known to be influenced by a considerable number of compounds (see review, Bhargava, 1994). In our study, both nandrolone pre-exposure and co-administration significantly decreased the development of antinociceptive tolerance. Interestingly, nandrolone effects on morphine tolerance were only observed in the hot plate test but not in the tail immersion test. These results are in agreement with results of previous studies showing that, in rhesus monkeys, testosterone propionate treatment for 14 days did not alter morphine antinociception in the warm-water tail-withdrawal test, the equivalent in monkey of the rodent tail immersion test (Negus et al., 2001). The lower degree of morphine tolerance in the jumping response of the hot plate test in mice receiving nandrolone was not related to an indirect nandrolone effect on locomotion. Indeed, nandrolone did not itself produce any change in locomotor activity and did not modify locomotor responses induced by acute or chronic morphine treatment. Since the tail-withdrawal response is predominantly mediated by spinal mechanisms whereas hot plate responses are thought to require the involvement of supraspinal structures (Grossman et al., 1982; Morgan et al., 1989), it can be suggested that nandrolone action on morphine tolerance would involve supraspinal mechanisms. In accordance with such a hypothesis, microinjection of morphine into the rostral ventromedial medulla and the ventrolateral periaqueductal gray in rats produced greater antinociception in males than in females (Boyer et al., 1998; Krzanowska and Bodnar, 1999). The observation that nandrolone treatment modifies the levels of endogenous opioid peptide immunoreactivity in the periacqueductal gray (Johansson et al., 2000a) further supports such a supraspinal steroid action on the development of tolerance to opioid antinociceptive responses.

The influence of nandrolone on opioid sensitisation and rewarding properties was also investigated. Dopamine activity in the nucleus accumbens appears to be critically involved in opioid rewarding and locomotor responses (Di Chiara, 1995; Koob, 1992). Anabolic–androgenic steroids have been found to either directly or indirectly elevate dopamine levels in the nucleus accumbens (Kindlundh et al., 2001) by interacting with the endogenous opioid system in the rat brain (Hallberg et al., 2000; Johansson et al., 1997, 2000a,b; Lukas, 1993; Menard et al., 1995). Nandrolone increases β-endorphin levels in the ventral tegmental area in the male rat brain, which is consistent with an increase in dopaminergic activity, leading to reward and euphoria (Johansson et al., 1997). Surprisingly, in our study, nandrolone pre-exposure was associated with a decrease in morphine-induced place preference and no changes were observed in the sensitisation to morphine locomotor effects after the different nandrolone treatments. A possible change induced by nandrolone in the imbalance of the different endogenous opioid peptides related to reinforcing/dysphoric effects of the opioids (Johansson et al., 2000a) may explain these discrepancies. Thus, nandrolone was reported to elevate levels of the endogenous κ-agonist, dynorphin, in the striatum, which could account for a dysphoric effect (Johansson et al., 2000a), through inhibition of the dopaminergic activity in this area (Steiner and Gerfen, 1998).

