Nuevas drogas psicoestimulantes. Estudio farmacológico y neurotoxicológico de la metilona

Raúl López Arnau

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Universitat de Barcelona

FACULTAD DE FARMACIA

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Raúl López Arnau

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FACULTAD DE FARMACIA

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“No tengo ningún talento especial,

sólo soy apasionadamente curioso”

Albert Einstein

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Acrónimos

[^{3}H]5-HT	serotonina (5-hidroxitriptamina) tritiada
[^{3}H]DA	dopamina tritiada
[^{3}H]NA	noradrenalina tritiada
[^{3}H]paroxetina	paroxetina tritiada
3-OH-4-MeO-MC	3-hidroxi-4-metoximetcatinona
3'-OH-MDMC	3'-hidroxi-metilendioximetcatinona
4-OH-3-MeO-MC	4-hidroxi-3-metoximetcatinona
5-HIAA	ácido 5-hidroxiindolacético
5-HT	serotonina (5-hidroxitriptamina)
B_{max}	concentración máxima de receptores
***bk*-MDMA**	β-ceto-MDMA
C_{max}	concentración máxima
C6-DAT	células gliales C6 con el DAT expresado
C6-NET	células gliales C6 con el NET expresado
CE_{50}	concentración eficaz 50
CHO	células de ovario de hámster chino
CHO/rDAT	células CHO con el DAT de rata expresado
CHO/rNET	células CHO con el NET de rata expresado
CHO/rSERT	células CHO con el SERT de rata expresado
CI_{50}	concentración inhibitoria 50
Clp	aclaramiento plasmático

CYP	citocromo P450
DA	dopamina
DAT	transportador de dopamina
DEA	Drug Enforcement Administration
E_{max}	efecto máximo
GFAP	proteína ácida fibrilar glial
HHA	3,4-dihidroxianfetamina
HHMA	3,4-dihidroximetanfetamina
HMA	4-hidroxi- 3-metoxianfetamina
HMMA	4-hidroxi- 3-metoximetanfetamina
k_a	constante de absorción
K_d	constante de disociación
K_i	constante de inhibición
L-dopa	levodopa (L-3,4-dihidroxifenilalanina)
LDH	lactato deshidrogenasa
LSD	dietilamida del ácido lisérgico
MAO-A	monoamino oxidasa A
MAO-B	monoamino oxidasa B
MDA	3,4-metilendioxianfetamina
MDE	3,4-metilendioxietilanfetamina
MDMA	3,4-metilendioximetanfetamina
MDC	3,4-metilendioxicatinona
MDMC	3,4-metilendioximetcatinona

MDPV	3,4-metilendioxipirovalerona
MPHP	4’-metil-α-pirrolidinohexofenona
MPPP	4’-metil-α-pirrolidinopropiofenona
NET	transportador de noradrenalina
NA	noradrenalina
PC12	línea celular de feocromocitoma
pCPA	para-cloro-fenilalanina
PET	tomografía por emisión de positrones
pKa	log negativo de la constante de disociación ácida
SNC	sistema nervioso central
SERT	transportador de serotonina
$\mathbf{t_{1/2abs}}$	tiempo de semivida de absorción
$\mathbf{t_{1/2\beta}}$	tiempo de semivida de eliminación
$\mathbf{T_{max}}$	tiempo máximo
TH	tirosina hidroxilasa
TPH-2	triptófano hidroxilasa 2
$\mathbf{V_{ss}}$	volumen de distribución en estado estacionario
VMAT-2	transportador vesicular de monoaminas 2

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Breve historia de las drogas

Desde siempre la especie humana ha buscado sustancias que fueran capaces de producir cambios en el estado de ánimo, el nivel de alerta y la percepción de lo que nos rodea, a través del consumo de plantas, hongos o compuestos sintéticos que, alterando el sistema nervioso central, le han permitido asomarse a paraísos artificiales o ficticios, a veces fabulosos. Los términos *drug* (en inglés) y *drogue* (en francés) se utilizan para definir tanto fármacos de prescripción como sustancias de abuso. Según la Organización Mundial de la Salud, droga es: “toda sustancia que, introducida en un organismo vivo, pueda modificar una o varias de sus funciones” (World Health Organization, 1969). El término “droga de abuso” define mejor lo que coloquialmente entendemos como droga: “sustancia de uso no médico con efectos psicoactivos (capaz de producir cambios en la percepción, el estado de ánimo, la conciencia y el comportamiento) y susceptible de ser autoadministrada”. De esta forma, la principal diferencia entre una droga y un fármaco viene dada por matices de tipo instrumental y social. Bien sea con fines religiosos, en rituales esotéricos, para huir de la realidad, para poder hacer frente a diversos problemas o por la incapacidad de afrontarlos, por placer y diversión o por fines médicos, el hombre ha hecho uso de una gran variedad de compuestos, hoy incluidos en el concepto de droga.

1.1.1. El alcohol

Es probable que el alcohol sea la droga más antigua consumida por el ser humano. La historia nos indica que quizá la fermentación de un fruto o de la miel sea el origen del primer compuesto psicoactivo. Es más, excavaciones arqueológicas revelan vasijas empleadas para el almacenamiento y fermentación de la miel alrededor del año 8000 a.C. De ello se deduce que la miel fermentada y diluida (hidromiel) haya sido la primera bebida alcohólica para el consumo humano (Rosenstingl, 1978). En la pirámide de Sakkara, en Egipto, se hallan pinturas, relieves y figuras de cerámica datadas entre el 4000 y el 2000 a.C. relacionadas todas ellas con la producción de cerveza (Figura 1).

Figura 1. Figura de cerámica de una mujer elaborando cerveza. Sakkara, Imperio Antiguo de Egipto. Dinastía V- (3000-2000 a.C.)

No sólo en la Antigua Babilonia y en Egipto existen evidencias del consumo de bebidas alcohólicas, sin ir más lejos, en el propio Antiguo Testamento, en el libro del Génesis se puede leer:" *Y Noé, que era labrador, comenzó a labrar la tierra y plantó una viña, y bebiendo de su vino quedó embriagado... "*. Con la revolución industrial el consumo de alcohol se expandió por todo el mundo occidental. A principios del s. XX, en Estados Unidos, empezó a proliferar el denominado Movimiento por la Templanza, en el cual se promovía la prohibición del uso libre del alcohol. El éxito de este movimiento llegó en el año 1920, con el establecimiento de la conocida como Ley Seca en los Estados Unidos, prohibiéndose la venta, importación, exportación, fabricación y transporte de bebidas alcohólicas. Sin embargo, dicha ley, produjo la creación de un mercado clandestino, el cual se encontraba dirigido por organizaciones mafiosas, provocando simultáneamente un crecimiento de las actividades criminales. Por todo ello, en el año 1933, el presidente Franklin D. Roosevelt, derogó finalmente la anteriormente ratificada como Ley Seca. A partir de la segunda Guerra Mundial, los patrones del consumo de alcohol empiezan a cambiar, vinculándose en gran medida a actividades de ocio y tiempo libre y posteriormente también a la ingesta de grandes cantidades en un breve espacio de tiempo.

1.1.2. Los opiáceos

Los opiáceos también poseen una antigua historia. El opio (del griego *opós*, que significa jugo [de la adormidera]), alcaloide extraído de la planta *Papaver somniferum* L., es una de las drogas más antiguas utilizadas por el hombre. Se cree que su uso se remonta al 3000 a.C., en el imperio sumerio, aunque la primera referencia al opio la encontramos en el Papiro de Ebers (s. XVI a.C.) que incluye más de 700 remedios y recetas (Dugarin and Nominé, 1988). Ya en la cultura griega surgieron los principios del uso sistemático del opio, tiempo en el que comienza la polémica sobre su uso, medicinal y como sustancia peligrosa. Los herederos de la cultura griega, los romanos, conocían, utilizaban e indicaban el opio con gran naturalidad, y grandes hombres de aquella época, cómo el emperador Marco Aurelio, lo empleaban con fines terapéuticos. En China ya se conocía el opio desde el s.VIII, pero no fue hasta el s. XVI que su consumo como medio de divertimento y placer fue incrementándose paulatinamente. En el s. XVIII ya existía un importante comercio de opio y el imperio británico se percató del enorme y apetecible negocio, y monopolizó la compraventa de opio en toda Asia, pasando de vender 300 en 1821 a más de 3000 toneladas en 1848. La rebelión del imperio chino no tardó en aparecer, dando lugar en 1839 a las conocidas como Guerras del Opio. Después de la victoria de los británicos, China se vio obligada a aceptar las condiciones de los vencedores, como el traspaso de todos los derechos de compraventa de opio, entre otras. Sin embargo, las consecuencias sociales fueron mayores, pasando de dos millones de chinos adictos al opio en 1850 a 120 millones en 1878. En Europa, su consumo se difunde lentamente, llegando incluso a ser arropado por grandes artistas como Baudelaire, Balzac o Picasso, entre otros (Dugarin and Nominé, 1988; Pelt, 1983).

El problema iría en aumento a partir, tanto del aislamiento de los alcaloides del opio, como de la invención de la aguja hipodérmica. Es en ese momento cuando el uso de la morfina como analgésico se expande en todo el mundo occidental. A partir de entonces, los médicos empiezan a observar un gran número de muertes, intoxicaciones y dependencias relacionadas con el uso de los alcaloides del opio. Para resolver este problema se creó otro aún mayor, la heroína, un derivado semisintético, obtenido por C.R. Alder Wright en 1874, a partir de la diacetilación de la morfina. La heroína se utilizó durante algunas décadas en el tratamiento del dolor, enfermedades

respiratorias y pulmonares, así como sustitutivo en casos de adicción a la morfina hasta que finalmente se consideró una sustancia prohibida gracias al Convenio de Viena sobre Estupefacientes en 1968. En esa misma época, gracias a la cultura *underground* y al movimiento *hippie*, el consumo de heroína se expande por todo el mundo, y perdura hasta hoy en día. Se puede decir que la heroína ha generado más muertes, directa o indirectamente, que cualquier otra sustancia química conocida, con excepción quizá del alcohol y el tabaco (Dugarin and Nominé, 1988).

1.1.3. La cocaína

Otra de las grandes drogas adictivas es la cocaína, alcaloide principal de la planta de coca (*Erythroxylum coca*), extraída por Albert Niemann en la segunda mitad del s. XIX. Sin embargo, el consumo de la hoja de la coca se remonta al 2000 a.C. en Chile y Perú. Fue en el imperio inca donde el consumo de esta planta fue máximo, situándose en el centro religioso y social de aquella época.

A partir del aislamiento de la cocaína, aumenta el interés clínico por sus efectos vigorizantes y estimulantes, expandiéndose por toda Europa, gracias en gran medida al padre del psicoanálisis, Sigmund Freud, quién, además de consumirla habitualmente, incidió en los efectos de la cocaína como anestésico local, para el tratamiento de la hipocondría, trastornos digestivos, así como su utilidad en el tratamiento de adicciones al alcohol o la morfina (Dugarin and Nominé 1988). La investigación sobre la cocaína y su uso se instalan en Europa y en la sociedad americana de forma rotunda, llegándose incluso a elaborar bebidas, preparados farmacéuticos y una gran variedad de formas galénicas con extractos de hoja de coca o cocaína. Es el caso de la conocida bebida Coca-Cola®, que contenía originalmente en su compleja fórmula extractos carbónicos de hoja de coca; una “bebida medicinal, intelectual y para el temperamento”, según los anuncios publicitarios de aquella época (Figura 2). Finalmente, se retiró el extracto de hoja de coca y se sustituyó por cafeína (Gold, 1997).

En 1914 fue promulgada la *Harrison Narcotic Act*, convirtiendo a la cocaína en una sustancia controlada. A mediados del s. XX, coincidiendo con el declive de los opiáceos, se extendió el consumo de cocaína por el mundo occidental, aumentando paralelamente su población adicta, llegando

a producir una de las epidemias de drogas más grandes de la historia, especialmente en Norteamérica (Cabrera, 2000). Hoy en día la adicción a la cocaína es extremadamente preocupante, siendo necesarias grandes inversiones en el tratamiento de desintoxicación y la prevención de su consumo.

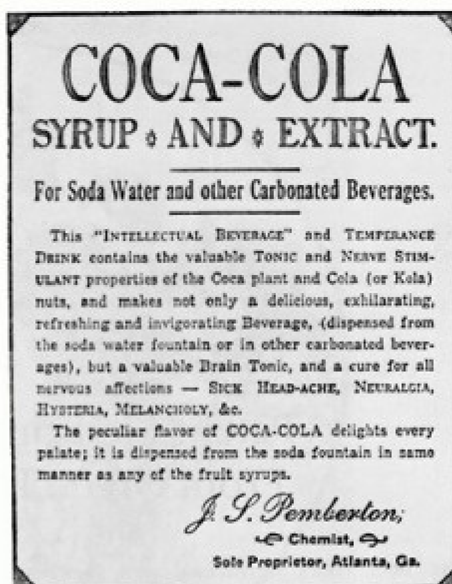


Figura 2. Anuncio de la conocida bebida Coca-Cola® de finales del siglo XIX

1.1.4. El cannabis

El uso y consumo del cannabis, como otras muchas drogas, también es relativamente antiguo. La planta *Cannabis sativa* es originaria de Asia, concretamente de China e India, donde se conoce desde hace milenios. En la antigua China, hace más de 5000 mil años, se utilizaba el cáñamo como elemento para elaborar fibras textiles, ropa, cuerdas, sacos y velas de barco. Fue en Oriente donde se extendió su consumo llegada la Edad Media, donde no solo utilizó el cannabis con fines religiosos, sino también bélicos. Se conoce que el líder de una secta chiita, Hasan ibn-al-Sabbah, arengaba a sus guerreros con el uso del cannabis como premio cada vez que una batalla se acercaba o en el caso que tuvieran que cometer algún asesinato, siempre eso si, por encargo y previo pago. Este grupo de guerreros se hicieron llamar

"haschischin" o "assassins", de donde deriva la palabra castellana asesinos, que significa fumadores de hachís (Khal and Basile, 1977). El auge del consumo de cannabis en la sociedad europea llegó entre los siglos XVIII y XIX, importado por las tropas napoleónicas de la campaña de Egipto. A finales del s. XIX, en Francia, nace el "Club de los Hachichins", creado por médicos, literarios y artistas, como De la Croix, Gautier o Baudelaire, promotores del consumo de cannabis. El cannabis es introducido en el continente americano por los conquistadores españoles, hacia el 1547. En 1930 se crea en Estados Unidos la Oficina Federal de Narcóticos, inicialmente para el control exclusivo del cultivo, uso y venta de la planta de cannabis, a consecuencia del agravio en su consumo a raíz de la inmigración mejicana. A partir de la II Guerra Mundial, arropado por el movimiento *hippie*, el consumo de cannabis se generaliza entre los jóvenes de toda Europa. Hoy en día es una de las sustancias de mayor consumo en todo el mundo, donde se han logrado un gran número de variedades con altos contenido del principal principio activo, el Δ-9-tetrahidrocannabinol (THC), aislado por primera vez en 1965 por Raphael Mechoulam (Khal and Basile, 1977; Cabrera, 1999).

1.1.5. Los alucinógenos (LSD)

Aunque se considera al cannabis como un alucinógeno moderado, existen una gran variedad de potentes enteógenos, que se caracterizan por producir en la persona fuertes alteraciones en la percepción de la realidad y una psicosis tóxica. El origen de estas sustancias puede ser natural o sintético, y su consumo se ha limitado a situaciones muy ocasionales. Se cree que algunos alucinógenos naturales poseen un origen milenario. Es el caso del uso del cactus peyote (*Lophophora williamsii)*, cuyo principio activo es la mescalina, utilizado por las civilizaciones americanas precolombinas, como parte de la espiritualidad tradicional.

Un gran número de autores relatan el descubrimiento de la LSD (dietilamida del ácido lisérgico) por el químico Albert Hofmann (Figura 3), en 1943, como un hecho accidental. No opina lo mismo su descubridor, quién nos describe en su libro, *"LSD, cómo descubrí el ácido y qué pasó después en el mundo",* la investigación que llevó a cabo sobre un gran número de derivados del ácido lisérgico, concretamente el número 25, de ahí su nombre, LSD-25. En 1938 Hofmann sintetizó por primera vez la LSD, con

la intención de crear un analéptico. Al probar el compuesto en animales en la sección farmacológica de Sandoz no se observó ningún efecto de los posibles predichos, sin embargo los animales se comportaban de una manera poco usual, por ejemplo, sobre los gatos, uno de los comportamientos más llamativos fue el temor que les producía la presencia de un ratón, o en chimpancés, dónde el LSD eliminaba cualquier norma de jerarquía, produciendo un desconcierto en el resto del grupo. Cinco años después de la primera síntesis de la LSD, Hofmann, retomó la investigación, y fue entonces cuando descubrió sus efectos psicodélicos en sí mismo. Durante su síntesis, una pequeña cantidad del derivado número 25 parece ser que fue absorbida por vía cutánea a través de la punta de sus dedos, al recristalizar dicha sustancia. A partir de ahí comenzó a sentirse mareado y a tener visiones extrañas, y tuvo que refugiarse en su domicilio, hasta que los efectos alucinógenos cedieron. Posteriormente, Hofmann se percató de la potente actividad de esta sustancia en cantidades muy pequeñas, y se decantó por el autoensayo, autoadministrándose dicha sustancia, conociendo de primera mano sus efectos (Hofmann, 1991).

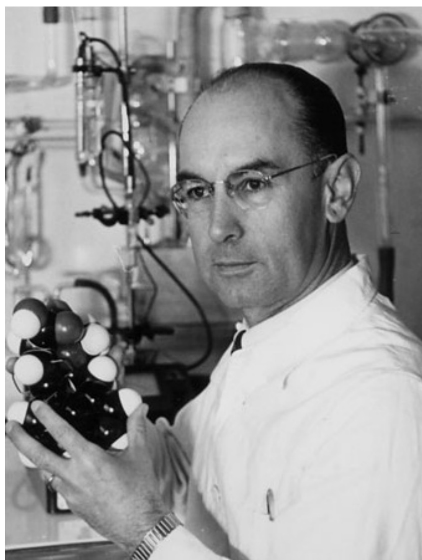


Figura 3. Albert Hofmann, (1906-2008), químico descubridor de la LSD.

En 1947, los laboratorios Sandoz empezaron a comercializar la LSD bajo el nombre de Delysid®, un fármaco indicado en la terapia psicoanalítica. En 1962, EEUU catalogó la LSD como droga experimental, prohibiendo su uso

clínico, aunque tanto la CIA como el ejército continuaron con sus experimentos (Cabrera and Cabrera, 1994). Gracias, nuevamente, al movimiento *hippie,* el consumo de LSD y otros alucinógenos vivió su máximo esplendor. Actualmente, la LSD se encuentra clasificada como ilegal gracias al Convenio de Viena de 1971.

1.1.6. Drogas de diseño o de síntesis

Bajo la denominación de “drogas de síntesis” o “drogas de diseño”, se agrupan una serie de sustancias de origen sintético que en los años sesenta comenzaron a ser objeto de tráfico ilegal. Son sustancias psicoactivas creadas a partir de la modificación de la estructura química de determinados productos naturales, como la efedrina, principio activo de la *Ephedra*, una planta milenaria utilizada ya en el s. III a.C. por los chinos como antiasmática y antiespasmódica. En 1887, el químico rumano L. Edeleano (1887), sintetizó por primera vez la anfetamina, y en 1893, en Japón, se sintetizó la metanfetamina, análogo N-metilado de la anfetamina, cristalizada en 1919 por Akira Ogata a partir de la reducción de la efedrina, utilizando fosforo rojo y yodo (Vermont Department of Health, 2012). A principios del s. XX, la industria alemana, empieza a sintetizar un gran número de moléculas cuyo núcleo principal se basa en una estructura de fenilisopropilamina. A partir de entonces se lleva a cabo la comercialización de la anfetamina (*Benzedrina*®) y la metanfetamina (*Methedrina*®) con fines tan variados como la narcolepsia, la descongestión nasal, la obesidad, la depresión o el trastorno de déficit de atención de niños y adultos.

Las anfetaminas han sido utilizadas, y siguen siéndolo hoy en día, como agentes para la mejora del rendimiento físico-deportivo o intelectual, consumidas con este último fin por muchos estudiantes , quiénes no sólo buscan una mejora cognitiva, sino también el objetivo de mantenerse despiertos y activos durante las largas fiestas nocturnas. A principios de la década de 1960 la fabricación clandestina de anfetamina y metanfetamina empezó a adquirir una importancia significativa, y durante la década de los 90 su producción casera vivió su punto álgido, provocando no sólo un delito de salud pública sino también un gran número de accidentes y explosiones, debidas al uso de peligrosas reacciones químicas para su síntesis.

En 1971, tanto la anfetamina como la metanfetamina, fueron sometidas a control internacional en el marco de la Convención Internacional de Psicotrópicos, incluidas en la Lista II de sustancias controladas, en la cual se acepta su uso médico en los Estados Unidos, bajo severas restricciones. A finales del s. XX y principios del s. XXI, las anfetaminas han invadido las discotecas y lugares de ocio nocturno de todo el mundo, produciendo grandes estragos en la sociedad juvenil de hoy en día.

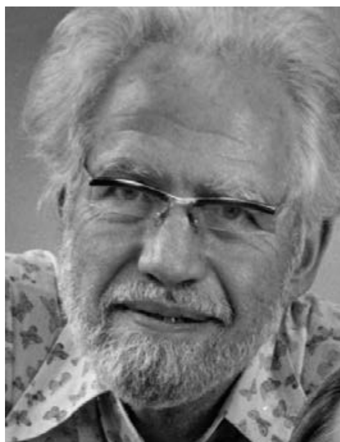


Figura 4. Alexander “Sasha” Shulgin (1925-2014)

Otro de los derivados anfetamínicos más extendido y consumido es la MDMA (3,4-metilendioximetanfetamina) más conocido como *éxtasis*. La MDMA cuenta con más de una década de abuso, aunque su verdadera historia es mucho más antigua, sintetizada por primera vez en diciembre de 1912, en los laboratorios Merck en Alemania, aunque existen informes que fue patentada en 1914, en realidad la sustancia patentada fue la MDA (3,4-metilendioxianfetamina), de estructura similar, sintetizada por Manis y Jacobsen, en 1910, con la intención de utilizarla como agente anorexígeno (Shulgin and Shulgin, 1990). Años más tarde, en 1965, el químico-farmacólogo Alexander Shulgin (Figura 4) resintetiza la MDMA y empieza a autoexperimentar con dicha sustancia, llegando a ser utilizada en el mundo de la psicoterapia gracias a sus propiedades favorecedoras de la comunicación interpersonal. Pero no fue hasta el 1976 cuando Alexander Shulgin y su colaborador, David Nichols, publican el primer informe sobre los efectos de la MDMA en humanos. Este hermetismo en torno a la psicoactividad de la MDMA fue debido al miedo de algunos profesionales a

que la MDMA se convirtiera en una "droga callejera", tal y como había pasado con la LSD años antes, que tras la difusión pública de sus efectos, pasó de ser un instrumento de ayuda para los psicoterapeutas a una droga ampliamente difundida dentro de la contracultura *hippie*, y rápidamente ilegalizada (Eisner, 1995).

La MDMA se fue introduciendo en los clubs más exclusivos de Chicago, New York, San Francisco y Detroit. Desde América, la música *house* y *techno,* de la mano del éxtasis, atraviesan el océano y llegan a Europa, primero a Ibiza y Valencia y posteriormente a Londres y Manchester, extendiéndose por todo el viejo continente.

Ralph Metzner fue el primero en designar a la MDA y la MDMA, con el nombre de empatógenos, en lugar de llamarlas drogas psicodélicas, ya que no producían verdaderas alucinaciones como sucede con la LSD, sino que, estas nuevas sustancias inducían en el consumidor un estado de empatía profundo por uno mismo y por los demás. En 1985, la DEA (Drug Enforcement Administration), incluyó la MDMA en la Lista I de sustancias prohibidas, quedando así prohibido su consumo tanto con fines terapéuticos como recreativos, basándose en un estudio realizado por Schuster, que demostraba que la MDA, análogo de la MDMA, era capaz de producir lesión cerebral en ratas (Ricaurte et al., 1985). Posteriormente, se produjeron algunos intentos, por parte de algunos psicoterapeutas, de incluir la MDMA en la Lista III, lo cual permitiría su uso con fines médicos. Sin embargo, la DEA no abandonó su propósito, y consiguió en 1988 que la MDMA estuviera incluida en la Lista I de la *Comprehensive Substances Act* de forma permanente.

1.1.7. Nuevas sustancias psicotrópicas

El mercado actual de las drogas parece ser más dinámico y basarse menos en una serie de sustancias a base de plantas. La globalización y los avances en la tecnología de la información son factores importantes al respecto. Están cambiando las pautas de consumo de drogas en los países de renta baja y media, y esto también puede tener consecuencias para la problemática de las drogas en un futuro. Internet presenta retos cada vez mayores, tanto como mecanismo para la difusión rápida de las nuevas tendencias como en su condición de mercado anónimo en rápido auge y de

alcance global. Internet crea una nueva interconexión entre el consumo y la oferta de drogas. No obstante, ofrece también oportunidades de búsqueda de vías innovadoras en la administración de tratamiento y en las intervenciones de prevención y de reducción de daños.

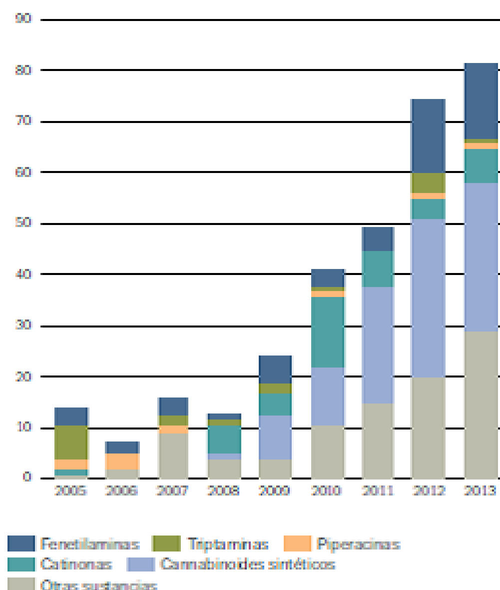


Figura 5. Principales grupos de sustancias psicotrópicas identificadas a través del sistema de alerta rápida desde 2005. *Fuente*: Observatorio Europeo de las Drogas y las Toxicomanías.

Las consecuencias de estos cambios para la salud pública no se conocen aún por completo. Los signos de éxito de las políticas actuales en algunos ámbitos deben examinarse a la luz de la cambiante situación de las drogas, que exigirá ajustes de las prácticas actuales si se quiere que sigan bien enfocadas y ajustadas a su objetivo. Cada vez es mayor en Europa el número de nuevas sustancias psicotrópicas, a menudo concebidas para imitar los efectos de las drogas controladas. Algunas se venden directamente en el mercado ilegal, mientras que otras, los “euforizantes legales” (*legal highs)*, se venden de forma más abierta. La evolución en este campo es muy rápida y aparecen nuevas sustancias a ritmo creciente.

El Sistema de alerta rápida de la UE sigue recibiendo notificaciones que apuntan a la aparición de una nueva sustancia más o menos cada semana en 2013. En los últimos años destaca la aparición de nuevos agonistas sintéticos de receptores de cannabinoides, de fenetilaminas y de catinonas,

que imitan en gran medida las drogas ilegales más conocidas (Figura 5). Más reciente aún es el aumento creciente de la presencia de grupos químicos menos conocidos. Muchos de los productos en venta contienen mezclas de sustancias y la falta de datos farmacológicos y toxicológicos dificulta cualquier especulación sobre las consecuencias de su consumo a largo plazo para la salud, aunque hay cada vez más datos de que algunos de ellos causan problemas que requieren tratamiento médico y se han registrado casos de muerte (EMCDDA, 2013).

La Comisión Europea está preparando una nueva propuesta para reforzar la respuesta de la UE a las nuevas sustancias psicotrópicas. Aunque el consumo de éstas se da principalmente con carácter recreativo entre jóvenes, se ha observado una cierta difusión en poblaciones de consumidores problemáticos. En países que han informado de problemas de escasez de heroína se observa una cierta sustitución de opiáceos por estimulantes sintéticos, especialmente catinonas. No está claro por qué se sustituye la heroína por vía parenteral por las catinonas, pero tal evolución se puede vincular a la fácil disponibilidad y la alta calidad de las nuevas drogas (EMCDDA, 2013). Hasta la fecha, los países han establecido diversas medidas de control para responder al problema de las nuevas drogas.

1.2. Anfetamina

Las anfetaminas son sustancias psicoestimulantes, que producen un aumento del nivel de alerta, del insomnio, de la energía y de la confianza, asociado a una disminución de la fatiga y el apetito, así como una sensación de bienestar y euforia. Dosis elevadas también pueden provocar convulsiones, movimientos estereotipados y psicosis. La fatiga, la ansiedad y el cansancio pueden aparecer cuando los efectos psicoestimulantes se desvanecen (Camí and Farré, 2003; Green et al., 2003). Todos estos efectos negativos se intensifican cuando se utilizan dosis repetidas. Un uso prolongado de este tipo de sustancias se caracteriza por la aparición de reacciones psicóticas, alucinaciones y paranoia, además muestran un alto potencial de abuso y pueden incluso llegar a inducir síntomas de dependencia y tolerancia (Westfall and Westfall, 2011).

1.2.1. Mecanismo de acción

Años después de la síntesis de la anfetamina por L. Edeleano, Gordon Alles, en un intento de desarrollar un potente descongestionante, volvió a sintetizar la anfetamina y fue uno de los primeros en mostrar sus efectos estimulantes (Hanson et al., 2011). Sin embargo, pasaron muchos años antes de que el mecanismo de acción de la anfetamina fuera completamente elucidado. Durante los años 50, muchos investigadores sugirieron que la anfetamina actuaba como una sustancia que liberaba noradrenalina (NA) (Burn and Rand, 1958). Años más tarde, algunos estudios demostraron que la anfetamina también promovía la liberación de otras catecolaminas, especialmente dopamina (DA). Desde entonces, un gran número de estudios se han centrado en el impacto que producía la anfetamina y sus análogos en los transportadores de membrana, incluyendo el transportador de dopamina (DAT), el de serotonina (SERT) y el de noradrenalina (NET). Aunque la anfetamina y algunos de sus derivados generalmente muestran una acción similar en los diferentes transportadores, el DAT es la proteína más frecuentemente implicada en sus propiedades reforzantes y de abuso (Kuczenski et al., 1995; Gulley and Zahniser, 2003; Sulzer et al., 2005).

Numerosas evidencias indican que los transportadores de membrana son las dianas a través de las cuales la anfetamina causa una liberación de monoaminas. Se conoce que la anfetamina libera DA mediante un

mecanismo que puede ser anulado por un inhibidor de la recaptación de DA, como es el caso de la nomifensina (Raiteri et al., 1979). Al mismo tiempo, Fischer y Cho sugirieron un modelo de difusión por intercambio en el cual la anfetamina extracelular era sustituida o intercambiada por DA y transportada dentro de la célula a través del DAT. Esto aumentó la probabilidad que la DA citosólica se uniera al DAT y fuera transportada hacia el espacio extracelular (Fischer and Cho, 1979). Más tarde, Liang y Rutledge propusieron un mecanismo dual dependiente de la concentración para la liberación de DA. Concretamente, a bajas concentraciones, la anfetamina extracelular es intercambiada por la DA citosólica a través del DAT. Sin embargo, a altas concentraciones, la anfetamina, un compuesto altamente lipofílico, difunde hacia el interior del terminal nervioso a través de la membrana plasmática y libera DA, permitiendo su salida del terminal mediante un transporte reverso mediado por el DAT (Liang and Rutledge, 1982). Para que tal modelo pueda cumplirse la anfetamina debe ser sustrato del DAT y ser transportada por éste, y así se demostró como la acumulación de anfetamina era saturable, y dependiente de la temperatura en sinaptosomas de estriado, además de indicar un transporte activo del ligando (Zaczek et al., 1991). Otras muchas evidencias por la cuales se cree que la anfetamina actúa como sustrato del DAT provienen de estudios donde se demuestra que la anfetamina inhibe la actividad del DAT en estriado por competición con la DA por un lugar de unión común, mecanismo contrario a la cocaína, que inhibe el DAT mediante un lugar de unión completamente diferente al de la DA (Wayment et al., 1998). Esta actuación como sustrato es fuertemente predecible si tenemos en cuenta la similitud estructural de la anfetamina con la DA (Figura 6).

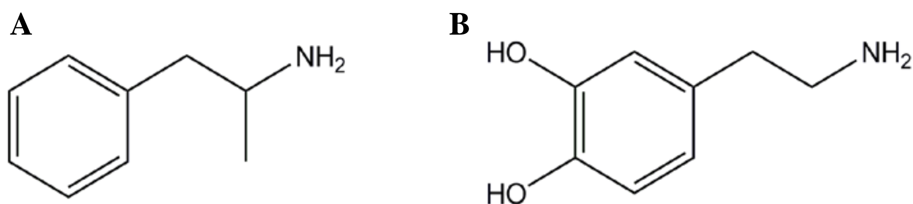


Figura 6. Estructura química de la anfetamina (**A**) y la dopamina (**B**)

Desde hace mucho tiempo se ha reconocido que además del DAT, el transportador vesicular de monoaminas (VMAT) es un mediador importante en la inducción por parte de la anfetamina de la liberación de

neurotransmisor (Sulzer et al., 2005) . Existen al menos dos hipótesis, no excluyentes entre ellas, que podrían explicar el mecanismo por el cual la anfetamina redistribuye las monoaminas vesiculares hacia el citosol; la hipótesis de la base débil y la competición por el VMAT.

La anfetamina es una base lipofílica débil, con un pKa de 9,9 (Mack and Bönisch, 1979) que puede ser protonada en los orgánulos ácidos, incluyendo las vesículas de catecolaminas (Sulzer and Rayport, 1990); una vez cargada, se convierte en una molécula con un poder de permeabilización de membrana mucho menor y es acumulada en el interior de la vesícula, provocando una alcalinización de dicho orgánulo. Este proceso produce una alteración en el gradiente electroquímico de protones necesario para el secuestro o almacenamiento de la DA vesicular, causando un aumento de la DA citosólica, que a su vez da lugar a una alteración en el gradiente de concentración y al transporte reverso de DA a través del DAT de membrana (Sulzer and Rayport, 1990; Sulzer et al., 1995). Independientemente de la acción de la anfetamina sobre el DAT de membrana, este modelo produce una liberación de DA a través del DAT gracias a un aumento de los niveles de DA citosólica y una alteración del secuestro vesicular. Diversos estudios han demostrado que bases débiles, las cuales producen una liberación de neurotransmisor similar a la anfetamina, no son sustratos del DAT y a pesar de ello son capaces de inducir un transporte reverso de DA en cultivos celulares (Sulzer et al., 1993; Sulzer et al., 1995). Sin embargo, no existe una estrecha relación entre la disipación del pH en las vesículas y la acumulación de monoaminas. Existen estudios en los cuales se ha probado dicha hipótesis mediante comparación con los efectos del pH vesicular. Floor y Meng demostraron como una concentración de 3μM de anfetamina era capaz de mermar más de un 50% las reservas de DA pero sólo era capaz de colapsar el gradiente de protones en un 12%, aunque al aumentar la concentración de anfetamina (100μM) el nivel de alcalinización correlacionaba con la liberación de neurotransmisor (Floor and Meng, 1996).

Otro indicador por el cual la alcalinización de las vesículas podría no ser suficiente para explicar la redistribución de la DA vesicular es que la bafilomicina, un inhibidor de la bomba de protones que no es sustrato del VMAT, disminuye dos veces más el gradiente de pH que la anfetamina, y en cambio libera la mitad de DA (Floor and Meng, 1996). Se conoce que el

estéreoisómero S-(+)-anfetamina es varias veces más eficaz en el efecto vesicular que el isómero R-(-) (Peter et al., 1994) además de exhibir una unión preferente por el transportador vesicular (Peter et al., 1994; Erickson et al., 1996). Todos estos resultados nos llevan a creer que la competición por el VMAT juega un papel complementario en el mecanismo de acción de la anfetamina (Figura 7).

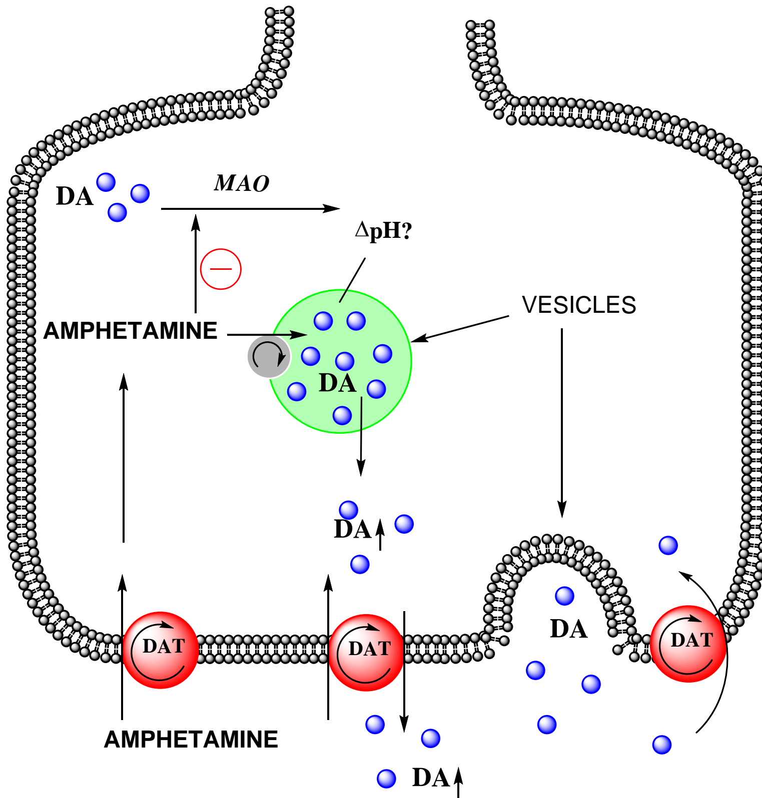


Figura 7. Esquema del mecanismo de acción de la anfetamina sobre los terminales dopaminérgicos

Mientras que la anfetamina compite con la reserpina por la unión con el VMAT (Peter et al., 1994) y con ligandos por el transportador de membrana plasmática, no es fácil demostrar que de hecho sean sustratos del transportador. Si realmente es transportada por el VMAT, es probable que el movimiento de la anfetamina del citoplasma al interior de la vesícula promueva un transporte reverso de monoaminas, ya que es un fenómeno general de las proteínas transportadoras. Independientemente de si la

anfetamina es sustrato del VMAT o simplemente se une sin ser transportado, debe haber una competición entre las monoaminas y la anfetamina por el lugar de captación. La unión de la anfetamina y sus derivados por el VMAT ya ha sido demostrada (Reith and Coffey, 1994; Erickson et al., 1996) pero la competición por la unión por sí misma no es un simple parámetro que se pueda medir ya que se requiere un gradiente de pH vesícula-citosol para la captación, y éste puede ser alterado a causa de los diferentes protocolos a utilizar. Schuldiner y colaboradores (Schuldiner et al., 1993) demostraron que una auténtica disipación del gradiente de pH puede producir una aparente inhibición competitiva. Esto nos llevaría a una sobreestimación del efecto provocado a causa de la unión con el VMAT y a una subestimación del efecto a causa de la acción de la base débil.

En resumen, existen numerosas preguntas sin respuesta sobre los efectos de la anfetamina a nivel de las vesículas sinápticas. El papel de la anfetamina en la competición por la captación de las catecolaminas puede ser deducido, particularmente cuando nos encontramos con bajos niveles de anfetamina, pero sigue siendo diferente al efecto producido por sustancias con propiedades de base débil.

Además de los efectos sobre la membrana plasmática y las vesículas sinápticas, existe otro medio por el cual la anfetamina puede influir en la liberación de catecolaminas, aumentando la cantidad de aminas disponibles para su liberación mediante la inhibición de la monoamino oxidasa (MAO) (Leitz and Stefano 1971; Blaschko et al., 1937; Mantle et al., 1976; Robinson et al., 1985; Scorza et al., 1997). La anfetamina, además de promover la liberación de catecolaminas, también activa los receptores pre-sinápticos α-2 adrenérgicos (Ritz and Kuhar, 1989) y los receptores nicotínicos de acetilcolina de placa motora (Liu et al., 2003).

1.2.2. Farmacocinética

Las principales vías por las cuales se consume la anfetamina son oral o intranasal, ya sea en su forma enantioméricamente pura ((S)-(+)-anfetamina) o mezcla racémica (por ejemplo, sulfato de anfetamina) (Quinn et al., 1997; Caldwell and Wainer, 2001). Una sola dosis de anfetamina por vía oral se absorbe muy rápidamente. Tanto la Cmax como el área bajo la curva registrada durante las primeras 24 horas, son proporcionales a la dosis

administrada (Angrist et al., 1987; Perez-Reyes et al., 1992; Pizarro et al., 1999). Cabe destacar que no se observan grandes diferencias en los parámetros farmacocinéticos durante la fase de absorción al administrar tanto la mezcla racémica como los dos enantiómeros individuales (Wan et al., 1978).Las anfetaminas, en general, poseen baja afinidad por las proteínas plasmáticas, confiriéndoles una alta biodisponibilidad y favoreciendo una fácil difusión del plasma hacia el compartimento

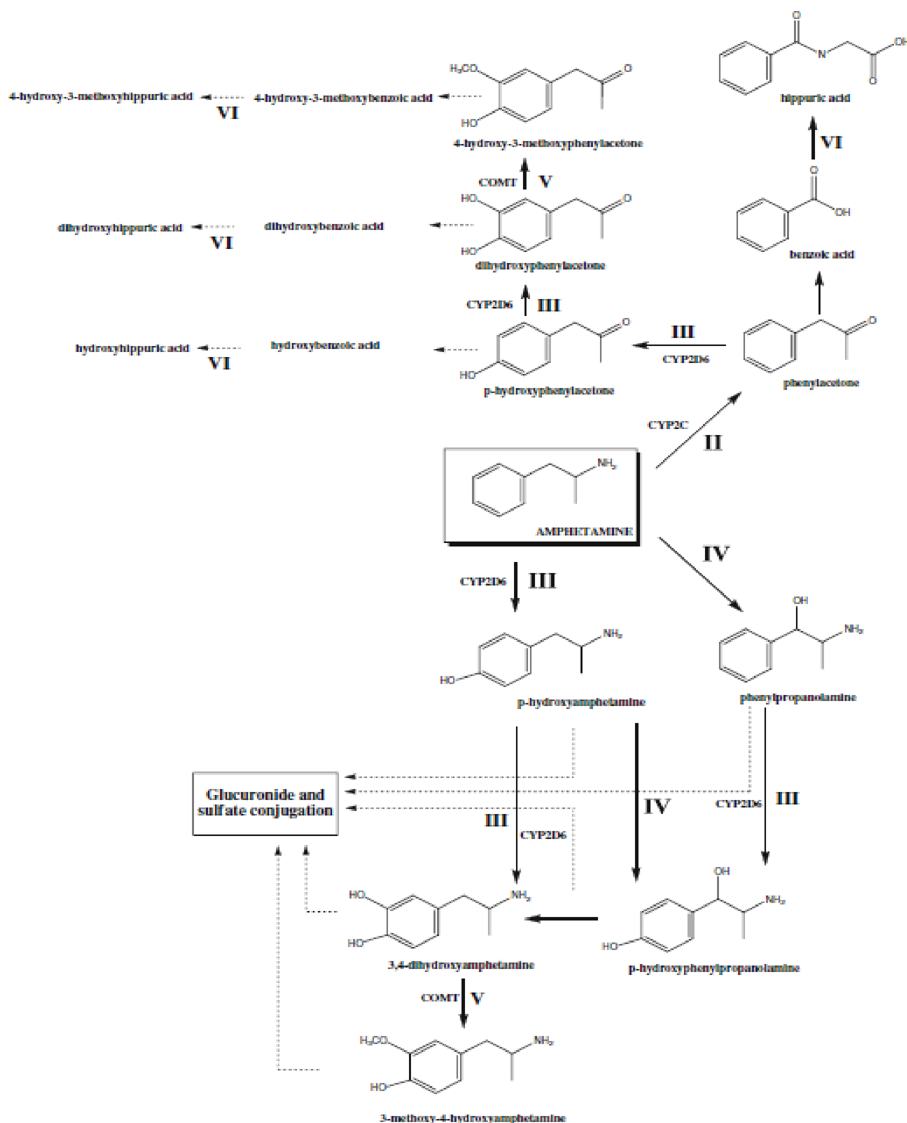


Figura 8. Esquema del metabolismo de la anfetamina (Carvalho et al., 2012).

extravascular (de la Torre et al., 2004). Los consumidores adictos a la anfetamina parecen poseer un mayor volumen de distribución y tiempo de semivida de eliminación ($t_{1/2\beta}$) que los individuos que nunca antes habían consumido dicha sustancia, probablemente como consecuencia del desarrollo de una tolerancia farmacocinética (de la Torre et al., 2004; Busto et al., 1989).

La anfetamina es metabolizada principalmente a través de una N-desaminación y una posterior oxidación para convertirse en el correspondiente ácido benzoico, que más tarde se conjuga con la glicina y es excretado en forma de ácido hipúrico. Otra vía consiste en una hidroxilación en el carbono número 4 del anillo aromático, generando la 4-hidroxianfetamina, seguida de una conjugación del fenol con ácido glucurónico o sulfato (Kraemer and Maurer, 2002). Existe una ruta metabólica minoritaria compuesta por una oxidación en el carbono β de la cadena lateral, permitiendo la formación de norefedrina, la cual a su vez, puede ser oxidada en el anillo aromático a hidroxinorefedrina (Kraemer and Maurer, 2002) (Figura 8). La N-desaminación de la anfetamina parece ser catalizada por la isoenzima CYP450 de la subfamilia del CYP2C (Yamada et al., 1997), mientras que el CYP2D6 está involucrado en la hidroxilación del anillo aromático (Bach et al., 2000).

1.2.3. Neurotoxicidad

Los efectos neurotóxicos de las anfetaminas se han evaluado principalmente a través de técnicas bioquímicas e histológicas o inmunocitoquímicas. Los marcadores neurotóxicos incluyen la disminución de los niveles de monoaminas y sus metabolitos, la disminución de los lugares de unión de los transportadores de monoaminas, así como también una alteración en la expresión o actividad de las enzimas involucradas en la biosíntesis y el metabolismo de los neurotransmisores monoaminérgicos en el cerebro. Los indicadores neurotóxicos histológicos o inmunocitoquímicos se basan en el uso de métodos de tinción o anticuerpos específicos dirigidos hacia marcadores neuronales concretos. Probablemente, el primer estudio que claramente describía que la anfetamina podía producir una depleción de monoaminas se publicó en 1961. McLean y McCartney descubrieron que las ratas tratadas con S-(+)-anfetamina presentaban niveles de NA inferiores

al grupo control, incluso algunos días después del tratamiento (McLean and McCartney, 1961).