The induction of physical dependence and consequent avoidance of an aversive withdrawal state represents one aspect of the motivational impetus maintaining opioid addiction (Koob and Le Moal, 2001). We now found that chronic treatment with nandrolone did not produce any sign of withdrawal after administration of naloxone in saline-treated mice. This result is consistent with the study of Negus et al. (2001) showing no manifestations of withdrawal in the rhesus monkey after injection of naloxone during a chronic testosterone treatment performed in the same way as in our study (once-daily administration of high doses for 14 days). However, clinical studies suggest that prolonged use of high doses of anabolic–androgenic steroids may induce physical dependence in humans (Brower et al., 1991; Pope and Katz, 1994). The experimental animal results suggest that such an anabolic–androgenic steroid dependence does not directly involve the endogenous opioid system and/or cannot be shown under the standard experimental conditions in rodents and monkeys. Recent reports also described a higher incidence of opioid consumption in anabolic–androgenic steroid abusers (Arvary and Pope, 2000; McBride et al., 1996; Wines et al., 1999). Our results showing that chronic nandrolone pre-exposure significantly enhances naloxone-precipitated morphine withdrawal are in accordance with such an observation in humans, and with biochemical studies indicating changes in the endogenous opioid system after chronic anabolic–androgenic steroid exposure (Harlan et al., 2000; Menard et al., 1995; Johansson et al., 1997). In this context, another possible explanation for the discrepancies between animal and clinical studies regarding the ability of nandrolone to induce dependence could be that this compound might enhance the endogenous opioid activity in a way that produces a level of dependence too subtle to be detected in experimental animals, but sufficient to increase the morphine withdrawal syndrome. Thus, a lower degree of opioid dependence was induced after chronic treatment with inhibitors of the enkephalin degrading enzyme, which increase endogenous opioid activity, than after chronic exogenous opioid administration (Maldonado et al., 1990). Morphine dependence is thought to result from complex adaptive changes. It is possible that nandrolone promotes the agonist action of morphine on neuronal systems that undergo plastic changes, thereby increasing withdrawal symptoms. A major involvement of the brain noradrenergic pathways in morphine physical withdrawal, especially the locus coeruleus, has been proposed (Maldonado et al., 1992b). Interestingly, there is a clinical case report of a hyperadrenergic withdrawal syndrome in an anabolic–androgenic steroid abuser after naloxone challenge (Tennant et al., 1988), which could have been due to a decrease of endogenous opioid activity during anabolic–androgenic steroid withdrawal (Kashkin and Kleber, 1989). The stress-responsive hypothalamic–pituitary–adrenal axis also participates in opioid withdrawal (Koob and Le Moal, 2001). It is relevant that anabolic–androgenic steroids may disrupt components of this axis by acting on the expression of proopiomelanocortin and corticotrophin-releasing hormone (CRF) mRNA in the brain and on the circulating levels of corticosterone and ACTH (Schlussman et al., 2000).

Of particular interest are the differential effects of nandrolone pre-exposure on morphine-induced place preference and withdrawal syndrome, i.e., decreased morphine rewarding properties and increased somatic manifestations of abstinence. Such a result is not surprising since different anatomical and neurochemical mechanisms are involved in opioid reward and physical dependence (Maldonado et al., 1992a,b; Van Ree et al., 1999). One possible explanation for this suppression of morphine reward and increased withdrawal syndrome could be that chronic nandrolone treatment would produce long-term changes in reward brain circuits leading to a progressive decrease in the basal hedonic level, which results in an unpleasant state that would render the organism more vulnerable to development of an addictive process (Koob and Le Moal, 2001).

In summary, we have clarified the consequences of nandrolone exposure on the acute and chronic effects of morphine on behaviour in rodents. These new data have identified the functional consequences of the biochemical changes previously reported in the endogenous opioid system after nandrolone administration. Although many aspects of steroids action on drug abuse still require intensive research, our results give further support to suggestions that the growing use of nandrolone by both athletes and nonathletes could lead to relevant public health problems.

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The role of the cannabinoid system in nicotine addiction

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Abstract

Nicotine, the main psychoactive component in tobacco smoke appears to play a major role in tobacco addiction, producing a high morbidity and mortality in the world. A great amount of research has been developed to elucidate the neural pathways and neurotransmitter systems involved in such a complex addictive behaviour. One possible candidate is the cannabinoid system, which has been reported to participate in the addictive properties of other drugs of abuse. This review is focused on the recent pharmacological and molecular studies assessing cannabinoid-nicotine interactions, with special attention to those studies evaluating the behavioural responses related to the development of nicotine addiction.

Key words: addiction, cannabinoid, dependence, nicotine, reward, withdrawal

Introduction

Nicotine is one of the main active components in tobacco smoke that initiates and sustains tobacco addiction. Nicotine induces its central pharmacological effects by acting on nicotinic acetylcholine receptors (nAChRs), which are pentameric complexes consisting of the combination of different α (α2-α10) and β (β2-β4) protein subunits (Le Novère et al., 2002). The nAChRs are ubiquitously distributed in the central nervous system (CNS), mainly at a pre-synaptic level, and they serve as ligand-gated ion channels that promote neurotransmitter release (Wonnacott, 1997). Thus, nAChR activation plays a neuromodulatory role in the CNS and is involved in a large number of physiological and pathological processes such as pain neurotransmission, control of movement, cognitive processes, emotional responses, and drug abuse (Buisson and Bertrand, 2002; File et al., 2002; Jain, 2004; Katner et al., 2004; Schochet et al., 2004).

An intense research has been developed to elucidate the neural pathways and neurotransmitter systems involved in nicotine addictive properties. Numerous candidates including GABA, glutamate, noradrenaline, serotonin, corticotropin-releasing factor (CRF), dopamine (DA) and endogenous opioids have been shown to play a role in nicotine addiction (Cryan et al., 2003). More recently, pharmacological and molecular studies have suggested that the endocannabinoid system could also play an important role in nicotine addictive properties. So far, two cannabinoid receptors have been identified and cloned, the CB1 cannabinoid receptor, mainly located in the CNS (Matsuda et al., 1990) and the CB2 receptor, which has a predominant distribution in immune cells (Munro et al., 1993). However, recent data suggest the presence of a third and still uncloned cannabinoid receptor in the brain, namely the "CB3" or "CBx" receptor (Breivogel et al., 2001; Di Marzo et al., 2000; Hájos and Freund, 2002; Járai et al., 1999). The activation of CB1 cannabinoid receptors mediates the main effects of

cannabinoids in the CNS (Ledent et al., 1999; Zimmer et al., 1999), and is responsible for the addicting properties of cannabinoids (Ledent et al., 1999). These cannabinoid receptors participate in similar physiological functions than nAChRs, such as nociceptive transmission, motor activity, learning and memory processes and emotional responses. Interestingly, CB1 cannabinoid receptors have been shown to be involved in the addictive properties of other drugs of abuse, such as opioids, ethanol, cocaine and MDMA (Braida and Sala, 2002; De Vries et al., 2001; Fattore et al., 2003; Mechoulam and Parker, 2003; Navarro et al., 2001), suggesting that the cannabinoid system may be a common neurobiological substrate for the addictive properties of drugs of abuse.

This review is focused on the involvement of the cannabinoid system in the different responses induced by acute and chronic administration of nicotine that are related to its addictive properties. We will examine the pharmacological and molecular studies concerning cannabinoid-nicotine interactions, with special attention to those studies evaluating the adaptive and motivational responses induced by chronic nicotine administration.

Acute cannabinoid-nicotine interactions

Two pharmacological studies have investigated the acute interaction between the effects induced by nicotine and cannabinoid agonists. Significant interactions between Δ^9-tetrahydrocannabinol (THC) and nicotine were reported on locomotion, heart rate, body temperature, anxiety and nociception (Pryor et al., 1978; Valjent et al., 2002). Thus, nicotine potentiated hypothermia, bradycardia, hypolocomotion and impaired rotarod performance induced by THC (Pryor et al., 1978). In agreement, a more recent study showed that nicotine strongly facilitated hypolocomotion, antinociception, hypothermia and anxiolytic-like effects induced by acute administration of THC

(Valjent et al., 2002). The facilitating effect of nicotine in THC acute responses was also observed at the biochemical level. Accordingly, co-administration of both nicotine and THC pontentiated the enhancement of c-Fos inmunoreactivity in several brain regions such as the shell of the nucleus accumbens (NAcc), central and basolateral amygdala, bed-nucleus of stria terminalis, cingular and piriform cortex and paraventricular nucleus of the hypothalamus (Valjent et al., 2002). Most of these areas are highly innervated by DA inputs, suggesting that the interaction between nicotine and cannabinoids could occur *via* the stimulation of mesolimbic and mesocortical dopaminergic system.

So far, only one study has evaluated the possible role of the CB1 cannabinoid receptors in nicotine acute pharmacological responses by using CB1 knockout mice (Castañé et al., 2002). Thus, nicotine-induced antinociceptive responses in the tail-immersion test, which are mainly mediated through a spinal mechanism, were enhanced in mice lacking CB1 cannabinoid receptors. However, the effects of acute nicotine administration on the hot-plate test and locomotor activity were not modified in these CB1 knockout mice (Castañé et al., 2002).

The role of the cannabinoid system in nicotine-induced reinforcing effects

From a neurobiological and behavioural point of view processes involved in the initiation and maintenance of drug addictive behaviour are complex. One important aspect for the initiation of the addictive process is the capacity of the drug to induce reinforcing effects. On the other hand, the negative consequences of drug abstinence have a crucial motivational significance for relapse and maintenance of the addictive behaviour (Koob and Le Moal, 2001).