El abuso crónico de anfetamina está asociado con una alteración de la atención y la memoria, problemas de aprendizaje, así como en la toma de decisiones (McKetin and Mattick, 1997; Rogers et al., 1999; Ornstein et al., 2000). Algunas de estas complicaciones neuropsiquiatricas se cree que están relacionadas con los efectos neurotóxicos producidos por el consumo de esta sustancia. Estos efectos neurotóxicos consisten en una disminución en la actividad o expresión de la tirosina hidroxilasa (TH) (Ellison et al., 1978), una depleción de DA a largo plazo (Wagner et al., 1980), la pérdida de DATs (Scheffel et al., 1996; Krasnova et al., 2001), o incluso una disminución del VMAT (Krasnova et al., 2001). Además de todos estos efectos a nivel del terminal monoaminérgico, la anfetamina puede causar muerte celular en cultivos primarios de células corticales y de células PC12 in vitro (Stumm et al., 1999; Lotharius and O'Malley, 2001; Oliveira et al., 2002) así como una degeneración en los cuerpos celulares de la corteza cerebral (Jakab and Bowyer, 2002).

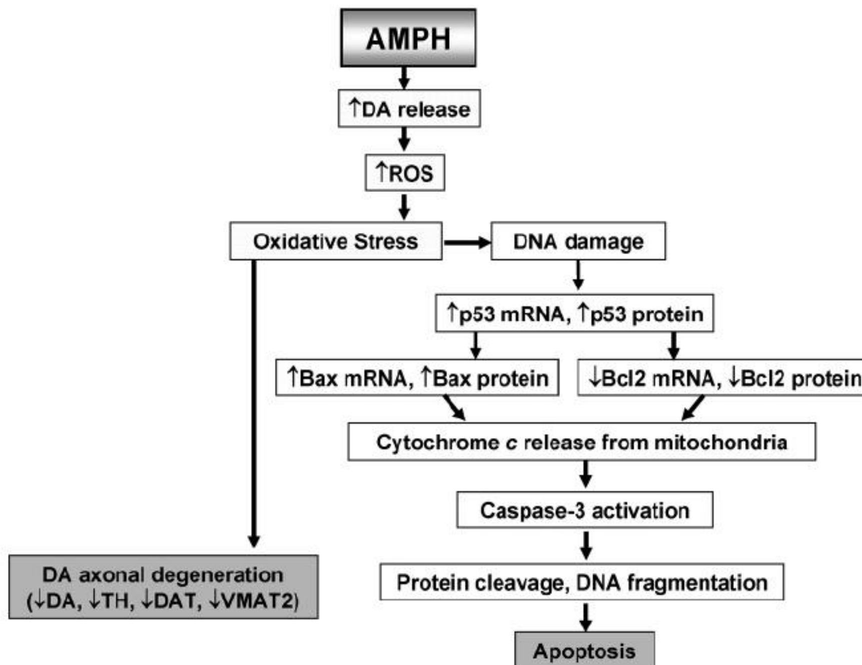


Figura 9. Esquema de los procesos responsables de la neurotoxicidad de la anfetamina (Cadet et al., 2007)

Como ya se ha explicado anteriormente, la anfetamina induce una redistribución de la DA de las vesículas sinápticas hacia el citosol, seguido por la liberación de dicha DA al espacio sináptico mediante transporte reverso a través del DAT, produciendo un aumento de los niveles de monoamina en la hendidura sináptica. El metabolismo de la DA puede suponer la producción de radicales hidroxilo y superóxido (Huang et al., 1997; Krasnova et al., 2001) que participan en los efectos tóxicos de la droga a través de una destrucción de los terminales monoaminérgicos mediada por la formación de dichos radicales (Figura 9) (Huang et al., 1997; Cadet and Brannock, 1998; Wan et al., 2000; Krasnova et al., 2001).

Se sabe que los astrocitos son los responsables de estabilizar y mantener la reparación homeostática de los tejidos y contribuyen a una rápida reparación del daño neuronal (Eddleston and Mucke, 1993). Por otro lado, las células microgliales están implicadas en numerosas formas de daño neuronal, incluyendo las asociadas con la isquemia, enfermedades neurodegenerativas o una lesión cerebral directa (Moore and Thanos, 1996; González-Scarano and Baltuch, 1999; Gebicke-Haerter, 2001). La activación de dicha microglia produce una variedad de citocinas proinflamatorias, prostaglandinas y especies reactivas de oxígeno como respuesta a una lesión cerebral, explicando cómo pueden contribuir o agravar el daño neuronal (Gebicke-Haerter et al., 2001; Pocock and Liddle, 2001). Un gran número de estudios realizados en roedores han demostrado que la anfetamina favorece la activación tanto de la astroglia como de la microglia en diferentes regiones cerebrales (Miller and O'Callaghan, 1996; Bowyer, 2000; Krasnova et al., 2001; Jakab and Bowyer, 2002; Armstrong et al., 2004; Thomas et al., 2004).

La regulación de la temperatura corporal parece ser también un factor importante en la respuesta neurotóxica de la anfetamina. En roedores, la anfetamina muestra un efecto bifásico. A dosis bajas (≤2,5 mg/kg) induce hipotermia, mientras que a dosis más elevadas (≥5 mg/kg) causa hipertermia a una temperatura ambiente de unos 20ºC (Seale et al., 1985; Krasnova et al., 2001; Baker and Meert, 2003). Dicha hipertermia puede favorecer la toxicidad inducida por la anfetamina, ya que un aumento de temperatura incrementa la formación de radicales libres (Kil et al., 1996) y porque la hipertermia también potencia los efectos citotóxicos de las especies reactivas de oxigeno (Lin et al., 1991).

1.3. MDMA (3,4-metilendioximetanfetamina) o *éxtasis*

Éxtasis es el nombre popular por el cual se conoce a la sustancia identificada químicamente como 3,4-metilendioximetanfetamina (MDMA) o N-metil-3,4-metilendioxi-anfetamina. La MDMA difiere de la anfetamina y la metanfetamina en un aspecto estructural importante. Como se puede observar en la figura 10B, la MDMA posee un grupo metilendioxi (-O-CH_2-O-) unido a las posiciones 3 y 4 del anillo aromático de la molécula de metanfetamina. Así, la estructura de la MDMA posee cierta similitud con la sustancia alucinógena mescalina (Figura 10). Como resultado de dicha semejanza, la MDMA comparte efectos farmacológicos tanto de la anfetamina como de la mescalina.

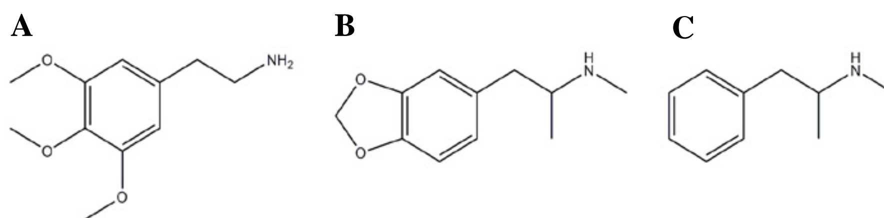


Figura 10. Estructura química de la mescalina (**A**), la MDMA (**B**) y la metanfetamina (**C**)

La MDMA se suele consumir por vía oral, en forma de comprimidos o como un polvo cristalino envuelto en papel de cigarrillo o disuelto en la bebida. El rango de dosis de la MDMA, para uso recreacional, varía entre los 50 y los 150 mg (Theune et al., 1999). Sin embargo, un elevado porcentaje de comprimidos se encuentran adulterados con análogos de la fenetilamina, como la MDE o la MDA, con otros psicoestimulantes como la anfetamina o la cafeína, e incluso muchos de ellos poseen sustancias que no producen ningún tipo de efecto psicoactivo (Parrott, 2004).

Tal y como ocurre con la mayoría de drogas de abuso, algunos de los efectos farmacológicos de la MDMA son efectos buscados o deseados por sus consumidores, sin embargo, existen además efectos claramente no deseados o adversos. Algunos de los efectos deseados son la euforia, la sensación de bienestar y placer, acompañados por un aumento de energía, locuacidad, disminución del cansancio y el apetito. Los consumidores relatan principalmente la inducción de un estado emocional positivo, caracterizado por un aumento de la empatía, una mayor facilidad para

comunicarse y para las relaciones interpersonales, es decir, un efecto entactógeno. La MDMA no es un compuesto afrodisíaco, sin embargo, parece ser que aumenta la sensualidad (aumento de la sensación de atracción sexual). A diferencia de la anfetamina, la MDMA no mejora el rendimiento psicomotor o la concentración, incluso puede llegar a empeorar dicho rendimiento cuando es administrada o consumida en dosis elevadas (Green et al., 2003).

La MDMA produce una estimulación del sistema nervioso simpático con un aumento del diámetro de la pupila (midriasis), el cual puede llegar a producir visión borrosa y una elevada sensibilidad a la luz. La MDMA también produce sequedad bucal, sudoración, temblor, tensión en la mandíbula y lo que se conoce como bruxismo (apretar o rechinar los dientes). Además de estos efectos, la MDMA también produce aumento de la temperatura corporal, aumento de la presión arterial y del ritmo cardíaco. La mayoría de todos estos efectos son considerados como efectos no deseados por los consumidores (Green et al., 2003).

1.3.1. Mecanismo de acción

El mecanismo de acción de la MDMA es similar al de la anfetamina, produciendo una liberación de monoaminas (NA, DA y 5-HT) en el espacio sináptico. La principal diferencia es que mientras la anfetamina posee mayor actividad adrenérgica y dopaminérgica que serotoninérgica, la MDMA parece ser más activa a nivel serotoninérgico que no dopaminérgico o adrenérgico (White et al., 1996).

En las neuronas monoaminérgicas, los neurotransmisores formados, por ejemplo la 5-HT, se almacenan en las vesículas situadas cerca de la sinapsis. Cuando llega un impulso nervioso el contenido de estas vesículas se libera hacia la sinapsis, y los neurotransmisores pueden unirse a receptores post-sinápticos. De la misma manera los neurotransmisores pueden unirse a los receptores pre-sinápticos situados en la misma neurona en la cual se ha producido la liberación y de esa manera regular la cantidad de neurotransmisor que deben liberar. La acción de estas monoaminas finaliza con la recuperación o recaptación de la mayoría de ellas mediante los transportadores responsables de su recogida del espacio sináptico e introduciéndolas de vuelta dentro del citoplasma. Una vez en el citoplasma,

estas monoaminas son introducidas al interior de las vesículas de almacenaje mediante otro sistema de transporte vesicular (VMAT-2). Tal y como ocurre con la anfetamina y otros derivados, la MDMA posee la capacidad de vaciar el contenido de las vesículas hacia el citoplasma y posteriormente, mediante un transporte reverso de las proteínas de membrana, las monoaminas son liberadas hacia la sinapsis (White et al., 1996). Además, la MDMA inhibe la triptófano hidroxilasa (TPH), enzima limitante de la biosíntesis de 5-HT (Che et al., 1995).

Resumiendo, la MDMA provoca una importante liberación de 5-HT, impide su recaptación y produce una depleción de las reservas de 5-HT situadas en las vesículas, todo ello combinado con una disminución de la síntesis de dicho neurotransmisor. El resultado es un aumento inicial de 5-HT seguido por una disminución, entre 2 y 4 horas más tarde, que puede persistir más de 24 horas. Como muchos otros derivados anfetamínicos, la MDMA posee cierta actividad como inhibidor de la MAO-A (Leonardi and Azmitia, 1994). La MDMA y algunos de sus derivados son también agonistas de los receptores α_2-adrenérgicos, los muscarínicos M_1 y los serotoninérgicos 5-HT_2 (White et al., 1996).

1.3.2. Farmacocinética

Aunque existen un gran número de estudios sobre las concentraciones de MDMA y sus metabolitos en animales, sólo unos pocos muestran valores farmacocinéticos a causa de la dificultad de la recogida en serie de muestras de plasma. Se han descubierto grandes diferencias en el metabolismo del MDMA entre especies, cepas, sexo y vía de administración.

En ratas Sprague-Dawley, después de una sola dosis de 10 mg/kg intravenosa, el $t_{1/2\beta}$ es de 1,7 h con un volumen de distribución de aproximadamente 7 L/kg. También se ha observado un metabolismo enantioselectivo, favoreciendo el aclaramiento de la (S)-MDMA sobre la (R)-MDMA (Cho et al., 1990). En primates, a una dosis de 1,5 mg/kg de MDMA por vía intravenosa, el pico de concentración máxima plasmática alcanza los 9000 μg/L aproximadamente en 1,5 h, con un $t_{1/2\beta}$ de 1,6 h y un volumen de distribución de 1,74 L/kg. Además la MDMA posee una farmacocinética no lineal en Saimiri (monos ardilla) (Mechan et al., 2006). En ratas, la principal vía metabólica es la N-desmetilación, dando lugar a la

3,4-metilendioxianfetamina (MDA). Otros metabolitos hallados en rata son la 3-hidroxi-4-metoximetanfetamina, la 4-hidroxi-3-metoximetanfetamina (HMMA), la 3,4-dihidroximetanfetamina (HHMA), la 4-hidroxi-3-metoxifenilacetona, la 3,4-metilendioxifenilacetona y la 3,4-dihidroxifenilacetona (Lim and Foltz, 1988). En humanos, la MDMA se absorbe fácilmente en el tracto intestinal y alcanza su concentración máxima plasmática alrededor de las 2 h posteriores a la administración oral (Mas et al., 1999; Farré et al., 2004). Sin embargo, algunos de los datos que provienen de administraciones orales difieren en el rango de absorción ya que, dicho proceso, depende estrechamente de la forma farmacéutica utilizada para la administración de MDMA (cápsula de gelatina, comprimido o solución oral).

La administración de dosis de 50, 75 y 125 mg de MDMA a voluntarios sanos produjeron un pico de concentración máxima en sangre de 106, 131 y 236 ng/ml, respectivamente (de la Torre et al., 2004; Mas et al., 1999). Estas concentraciones son relativamente bajas, ya que la biodisponibilidad de la MDMA en humanos se ve afectada por un importante efecto de primer paso hepático (de la Torre et al., 2000).

En humanos, existen dos principales vías en el metabolismo de la MDMA (Figura 11); la O-demetilenación es la principal vía, regulada por un gran número de isoformas del citocromo P450, dando lugar a la 3,4-dihidroximetanfetamina (HHMA), por otro lado, la N-dealquilación es la ruta metabólica minoritaria, que da lugar al metabolito MDA, el cual puede ser posteriormente también O-demetilenado para dar lugar a la 3,4-dihidroxianfetamina (HHA). Tanto la HHMA como la HHA son O-metiladas (HMMA y HMA) en una reacción regulada por la catecol-O-metiltransferasa (COMT) o formar los glucorono/sulfato conjugados. (de la Torre et al., 2000a; de la Torre et al., 2000b; de la Torre et al., 2004; Wenk et al., 2004). Una fracción de la HHMA y la HHA puede sufrir posteriores autooxidaciones a las correspondientes orto-quinonas, las cuales son posteriormente conjugadas para formar aductos glutationiles (Perfetti et al., 2009).

Después de una administración oral única de 100 mg de MDMA, las concentraciones plasmáticas de HHMA y HMMA son similares a las de la MDMA inalterada, con unos valores de concentración máxima plasmática

(C_{max}) de aproximadamente 180µg/L y 160µg/L a la hora y dos horas post-administración, respectivamente (Segura et al., 2005). El pico de concentración máxima para el metabolito MDA es de aproximadamente 13µg/L entre las 5 y las 7 horas después de la administración oral.

La MDMA posee en humanos una cinética no lineal por encima de ciertas dosis, ya que las concentraciones plasmáticas no son proporcionales a las dosis administradas, y parece ser que tiende a acumularse a altas dosis. Muchas de las intoxicaciones agudas producidas por la MDMA se han asociado a esta tendencia de acumulación de concentraciones plasmáticas elevadas (Gilhooly and Daly, 2002). Finalmente, tal y como ocurre en rata, la MDMA posee una farmacocinética enantioselectiva en humanos, siendo elminada la (S)-MDMA más rápidamente que la (R)-MDMA (Pizarro et al., 2004).

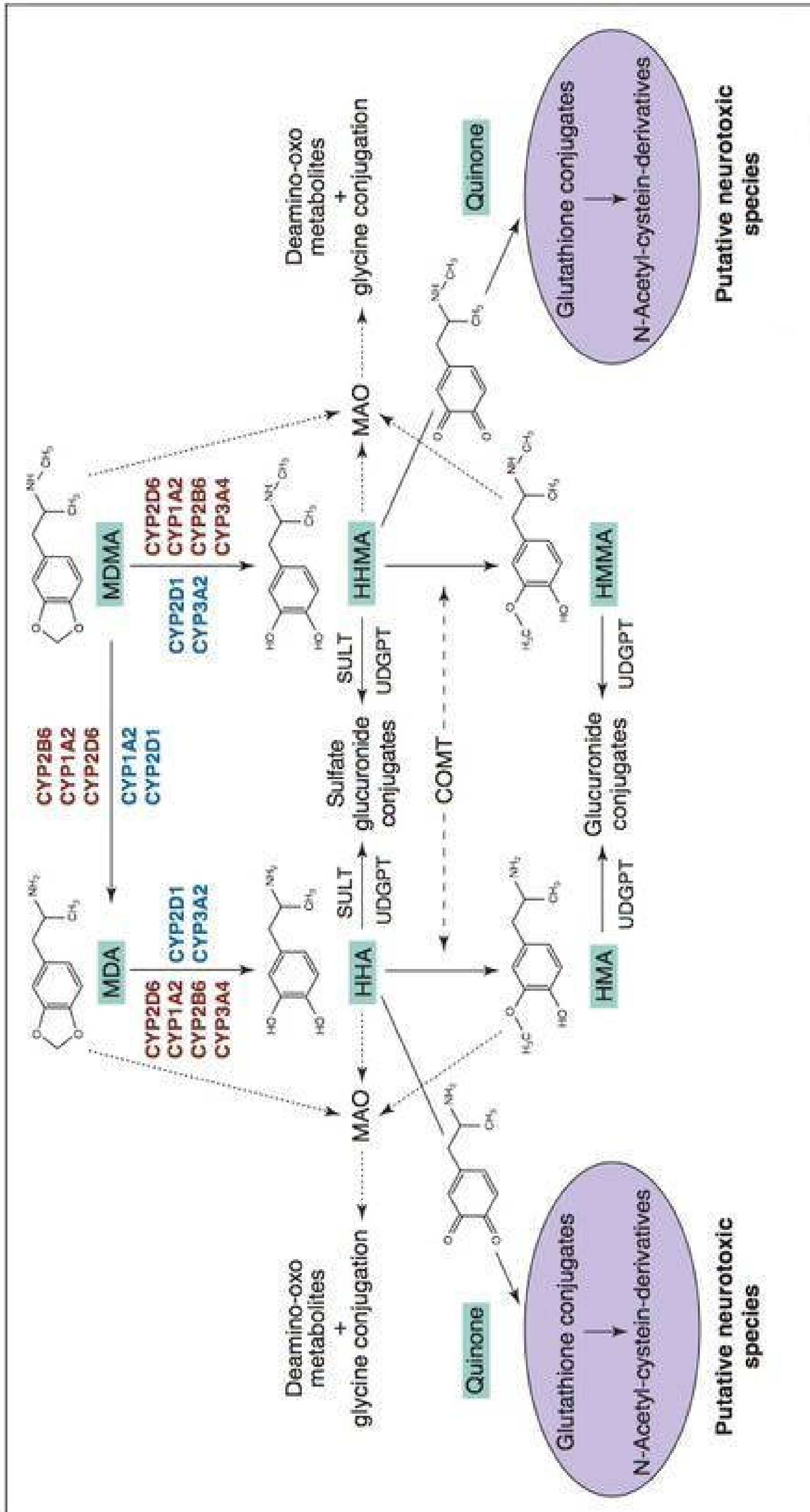


Figura 11. Ruta metabólica de la MDMA en rata y humano. Las isoenzimas del citocromo P450 involucradas en la N-demetilación y la O-demetilenación en ratas se encuentran resaltadas de color azul, mientras que en humanos se muestran en rojo. Adaptado de (de la Torre et al., 2004)

1.3.3. Neurotoxicidad

La neurotoxicidad de la MDMA está asociada a la reducción, a largo plazo, de los marcadores de la actividad del sistema serotoninérgico. Estas reducciones se han observado tanto en ratas como en primates no humanos, siendo estos últimos mucho más sensibles a los efectos neurotóxicos. La administración repetida de MDMA induce una reducción de las concentraciones tisulares de 5-HT, así como de su metabolito, el ácido 5-hidroxi-indol-acético (5-HIAA), y de la actividad de la TPH, enzima limitante en la biosíntesis de 5-HT (Schmidt, 1987). Todos estos déficits pueden persistir meses y dependen del área estudiada y del tipo de animal de experimentación utilizado (Ricaurte et al., 2000; Gudelsky and Yamamoto, 2003). La MDMA también produce una disminución de los lugares de recaptación de 5-HT en distintas áreas cerebrales (córtex, núcleo caudado e hipocampo). Asimismo, se ha descrito, en cerebro de rata, una regulación al alza de los receptores postsinápticos 5-HT_{2A} en la corteza occipital, debido, posiblemente, a un efecto compensador a causa de la depleción de 5-HT inducida por la MDMA (Reneman et al., 2002).

En ratas, la MDMA es selectivamente neurotóxica a nivel del sistema serotoninérgico a dosis que no tienen ningún efecto persistente sobre el sistema dopaminérgico (Pubill et al., 2003). Sin embargo, en ratones, la MDMA es una neurotoxina fundamentalmente dopaminérgica, produciendo una disminución de los niveles de DA y de sus metabolitos varios días después del tratamiento (Green et al., 1995).

Como se ha comentado anteriormente, los primates no humanos son una especie muy sensible a los efectos neurotóxicos de la MDMA. La depleción serotoninérgica y la disminución de los lugares de recaptación de 5-HT en la corteza cerebral de monos ocurre de forma más acusada y a dosis inferiores a las utilizadas en la rata. Por ejemplo, una dosis repetida de 2,5mg/kg de MDMA ocasiona una reducción de la densidad de axones serotoninérgicos en el primate similar a la producida en la rata a una dosis repetida de 4mg/kg (Ricaurte et al., 1988). Asimismo, los cambios neurohistológicos son más pronunciados y permanentes en primate, mientras que en rata se ha descrito una regeneración axonal y una reinervación de la región afectada (Fischer et al., 1995).

En la lesión de los axones serotoninérgicos inducida por MDMA, observada en ratas y primates, la DA parece jugar un papel importante. Sustancias que disminuyen la liberación de DA inducida por MDMA (como la α-metil-*p*-tirosina, la reserpina, antagonistas D_2 como el haloperidol, o los antagonistas 5-HT_2) disminuyen la toxicidad serotoninérgica producida por la MDMA (Stone et al., 1988; Hewitt and Green, 1994). Por el contrario, aquellas sustancias que potencian la liberación de DA inducida por MDMA, como pueden ser la L-dopa o los agonistas 5-HT_2, aceleran e incrementan la lesión serotoninérgica producida por dicho derivado anfetamínico.

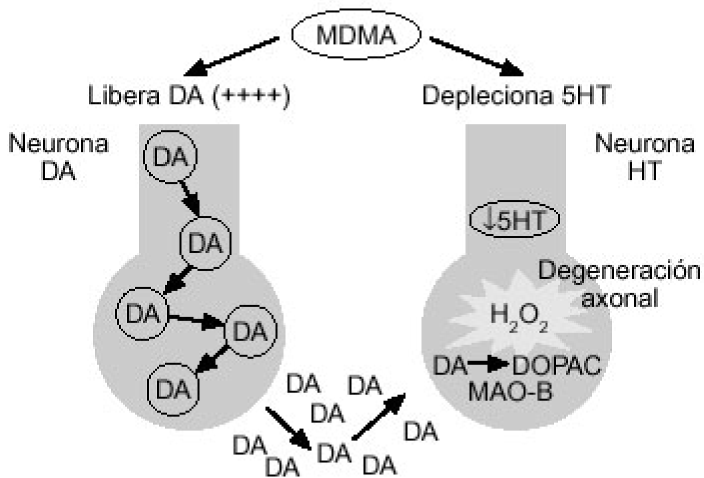


Figura 12. Mecanismo hipotético de toxicidad por MDMA sobre las neuronas (López-Muñoz et al., 2004).

Existen estudios que sugieren que el mecanismo por el cual se produce la degeneración axonal serotoninérgica podría consistir, en un primer momento, en la inducción de liberación masiva de 5-HT, que a su vez provocaría una depleción de los terminales serotoninérgicos, haciendo a estos más vulnerables al proceso tóxico. A su vez, la MDMA aumentaría la liberación de DA, que sería captada hacia el interior de los terminales serotoninérgicos mermados, en los cuales la DA sería desaminada por la MAO-B, generando especies reactivas de oxígeno, como el peróxido de hidrogeno (Figura 12). Este último proceso ocasionaría el fenómeno de degeneración y destrucción de los axones serotoninérgicos (Sprague et al., 1994; López-Muñoz et al., 2004).

Existe también una estrecha relación entre la hipertermia y la neurotoxicidad inducida por la MDMA (Broening et al., 1995; Malberg and Seiden, 1998; Johnson et al., 2004). Además, pequeños cambios en la temperatura ambiente producen importantes cambios en la neurotoxicidad serotoninérgica y la temperatura corporal en rata inducidas por la MDMA (Schmidt et al., 1990; Malberg and Seiden, 1998).

Si bien la MDMA y la MDA son potentes neurotoxinas serotoninérgicas, su administración directa al cerebro en modelos animales no produce neurotoxicidad (Esteban et al., 2001). Este hecho sugiere que es imprescindible una activación metabólica previa (Figura 11) de estas sustancias para poder desarrollar dicha neurotoxicidad (McCann and Ricaurte, 1991; Chu et al., 1996).

Aunque es difícil extrapolar los resultados obtenidos en animales de experimentación a humanos, existe un creciente consenso en el cual se cree que la MDMA también es neurotóxica en humanos. Se han observado diferencias en las concentraciones de 5-HT y sus metabolitos en líquido cefaloraquídeo de consumidores de MDMA (McCann et al., 1994). Además, técnicas de imagen PET han demostrado cambios sobre los lugares de recaptación de 5-HT en ex-consumidores de MDMA (McCann et al., 1998).

En estudios mediante resonancia magnética, en consumidores habituales de MDMA, se ha observado una hipertrofia glial en el cerebro (Chang et al., 1999). En animales de experimentación, un gran número de estudios han demostrado una activación de la microglia y de la astroglia en ratones después de un tratamiento con MDMA (O'Callaghan and Miller, 1994; Granado et al., 2008; Khairnar et al., 2010). Sin embargo, parece ser que la MDMA no induce astrogliosis en el hipocampo, estriado o corteza de ratas Long-Evans o Sprague-Dawley (O'Callaghan and Miller, 1993; Wang et al., 2004), aunque Pubill y col. (2003) observaron un leve aumento de la immunoreactividad a GFAP (proteína ácida fibrilar glial) en la región CA1 del hipocampo 7 días después del tratamiento. Además, Aguirre y col. ya habían observado años antes este mismo hecho en hipocampo de rata después de una sola dosis de MDMA (Aguirre et al., 1999). Adori y col. también observaron un aumento significativo de la densidad de la GFAP de astrocitos protoplásmicos en la subregión CA1 del hipocampo acompañado

de un aumento del número de células inmunoreactivas a GFAP comparado con el grupo control (Adori et al., 2006).

1.4. *Khat*

El khat (*Catha edulis*) es un árbol de hoja perenne que crece de forma espontánea en el este del continente africano y en el sudeste de la Península Arábica. La masticación de las hojas frescas de khat se ha convertido en una tradición en ciertas comunidades, especialmente en ceremonias religiosas y culturales por sus efectos estimulantes. Dicha práctica también se encuentra extendida de manera diaria y es un importante fenómeno tanto social como cultural, sobre todo en países como Yemen, dónde se estima que alrededor del 90% de los hombres adultos y aproximadamente el 50% de la población adulta consume hojas de khat diariamente. También se ha observado una elevada prevalencia en su consumo en países del este africano, como Somalia, Etiopia, Uganda y Kenia (Al-Motarreb et al., 2002; Carvalho, 2003; Balint et al., 2009). Las hojas del khat son conocidas por diferentes nombres, como por ejemplo qat, qaat, chat o miraa, y son recolectadas a primera hora de la mañana y vendidas posteriormente en paquetes o manojos de alrededor de 100-200 gramos de hojas frescas, envueltas en hojas de plátano (Figura 13). Se considera un uso moderado y usual el consumo de uno de estos paquetes o manojos al día.

Figura 13. Hojas frescas de khat (Catha Edulis) envueltas en hojas de plátano

La planta del khat fue descubierta por primera vez durante una expedición a Egipto y Yemen entre 1761 y 1763, por el botánico sueco Peter Forskal. Actualmente, gracias a las mejoras en las rutas de transporte y distribución, la disponibilidad de las hojas del khat ha dejado de ser exclusiva a las regiones en las cuales se cultiva, y la inmigración ha propagado su uso a los países occidentales (Alem et al., 1999; Griffiths et al., 2010), aunque con la

limitación que sólo las hojas frescas del khat son las responsables de sus efectos psicotrópicos.

La planta del khat es actualmente ilegal en los Estados Unidos y Canadá, y fue prohibida en un gran número de países europeos, como Irlanda, Francia, Dinamarca, Alemania, Suecia, Noruega y Holanda. Sin embargo, todavía no se encuentra ilegalizada en el Reino Unido, Portugal, Australia y en muchos países del Este de África (Arunotayanun and Gibbons, 2012; Gezon, 2012; Klein et al., 2012).

Las hojas frescas del khat contienen alrededor de 40 compuestos, incluyendo alcaloides, taninos, flavonoides, terpenos, esteroles, glucósidos, aminoácidos, vitaminas y minerales (Halbach, 1972; Kalix, 1984; Cox and Rampes, 2003; Balint et al., 2009). En un primer intento de identificar el principio activo o principios activos del khat, Fluckiger y Gerock (1887) detectaron un compuesto psicoactivo, al cual llamaron catina, que posteriormente fue identificado por Wolfes (1930) como (+)-norpseudoefedrina.

Durante las siguientes décadas, se creyó que la catina era el principal compuesto activo del khat. Sin embargo, existían evidencias acerca de que el efecto estimulante de la catina era insuficiente para explicar los efectos farmacológicos del khat (Halbach, 1972; Szendrei, 1980; Zelger et al., 1980; Kalix, 1984). Por ello, en 1975 el Laboratorio de Narcóticos de las Naciones Unidas aisló la catinona, (-)-α-aminopropiofenona, precursor de la catina. Estudios posteriores demostraron que la catinona poseía una potencia entre 7 y 10 veces mayor que la catina, pero se degradaba rápidamente, ya que la catinona es un intermedio en la biosíntesis de la catina, la cual cosa explica la necesidad de masticar las hojas del khat cuando son frescas, ya que éstas acumulan niveles de catinona mucho más elevados que las maduras (Knoll, 1979; Kalix and Khan, 1984; Nencini and Ahmed, 1989; Cox and Rampes, 2003; Kelly, 2011).

Tal y como ocurre con la anfetamina, el khat, posee efectos tanto a nivel del sistema nervioso central como periférico. Los efectos periféricos son principalmente simpaticomiméticos: aumento de la frecuencia respiratoria, de la temperatura corporal, de la presión sanguínea, ritmo cardíaco y midriasis. Los efectos a nivel del sistema nervioso central son la euforia, el

aumento del estado de alerta y de la sensación de bienestar. Además produce insomnio, anorexia, y a dosis elevadas puede provocar hiperactividad. Se pueden llegar a producir manifestaciones del tipo psiquiátrico como consecuencia de una intoxicación por consumo de khat, como por ejemplo, un comportamiento maníaco (Giannini and Castellani, 1982), psicosis (Gough and Cookson, 1984) o incluso paranoia (Critchlow and Seifert, 1987).

Como se puede observar en la figura 14, la catinona es el análogo β-cetónico de la anfetamina. Estudios sobre la actividad farmacológica de la catinona han demostrado que dicha sustancia es capaz de inducir una inhibición de la recaptación y favorecer la liberación de DA, 5-HT y NA, en el sistema nervioso central, de manera similar, aunque con una potencia inferior a la anfetamina (Kalix, 1983, 1984; Kalix and Braenden, 1985; Fleckenstein et al., 1999; Rothman et al., 2003). Sin embargo, la afinidad de la catinona por los receptores serotoninérgicos parece ser 4 veces superior en comparación a la de la anfetamina (Glennon and Liebowitz, 1982). Además, se ha demostrado que la catinona también inhibe la MAO, de manera similar a la anfetamina, pero con una potencia superior (Nencini et al., 1984). La catinona, a la dosis de 15 mg/kg, aumenta de manera significativa tanto la actividad locomotora como el comportamiento agresivo de ratas Sprague-Dawley (Banjaw et al., 2006).

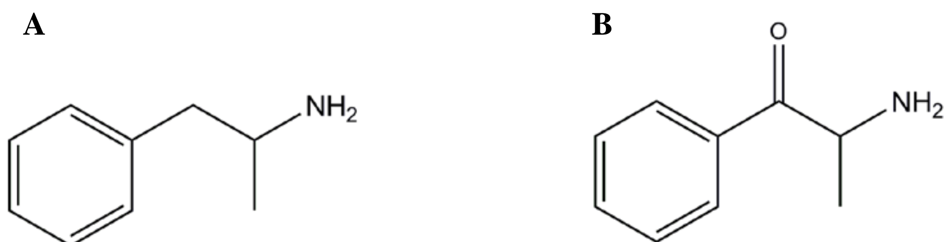


Figura 14. Estructura química de la anfetamina (**A**) y la catinona (**B**)

Tal y como ocurre con la anfetamina, la catinona induce hipertermia. Dicho efecto en la temperatura corporal es similar también al del MDMA o éxtasis; produce hipertermia a una temperatura ambiente de 22ºC o superior, en cambio produce hipotermia cuando los animales se encuentran a una temperatura ambiente inferior (Kalix and Braenden, 1985). Además, se ha demostrado como la administración de dosis elevadas y de forma repetitiva

pueden producir depleción de DA en diferentes áreas cerebrales de rata, de una manera bastante similar a la producida por administraciones repetidas de anfetamina (Wagner et al., 1982). La catinona no sólo comparte el perfil farmacológico de la anfetamina, sino también las características de sus enantiómeros, ya que el enantiómero S es más potente, a nivel de los terminales dopaminérgicos del sistema nervioso central, que el enantiómero R (Kalix, 1986).

La autoadministración de drogas psicoactivas en modelos animales ha sido utilizada como técnica predictiva del abuso de dichas sustancias por humanos. Tal y como se esperaba, la catinona induce un comportamiento de autoadministración en ratas (Gosnell et al., 1996). Otra de las técnicas más ampliamente utilizadas para medir las propiedades motivacionales del consumo de drogas de abuso es el paradigma de la preferencia condicionada de lugar. En dicho test, el animal aprende a asociar un particular entorno con el estado inducido por el efecto de una droga, y otro entorno diferente con un estado libre de sustancia. Dependiendo del efecto de la droga, el animal puede preferir el entorno relacionado con la droga o, por el contrario, inducirle un estado de aversión hacia dicha sustancia. La catinona, además de poseer propiedas reforzantes o favorecer la autoadministración, también posee propiedades motivacionales, como se ha demostrado en estudios realizados mediante la técnica anteriormente mencionada, en las cuales la administración a ratas, tanto a nivel periférico como a nivel central, ha reflejado un resultado positivo en dicho paradigma.

En una sola sesión, se suelen masticar entre 100 y 500 gramos de hojas de khat durante horas (Feyissa and Kelly, 2008). Tal y como se ha comentado, la catinona es el principal alcaloide presente en el khat, y su concentración se encuentra comprendida entre los 78 y los 343 mg de catinona por cada 100 gramos de hoja fresca (Sakitama et al., 1995; Arunotayanun and Gibbons, 2012). Brenneisen y Kalix (1990) fueron los primeros en obtener datos sobre la farmacocinética de la catinona en estudios clínicos comparativos. Observaron como la administración de catinona a una sola dosis única por vía oral de 0,5 mg/kg (que corresponde a masticar unos 100 gramos de khat) a voluntarios sanos, producía un pico de concentración plasmática de aproximadamente 100 ng/ml después de 72 ± 33 minutos. Los resultados demostraron además que la catinona poseía un $t_{1/2\beta}$ relativamente corto, alrededor de 1,5 horas (Brenneisen et al., 1990). Aquí nos

encontramos con una de las pocas diferencias respecto a la anfetamina, sustancia cuyos niveles plasmáticos persisten durante un periodo mucho más largo. Sin embargo, cuando se administra directamente hojas de khat, a una dosis estandarizada (que equivale a una dosis de 0,8mg/kg), el pico de concentración plasmática aparece a los 127 minutos, correspondiente al tiempo presumiblemente necesario para la extracción de los alcaloides tras la masticación (Widler et al., 1994). Menos del 7% de la dosis ingerida de catinona aparece inalterada en la orina, siendo principalmente eliminada en forma de sus dos metabolitos norefedrina y norpseudoefedrina o catina. El metabolismo de fase I de la catinona, como muchas otras catinonas sintéticas, se caracteriza por la reducción del grupo cetona, en posición β, a un grupo alcohol, catalizada por las enzimas microsomales hepáticas (Guantai and Maitai, 1983; Brenneisen et al., 1986). Existe incluso estereoselectividad en el metabolismo de la catinona, siendo la norefedrina el principal metabolito de la S-(-)-catinona, mientras que la norpseudoefedrina lo es de la R-(-)-catinona (Figura 15) (Brenneisen et al., 1986; Toennes et al., 2003).

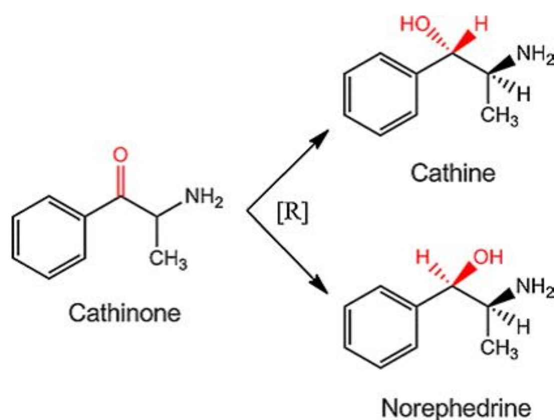


Figura 15. Esquema del metabolismo estereoselectivo de la catinona (Brenneisen et al., 1986).

1.5. Catinonas sintéticas

La estructura química de la catinona se puede considerar como el prototipo a partir del cual se han desarrollado un gran número de derivados. En la figura 16 se muestra la estructura de la catinona a la cual se han añadido diferentes sustituyentes que dan lugar a un amplio rango de derivados. Actualmente, existen alrededor de 30 catinonas sintéticas, y todas ellas pueden ser consideradas como derivados de fenetilamina con un grupo cetona en la posición β de la cadena lateral. Químicamente, los derivados de la catinona pertenecen a una serie de "clústers" relacionados.

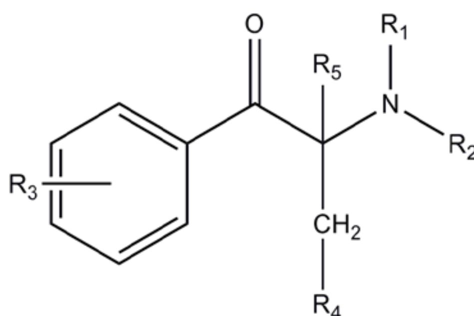


Figura 16. Estructura química genérica de la catinona cuyas sustituciones dan lugar a sus diferentes derivados.

La N-alquilación en las posiciones R1 y/o R2 (con grupos metilo o etilo) dan lugar a derivados de la catinona alquilados. La N-metilación da lugar a la metcatinona, y posteriores metilaciones en la posición 4 del anillo dan lugar a la mefedrona. La N-metilación tanto en la posición R1 como en la posición R2 da lugar a la metamfepramona, mientras que la introducción de grupos etilo da como resultado la dietilpropion o amfepramona. La adición de un grupo metilo en la posición 4 del anillo de la N-etilcatinona da lugar a la 4-metil-N-etilcatinona. Metilaciones en R1 y R4 darían lugar a la bufedrona. La metedrona en cambio posee un grupo metilo en la posición R1 con un grupo metóxido en la posición 4 del anillo. El bupropion (Figura 17) posee un grupo N-tert-butilo y un cloruro en la posición 3 del anillo; modificaciones de este grupo tert-butilo del bupropion están siendo investigadas con finalidad terapéutica (Foley and Cozzi, 2003). Existen

además una serie de derivados fluorados de la catinona, siendo el más común la 4-fluorometcatinona (flefedrona) (Figura 17) (Archer, 2009).
Una sustitución en el anillo en la posición R3 con un grupo metilendioxi da lugar también a un gran número de análogos de las MDAs. Por ejemplo, la metilación en la posición R1 en este tipo de compuestos da lugar a la **METILONA** (Figura 17), motivo de estudio en esta Tesis Doctoral, un derivado β-ceto de la MDMA, cuya elongación de la cadena con un grupo etilo en la misma posición R1 da lugar a la etilona (Figura 17). En el caso que se añadiera un metilo en la posición R4 obtendríamos la pentilona. En el caso que utilizásemos la molécula de metilona como prototipo, la inserción de un grupo metilo en la posición R4 daría lugar a la butilona (Figura 17).

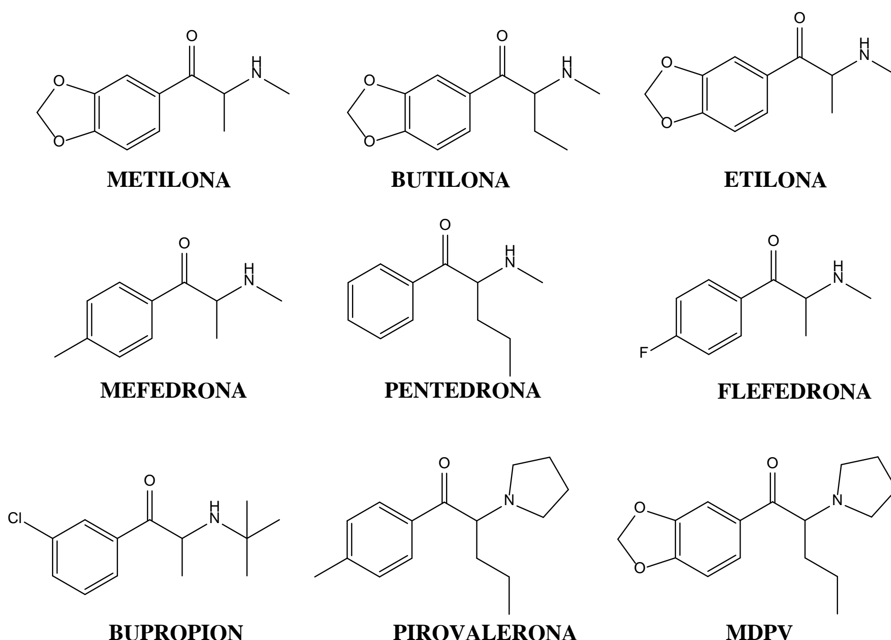


Figura 17. Estructura química de algunas de las catinonas sintéticas.

Además de todos estos compuestos, existen también un gran número de derivados de la catinona que poseen en el átomo de nitrógeno de la estructura de la catinona un grupo pirrolidinilo. Estos tienen como prototipo la α-pirrolidinapropiofenona (PPP), la cual carece de sustituyentes en la posiciones R3 o R5. La sustitución más común es la inserción de un metilo en la posición 4 del anillo, dando lugar a la 4-metil-α-

pirrolidinapropiofenona (MPPP). La inserción de un grupo etilo o propilo en la posición R4 da lugar a la pirovalerona (Figura 17) y a la 4-metil-α-pirrolidinahexafenona (MPHP), respectivamente. Finalmente también existen derivados pirrolidinicos de la 3,4-metilendioxianfetamina, dónde la más común es la MDPV (3,4-metilendioxipirovalerona) (Figura 17), que posee además un grupo etilo en la posición R4. Tal y como ocurre con las fenetilaminas, todos estos compuestos pueden existir en dos formas estereoisoméricas (enantiómeros).

La estrecha relación estructural de estas sustancias con las anfetaminas y sus análogos ha propiciado el interés en el desarrollo de algunos de estos compuestos para un uso terapéutico. Es el caso de la metcatinona (efedrona), sintetizada por primera vez en el año 1928 (Hyde et al., 1928) y utilizada como antidepresivo en la antigua Unión Soviética durante los años 30 y 40, y posteriormente desarrollada por la compañía Parke Davis como un potente estimulante del sistema nervioso central (Cozzi and Foley, 2003). También la amfepramona (dietilpropion) fue comercializada como agente supresor del apetito a finales de los años 50 (Ioannides-Demos et al., 2011). Actualmente, el bupropion es el único derivado de la catinona que está indicado para el tratamiento de la depresión y la adicción al tabaco (ACMD, 2010). Por otro lado, la pirovalerona también fue investigada para su uso en el tratamiento de la fatiga crónica, la letargia y la obesidad, pero sin éxito a causa del abuso y dependencia que genera en sus consumidores (Gardos and Cole, 1971; Goldberg et al., 1973; Kriikku et al., 2011).

Recientemente las catinonas sintéticas han proliferado como drogas de abuso. El dramático aumento e interés de estos compuestos como drogas de abuso ha sido propiciado en parte por su amplia y fácil disponibilidad, así como debido a una disminución de la pureza y accesibilidad de las drogas más comunes, como la MDMA o la cocaína. A modo de ejemplo, en 2009, la policía del Reino Unido detectó una disminución de la pureza en el mercado de la cocaína, del 60% al 22%, atribuida principalmente a un aumento de sus incautaciones (Measham et al., 2010). En Holanda, durante el mismo periodo, se observó un cambio en la composición de las pastillas que contenían MDMA. Antes del 2009, más del 90% de las pastillas de "éxtasis" contenían MDMA. Sin embargo, en muestras analizadas posteriormente, menos de la mitad de las pastillas contenían dicha sustancia (Brunt et al., 2011).