Similar to other drugs of abuse, nicotine induces reinforcing effects, as revealed by conditioned place preference (CPP), intracranial self-stimulation (ICSS) and intravenous self-administration (SA) paradigms (Laviolette and van der Kooy, 2004; Malin, 2001). The possible involvement of the cannabinoid system in the rewarding effects of nicotine has been evaluated by using CPP and SA paradigms. The CPP paradigm measures a learning process where the animal shows a preference for a context due to the contingent association between the context and a drug-associated stimulus. Therefore, after the conditioning period, drug free animals spend more time in a previously drug-paired compartment in comparison with a neutral vehicle-paired compartment (Fig. 1a). On the other hand, the SA procedure directly evaluate the reinforcing properties of a drug and provide a unique model to reveal drug consumption in animals (Fig. 1b). In this paradigm, the reinforcing aspects of the drug are reflected by the number of injections that the animal self-administers. On the CPP paradigm, a first pharmacological study showed that the co-administration of sub-threshold doses of THC and nicotine induced rewarding effects (Valjent et al., 2002) (Fig. 2a). In addition, the previous priming of THC usually needed to induce this response (Valjent and Maldonado, 2000) was not required when low doses of THC were co-administered with nicotine. This result indicates that low doses of cannabinoids associated with nicotine could have a higher capability to induce behavioural responses related to addictive processes than THC administration alone (Valjent et al., 2002).

Recent studies using knockout mice have attempted to clarify the involvement of CB1 cannabinoid receptors in nicotine rewarding properties, although the results obtained with these animals have provided conflicting data. Indeed, the rewarding effects of nicotine, assessed in the CPP paradigm, were abolished in CB1 receptor knockout mice (Castañé et al., 2002) (Fig. 2b), while the absence of CB1 cannabinoid

receptors did not modify the acquisition of nicotine self-administration in an acute reinforcement paradigm (Cossu et al., 2001). However, the acquisition and maintenance of a stable operant self-administration responding for nicotine has not been yet evaluated in mice lacking CB1 cannabinoid receptors. Although data from these mutant mice show apparent discrepancies, the CB1 receptor antagonist SR 141716 has been shown to decrease nicotine stable operant self-administration in rats (Cohen et al., 2002), suggesting an involvement of CB1 cannabinoid receptors in nicotine rewarding effects. Therefore, CB1 cannabinoid receptor antagonists could be of interest to reduce the reinforcing value of nicotine in order to facilitate tobacco smoking cessation. In this sense, SR 141716A (Rimonabant) has been tested in Phase II clinical trials as a new possible therapeutic treatment for reduction of tobacco intake with promising results, and this cannabinoid antagonist is now being tested in Phase III clinical trials (Fernandez and Allison, 2004).

Nicotine produces its rewarding action by stimulating mesolimbic dopaminergic transmission (Dani and De Biasi, 2001; Di Chiara, 2000; Pontieri et al., 1996), a common feature of all the prototypical addictive drugs (Koob and Le Moal, 2001). The activation of DA activity by nicotine depends on a functional balance between excitatory and inhibitory inputs to the ventral tegmental area (VTA) DA neurons, in addition to the direct nicotine effects on DA neurons themselves (Mansvelder and McGehee, 2002). The cannabinoid system may also contribute to the regulation of this balance. Indeed, in vivo brain microdialysis studies have revealed that SR 141716 blocked nicotine-induced DA release in the shell of the NAcc (Cohen et al., 2002). Different nAChR subtypes modulate GABAergic and glutamatergic inputs to VTA DA neurons. Indeed, while heteromeric $\alpha 4\beta 2$-nAChRs modulate GABA release, homomeric $\alpha 7$-nAChRs influence glutamate transmission (Mansvelder and McGehee,

2002). Recently, endocannabinoids have been shown to inhibit the function of α7-nAChRs expressed in *Xenopus oocytes* (Oz et al., 2004). Therefore, the activity of α7-nAChRs could be also modulated by endocannabinoids *in vivo*, thus contributing to the regulation of the rewarding properties of nicotine.