Las catinonas sintéticas son consideras como “legal highs”, en castellano estimulantes legales, y son vendidas como sales de baño (“bath salts”) o abono para plantas (“plant food”) (Figura 18) con la indicación de “no aptas para el consumo humano” para esquivar la legislación referente a las drogas de abuso. Este tipo de sustancias se pueden obtener con gran facilidad a través de internet, “head shops” o “smart shops” y gasolineras, y generalmente en forma de un polvo cristalino o amorfo blanco o amarillento, o incluso ya encapsulado. Se pueden encontrar en paquetes desde 200mg hasta los 10 gramos, con un coste aproximado de entre 10 y 20 dólares el gramo (Yohannan and Bozenko, 2010; Dargan et al., 2011; Karila and Reynaud, 2011; Kelly, 2011; Coppola and Mondola, 2012; Fass et al., 2012; Prosser and Nelson, 2012; Bretteville-Jensen et al., 2013). La producción de la mayoría de las catinonas sintéticas proviene de China y de los países del sureste asiático. Sus productores reivindican que la pureza de dichas sustancias se encuentra por encima del 99%. Sin embargo, los análisis de estos productos demuestran que dicha pureza no supera el 95%, siendo adulterados con sustancias como la benzocaína, la lidocaína, la cafeína, piperacinas y paracetamol (Davies et al., 2010; EMCDDA, 2011). Su abuso se asocia a razones sociales y económicas además de por sus propiedades estimulantes y/o alucinógenas, utilizadas en muchos casos como sustitutivos de la MDMA, la cocaína y la metanfetamina.

Figura 18. Fotografía de algunos de los productos, comercializados vía on-line o *smart-shops*, cuyos principales ingredientes son las catinonas sintéticas.

Las catinonas sintéticas más ampliamente consumidas son la 4-metilmetcatinona (mefedrona), la 3,4-metilendioxipirovalerona (MDPV) y la 3,4-metilendioximetcatinona (metilona). De hecho la mefedrona es la catinona sintética más consumida en Europa, mientras que la MDPV y la metilona son más frecuentes en los Estados Unidos.

En el 2010, la mefedrona fue la tercera droga de abuso más consumida en el Reino Unido (The NHS Information Centre, 2011) y aproximadamente el 20% de los jóvenes de entre 14 y 20 años de los colegios y universidades del Reino Unido reconocieron haber consumido mefedrona ese mismo año (Dargan et al., 2010). En Estados Unidos, los informes de drogas sobre catinonas sintéticas al *National Forensic Laboratory Information System* aumentaron de 34 en 2009 a 628 en 2010 (DEA, 2011). Mientras tanto, las llamadas al servicio de información toxicológica en relación a la exposición a sales de baño aumentaron de 304 en 2010 a 6136 en 2012 (German et al., 2013) y el *Toxicology Investigators Consortium* informó que las sales de baño formaban parte del 12% de todos los casos toxicológicos en 2011 (Wiegand et al., 2012).

Durante los últimos cuatro o cinco años los derivados de la catinona están siendo sujetos a una intensa legislación. Sin embargo, el estatus legal de las catinonas sintéticas varía entre países y se encuentra en continuo cambio. Probablemente estos cambios vienen producidos por la prohibición de ciertas catinonas sintéticas de manera individual en determinados países. En Diciembre del 2008, el gobierno de Suecia, ilegalizó la mefedrona y desde entonces países como Dinamarca, Alemania, Estonia, Noruega, Holanda, Finlandia e Irlanda han creado una legislación mucho más restrictiva hacia este tipo de sustancias. En Abril del 2010, el Reino Unido dio un paso sin precedentes recomendando que todo un amplio abanico de derivados de la catinona fuera incluido en la Clase B de sustancias controladas, compartiendo clasificación con las anfetaminas (ACMD, 2010; Kmietowicz, 2010; Morris, 2010). Desde entonces la mefedrona se encuentra controlada en todos los estados miembros de la Unión Europea (EMCDDA, 2010).

Hasta hace relativamente poco tiempo, en los Estados Unidos, las catinonas sintéticas se encontraban desclasificadas, pero era ilegal su consumo gracias a la ley de análogos de 1986. Esta ley se promulgó con la intención de

prevenir el uso de drogas de diseño que fueran análogos de otras drogas de abuso ilegales, creadas a partir de pequeños cambios en la estructura química. Dicha ley no criminaliza la posesión o la fabricación de este tipo de sustancias, a menos que su intención se dirija hacia el consumo humano. En 2011, la DEA, clasificó temporalmente en la Lista I de sustancias controladas a la mefedrona, la MDPV y la metilona, penalizando su venta y posesión. Recientemente, en 2013, estas tres catinonas sintéticas se encuentran incluidas de forma permanenente en dicha lista (DEA, 2011 and 2013a).

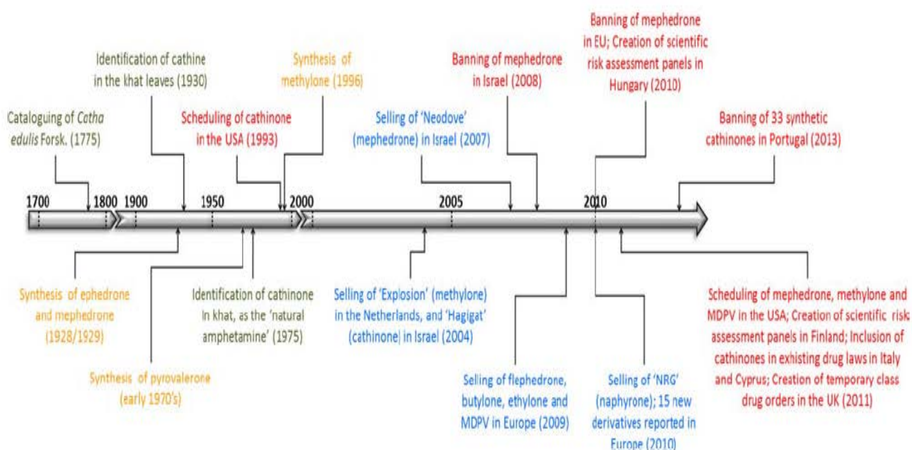


Figura 19. Cronología de los principales acontecimientos relacionados con la planta del *khat* y las catinonas sintéticas (Valente et al., 2014).

Los signos simpaticomiméticos son los más frecuentes asociados a la toxicidad de las catinonas sintéticas, lo cual no es sorprendente dado el hecho que estas sustancias podrían actuar sobre el sistema monoaminérgico. Excitación, paranoia, alucinaciones, psicosis, mioclonías y dolores de cabeza son los síntomas más comunes que refieren los pacientes que experimentan algún efecto tóxico por el consumo de las catinonas sintéticas (James et al., 2011; Spiller et al., 2011; Kasick et al., 2012; Thornton et al., 2012; Stoica and Felthous, 2013). A nivel periférico, los síntomas toxicológicos de las catinonas sintéticas más comunes son la hipertermia, la hipertensión, taquicardias, hiponatremia, nauseas, vómitos y dolor pectoral. Los síntomas más serios, que requieren un tratamiento médico prolongado y en algunos casos incluso llevan a la muerte, incluyen el fallo renal, el fallo hepático, rabdomiolisis y el desarrollo del síndrome compatimental (Adebamiro and Perazella, 2012; Borek and Holstege, 2012; Levine et al.,

2013; Stoica and Felthous, 2013). La toxicidad aguda de este tipo de sustancias es la principal causa de muertes y hospitalizaciones, aunque en numerosos casos se ha observado un policonsumo de estas catinonas sintéticas con otras drogas de abuso (Maskell et al., 2011; Aromatario et al., 2012; Schifano et al., 2012; Marinetti and Antonides, 2013). La segunda causa de muerte asociada al consumo de catinonas sintéticas son las autolesiones y una conducta de riesgo, sin evidencias de psicosis o depresión. Los suicidios son la forma más común de autolesión fatal, ya sea a través de disparos con arma de fuego, autolesión con arma blanca o precipitándose de puentes (Schifano et al., 2012; Marinetti and Antonides, 2013) aunque las catinonas sintéticas no son los únicos estimulantes que producen un comportamiento de auto-mutilación (Shishido et al., 2000; Wagner et al., 2004).

1.6. Metilona

La metilona (3,4-metilendioximetcatinona), también conocida como M1, MDMC, bk-MDMA o "Ease" es un psicoestimulante y entactógeno de la familia de las catinonas, también incluida dentro del grupo de las fenetilaminas o de las β-ceto anfetaminas. Cabe destacar que la metilona posee una gran similitud estructural con la MDMA o "éxtasis" (Figura 20), de la cual sólo difiere por la presencia de un grupo cetona en la posición β del anillo de fenetilamina y se suele presentar en forma de clorhidrato, con aspecto de un polvo blanco o amarillento. Tal y como ocurre con muchos otros derivados anfetamínincos, la metilona posee un centro quiral, obteniéndose por lo tanto sus dos enantiómeros (R-) y (S-).

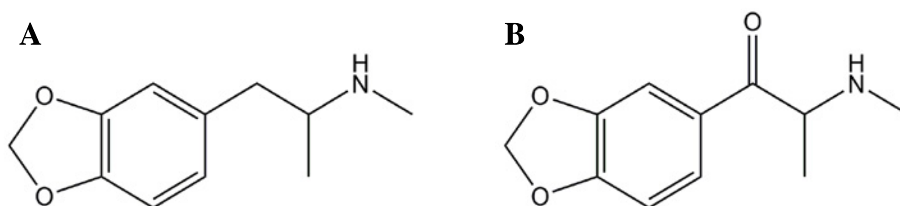


Figura 20. Estructura química de la MDMA (**A**) y la metilona (**B**)

La metilona es una sustancia relativamente nueva, con muy pocos años de historia, y por ello, la información sobre dicha sustancia es muy limitada. Fue sintetizada y patentada por Jacob y Shulgin, en 1996, como posible agente antiparkinsoniano y antidepresivo (Jacob and Shulgin, 1996), sin embargo nunca se desarrolló como tal. Posteriormente, a finales del 2004 en Holanda, una nueva droga de diseño llamada "Explosion" apareció en el mercado. Se trataba de un líquido que se vendía a través de internet o en las conocidas como "smartshops" holandesas. Dicho producto se anunciaba como ambientador para el hogar, y se vendía en tubos de plástico que contenían unos 5 mL de líquido. El precio de cada tubo estaba comprendido entre los 10 y los 15 euros, y no mostraba ningún tipo de información sobre su composición, simplemente una etiqueta en la cual se advertía que era un ambientador con olor a vainilla, que no se debía ingerir, que se mantuviera alejado de los niños y que no se utilizara más de una botella. Los consumidores que ingerían "Explosion" relataban una sensación de euforia similar a la producida por la MDMA. Análisis posteriores del producto confirmaron que el ingrediente activo de este ambientador era la metilona,

un análogo de la MDMA (Bossong et al., 2005). Actualmente la metilona se vende a través de internet como “research chemical” o producto para la investigación, en “headshops” o “smartshops” y es uno de los tres principales ingredientes de las conocidas como “bath salts” o sales de baño, anteriormente ya comentadas en el apartado 1.5. Incluso un gran número de narcotraficantes han utilizado el vacío legal que existe sobre la metilona en algunos países y su bajo precio, para adulterar o directamente sustituir a las pastillas de MDMA, sin embargo, se sigue vendiendo como MDMA o “Molly” (nombre por el cual se conoce a las pastillas de MDMA puro en EEUU) (Blanding, 2013; Hamblett, 2014).

El estado legal de la metilona difiere según el país. Se encuentra fiscalizada en algunos países europeos como el Reino Unido, Suecia, Dinamarca u Holanda. No es el caso de España, dónde la metilona no se encuentra todavía ilegalizada. En Octubre del 2011 la DEA incluyó temporalmente a la metilona y a dos catinonas sintéticas más (mefedrona y MDPV) en la Lista I de la Controlled Substances Act como consecuencia de la necesidad inmediata de evitar un daño inminente a la salud pública (DEA, 2011). En Octubre de 2012 se amplió la inclusión de la metilona en la Lista I de la Controlled Substances Act durante seis meses más (DEA, 2012). Finalmente, en Abril de 2013, la metilona fue incluida de manera permanente por la DEA en la Lista I de sustancias controladas, basándose en su potencial de abuso, su posible uso médico y el grado de dependencia que dicha sustancia podría provocar, penalizando el consumo, posesión y distribución tanto de la metilona como de cualquiera de sus sales y/o isómeros (DEA, 2013b).

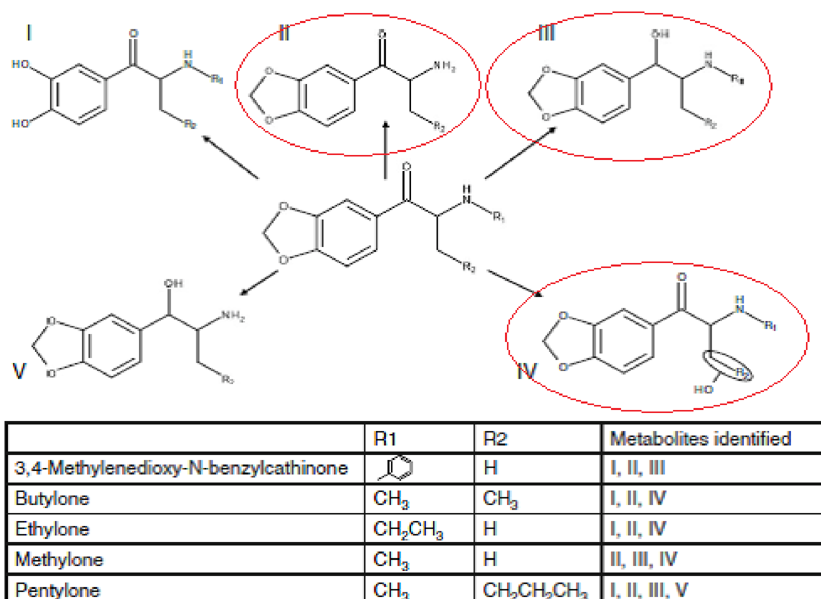
1.6.1. Mecanismo de acción

A causa de la similitud estructural que posee la metilona respecto a la MDMA, se cree que pueda actuar sobre el sistema monoaminérgico. Hasta la fecha de realización de la presente Tesis Doctoral, poco o muy poco se conocía sobre el mecanismo de acción de la metilona y de muchas catinonas sintéticas. En 1999, Cozzi y col. publicaron un estudio comparativo sobre la inhibición de los transportadores de monoaminas, entre la MDMA y la metanfetamina y sus homólogos β-cetónicos (metcatinona y metilona). Utilizaron plaquetas humanas como fuente de SERT y dos líneas celulares transfectadas (C6-DAT y C6-NET) como fuente de DAT y NET,

respectivamente. En cambio, para evaluar los efectos de las drogas a nivel vesicular (VMAT-2), utilizaron células cromafines. A nivel del DAT y el NET, los compuestos β-cetoanfetamínicos mostraron una potencia similar, respecto a sus análogos no cetónicos, inhibiendo la acumulación de monoaminas. Sin embargo, la potencia al inhibir la acumulación de 5-HT por los derivados anfetamínicos fue mayor en comparación con las dos β-cetoanfetaminas. La mayor diferencia entre estos compuestos tiene lugar sobre el VMAT-2, donde se observó una reducción de la potencia de inhibición por parte de la metcatinona y la metilona al compararse con la metanfetamina y la MDMA, respectivamente (Cozzi et al., 1999). En 2007, Nagai y col. realizaron un estudio *in vitro* sobre la inhibición y liberación de monoaminas usando una batería de compuestos psicoactivos. De todos los análogos de la MDMA examinados, la metilona era el compuesto con mayor efecto sobre la captación de monoaminas (Nagai et al., 2007).

1.6.2. Farmacocinética

Existe muy poca información sobre la farmacocinética y el metabolismo de la metilona tanto en humanos como en animales de experimentación. Kamata y colaboradores (2006) identificaron los metabolitos característicos (Fase II) de la metilona en muestras de orina de rata y humana (Figura 21).



	R1	R2	Metabolites identified
3,4-Methylenedioxy-N-benzylcathinone		H	I, II, III
Butylone	CH_3	CH_3	I, II, IV
Ethylone	CH_2CH_3	H	I, II, IV
Methylone	CH_3	H	II, III, IV
Pentylone	CH_3	$CH_2CH_2CH_3$	I, II, III, V

Figura 21. Estructuras para los metabolitos propuestos para la 3,4-metilendioxi-N-benzilcatinona, butilona, etilona, **metilona** y pentilona. (Mueller and Renstch 2012)

Según dichos autores, la especie mayoritaria en el metabolismo de la metilona en rata fue la forma conjugada de la 4-hidroxi-3-metoximetcatinona (Kamata et al., 2006). Posterior fue el estudio de Mueller y Renstch (2012) en el cual demostraron, mediante ensayos in vitro, que la metilona era metabolizada en microsomas hepáticos humanos a nor-metilona, dihidro-metilona e hidroxi-metilona (Figura 22) (Mueller and Rentsch, 2012).

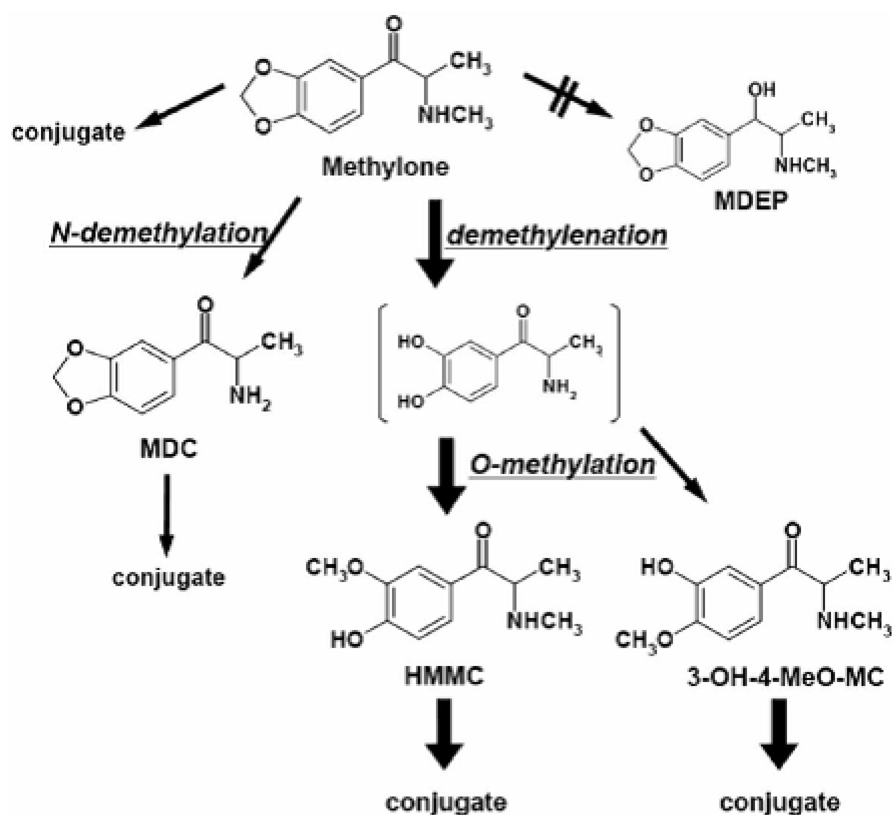


Figura 22. Ruta metabólica propuesta, en rata y humano, para la metilona (Kamata et al., 2006).

Un gran número de análogos anfetamínicos son inhibidores y sustratos de la isoforma CYP2D6 (Lin et al., 1997; Wu et al., 1997; Kreth et al., 2000; Maurer et al., 2000). Además, la MDMA es un inhibidor del CYP2D6 (Wu et al., 1997; Heydari et al., 2004). En estudios posteriores al comienzo de esta tesis doctoral, Pedersen y colaboradores concluyeron, en su trabajo

sobre el metabolismo de la metilona, que el 95% de dicha catinona era metabolizada por el CYP2D6, con la contribución de otras isoformas como la CYP1A2, CYP2B6 y CYP2C19. Además, sus experimentos indicaban que la metilona es un inhibidor del CYP2D6, tal y como ocurre con la MDMA (Pedersen et al., 2013).

1.6.3. Toxicidad

A día de hoy, la metilona ha demostrado ser la causa directa de la intoxicación y muerte de un gran número de jóvenes en todo el mundo, especialmente en los Estados Unidos (Boulanger-Gobeil et al., 2012; Cawrse et al., 2012; Pearson et al., 2012; Warrick et al., 2012; Carbone et al., 2013). A modo de ejemplo, en algunos de los casos publicados, la víctima ingresaba en estado febril, comatoso, con taquicardias e hipertensión y, a pesar de los cuidados recibidos, desarrollaba un fallo multiorgánico. De acuerdo con los exámenes médicos, la causa de la muerte se atribuyó al síndrome serotoninérgico provocado por el consumo de metilona y butilona. Pearson y col. también informaron del caso de tres jóvenes hospitalizados como consecuencia del consumo de metilona (Pearson et al., 2012). Cabe destacar que los tres casos compartían un síntoma común, una elevada temperatura corporal. Al revisar los fallecimientos relacionados con el consumo de MDMA, Patel y col. llegan a la conclusión que la concentración media en sangre de MDMA necesaria para producir la muerte es de alrededor de 0,172 mg/dl (Patel et al., 2004). Dicha concentración duplica la concentración hallada, por Carbone y colaboradores, en uno de los casos de fallo cardíaco súbito asociado al consumo de metilona (Carbone et al., 2013). Tal y como concluyen los autores, este caso sugiere la posibilidad de que la toxicidad inducida por la metilona haya sido infravalorada o subestimada entre los consumidores de este tipo de compuestos. Sin embargo, es extremadamente difícil relacionar las cantidades de metilona en sangre detectadas *post-mortem* y las dosis ingeridas, ya que no existen suficientes evidencias forenses para determinar cómo, cuánto y cuándo se ha consumido la metilona y la correspondiente cantidad detectada *post-mortem*.

Se considera que la citotoxicidad producida por la MDMA es una consecuencia de un déficit monoaminérgico a través de los efectos que posee la droga sobre los transportadores de monoaminas de membrana y

vesiculares. La metilona, por su parte, al poseer una similitud estructural podría compartir su perfil citotóxico. Sin embargo, Nakagawa y col. demostraron que la metilona no inducía efectos citotóxicos en hepatocitos aislados de rata (Nakagawa et al., 2009). Posteriormente, Sogawa y col. realizaron un estudio sobre la viabilidad *in vitro* de la metilona, mediante células CHO, en las cuales se encontraban expresados los transportadores de monoaminas (CHO/rDAT, CHO/rNET y CHO/rSERT). La metilona sólo demostró ser citotóxica a la concentración más alta ensayada (100µM), especialmente en las células en las cuales se encontraba expresado el SERT. Además, al combinar metilona y metanfetamina aparecía un efecto supra-aditivo en la liberación de LDH en las células CHO, es decir, una disminución de la viabilidad de dichas células (Sogawa et al., 2011).

1.6.4. Otros estudios

A dosis elevadas (≈56 mg/kg), la metilona produce una disminución de la coordinación motora y del equilibrio en ratones evidenciados mediante la técnica del *rotarod*. Además, a dichas dosis, un gran número de animales presentaban fuertes temblores y convulsiones. Otro de los efectos anómalos observados sobre el comportamiento de los animales es una excesiva salivación (Marusich et al., 2012).

Recientemente se ha demostrado que la metilona ejerce como reforzante en el modelo de autoadministración endovenosa en animales de laboratorio (Watterson et al., 2012). Existen estudios que demuestran que la MDMA actúa como reforzante en ratas de laboratorio. Sin embargo, sus bajos e inconsistentes índices de respuesta indican que la MDMA es un reforzante débil-moderado (Ratzenboeck et al., 2001; Cole and Sumnall, 2003). Al comparar los resultados anteriores con los estudios sobre la autoadministración de MDMA, la metilona parece poseer una respuesta a la autoadministración mucho más robusta que la observada con la MDMA. Anterior al estudio de Watterson y col. es el realizado por Dal Cason y col. en el cual demostraban que la metilona era preferida a la MDMA por ratas entrenadas para discriminar la MDMA de una solución salina (Dal Cason et al., 1997).

OBJETIVOS

2. OBJETIVOS

Al inicio de los trabajos conducentes a la presente Tesis Doctoral, nos planteamos los siguientes objetivos generales;

2.1. Objetivos generales

- Caracterizar las dianas farmacológicas así como el mecanismo de acción de la metilona y otras catinonas sintéticas, de las que se pudiera derivar su efecto psicoestimulante (Publicación **I**).

- Determinar el perfil farmacocinético de la metilona, a diferentes dosis y vías de administración, así como establecer las posibles vías de metabolismo de Fase I (Publicación **II**).

- Establecer un modelo farmacocinético-farmacodinámico para la metilona (Publicación **II**).

- Caracterizar el perfil neurotoxicológico de la metilona en rata y ratón, así como las consecuencias conductuales de dicha toxicidad (Publicación **III** y **IV**).

Estos objetivos generales se concretan en los siguientes objetivos específicos;

2.2. Objetivos específicos

- Establecer el perfil neuroquímico de la metilona en comparación con otras catinonas sintéticas, centrándonos en su efecto sobre la recaptación de monoaminas y en su interacción con receptores y transportadores de dopamina y serotonina.

- Estudiar el efecto psicoestimulante de la metilona en comparación con otras catinonas sintéticas.

- Caracterizar el perfil farmacocinético de la metilona en rata tras la administración oral e intravenosa, estableciendo el correspondiente modelo PK/PD.

- Determinar en sangre los metabolitos de fase I tras la administración oral de metilona a la rata.

- Estudiar, en el ratón, una serie de marcadores indicativos de neurotoxicidad, tras la administración de diferentes regímenes de metilona.

- Estudiar la neurotoxicidad en rata tras un régimen de administración similar al utilizado en ratón a fin de determinar posibles diferencias entre especies.

- Estudiar las posibles consecuencias conductuales en rata y ratón tras un régimen neurotóxico de metilona.

MÉTODOS Y RESULTADOS

3.1. Publicación I

Comparative neuropharmacology of three psychostimulant cathinone derivatives: butylone, mephedrone and methylone

Raúl López-Arnau, Jose Martínez-Clemente, David Pubill, Elena Escubedo, Jorge Camarasa (2012). *British Journal of Pharmacology* 167: 407-20

Resumen

Una disminución en la disponibilidad de los precursores utilizados en la síntesis de la MDMA ha provocado la aparición de una nueva generación de drogas de diseño, conocidas como catinonas o β-cetoanfetaminas, caracterizadas por la presencia de un grupo cetona en la cadena lateral de la molécula de fenetilamina.

El objetivo principal de este trabajo fue estudiar la neurofarmacología de tres de estas nuevas catinonas: butilona, mefedrona y metilona. Se caracterizó su capacidad de inhibir los transportadores de membrana y vesicular de monoaminas. También se estudió su afinidad por los receptores de serotonina (5-HT) y dopamina (DA), así como su efecto psicoestimulante.

Las catinonas fueron sintetizadas en nuestro laboratorio y se estudió la actividad locomotora en ratones Swiss CD-1 a diferentes dosis. Los ensayos de captación de monoaminas se llevaron a cabo en sinaptosomas de estriado y corteza frontal de ratas Sprague-Dawley. Para estudiar la afinidad de estos compuestos por los transportadores o receptores de monoaminas se realizaron ensayos de competición con radioligandos.

Butilona, mefedrona y metilona (5-25 mg/kg s.c.), produjeron un aumento dosis-dependiente de la actividad locomotora el cual fue inhibido por un pre-tratamiento con ketanserina (antagonista $5\text{-}HT_{2A}$) o haloperidol (antagonista D_2). Concretamente, butilona y metilona produjeron un aumento de actividad locomotora a través de la activación directa de los receptores $5\text{-}HT_{2A}$ y un aumento de la dopamina extracelular. En cambio, la mefedrona fue la única catinona en producir un aumento de la actividad locomotora dependiente de la 5-HT endógena. Por otro lado, la metilona fue el compuesto más potente inhibiendo la captación tanto de 5-HT como de DA por competición con el sustrato. La inhibición en la captación de 5-HT y DA inducido por la metilona persistió parcialmente incluso después de la retirada del compuesto del medio. La mefedrona fue la catinona que mostró la afinidad más alta por el transportador vesicular de monoaminas inhibiendo la captación de DA. Además, el contenido vesicular parece jugar un papel importante en el efecto final de la mefedrona.

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RESEARCH PAPER

Comparative neuropharmacology of three psychostimulant cathinone derivatives: butylone, mephedrone and methylone

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Keywords
butylone; mephedrone; methylone; dopamine; serotonin; locomotor activity

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BACKGROUND AND PURPOSE
Here, we have compared the neurochemical profile of three new cathinones, butylone, mephedrone and methylone, in terms of their potential to inhibit plasmalemmal and vesicular monoamine transporters. Their interaction with 5-HT and dopamine receptors and their psychostimulant effect was also studied.

EXPERIMENTAL APPROACH
Locomotor activity was recorded in mice following different doses of cathinones. Monoamine uptake assays were performed in purified rat synaptosomes. Radioligand-binding assays were carried out to assess the affinity of these compounds for monoamine transporters or receptors.

KEY RESULTS
Butylone, mephedrone and methylone (5–25 $mg \cdot kg^{-1}$) caused hyperlocomotion, which was prevented with ketanserin or haloperidol. Methylone was the most potent compound inhibiting both [^{3}H]5-HT and [^{3}H]dopamine uptake with IC_{50} values that correlate with its affinity for dopamine and 5-HT transporter. Mephedrone was found to be the cathinone derivative with highest affinity for vesicular monoamine transporter-2 causing the inhibition of dopamine uptake. The affinity of cathinones for 5-HT_{2A} receptors was similar to that of MDMA.

CONCLUSIONS AND IMPLICATIONS
Butylone and methylone induced hyperlocomotion through activating 5-HT_{2A} receptors and increasing extra-cellular dopamine. They inhibited 5-HT and dopamine uptake by competing with substrate. Methylone was the most potent 5-HT and dopamine uptake inhibitor and its effect partly persisted after withdrawal. Mephedrone-induced hyperlocomotion was dependent on endogenous 5-HT. Vesicular content played a key role in the effect of mephedrone, especially for 5-HT uptake inhibition. The potency of mephedrone in inhibiting noradrenaline uptake suggests a sympathetic effect of this cathinone.

Abbreviations
AUC, area under the curve; pCPA, p-chlorophenylalanine; VMAT2, vesicular monoamine transporter

Introduction

A decrease in the illegal availability of chemical compounds used for the synthesis of methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) or Ecstasy, coupled with a more than 50% decrease in the purity of ecstasy or cocaine (Measham *et al.*, 2010; Winstock *et al.*, 2011), has resulted in the appearance on the black market of

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a new generation of designer drugs known as 'cathinones' or 'beta-keto amphetamines' (the latter name deriving from the characteristic presence of a ketone in the side chain). These derivatives include a wide range of substances such as butylone, ethylone, methylone and mephedrone (4-methylmethcathinone). The most commonly available cathinones sold on the illegal market up until 2010 were mephedrone and methylone (Brunt *et al.*, 2011).

The popularity of mephedrone (with street names such as 'meow meow', 'plant food', 'bubbles' and 'MCAT') rose sharply in 2009 after it came to be seen as a legal, cheap and easily available alternative to MDMA. Mephedrone is predominantly used by teenagers and young adults (Vardakou *et al.*, 2011). It is sometimes sold mixed with methylone in a product called 'bubbles' and may also be mixed with butylone.

Methylone emerged under the trade name 'Explosion' around 2004 and was one of the first products to be marketed online and via head shops (Bossong *et al.*, 2005). In recreational users, a subjective comparison of the effects of this new drug suggested that it exhibited subtle differences when compared with MDMA. Little is known about any potential detrimental effects of methylone, however, given the similarities between this drug and MDMA, risks commonly associated with MDMA cannot be excluded. Butylone is also closely related to methylone. Butylone acts as an entactogen, psychedelic and stimulant and shares the same relationship to methylbenzodioxylbutanamine (MBDB) as methylone does to MDMA.

The first new beta-keto amphetamine to be banned (in 2009) was mephedrone; following this ruling, different European countries banned some of these derivatives, such as methylone but butylone was banned only in the UK and Denmark. Very recently, the Drug Enforcement Administration (DEA) temporarily classified mephedrone and methylone, but not butylone, as Schedule I under the Controlled Substances Act (Drug Enforcement Administration, 2011).

Previous reports have noted that users of cathinone derivatives loosely compared the effects with those of amphetamines and cocaine (Winstock *et al.*, 2011), but there was a greater similarity of effects with MDMA. These observations may explain the drug's rapid rise in popularity before its ban. In fact, many users consider the effects of cathinones to be superior to cocaine and MDMA (Winstock *et al.*, 2010; Vardakou *et al.*, 2011). Moreover, the abuse potential of cathinone derivatives is comparable with that of cocaine or Ecstasy (McElrath and O'Neill, 2011).

Based on their chemical structure, it could be postulated that the stimulant and empathogenic effects of cathinones are similar to those of amphetamine derivatives (Schifano *et al.*, 2011). However, very little is known about the pharmacology of the new cathinones. Cozzi *et al.* (1999) performed some *in vitro* studies with methcathinone and methylone, resulting in a hypothesis that the mechanism of action was similar to that of amphetamine. In addition, some cathinones, such as methylone, are able to bind to some monoamine transporters (Nagai *et al.*, 2007). Recently, some preliminary studies on the pharmacological targets of mephedrone have been published (Martínez-Clemente *et al.*, 2012; Hadlock *et al.*, 2011; Kehr *et al.*, 2011; Motbey *et al.*, 2012). Cathinone overdose results in cardiovascular disturbances (Wood and Dargan, 2010; Regan *et al.*, 2011) and the studies of Meng *et al.* (2012), characterizing the effects of mephedrone on cardiac ion channels, concluded that this drug acts as a sympathomimetic agent. Given what is known about the neuropharmacology of amphetamine derivatives, primarily methamphetamine and MDMA, and because of the chemical structural similarity of the cathinone derivatives, it has been suggested that these new compounds possess a monoaminergic mechanism of action.

In order to understand the effects of these psychostimulant beta-keto amphetamines, it is essential to determine which transporter or neurotransmitter systems are most affected. A thorough understanding of the pharmacological profile for each psychostimulant drug is essential in the development of treatment protocols for stimulant overdose and dependence.

The aim of the present study was to compare the neurochemical profile of butylone, mephedrone and methylone in terms of their abilities to inhibit plasma membrane and vesicular monoamine uptake transporters. We focused our attention on the dopamine and 5-HT transporters because these proteins are the most frequently implicated in the reinforcing properties and abuse potential of amphetamines. In addition, a comparative study of the interaction of these drugs with 5-HT and dopamine receptors and their psychostimulant effect was also carried out. In summary, this study represents the first comparison of the neuropharmacology of the three cathinone derivatives and suggests the likely effects of these compounds on individuals who abuse them.

Methods

Animals

All animal care and experimental protocols in this study complied with the guidelines of the European Community Council (86/609/ECC) and were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia. Efforts were made to minimize suffering and reduce the number of animals used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Adult Swiss CD-1 mice (Charles River, Lyon, France) weighing 25–30 g (total = 215) and male Sprague Dawley rats (Janvier, Le Genest, France) weighing 225–250 g (total = 58) were used. The animals were housed at 22 ± 1°C under a 12 h light/dark cycle with free access to food (standard laboratory diet, Panlab SL, Barcelona, Spain) and drinking water.

Synthesis of butylone, mephedrone and methylone

We synthesized cathinones in our organic chemistry laboratory, with permission from our University, following the procedures described by Chad and Copeland (2010) with minor modifications. Briefly, butylone, mephedrone and methylone were obtained by adding 3,4-methylenedioxybutyrophenone, 4-methylpropiophenone and 3,4-methylenedioxypropiophenone dissolved in CH_2Cl_2 to bromine, to give 3′,4′-methylenedioxy-2-

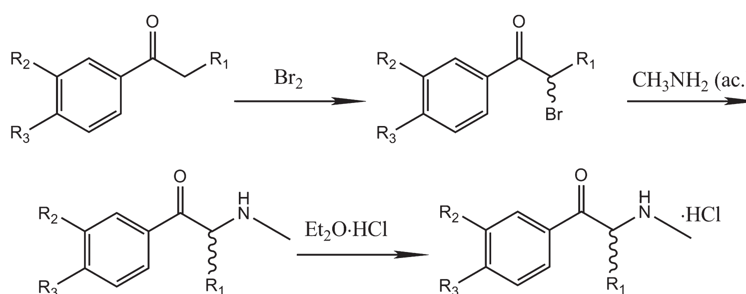

Figure 1

Chemical synthesis of butylone from 3,4-methylenedioxybutyrophenone, mephedrone from 4-methylpropiophenone and methylone from 3,4-methylenedioxypropiophenone. Butylone: R_1 = $-CH_2CH_3$; R_2/R_3 = $-OCH_2O-$. Mephedrone: R_1 = $-CH_3$; R_2 = $-CH_3$; R_3 = $-CH_3$. Methylone: R_1 = $-CH_3$; R2/R3 = $-OCH_2O-$.

bromobutyrophenone, 4′methyl-2-bromopropiophenone and 3′,4′-methylenedioxy-2-bromopropiophenone respectively. These compounds were dissolved in CH_2Cl_2 and added to an aqueous solution of methylamine (40%). HCl was then added. The aqueous layer was removed and made alkaline using sodium bicarbonate. Ether was used to extract the amine. Finally, a drop of ether–HCl solution was added to produce β-keto-*N*-methylbenzodiololylpropylamine hydrochloride (butylone), 4-methylmethcathinone hydrochloride (mephedrone) and 3,4-methylenedioxy-*N*-methylcathinone hydrochloride (methylone) (Figure 1). The identification of the three compounds obtained was assessed by proton nuclear magnetic resonance (^{1}H NMR) (CD_3OD) yielding the following results: butylone: δ 7.87 (dd, 1H) J = 8.1 Hz J = 1.8 Hz; 7.67 (d, 1H) J = 1.8 Hz; 7.18 (d, 1H) J = 8.1 Hz; 6.29 (s, 2H); 5.19 (t, 1H) J = 5.1 Hz; 2.88 (s, 3H); 2.22 (m, 2H); 1.07 (t, 3H) J = 7.5 Hz; mephedrone: δ 7.62 (d, 2H) J = 8.5 Hz; 7.42 (d, 2H) J = 8.5 Hz; 5.09 (q, 1H) J = 7.2 Hz; 2.77 (s, 3H); 2.45 (s, 1H); 1.57 (d, 3H) J = 7.2 Hz; methylone: δ 7.86 (dd, 1H) J = 8.1 Hz J = 1.8 Hz; 7.66 (d, 1H) J = 1.8 Hz; 7.18 (d, 1H) J = 8.1 Hz; 6.29 (s, 2H); 5.15 (q, 1H) J = 7.2 Hz; 2.91 (s, 3H); 1.73 (d, 3H) J = 7.2 Hz.

Chemical purity of the obtained compounds was also assessed by melting point determination, thin layer chromatography, ^{1}H NMR and mass spectrometry. All analytical data were consistent with the assigned structures with over 98% purity for the three cathinone derivatives.

Drug-induced spontaneous locomotor activity in mice

Before experimentation, all the mice (n = 9 per group) received two habituation sessions (48 and 24 h before testing) that were intended to reduce the novelty and stress associated with handling and injection. During these sessions, each mouse was given a subcutaneous injection of saline and placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of two sets of 16 infrared photocells (LE8811, Panlab SL) mounted according to the x, y axis coordinates and 1.5 cm above the wire mesh floor. Occlusions of the photo beams were recorded and sent to a computerized system (SedaCom32, Panlab SL). The interruption counts, over a 10 min period, were used as a measure of horizontal locomotor activity. The locomotor activity was monitored for 360 min. All experiments were conducted between 8:30 a.m. and 2:30 p.m. On the testing day, the animals received the cathinone derivative subcutaneously, at different doses, and were immediately placed in the activity box. Registration of horizontal locomotor activity then began. Antagonists were administered i.p., 20 min before cathinone derivative treatment. Treatment with p-chlorophenylalanine (pCPA) was carried out at a dose of 300 mg·kg^{-1} given daily i.p., over the course of 3 days. Eighteen hours after the last administration of pCPA, the animals received the cathinone. Each animal was used only once. Results are expressed as area under the curve (AUC), which was measured as the total changes from baseline at each recorded interval over 360 min or cumulative breaks for 120 min.

Rat synaptosome preparation

Pure synaptosome suspensions were prepared as described elsewhere (Pubill *et al.*, 2005) with minor modifications. Briefly, on the morning of each day of the experiment, two rats were decapitated and their striata or cortex were homogenized and centrifuged at 1000× g at 4°C for 10 min. The supernatant was recovered and sucrose buffer was added to a final sucrose concentration of 0.8 M. Samples were then centrifuged at 13 000× g for 30 min at 4°C. The supernatant was discarded and the synaptosome layer was separated from mitochondria by carefully adding 1 mL of ice-cold 320 mM sucrose buffer and gently shaking. Finally, the synaptosome fraction was diluted in HEPES-buffered solution (composition in mM: 140 NaCl, 5.37 KCl, 1.26 $CaCl_2$, 0.44 KH_2PO_4, 0.49 $MgCl_2.6H_2O$, 0.41 $MgSO_4.7H_2O$, 4.17 $NaHCO_3$, 0.34 $Na_2HPO_4.7H_2O$, 5.5 glucose and 20 HEPES-Na) containing pargyline (20 μM) and ascorbic acid (1 mM) [HEPES pargyline ascorbic buffer (HPAB)].

Plasmalemmal 5-HT, dopamine and noradrenaline uptake

In order to obtain evidence of the direct blockade (competitive inhibition) of [^{3}H]5-HT uptake in the presence of cathinone derivatives, synaptosomes from rat cortex were prepared as described earlier so that the final protein content

was approximately equivalent to 10 mg of tissue (wet weight) per mL. Reaction tubes were composed of 0.85 mL of butylone, mephedrone or methylone at different concentrations in buffer and 0.1 mL of synaptosome suspension. Tubes were warmed 10 min at 37°C before the addition of 0.05 mL of [^{3}H]5-HT (final concentration 15 nM), after which incubation was carried out for a further 5 min. The uptake reaction was stopped by rapid vacuum filtration through Whatman GF/B glass fibre filters (Whatman Intl Ltd., Maidstone, UK) pre-soaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly three times with 4 mL ice-cold 50 mM Tris–HCl. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Non-specific uptake was determined at 4°C in parallel samples containing fluoxetine (10 μM).

Synaptosomes from the rat striatum were obtained in order to measure the direct blockade of [^{3}H]dopamine uptake by cathinone derivatives. Competitive blockade of [^{3}H]dopamine uptake was assessed in the presence of butylone, mephedrone and methylone at different concentrations. The experiments were carried out as described earlier, using a final concentration of [^{3}H]dopamine of 5 nM. Non-specific uptake was determined at 4°C in parallel samples containing cocaine 100 μM.

Similarly, the direct blockade of [^{3}H]noradrenaline uptake by cathinone derivatives was measured in synaptosomes from the rat cortex. The experiments were carried out as described earlier, using a final concentration of [^{3}H]noradrenaline of 20 nM. Non-specific uptake was determined at 4°C in parallel samples containing 10 μM desipramine.

To measure persistent inhibition of [^{3}H]5-HT or [^{3}H]dopamine uptake, fresh synaptosomes were pre-incubated in a shaking water bath at 37°C for 1 h with butylone, mephedrone or methylone at different concentrations. Following pre-incubation, synaptosomes were centrifuged at 13 000× *g* for 20 min, resuspended in 5 mM Tris–HCl/ 320 mM sucrose buffer and re-centrifugated. Final pellets were resuspended in HPAB buffer. The suspension was warmed for 10 min at 37°C. Then, incubation was carried out for 5 min as described. The remaining synaptosomal preparation (i.e. that was not used for the uptake assay) was kept and the protein concentration was determined using a Bio-Rad protein reagent (Bio-Rad Labs, Inc., Hercules, CA, USA). Specific [^{3}H]5-HT or [^{3}H]dopamine uptake for each condition was normalized by dividing by the protein concentration.

Previous studies (Hrometz *et al*., 2004; Jones *et al*., 2004) demonstrated that dopamine can enter 5-HT nerve terminals via the 5-HT transporter. To measure this uptake, experiments were carried out as described but using synaptosomes from the rat cortex. In these experiments [^{3}H]dopamine (5 nM), d-amphetamine (1 μM) and cathinone derivatives were present in the medium. Inhibition by fluoxetine (10 μM) was used to confirm that, in this preparation, [^{3}H]dopamine uptake was carried out only by the 5-HT transporter.

Vesicular dopamine uptake

In order to measure [^{3}H]dopamine uptake via the vesicular monoamine transporter 2 (VMAT2; transporter and receptor nomenclature follows Alexander *et al*., 2011), the method described by Hansen *et al*. (2002) was used with minor modifications. Briefly, rat striatal synaptosomes were obtained as previously described and resuspended and lysed in ice-cold deionized water. Osmolarity was restored by the addition of HEPES and potassium tartrate to final concentrations of 245 and 100 mM, respectively, and samples were centrifuged for 20 min at 20 000× *g* (4°C) to remove synaptosomal membranes. $MgSO_4$ (1 mM) was added to the supernatant, which was then centrifuged for 45 min at 100 000× *g* (4°C). The resulting vesicular pellet was resuspended in wash buffer (see later section for composition), at a concentration of 50 mg·mL^{-1} (wet tissue weight). Vesicular [^{3}H]dopamine uptake measurement was performed by incubating 100 μL of vesicles at 30°C for 3 min in assay buffer (25 mM HEPES, 100 mM potassium tartrate, 1.7 mM ascorbic acid, 0.05 mM EGTA, 0.1 mM EDTA, and 2 mM ATP-Mg, pH 7.5) in the presence of 30 nM [^{3}H]dopamine. The reaction was terminated by addition of 1 mL of ice-cold wash buffer (assay buffer containing 2 mM $MgSO_4$ instead of ATP-Mg, pH 7.5) and rapid filtration followed by three 1 mL washes. Reserpine (10 μM) was tested in each experiment as a positive control of vesicular uptake inhibition. Non-specific incorporation was determined by measuring uptake at 4°C in wash buffer containing reserpine (20 μM).

Rat tissue membrane preparation

The rats were killed by decapitation under isoflurane anaesthesia and the brains were removed rapidly from the skull and the striatum and cortex were quickly excised, dissected out, frozen on dry ice and stored at –80°C until later use (Chipana *et al*., 2008a). When required, samples were thawed and homogenized in a 10-volume buffer: 5 mM Tris–HCl, 320 mM sucrose and protease inhibitors (aprotinin 4.5 μg·μL^{-1}, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer (Heidolph, Schwabach, Germany). The homogenates were centrifuged at 15 000× *g* for 30 min at 4°C. The resulting pellets were washed twice and the final pellets were resuspended in the appropriate buffer and stored at –80°C for use in radioligand binding experiments.

Interaction with 5-HT and dopamine transporters

[^{3}H]Paroxetine binding was used to label the cortical 5-HT transporter. Competition binding experiments were carried out using the membrane preparations from rat cortex. These experiments were performed in glass tubes containing 0.05 nM [^{3}H]paroxetine, butylone, mephedrone or methylone at increasing concentrations, and 150 μg of brain membranes. Incubation was carried out at 25°C for 2 h in a Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 mL. Clomipramine (100 μM) was used to determine non-specific binding. Binding was terminated by filtration and data were treated as explained earlier.

[^{3}H]WIN35428 binding was used to label striatal dopamine transporters. Membrane preparations from rat striatum (Chipana *et al*., 2008b) were resuspended in phosphate-buffered 0.32 M sucrose, pH 7.9 at 4°C to a concentration of 1 mg·mL^{-1}. Binding assays were performed in glass tubes containing 200 μL of [^{3}H]WIN35428 dilution in phosphate-buffered 0.32 M sucrose (final radioligand concen-

tration: 5 nM), butylone, mephedrone or methylone at increasing concentrations and 50 µL of membrane suspension. Samples were incubated for 2 h at 4°C. Non-specific binding was determined in the presence of 30 µM bupropion. Binding was terminated by filtration and data were analysed as previously described.

Interaction with 5-HT and dopamine receptors

[^{3}H]Ketanserin binding was used to label cortical $5\text{-}HT_{2A}$ receptors. Competition binding experiments were carried out using the membrane preparations from rat cortex. These experiments were performed in tubes containing 1 nM [^{3}H]ketanserin, cathinone derivatives at increasing concentrations and 100 µg of brain membranes. Incubation was carried out at 37°C for 30 min in a Tris–HCl buffer to a final volume of 0.5 mL. Methysergide (10 µM) was used to determine non-specific binding. Binding was terminated by filtration and data were analysed as previously described.

[^{3}H]Raclopride binding was used to label striatal dopamine D_2 receptors. Competition binding experiments were carried out using the membrane preparations from rat striatum. These experiments were performed in tubes containing 2 nM [^{3}H]raclopride, cathinone derivatives at increasing concentrations and 50 µg of brain membranes. Incubation was carried out at 25°C for 1 h in a Tris–HCl buffer to a final volume of 0.5 mL. Sulpiride (300 µM) was used to determine non-specific binding. Binding was terminated by filtration and data were analysed as previously described.

Data analysis

All data are expressed as mean ± SEM. One-way ANOVA was used to determine overall treatment effects on locomotor activity. The Tukey–Kramer multiple comparisons test was used for *post hoc* analysis in all instances following a significant ANOVA ($P < 0.05$) to assess the difference between treatment groups and the control group. Competition-binding curves were plotted and calculated by nonlinear regression using GraphPAD Prism (GraphPAD software, San Diego, CA, USA). Data were best fitted to a one-site competition model and an IC_{50} value (the concentration that induces 50% displacement) and the Hill coefficient (n_H) was obtained. The K_i values (the concentration that occupies 50% of the receptor population) for competing drugs were calculated using the equation by Cheng and Prusoff: $K_i = IC_{50} / [1 + (L/K_d)]$, where L is the total radioligand concentration and K_d is the dissociation constant of the radioligand. The Hill coefficient (n_H) was calculated by linear regression with data transformed according to Hill function.

Materials

All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) with the exception of cocaine and MDMA (National Health Laboratory, Barcelona, Spain). SB-216 641 was from Tocris Bioscience (Bristol, UK). 3,4-Methylenedioxybutyrophenone and 3,4-methylenedioxypropiophenone were from Alfa Aesar GmbH (Karlsruhe, Germany). [^{3}H]dopamine, [^{3}H]5-HT, [^{3}H]ketanserin, [^{3}H]noradrenaline, [^{3}H]paroxetine, [^{3}H]raclopride and [^{3}H]WIN35428 were from Perkin Elmer Life Sci. (Boston, MA, USA). All buffer reagents were of analytical grade.

Results

Effect of cathinone derivatives on spontaneous locomotor activity

Overall, analysis of the data (by ANOVA) demonstrated an extremely significant effect of cathinone treatment in the increase of spontaneous locomotor activity (expressed as AUC value) in mice [$F(9,35) = 48.892$, $P < 0.001$]. The *post hoc* Tukey–Kramer test to analyse each drug treatment revealed that subcutaneous administration of butylone [$F(3,17) = 98.267$, $P < 0.001$], mephedrone [$F(3,17) = 37.606$, $P < 0.001$] and methylone [$F(3,17) = 96.515$, $P < 0.001$] increased the locomotor activity in a dose-dependent manner (Table 1).

At the highest dose tested, both butylone and methylone induced a very significant hyperlocomotion compared with mephedrone. Furthermore, at 10 mg·kg^{-1}, a similar hyperlocomotion effect for methylone and mephedrone was found, whilst butylone induced a greater stimulant effect. The duration of the hyperlocomotive effect induced by cathinone

Table 1

Effect of butylone, mephedrone and methylone on the behavioural parameters in mice

Drug (dose in mg kg^{-1})	Locomotor activity	Rearings
Saline	72 829 ± 9524	8657 ± 1764
MDMA (5)	164 408 ± 29 558[a]	3405 ± 1695
Methamphetamine (5)	723 295 ± 35 137[c,h]	40 025 ± 7860[b,h]
Butylone (5)	229 910 ± 22 684[c]	12 945 ± 2465
Butylone (10)	400 775 ± 4026[c,f,g]	24 800 ± 1571[a]
Butylone (25)	756 330 ± 84 909[c,e]	26 735 ± 7686[a]
Mephedrone (5)	201 806 ± 21 894[c]	11 470 ± 1457
Mephedrone (10)	203 650 ± 35 616[b]	7185 ± 2703
Mephedrone (25)	319 265 ± 19 834[c]	7010 ± 1450
Methylone (5)	163 340 ± 20 019[a]	13 295 ± 3523
Methylone (10)	227 780 ± 10 635[b]	13 685 ± 6825
Methylone (25)	689 705 ± 73 385[c,d]	13 785 ± 4604

Results are expressed as mean ± SEM and represent the measurement of the area under the curve (total AUC) over a period of 360 min.
The number of animals in each group was nine with the exception of saline, which numbered 15.
[a]$P < 0.05$ versus saline.
[b]$P < 0.01$ versus saline.
[c]$P < 0.001$ versus saline.
[d]$P < 0.05$ versus mephedrone (25).
[e]$P < 0.01$ versus mephedrone (25).
[f]$P < 0.01$ versus mephedrone (10).
[g]$P < 0.01$ versus methylone (10).
[h]From Camarasa *et al.* (2009).

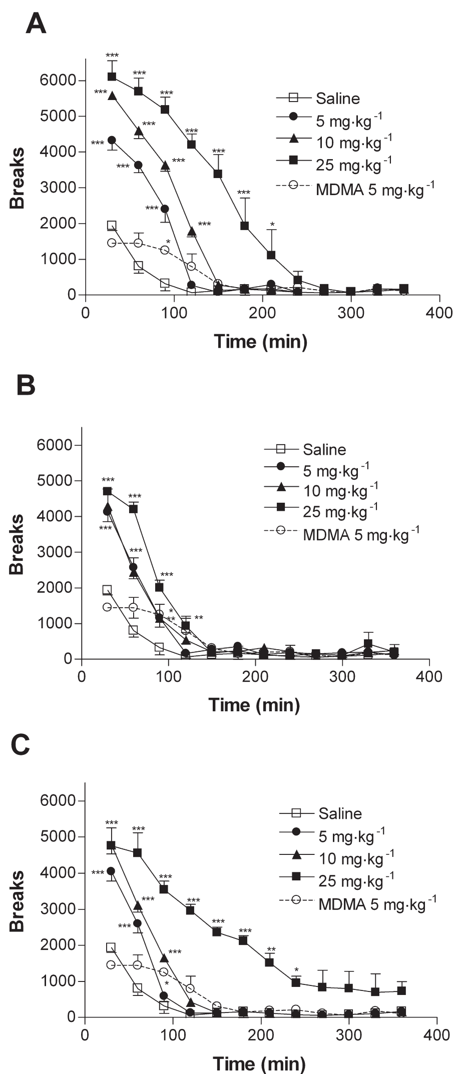

Figure 2

Effect of a single s.c. administration of (A) butylone, (B) mephedrone (B) and (C) methylone (5, 10 and 25 mg·kg^{-1}) compared with MDMA (5 mg·kg^{-1}) and saline. For locomotor activity, the interruption counts in the lower frame of the apparatus were registered, displayed in a 30 min block and monitored for 360 min. Data are expressed as the mean ± SEM of values from 9 mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; significantly different from saline.

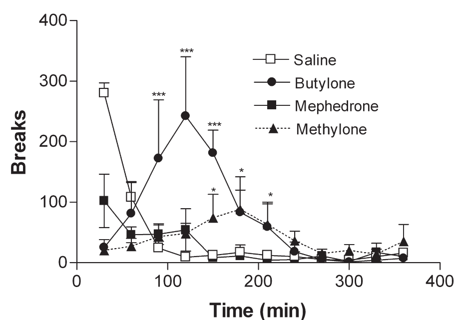

Figure 3

Time course of rearings induced by a single s.c. administration of 25 mg·kg^{-1} of butylone, mephedrone and methylone compared with saline. For this behaviour, the interruption counts in the upper frame of the apparatus were registered, displayed in a 30 min block and monitored for 360 min. Vertical axis shows these counts per animal in 30 min intervals. Data are expressed as the mean ± SEM of values from nine mice. **P* < 0.05, ****P* < 0.001; significantly different from saline. Significance at 180 min refers only to butylone.

treatment was also dose-dependent. At the highest dose tested, the stimulant effect of mephedrone lasted for 150 min after administration, whilst the effect of butylone and methylone persisted for 240 min and 270 min respectively (Figure 2).

In addition to our analysis of locomotor activity, we simultaneously recorded the number of rearings as a measure to determine the habituation of animals to the new environment (Figure 3). As Table 1 indicates, the animals treated with different doses of mephedrone or methylone showed no change in this behavioural activity (the total AUC rearing values were not significantly different from those of saline-treated mice). This observation contrasts with the results found for butylone at doses of 10 and 25 mg·kg^{-1}.

Pretreatment with ketanserin (0.5 and 4 mg·kg^{-1}), a 5-HT_2 receptor antagonist, and haloperidol (0.1 and 0.25 mg·kg^{-1}), a non-selective dopamine receptor antagonist, at doses that did not affect basal locomotor activity, fully and dose-dependently inhibited the hyperlocomotion induced by butylone and methylone. However, these antagonists partly inhibited the effect of mephedrone on locomotor activity (by about 53% and 65% respectively). Furthermore, pretreatment of animals with SB-216 641, a selective antagonist of 5-HT_{1B} receptors, at a dose of 8 mg·kg^{-1}, significantly reduced (by about 37%) the effect of butylone and fully inhibited the increase in number of rearings induced by this cathinone. In contrast, SB-216 641 failed to antagonize the increase in locomotor activity induced by mephedrone and methylone. Additionally, pCPA, an inhibitor of 5-HT synthesis, administered in a regime that fully inhibited the effect of MDMA (5 mg·kg^{-1}) (data not shown), significantly reduced the effect of mephedrone (by about 53%) and failed to antagonize the effect of butylone and methylone (Figures 4–7). Surprisingly, this antagonist potentiates the methylone effect at later test times (Figure 6).

Effect of cathinone derivatives on plasmalemmal 5-HT, dopamine and noradrenaline uptake

Butylone and methylone reduced [^{3}H]5-HT uptake in synaptosomes in a concentration-dependent manner, similar to mephedrone (Martínez-Clemente *et al.*, 2012). At the same

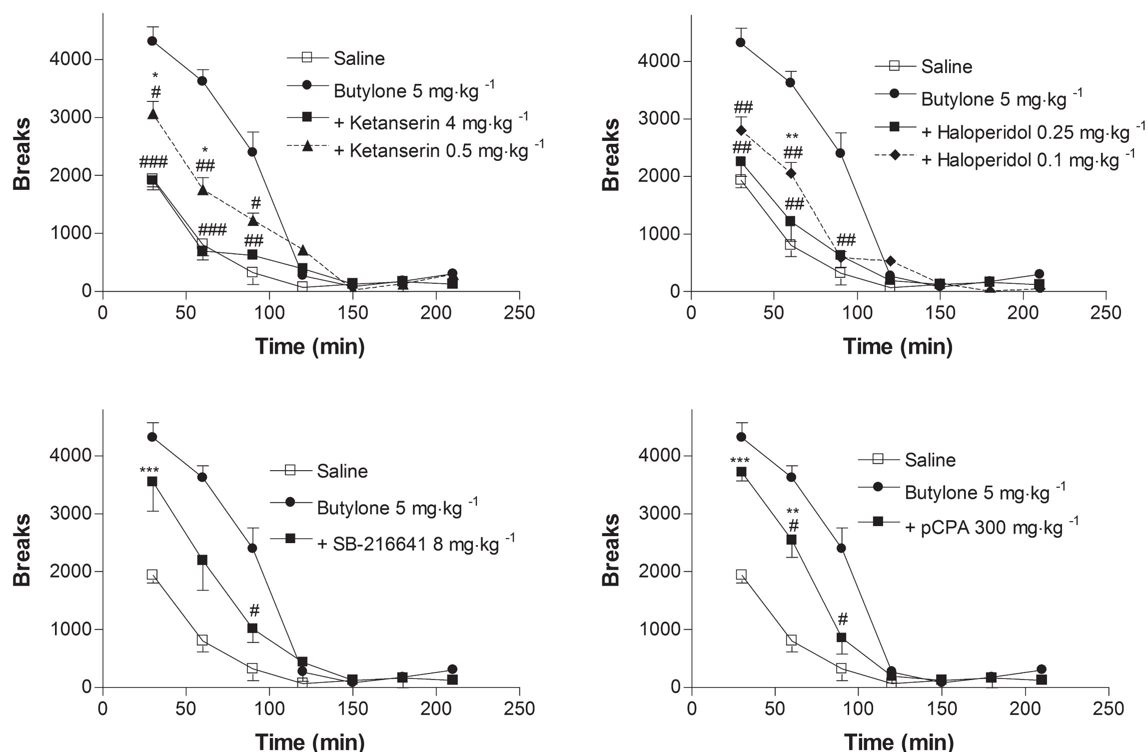

Figure 4

Effect of ketanserin, haloperidol, SB-216 641 and p-chlorophenylalanine (pCPA) on the time course of butylone-induced hyperlocomotion. Data are expressed as the mean ± SEM of values from nine mice. *$P < 0.05$, ***$P < 0.001$; significantly different from saline; #$P < 0.05$, ##**$P < 0.01$, ###$P < 0.001$; significantly different from butylone. Differences between butylone data and saline are displayed in Figure 2A.

Table 2

Calculated IC_{50} values (μM) of the three cathinone derivatives inhibiting the plasmalemmal and vesicular monoamine uptake

Drug	Monoamine transporter 5-HT	Dopamine	Noradrenaline	VMAT2
Butylone (10^{-8} M–10^{-4} M)	0.68 ± 0.13	1.71 ± 0.32	0.92 ± 0.13	81.84 ± 3.33
Mephedrone (10^{-8} M–10^{-4} M)	0.31 ± 0.08[a]	0.97 ± 0.05[a]	0.18 ± 0.01	3.40 ± 0.20
Methylone (5×10^{-8} M–10^{-4} M)	0.23 ± 0.03	0.56 ± 0.05	0.53 ± 0.05	21.83 ± 1.77

Data are mean ± SEM from three different experiments.
[a]From Martínez-Clemente *et al.* (2012).

concentration range, the three cathinone derivatives also reduced both [^{3}H]dopamine and [^{3}H]noradrenaline uptake (Table 2)

When measuring persistent inhibition of monoamine uptake, only at the highest concentration tested (1 mM) was [^{3}H]5-HT uptake inhibited by mephedrone (22 ± 1% $P < 0.01$) but not by butylone. Methylone inhibited [^{3}H]5-HT uptake at a concentration of 10 μM and reached a maximum of 71 ± 0.8% inhibition ($P < 0.001$) at a concentration of 1 mM. [^{3}H]dopamine uptake was also persistently inhibited by 1 mM butylone (35 ± 1.3%, $P < 0.05$) and methylone (75 ± 1.8%, $P < 0.01$) but not by mephedrone.

[^{3}H]Dopamine at a concentration of 5 nM enters the serotonergic terminal through the 5-HT transporter (90% inhibition was found with fluoxetine 1 μM). Butylone inhibited (by 27 ± 2.2% $P < 0.01$) this uptake only at the highest

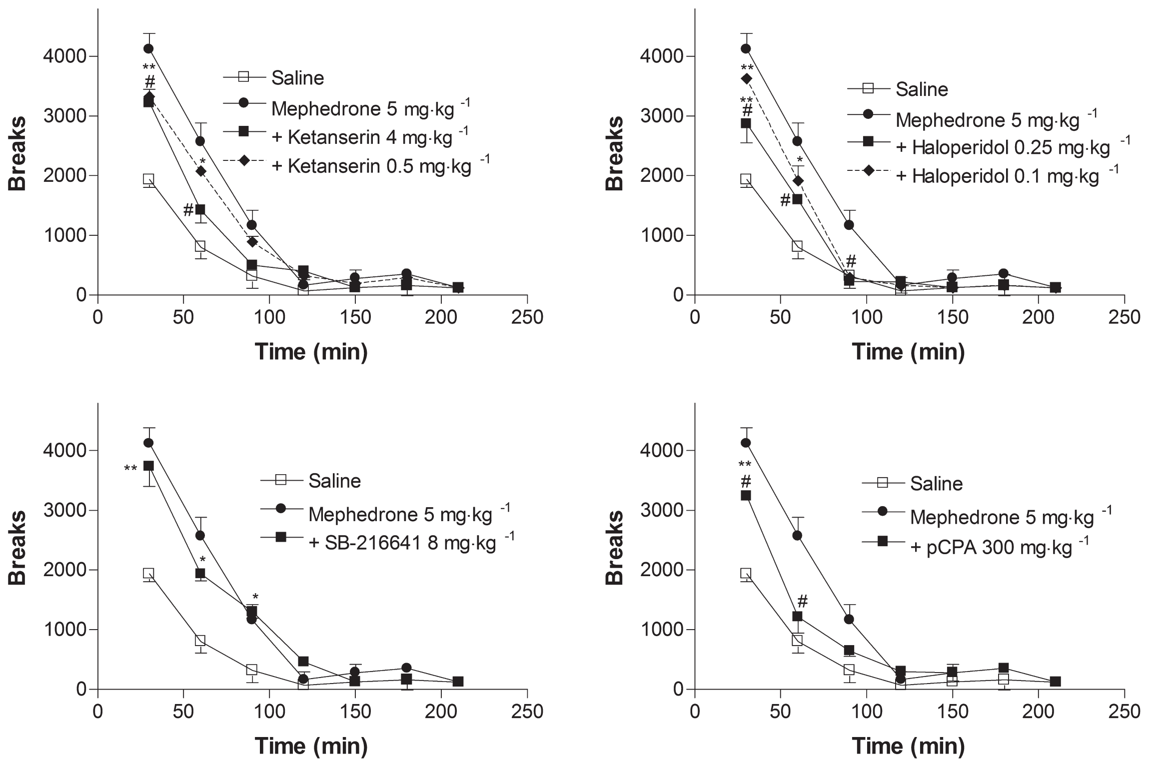

Figure 5

Effect of ketanserin, haloperidol, SB-216 641 and p-chlorophenylalanine (pCPA) on the time course of mephedrone-induced hyperlocomotion. Data are expressed as the mean ± SEM of values from nine mice. *$P < 0.05$, ***$P < 0.001$, significantly different from saline; #$P < 0.05$, significantly different from mephedrone. Differences between mephedrone data and saline are displayed in Figure 2B.

Table 3

Inhibition of monoamine uptake (in %) by the three cathinone derivatives, at a concentration of 10^{-4} M, in rat synaptosome preparations in the presence or the absence of reserpine (20 µM)

Drug	5-HT Without reserpine	With reserpine	Dopamine Without reserpine	With reserpine
Butylone	94 ± 1.6	57 ± 1.6[a]	97 ± 0.7	54 ± 0.9[a]
Mephedrone	95 ± 0.1	28 ± 3.9[a]	98 ± 0.1	35 ± 1[a]
Methylone	96 ± 0.5	64 ± 0.7[a]	100 ± 0.2	67 ± 0.7[a]

Data are mean ± SEM from three different experiments.
[a]$P < 0.001$ significantly different from the corresponding value without reserpine. Student's *t*-test (independent samples).

concentration tested (500 µM). Mephedrone and methylone inhibited this process at a lower concentration (100 µM: by 28 ± 3.2% $P < 0.05$ and 17 ± 0.5% $P < 0.05$ respectively; 500 µM: by 57 ± 0.8% $P < 0.001$ and 28 ± 3.6% $P < 0.05$ respectively).

We also wished to determined the role of endogenous neurotransmitters in our studies and so we measured [^{3}H]5-HT and [^{3}H]dopamine uptake in synaptosomes from cortex and striatum in the presence of reserpine (20 µM), thereby ensuring the blockade of VMAT2. Under these experimental conditions, we measured the effect of butylone, mephedrone and methylone at a concentration range from 10^{-7} to 10^{-4} M. The three cathinone derivatives inhibited both [^{3}H]5-HT and [^{3}H]dopamine uptake; nonetheless, this inhibition was lower than in the absence of reserpine (Table 3). The

Figure 6

Effect of ketanserin, haloperidol, SB-216 641 and p-chlorophenylalanine (pCPA) on the time course of methylone-induced hyperlocomotion. Data are expressed as the mean ± SEM of values from nine mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; significantly different from saline; $\#P < 0.05$, $\#\#**P < 0.01$, $\#\#\#P < 0.001$; significantly different from methylone. Differences between methylone data and saline are displayed in Figure 2C.

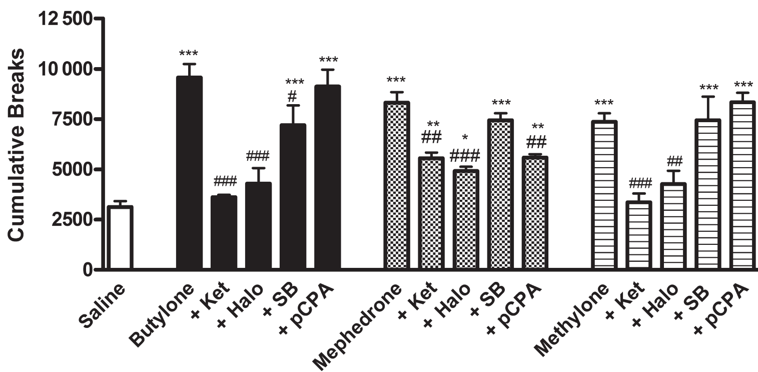

Figure 7

Cumulative breaks after 120 min for the effect of ketanserin (Ket) (4 mg·kg^{-1}), haloperidol (Halo) (0.25 mg·kg^{-1}), SB-216 641 (SB) (8 mg·kg^{-1}) and p-chlorophenylalanine (pCPA) (300 mg·kg^{-1}·day^{-1} for 3 days) on butylone, mephedrone and methylone (5 mg·kg^{-1})-induced hyperlocomotion. Data are expressed as and represent mean ± SEM from three separate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, significantly different from saline and $\#\#P < 0.01$, $\#\#\#P < 0.001$, significantly different from the corresponding cathinone derivative. One-way ANOVA and *post hoc* Tukey–Kramer multiple comparisons test.

greatest difference in results obtained with the two protocols was found with mephedrone.

Effect of cathinone derivatives on vesicular dopamine uptake

Vesicular uptake of dopamine through the VMAT2 was assayed in the presence of different concentrations of butylone, mephedrone and methylone, using reserpine 10 μM as positive control. Under these experimental conditions, butylone, mephedrone and methylone significantly inhibited [³H]dopamine uptake in a concentration-dependent manner. The corresponding IC_{50} calculated values for each compound are shown in Table 2.

Interaction of butylone, mephedrone and methylone with the 5-HT and dopamine transporters

All three cathinones displaced bound [³H]paroxetine in a concentration-dependent manner. This displacement occurred with K_i values in the low micromolar range for butylone (K_i = 2.86 ± 1.48 μM; n_H = 0.86 ± 0.04), mephedrone (K_i = 17.55 ± 0.78 μM; n_H = 0.72 ± 0.04, $P < 0.05$ vs. unity) and methylone (K_i = 6.49 ± 1.66 μM; n_H = 0.47 ± 0.08, $P < 0.05$ vs. unity) (Figure 8A).

Butylone, methylone and mephedrone displaced bound [³H]WIN35428 in a concentration-dependent manner. This displacement occurred with K_i values in the very low micromolar range for all drugs: butylone (K_i = 0.39 ± 0.06 μM; n_H = 1.28 ± 0.15); mephedrone (K_i = 1.53 ± 0.47 μM; n_H = 0.93 ± 0.04); and methylone (K_i = 0.86 ± 0.24 μM; n_H = 0.94 ± 0.09) (Figure 8B).

Interaction of butylone, mephedrone and methylone with 5-HT and dopamine receptors

Butylone, mephedrone and methylone displaced [³H]ketanserin binding in a concentration-dependent manner. Mephedrone showed the highest affinity for this receptor type followed by methylone and lastly by butylone. Calculated values of K_i for these drugs were: butylone (K_i: 37.49 ± 6.41 μM; n_H = 0.84 ± 0.03, $P < 0.05$ vs. unity), mephedrone (K_i: 3.96 ± 0.22 μM, n_H = 0.94 ± 0.09) and methylone (K_i: 11.12 ± 2.89 μM; n_H = 0.77 ± 0.03, $P < 0.05$ vs. unity) (Figure 9A).

The three cathinone derivatives displaced bound [³H]raclopride in a concentration-dependent manner with K_i values in the high micromolar range, mephedrone being the compound with highest affinity. K_i values obtained for these drugs were: butylone (K_i: 57.09 ± 11.46 μM; n_H = 1.00 ± 0.03), mephedrone (K_i: 50.86 ± 3.45 μM; n_H = 0.83 ± 0.21) and methylone (K_i: 191.28 ± 9.44 μM; n_H = 0.63 ± 0.06, $P <$ 0.05 vs. unity) (Figure 9B).

Discussion

In this study, we compared the *in vitro* neuropharmacology of three cathinone derivatives as well as demonstrating, in laboratory animals, the psychostimulant effect which has been described by recreational users. The mesolimbic dopamine system is a final common pathway through which amphetamines exert their psychostimulant effect. It is well known that amphetamine derivatives produce dose-dependent increases in locomotor activity in rodents (Izawa *et al*., 2006), which reflects increased dopaminergic transmission in the nucleus accumbens (Ljungberg and Ungerstedt, 1985). This hyperlocomotor activity of amphetamines is directly correlated with blockade of dopamine uptake and with a non-exocytotic, transporter-mediated, dopamine release (Leviel, 2001; Escubedo *et al*., 2005). Our results demonstrate that all three cathinone derivatives, given subcutaneously to mice, induced a dose-dependent increase in locomotor activity that reached its peak shortly after administration.

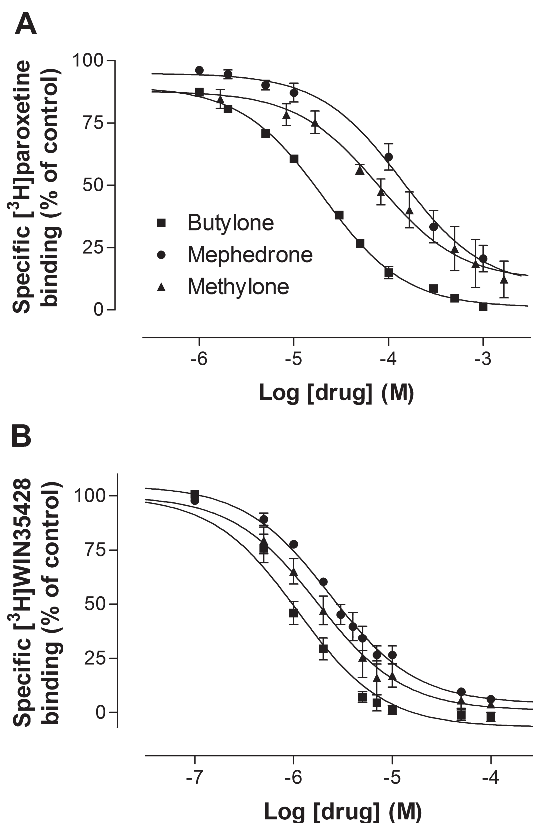

Figure 8

(A) Competition curves of the inhibition of [³H]paroxetine binding by butylone, mephedrone and methylone in cortical membranes from Sprague Dawley rats. Membranes were incubated at 25°C for 2 h with 0.05 nM [³H]paroxetine in the presence of increasing concentrations of cathinone derivatives. (B) Inhibition of [³H]WIN35428 binding by these drugs in striatal rat membranes incubated for 2 h at 4°C with 5 nM [³H]WIN35428 also in the presence of increasing concentrations of cathinone derivatives. Inhibition curves were calculated using the nonlinear least squares method and adjusted to a one-site model. Data shown are mean ± SEM of duplicates and the experiments were performed in triplicate.

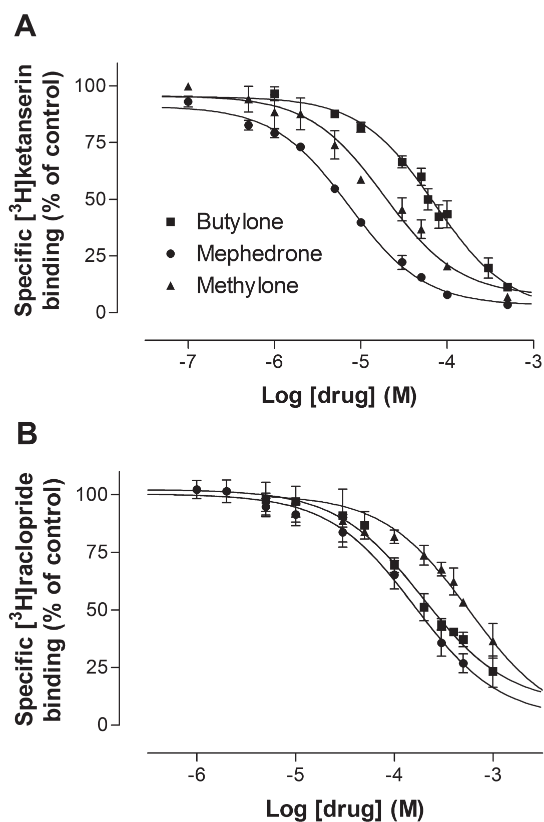

Figure 9

(A) Competition curves of the inhibition of [^{3}H]ketanserin binding by butylone, mephedrone and methylone in cortical membranes from Sprague-Dawley rats. Membranes were incubated at 25°C for 2 h with 1 nM [^{3}H]ketanserin in the presence of increasing concentrations of cathinone derivatives. (B) Inhibition of [^{3}H]raclopride binding by these drugs in striatal rat membranes incubated for 2 h at 4°C with 2 nM [^{3}H]raclopride also in the presence of increasing concentrations of cathinone derivatives. Inhibition curves were calculated using the nonlinear least squares method and adjusted to a one-site model. Data shown are mean ± SEM of duplicates and the experiments were performed in triplicate.

Mephedrone elicits a moderate increase in locomotor activity lasting for 150 min. The limited information available in humans comes from user self-reports, which suggest that the onset of the psychostimulant effects of mephedrone occurs 15–45 min after oral ingestion, and the duration of the desired effects appears to last 2–3 h. Whilst we used a different route of administration in our studies, our results are in general agreement with the psychostimulant effects of mephedrone seen in humans. To our knowledge, there are no published data about the locomotor stimulatory activity of butylone or methylone. In this regard, our results demonstrate a dose-dependent psychostimulant effect induced by these drugs which is greater than that of mephedrone. This effect persists for 4 h after administration at the highest dose. It should be noted that the methylone effect dramatically increases between the doses of 10 and 25 mg·kg^{-1}. Although we have no data on methylone metabolism, this result could be explained by a saturation of the metabolic pathways of methylone leading to increased and persistent plasma levels of this drug. In our mouse locomotor studies, cathinones exhibited a different profile of activity from that of MDMA in that they showed a higher maximal activity which returned more rapidly to basal values. These observations are in agreement with results obtained by Kehr *et al.* (2011) in rats.

High doses (25 mg·kg^{-1}) of amphetamine induces a climbing effect in test animals that impairs the proper measurement of locomotor activity. However, the beta-keto amphetamines, at this high dose, show a marked increase in locomotor activity, with an AUC value for butylone and methylone that does not differ from that of methamphetamine at 5 mg·kg^{-1}.

MDMA increased dopamine release by activating 5-HT$_2$ receptors, which may also contribute to the hyperlocomotion effect (Yamamoto *et al.*, 1995). When the animals were pretreated with ketanserin or haloperidol (at doses that did not affect basal locomotor activity), the hyperlocomotion following cathinone derivatives was inhibited, suggesting a 5-HT and dopamine involvement. The affinity of these cathinones for 5-HT$_{2A}$ receptors supports this hypothesis. However, their relatively low affinity for dopamine D$_2$ receptors rules out a direct effect on this receptor type and hence, we suggest that these drugs can induce an increase in the synaptic dopamine concentration that finally interacts with this dopamine receptor. Only mephedrone displayed a pCPA-dependent effect, suggesting the involvement of endogenous 5-HT. The hyperactivity of test animals treated with methylone in the presence of pCPA and seen at later time points could be due to an interaction of pCPA with the proposed cathinone-saturable metabolism. Kot and Daniel (2011) described an alteration of liver CYP activity when animals were treated with pCPA.

An activation of 5-HT$_{1B}$ receptors is suggested to be responsible for the increase in the locomotor and rearing behaviour induced by MDMA in mice (McCreary *et al.*, 1999). These terminal autoreceptors regulate 5-HT release from dorsal raphe nucleus projections throughout the rat forebrain (Clark *et al.*, 2002). The results obtained with a selective 5-HT$_{1B}$ receptor antagonist suggest a role for these receptors in the hyperlocomotive effect of butylone but not in the effects of mephedrone and methylone.

Mice treated with mephedrone or with methylone did not demonstrate novel-environment behaviour (absence of significant difference in rearings from saline-treated animals), but animals treated with butylone, demonstrated an increase in the number of rearings that appeared when hyperlocomotion was decreasing. This behaviour seems to be a consequence of the activation of 5-HT$_{1B}$ receptors by this drug, given that the increase was fully inhibited by pretreating the animals with SB-216 641.

Amphetamines are substrates for the dopamine and 5-HT transporters, but the action of amphetamines is not a mere inhibition of the uptake transporter. Following its transport into the synaptic terminal, amphetamines stimulate a reversal of the transporter thereby eliciting monoamine

efflux (Fischer and Cho, 1979; Fleckenstein *et al.*, 2007). This reverse operation results in a net inhibition of monoamine uptake into the terminal and a prolonged extra-cellular concentration, which is responsible for their psychostimulant effects (Levi and Raiteri, 1993; Leviel, 2001). Incubation of synaptosomes with low concentrations of butylone, mephedrone and methylone induced a significant concentration-dependent reduction in all [^{3}H]5-HT, [^{3}H]noradrenaline and [^{3}H]dopamine uptake. It is important to stress the low IC_{50} value of mephedrone inhibiting [^{3}H] NA uptake. Methylone is the most potent compound inhibiting both [^{3}H]5-HT and [^{3}H]DA uptake. This cathinone also induced the most persistent inhibition of 5-HT uptake. Nagai *et al.* (2007) reported higher IC_{50} values for methylone than those reported in our study. However, the higher purity of the synaptosomal preparation used in our study could result in greater sensitivity to the compound.

The direct interaction of cathinones with both plasmalemmal dopamine and 5-HT transporters can be correlated with the displacement of [^{3}H]WIN35428 or [^{3}H]paroxetine binding. In our studies, butylone and methylone demonstrated a close relationship between the IC_{50} values of their inhibition of [^{3}H]5-HT and [^{3}H]dopamine uptake and the K_i values obtained for their interaction with the respective transporters. Consequently, the inhibition of these transporters by cathinones is the result of a direct interaction with these proteins, competing with the endogenous substrate. However, mephedrone does not seem to interact directly with the 5-HT transporter, suggesting an additional mechanism.

We have limited information about pharmacokinetics of cathinones. Hadlock *et al.* (2011) found plasma mephedrone levels of about 5 μM one hour after a dosing regimen that mimics binge consumption. Present results from *in vitro* uptake experiments demonstrate that monoamine uptake inhibition is a probable mechanism of action of the *in vivo* psychostimulant effect of cathinones.

Cathinones also inhibit the entry of dopamine through the 5-HT transporter, but only at very high concentrations. Consequently, at concentrations reached *in vivo*, cathinones cannot prevent the excessive dopamine released from being transported and oxidized in the depleted 5-HT terminals.

The biggest differences among these compounds were observed in their effects on the transporter VMAT2. When measuring dopamine uptake through this transporter, we found a sevenfold and 27-fold reduction in potency of methylone and butylone, respectively, as compared with mephedrone. The inhibition of VMAT2-mediated uptake by amphetamines has been attributed to the ability of these compounds to act as substrates for VMAT2 (Schuldiner *et al.*, 1993), although another potential inhibitory mechanism could be through dissipation of the pH gradient. Given that the IC_{50} values obtained for cathinone derivatives are substantially lower than the concentration of MDMA required for this dissipation to take place (Rudnick and Wall, 1992), this mechanism can be ruled out. The results on VMAT2 inhibition and those obtained with reserpine-treated synaptosomes suggest that plasmalemmal and vesicular components are involved in the final inhibitory effect of cathinones. The vesicular component is especially important in explaining the effects of mephedrone. This suggestion is in agreement with results obtained by Kehr *et al.* (2011) in rats.

Putative phosphorylation sites for protein kinases exist in dopamine and 5-HT transporters. For example, protein kinases A and C have been implicated in various aspects of dopamine transporter function and regulation, such as trafficking, transport activity and direct phosphorylation (Jayanthi *et al.*, 2005; Foster *et al.*, 2006; Ramamoorthy *et al.*, 2011). Thus, a substance acting on specific intracellular pathways can induce a persistent inhibition of the transporter. We found that methylone caused prolonged inhibition of [^{3}H]5-HT uptake suggesting that alterations in the transporter functionality are more complex than a simple blocking of the carrier. In our *in vitro* model, mephedrone did not inhibit dopamine uptake. Nonetheless, it persistently inhibited 5-HT uptake, by about 22%, but only at a high concentration. This observation is in accordance with recent reports stating that repeated mephedrone administrations causes persistent 5-hydroxytryptaminergic, but not dopaminergic deficits (Hadlock *et al.*, 2011).

Results obtained in the present work, using radioligand binding assays, confirm our initial hypothesis that cathinone derivatives interact with both dopaminergic and 5-hydroxytryptaminergic targets in the CNS. We demonstrate a great affinity of butylone, mephedrone and methylone for the 5-HT transporter as these drugs displaced [^{3}H]paroxetine binding with K_i values in the micromolar range, with the following order of affinity: butylone > methylone >> mephedrone. The interaction of butylone with the plasma membrane 5-HT transporter occurs in a single class of binding sites (the Hill coefficient value did not differ from unity), whilst the interaction of mephedrone and methylone occur in a more complex manner. Similarly, we demonstrate a high affinity of butylone, mephedrone and methylone for the dopamine transporter. This interaction occurs in a single class of binding sites.

5-HT_{2A} receptors are a pharmacological target of amphetamine compounds, mediating not only dopamine release but also locomotor responses (Auclair *et al.*, 2004). Hallucinogens potently stimulate the 5-HT_{2A} receptors, and their agonistic properties are responsible for the behavioural effects of these compounds (Erritzoe *et al.*, 2011). All three compounds displaced bound [^{3}H]ketanserin, with the lowest K_i value being for mephedrone, which interacts with a single class of binding sites. This low value is similar to that of MDMA acting as a 5-HT_{2A} receptor agonist. Moreover, the affinity values of cathinone derivatives for dopamine D_2 receptors are in the high micromolar range. This feature is shared with MDMA, which has an affinity for this dopamine receptor of 95 μM (Battaglia *et al.*, 1988), and rules out the possibility of a direct activation of these receptors *in vivo,* by these cathinones.

In summary, we provide evidence that some cathinone derivatives interact with dopamine and 5-HT transporters and receptors, suggesting a pharmacological profile similar to other psychoactive drugs such as the amphetamine-like compounds.

Butylone particularly induces intense hyperlocomotion through an activation of 5-HT_{2A} receptors and an increase in extra-cellular dopamine concentration. The activation of 5-HT_{1B} receptors also contributes to this psychostimulant effect. This cathinone inhibits 5-HT and dopamine uptake as a consequence of a competition with the substrate. At high doses, its effect lasts for 4 h after its administration.

The locomotor profile of methylone, similar to that of butylone, points to a saturation of its metabolism. *In vitro*, this cathinone derivative is the most potent 5-HT and dopamine uptake inhibitor. This inhibition partially persists after its withdrawal.

Finally, mephedrone also induced endogenous 5-HT-dependent hyperlocomotion, which was prevented by 5-HT_{2A} and dopamine D_2 receptor antagonists. Vesicular content plays a key role in the effect of mephedrone, especially in the 5-HT uptake. Its potency in inhibiting noradrenaline uptake is suggestive of a significant sympathetic effect of this drug.

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Conflicts of interest

None.

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3.2. Publicación II

An integrated pharmacokinetic and pharmacodynamics study of a new drug of abuse, methylone, a synthetic cathinone sold as "bath salts"

Raúl López-Arnau, Jose Martínez-Clemente, Marcel·lí Carbó, David Pubill, Elena Escubedo, Jorge Camarasa (2013). *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 45: 64-72

Resumen

En los últimos años una nueva clase de drogas de diseño ha aparecido en el mercado de las drogas de abuso, las llamadas catinonas sintéticas, como la metilona, una nueva sustancia psicoactiva e ingrediente activo de las conocidas como "legal highs" o "bath salts". La relación concentración-tiempo-efecto de dicha sustancia en animales de laboratorio y/o humanos a diferentes dosis y vías de administración todavía no ha sido totalmente caracterizada. Por ello, son del todo necesarios estudios, inicialmente a nivel preclínico, para desarrollar directrices de prevención y tratamiento. El principal objetivo del presente estudio fue caracterizar el perfil farmacocinético de la metilona en rata después de la administración intravenosa y oral y relacionarlo con su efecto psicoestimulante. Además se caracterizaron *in vivo* los metabolitos de fase I en sangre después de la administración oral de metilona. El análisis se realizó mediante HPLC acoplada a espectrometría de masas.

Las concentraciones plasmáticas de metilona tras la administración intravenosa (10mg/kg) se ajustaron a un modelo bicompartimental (α=1.95h^{-1} y β=0.72h^{-1}). Por otro lado, tras la administración oral de metilona (15-30mg/kg), el pico de concentración máxima se alcanzó entre 0.5 y 1 h después de la administración. En este caso, el perfil farmacocinetico por vía oral se ajustó a un modelo *flip-flop*, en el cual la tasa de absorción es más lenta que la de eliminación. En el metabolismo de fase I de la metilona se identificó una degradación de la cadena lateral a través de una N-desmetilación para dar lugar a la correspondiente amina primaria. Además se identificó una desmetilenación de la metilona seguida de una O-metilación para dar lugar al metabolito 4-hidroxi-3-metoximetcatinona o al 3-hidroxi-4-metoximetcatinona. Se identificó también una hidroxilación en la posición β al grupo cetona. El análisis farmacocinético/farmacodinámico mostró una relación entre las concentraciones plasmáticas y el aumento de la actividad locomotora. Después de dicho análisis, se sugirió además la posible participación en el efecto hiperlocomotor de algún metabolito, similar a lo que ocurre con la MDMA. Además, a dosis elevadas, una activación directa de los receptores 5-HT$_{2A}$ podría también contribuir en el efecto psicoestimulante final.

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An integrated pharmacokinetic and pharmacodynamic study of a new drug of abuse, methylone, a synthetic cathinone sold as “bath salts”

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ABSTRACT

Introduction: Methylone (3,4-methylenedioxymethcathinone) is a new psychoactive substance and an active ingredient of “legal highs” or “bath salts”. We studied the pharmacokinetics and locomotor activity of methylone in rats at doses equivalent to those used in humans.
Material and methods: Methylone was administered to male Sprague–Dawley rats intravenously (10 mg/kg) and orally (15 and 30 mg/kg). Plasma concentrations and metabolites were characterized by LC/MS and LC–MS/MS fragmentation patterns. Locomotor activity was monitored for 180–240 min.
Results: Oral administration of methylone induced a dose-dependent increase in locomotor activity in rats. The plasma concentrations after i.v. administration were described by a two-compartment model with distribution and terminal elimination phases of $\alpha = 1.95\ h^{-1}$ and $\beta = 0.72\ h^{-1}$. For oral administration, peak methylone concentrations were achieved between 0.5 and 1 h and fitted to a flip-flop model. Absolute bioavailability was about 80% and the percentage of methylone protein binding was of 30%. A relationship between methylone brain levels and free plasma concentration yielded a ratio of 1.42 ± 0.06, indicating access to the central nervous system. We have identified four Phase I metabolites after oral administration. The major metabolic routes are *N*-demethylation, aliphatic hydroxylation and *O*-methylation of a demethylenate intermediate.
Discussion: Pharmacokinetic and pharmacodynamic analysis of methylone showed a correlation between plasma concentrations and enhancement of the locomotor activity. A contribution of metabolites in the activity of methylone after oral administration is suggested. Present results will be helpful to understand the time course of the effects of this drug of abuse in humans.

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1. Introduction

A new generation of designer phenethylamine derivatives, known as “legal highs” or “research chemicals” has emerged and has been marketed as a legal alternative to “ecstasy” (3,4-methylenedioxymethamphetamine or MDMA) or cocaine. Their chemical structure is related to cathinone, the main psychoactive constituent of khat (Sørensen, 2011). These products are also advertised as “bath salts”, the active ingredients of which include mephedrone, methylone and MDPV (3,4-methylenedioxypyrovalerone) alone or mixed (Spiller et al., 2011), available for purchase on line, at convenience stores or truck stops. In October 2011, the Drug Enforcement Administration (DEA) placed these three synthetic cathinones (mephedrone, methylone and MDPV) and their salts and isomers into the Schedule I of the Controlled Substances Act, to avoid an imminent hazard to the public safety. In October 2012, the DEA extended the temporary placement of methylone into this schedule.

The most commonly available cathinone derivatives sold on the recreational market appear to be mephedrone (4-methyl-methcathinone) and methylone (3,4-methylenedioxymethcathinone) (Brunt et al., 2010). Methylone was first synthesized as an antidepressant and taken orally or nasally, as drug of abuse, emerged under the trade name “explosion” around 2004 thus making it one of the first products to be marketed via on-line (Bossong et al., 2005).

Previous studies have described that cathinone users consider the effects of these new drugs of abuse to be superior to those of cocaine and MDMA (Vardakou et al., 2011; Winstock et al., 2010) and it may explain their rapid rise in popularity.