The role of the cannabinoid system in nicotine-induced physical dependence

Clinical and animal studies have shown that chronic nicotine administration develops physical dependence revealed by the presence of a withdrawal syndrome when the treatment is disrupted. Thus, both spontaneous (Damaj et al., 2003) and mecamylamine-precipitated nicotine withdrawal (Malin, 2001) have been reported in several animal species by using different experimental protocols (Fig. 1c). The most characteristic somatic manifestations of nicotine withdrawal in rodents are tremors, wet dog shakes, teeth chatters, ptosis, abdominal constrictions and scratching (Isola et al., 1999). The first evidence demonstrating an interaction between nicotine and cannabinoids in the development of physical dependence processes was reported by Valjent et al. (2002). Indeed, mice co-treated with nicotine and THC displayed an enhancement in the somatic expression of cannabinoid antagonist-precipitated THC withdrawal syndrome.

The involvement of CB1 cannabinoid receptors in nicotine dependence has been studied by using CB1 knockout mice. Thus, the nicotinic antagonist mecamylamine precipitated a withdrawal syndrome in nicotine-treated animals that was similar in wild-type and CB1 knockout mice (Castañé et al., 2002) (Fig. 3a). In agreement, the CB1 antagonist SR 141716A, was not able to precipitate a withdrawal syndrome in nicotine-dependent animals (Balerio et al., 2004) (Fig. 3b), suggesting that the endogenous cannabinoid system, through CB1 cannabinoid receptors, does not participate in the

development and expression of nicotine physical dependence. Biochemical studies also support these findings since no modification in CB1 cannabinoid receptor levels was reported following chronic nicotine exposure (Balerio et al., 2004; González et al., 2002). However, Izenwasser et al., (unpublished data) have recently observed that this treatment induces changes in cannabinoid receptor density in adolescent male, but not in female or adult rats. Therefore, we cannot exclude the participation of cannabinoid receptors in the effects of nicotine when administered at younger animals. On the other hand, cannabinoid agonists seem to attenuate the severity of the somatic manifestations of nicotine withdrawal (Balerio et al., 2004) (Fig. 3c). Similar to other drugs of abuse, nicotine abstinence is associated with a selective up-regulation of the cyclic AMP pathway (Tzavara et al., 2002) pointing to this cascade as a possible target for cannabinoids in ameliorating nicotine withdrawal symptoms. Further studies must be performed to clarify the neurobiological substrate of nicotine dependence, and the possible role of the cannabinoid system in this nicotine behavioural response.

Final remarks

Nicotine addiction is a complex behavioural and neurochemical process in which many neuroanatomical pathways and neurotransmitters are involved. The pharmacological and molecular studies described in the present review support the specific role of the endogenous cannabinoid system in the modulation of nicotine responses related to its addictive properties. These findings improve our understanding of nicotine addiction and could open new possibilities in the treatment of this major public health disorder.

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Figure Legends

Figure 1. Experimental procedures for evaluating behavioural responses related to nicotine addiction in rodents. a) The conditioned place preference procedure is commonly used to reveal the rewarding properties of nicotine. This paradigm consists of three different phases. In the first phase, namely pre-conditioning, animals are allowed to freely explore a box with two compartments, that have distinct visual and tactile characteristics and the time spent in each compartment is recorded. In the conditioning phase, mice receive alternative injections of nicotine in one compartment and vehicle in the opposite compartment during several days. The last phase, namely post-conditioning, is conducted exactly as the pre-conditioning phase, animals have free access to both compartments of the conditioning apparatus and the time spend in each compartment is recorded. Results are usually expressed as a score calculated by the time spent in the drug associated compartment during the post-conditioning minus the time spent in the same compartment during the pre-conditioning. A positive score means that the compound tested induces rewarding effects. CPP paradigm also allows to evaluate the aversive properties induced by a stimulus and has been used to reveal the dysphoric aspects of nicotine withdrawal. **b)** Intravenous self-administration is another common model used to study the reinforcing properties of nicotine. This model resembles the drug-taking behaviour in humans. Animals are first implanted with indwelling vein catheters. After several days of recovery, animals are trained to make an operant response (nose-poke or lever-press) in order to receive an infusion of nicotine under a fixed ratio or progressive ratio schedule of reinforcement. **c)** Nicotine physical dependence can be induced by using two different strategies: the subcutaneous implantation of osmotic mini pumps that deliver a constant flow of nicotine solution or a discontinuing treatment based on intermittent administration of this drug during

several days. The spontaneous or antagonist-precipitated interruption of nicotine chronic treatment precipitates several somatic signs of withdrawal which are usually evaluated placing mice inside a circular clear plastic area.