Abbreviations: MDMC, Methylone (3,4-methylenedioxymethcathinone); LC/MS, liquid chromatography/mass spectrometry; PK/PD, Pharmacokinetic/Pharmacodynamics analysis; MDC, 3,4-Methylenedioxycathinone; 4-OH-3-MeO-MC, 4-Hydroxy-3-methoxymethcathinone; 3-OH-4-MeO-MC, 3-Hydroxy-3-methoxymethcathinone; 3’-OH-MDMC, 3’-Hydroxy-methylenedioxymethcathinone; AIC, Akaike's information criterion.

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To our knowledge, very little is known about the pharmacology of methylone. Results from *in vitro* studies hypothesized that the mechanism of action of methylone, was similar to that of d-amphetamine (Baumann et al., 2012; Cozzi et al., 1999), by binding to the monoamine transporters (Nagai et al., 2007; Simmler et al., 2012). More recently, some studies on the pharmacological targets of cathinones have been published by our group (Martínez-Clemente et al., 2012) and others (Hadlock et al., 2011; Kehr et al., 2011; Motbey et al., 2011; Simmler et al., 2013). It has been demonstrated that methylone increased the spontaneous locomotor activity in mice in a dose-dependent manner, this effect is prevented by ketanserin or haloperidol pre-treatment. Methylone compared to mephedrone and butylone, was the most potent cathinone to inhibit serotonin and dopamine uptake, an effect which partially persists after withdrawal (López-Arnau et al., 2012).

Human pharmacokinetics data on methylone are obtained from consumer reports (Boulanger-Gobeil et al., 2012; Shimizu et al., 2007), on-line reports also indicate that 150–300 mg is a common oral dose of methylone. Some reports describe that in humans the onset of effect of methylone appears at 15–30 min with a 2–3.5 h duration, but 6–24 h to return to "normal" status. Kamata et al. (2006) identified the characteristic human and rat urinary metabolites of methylone that were of great importance in forensic analysis (Zaitsu et al., 2009). Moreover, some human sudden deaths related to methylone intake have been reported (Cawrse et al., 2012; Pearson et al., 2012) and some reported cases met the Hunter criteria for serotonin syndrome (Boyer and Shannon, 2005; Warrick et al., 2012). Methylone concentration- and time-effect relationships in laboratory animals and/or humans after different doses and routes of administration have not been adequately characterized and further research will be necessary to develop adequate prevention and treatment policies.

The aim of the present study was to characterize the pharmacokinetic profile of methylone in rats after intravenous and oral administration and to correlate it with a pharmacodynamic evaluation of the psychostimulant effect of this drug of abuse, thus establishing a PK/PD model. Furthermore, another goal of this study was to analyze the *in vivo* Phase I metabolites in rat blood and the brain/plasma concentration of this cathinone after an oral administration to assess its penetration into the central nervous system. Because ethical considerations considerably limit the administration of this addictive substance to humans, animal models that mimic human use are essential. Accordingly, the oral doses used in this study were selected in the attempt to emulate those used by human drug abusers. To our knowledge, all published papers on pharmacokinetics of methylone are of a forensic nature (urinary collected specimens) (Crawse et al., 2012; Kamata et al., 2006) or from *in vitro* experiments (Mueller and Rentsch, 2012). This study constitutes the first approach to the kinetics of methylone based on *in vivo* blood sample data from laboratory animals and will be helpful to design different treatment regimes in rodents in order to evaluate methylone's effects as well as to understand the time course of the effects of this drug of abuse in humans.

2. Material and methods

2.1. Drugs and reagents

Pure racemic methylone and mephedrone HCl were synthetized and characterized by us, as described previously (López-Arnau et al., 2012). Methylone solutions for injection were prepared in saline immediately before administration. Isoflurane was from Laboratories Dr. Esteve (Barcelona, Spain). Reagents required for LC/MS assays were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

The experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona, following the 86/609/EEC guidelines. Male Sprague–Dawley rats (Janvier, Le Génest, France) weighing 225–250 g were used. Animals were housed at 22 ± 1 °C under a 12-h light/dark cycle with free access to food and drinking water.

2.3. Pharmacokinetic experiments

For oral pharmacokinetic experiments, methylone was administered at doses of 15 and 30 mg/kg to animals previously fasted for 18 h and for intravenous pharmacokinetic analysis, methylone was administered at a dose of 10 mg/kg. A total of 8–10 animals per dose were used.

Blood samples (150–200 μl) were collected from isoflurane anesthetized rats through a venipuncture of the external jugular vein in a time schedule from 5 min to 8 h (or 24 h in some cases) and transferred to 1 ml glass tubes (containing 10 μl EDTA 20 mg/ml) on ice. A total of 4–5 blood samples were obtained from one animal. After each blood extraction an equal volume of sterile saline was infused to maintain volume and osmotic homeostasis.

Blood samples were centrifuged at 1000 ×*g* for 10 min to obtain the plasma. 90 μl of plasma samples were mixed with 10 μl of internal standard (IS) solution (methylone, 200 ng/ml). The mixture was extracted by adding 250 μl of methanol up to a final concentration of 70%. The denatured proteins were precipitated by centrifugation at 10,000 ×*g* for 5 min. 250 μl of clear supernatant was acidified with formic acid (50% v/v) to a pH of 2.5–3.0 to obtain stable extracts, because in non-acidified live-blood extracts, cathinones degraded relatively fast (Sørensen, 2011). The mixture was transferred to an ultrafiltration filter cup and high-molecular-weight components were removed by means of filtration through a 30-kDa regenerated cellulose membrane (Microcon 30®, Millipore, Bedford, MA, USA). The ultrafiltration unit was centrifuged at 20,000 ×*g* for 10 min and 100 μl of the filtrate were transferred to an auto sampler vial.

An HP 1100 Liquid Chromatography (LC) system equipped with an autosampler, a column oven set to 40 °C and coupled API 3000 triple-quadrupole mass spectrometer (MS), with a turbo ion spray source was used to quantify the corresponding cathinone. Chromatographic separation was achieved on a Luna HST C18 (100 × 2 mm, i.d., 2.5 μm) column. The mobile phase was water (A) and methanol (B) with 0.1% of formic acid in both solvents. An increasing linear gradient (v/v) of B was used (t(min),%B), as follows, (0, 5), (20, 95), (22, 95), (22.5, 5) and (32.5, 5), at a constant flow rate (150 μl/min). The biological samples were refrigerated at 4 °C and 5 μl were injected into the LC-MS/MS system. The LC-ESI (electrospray ionization)-MS/MS conditions were optimized by direct infusion of cathinone standards (1 μg/ml) dissolved in 50,50 (v/v) water (0.1% formic acid)/methanol (0.1% formic acid) into the MS at a constant flow rate (5 μl/min). Two transitions were followed for methylone (*m/z* 208.1 → 190.1 and 208.1 → 160.0) (collision energies of 17 and 22 V) and both were used for the quantification. For mephedrone one transition was followed (*m/z* 178.1 → 160.0, 17 V).

2.4. Blood metabolite determination

Blood samples were collected at 60, 120 and 180 min after oral administration at a dose of 30 mg/kg. Samples were treated as described above, without IS. For metabolite identification, a Linear Trap Quadrupole Orbitrap Velos MS equipped with an ESI source was used. This system was coupled to an Accela chromatograph, a refrigerated auto sampler and a photodiode array detector. Chromatographic separation was achieved on a Luna C18 (100 × 2.1 mm, i.d., 3 μm) column. The mobile phase was the same as the one used in the pharmacokinetic studies. In this case, an increasing linear gradient (v/v) of B was used (t(min),%B), as follows, (0, 2), (20, 95), (22, 95), (25, 2) and (30, 2) at a constant flow rate (150 μl/min). The injection volume was 10 μl. The data were acquired in Fourier transform mass spectrometry mode (FT MS) and ranged from *m/z* 50 to 1000 in both positive and negative ion modes. Operation parameters were as follow, source voltage, 3.5

(kV) in positive mode, S-Lens RF levels, 60%, capillary temperature was fixed at 275 °C, sheath gas at 40 (arbitrary units) and auxiliary gas at 10 (arbitrary units). MS^2 acquisition was carried out under collision-induced dissociation conditions using collision energy between 35 and 50%.

2.5. Protein binding and brain levels

Blood samples were obtained 45 min after oral administration (dose of 30 mg/kg) followed by decapitation and removal of the whole brains. In protein binding experiments blood samples were divided by half. One half was filtered through centrifugal filter units (Centrifree® YM-30, Millipore, Bedford, MA, USA) for comparison with the other unfiltered half. Plasma samples were extracted as described above. The extraction of brain samples was carried out as described by Hadlock et al. (2011) and brain methylone levels and protein binding assays were quantified as described in the pharmacokinetic experiments.

2.6. Calibration

Plasma and brains from untreated rats were used to obtain the calibration curves. In the plasma analysis, seven standards were prepared daily in 100 μl of blank plasma (from 10 to 6000 ng/ml). To determine brain methylone levels, five standards were prepared, also daily, in 0.5 ml of brain homogenate (from 10 to 250 ng/ml). Mephedrone was used as IS at the final concentration of 200 ng/ml for plasma levels and 50 ng/ml for brain levels. The method showed linearity within the concentration range studied and the limit of quantification was considered lower than 10 ng/ml. Quality control samples were prepared at 50, 1000, 5000 ng/ml and 20, 50, and 200 ng/ml for plasma and brain analysis, respectively. The accuracy of the assay was 90–110%. The intra- and inter-assay coefficients of variation (CV) were less than 15%.

2.7. Pharmacokinetic analysis.

Mean plasma concentration time profiles were analyzed by bi-compartmental modeling. The distribution and elimination characteristics of methylone were determined after the i.v. administration. Fixing the parameters obtained in the i.v. model, oral methylone profiles were analyzed simultaneously by using a bi-compartmental model with oral delay and Michaelis–Menten metabolism kinetics. The best fit line was selected after visual inspection of the fitting, the analysis of the objective function and the AIC (Akaike's information criterion), the precision of the estimates (mean and CV) and the weighted residuals plot analysis.

The i.v. data were described by and open two-compartmental model and fit to the following equation,

$$Cp = Ae^{-at} + Be^{-\beta t}$$

where Cp is the total plasma drug concentration at time t, A and B are the extrapolated zero intercepts, and α and β represent the apparent first-order elimination rate constants. The half-life ($t_{1/2\beta}$) for the elimination phase and the volume of distribution in the central compartment (*V*c) were calculated as follows, $t_{1/2\beta} = 0.693/k_{10}$ where k_{10} is an overall elimination rate constant, $Vc = Dose/(A + B)$. For the oral route, absorption rate constant, k_a, was fitted. The parameters Clp (total plasma clearance) and *V*ss (steady state apparent volume of distribution) were calculated using non-compartmental methodology. The area under the concentration-time curve ($AUC_{0\text{-}\infty}$) and area under the first moment of the plasma drug concentration-time curve ($AUMC_{0\text{-}\infty}$) were calculated by the following equations,

$$AUC_{0\rightarrow\infty} = \frac{A}{a} + \frac{B}{\beta}$$

$$AUMC_{0\rightarrow\infty} = \frac{A}{a^2} + \frac{B}{\beta^2}$$

The values reported as the Cmax and Tmax are the actual observed values. The *F* (absolute bioavailability) value for oral administration can be calculated by the following formula,

$$F = \frac{D_{iv} \cdot AUC_{oral(0\rightarrow\infty)}}{D_{oral} \cdot AUC_{iv(0\rightarrow\infty)}}$$

where, for the oral and i.v. routes D_{oral} and D_{iv} are the respective doses, ($AUC_{0-\infty}$) oral and ($AUC_{0-\infty}$) i.v. are the respective AUCs from 0 to infinity.

Oral pharmacokinetic parameters were calculated with the following equations,

$$t^{1}/_{2abs} = \frac{0.693}{k_a}$$

$$AUC_{0\rightarrow\infty} = \frac{F \cdot D}{V_c \cdot k_{10}}$$

$$AUMC_{0\rightarrow\infty} = \frac{F \cdot D}{V_c \cdot k_{10}^2}$$

$$MRT = \frac{AUMC_{0\rightarrow\infty}}{AUC_{0\rightarrow\infty}}$$

$$MAT = \frac{1}{k_a}$$

$$Cl_p = V_c \cdot k_{10}$$

$$V_{ss} = V_c \cdot \left(\frac{k_{12} + k_{21}}{k_{21}}\right)$$

where k_a and $t_{1/2abs}$ are the absorption constant and the absorption constant half-life obtained after oral administration. MRT and MAT are the mean resident time and the mean absorption time, respectively. Initial estimates of Clp and Vss were those of i.v. route. The microconstants k_{12} and k_{21} used for the calculation of *V*ss are the terms that describe the distribution of the drug between the central and peripheral compartments. When Michaelis–Menten fitting was applied, the first order elimination constant from the central compartment was substituted by the following equation,

$$C_t = \frac{V_{\max} \cdot C_t}{K_m + C_t}$$

where C_t is the methylone concentration at time t, the *Vmax* the maximum metabolic capacity achieved and K_m the Michaelis–Menten constant. Cl_{met} is the metabolic clearance calculated as follows (Barrett et al., 1998),

$$Cl_{met} = \frac{V_{\max}}{K_m}$$

2.8. Locomotor activity experiments

Prior to experiments, all rats received two habituation sessions (48 and 24 h before testing). During these sessions, each rat received saline and was placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of 16 infrared photocells (LE8811, PANLAB, Barcelona, Spain) mounted according to the *x*, *y* axis coordinates and 2.5 cm above the wire mesh floor.

Occlusions of the photo beams (breaks) were recorded and sent to a computerized system (SedaCom32, PANLAB, Barcelona, Spain). The interruption counts, over a 10 min-block, were measured. After intravenous or oral drug administration, locomotor activity was monitored for 180 min and 360 min, respectively. On the testing day, the animals received methylone intravenously (10 mg/kg), or orally (15 or 30 mg/kg), and were immediately placed in the activity box. Registration of horizontal locomotor activity then began. Results are expressed as area under the curve (AUC), which was measured as the total changes from baseline at each recording interval over 360 min.

2.9. Pharmacokinetic/pharmacodynamics modeling

PK/PD analysis was carried out on mean and standard deviation data. Because experimental observed data were obtained in parallel assays, data fitting were performed with the aggregates of the different doses (data pooling) in order to estimate a unique set of parameters. PK and PK/PD analysis was achieved by use of the compartmental modeling SAAM II software system (SAAM Institute, Seattle, WA, USA).

2.10. Pharmacokinetic/pharmacodynamics analysis.

A link compartment representing stimulation of the locomotor behavior was used to describe the data (Sheiner et al., 1979). Integration of methylone pharmacokinetics and pharmacodynamics was based on the relationship between mean plasma methylone concentration-time profile for i.v. and oral dosages. PK/PD modeling was also performed by using SAAM II. The pharmacokinetic model includes central and peripheral compartments, the inter-compartmental rate constants of absorption and elimination and the input rate for i.v. and oral administration. The effect site was connected by a fixed rate constant from the central plasma compartment. A dummy compartment provides the concentrations in the effect site (C_e). The simulation PK/PD model proposed is an additive sigmoid E_{max} equation expressed in terms of C_e such that,

$$E = E_0 = \frac{E_{max} \cdot C_e^n}{C_e^n + EC_{50}^n}$$

The baseline value E_0 is the effect when methylone concentration is zero. EC_{50} is the concentration that increases E_0 to 50% of the Emax or maximal response and “n” determines the sigmoid shape of the function (Hill coefficient).

3. Results

3.1. Methylone pharmacokinetics

The observed and model-fitted plasma concentrations of methylone at each time point are shown in Fig. 1. The plasma concentrations versus time curve after intravenous administration of methylone were adequately described by a two-compartment model ($\alpha = 1.95\ h^{-1}$ and $\beta = 0.72\ h^{-1}$) (Fig. 1). Pharmacokinetic parameters (Table 1) showed that the $t_{1/2\beta}$ was about 1 h. The Vss and Clp resulted in values of 2.39 l/kg and 0.53 l/h respectively.

For oral dosing conditions, pharmacokinetic parameters derived from the methylone curves are summarized in Table 2. Cmax values were achieved rapidly, usually within 0.5 to 1 h, and declined to undetectable levels at 24 h. As might be expected, absolute AUC value increased proportionally with dose. Bioavailability was calculated of 78–89% after oral administration of the two doses. The standard errors of the majority of pharmacokinetic parameters were relatively small (coefficients of variation < 20%). Interindividual variability in the experimental Cmax value was evident from the high value of CV.

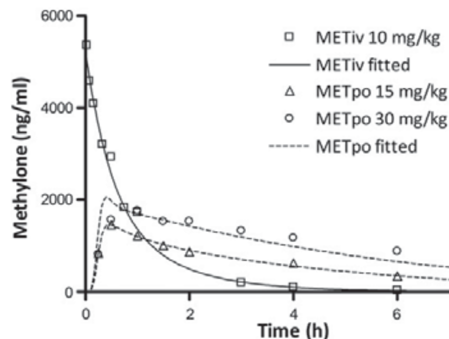

Fig. 1. Time-course of experimental and fitted plasma methylone levels after intravenous (10 mg/kg) and oral (15 and 30 mg/kg) administration. Rats received methylone at time 0, and blood specimens (0.2 mL) were collected through the external jugular vein from 0.08 to 8 h after administration. Plasma levels of methylone were quantitated by LC-MS as described in *Materials and Methods section*. Data are expressed as mean for *n*, 4 to 5 rats/group. SEM values are not displayed for clarity.

3.2. Methylone protein binding and brain levels

Results from blood samples obtained near Tmax yielded a percentage of methylone protein binding of 30.82 ± 2.23%. Whole brain levels of 349.2 ± 26.7 ng methylone/g tissue (n = 3) were found. The relationship between brain levels and free plasma concentration yielded a ratio of 1.42 ± 0.06, indicating access of methylone to the central nervous system.

3.3. Identification of methylone and metabolites in rat blood

In the present study, we have identified four metabolites that are detected in all collected samples at 60, 120 and 180 min. The identification of methylone and the observed metabolites by mass spectrometry is provided below.

3.3.1. Methylone

The calculated [M + H$^+$] m/z for methylone ($C_{11}H_{13}NO_3$) was 208.09737, the found [M + H$^+$] m/z was 208.09720 (0.17 mDa). The peak at m/z 190 is attributable to the typical H_2O loss (18 Da). The loss of methylamine group (31 Da) gave a fragment with low intensity at m/z 177. We also observed the loss of the methylenedioxy group (CH_4O_2, 48 Da). The presence of the fragment at m/z 149 indicates the loss of C_3H_9N (59 Da), the intensity of which was found to be considerably low.

Table 1
Main pharmacokinetic parameter estimates of methylone after i.v. administration (10 mg/kg) to male Sprague–Dawley rats.

Parameter	Units	Estimate	CV(%)
A	ng/ml	3483.84	12.3
B	ng/ml	1787.75	21.8
α	h^{-1}	1.95	11.7
β	h^{-1}	0.72	7.1
K_{10}	h^{-1}	1.22	4.2
K_{12}	h^{-1}	0.28	24.7
K_{21}	h^{-1}	1.15	16.4
AUC 0 − ∞	ng.h/ml	4251.89	1.9
AUC 0 − t	ng.h/ml	4241.6	0.2
C^o_p	ng/ml	5271.60	4.7
V_c	ml	426.81	4.7
V_{ss}	ml	537.68	1.54
$t_{1/2\beta}$	h	0.95	7.1
CLp	ml/h	529.18	1.9

Table 2
Main pharmacokinetic parameter estimates of methylone after oral administration to male Sprague–Dawley rats at a dose of 15 mg/Kg (Value 15) and 30 mg/Kg (Value 30). Results are expressed as mean and the corresponding coefficient of variation (CV) in %.

Parameter	Units	Value 15 (CV)	Value 30 (CV)
Cmax obs	ng/ml	1456.67 (15.8)	1896.00 (69.2.)
Tmax obs	h	0.50 (0.0)	0.97 (55.3)
AUC 0 − ∞	ng.h/ml	5740.30 (24.4)	9988.80 (10.1)
AUC 0 − t	ng.h/ml	4942.50 (2.4)	8092.60 (9.9)
$t_{1/2abs\ app}$	h	2.15 (7.0)	3.14 (10.7)
$t_{1/2\beta\ app}$	h	0.55 (4.5)	0.55 (4.5)
Tlag	h	0.17 (7.7)	0.28 (9.8)
V_{ss}	ml	433.31 (4.9)	
F*	%	89.00 (−)	78.40 (−)
CLmet	ml/h	154.80 (18.7)	154.80 (18.7)
MRT	h	0.79 (4.5)	0.82 (4.2)
MAT	h	0.46 (7.0)	0.20 (10.7)
Km	µ/ml	2.68 (122)	2.68 (122)
Vmax	µg/h	414.30 (104)	414.30 (104)
CLp	ml/h	529.52 (2.05)	530.96 (1.9)

* Calculated as F = (AUC_{0-}oral *x* Dose i.v.)/(AUC_{0-}i.v. *x* Dose oral).

3.3.2. 3,4-Methylenedioxycathinone (MDC)

We identified the corresponding N-demethylation metabolite, MDC, with formula $C_{10}H_{11}NO_3$. The calculated $[M + H^+]$ m/z was 194.08117, the found $[M + H^+]$ m/z was 194.08142 (0.25 mDa). The peak at m/z 176 is corresponding to the H_2O loss (18 Da). The C_2H_6N and CH_4O_2 loss (45 Da and 48 Da) gave two peaks at m/z 149 and 146, respectively, suggesting that this mass spectrum is in accordance with the metabolite structure proposed.

3.3.3. 4-Hydroxy-3-methoxymethcathinone (4-OH-3-MeO-MC) and 3-hydroxy-3-methoxymethcathinone (3-OH-4-MeO-MC)

Two metabolites were detected with the same chemical formula $C_{11}H_{15}NO_3$ and mass spectrum, but with different retention times (Fig. 2). For 4-OH-3-MeO-MC and 3-OH-4-MeO-MC the calculated $[M + H^+]$ m/z was 210.11247, the found $[M + H^+]$ m/z was 210.11319 (0.72 mDa) and 210.11355 (1.08 mDa) respectively. Both compounds gave a peak at m/z 192 (H_2O loss). In this case, the loss of the methylenedioxy group was not found, however the peak at m/z 160 shows the CO_2H_6 loss (50 Da) reflecting the aperture of the methylenedioxy ring. Based on studies by Kamata et al. (2006), we assumed that 4-OH-3-MeO-MC was the first compound to be eluted out of the two, due to that the chromatography conditions were similar.

3.3.5. 3′-Hydroxy-methylenedioxymethcathinone (3′-OH-MDMC)

A compound with chemical formula $C_{11}H_{13}NO_4$ was detected, and the calculated $[M + H^+]$ m/z was 224.09173, the found $[M + H^+]$ m/z was 224.09154 (0.19 mDa). Typical loss of water was also observed giving a peak at m/z 206. Furthermore, a double water loss (36 Da) was detected, indicating the possible presence of a hydroxyl group in this structure. The peak at m/z 176, which shows the loss of 48 Da, can be attributed to the methylenedioxy group. In order to confirm the proposed structure, the loss of the methylenedioxybenzoylcation fragment (149 Da) gave a peak at m/z 74, which corresponds to a hydroxylated immonium cation (Fig. 2).

We ensured that the mass found did not correspond to endogenous compounds by comparing each metabolite mass from treated and untreated rat blood samples. From the metabolites detected, the proposed *in vivo* phase I metabolic pathway for this cathinone is displayed in Fig. 3.

3.4. Locomotor activity

Intravenous administration of methylone induced an increase in the rat locomotor activity (AUC Saline, 8683 ± 98, Methylone, 95078 ± 19953, n = 3, p < 0.01, Student-t test, independent samples) that lasted for 150 min.

Similarly, an overall ANOVA demonstrated a significant effect of oral methylone in the locomotor activity in rats ($F_{2,8} = 6.015$, $p < 0.05$). The post-hoc Tukey–Kramer test revealed that methylone increased the locomotor activity in a dose-dependent manner (AUC saline, 20760 ± 2002, methylone 15 mg/kg, 95767 ± 23537, p < 0.05, methylone 30 mg/kg, 133354 ± 32878, p < 0.05, n = 3). As can be seen in Fig. 4, this increase is due mainly to a different time-course profile. The higher dose (30 mg/kg) induced a maximum break response (2011 ± 750, n = 3) that did not differ significantly from that of 15 mg/kg (2005 ± 344, n = 3), but the disappearance of the effect becomes much slower. After the dose of 15 mg/kg, break values were not significantly different from animals treated with saline after 180 min. At the highest dose

Drug and Metabolites	R_t (min)	Monoisotopic mass	Precursor ion (m/z)	Product ions (m/z)
MDMC	7,54	208,09720	208	190,177,160
MDC	7,14	194,08142	194	176,149,146
4-OH-3-MeO-MC	5,84	210,11319	210	192,160
3-OH-3-MeO-MC	6,16	210,11355	210	192,160
3′-OH-MDMC	10,34	224,09154	224	206,188,176,149,74

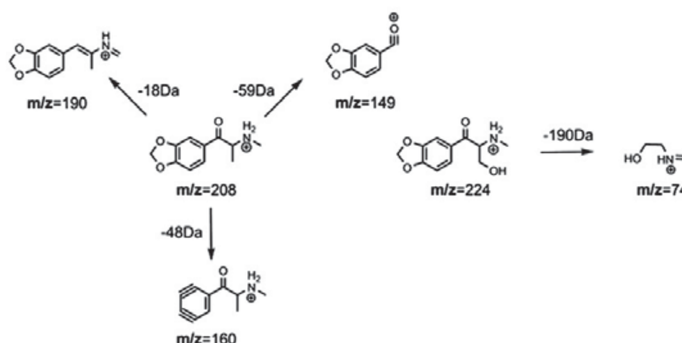

Fig. 2. LC-MS Orbitrap data for fragmentation of methylone and their metabolites in rat plasma after a single oral dose of 30 mg/kg at three different times after administration (60, 120 and 180 min). Scheme of proposed fragmentation patterns for methylone and its metabolite 3′-OH-MDMC.

tested, the psychostimulant effect of methylone persisted for 270 min (Fig. 4).

3.5. Pharmacokinetic/pharmacodynamic analysis.

A plot of locomotor activity versus methylone concentrations over time shows a direct relationship between concentrations and pharmacological effect after i.v. administration of methylone (Fig. 5A). A counterclockwise hysteresis behavior was observed after the oral administration (Fig. 5B and 5C). With the developed PK/PD model the mean EC_{50} and E_{max} values obtained ranged from 3.1 to 6.7 ng/ml and from 508.42 to 1280.86 breaks, respectively (Table 3). Good agreement between the predicted and observed values was noted for the locomotor activity data (mean objective function of 7.60 ± 0.52 and mean AIC of 7.04 ± 0.57). Methylone plasma concentration profile and predicted %Emax versus time are shown in Fig. 6. A delay between these plasma concentrations and the effect can be observed at the dose of 15 mg/kg, but this delay is reduced at the highest dose. This does not occur when effect site-concentration is displayed. The values of methylone concentration in the effect-site were approximately twice when doubling the dose.

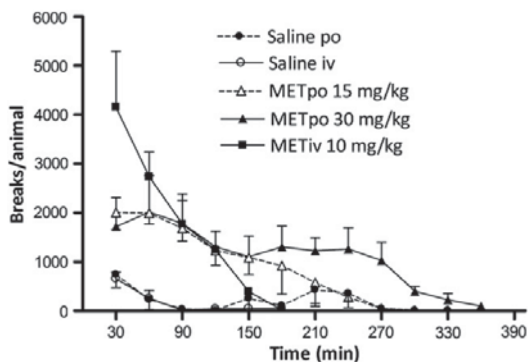

Fig. 4. Time-course of locomotor activity induced after oral (15 and 30 mg/kg) and intravenous (10 mg/kg) administration of methylone. For this behavior, the interruption counts in the frame of the apparatus were registered and displayed in a 30 min-block. Vertical axis shows breaks/animal in 30 minutes intervals. Locomotor activity was monitored for 360 min and 180 min for oral and intravenous administration, respectively. Data are expressed as the mean ± SEM of values from 3 rats.

4. Discussion

To our knowledge, no research on the pharmacokinetics of methylone in rats is presently available. Therefore, in this study we have characterized the pharmacokinetics and pharmacodynamics of methylone in male Sprague–Dawley rats. High levels of locomotor activity, which measures the psychostimulant effect, occurred after methylone administration and are consistent with the onset of subjective and physiological effects in humans. The oral doses used, 15 and 30 mg/kg, are equivalent to 158 and 315 mg respectively, according to the FDA guidelines (Food Drug Administration Center for Drug Evaluation Research, 2005), to those frequently declared as very usual in humans.

Our results show that the blood levels of methylone in rats declined in a biphasic fashion after intravenous administration at a dose of 10 mg/kg. The large V_{ss} indicates that methylone is distributed extensively into tissues and the Clp value explains the rapid elimination half-life (1 h). At administered oral doses, methylone displayed linear pharmacokinetics since the observed concentrations in blood were directly proportional to the administered dose. This observation suggests that, at present doses, the processes involved in the disposition of methylone were not saturated. At the highest dose tested, the reduction in the k_a value and increased tlag explained that Tmax moves from 30 to 60 min.

The terminal plasma half-life of methylone after oral administration was significantly higher than after i.v. administration, suggesting

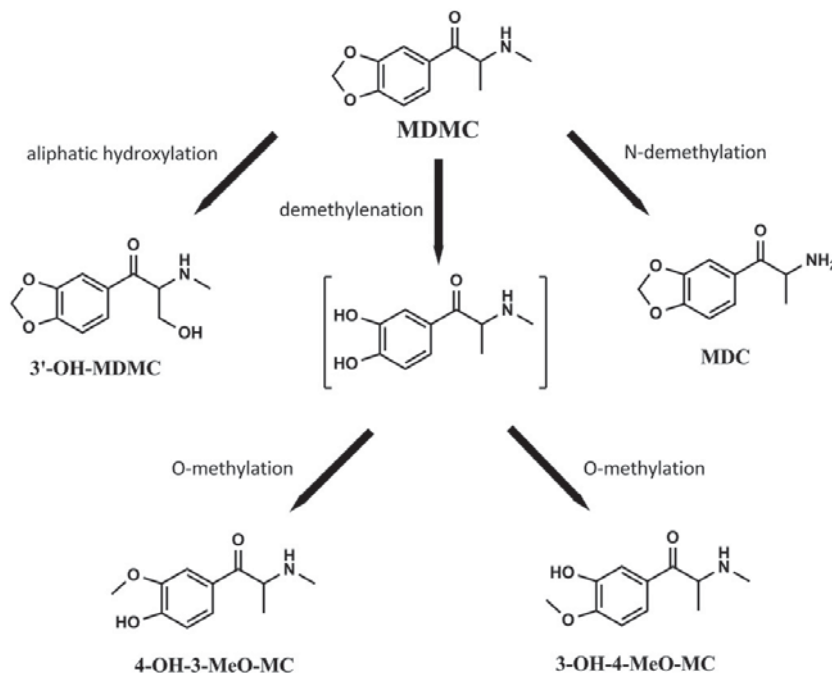

Fig. 3. *In vivo* metabolic pathways proposed for methylone in rat plasma after a single oral administration of 30 mg/kg.

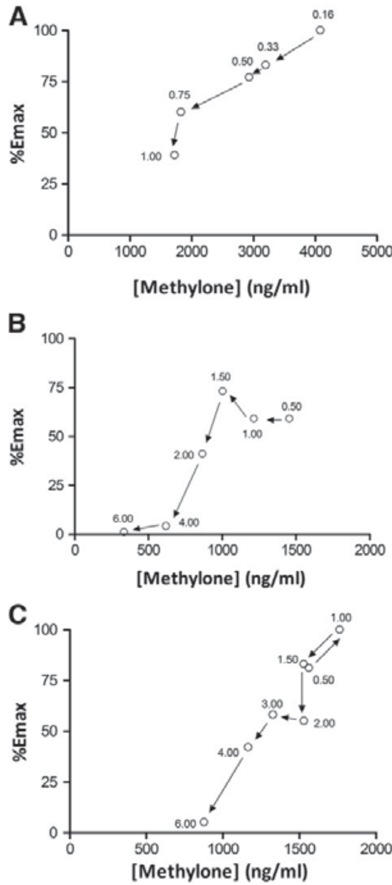

Fig. 5. Observed plasma concentrations of methylone *versus* observed %Emax in 10 min-block. Panel A, after intravenous administration (10 mg/kg). Panel B, after oral administration (15 mg/kg). Panel C, after oral administration (30 mg/kg). Data points show experimental time (in h) of pharmacokinetic and pharmacodynamic data.

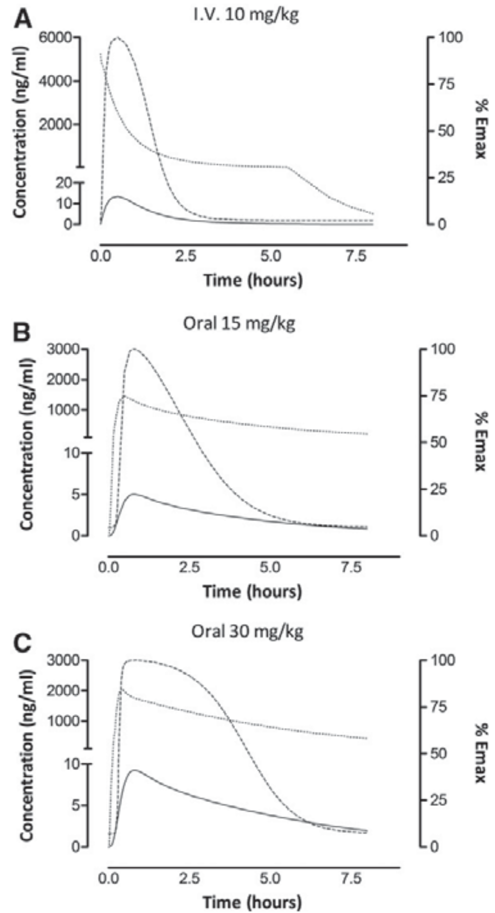

Fig. 6. Methylone plasma concentration profile (······), effect site-concentration curve (—) and predicted %Emax (— — — —) *versus* time. Panel A, after intravenous administration (10 mg/kg). Panel B, after oral administration (15 mg/kg). Panel C, after oral administration (30 mg/kg).

a pharmacokinetic flip-flop model. Flip-flop pharmacokinetics is a phenomenon often encountered with extravascular administered drugs (Yáñez et al., 2011). Flip-flop occurs when the rate of absorption is slower than the rate of elimination. When flip-flop is expected, a longer duration of sampling may be necessary. Accordingly, in some animals, we collected blood samples 24 h after drug administration. Our fitted model confirmed that a flip-flop phenomenon was taken place, since absorption rate ($t_{1/2abs}$ between 2–3 h) of methylone is considerably slower than its elimination rate ($t_{1/2\beta}$ of 0.55 h).

It is well recognized that compounds with a brain/plasma concentration ratio greater than 1 freely cross the blood–brain-barrier (Hitchcock and Pennington, 2006). Hence, the obtained brain/plasma ratio for methylone at Tmax of 1.42 demonstrates the access to central nervous system.

The rate of clearance and protein binding are acknowledged could cause discrepancy between *in vitro* and *in vivo* values (Halifax and Houston, 2012). In this study, methylone, at a plasma concentration near T max, binds to serum proteins at a low rate of about 30% and the metabolic clearance averages a 29% of total clearance. The calculated Clp for methylone averaged 0.5 l/h after both oral doses. Because it appeared to exceed both hepatic and renal blood flow in the rat (Birnie and Grayson, 1952; Heller and Hollyová, 1977), these *in vivo* data suggest that the liver, kidney, and possibly other clearing organs are involved in methylone elimination at these two oral doses.

We have investigated the *in vivo* Phase I metabolism of methylone after oral administration of a 30 mg/kg dose. We identified the existence of four metabolites in rat blood at three times after administration (60, 120 and 180 min) and we propose a first step phase I metabolism for methylone consisting of demethylation reaction,

Table 3
Estimates of the pharmacodynamic parameters, according to the proposed additive sigmoid E_{max} equation PKPD model. In parentheses the corresponding coefficient of variation (CV) in %.

Parameter	Units	Dose		
		10 mg/kg i.v.	15 mg/kg p.o.	30 mg/kg p.o.
E_0	Breaks	29.43 (112)	185.63 (28.20)	10.80 (*)
EC_{50}	ng/ml	6.68 (11.3)	1.32 (6.05)	0.90 (6.50)
E_{max}	Breaks	1280.86 (8.97)	1985.30 (8.90)	1733.84 (6.60)
N	–	3.13 (**)	5.23 (**)	4.96 (**)
O.F.		6.91	5.68	5.25
AIC		7.77	6.60	7.85

O.F.: Objective Function; AIC: Akaike information criterion; (*): CV > 150%; (**) CV not determined.

yielding the corresponding methylenedioxycathinone metabolite. Moreover, we propose that, in rats, methylone is a substrate of an aliphatic hydroxylation process resulting in the corresponding 3′-hydroxy-methylenedioxymethcathinone. Mueller and Rentsch (2012) proposed these methylone metabolites by an automated online metabolism method using human liver microsomes, but this is the first time that 3′-hydroxy-methylenedioxymethcathinone metabolite has been identified *in vivo*. The same authors found a third metabolite corresponding to the reduction of the beta-keto group which we did not detect in rat blood probably due to its low half-life.

We have also identified two hydroxylated metabolites (4-hydroxy-3-methoxymethcathinone and 3-hydroxy-4-methoxymethcathinone) with the same chemical formula and mass spectrum, resulting from a demethylenated intermediate that had lost the methylenedioxy group to the respective diol and which has not been found, probably due to its short half-life. Moreover, the amounts of these metabolites were very low. This is also in agreement with results from the same authors who found that over 80% of these two metabolites were conjugated when excreted through urine. Surprisingly, we have not found the corresponding demethyl compounds of the hydroxylated metabolites but these was also not found by Mueller and Rentsch (2012), which studied Phase I metabolites. The results indicate that the metabolism of methylone contributes significantly to its plasmatic clearance and this contribution is especially evident when the drug is administered orally, as a result of a hepatic first-pass effect, as is suggested by the differential pharmacokinetic profile observed in both routes of administration.

Pharmacodynamic experiments demonstrated that intravenous and oral administration of methylone induced a psychostimulant effect, measured as an increase in locomotor activity in rats. It is important to note that the increase in the locomotor activity elicited by oral administration of methylone is due mainly to a different time-course profile. The 30 mg/kg dose induced a maximum break response which was not significantly different to that of 15 mg/kg, but the psychostimulant effect lasted longer. This is in agreement with previous published results in mice (López-Arnau et al., 2012; Marusich et al., 2012).

The PK/PD relationship established in the study allows performing an estimation of the EC_{50} and Emax parameters, and provides information about the onset, magnitude and duration of the locomotor activity with relation to the time course of methylone plasma concentrations. An increase in locomotor activity was observed immediately after methylone administration in accordance with the immediate onset of its effects in humans (Shimizu et al., 2007). There is a delay between drug response and methylone plasma levels after oral administration. Thus, in presence of delay, the methylone plasma concentration profile may not be directly related to the pharmacological effect. When defining in the model an effect site-compartment, a close relationship between the effect and the obtained concentration curve becomes evident.

At the dose of 15 mg/kg, the plot of locomotor activity as a function of plasma methylone concentrations shows a counter-clockwise hysteresis loop. This hysteresis can be explained by the appearance of active metabolites (Mandema et al., 1992); by indirect mechanisms of drug action (Dayneka et al., 1993) or by an imbalance between the site of action (the brain) and the plasmatic compartment (Sheiner et al., 1979).

In this study the presence of some metabolites of methylone, similar to active metabolites of MDMA (De la Torre and Farré, 2004), strongly suggests their participation in the overall locomotor activity, but other possibilities cannot be discarded (Csajka and Verotta, 2006). Further studies with individual metabolites will determine which structural species have the highest likelihood of contributing to the locomotor activity caused by methylone.

Additionally, we have previously described that methylone inhibits monoamine uptake by competing with the substrate (López-Arnau et al., 2012). Consequently, it induces hyperlocomotion mainly by an indirect mechanism (increasing extra-cellular dopamine) and by also a direct mechanism through activation of 5-HT_{2A} receptors could be evidenced. The lower affinity of methylone for 5-HT_{2A} receptors than for dopamine transporter lead us to hypothesize that direct activation of this receptor type can contribute to the final effect when high concentrations in the effect-site compartment are achieved. Then, at the oral dose of 30 mg/kg, the lack of hysteresis can be explained by a) this additional direct mechanism of action or b) the low imbalance between brain and plasma concentrations at this dose.

The variability obtained in the pharmacodynamic estimates can be explained by the use of an E_{max} model without a clear maximum effect, seeing as it was obtained experimentally and taking into account that in this model maximum effect must be reached (Schoemaker et al., 1998). The experimental design followed in this study does not allow the assessment of the within and between variability of the PK/PD relationship and this drawback may contribute to the overall variability of the pharmacodynamic parameter estimates.

5. Conclusion

PK/PD analysis of methylone showed a correlation between plasma concentrations and enhancement of the locomotor activity. The identification of some metabolites of methylone, similar to active metabolites of MDMA strongly suggests their participation in the overall locomotor activity. We have previously described that methylone induces hyperlocomotion mainly by an indirect mechanism (increasing extra-cellular dopamine). At the highest oral dose assayed, direct activation of 5-HT_{2A} receptor seems also to contribute to the final psychostimulant effect. The present research provides, for a first time, useful information on the *in vivo* pharmacokinetics of methylone, and can help design new experiments in rodents with kinetics data as well as provide a better understanding of the effects of this cathinone in humans.

Contributors

EE and JC were responsible for the study concept and design. RLA, JMC. MC and DP assisted with data analysis. All authors critically reviewed content and approved final version for publication.

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3.3. Publicación III

Repeated doses of methylone, a new drug of abuse, induce changes in serotonin and dopamine systems in the mouse

Raúl López-Arnau, Jose Martínez-Clemente, Sònia Abad, David Pubill, Jorge Camarasa, Elena Escubedo (2014). *Psychopharmacology (Berl.)* 231: 3119-29

Resumen

El estado ilegal de los psicoestimulantes clásicos ha empujado a los usuarios de drogas de abuso a buscar nuevas drogas, disponibles a través de internet, como es el caso de la metilona, un derivado de la catinona. No sólo preocupa el aumento del consumo de metilona, sino también la falta de datos experimentales sobre la neurotoxicidad de este tipo de nuevas sustancias. Se sabe que la MDMA produce neurotoxicidad especie-dependiente (Logan et al., 1988), y existen razones para creer que la metilona, estructuralmente relacionada con la MDMA, podría mostrar efectos similares. Sin embargo, las evidencias existentes eran contradictorias, difiriendo según el modelo animal (rata/ratón), dosis y esquema de dosificación y tiempos a la cual se evaluaban. Por todo ello, el objetivo de este estudio fue examinar los cambios neuroquímicos, indicativos de neurotoxicidad, inducidos por la metilona en ratón.

Dosis repetidas de metilona produjeron hipertermia y una pérdida significativa de peso en ratones. Después del tratamiento A (25mg/kg s.c. x3, 3.5h intervalo, 2 días consecutivos) se observó un deterioro dopaminérgico y serotoninérgico transitorio. Tras la administración del tratamiento B (25mg/kg s.c. x4, 3h intervalo) también se observó un deterioro dopaminérgico transitorio y cambios a nivel serotoninérgico que perduraron hasta 7 días después del tratamiento. Además, tras el tratamiento B, observamos evidencias de una activación astroglial en la región de la CA1 y el giro dentado del hipocampo. Los ratones mostraron un aumento en el tiempo de inmovilidad en el test de natación forzada, indicándonos un posible estado depresivo, tras la administración de ambos tratamientos.

En conclusión, los efectos a nivel neuronal producidos por la administración repetida de metilona parecen diferir según el tipo de tratamiento utilizado. Estos cambios neuroquímicos son evidentes cuando la metilona es administrada 4 veces al día con un intervalo entre dosis de 3 horas, en concordancia con el corto tiempo de semivida que posee la metilona.

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ORIGINAL INVESTIGATION

Repeated doses of methylone, a new drug of abuse, induce changes in serotonin and dopamine systems in the mouse

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Abstract
Rationale Methylone, a new drug of abuse sold as "bath salts," has similar effects to ecstasy or cocaine.
Objective We have investigated changes in dopaminergic and serotoninergic markers, indicative of neuronal damage induced by methylone in the frontal cortex, hippocampus, and striatum of mice, according to two different treatment schedules.
Methods Methylone was given subcutaneously to male Swiss CD1 mice at an ambient temperature of 26 °C. Treatment A consisted of three doses of 25 mg/kg at 3.5-h intervals between doses for two consecutive days, and treatment B consisted of four doses of 25 mg/kg at 3-h intervals in 1 day.
Results Repeated methylone administration induced hyperthermia and a significant loss in body weight. Following treatment A, methylone induced transient dopaminergic (frontal cortex) and serotoninergic (hippocampus) impairment. Following treatment B, transient dopaminergic (frontal cortex) and serotonergic (frontal cortex and hippocampus) changes 7 days after treatment were found. We found evidence of astrogliosis in the CA1 and the dentate gyrus of the hippocampus following treatment B. The animals also showed an increase in immobility time in the forced swim test, pointing to a depressive-like behavior. In cultured cortical neurons, methylone (for 24 and 48 h) did not induce a remarkable cytotoxic effect.
Conclusions The neural effects of methylone differ depending upon the treatment schedule. Neurochemical changes elicited by methylone are apparent when administered at an elevated ambient temperature, four times per day at 3-h intervals, which is in accordance with its short half-life.

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Keywords Methylone · Neurotoxicity · Striatum · Frontal cortex · Hippocampus · Mice

Introduction

The illegal status of the classic psychostimulants (particularly MDMA and cocaine) has encouraged users to seek newer drugs that have become increasingly available through the Internet, which allows effective marketing, sale, and distribution and is the major reason for the increase in their availability (Brandt et al. 2010; McElrath and O'Neill 2011; Karila and Reynaud 2010).

Synthetic modifications of cathinone, structurally similar to amphetamine and extracted from the leaves of khat, have led to a number of so-called designer cathinone derivatives sold as "bath salts." The most commonly available cathinones appear to be mephedrone (4-methyl-methcathinone) and methylone (3,4-methylene-dioxymethcathinone) (Brunt et al. 2011). Methylone was first synthesized as an antidepressant but, around 2004, emerged as a recreational drug under the trade name "explosion" and was one of the first products of this nature to be marketed online (Bossong et al. 2005). It is taken by the oral or intranasal route.

Methylone shows a strong structural and pharmacological similarity to MDMA, but little is known about its particular pharmacology. Results from in vitro studies hypothesized that methylone acted similarly to *d*-amphetamine (Cozzi et al. 1999; Baumann et al. 2012), by binding to monoamine transporters (Nagai et al. 2007; Simmler et al. 2013). Recently, studies have been published on the pharmacological targets of cathinones by our group (Martínez-Clemente et al. 2012; López-Arnau et al. 2012) and others (Kehr et al. 2011;

Hadlock et al. 2011; Motbey et al. 2011) demonstrating that methylone acts on monoaminergic systems. Moreover, in vitro studies reveal that cathinone derivatives are nonselective substrates for monoamine transporters, which lead to reuptake blockage and enhancement of the release of monoamines by reversing the flow of the transporter, which results in elevated synaptic neurotransmitter levels (Sogawa et al. 2011; Baumann et al. 2012; Simmler et al. 2013; Eshleman et al. 2013). This is a critical point since only transporter substrates (and not uptake inhibitors) are capable of causing long-term deficits in monoamine cells (Fleckenstein et al. 2007).

Human data on methylone abuse are obtained from consumer reports (Shimizu et al. 2007; Boulanger-Gobeil et al. 2012). Online reports indicate that 100–250 mg in each intake is a common oral dose of methylone, and users evidenced a desire to redose, leading them to ingest large quantities of the drug. Fatal intoxications due to methylone have been described (Pearson et al. 2012) with elevated body temperature and symptoms similar to sympathomimetic toxicity, including metabolic acidosis, rhabdomyolysis, acute renal failure, and disseminated intravascular coagulation. The authors concluded that peripheral blood methylone concentrations greater than 0.5 mg/l may be lethal.

Not only is the rise in abuse of methylone of concern, but so is the lack of experimental data on the neurotoxicity of methylone in rodents. MDMA produces species-dependent neurotoxicity (Logan et al. 1988), and there is reason to suspect that methylone would display a similar effect. In keeping with this, some species differences in the sensitivity to long-term neurochemical effects of methylone have been shown. den Hollander et al. (2013) described no effects of methylone on serotonin (5-HT) levels in mice, but a widespread depletion of 5-HT and 5-HT transporter levels in rats was evidenced. Moreover, Baumann et al. (2012) found no effect of methylone on monoamine levels 2 weeks after treatment, also in rats, although they concluded that the effects of methylone and other cathinones should be evaluated in assays measuring 5-HT deficits, for example, with high-dose administrations.

The aim of this paper is to investigate methylone-induced neurochemical changes in mice that are indicative of neurotoxicity, addressing some of the limitations found in the literature on the subject to date. Any information that may lead to suspicion regarding the neurotoxicity or safety of methylone is critical. Experiments were carried out at a high ambient temperature simulating hot conditions found in dance clubs where amphetamine derivatives are usually consumed (Senn et al. 2007). We have evaluated the in vivo effect of this cathinone using different dosage schedules and in different brain areas assessed by decreases in the density of dopamine (DA) or 5-HT uptake sites and the enzyme that catalyzes the first and rate-limiting step in the biosynthesis of both neurotransmitters. Other outcome measures included drug-induced changes in body weight, core body temperature, depressive behavior, and glial activation. Although mice or rats do not provide an exact model of methylone-induced neurotoxicity for humans, the present study intends to broaden our understanding of the adverse effects of this cathinone derivative.

Materials and methods

Drugs and reagents

Racemic and pure methylone HCl was synthetized and characterized by us in our department's organic chemistry laboratory, under authorization from the University of Barcelona as described previously (López-Arnau et al. 2012). The rest of the drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]ketanserin, [^{3}H]paroxetine, and [^{3}H]WIN35428 were from PerkinElmer (Boston, MA, USA). All buffer reagents were of analytical grade.

Animals

The experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia, following the guidelines of the European Community Council (86/609/EEC). Male Swiss CD-1 mice (Charles River, Spain) weighing 25–30 g and aged 4–5 weeks were used. Animals were housed at 22±1 °C under a 12-h light/dark cycle with free access to food and drinking water. All the end points were taken in different animals.

In vivo neurotoxicity assays

The doses used in the present study were chosen according to available Internet information (www.erowid.org) and literature (den Hollander et al. 2013; Simmler et al. 2013) and were calculated following the FDA guidelines (Food and Drug Administration Center for Drug Evaluation and Research 2005). No information is available on subcutaneous doses in humans. A dose of 150–200 mg in a 60–70-kg human yields a 2–3-mg/kg dose equivalent to 25–35 mg/kg in mice.

Mice (8–12 animals per group) were treated with methylone applying a regimen of three subcutaneous doses of 25 mg/kg, with a 3.5-h interval between each injection for two consecutive days (treatment A; total daily dose, 75 mg/kg) or four subcutaneous injections of 25 mg/kg in 1 day with a 3-h interval between each injection (treatment B; total daily dose, 100 mg/kg). Another group also received saline (5 ml/kg). Rectal temperatures were measured using a lubricated, flexible rectal probe (1.5 cm) attached to a digital

thermometer (0331 Panlab, Barcelona, Spain). In preliminary studies, we found that maximum hyperthermia was achieved 45 min after administration. However, in order to reduce animal stress, we chose to record body temperature after the second dose of each day's treatment. Mice were lightly restrained by hand during the procedure, with a steady readout of temperature obtained approximately 40 s after the probe insertion. During the treatments, the animals were maintained in an ambient temperature of 26±2ºC and kept under these conditions until 1 h after the last daily dose.

Tissue sample preparation

Crude membrane preparations were prepared as described elsewhere (Escubedo et al. 2005) with minor modifications. Mice were killed by cervical dislocation at 3 or 7 days post-treatment, and the brains were rapidly removed from the skull. The hippocampus, striatum, and frontal or parietal cortex were quickly dissected out, frozen on dry ice, and stored at −80 °C until use. When required, tissue samples were thawed and homogenized at 4 °C in 20 volumes of buffer consisting of 5 mM Tris–HCl, 320 mM sucrose, and protease inhibitors (aprotinin 4.5 μg/μl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), pH 7.4. The homogenates were centrifuged at 1,000×*g* for 15 min at 4 °C. Aliquots of the resulting supernatants were stored at −80 °C until use for Western blot experiments. The rest of the samples were resuspended and centrifuged at 15,000×*g* for 30 min at 4 °C. The pellets were resuspended in buffer and incubated at 37 °C for 5 min to remove endogenous neurotransmitters. The protein samples were then recentrifuged and washed two more times. The final pellets (crude membrane preparation) were resuspended in the appropriate buffer and stored at −80 °C until use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent.

DA and 5-HT transporter densities

The density of DA transporters in striatal or frontal cortex membranes was measured by [^{3}H]WIN35428 binding assays. Assays were performed in glass tubes containing 250 or 500 μl of [^{3}H]WIN35428 diluted in phosphate-buffered 0.32 M sucrose (final radioligand concentration, 5 nM) and 50 or 100 μg of membranes, respectively. Incubation was done for 2 h at 4 °C, and nonspecific binding was determined in the presence of 30 μM bupropion. All incubations were finished by rapid filtration under vacuum through GF-B glass fiber filters (Whatman, Maidstone, UK) pre-soaked in 0.5 % polyethyleneimine. Tubes and filters were washed rapidly three times with 4 ml of ice-cold buffer, and the radioactivity in the filters was measured by liquid scintillation spectrometry.

The density of 5-HT transporters in the hippocampal and frontal cortex membranes was quantified by measuring the specific binding of 0.05 nM [^{3}H]paroxetine after incubation with 150 μg of protein at 25 °C for 2 h in a Tris–HCl buffer (50 mM, pH 7.4), containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 μM) was used to determine nonspecific binding.

5-HT$_{2A}$ receptor density

The density of 5-HT$_{2A}$ receptors in mice parietal or frontal cortex membranes was measured by [^{3}H]ketanserin binding assays. Membranes were resuspended in 50 mM Tris–HCl buffer, pH 7.4 at 4 °C to a concentration of 1 μg/μl. Assays were performed in glass tubes containing 1 nM [^{3}H]ketanserin and 100 μg of membranes. Incubation was carried out at 37 °C for 30 min in a 50 mM Tris–HCl buffer to a final volume of 0.5 ml. Methysergide (10 μM) was used to determine nonspecific binding.

Western blotting and immunodetection

A general Western blotting and immunodetection protocol was used to determine tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2) levels. For each sample, 20 μg of protein was mixed with sample buffer [0.5 M Tris–HCl, pH 6.8, 10 % glycerol, 2 % (*w*/*v*) SDS, 5 % (*v*/*v*) 2-β-mercaptoethanol, 0.05 % bromophenol blue, final concentrations], boiled for 5 min, and loaded onto a 10 % acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P; Millipore, USA). PVDF membranes were blocked overnight with 5 % defatted milk in Tris-buffered saline buffer plus 0.05 % Tween-20 and incubated for 2 h at room temperature with a primary mouse monoclonal antibody against TH (Transduction Laboratories, Lexington, KY, USA) diluted 1:5,000 or with a primary rabbit polyclonal antibody against TPH2 (Millipore, USA) diluted 1:1,000. After washing, membranes were incubated with a peroxidase-conjugated antimouse IgG antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:2,500 or with a peroxidase-conjugated antirabbit IgG antibody (GE Healthcare) diluted 1:5,000. Immunoreactive protein was visualized using a chemiluminescence-based detection kit following the manufacturer's protocol (Immobilon Western, Millipore, USA) and a Bio-Rad ChemiDoc XRS gel documentation system (Bio-Rad Labs., Hercules, CA, USA). Scanned blots were analyzed using Bio-Rad Image Lab software, and dot densities were expressed as a percentage of those taken from the control. Immunodetection of β-actin (mouse monoclonal antibody, dil. 1:2,500) served as a control of load uniformity for each lane and was used to normalize differences in TH or TPH2 expression due to protein content.

Immunohistochemistry

Seven days after treatment B, animals were anesthetized with pentobarbital sodium (60 mg/kg) and perfused through the heart with 4 % paraformaldehyde in 0.1 M phosphate buffer (1 ml/g of body weight). Brains were removed and postfixed for 2 h in the same solution, cryoprotected by immersion in 30 % sucrose/phosphate buffer solution for 24 h, and frozen in dry ice-cooled isopentane. Serial coronal sections (30 μm thick) through the whole brain were cut in a cryostat and collected in phosphate buffer solution. Free-floating coronal sections were incubated for 15 min at room temperature in H_2O_2 (0.3 % in phosphate buffer with 10 % methanol). Thereafter, sections were incubated in a blocking solution (1 % of fetal bovine serum and 0.2 M glycine plus 0.5 % Triton X-100 in phosphate buffer). After blocking with 10 % normal serum and 0.2 % bovine serum albumin, sections were rinsed and incubated overnight at 4 °C using a monoclonal antibody against glial fibrillary acidic protein (GFAP, 1:1,000) (Dako, Denmark). Following this, sections were washed and incubated with a biotinylated secondary antibody (1:200, Sigma-Aldrich) for 2 h at room temperature. Afterwards, sections were incubated with avidin–biotin–peroxidase complex (ABC; 1:200; Vector, Burlingame, CA). A peroxidase reaction was developed with 0.05 % diaminobenzidine in 0.1 M phosphate buffer and 0.02 % H_2O_2, and immunoreacted sections were mounted on gelatinized slides. Stained sections were examined under a light microscope (Olympus BX61).

Neuronal cell cultures

Primary neuronal cultures of the cerebral cortex were obtained from mouse embryos (E-16-18). The cerebral cortex was dissected, meninges were removed, and tissue was incubated for 20 min in trypsin (0.05 %) at 37 °C. Trypsin was inactivated with fetal bovine serum, and tissue was triturated with a fire-polished Pasteur pipette. Dissociate cells were washed with phosphate buffer containing 0.6 % glucose and centrifuged at 500×*g* for 5 min to remove debris. The cells were redissociated in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) with 0.5 mM L-glutamine, sodium bicarbonate (0.04 %), and 1 μg/ml penicillin and streptomycin, containing B27 supplement and 10 % horse serum. Neurons were plated at 0.4 million cells/ml in 96-well plates precoated with 1 mg/ml poly-L-lysine. Cultures were maintained at 37 °C in a humidified incubator with 5 % CO_2 and 95 % air. Twenty-four hours later, cells were treated with arabinosylcytosine (10 μM) to prevent the growth of glial cells. The culture medium was changed every 3 days to Neurobasal medium with B27 without antioxidants, and the concentration of horse serum was reduced gradually down to 1 %. The cultures were used for experiments after 8–9 days in vitro with different concentrations of methylone (80–1,000 μM) and different times of drug exposure (24 and 48 h).

Cell viability assay

Cell viability was assessed by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide) assay. MTT was added to the cells to a final concentration of 250 μM and incubated for 2 h (Hansen et al. 1989). The media were removed, and cells were dissolved in dimethyl sulfoxide. Formation of formazan was tested by measuring the amount of reaction product by absorbance change (595 nm) using a microplate reader (Bio-Rad Laboratories, CA, USA). Viability results were expressed as a percentage of the absorbance measured in untreated cells.

Forced swimming test

The immobility time in the forced swimming test (FST) was measured by an observer blind to the treatment using the procedure described by Porsolt et al. (1978). Briefly, mice were placed individually in a glass cylinder (height 21 cm, diameter 12 cm) containing water at 25±1 °C up to a height of 15 cm. Mice do not try to dive or explore the water surface, which explains the ease in use of mice that do not need a previous session (Petit-Demouliere et al. 2005). Animals were randomly divided into two groups (12–16 animals per group), treated with saline or methylone, and tested 3 or 7 days after treatment. Each animal was recorded for 6 min, and the total period of immobility, in seconds, was measured. A mouse was judged to be immobile when it remained floating in water, making only the necessary movements to keep its head above water. Each mouse was only tested once (Calapai et al. 2001). Increase in the duration of immobility was considered to reflect a depressant-like effect of the drug.

Statistical analysis

All data are expressed as mean±standard error of the mean (S.E.M.). Differences between groups were compared using one-way ANOVA or Student's *t* test for independent samples where appropriate. Significant ($P<0.05$) differences were then analyzed by Tukey's post hoc test for multiple mean comparisons where appropriate. All statistic calculations were performed using GraphPad Instat (GraphPad Software, San Diego, USA).

Results

Lethality

Initial experiments were carried out with four to six animals per cage (48×25×13 cm). Under these conditions, lethality was approximately at 75 % level. Given that, all the treatments carried out in this study were performed with a single animal per cage (35×14×13 cm) housed 4 days before dosing.

The number of fatalities of methylone-treated mice was similar in treatment A (of about 25 %) and treatment B (of about 20 %) and occurred 1–2 h after the third or the fourth dose. To obtain an accurate cause of death, a veterinarian necropsy was performed. Immediately after the animals' death, the veterinarian did an overall examination of some of the animals and looked at individual organs within the body. Final diagnostics evidenced hepatomegaly and acute hemorrhagic pericarditis as the cause of death.

Effect of methylone on body temperature and body weight

Methylone induced a significant increase in the body temperature in treatment A and treatment B (Fig. 1a). Since the hyperthermia induced by methylone at the second day of treatment A was significantly lower than that induced at the first day, tolerance to hyperthermic effects of methylone can be concluded.

In both treatments, methylone produced significant loss in body weight (Fig. 1b). At the end of treatment A, methylone-treated animals showed an overall decrease in body weight of −0.60±0.16 g.

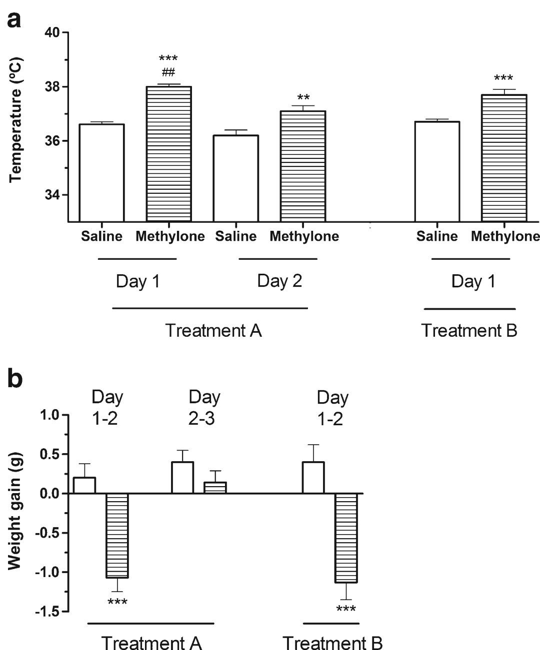

Fig. 1 Effect of methylone treatments *A* and *B* in body temperature measured after the second dose of each day's treatment (**a**) and in body weight (**b**; *empty bars* represent saline, and *striped bars* represent methylone). Results are expressed as mean±S.E.M. of 8–10 animals. **$P<0.01$ and ***$P<0.001$ vs. saline. ##$P<0.01$ vs. methylone treatment A day 2

Effect of methylone on different in vivo markers of DA and 5-HT terminals

In treatment A, methylone-treated mice showed a transient decrease in [^{3}H]WIN35428 specific binding in the frontal cortex that resumed a normal level four days later (Fig. 2a). This decrease was not accompanied by a change in TH expression (3 days: saline, 100.00±22.60 %; methylone, 110.06±12.70 %; 7 days: saline, 100.00±11.72 %; methylone, 116.00±9.49 %).

In contrast, in the striatum, methylone neither affected [^{3}H]WIN35428 binding (saline, 100.00±10.27 %; methylone, 94.38±8.20 %) nor TH expression (saline, 100.00±11.10 %; methylone, 82.40±4.20 %), even after 3 days following the end of exposure. Thus, no further determinations were performed at 7 days.

With regard to 5-HT transporters, methylone did not modify [^{3}H]paroxetine binding in the frontal cortex (Fig. 2b). However, in the hippocampus, methylone induced a slight reduction in [^{3}H]paroxetine binding, measured 3 days after treatment, which was reverted 4 days later (Fig. 2c). The levels of THP2 remained unchanged after the treatment (saline, 100.00±3.80 %; methylone, 83.20±5.70 %, $P>0.05$).

In treatment B, as above, methylone treatment induced a transient decrease in the specific binding of [^{3}H]WIN35428 of about 27 % in the frontal cortex that returned to control values 4 days later (Fig. 3a). Similarly, no changes in TH expression were evidenced (3 days: saline, 100.00±24.47 %; methylone, 94.75±15.47 %; 7 days: saline, 100.00±10.52 %; methylone, 97.43±29.88 %). As in treatment A, methylone did not affect any of these dopaminergic markers in the striatum ([^{3}H]WIN35428 binding: saline, 100.00±7.57 %; methylone, 93.91±4.26 %; TH expression: saline, 100.00±3.53 %; methylone, 97.06±4.70 %; 3 days after drug exposure).

As regards serotonergic markers, at 3 and 7 days post-treatment, methylone induced a diminution of 5-HT reuptake sites of about 30–20 % in the frontal cortex and 20–12 % in the hippocampus, respectively (Fig. 3b, c). Additionally, 7 days after treatment, TPH2-immunoreactivity levels were decreased in both brain areas in the methylone-treated mice that correlates with the diminution of 5-HT reuptake sites (Fig. 4a, b).

Effect of methylone on 5-HT$_{2A}$ receptor density

According to the treatment A schedule, methylone-treated animals showed a decrease in the number of 5-HT$_{2A}$ receptors in the frontal (saline, 100.00±4.80 %; methylone, 81.69±2.65 %, $P<0.01$) and parietal cortex (saline, 100.00±4.75 %; methylone, 80.60±5.35 %, $P<0.05$), measured as [^{3}H]ketanserin binding, 3 days after treatment, which returned to basal values 4 days later (105.26±10.23 and 119.13±8.49 %, respectively), pointing to a homeostatic process. In

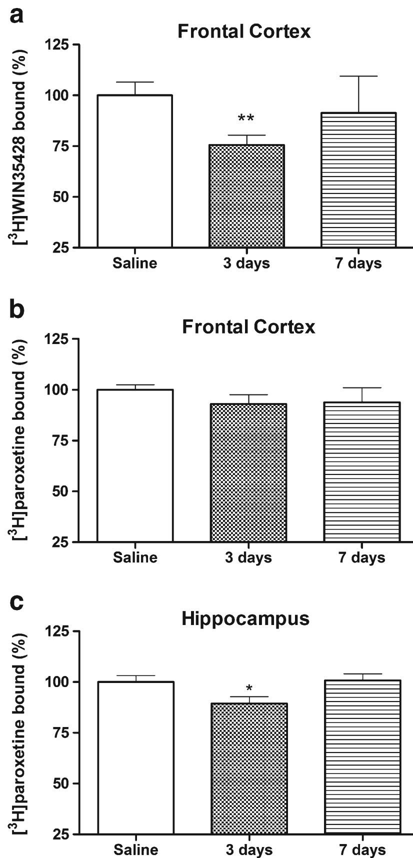

Fig. 2 Effect of methylone treatment (three doses of 25 mg/kg, sc at 3.5-h interval for 2 days) in dopamine transporter density, measured as [^{3}H]WIN35428 binding in the mouse frontal cortex (**a**), and serotonin transporter density, measured as [^{3}H]paroxetine binding in the frontal cortex (**b**) and hippocampus (**c**). Results are expressed as mean±S.E.M. of 8–10 animals. *P<0.05 and **P<0.01 vs. saline

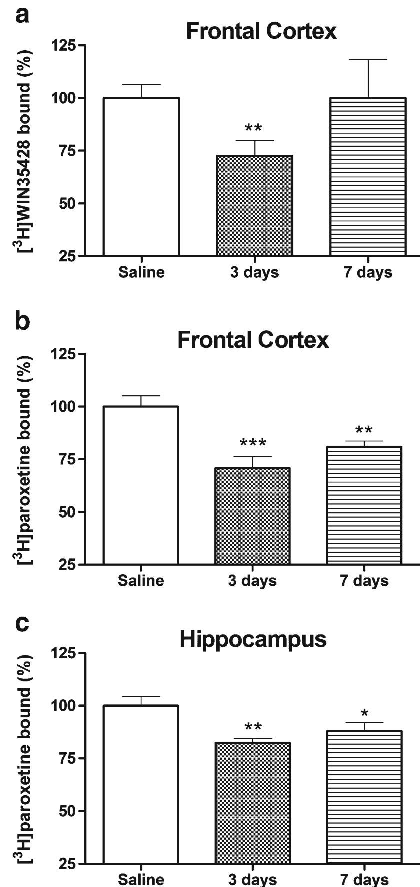

Fig. 3 Effect of methylone treatment (four doses of 25 mg/kg, sc at 3-h interval) in dopamine transporter density, measured as [^{3}H]WIN35428 binding in the mouse frontal cortex (**a**), and serotonin transporter density, measured as [^{3}H]paroxetine binding in the frontal cortex (**b**) and hippocampus (**c**). Results are expressed as mean±S.E.M. of 8–10 animals. *P<0.05; **P<0.01, and ***P<0.001 vs. saline

contrast, following schedule B, methylone did not modify the density of 5-HT$_{2A}$ receptors in the two cortical areas either at 3 (frontal cortex, 85.79±5.40 %; parietal cortex, 96.40±3.58 %) or 7 days after drug exposure (frontal cortex, 111.23±12.96 %; parietal cortex, 113.16±3.45 %).

Effect of methylone on astroglial activation

Because methylone induced neuronal damage 7 days after treatment B schedule, the next experiment was carried out to assess the presence of astroglial activation. Accordingly, immunohistochemistry studies were performed with the glia-specific marker GFAP in brains of animals killed 7 days after the treatment. There were no signs of striatal or cortical astroglial activation in methylone-treated animals. However, in the hippocampus, an increase in GFAP immunoreactivity was observed in the CA1 and dentate gyrus of the methylone group, compared with that from saline-tested mice. This suggests the presence of a slight reactive astrocytosis (Fig. 5).

Effect of methylone on cultured cortical neuron viability

The exposure of cultured cortical mouse cells to various concentrations of methylone (from 80 μM to 1 mM) for 24 or 48 h caused a weak concentration- or time-dependent decrease in metabolically active cells, as assessed by the MTT assay (Fig. 6). Cell viability was only affected by methylone concentrations above 300 μM, and the corresponding calculated LD$_{50}$ value for methylone after 24 or 48 h of exposure was over 1 mM, ruling out cell toxicity in cortical neurons.

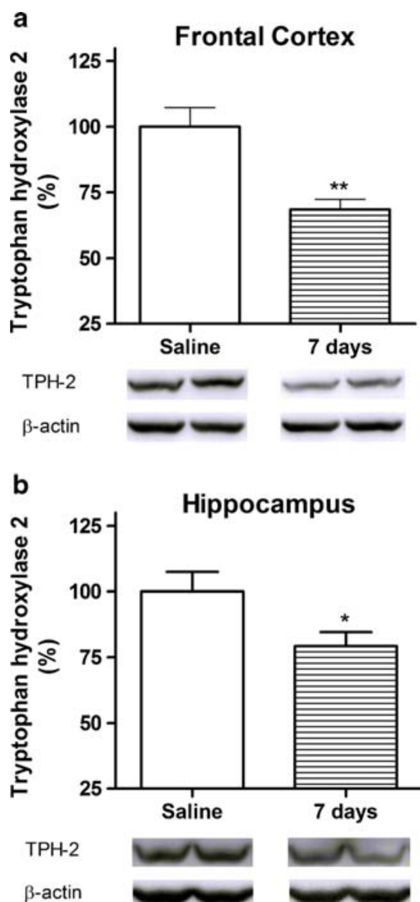

Fig. 4 Effect of methylone treatment (four doses of 25 mg/kg, sc at 3-h interval) on tryptophan hydroxylase 2 expression in the mouse frontal cortex (**a**) and hippocampus (**b**) 7 days after treatment. Below each *bar graph*, representative Western blots of TPH-2 expression in the frontal cortex and hippocampus, respectively. $^{**}P<0.01$ and $^{*}P<0.05$ vs. saline

Depressant-like effect of methylone

Methylone administration (treatments A and B) increased the immobility time in the FST 3 days posttreatment as compared with the saline group. This time period coincided with the observed impairment of both dopaminergic and serotonergic markers. These effects vanished in both schedules 1 week following drug exposure, when only the serotonergic markers remained decreased (Fig. 7).

Discussion

There is little information regarding methylone and its potential toxicity. The initial status of cathinones as legal highs may have contributed to their increasing popularity as drugs of abuse. Because of the relatively short history of the use of cathinones as recreational drugs, their long-term effects have not yet been determined.

Very few studies exist, even in rodents, on the dosing schedules or doses required to induce damage (Baumann et al. 2012; den Hollander et al. 2013). Therefore, the primary goal of this study was to evaluate the risk of neuronal changes linked to methylone abuse in mice. Methylone is a close structural analogue of MDMA, differing only by the addition of a β-ketone group. Consequently, it is also known as beta-keto-MDMA. As with MDMA, it might affect the DA or 5-HT system differently, depending on the animal species used for the experiment. Most authors described the maximum neurotoxic effects of methamphetamine 3 days after treatment (Pu and Vorhees 1993) and those of MDMA 7 days after treatment (Battaglia et al. 1988). Thus, we examined the neurotoxic injury induced by methylone at 3 and 7 days after following the end of the treatment. In addition, a close relationship was already established between the hyperthermic response and the severity of the brain lesion induced by amphetamines (Sánchez et al. 2004), supporting the hypothesis that MDMA is neurotoxic when a binge dosing schedule is employed and the animals are in a hot environment. Accordingly, present experiments were carried out at a high ambient temperature simulating hot conditions found in dance clubs. We administered the drug at 3–3.5-h intervals, in accordance with our previous paper characterizing the pharmacokinetics of methylone, distinguished by its short half-life (López-Arnau et al. 2013). To model recreational methylone use, we simulated the widespread practices of “stacking” (taking multiple doses at once in order to increase the desired effect and/or offset tolerance from prior use) and “boosting” (taking supplemental doses over time in order to maintain the drug’s effect). Thus, we chose to administer multiple doses/day of methylone during each treatment.

Overall, our data demonstrate a slight serotonergic toxicity of methylone, 1 week after treatment, only when four doses are administered in a day. This toxicity is substantiated by decreases in density of the 5-HT terminal marker and reduction in TPH2 expression, both more apparent in the frontal cortex than in the hippocampus. Postmortem necropsies showed evident signs of hepatomegaly and hemorrhagic pericarditis that could be the cause of death. Further pathology studies are needed to affirm that these signs resulted directly from methylone toxicity, but methylone has been described as a cause of cardiac arrest leading to human death (Cawrse et al. 2012).

Our initial experiments indicated that methylone toxicity is exacerbated in group-housed animals, as with other amphetamines (Fantigrossi et al. 2003). Baumann et al. (2013) already described that, in humans, adverse effects of bath salts could be intensified in hot crowded spaces, such as rave party

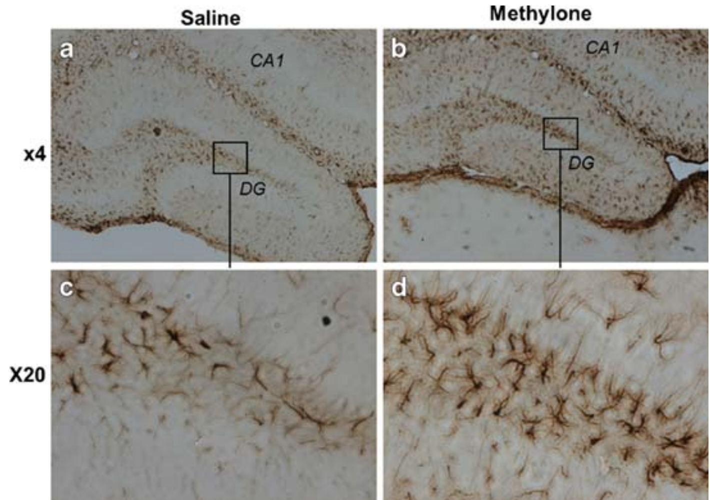

Fig. 5 Representative hippocampal expression of glial fibrillary acidic protein (GFAP). Sections of the dentate gyrus from mice treated with saline (**a**, **c**) or methylone (four doses of 25 mg/kg, sc 3-h interval) (**b**, **d**). The animals were sacrificed 7 days after treatment

venues where these drugs are often used. Present experiments were performed in singly housed mice. We have studied the evolution of body weight during methylone treatments. Like other amphetamine derivatives such as MDMA, the animals treated with methylone, but not with saline, showed weight loss, probably due to an anorectic effect of the drug.

Several factors, particularly hyperthermia, contribute to MDMA-induced neurotoxicity. In this regard, the influence of ambient temperature on MDMA-induced thermal responses has been shown in earlier studies that noted a hyperthermic response when experiments were carried out at high ambient temperatures (26–28 °C). Hyperthermia is, in fact, a commonly reported acute adverse effect of beta-ketoamphetamine ingestion in humans (Borek and Holstege 2012; Prosser and Nelson 2012). In the present study, experiments were performed at the ambient temperature of 26±2 °C. Under these conditions, methylone induced hyperthermia. This effect was more

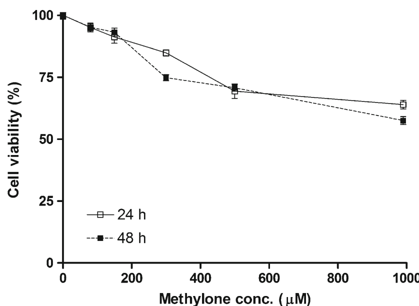

Fig. 6 Effect in cell viability of methylone on mouse cortical cultured neurons. Cells were exposed to different concentrations of methylone for 24 or 48 h, and cell viability was assessed by the MTT assay. Data are expressed as mean±S.E.M. from three different cultures

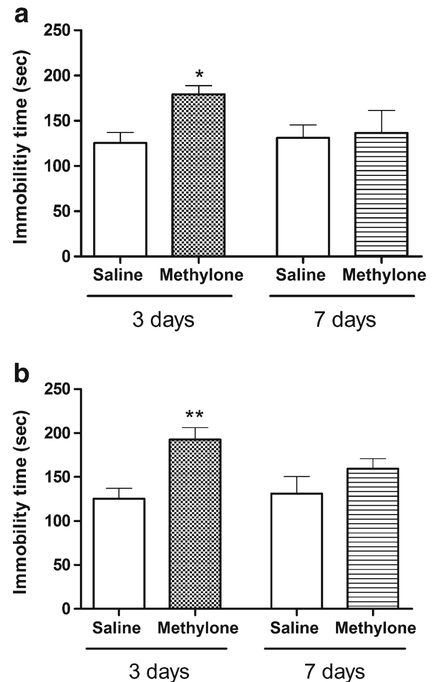

Fig. 7 Effect of methylone on immobility time in the mouse forced swim test. Animals (12–16 animals/group) were randomly divided and treated subcutaneously with saline (5 ml/kg) or methylone (three doses of 25 mg/kg, sc at 3.5-h interval for 2 days (**a**) or four doses of 25 mg/kg, sc 3-h interval (**b**)) and tested 3 or 7 days after treatment. Each animal was recorded for 6 min, and the total period of immobility was registered. Each mouse was used only once for each experimental session. Each *bar* represents mean±S.E.M. immobility time in seconds. $^{**}P<0.01$ and $^{*}P<0.05$ as compared with respective saline-treated group (one-way ANOVA and post hoc Tukey's test)

apparent on the first day of treatment and diminished when the drug was administered on the second day, indicating the possibility of tolerance that could be due to a depletion of neurotransmitter stores.

Following the two treatments performed in this study, methylone induced a transient loss of the DA transporter in the frontal cortex. The initial decline and later recovery of DA transporter points to a biochemical downregulation in the absence of tissue damage, but we can also assume that a methylone-induced dopamine transporter structural modification could occur, explaining the reduction in binding experiment. This hypothesis is in agreement with our previous results, which demonstrate that methylone inhibits [^{3}H]DA uptake after drug withdrawal, pointing to alterations in the transporter that are more complex than a simple blocking of the carrier (López-Arnau et al. 2013). Unlike MDMA (Chipana et al. 2006), methylone did not alter DA transporter radioligand binding or TH levels in the striatum in any of the performed treatments. The main difference between treatments was found in 5-HT terminal markers. When exposure to methylone was performed over two consecutive days with three doses per day, we registered a transient reduction of these markers, but when treatment consisted of four doses in a single day, a more persistent effect appeared, affecting the frontal cortex and hippocampus. The final reduction in the 5-HT transporter marker correlates with the decrease in TPH2 expression. Astrocytes stabilize and maintain homeostatic tissue repair and contribute to early wound repair (Eddleston and Mucke 1993). In the present study, methylone-treated animals with schedule B showed an increase in GFAP immunoreactivity in hippocampal CA1 and dentate gyrus that corresponds to real terminal injury in these areas.

Only one study has been published concerning the neurotoxic effect of methylone in the DA and 5-HT systems in mice (den Hollander et al. 2013). Authors demonstrated that methylone exposure (30 mg/kg, twice daily for 4 days) had no effect on neurotransmitter levels in C57BL/J6 mice 2 weeks after treatment. Our results demonstrate that methylone neurotoxicity in mice depends on the number of doses and intervals between each dose, as occurs with other cathinones. Furthermore, we suspect that the high room temperature, used in this study, could play an important role in methylone-induced neurotoxicity if we compare our findings with those of den Hollander et al. (2013). Nonetheless, further research is necessary in order to assess whether the role of hyperthermia and room temperature is complementary or essential in the advent of methylone-induced neurotoxicity. Some differences among cathinone neurotoxicity literature can be partially due to differences in the employed dosing regimen and the time of sacrifice. However, due to the mechanism of action and structural similarity between methylone and MDMA, we used similar doses and time of sacrifice assessed in MDMA neurotoxicity studies (Chipana et al. 2006; Granado et al. 2008; Sánchez et al. 2003; Mueller et al. 2013).

Mice differ from other animal species because they display deficits in DA neurotransmission greater than 5-HT after binge MDMA exposure. The present results demonstrated that methylone acts contrarily. The methylone neurochemical profile could be explained by the fact that this drug acts preferentially as an inhibitor for the 5-HT transporter than for the DA transporter (Baumann et al. 2012; López-Arnau et al. 2012; Sogawa et al. 2011), implying a better access of this drug to the 5-HT nerve terminals leading to the corresponding injury. Moreover, a well-recognized hypothesis of MDMA neurotoxicity involves some metabolite participation that has not been demonstrated for methylone.

Methylone increased the immobility time in the FST following both treatments, which indicates an increase in stress-related depressive behavior. This effect was evidenced 3 days after treatment and correlates with the reduction of DA and 5-HT markers assayed. This is in accordance with results from McGregor et al. (2003), who demonstrated that MDMA-treated animals show a higher immobility and fewer active escape attempts in the forced swimming model. To our knowledge, the present studies provide the first preclinical data to shed light on this issue, suggesting that mice exposed to a stacking and boosting regime of methylone could be more prone to suffering from depressive-like symptoms. This effect disappeared 7 days after treatment, when only serotonergic neurotransmission remained impaired. It must be noted that depression pathophysiology may also involve changes in the 5-HT$_2$ receptors in brain regions selectively implicated in mood regulation. In this regard, in treatment A, we found a significant transient decrease in the number of cortical 5-HT$_{2A}$ receptors 3 days after administration, possibly resulting from a neuroadaptive response to the massive 5-HT release induced by methylone. These results hark back to those published by Scheffel et al. (1992) regarding MDMA. In treatment B, we detected a similar but nonsignificant reduction in the frontal cortex.

The impairment induced by methylone on 5-HT and DA terminals is limited to the frontal cortex and hippocampus when exposure is clustered in four doses in a day. This mild cathinone neurotoxicity correlates with results of our in vitro studies in cortical cultured cells, where we describe that methylone did not show concentration- and time-dependent deleterious effects on neuronal viability. The data reveal that doses up to 1,000 μM for 24 to 48 h do not appreciably affect cell viability. This is a remarkable finding, which confirms previous studies that found methylone alone is not cytotoxic even at high doses (Nakagawa et al. 2009; Sogawa et al. 2011). In this regard, other studies assessing the effects of MDMA on cortical or hippocampal cultured cell viability reported no or low cell death following exposure to high MDMA concentrations (Capela et al. 2006).

In conclusion, our results demonstrate that methylone-induced brain consequences differ according to treatment schedule (dose, number of doses, and dose interval). Neurochemical changes elicited by methylone are apparent when administered at an elevated ambient temperature, four times per day at 3-h intervals. This schedule is related with patterns used by humans and agrees with methylone's half-life in rodents (López-Arnau et al. 2013). Following this, we found a decrease in frontal cortex and hippocampal serotoninergic nerve ending markers around 20–25 % together with hippocampal astrogliosis suggesting nerve ending injuries. No effect in the striatum was evidenced. Methylone did not show a cytotoxic effect in cortical cultured neurons. The limited neurotoxicity found in this study, however, should not preclude advice concerning the high risk of acute fatal effects affecting the cardiovascular system and thermoregulation.

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Conflict of interest The authors declare that they have no financial or commercial conflicts of interest.

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3.4. Publicación IV

Serotonergic impairment and memory deficits in adolescent rats after binge exposure of methylone

Raúl López-Arnau, Jose Martínez-Clemente, David Pubill, Elena Escubedo, Jorge Camarasa (2014). *Journal of Psychopharmacology* DOI: 10.1177/0269881114548439

Resumen

La 3,4-metilendioximetcatinona (metilona) es un derivado de la catinona que recientemente ha aparecido como droga de diseño en Europa y los Estados Unidos, vendida a través de internet o "smart shops" bajo la denominación de "legal high" o "bath salts". La MDMA induce toxicidad sobre el sistema serotoninérgico tanto en ratas como en humanos, mientras que ésta afecta principalmente al sistema dopaminérgico en ratón (Logan et al., 1988; Reneman et al., 2001). A causa de la similitud estructural con la MDMA, hay razones para creer que la metilona podría mostrar una neurotoxicidad dependiente de la especie. En la publicación **III** describimos alteraciones neuronales en los ratones tratados con metilona dependiendo del tipo de tratamiento administrado, pero era necesario estudiar si estos cambios también ocurrían en rata utilizando un régimen de administración similar.

El tratamiento con metilona (20mg/kg s.c. x4, 3h intervalo) produjo un efecto hipertérmico, el cual fue aumentando tras cada administración. Además se observó una disfunción serotoninérgica 7 días después del tratamiento, producida por una disminución tanto del número de lugares de recaptación como de la enzima limitante en la biosíntesis de serotonina (5-HT), especialmente en la corteza frontal, donde también se observó una activación astroglial. También se observaron alteraciones a nivel serotoninérgico en el hipocampo y estriado de rata. Sin embargo, no se evidenció un efecto neurotóxico a nivel dopaminérgico, como tampoco una activación microglial en ninguna de las áreas cerebrales estudiadas. Además, los animales tratados con metilona mostraron, 7 días después del tratamiento, una alteración cognitiva en el test del laberinto acuático de Morris.

En conclusión, nuestros resultados han demostrado que la metilona induce en la rata, a una temperatura ambiente elevada, una pérdida de marcadores neuronales serotoninérgicos a nivel del estriado, hipocampo y corteza frontal, juntamente con una activación astroglial, indicando la posible lesión de los terminales neuronales. La disfunción producida por el tratamiento con metilona sobre la memoria espacial podría ser consecuencia de los cambios neuroquímicos observados.

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Serotonergic impairment and memory deficits in adolescent rats after binge exposure of methylone

Raúl López-Arnau, José Martínez-Clemente, David Pubill, Elena Escubedo and Jorge Camarasa

Abstract

Methylone is a cathinone derivative that has recently emerged as a designer drug of abuse in Europe and the USA. Studies on the acute and long-term neurotoxicity of cathinones are starting to be conducted. We investigated the neurochemical/enzymatic changes indicative of neurotoxicity after methylone administration (4 × 20 mg/kg, subcutaneously, per day with 3 h intervals) to adolescent rats, to model human recreational use. In addition, we studied the effect of methylone on spatial learning and memory using the Morris water maze paradigm. Our experiments were carried out at a high ambient temperature to simulate the hot conditions found in dance clubs where the drug is consumed. We observed a hyperthermic response to methylone that reached a peak 30 min after each dose. We determined a serotonergic impairment in methylone-treated rats, especially in the frontal cortex, where it was accompanied by astrogliosis. Some serotonergic alterations were also present in the hippocampus and striatum. No significant neurotoxic effect on the dopaminergic system was identified. Methylone-treated animals only displayed impairments in the probe trial of the Morris water maze, which concerns reference memory, while the spatial learning process seemed to be preserved.

Keywords

Methylone, neurotoxicity, striatum, frontal cortex, hippocampus, rat

Introduction

Methylenedioxymethcathinone (methylone), together with other substances such as methylenedioxypyrovalerone and 4-methylmethcathinone (mephedrone), is a derivative of cathinone, a naturally occurring stimulant found in the leaves of the khat plant. These compounds have recently emerged as designer drugs of abuse in Europe and the USA (McElrath and O'Neill, 2011; Spiller et al., 2011; Vardakou et al., 2011) and are marketed under deceptively benign names, including the term 'bath salts', and sold over the internet and in head shops worldwide (Davies et al., 2010). Synthetic cathinones are also phenylalkylamine derivatives often termed 'bk-amphetamines' for the beta-ketone moiety. Methylone was first synthesized as an antidepressant, but it emerged as a recreational drug in about 2004 under the trade name 'Explosion' and was one of the first products of this type to be marketed on-line (Bossong et al., 2005). In April 2013, the Drug Enforcement Administration included it in Schedule I of the Controlled Substances Act (Drug Enforcement Administration, Department of Justice, 2013).

Methylone has a strong structural and pharmacological similarity to 3,4,-methylenedioxymethamphetamine (MDMA). Studies on the pharmacological targets of cathinones have recently been published by our group (Martínez-Clemente et al., 2012) and by others (Hadlock et al., 2011; Kehr et al., 2011; Motbey et al., 2012), and have demonstrated that methylone acts on monoaminergic systems. This substance, like MDMA, stimulates the non-exocytic release of serotonin (5-HT) and, to a lesser degree, dopamine (Hadlock et al., 2011; Sogawa et al., 2011). Furthermore, it is a potent nonselective monoamine uptake inhibitor that blocks 5-HT and dopamine uptake competing with the substrate, and its effects partially persist after drug withdrawal (López-Arnau et al., 2012).

Methylone has been shown to increase spontaneous locomotor activity in mice and rats in a dose-dependent manner (Baumann et al., 2013; Kehr et al., 2011; Marusich et al., 2012; Motbey et al., 2012). This effect is prevented by ketanserin or haloperidol pre-treatment (López-Arnau et al., 2012).

The repeated administration of amphetamine derivatives such as methamphetamine and MDMA to rodents has also been found to cause neurotoxicity (Halpin et al., 2014). Because cathinones are relatively new, research on their acute and long-term neurotoxicity of cathinones is starting to be conducted. A recent report (Blum et al., 2013) stated that cathinones 'have been known to cause intensive cravings for the substances and users have been reported to go on multiday binges that often cause medical problems necessitating medical intervention'.

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Due to its similarity to MDMA, there is reason to suspect that methylone could also display a species-dependent neurotoxicity. MDMA is toxic to the 5-HT system in both rats and humans, whereas it affects the dopamine system in mice (Logan et al., 1988; Reneman et al., 2001). For this reason, the rat is generally considered to be a better rodent model for amphetamines than the mouse, although it is currently unclear whether rats are also a better model for methylone neurotoxicity, as the long-term behavioural and neurochemical effects of this drug have not yet been characterized in humans. Den Hollander et al. (2013) found that methylone had no effects on 5-HT levels in mice, but demonstrated a widespread depletion of 5-HT and 5-HT transporter (SERT) levels in rats two weeks after the last administration. By contrast, Baumann et al. (2012) found that methylone had no effects on monoamine levels in rats two weeks after treatment. These inconsistencies may be partially due to differences in the dosage-regimen and recovery period used. In a recent paper (López-Arnau et al., 2014), we described neuronal disturbances in methylone-treated mice depending on the drug schedule, but it is necessary to study whether these changes also occur when rats are treated using a similar regimen.

There is also ample evidence linking the use of amphetamines with decreased memory function and increased neuropsychiatric symptoms in animal models and humans (McCardle et al., 2004; McGregor et al., 2003; Moon et al., 2007; Parrott et al., 2000; Piper and Meyer, 2004; Volkow et al., 2001); this could be a consequence of neurotoxicity or robust neurochemical changes.

The aim of this study was to investigate the neurochemical changes indicative of neurotoxicity after methylone administration in rats and to address some of the limitations found in the current literature. We administered methylone to rats using a schedule intended to model human recreational use, and assessed the possible changes in the density of dopamine or 5-HT uptake sites and tyrosine hydroxylase and tryptophan hydroxylase-2 (TPH-2) expression. In addition, we studied whether a glial response took place as a result of cathinone-induced neuroinflammation (Blum et al., 2013). Since a close relationship between the hyperthermic response, which increases in a high ambient temperature, and the severity of the brain lesion induced by MDMA has been established (Sanchez et al., 2004), our experiments were carried out in a raised ambient temperature to simulate the hot conditions found in dance clubs, where the drug is consumed. Finally, we also assessed the effect of methylone on spatial learning and memory using the Morris water maze (MWM).