Figure 2. Cannabinoid-nicotine interactions on the conditioned place preference paradigm. a) ★★ $p < 0.01$ versus vehicle (VEH) group (Newman-Keuls test). Adapted with permission from Valjent et al., 2002. **b)** ★★ $p < 0.01$ versus control group of the same genotype (Dunnett test). Adapted with the permission from Castañé et al., 2002.

Figure 3. The role of the cannabinoid system in nicotine-induced physical dependence. a) Abstinence was precipitated by acute administration of mecamylamine (1 mg/kg, s.c.) after a 6-day period of nicotine (10 mg/kg/day) or saline infusion. Adapted with the permission from Castañé et al., 2002. **b)** Abstinence was precipitated by acute administration of mecamylamine (MEC) (1 mg/kg, s.c.) or SR 141716A (SR) (1 and 3 mg/kg, i.p.) after a 6-day period of nicotine (25 mg/kg/day) or saline infusion. ★★★ $p < 0.001$ versus chronic saline (SAL) (one-way ANOVA). ☆☆☆ $p < 0.001$ versus nicotine-mecamylamine group (Dunnett test). Δ $p < 0.05$, Δ Δ Δ $p < 0.001$ versus saline-mecamylamine group (Dunnett test). Adapted with the permission from Balerio et al., 2004. **c)** Abstinence was precipitated by acute administration of mecamylamine (MEC) (1 mg/kg, s.c.) after a 6-day period of nicotine (25 mg/kg/day) or saline infusion. THC (0, 0.3, 1 and 3 mg/kg, i.p.) was administered 15 min before withdrawal. ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ versus chronic saline (SAL) (one-way ANOVA). ☆☆☆ $p < 0.001$ versus vehicle (VEH) group (Dunnett test).

Figure 1

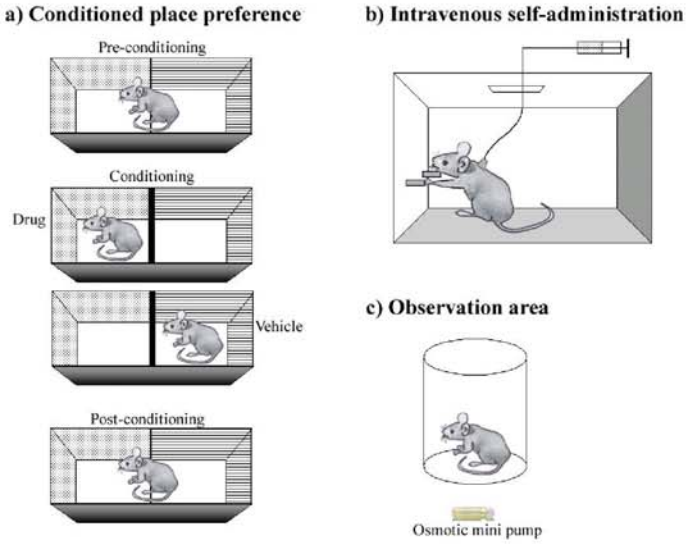

Figure 2

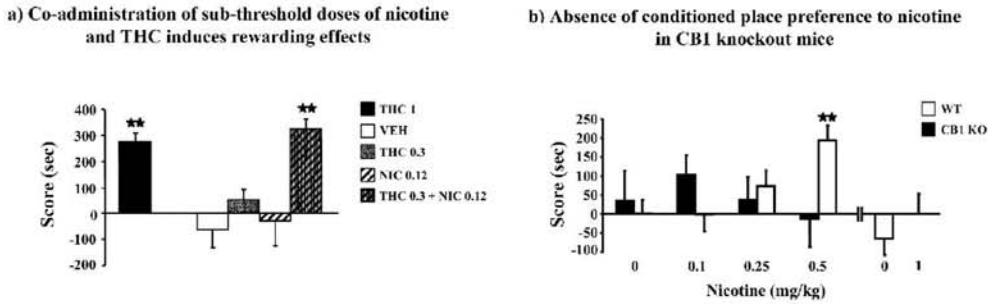

Figure 3