Materials and methods

Animals and treatment

Male Sprague-Dawley rats (125–175 g, aged 46 weeks) (Janvier, Lé Genest, France) were used. The animals were housed in a regulated temperature environment (21 ± 1°C; 12 h light/dark cycle, lights on at 08:00 h) with free access to food and water. With the exception of experiments registering body temperature, other experiments took place between 09:00 and 18:00 h. Experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia, and following the guidelines of the European Communities Council (86/609/EEC). Efforts were made to minimize suffering and reduce the number of animals used. Animals were administered subcutaneously with four doses of saline (1 mL/kg) or methylone (20 mg/kg) per day with 3 h intervals. There is good evidence that, at least from the perspective of neurotoxicity of MDMA, the subcutaneous (s.c.) injection route yields results similar to those observed after oral administration (Finnegan et al., 1988; Slikker et al., 1988). Therefore, for the sake of convenience, ease of drug delivery and safety we chose to dose the animals by s.c. injection. During the treatments, the animals remained one per cage in a room with a controlled ambient temperature of 26 ± 1°C until 1 h after the last dose. A different set of animals was used for behavioural, neurochemical or immunohistochemistry experiments respectively. No information about s.c. doses in humans is available. In our case, the dose of 20 mg/kg in rats corresponds to 3 mg/kg in a human. This rat equivalent dose was calculated following the body surface area normalization method (Reagan-Shaw et al., 2008). Moreover, the interval of 3 h between doses was chosen according to our previously published paper on methylone pharmacokinetics in rats (López-Arnau et al., 2013).

Drugs and reagents

Pure racemic methylone hydrochloride was synthesized and characterized by us as described previously (López-Arnau et al., 2012). Methylone solutions for injection were prepared in sterile 0.9% NaCl (saline) immediately before administration. Isoflurane was from Laboratorios Dr Esteve (Barcelona, Spain). The other drugs were obtained from Sigma-Aldrich (St Louis, MO, USA). [^{3}H]ketanserin, [^{3}H]paroxetine and [^{3}H]WIN35428 were from Perkin Elmer (Boston, MA, USA). All buffer reagents were of analytical grade.

Surgical procedures and telemetric acquisition of body temperature

The animals (*n*=6–8/group) were allowed one week after arrival to acclimatize before surgery. Subsequently, they had implanted an electronic device (Thermo Tracker, IDC, Barcelona, Spain), enabling continuous measurement of core body temperature. The implant was placed in the abdominal cavity as follows: the rats were anesthetized with isoflurane, the abdomen was opened by making a 2-cm midline incision and the device was placed in the abdominal cavity, along the sagittal plane. The abdominal and the skin wound were then closed with absorbable suture material. After surgery, animals were individually housed, received analgesic therapy and were allowed to recover for seven days before saline or methylone administration, avoiding a possible influence of this type of manipulation. The device registered the core temperature every 5 min and values were downloaded to a computer after removal of the device, once the animals had been sacrificed, using the interface and software provided by the manufacturer. Data were acquired from 24 h prior to until 24 h after drug administration.

MWM

Spatial learning and memory were assessed in a MWM one week after treatment. Male Sprague-Dawley rats (*n*=9–11/group) were trained in the water maze, which consisted of a circular pool (160 cm in diameter and 45 cm high) that was filled with water (22 ±

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1°C) to a depth of 25 cm and rendered opaque by the addition of a non-toxic latex solution. The pool was in an isolated room and black curtains were closed around it to minimize static room cues. Four positions around the edge of the tank were designated as north (N), south (S), east (E) and west (W) and also defined the division of the tank into four quadrants: NE, SE, SW and NW, providing alternative start positions. A Plexiglas escape platform (11 cm diameter) was submerged to a depth of 1 cm from the water surface and was not visible at water level. The escape latency or total time needed by the rats to find the platform (in seconds) was measured using appropriate computer software (Smart, Panlab SL, Barcelona, Spain) connected to a video camera placed over the pool that allowed to trace the tracking of each animal.

In the spatial learning (acquisition trials) task, four objects or landmarks were suspended from a false ceiling at 30 cm above the water surface in N, S, E and W positions. The platform was always in the NW quadrant. Rats received a training session, consisting in five trials per day, by using a semi-random set of start locations that were not equidistant from the goal, thus creating short and long paths to the platform. Moreover, they were designed so that the animal was not able to learn a specific order of right or left turns to locate the platform. Animals were tested on five consecutive days (a total of 25 trials per animal were performed to reach asymptotic performance).

A trial was started by placing a rat in the desired start position in the maze, facing the tank wall. Rats were allowed to swim to the hidden platform, and the escape latency was measured. If an animal did not escape within 120 s, it was gently placed on the platform or guided to it. Rats were allowed to rest for 30 s (intertrial interval) on the platform (even those that failed to locate it). This procedure was repeated with each animal over the trials (Vorhees and Williams, 2006).

To assess reference memory at the end of learning, a probe trial (free swimming without platform for 60 s), was carried out 24 h after the last training session. We used a novel start position during the probe trial to ensure that its spatial preference is a reflection of the memory of the goal location rather than for a specific swim path. We used as start position the opposite quadrant (SE) to the original platform position. Different parameters of the rat's performance were analysed: the total time spent swimming and the number of entries in the target (where the platform should be located) and the opposite quadrants.

Tissue sample preparation

Crude membrane preparation was prepared as described (Escubedo et al., 2005) with minor modifications. Animals (n=5–8/group) were killed by decapitation under isoflurane anaesthesia at seven days after treatment, and the brains were rapidly removed from the skull. Hippocampus, striatum and frontal cortex were quickly dissected out, frozen on dry ice and stored at −80°C until use. When required, tissue samples were thawed and homogenized at 4°C in 20 volumes of buffer (5 mM Tris–HCl, 320 mM sucrose) with protease inhibitors (aprotinin 4.5 µg/µL, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), pH 7.4, using a Polytron homogenizer. The homogenates were centrifuged at 1000 × g for 15 min at 4°C. Aliquots of the resulting supernatants were stored at −80°C until use for Western blot experiments. The rest of the samples were resuspended and centrifuged at 15,000 × g for 30 min at 4°C. The pellets were resuspended in buffer and incubated at 37°C for 5 min to remove endogenous neurotransmitters. The protein samples were recentrifuged. The final pellets (crude membrane preparations) were resuspended in the appropriate buffer and stored at −80°C until use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent, according to the manufacturer's instructions.

Dopamine and 5-HT transporter density

The density of the dopamine transporter (DAT) in striatal or frontal cortex membranes was measured by [^{3}H]WIN35428 binding assays, as a marker of dopaminergic nerve terminals. Assays were performed in glass tubes containing 250 or 500 µL of [^{3}H]WIN35428 diluted in phosphate-buffered 0.32 M sucrose (final radioligand concentration, 5 nM) and 50 or 100 µg of membranes, respectively. Incubation was carried out for 2 h at 4°C and non-specific binding was determined in the presence of 30 µM bupropion.

The density of SERT, a marker of serotonergic nerve terminal, was assessed in membranes from hippocampus, striatum and frontal cortex by measuring the specific binding of 0.1 nM [^{3}H]paroxetine after incubation with 150 µg of protein at 20–22°C for 2 h in a Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 mL. Clomipramine (100 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration under vacuum through Whatman GF/B glass fibre filters, previously pre-treated with 0.05% polyethylenimine. Tubes and filters were washed rapidly three times with 4 mL of ice-cold buffer, and the radioactivity in the filters was measured by liquid scintillation spectrometry.

[^{3}H]-paroxetine saturation binding assay

This assay was performed to assess whether the decrease in [^{3}H]paroxetine binding is due to a change in affinity or a decrease in SERT density. Saturation binding assay was measured as described by Hewitt and Green (1994) with some modifications. Frontal cortex membrane preparation was prepared as described above. The density of SERT in frontal cortex membranes was quantified by measuring the specific binding of [^{3}H]paroxetine (0.01–3 nM) after incubation with 150 µg of protein at 20–22°C for 2 h in a Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl and 5 mM KCl to a final volume of 2 mL. 5-HT (100 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration as described for [^{3}H]paroxetine and [^{3}H]WIN35428 binding assays.

Western blotting and immunodetection

A general Western blotting and immunodetection protocol was used to determine the expression of tyrosine hydroxylase and TPH-2 in animals exposed to the treatment. For each sample, 15 µg of protein was mixed with sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min and loaded onto a 10% acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P, Millipore, Billerica, MA, USA). PVDF membranes were blocked overnight with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated for 2 h at room temperature with a primary

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mouse monoclonal antibody against tyrosine hydroxylase (dilution 1:5000) (Transduction Labs, Lexington, KY, USA) or a primary rabbit polyclonal antibody TPH-2 (dilution 1:1000) (Millipore, USA).

After washing, membranes were incubated with a peroxidase-conjugated antimouse IgG antibody (dilution 1:2500) or a peroxidase-conjugated antirabbit IgG antibody (dilution 1:5000) (GE Healthcare, Buckinghamshire, UK). Immunoreactive protein was visualized using a chemoluminescence-based detection kit following the manufacturer's protocol (Immobilon Western, Millipore, USA) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Hercules, CA, USA). Scanned blots were analysed using BioRad Image Lab software and dot densities were expressed as a percentage of those taken from the control. Immunodetection of beta-actin (dilution 1:2500) (mouse monoclonal antibody, Sigma-Aldrich) served as a control of load uniformity for each lane and was used to normalize differences in the corresponding enzyme expression due to protein content.

5-HT$_{2A}$ receptor density

The density of 5-HT$_{2A}$ receptors was measured in frontal cortex membranes in rat one week after their exposure to the treatment, using [^{3}H]ketanserin binding. Assays were performed in glass tubes containing 1 nM [^{3}H]ketanserin and 100 µg of membranes. Incubation was carried out at 37°C for 30 min in a Tris–HCl buffer to a final volume of 0.5 mL. Methysergide (10 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration under vacuum, washed rapidly and the radioactivity was measured by liquid scintillation spectrometry as described previously.

Ionized calcium binding adaptor molecule 1 and glial fibrillary acidic protein immunohistochemistry

Animals (*n*=3/group) used in ionized calcium binding adaptor molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) immunohistochemistry were anaesthetized with pentobarbital sodium (60 mg/kg) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (1 mL/g body weight) 48 h and seven days after treatment, respectively. Brains were removed and postfixed for 2 h in the same solution, cryoprotected by immersion in 30% sucrose/phosphate buffer solution for 24 h and frozen in dry ice-cooled isopentane. Serial coronal sections (30 µm thick) through the whole brain were incubated for 15 min at room temperature in H_2O_2 (0.3% in phosphate buffer with 10% methanol). Thereafter, sections were incubated in a blocking solution (1% of foetal bovine serum, and 0.2 M glycine plus 0.5% Triton X-100 in phosphate buffer). After blocking with 10% normal serum and 0.2% bovine serum albumin, sections were rinsed and incubated overnight at 4°C using a polyclonal antibody against Iba-1 (dilution 1:1000) (Wako, USA) or monoclonal antibody against GFAP (dilution 1:1000) (Dako, Denmark). Following this, the sections were washed and incubated with a biotinylated secondary antibody (dilution 1:200) for 2 h at room temperature. Afterwards they were incubated with avidin–biotin–peroxidase complex (dilution 1:200; Vector, Burlingame, CA). Peroxidase reaction was developed with 0.05% diaminobenzidine in 0.1 M phosphate buffer and 0.02% H_2O_2 and immunoreacted sections were mounted on gelatinized slides. Stained sections were examined under a light microscope (Olympus BX61) and quantified using Image J software.

Data analysis

Results are given as the mean ± standard error of the mean (SEM). One-way or two-way (repeated measures) ANOVA, followed by Tukey's post-hoc tests were used. Saturation-binding curves were fitted by nonlinear regression using GraphPAD Prism (GraphPAD software, San Diego, CA, USA); *p* values less than 0.05 were considered significant.

Results

Lethality

Adolescent rats were used in all experiments. Under these conditions, the number of fatalities in methylone-treated rats was 16%. Deaths were mostly produced after the fourth dose but never after the first or second dose. It is important to note that all methylone treatments induced the appearance of head-dipping stereotypy and salivation.

Effect of methylone on body weight and core temperature

Like amphetamines and MDMA, methylone produced a significant loss in body weight. In the saline group, weight gain was found to be 3.2 ± 0.9%, while the weight of methylone-treated rats decreased by 4.0 ± 1.5% ($p<0.001$) 24 h after treatment.

We used an electronic implant that recorded core temperature every 5 min. Through this system we observed a robust hyperthermic response that reached a peak between 25 and 35 min after each drug administration. Analysis of the average core temperature demonstrated a significant effect of treatment ($p<0.001$). When the curve profile analysis was carried out, it showed four significant peaks corresponding to the four maximal increases after drug administration (Figure 1).

Analysis of the core body temperature demonstrated an overall effect of treatment ($F_{1,36} = 20.14$; $p<0.001$) and treatment × dose ($F_{3,36} = 6.84$; $p<0.001$). Saline-treated rats exhibited a non-significant ($p=0.074$) increase in temperature after each injection, probably due to stress, and this decreased slightly in the two last administrations. Conversely, the effect of methylone increased significantly with the dose, so that the last dose induced a higher increase in body temperature than the first one ($p<0.05$) (Table 1).

Effect of methylone on different in vivo markers of dopamine and 5-HT terminals

Methylone treatment did not affect the specific binding of [^{3}H] WIN35428 either in the striatum (saline: 100.00 ± 15.83%; methylone: 105.67 ± 6.35%, NS) or in the frontal cortex (saline: 100.00 ± 15.72%; methylone: 81.79 ± 11.20%, NS) of animals killed seven days after drug administration. Also, although tyrosine hydroxylase expression decreased, it showed no significant differences between the treatment groups in any of the areas studied (striatum: saline: 100.00 ± 3.35%; methylone: 90.62 ±

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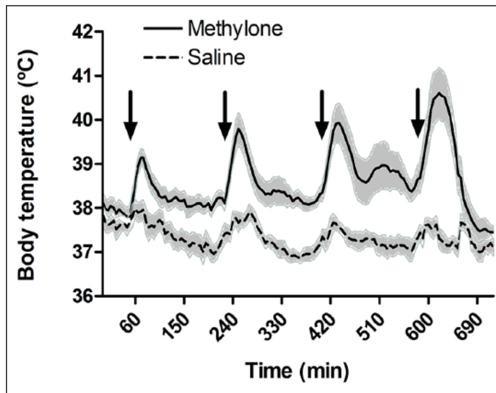

Figure 1. Effect of methylone on core body temperature. Rats (n=6–8 per group) were treated with saline (—) (1 mL/kg, sub-cutaneously (s.c.)) or methylone (—) (4 × 20 mg/kg, s.c., 3 h intervals) and core body temperature was recorded using an electronic implant at 5 min intervals for 12 h. Results are presented as group means. SEM values are omitted for the sake of clarity and displayed as a grey cloud. Injection times are indicated by arrows.

4.83%, NS; frontal cortex: saline: 100.00 ± 19.69%; methylone: 69.09 ± 16.85%, NS).

In terms of 5-HT markers, methylone induced a decrease in 5-HT reuptake sites assessed by the specific binding of [^{3}H]paroxetine: 13% in the striatum, 24% in the hippocampus and 48% in the frontal cortex (Figure 2(a), (c) and (e)). In the same animals, TPH-2-immunoreactivity levels, also a marker of serotonergic terminal integrity, decreased significantly in the three areas of the methylone-treated rats, compared with saline-injected animals (Figure 2(b), (d) and (f)). The decrease in these parameters ran in parallel, except in the frontal cortex, where the SERT reduction was higher than that of TPH-2.

Effect of methylone treatment on the saturation binding isotherm of [^{3}H] paroxetine in the frontal cortex

To assess the nature of the reduction in [^{3}H]paroxetine binding sites, saturation binding experiments were performed. Figure 3 shows a representative saturation binding isotherm of [^{3}H]paroxetine in the frontal cortex of saline- and methylone-treated rats. In both groups, a non-linear regression analysis of specific binding revealed a single saturable site (r^2 = 0.99). Methylone administration produced no changes in the dissociation constant (K_D). However, a reduction in the maximal number of binding sites (B_{max}) was evidenced seven days after treatment.

Effect of methylone treatment on 5-HT$_{2A}$ receptor density

Based on the reduction in serotonergic parameters, we determined the density of 5-HT$_{2A}$ receptors in the hippocampus and frontal cortex by measuring the specific binding of [^{3}H]ketanserin. None of the areas studied showed a significant change in this parameter (hippocampus: saline 100.00 ± 16.05%; methylone: 83.33 ± 6.30%, NS; frontal cortex: saline: 100.00 ± 5.11%; methylone: 112.00 ± 3.63%, NS).

Effect of methylone on microglial and astroglial activation

Because methylone exposure induces disturbances in 5-HT terminal markers, the next experiment was carried out to assess the presence of microglial and astroglial activation. In previous studies we have characterized the glial response to amphetamine derivatives (Pubill et al., 2003) and no microglial activation was noticeable at either three or seven days after treatment. On the contrary, astrogliosis was evidenced at seven days after treatment. To this end, immunohistochemistry studies were performed with the microglial- and glial-specific markers Iba-1 and GFAP on the brains from animals killed two or seven days after treatment, respectively. There were no signs of striatal, hippocampal or cortical microgliosis in methylone-treated animals (data not shown).

However, we found a significant increase (65%) in this marker in the frontal cortex of methylone-treated rats when assaying GFAP (Figure 4). By contrast, no significant differences were found in the striatum or any subregion of the hippocampus (dentate gyrus, CA1 or CA3) in the GFAP-immunohistochemistry study (data not shown).

Effect of methylone on performance in the MWM

We investigated the effect of treatment on spatial learning and reference memory seven days after the end of the binge exposure to saline or methylone using the MWM paradigm. An analysis of the mean swimming speed in the overall maze presented no differences between groups (saline: 22.43 ± 1.04 cm/s; methylone: 22.67 ± 1.24 cm/s), so the latency time to reach the hidden platform was used as the variable to measure performance.

Overall, both saline- and methylone-treated rats demonstrated a good ability to learn the task. In the acquisition phase, a repeated measures ANOVA showed a significant effect of the variable training days ($F_{4,72}$ = 12.29, p<0.001). There was no effect of the treatment ($F_{1,18}$ = 0.54, NS) or treatment × training days ($F_{4,72}$ = 0.68, NS). These results indicate that the escape latency diminished as the trials progressed (from day 1 to day 5) and that the animals learned the platform location (Figure 5), but the treatment did not affect the rats' ability to learn such a location.

A probe test in which the escape platform was removed from the pool was performed on day 6. Two-way ANOVA (repeated measures) analysis of time spent in the target or the opposite quadrant showed a significant effect of the quadrant ($F_{1,18}$ = 4.42, p<0.05) and the interaction quadrant × treatment ($F_{(1,18)}$ = 8.29, p<0.01). This allowed us to conclude that the treatment only has an effect in the target quadrant. Post-hoc comparisons showed that the saline group displayed better reference memory, as the animals in this group swam in the target quadrant for 37.88 ± 3.68% of total time while methylone-treated rats did so for 22.96 ± 2.82% of the time (Figure 6(a)) (p<0.01 *vs*. saline).

There was a similar behavioural profile of memory performance when we analysed the number of entries in the target and

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Table 1. Peak rat core body temperatures, in centigrade, after each saline (1 mL/kg, sub-cutaneously (s.c.)) or methylone (4 × 20 mg/kg, s.c.) administration at 3 h intervals. Results are expressed as mean ± SEM from *n*=6–8 animals.

Treatment	Basal	1st dose	2nd dose	3rd dose	4th dose
Saline	37.69 ± 0.24	37.98 ± 0.21	37.90 ± 0.20	37.67 ± 0.20	37.63 ± 0.16
Methylone	37.98 ± 0.23	39.14 ± 0.21*	39.80 ± 0.38*	39.94 ± 0.45*	40.61 ± 0.59**

*$p<0.01$ versus saline (two-way ANOVA followed by post-hoc test).
**$p<0.001$ versus saline (two-way ANOVA followed by post-hoc test).

the opposite quadrant ($F_{1,18} = 4.50$, $p<0.05$). Post-hoc comparisons revealed significantly fewer entries in the target quadrant in the methylone group ($p<0.05$) (Figure 6(b)).

Discussion

Excessive consumption of cathinones may lead to toxicity with severe neurologic and peripheral symptoms, including death. There is no study devoted to the mortality rate induced by methylone in humans. However, some reports identified the relationship between methylone and sudden death associated with an elevated body temperature and seizure activity (Carbone et al., 2013; Cawrse et al., 2012; Pearson et al., 2012). It is very difficult to know the correlation between the amount of methylone detected post-mortem in blood and the ingested doses. So, there is insufficient forensic evidence to determine how, how many and when methylone has been consumed and the corresponding amount found post-mortem. It is therefore important to study these effects in depth in order to provide society with evidence-based information on the risks involved.

Some studies reveal that methylone is non-selectively taken up by monoamine transporters leading to competitive monoamine uptake inhibition and this stimulates the non-exocytic release of 5-HT and, to a lesser extent, dopamine, resulting in elevated synaptic neurotransmitter levels (Baumann et al., 2012; Eshleman et al., 2013; Simmler et al., 2013; Sogawa et al., 2011). This is a critical point, since only uptake inhibitors that are transporter substrates (as amphetamines), but not direct transport blockers (as cocaine), cause long-term deficits in monoamine cells (Fleckenstein et al., 2007). In this sense, the potential for this cathinone to produce neurotoxic effects in various brain regions requires intensive investigation.

In the present study, we found that the neuronal disturbances induced by methylone in rats are closely related to those found in mice (López-Arnau et al., 2014), using a similar drug schedule.

To model human recreational methylone use, we simulated the widespread practices of ‘stacking’ and ‘boosting’. Thus, we chose to administer four doses per day of methylone during each treatment period. It is important to note that, in contrast to previous published papers (den Hollander et al., 2013), we performed our experiments in adolescent rats, to establish a relationship with human abuse.

Rats receiving methylone showed an incidence of death around 16%. This mortality rate was similar or lower to that reported in MDMA neurotoxicity studies (Ho et al., 2004; Itzhak et al., 2003; Mueller and Korey, 1998). Furthermore, there is only a single description of methylone-induced lethality in rats that reached 100% at 60mg/kg (den Hollander et al., 2013).

It is known that hyperthermia plays a key role in MDMA-induced neurotoxicity (Sanchez et al., 2004). For this reason, we performed the experiments at an ambient temperature of 26 ± 1°C to simulate the hot conditions found in dance clubs. Under these conditions methylone induced hyperthermia.

In our study we used electronic implants, which allowed us to measure body temperature more accurately over time. This revealed a hyperthermic response to methylone that peaked 30 min after administration.

Using our drug schedule, methylone caused significant changes in the specific marker of 5-HT terminals (SERT density) in the three brain areas studied, but a higher extent in the frontal cortex. In the same areas, methylone significantly decreased TPH-2 expression. With regard to dopamine terminal markers, we only detected a slight decrease in dopamine transporter density and tyrosine hydroxylase expression in the frontal cortex, but no change was found in the striatum. We have reported that the increase in locomotor activity induced by methylone is directly correlated with blockade of dopamine uptake and with a non-exocytotic, transporter-mediated, dopamine release (López-Arnau et al., 2012). In the present work we do not see a neurotoxic injury in dopamine nerve terminals keeping intact these neuronal pathways.

Previous to the present study, den Hollander et al. (2013) studied the neurotoxicity of methylone in rats, using a schedule of 30 mg/kg twice daily for four days, and reported a decrease in SERT markers in the frontal cortex and hippocampus, as well as a profound decrease in 5-HT levels in these brain areas plus the striatum. However, Baumann et al. (2012) examined monoamine levels after two weeks’ treatment and reported that there were no changes. This contrasts with the results of our experiment, but this might be explained by the fact that the doses used in that study were lower than in ours, and these authors did not observe a pronounced hyperthermic response, as occurs with MDMA. We can conclude from these comparisons that hyperthermia, together with dosing (both variables probably related), plays a crucial role in methylone’s impairment of 5-HT.

There is evidence that MDMA doses that produce substantial, long-term reductions in rat brain 5-HT and other serotonergic markers do not reliably provoke astroglial (Biezonski and Meyer, 2011) or microglial (Herndon et al., 2014; Pubill et al., 2003) responses, and thus it is not clear whether such reductions truly reflect structural damage to the serotonergic system. We did not detect any significant microglial responses in any of the brain regions processed. Nevertheless, methylone induced significant astroglial activation in the frontal cortex, the area where changes in the biochemical parameters of the serotonergic system are more apparent.

Whether these biochemical changes reflect an actual serotonergic injury requires further discussion. SERT depletion may occur

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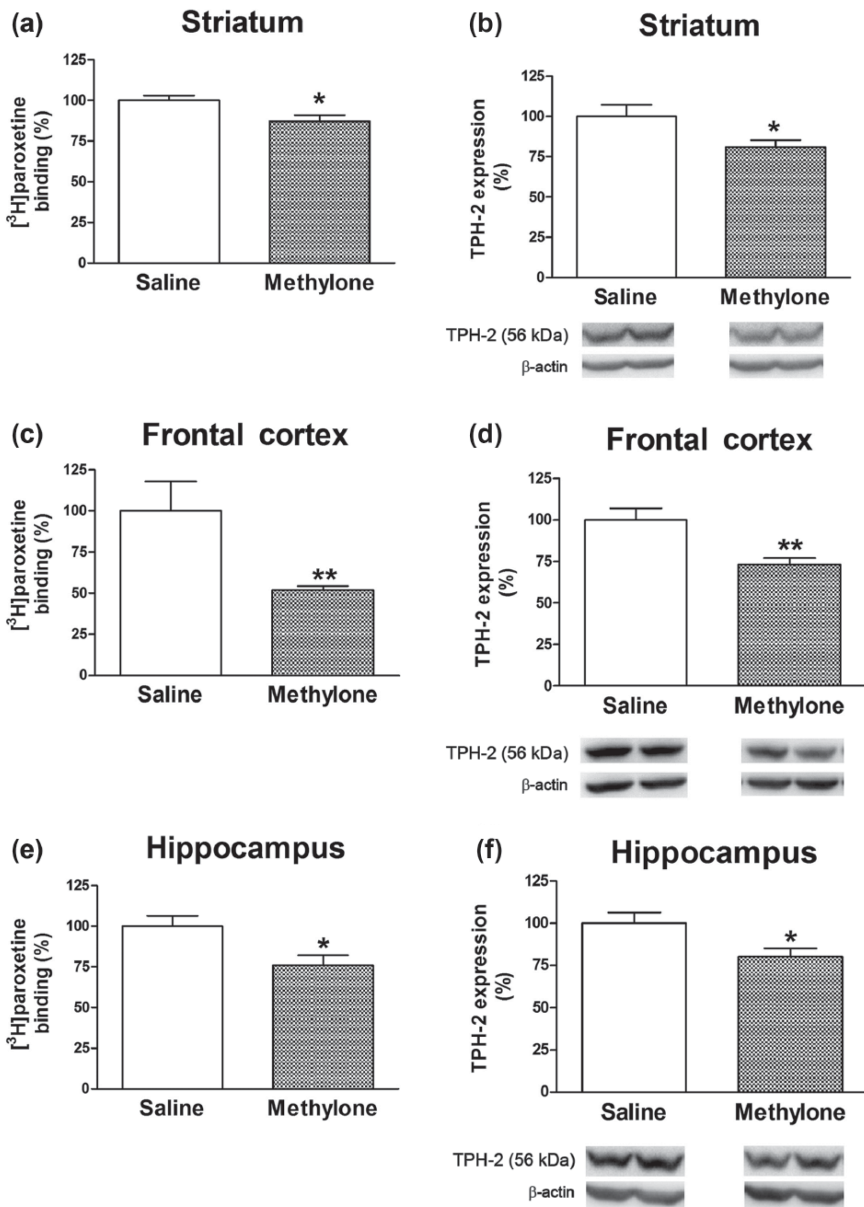

Figure 2. Effect of methylone treatment (4 × 20 mg/kg, sub-cutaneously, 3 h intervals) in serotonin transporter density, measured as [^{3}H]-paroxetine binding in the striatum (a), frontal cortex (c) and hippocampus (e); effect in tryptophan hydroxylase 2 (TPH-2) expression in the striatum (b), frontal cortex (d) and hippocampus (f). The values correspond to animals killed seven days after treatment. Below each bar graph, the corresponding representative Western blots of TPH-2 expression in the striatum, frontal cortex and hippocampus are shown. Results are expressed as mean ± SEM from 5–8 animals per group.
*$p<0.05$ versus saline.
**$p<0.01$ versus saline.

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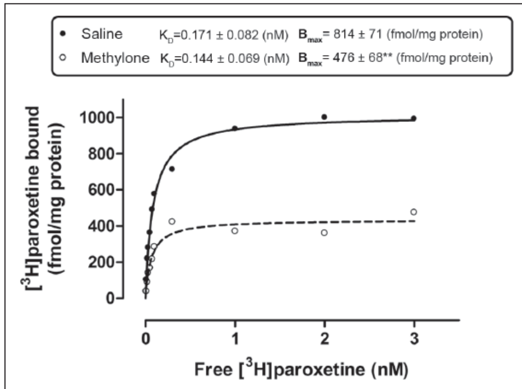

Figure 3. Representative saturation binding assay of [3H]-paroxetine in the frontal cortex of saline- and methylone-treated rats. B_{max} and K_D values are expressed as mean ± SEM of 4–5 experiments.
**p<0.01 versus saline (see box).

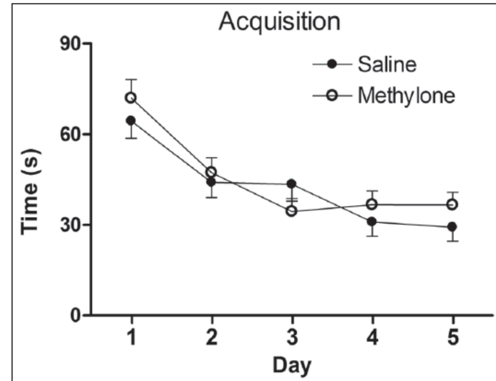

Figure 5. Effect of treatment with saline or methylone in the acquisition phase of spatial learning in the Morris water maze. The escape latency (the time required for rats to locate the platform) for the five different days is expressed as mean ± SEM of 9–11 animals per group.

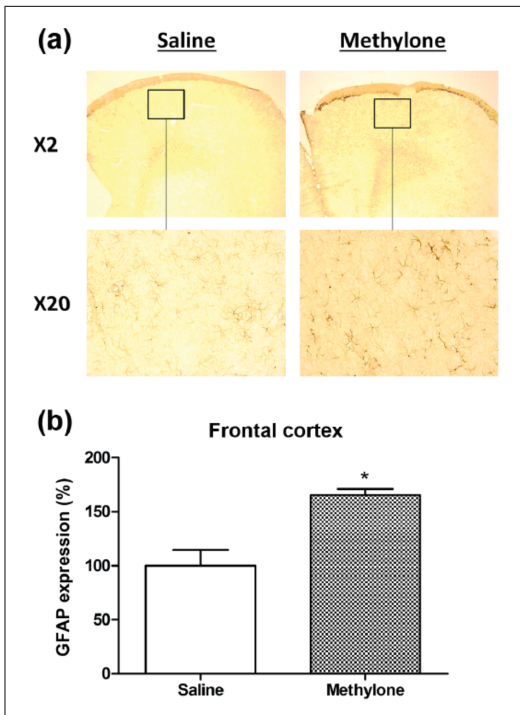

Figure 4. Representative immunohistochemistry for glial fibrillary acidic protein (GFAP) expression in horizontal sections of the frontal cortex for animals treated with saline or methylone (× 2 and × 20) (a). Effect of methylone treatment on GFAP expression in rat frontal cortex (b) seven days after treatment. Quantification was carried out using Image J software. Results are expressed as mean ± SEM from three animals per group.
*p<0.05 versus saline.

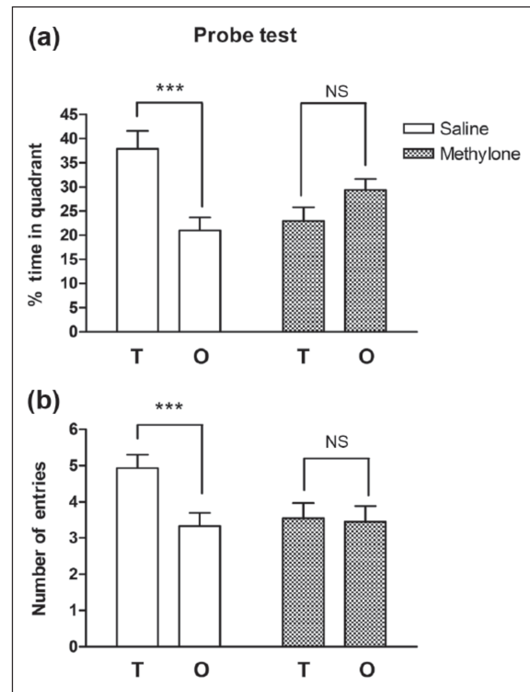

Figure 6. Effect of methylone treatment on the probe trial in the Morris water maze. (a) Per cent of time spent by the animals in the target (T) and the opposite (O) quadrant. (b) Number of entries in the target and opposite quadrant. Results are expressed as mean ± SEM from 9–11 animals per group.
***p<0.001 versus saline (two-way ANOVA of repeated measures followed by Tukey's post-hoc test).

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in the absence of axotomy. In fact, SERT can be regulated by endocytic translocation (Carneiro and Blakely, 2006). In early studies we corroborated that the membrane preparation used also contains the transporter internalized by endocytosis, despite the fact that the loss of a small amount of this fraction cannot be completely ruled out. Consequently, the decrease in binding density is not the result of transporter redistribution. Moreover, the parallel decrease in TPH-2, a cytosolic serotoninergic terminal marker, leads us to suggest that a real terminal injury exists.

Furthermore, the actual number of transporters could be higher than the apparent score because of a decrease in the affinity for [^{3}H]paroxetine induced by the interaction of methylone with SERT. In fact we previously found that the inhibition of 5-HT uptake by methylone can persist after drug withdrawal (López-Arnau et al., 2012). To clarify this point we carried out the saturation binding experiments with [^{3}H]paroxetine in samples from methylone- and saline-treated animals. This study revealed that the affinity for the radioligand remains unchanged, whereas the B_{max} value reduced significantly in the methylone group. This allowed us to conclude that the decrease in [^{3}H]paroxetine binding is due not to a decrease in affinity, but to a decrease in SERT density.

Moreover, other authors (Cuyas et al., 2014; Kirilly et al., 2008) have suggested that brain SERT gene expression may be negatively regulated seven days after MDMA exposure, which could lead to a reduction in paroxetine binding in the absence of physical damage. Conversely, they found an increase in the expression levels of the TPH-2 gene (Cuyas et al., 2014). Both regulations are considered a compensatory mechanism in order to overcome the decrease in 5-HT induced by MDMA treatment. In this study we have shown that both the density of SERT and the expression of TPH-2 are reduced in all of the brain areas studied. Hence the fact that TPH-2 decrease precludes a regulatory response to 5-HT reduction and reflects damage to serotonergic terminals.

As a result of an initial massive release of 5-HT followed by a damage-induced serotonergic depletion (Simmler et al., 2013), the compensatory regulation of post-synaptic receptor could be expected. For this reason, we assessed the 5-HT$_{2A}$ receptor density. Although a slight increase was observed in the frontal cortex, non-significant changes were ascertained, which ruled out a compensatory mechanism seven days after drug exposure.

In the MWM, methylone-treated rats do not show spatial learning deficits one week after binge exposure. However, when a single probe trial was conducted 24 h after the acquisition phase to assess reference memory, the methylone group displayed a poorer performance, which would suggest that this drug affects this memory type.

Den Hollander et al. (2013) observed an improved performance in methylone-treated mice during a reversal probe trial eight weeks after treatment. The authors explained this result by suggesting that the drug-treated mice forgot the former location of the platform more quickly, so they had no preference for either quadrant. This assertion is consistent with our results in the probe trial with methylone-treated rats.

MWM performance appears to depend on the coordinated action of different brain regions and neurotransmitter systems (D'Hooge and De Deyn, 2001). These systems do not support learning and memory in a task-dependent manner, but some predominate over others in a specific task. Glutamate, GABA and dopamine seem to be the most critical in MWM performance (Myhrer, 2003), while reducing 5-HT synthesis with p-chlorophenylalanine has no effect on such performance unless combined with a treatment that reduces cholinergic transmission (Richter-Levin and Segal, 1989).

We found serotonergic impairment in methylone-treated rats, especially in the frontal cortex, where it was accompanied by astrogliosis. Some serotonergic alterations were also present in the hippocampus and striatum. However, these animals only displayed impairments in the probe trial of the MWM. Further investigation is needed to determine the effects of binge administration of methylone in rats on different memory tasks, but the present results indicate that learning processes are unaffected, although there are some memory deficits.

In conclusion, the present results demonstrate that, in contrast to the species-dependent neurotoxicity of MDMA, methylone-induced brain alterations in rats do not qualitatively differ from those observed in mice using a similar drug schedule. Methylone elicited neurochemical changes when it was administered in an elevated ambient temperature, four times per day at 3 h intervals. These include loss of 5-HT neuronal markers in the frontal cortex, hippocampus and striatum, as well as cortical astrogliosis, which would indicate the presence of injuries in nerve endings in this area. However, other, additional, mechanisms, such as the regulation of SERT gene expression or transporter recycling, cannot be completely ruled out. No significant effect on the dopaminergic system was observed. While these changes occur, the animals perform well during a spatial learning task, but show memory impairment in the probe trial.

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Conflict of interest

The authors declare that there is no conflict of interest.

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DISCUSIÓN

4. DISCUSIÓN

Como ya se ha comentado en la introducción de la presente Tesis Doctoral, recientemente una nueva generación de drogas de diseño, las catinonas sintéticas, ha proliferado como drogas de abuso. La mayoría de estas catinonas poseen una similitud estructural con otros derivados anfetamínicos, como la MDMA o la metanfetamina por lo que sus efectos psicoestimulantes y empatógenicos podrían ser similares a los producidos por éstos (Schifano et al., 2011). Concretamente, la metilona, objeto de la presente Tesis Doctoral, es una sustancia relativamente nueva, y por ello, la información disponible es muy limitada. El rápido aumento del consumo/abuso de esta sustancia, juntamente con los serios efectos sobre la salud, exigen un mejor conocimiento tanto de su perfil farmacocinético como farmacodinámico. No podemos subestimar tampoco el potencial de la metilona para producir efectos a largo plazo de índole neurotóxica.

4.1. Neurofarmacología de la metilona y otras catinonas

Para poder entender los efectos de estas β-ceto anfetaminas psicoestimulantes, es esencial determinar sobre qué transportadores y/o receptores actúan así como el sistema de neurotransmisión implicado en su mecanismo de acción. Un profundo conocimiento del perfil farmacológico para cada una de estas drogas es básico para el desarrollo de tratamientos frente a problemas derivados de la sobredosis y dependencia a este tipo de sustancias. En la publicación **I** realizamos un estudio comparativo en animales de laboratorio de la neurofarmacología de tres de las catinonas sintéticas más importantes, butilona, mefedrona y metilona.

Tal como ya se ha comentado, las anfetaminas son sustratos de los transportadores de 5-HT y DA, pero su acción no es una mera inhibición de la captación del neurotransmisor. Las anfetaminas producen un transporte reverso de las monoaminas desde el interior del terminal hacia el espacio sináptico, dando lugar una inhibición neta de la captación de monoaminas y un aumento prolongado de las concentraciones de éstas en la hendidura sináptica, que es responsable de su efecto psicoestimulante (Levi and Raiteri, 1993; Leviel, 2001). En nuestro modelo *in vitro*, utilizando sinaptosomas de rata, tanto la butilona como la mefedrona y la metilona

produjeron una reducción concentración-dependiente de la captación de [^{3}H]5-HT, [^{3}H]DA y [^{3}H]NA. Se observó una cierta relación entre los valores de CI_{50} para la recaptación de monoaminas y los valores de la constante de afinidad (K_i) obtenidos de los ensayos de fijación a los distintos transportadores. Por lo tanto, la inhibición de estos transportadores por las catinonas parece ser el resultado de una interacción directa con dichos transportadores, compitiendo con el sustrato endógeno. Sin embargo, en el caso de la metilona, y sobre todo la mefedrona, más que una unión directa que bloquea el transportador, se apunta a un mecanismo adicional, como por ejemplo un cambio en la funcionalidad del transportador, para la inhibición de la recaptación de este neurotransmisor. Estudios posteriores (Baumann et al., 2012; Simmler et al., 2013) han confirmado tal hipótesis, en la cual tanto metilona como mefedrona actúan como sustratos del transportador, liberando neurotransmisor desde el interior del terminal hacia el espacio sináptico. Finalmente, cabe destacar, por un lado, la potencia de la mefedrona en cuanto a la inhibición de la recaptación de NA así como el efecto de la metilona inhibiendo tanto la recaptación de 5-HT como de DA, siendo esta catinona la más potente inhibiendo la recaptación de ambos neurotransmisores.

Otra cuestión importante es la inhibición que producen las anfetaminas sobre la captación de monoaminas a través del transportador vesicular (VMAT-2). Ésta es atribuida a la capacidad de estos compuestos para actuar como sustratos de este transportador, aunque existe también otro mecanismo inhibitorio a través de la disipación del gradiente de pH, como se ha descrito anteriormente. Al analizar el efecto de butilona, mefedrona y metilona, sobre el VMAT-2, observamos importantes diferencias. Al medir la captación de DA a través del VMAT-2 se observó que la potencia de metilona y butilona para inhibir el VMAT fue 7 y 27 veces, respectivamente, menor que la de la mefedrona. Estos resultados sobre la inhibición del VMAT-2 y los obtenidos tras la incubación de sinaptosomas con reserpina, en los cuales se observó una disminución de la inhibición de la captación, sugieren que tanto el componente de membrana como el componente vesicular están involucrados en el efecto inhibitorio final de estas catinonas. Concretamente, el componente vesicular es de especial importancia en el efecto de la mefedrona.

En los transportadores de 5-HT y DA existen supuestos lugares de fosforilación por proteína cinasas. Por ejemplo, las proteína cinasas A y C se encuentran implicadas en varios aspectos de la función y regulación de los transportadores, como el tráfico, transporte y la fosforilación directa (Jayanthi et al., 2005; Foster et al., 2006; Ramamoorthy et al., 2011). Por ello, una sustancia que produzca fosforilación en el transportador provocará cambios persistentes en el mismo. En nuestro estudio, la metilona indujo una inhibición persistente, especialmente en el caso de la captación de [^{3}H]5-HT, lo que indica que esta sustancia produce alteraciones en la funcionalidad del transportador.

Muchos compuestos anfetamínicos interaccionan con el receptor post-sináptico 5-HT_{2A}, mediando no sólo la liberación de DA, sino también la respuesta locomotora (Auclair et al., 2004). Por otro lado, los alucinógenos, actúan como agonistas de este tipo de receptores, siendo los responsables de sus efectos sobre el comportamiento. Como ya se ha descrito en el apartado de resultados, las tres catinonas desplazaron la unión de la [^{3}H]ketanserina de manera concentración-dependiente, con valores de K_i en el rango micromolar bajo, al igual que ocurre con la interacción MDMA-5HT_{2A}, lo que permite deducir que la activación directa de estos receptores puede contribuir en la activación serotoninérgica que producen. Por otro lado, los valores de afinidad de las catinonas sobre los receptores D_2 se encontraron en el rango micromolar alto, hecho que también coincide con la MDMA (K_i=95μM) (Battaglia et al., 1988), permitiendo descartar la posibilidad de una activación directa de dichos receptores por estas catinonas a las dosis utilizadas.

Como ya se ha descrito, la depleción de los terminales serotoninérgicos juntamente con el aumento de la liberación de DA inducida por la MDMA, podría provocar que dicha DA fuera captada hacia el interior de los terminales serotoninérgicos depleccionados, en los cuales la DA sería desaminada por la MAO-B, generando especies reactivas de oxígeno y produciendo el fenómeno de degeneración y destrucción de los axones serotoninérgicos (Sprague et al., 1994; López-Muñoz et al., 2004). Un fenómeno similar podría darse con las catinonas, a menos que éstas también inhibieran dicha incorporación. Sin embargo, las tres catinonas estudiadas inhibieron la entrada de DA a través del SERT sólo a concentraciones muy elevadas, las cuales no suelen llegar a alcanzarse *in vivo*, por lo que

podemos hipotetizar que también es posible que la DA sea incorporada mediante el SERT y oxidada en el interior de los terminales. En parte por ello, se decidió posteriormente realizar un estudio sobre la neurotoxicidad inducida por metilona en roedores (Publicación **III** y **IV**).

El sistema dopaminérgico mesolímbico es la ruta común a través de la cual las anfetaminas ejercen su efecto psicoestimulante. Los derivados anfetamínicos producen un aumento de la actividad locomotora en roedores que refleja un aumento de la transmisión dopaminérgica en el núcleo acumbens (Ljunberg and Ungerstedt, 1985; Izawa et al., 2006). Este efecto hiperlocomotor de las anfetaminas está directamente relacionado con el bloqueo de la captación de DA y con un aumento en la liberación de dicho neurotransmisor (Leviel, 2001; Escubedo et al., 2005). Nuestros resultados demuestran que las tres catinonas producen un aumento de la actividad locomotora en ratones dependiente de la dosis. Aunque utilizamos una vía de administración diferente a la utilizada por los consumidores de este tipo de sustancias, nuestros resultados se encuentran en concordancia con la cinética descrita por dichos consumidores, quienes describen que los efectos aparecen entre 15-45 minutos y la duración de sus efectos perduran hasta las 2-3 horas tras la ingesta por vía oral (*www.erowid.org*). Hasta la publicación de este primer estudio no existían datos sobre la actividad locomotora inducida por butilona o metilona. Nuestro estudio demostró que tanto la butilona como la metilona producen un efecto psicoestimulante que persiste incluso hasta 4 horas después de la administración, a la dosis más alta estudiada (25 mg/kg s.c.). Cabe destacar el notable aumento en el efecto de la metilona observado entre las dosis de 10 y 25 mg/kg, posiblemente debido a una saturación de su metabolismo, dando lugar a un aumento persistente de los niveles plasmáticos. En la publicación **II** estudiamos de una manera más profunda la relación entre los niveles plasmáticos y el efecto hiperlocomotor inducido por la metilona. Finalmente decir que, mefedrona produjo un moderado aumento de la actividad locomotora, que perduró hasta los 150 min a la dosis más alta estudiada (25 mg/kg s.c.).

Sabemos que la MDMA aumenta la liberación de DA, en parte, por una activación de los receptores 5-HT_{2A}, la cual podría contribuir en el efecto hiperlocomotor final (Yamamoto et al., 1995). Cuando a los animales se les administró, 30 minutos antes de la catinona, ketanserina (antagonista 5-HT_{2A}) o haloperidol (antagonista no-selectivo dopaminérgico), se inhibió el

aumento de la actividad locomotora inducido por las catinonas, sugiriendo la implicación tanto del sistema serotoninérgico como del dopaminérgico. La afinidad de estos compuestos por los receptores $5\text{-}HT_{2A}$, discutida al inicio de este apartado, respalda tal hipótesis. Sin embargo, su relativa baja afinidad por los receptores D_2 nos permite descartar un efecto directo sobre este tipo de receptores, lo cual nos lleva a creer que este tipo de sustancias al producir un aumento de la concentración de DA extracelular, será ésta la que finalmente interactúe con este tipo de receptores. Por otro lado, al tratar los animales con pCPA, un inhibidor de la síntesis de 5-HT, se observó que sólo aquellos animales tratados posteriormente con mefedrona mostraban una disminución significativa del efecto hiperlocomotor inducido por dicha catinona, lo que sugiere una importante participación de la 5-HT endógena en el caso de la mefedrona.

Se cree que el aumento de la actividad locomotora y, principalmente, del número de "rearings" (término que se usa para describir cuando el animal se encuentra posado tan sólo sobre sus dos patas traseras), inducido por la MDMA en ratones, se produce a causa de una activación de los receptores $5\text{-}HT_{1B}$ (McCreary et al., 1999), involucrados en la liberación de 5-HT (Clark et al., 2002). En el caso de la butilona, se observó un claro aumento del número de "rearings" en el momento en el cual la actividad locomotora empezaba a disminuir. Al pre-tratar los animales con SB-216 641 (un antagonista selectivo de los receptores $5\text{-}HT_{1B}$) se observó tanto una inhibición total del aumento del número de "rearings", como una inhibición parcial del aumento de la actividad locomotora. Por ello, parece corroborarse que este comportamiento es consecuencia de la activación de los receptores $5\text{-}HT_{1B}$, los cuales parecen jugar también un papel en el efecto hiperlocomotor inducido por la butilona.

En resumen, los resultados hasta aquí comentados proporcionan claras evidencias que algunos derivados de la catinona interaccionan con los transportadores y receptores de 5-HT y DA, sugiriendo un perfil neurofarmacológico similar al de otras drogas psicoactivas como los compuestos de tipo anfetamínico.

4.2. Farmacocinética y modelización PK/PD de la metilona

Los datos que existen sobre la farmacocinética de la metilona en humanos provienen básicamente de la información proporcionada por los consumidores a través de foros de internet (Boulanger-Gobeil et al., 2012; Shimizu et al., 2007). Según esta información, la dosis más común se encuentra comprendida entre los 150 y los 300 mg de metilona ingeridas por vía oral, cuyos efectos aparecen, aproximadamente, 30 minutos después de su ingesta, con una duración de entre 2 y 3,5 horas. Sin embargo, la gran mayoría de estudios realizados sobre la farmacocinética de la metilona en humanos provienen de informes forenses (Crawse et al., 2012; Kamata et al., 2006).

En nuestros estudios, la metilona ejerció un fuerte efecto psicoestimulante en ratas Sprague-Dawley, medido a través de la actividad locomotora. La aparición y duración de dicho efecto no se aleja del efecto subjetivo descrito por los consumidores. Las dosis orales en rata utilizadas en este estudio, 15 y 30 mg/kg p.o., equivalen en humanos, si tenemos en cuenta las directrices de la FDA (Food Drug Administration Center for Drug Evaluation Research, 2005), a dosis de aproximadamente 158 y 315 mg, respectivamente. Cabe destacar el aumento de la actividad locomotora entre las dosis de 15 y 30 mg/kg por vía oral, a causa principalmente de diferencias sobre el perfil hiperlocomotor. La dosis de 30 mg/kg p.o. produjo una respuesta locomotora máxima la cual no fue significativamente diferente de la producida tras la administración de una dosis de 15 mg/kg p.o., sin embargo, se observó una prolongación de sus efectos. Tal aumento en la duración de los efectos, pero no en la respuesta máxima, se ha descrito en ratones tanto en la publicación **I**, como en otros estudios (Marusich et al., 2012).

Tras la administración por vía intravenosa (10 mg/kg), los niveles en sangre de la metilona se ajustaron a un modelo bifásico (α=1.95 h^{-1}; β=0.72 h^{-1}). El elevado valor de V_{ss} indica que la metilona se distribuye de manera extensa y el valor de Clp explica además su corto tiempo de semivida (1h). Por otro lado, la administración oral de metilona se ajustó a una farmacocinética lineal ya que los niveles en sangre fueron directamente proporcionales a la dosis administrada. El movimiento de la Tmax de 30 a 60 minutos al utilizar

la dosis más alta estudiada (30 mg/kg p.o.) se explica en parte por el valor de la k_a y el aumento del tiempo de latencia.

El tiempo de semivida de la metilona tras la administración oral fue significativamente más elevado que tras la administración intravenosa, lo que nos lleva a pensar que su farmacocinética se ajusta a un modelo *flip-flop*. Dicho modelo es un fenómeno que suele aparecer cuando la tasa de absorción del compuesto es mucho más lenta que su tasa de eliminación. Nuestro modelo confirmó la presencia de dicho fenómeno, ya que la tasa de absorción de la metilona (con un valor de $t_{1/2abs}$ de entre 2 y 3 horas) es considerablemente más lenta que la de eliminación ($t_{1/2\beta}$ de 0.55 horas).

En nuestro estudio, la metilona se unió un 30% a proteínas plasmáticas, a concentraciones cercanas a la Tmax, y el aclaramiento metabólico constituyó un 29% del aclaramiento total. El Clp calculado para la metilona para ambas dosis orales se encuentra alrededor de los 0.5 l/h. Este valor parece exceder tanto el flujo sanguíneo hepático como renal en rata (Birnie and Grayson, 1952; Heller and Hollyová, 1977). Tales valores *in vivo* hacen suponer que tanto el riñón, como el hígado, y posiblemente otros órganos se encuentran involucrados en la eliminación de la metilona a las dosis estudiadas.

Al estudiar en sangre los metabolitos de Fase I de la metilona, tras la administración oral de 30 mg/kg, se identificaron 4 metabolitos, todos ellos detectados a tres tiempos diferentes (60, 120 y 180 minutos post-administración). La ruta metabólica propuesta consta de, en primer lugar, una reacción de desmetilación, dando lugar a la correspondiente amina primaria, la metilendioxicatinona (**MDC**), estructuralmente relacionada con uno de los metabolitos activos de la MDMA, la MDA. Además creemos que la metilona puede ser sustrato de una hidroxilación alifática, dando lugar a la 3'-hidroxi-metilendioximetcatinona (**3'-OH-MDMC**). Como ya se ha descrito en la introducción, Mueller y Rentsch (2012), utilizando microsomas hepáticos humanos, también propusieron a la 3'-OH-MDMC como un metabolito de la metilona, aunque nuestro estudio ha sido el primero en identificar *in vivo* dicho metabolito. Estos mismos autores identificaron además un tercer metabolito ciertamente previsible, la correspondiente reducción del grupo cetónico de la metilona, sin embargo, ni nuestro estudio *in vivo* en sangre de rata ni el publicado por otros autores

en orina (Kamata et al., 2006) han podido detectar tal metabolito. También se identificaron dos metabolitos hidroxilados, la 4-hidroxi-3-metoximetcatinona (**4-OH-3-MeO-MC**) y la 3-hidroxi-4-metoximetcatinona (**3-OH-4-MeO-MC**), con la misma fórmula molecular y espectro de masas, provenientes de una desmetilenación (apertura del anillo metilendioxi) y posterior O-metilación. Sin embargo, el metabolito intermedio correspondiente a dicha apertura del anillo no pudo ser identificado, probablemente a causa de su corto tiempo de vida. Ello se corrobora porque las cantidades de estos dos metabolitos hidroxilados, medida a través del área bajo el pico cromatográfico, fueron realmente bajas. Esta observación no fue nada sorprendente, ya que según Kamata y col. (2006), estos dos metabolitos se encuentran conjugados en más de un 80% cuando son excretados en orina. Todo ello parece indicar que el metabolismo de la metilona contribuye en gran medida a su aclaramiento plasmático, contribución especialmente evidente cuando la sustancia es administrada por vía oral, como resultado de un importante efecto de primer paso hepático, tal y como se puede observar por el diferente perfil farmacocinético entre ambas rutas de administración.

El análisis farmacocinético-farmacodinámico nos permitió realizar una estimación de los parámetros CE_{50} y Emax, como también información sobre el momento de aparición, magnitud y duración de la actividad locomotora inducida por la metilona en relación a sus concentraciones plasmáticas. Inicialmente, tras su administración oral se observó un retraso entre la respuesta psicomotora y los niveles plasmáticos, por lo que dichos niveles parecían no estar directamente relacionados con su efecto farmacológico. Sin embargo, al definir en el modelo un lugar-compartimiento de efecto se evidenció una estrecha relación entre las concentraciones obtenidas en el hipotético lugar de efecto y la respuesta motora.

El ajuste de los valores de actividad locomotora en función de las concentraciones plasmáticas de metilona, a la dosis de 15 mg/kg p.o., mostró lo que se conoce como un ciclo antihorario de histéresis. Este tipo de ciclo de histéresis se puede explicar por: a) la presencia de metabolitos activos (Mandema et al., 1992); b) un mecanismo de acción indirecto (Dayneka et al., 1993); c) un desequilibrio entre el lugar de acción (el cerebro en este caso) y el compartimiento plasmático (Sheiner et al., 1979).

Debido a la presencia de algún metabolito de la metilona, similar estructuralmente a algún metabolito activo de la MDMA (De la Torre and Farré, 2004), es probable que pueda participar en el efecto hiperlocomotor final, aunque no podemos descartar otras posibilidades (Csajka and Verotta, 2006) hasta que no se determine individualmente qué metabolito o metabolitos son los responsables de contribuir al aumento de la actividad locomotora inducida por la metilona.

Como ya se ha descrito en la publicación **I**, la metilona inhibe la recaptación de monoaminas por competición con el sustrato. Por ello, la metilona produce un aumento de la actividad locomotora principalmente a través de un mecanismo indirecto, un aumento de la concentración de DA extracelular, como también por un mecanismo directo a través de la activación de los receptores 5-HT_{2A}. La menor afinidad de la metilona por los receptores 5-HT_{2A} que por los DATs nos permite deducir que la activación directa de estos receptores puede contribuir al efecto final cuando se alcanzan concentraciones más elevadas en el compartimiento del lugar de acción. Por ello, a la dosis más elevada estudiada (30 mg/kg p.o.), la aparente ausencia del ciclo antihorario de histéresis se puede explicar por: a) un mecanismo de acción directo adicional (a través de la activación de los receptores 5-HT_{2A}); b) un menor desequilibrio entre las concentraciones en cerebro y plasma a esta dosis.

4.3. Neurotoxicología de la metilona en roedores

Ya hemos comentado que existen estudios que demuestran que la metilona es un inhibidor competitivo y no selectivo de la recaptación de monoaminas, promoviendo además la liberación de 5-HT, y en menor medida, de DA (Publicación **I**; Baumann et al., 2012; Eshleman et al., 2013; Simmler et al., 2013; Sogawa et al., 2011). Este mecanismo es de especial importancia, ya que sólo los inhibidores de la recaptación que son sustratos del transportador, como en el caso de las anfetaminas, y no los bloqueadores del transportador, como la cocaína, producen disfunciones a largo plazo en las células monoaminérgicas (Fleckenstein et al., 2007).

A menudo se afirma en foros de consumidores de MDMA y otras drogas de abuso, que los datos sobre los efectos adversos a largo plazo obtenidos en

animales de experimentación no son relevantes al uso en humanos, ya que la dosis administrada es mucho mayor que la utilizada por los humanos. En nuestros estudios de neurotoxicidad, para simular el consumo recreacional de la metilona en humanos, hemos utilizado la pauta ampliamente generalizada de consumir grandes cantidades y dosis adicionales ("binge"), con la intención de mantener el efecto psicoestimulante concentrado en un espacio de tiempo limitado. Por ello hemos administrado múltiples dosis en uno o varios días en cada tratamiento.

Es cierto que un gran número de estudios han utilizado o utilizan elevadas dosis, aunque parece ser que dependiendo de la cepa la susceptibilidad a la droga varía. Como se ha mencionado anteriormente, es difícil extrapolar o comparar los resultados obtenidos en animales de laboratorio. Sin embargo, se ha propuesto que la técnica de escalado entre especies permitiría predecir la eliminación de la droga en diferentes especies, basándose en las similitudes anatómicas, fisiológicas y bioquímicas entre mamíferos. Por ello, para conseguir un efecto similar a los observados en humanos, los animales más pequeños requieren dosis más elevadas. Sin embargo, la validez sobre el escalado entre especies en relación a la MDMA y otras drogas de abuso ha sido discutida por algunos autores, quienes opinan que la equivalencia de dosis debe basarse en la mínima dosis que pueda causar un efecto farmacológico. Sin embargo, las cantidades que ingieren los consumidores de este tipo de sustancias no suelen ser las mínimas capaces de producir el efecto deseado, sino que se tiende a abusar de su consumo. Existen además muchas otras diferencias, como puede ser la vía de administración, la respuesta farmacodinámica, la farmacocinética y el metabolismo, entre los estudios en animales y el uso de la MDMA y otras drogas en humanos. En nuestros estudios sobre la neurotoxicidad inducida por metilona, hemos utilizado unas dosis en animales de experimentación que equivalen a dosis en humanos de entre 2-3 mg/kg (Food Drug Administration Center for Drug Evaluation Research, 2005; Reagan-Shaw et al., 2008), o lo que es lo mismo, dosis de 150-200 mg en individuos de 60-70 kg de peso aproximadamente, consideradas muy habituales entre los consumidores de esta catinona.

Baumann y col. (2013) describen que, en humanos, los efectos adversos de las conocidas como "bath salts", se pueden ver incrementados en espacios abarrotados de gente y con una temperatura ambiente relativamente elevada,

tal y como ocurre en las llamadas fiestas “rave”, donde se suelen consumir este tipo de psicoestimulantes. Además, muchos factores, y especialmente la hipertermia, parecen contribuir en la neurotoxicidad producida por las anfetaminas (Sánchez et al., 2004). Parece existir una influencia de la temperatura ambiente sobre la respuesta producida/inducida por la MDMA, ya que se observa una notable hipertermia tras la administración de dicha sustancia cuando los experimentos se llevan a cabo en espacios con una temperatura ambiente elevada. De hecho, la hipertermia es un efecto adverso muy común del consumo de estas β-ceto anfetaminas en humanos (Borek and Holstege, 2012; Prosser and Nelson, 2012). En todos nuestros estudios de neurotoxicidad, tanto en rata como en ratón, los experimentos se realizaron bajo una temperatura ambiente elevada (26 ±1 ºC). Bajo estas condiciones, la metilona produce una clara hipertermia en ambos animales de experimentación. En el caso del ratón, dicho efecto es más notable en el primer día de tratamiento, y disminuye cuando la droga se vuelve a administrar al día siguiente, por lo que parece existir una cierta tolerancia, probablemente a causa de la merma de las reservas de neurotransmisor.

En el caso de la rata, pudimos utilizar unos implantes electrónicos que registraron la temperatura corporal a intervalos de 5 minutos, permitiéndonos obtener un perfil de temperatura mucho más preciso. En este caso, observamos que la hipertermia, producida tras la administración de metilona, alcanzaba niveles máximos 30 minutos después de cada administración. Además, dicho efecto aumentó en cada administración, llegando en algunos animales a alcanzarse temperaturas corporales superiores a los 40ºC tras la última administración. Al estudiar la evolución del peso durante los tratamientos con metilona, ambas especies mostraron también una importante pérdida de peso, tal y como ocurre con otros derivados anfetamínicos, posiblemente a causa de un efecto anorexígeno de la droga.

Como ya se ha descrito anteriormente, la metilona es un análogo estructural de la MDMA, y en consecuencia podría afectar de un modo diferente al sistema dopaminérgico o serotoninérgico, dependiendo de la especie animal utilizada en los experimentos. Concretamente, los ratones difieren de otras especies animales ya que muestran mayores daños dopaminérgicos que serotoninérgicos tras un tratamiento con MDMA. A diferencia de la MDMA (Chipana et al., 2006), la metilona no produjo alteraciones en la densidad de

transportadores de DA, como tampoco en los niveles de expresión de tirosina hidroxilasa (TH) en estriado, tanto de ratón como de rata. Sin embargo, en ratón, la metilona produjo una perdida transitoria de transportadores de DA, en corteza frontal. La disminución inicial y posterior recuperación de transportadores de DA, acompañada de la ausencia de diferencias significativas sobre los niveles de expresión de tirosina hidroxilasa, respecto al grupo control, indican una posible regulación bioquímica en ausencia de daño o lesión sobre los terminales dopaminérgicos. En rata, sólo se detectó una leve disminución, aunque no significativa, de la densidad de DATs y de los niveles de expresión de TH en corteza frontal, 7 días después del tratamiento.

La principal diferencia que se observó, entre los dos tipos de tratamientos administrados al ratón, fue sobre los terminales serotoninérgicos. Cuando la metilona es administrada, durante dos días consecutivos, 3 dosis por día, con un intervalo entre dosis de 3,5 horas (tratamiento A), se produce una disminución muy leve y transitoria de dichos marcadores. Sin embargo, cuando se administran 4 dosis en un solo día, con un intervalo entre dosis inferior (3 horas) (tratamiento B), se produce un efecto persistente sobre los marcadores serotoninérgicos en hipocampo y corteza frontal, con una disminución tanto de la densidad de SERTs como de los niveles de expresión de triptófano hidroxilasa 2 (TPH-2). Ello se acompaña de una activación astroglial en las regiones CA1 y giro dentado del hipocampo como consecuencia de una posible lesión de los terminales neuronales en estas áreas. Hasta la fecha de realización de la presente Tesis Doctoral, sólo existía un estudio publicado por den Hollander y col. (2013) sobre los efectos neurotóxicos en el sistema dopaminérgico y serotoninérgico de ratón. A diferencia de nuestros resultados, dichos autores no observaron ningún efecto sobre los niveles de neurotransmisores en ratones C57BL/J6 dos semanas después del tratamiento (30 mg/kg, dos veces al día, durante 4 días). Tal divergencia de resultados se podría explicar tanto por el uso de cepas de ratón diferentes y un tiempo de sacrificio distinto, como también por las condiciones de temperatura ambiente elevada utilizadas en nuestros estudios.

En la rata, administrando un régimen similar al utilizado en ratón (20 mg/kg, 4 veces al día, intervalo 3h), la metilona produjo una disminución de los marcadores específicos de los terminales serotoninérgicos (SERTs y

niveles de expresión TPH-2) en estriado, hipocampo y sobretodo en corteza frontal y ausencia de activación microglial. En cambio sí que se detectó una activación astroglial en corteza frontal, área en la cual los cambios neuroquímicos eran mucho más pronunciados. Existía la posibilidad de que la aparente disminución en el número de SERTs fuera provocada por una disminución en la afinidad a la unión a [^{3}H]paroxetina, producida por la interacción de la metilona con el SERT. De hecho, previamente ya habíamos descrito que la inhibición sobre la recaptación de 5-HT producida por la metilona puede persistir incluso después de la retirada del compuesto del medio (Publicación **I**). Con la intención de aclarar este punto realizamos los correspondientes ensayos de saturación con [^{3}H]paroxetina, observando una disminución significativa de los valores de B_{max}, pero no en la afinidad por el radioligando (K_d) en el grupo tratado con metilona. Este resultado nos permite concluir que la disminución observada en los ensayos de unión con radioligando no se debe a una disminución en la afinidad, sino a una disminución de la densidad de SERTs. Den Hollander y col. (2013) también observaron una disminución de los SERTs en hipocampo y corteza frontal de rata, así como una clara disminución de los niveles de 5-HT en estriado, hipocampo y corteza frontal. Sin embargo, Baumann y col. (2012), al examinar los niveles de neurotransmisor en rata, 2 semanas después del tratamiento con metilona, no observaron cambios. Cabe decir que, en este último estudio, los autores, no observaron una marcada hipertermia, y tanto la dosis como el número de dosis fue inferior al utilizado en nuestro estudio y en el de den Hollander y col. (2013), lo cual podría explicar las diferencias existentes.

Las conclusiones que se pueden extraer de todas estas comparaciones son, que tanto la hipertermia como el régimen de administración (dosis, número de dosis e intervalo entre dosis) parecen jugar un papel crucial en la disfunción serotoninérgica producida por la metilona. Además, creemos que las condiciones de temperatura ambiente elevada podrían jugar también un papel importante sobre sus efectos neurotóxicos, aunque serían necesarios futuros estudios para poder evaluar si la hipertermia y dichas condiciones son complementarias o esenciales en la neurotoxicidad inducida por la metilona en ambos animales de experimentación.

La participación de algún metabolito de la metilona sobre su efecto neurotóxico no ha sido aún demostrada, sin embargo, tal y como se ha

expuesto en la Publicación **II,** la ruta metabólica de la metilona se asemeja a la de la MDMA, dando lugar a metabolitos estructuralmente similares y probablemente con potencial neurotóxico.

La predominante lesión de los terminales serotoninérgicos sobre los dopaminérgicos observada, concuerda con el estudio publicado por Sogawa y col. (2011), cuyos resultados *in vitro* demostraron que la metilona es citotóxica en células CHO en las cuales se encuentra expresado el SERT, sin producir ningún efecto sobre aquellas en las cuales se encuentran expresados el DAT o el NET.

Al estudiar las consecuencias conductuales en ratón, derivadas de los tratamientos con metilona, se observó un aumento del tiempo de inmovilidad en el test de natación forzada, lo que indica la aparición de un comportamiento depresivo, tal y como ocurre con la MDMA (McGregor et al., 2003). Este efecto fue evidente 3 días después del tratamiento, momento en el cual la reducción de los marcadores dopaminérgicos y serotoninérgicos fue mayor. Cabe destacar que, en la patofisiología de la depresión, pueden estar también involucrados cambios sobre los receptores 5-HT_2 en algunas regiones del cerebro implicadas en la regulación del estado de ánimo. Tras ambos tratamientos (A y B) en ratón, se detectó una disminución similar y transitoria de los receptores 5-HT_{2A} corticales, aunque en el caso del tratamiento B dicha disminución no fue significativa. Esas disminuciones podrían ser el resultado de una respuesta neuroadaptativa a causa de la liberación masiva de 5-HT inducida por la metilona (Scheffel et al., 1992).

Finalmente decir que, al estudiar las consecuencias conductuales en rata, no se evidenciaron deficiencias en el aprendizaje espacial. Sin embargo, sí que se observaron alteraciones sobre la memoria espacial. La memoria espacial evidenciada en el laberinto acuático de Morris responde a una integridad de la neurotransmisión en hipocampo (Squire, 1993). Los resultados conductuales podrían correlacionarse con la lesión neuroquímica evidenciada en el hipocampo de rata.

El abuso de drogas sigue siendo, hoy en día, motivo de gran preocupación debido, en gran medida, a sus efectos perjudiciales sobre la salud y sus consecuencias sociales. A pesar del actual estado ilegal de estas nuevas

drogas psicoestimulantes en algunos países, las catinonas sintéticas prevalecen como drogas de abuso. Tanto los datos aquí expuestos, como los publicados por otros investigadores claramente demuestran el potencial toxicológico a nivel del SNC y cardiovascular, además del elevado riesgo de muerte, abuso, tolerancia y dependencia producido por el consumo de estas nuevas catinonas. Sin embargo, existe todavía un amplio desconocimiento del perfil farmacológico y toxicológico de este tipo de sustancias. Los resultados de esta Tesis representan una aportación significativa al mejor conocimiento de estas sustancias pero todavía queda mucho por investigar. Por ello, no cabe duda que es necesario incentivar su estudio, tanto a nivel clínico como pre-clínico, así como la prevención de su consumo en humanos.

CONCLUSIONES

5. CONCLUSIONES

La metilona, y el resto de catinonas estudiadas, interaccionan con los transportadores y receptores dopaminérgicos y serotoninérgicos, sugiriendo un perfil farmacológico similar al de otras drogas psicoestimulantes como las anfetaminas.

5.1. Perfil psicoestimulante (A)

A-1) La metilona, al igual que el resto de catinonas estudiadas produce un aumento de la actividad locomotora dosis-dependiente.

A-2) La metilona y la butilona producen dicho efecto debido a una activación directa de los receptores 5-HT_{2A} y a un aumento de la dopamina extracelular.

A-3) La mefedrona produce un aumento de la actividad locomotora dependiente de la serotonina endógena. El contenido vesicular juega un papel importante, especialmente en la recaptación de serotonina.

A-4) *In vitro*, la metilona es la catinona estudiada más potente inhibiendo la recaptación de dopamina y serotonina, por competición con el sustrato. Tal inhibición persiste incluso después de la retirada del compuesto.

5.2. Perfil farmacocinético (B)

B-1) El perfil farmacocinético de la metilona por vía oral en rata se ajusta a un modelo *flip-flop*, en el cual la tasa de absorción es relativamente más lenta que la de eliminación.

B-2) El análisis PK/PD de la metilona muestra una estrecha correlación entre los niveles plasmáticos y el aumento de actividad locomotora.

B-3) La identificación de algunos de los metabolitos de la metilona, similares a los metabolitos activos de la MDMA, sugiere su posible participación en el efecto psicoestimulante.

5.3. Perfil neurotóxico (C)

C-1) Los cambios neuroquímicos inducidos por metilona son más aparentes cuando ésta es administrada 4 veces al día cada 3 horas, mostrando una disminución de los marcadores de los terminales neuronales serotoninérgicos en corteza frontal e hipocampo, juntamente con una activación astroglial en la región del giro dentado y la CA1 del hipocampo una semana después del tratamiento.

C-2) A diferencia de la neurotoxicidad especie-dependiente de la MDMA, la metilona induce alteraciones en el cerebro de rata que no difieren cualitativamente de las observadas en ratón utilizando un régimen de administración similar.

C-3) La metilona produce, en ratón, efectos depresivos tras un régimen de administración neurotóxico. Tras un tratamiento similar en rata, la metilona provoca una disfunción en la memoria referencial.

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ANEXOS

7.1. Anexo I (Publicación V)

Mephedrone pharmacokinetics after intravenous and oral administration in rats: relation to pharmacodynamics

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ORIGINAL INVESTIGATION

Mephedrone pharmacokinetics after intravenous and oral administration in rats: relation to pharmacodynamics

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Abstract

Rationale Mephedrone (4-methylmethcathinone) is a still poorly known drug of abuse, alternative to ecstasy or cocaine.

Objective The major aims were to investigate the pharmacokinetics and locomotor activity of mephedrone in rats and provide a pharmacokinetic/pharmacodynamic model.

Methods Mephedrone was administered to male Sprague–Dawley rats intravenously (10 mg/kg) and orally (30 and 60 mg/kg). Plasma concentrations and metabolites were characterized using LC/MS and LC-MS/MS fragmentation patterns. Locomotor activity was monitored for 180–240 min.

Results Mephedrone plasma concentrations after i.v. administration fit a two-compartment model (α=10.23 h^{-1}, β= 1.86 h^{-1}). After oral administration, peak mephedrone concentrations were achieved between 0.5 and 1 h and declined to undetectable levels at 9 h. The absolute bioavailability of mephedrone was about 10 % and the percentage of mephedrone protein binding was 21.59±3.67 %. We have identified five phase I metabolites in rat blood after oral administration. The relationship between brain levels and free plasma concentration was 1.85±0.08. Mephedrone induced a dose-dependent increase in locomotor activity, which lasted up to 2 h. The pharmacokinetic–pharmacodynamic model successfully describes the relationship between mephedrone plasma concentrations and its psychostimulant effect.

Conclusions We suggest a very important first-pass effect for mephedrone after oral administration and an easy access to the central nervous system. The model described might be useful in the estimation and prediction of the onset, magnitude, and time course of mephedrone pharmacodynamics as well as to design new animal models of mephedrone addiction and toxicity.

Keywords Mephedrone · Pharmacokinetics · Locomotor activity · PK/PD modeling · Rat

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Introduction

Mephedrone is a synthetic ring-substituted cathinone closely related to the phenethylamine family, differing only by a keto functional group at the beta carbon. It can be purchased online or from street dealers. On the Internet, mephedrone is often marketed as “plant food,” “bath salts,” or “research chemical.” The rapid rise in the popularity of mephedrone may reflect growing user dissatisfaction with the purity and availability of MDMA (ecstasy) or its chemical precursors and cocaine. Mephedrone appears to be used by several population groups, including people involved in the dance and music scene as well as mainstream young adults and adolescents (Schifano et al. 2011).

Mephedrone is known to have similar effects to other psychostimulant drugs (Brunt et al. 2012; Varner et al. 2013). There are two reported fatalities in the European Union in which mephedrone appears to be the sole cause of death, and there are at least another 37 deaths in which mephedrone has been detected in postmortem samples (Maskell et al. 2011).

Mephedrone was the first cathinone derivative to be "risk-assessed" by the extended Scientific Committee of the European Monitoring Centre for Drugs and Drug Addiction.

Information on mephedrone human pharmacokinetics is limited to user reports. From these, it appears that both the desired and adverse effects of mephedrone are similar to those found for MDMA and cocaine (Wood et al. 2010b). However, its relatively short duration of action, which leads to repeated dosing, is more similar to cocaine (Wilkinson et al. 1980). In a recent study, 70 % of mephedrone users reported to be using it by nasal insufflation, while 30 % admitted to be taking it orally (Dickson et al. 2010; Winstock et al. 2011). There are numerous reports of individuals using mixed routes during a single session. Moreover, there are increasing reports of intravenous injection, and there is also one case report from the UK of an individual who developed acute mephedrone toxicity after an intramuscular injection of dissolved powder (Wood et al. 2010a).

Users report on Internet forums that the desired effects are typically seen within 15–45 min after oral ingestion and stated that the onset of action is slower when mephedrone is taken orally on a full stomach. Users also report onset of action to be within a few minutes following nasal insufflation, with peak desired effects after 30 min. The desired effects last approximately 2–3 h; this leads users to redose during a single session to extend the duration of the desired effects.

Studies on the pharmacokinetics of mephedrone in rats are very scarce. Meyer et al. (2010) suggested that the most probable metabolites of mephedrone are present in the urine. In this study, mephedrone was administered orally and urine was collected over a 24-h period. Hadlock et al. (2011) assessed mephedrone concentrations in rat brain and plasma 1 h after binge treatment and more recently (Khreit et al. 2013) investigated the in vitro metabolism of mephedrone in rat-isolated hepatocytes.

The current studies were designed to investigate the pharmacokinetic profile and pharmacodynamic response to mephedrone in rats after its acute administration by intravenous and oral route at different doses using a highly sensitive and specific analytical methodology based on liquid chromatography–tandem mass spectrometry (LC-MS/MS). Results from this study might provide useful tools in order to design new animal models of mephedrone addiction and toxicity. This objective was first pursued by constructing a pharmacokinetic/pharmacodynamics (PK/PD) model for mephedrone in rats in order to obtain basic guidelines for human risk assessment. In this study, the following main pharmacokinetic parameters were estimated: total area under the curve ($AUC_{0-\infty}$), apparent volume of distribution at steady state (V_{ss}), total plasma clearance (Cl_p), metabolic clearance (Cl_{met}), and elimination half-life ($t_{1/2\beta}$). The pharmacodynamic studies described herein focused on the evaluation of the psychostimulant effect of this drug of abuse, trying to establish a correlation between the hyperlocomotion induced by mephedrone and its plasma levels. Furthermore, other goals of this study were to analyze the metabolic pathway of mephedrone and its brain concentration after an oral administration as well as to establish a relationship between mephedrone brain concentration and its psychostimulant effect.

Materials and methods

Drugs

Pure racemic mephedrone and methylone HCl were synthetized and characterized by us as described previously (López-Arnau et al. 2012). Mephedrone solutions for injection were prepared in saline immediately before administration. Isoflurane was from Laboratorios Dr. Esteve (Barcelona, Spain). Reagents required for LC-MS assays were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

The experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona, following the guidelines of the European Community Council (86/609/EEC). Male Sprague–Dawley rats (Janvier, Le Génest, France) weighing 225–250 g were used. Animals were housed at 22±1 °C under a 12-h light/dark cycle with free access to food and drinking water.

Pharmacokinetic experiments

For intravenous pharmacokinetic analysis, mephedrone was administered at a dose of 10 mg/kg. For oral pharmacokinetic experiments, mephedrone was administered at doses of 30 and 60 mg/kg to rats previously fasted for 18 h. A total of seven to nine animals per dose were used.

Blood samples (150–200 μl) were collected from isoflurane-anesthetized rats through a venipuncture of the external jugular vein in a time schedule from 0.08 to 9 h and transferred to 1-ml glass tubes (containing 10 μl EDTA 20 mg/ml) on ice. A total of four to five blood samples were obtained from one animal. After each blood extraction, an equal volume of sterile saline was infused to maintain volume and osmotic homeostasis. In some animals, blood samples were obtained 45 min after oral administration (dose of 30 mg/kg), followed by decapitation and removal of the whole brains. Blood samples from these animals were also used to perform protein binding studies.

Blood samples were centrifuged at 1,000×*g* for 10 min to obtain the plasma. Of the plasma samples, 90 μl was mixed with 10 μl of internal standard (IS) solution (methylone, 200 ng/ml). The mixture was extracted by adding 250 μl of methanol up to a final concentration of 70 %. The denatured proteins were precipitated by centrifugation at 10,000×*g* for 5 min. Of the clear supernatant, 250 μl was acidified with formic acid (50 %, *v*/*v*) to a pH of 2.5–3.0 to obtain stable extracts because in non-acidified live blood extracts, cathinones degraded relatively fast (Sørensen 2011). The mixture was transferred to an ultrafiltration filter cup and high-molecular-weight components were removed by means of filtration through a 30-kDa regenerated cellulose membrane (Microcon 30®, Millipore, Bedford, MA, USA). The ultrafiltration unit was centrifuged at 20,000×*g* for 10 min and 100 μl of the filtrate was transferred to an autosampler vial.

An HP Agilent Technologies 1100 LC system equipped with an autosampler and a column oven set to 40 °C and coupled API 3000 triple-quadrupole MS (AB Sciex) with a turbo ion spray source was used to quantify the corresponding cathinone. Chromatographic separation was achieved on a Luna HST C18 (100×2-mm i.d., 2.5 μm) column and controlled by Analyst software. The mobile phase was water (A) and methanol (B) with 0.1 % of formic acid in both solvents. An increasing linear gradient (*v*/*v*) of B was used (*t*(min), %B) as follows—(0, 5), (20, 95), (22, 95), (22.5, 5), and (32.5, 5)—at a constant flow rate (150 μl/min). The biological samples were refrigerated at 4 °C in the autosampler and 5 μl was injected into the LC-MS/MS system. The LC–electrospray ionization (ESI)–MS/MS conditions were optimized by direct infusion of cathinone standards (1 μg/ml) dissolved in 50:50 (*v*/*v*) water (0.1 % formic acid)/methanol (0.1 % formic acid) into the MS at a constant flow rate (5 μl/min). The turbo ion spray source was used in positive mode with a capillary voltage of +4,500 V and N_2 as the nebulizer gas. For mephedrone, one transition was followed (*m/z* 178.1→160.0, collision energy of 17 V). Two transitions were followed for methylone (*m/z* 208.1→190.1 and 208.1→160.0, collision energies of 17 and 22 V respectively), and both were used for the quantification.

Blood metabolite determination

Blood samples were collected at 30, 60, and 120 min after oral administration at a dose of 30 mg/kg. The metabolite samples were treated as described above, without IS. For the metabolite identification, a Linear Trap Quadrupole Orbitrap Velos MS equipped with an ESI source was used, coupled to an Accela chromatograph, a refrigerated autosampler, and a photodiode array detector (Thermo Scientific, Hemel Hempstead, UK). Chromatographic separation was achieved on a Luna C18 (100×2.1-mm i.d., 3 μm) column. The mobile phase was the same as the one used in the pharmacokinetic studies, with an increasing linear gradient (*v*/*v*) of B (*t*(min), %B) as follows—(0, 2), (20, 95), (22, 95), (25, 2), and (30, 2)—at a constant flow rate (150 μl/min). The injection volume was 10 μl. Data were acquired in Fourier transform mass spectrometry mode. Operation parameters were as follow: source voltage, 3.5 kV in positive or negative ion mode; S-Lens RF levels, 60 %; capillary temperature was fixed at 275 °C; sheath gas, 40 arbitrary units; and auxiliary gas, 10 arbitrary units. MS^2 acquisition was carried out using collision energy between 35 and 50 %.

Protein binding and brain levels

In protein binding experiments, samples were divided into two. One half was filtered through centrifugal filter units (Centrifree YM-30, Millipore) for comparison with the other unfiltered half. Plasma samples were extracted as described above. The extraction of brain samples was carried out as described by Hadlock et al. (2011), and brain mephedrone levels and protein binding assays were quantified as described in the pharmacokinetic experiments.

Calibration

The plasma and brains from untreated rats were used to obtain the calibration curves. In the plasma analysis, seven standards were prepared daily in 100 μl of blank plasma (from 10 to 6,000 ng/ml). To determine brain mephedrone levels, five standards were prepared, also daily, in 0.5 ml of brain homogenate (from 10 to 250 ng/ml). Methylone was used as the IS at the final concentration of 200 ng/ml for plasma levels and 50 ng/ml for brain levels. The method showed linearity within the concentration range studied, and the limit of quantification was considered lower than 10 ng/ml. Quality control samples were prepared at 50, 1,000, and 5,000 ng/ml for plasma and 20, 50, and 200 ng/ml for brain analyses. The accuracy of the assay was 90–110 % and the intra- and inter-assay coefficients of variation (CV) were <15 %.

Locomotor activity experiments

Prior to experiments, animals received two habituation sessions (48 and 24 h before testing). During these sessions, each rat was intravenously or orally administered saline and placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of two sets of 16 infrared photocells (LE8811, PANLAB, Barcelona, Spain) mounted according to the *x*- and *y*-axis coordinates and 2.5 cm above the wire mesh floor. Occlusions of the photo

beams were recorded and sent to a computerized system (SedaCom32, PANLAB). The interruption counts, over a 10-min block, were used as a measure of horizontal locomotor activity. After intravenous or oral drug administration, locomotor activity was monitored for 180 and 240 min, respectively. On the testing day, the animals received mephedrone intravenously (10 mg/kg) or orally (30 or 60 mg/kg) and were immediately placed in the activity box. Registration of horizontal locomotor activity then began.

Pharmacokinetic/pharmacodynamic modeling

PK/PD analysis was carried out on mean and standard deviation data. Because experimental data were obtained in parallel assays, data fitting was performed with the aggregates of the different doses (data pooling) in order to estimate a unique set of parameters. Assessment of the relative goodness of fit of each proposed model to the observed data was based on the objective function value, the Akaike's information criterion (AIC), the residual and weighted residual plots, and the errors in parameter estimation, expressed as the CV (in percent). PK and PK/PD analyses were achieved using the compartmental modeling SAAM II software system (SAAM Institute, Seattle, WA, USA).

Pharmacokinetic analysis

The distribution and elimination characteristics of mephedrone were determined after i.v. administration. Fixing the parameters obtained in the i.v. model, 30 and 60 mg/kg (p.o.) mephedrone profiles were analyzed simultaneously using a bicompartmental model with oral delay and Michaelis–Menten metabolism kinetics. The best-fit line was selected after visual inspection of the fitting, the analysis of the objective function and the AIC, the precision of the estimates (mean and CV), and the weighted residual plot analysis.

The i.v. data were described by an open two-compartment model and fit to the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p is the total plasma drug concentration at time t, the terms A and B are the extrapolated zero intercepts, and α and β represent the apparent first-order elimination rate constants. The half-life ($t_{1/2\beta}$) for the elimination phase and the volume of distribution in the central compartment (V_c) were calculated as follows: $t_{1/2\beta}$=0.693/k_{10}, where k_{10} is an overall elimination rate constant, $V_c = \mathrm{Dose}/(A+B)$. For the oral route, an absorption rate constant, k_a, was fitted. The area under the concentration–time curve ($AUC_{0-\infty}$) and area under the first moment of the plasma drug concentration–time curve ($AUCM_{0-\infty}$) were calculated using the following equations:

$$AUC_{0-\infty} = \frac{A}{\alpha} + \frac{B}{\beta}$$

$$AUMC_{0-\infty} = \frac{A}{\alpha^2} + \frac{B}{\beta^2}$$

The values reported as the C_{max} and T_{max} are the actual observed values. The F (bioavailability) value for oral administration can be calculated using the following formula:

$$F = \frac{D_{iv} \cdot AUC_{oral(0\to\infty)}}{D_{oral} \cdot AUC_{iv(0\to\infty)}}$$

where, for the oral and i.v. routes, D_{oral} and D_{iv} are the respective doses; $AUC_{0-\infty}$ oral and $AUC_{0-\infty}$ i.v. are the respective AUCs from 0 to infinity.

Oral pharmacokinetics parameters were calculated with the following equations:

$$\frac{t1}{2abs} = \frac{0.693}{k_a}$$

$$AUC_{0\to\infty} = \frac{F \cdot D}{V_c \cdot k_{10}}$$

$$AUMC_{0\to\infty} = \frac{F \cdot D}{V_c \cdot k_{10}^2}$$

$$MRT = \frac{AUMC_{0-\infty}}{AUC_{0-\infty}}$$

$$MAT = \frac{1}{k_a}$$

$$Cl_p = V_c \cdot k_{10}$$

$$V_{ss} = V_c \cdot \left(\frac{k_{12} + k_{12}}{k_{21}}\right)$$

where k_a and $t_{1/2,abs}$ are the absorption constant and the absorption constant half-life obtained after oral administration, respectively. MRT and MAT are the mean resident time and the mean absorption time, respectively. The microconstants k_{12} and k_{21} used for the calculation of V_{ss} are the terms that describe the distribution of the drug between the central and peripheral compartments. When Michaelis–

Menten fitting was applied, the first-order elimination constant from the central compartment was substituted by the following equation:

$$C_t = \frac{V_{\max} \cdot C_t}{K_m + C_t}$$

where C_t is the mephedrone concentration at time t, $V_{\max}$ the maximum metabolic capacity achieved by the metabolic system, and K_m the Michaelis–Menten constant. Cl_{met} is the metabolic clearance calculated as follows (Barrett et al. 1998):

$$Cl_{met} = \frac{V_{\max}}{K_m}$$

Pharmacokinetic/pharmacodynamic analysis

A link compartment representing stimulation of the locomotor behavior was used to describe the data (Sheiner et al. 1979). Integration of mephedrone pharmacokinetics and pharmacodynamics was based on the relationship between mean plasma mephedrone concentration–time profiles for i.v. and oral dosages. PK/PD modeling was also performed using SAAM II. The effect site was connected by a fixed rate constant from the central plasma compartment. A dummy compartment provides the concentrations in the effect site (C_e). The stimulation PK/PD model proposed by the sigmoid $E_{\max}$ equation is expressed in terms of C_e such that

$$E = E_0 + \frac{E_{\max} \cdot C_e^n}{C_e^n + EC_{50}^n}$$

The baseline value E_0 is the effect when mephedrone concentration is zero. EC_{50} is the concentrations that increase E_0 to 50 % of the $E_{\max}$ or maximal response, and "n" determines the sigmoid shape of the function (Hill coefficient) and contributes to the steepness of the slope. This produces a PD model that describes the effect as a function of time.

Results

Mephedrone pharmacokinetics

The observed plasma concentrations after i.v. administration of mephedrone at each time point are shown in Fig. 1. Plasma mephedrone was almost undetectable at 4 h after administration of this dose. Estimated values for the main pharmacokinetic parameters of intravenous administration in rats are presented in Table 1.

The plasma concentrations versus time curves after intravenous administration of mephedrone were described by a two-compartment model with a significantly different distribution and terminal elimination phases (α=10.23 h^{-1}, β=1.86 h^{-1}). The pharmacokinetic parameters showed that $t_{1/2\beta}$ was 0.37 h. Cl_p and V_{ss} were 1.69 l/h and 2.60 l/kg, respectively (Table 1).

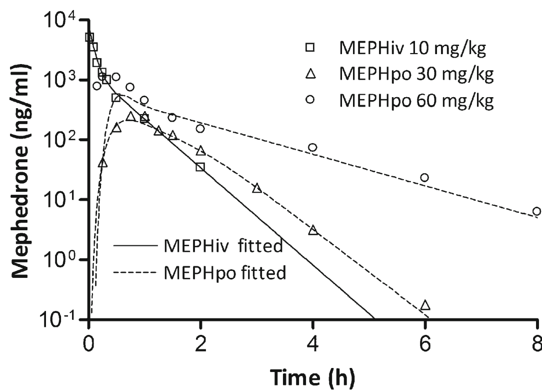

Fig. 1 Semi-logarithmic plot of the experimental and fitted mephedrone plasma levels after intravenous (10 mg/kg) and oral (30 and 60 mg/kg) administrations. Rats received mephedrone at time 0 and blood specimens (0.2 ml) were collected through the external jugular vein from 0.08 to 8 h after administration. Mephedrone plasma levels were quantitated by LC-MS as described in "Materials and methods." Data are the mean for n=4–5 rats/group

For oral dosing conditions, the pharmacokinetic parameters derived from the mephedrone plasma concentration curves are summarized in Table 2. $C_{\max}$ values were achieved rapidly, showing a $T_{\max}$ within 0.43–0.93 h; the plasma concentrations declined to undetectable levels at 9 h. Dose-normalized $C_{\max}$ at 60 mg/kg (16.00±5.70) was greater than that observed at 30 mg/kg (11.00±

Table 1 Main pharmacokinetic parameter estimates of mephedrone after i.v. administration (10 mg/kg) to male Sprague–Dawley rats

Parameters	Units	Estimate	CV (%)
A	ng/ml	5,808.17	9.5
B	ng/ml	1,412.83	7.0
α	h^{-1}	10.23	11.6
β	h^{-1}	1.86	2.0
K_{01}	h^{-1}	5.43	8.0
K_{12}	h^{-1}	3.49	6.5
K_{21}	h^{-1}	3.15	19.6
$AUC_{0-\infty}$	ng.h/ml	1,331.80	6.3
C_p	ng/ml	7,221.00	8.0
V_c	ml	311.59	25.0
V_{ss}	ml	584.11	2.21
$t_{1/2\beta}$	h	0.37	2.0
Cl_p	ml/h	1,694.97	1.3

6.93), but did not reach significance. The absolute oral bioavailability values were 7.30 and 11.20 % for 30 and 60 mg/kg, respectively.

Normalized $AUC_{0-\infty}$ values by the dose (9.82±0.72 and 14.92±2.87 for 30 and 60 mg/kg, respectively, $p<0.05$) show a non-lineal increase. V_{max} and K_m parameters and the calculated Cl_{met} were different for the two oral doses (Table 2). After oral administration, the value of Cl_{met} decreased from 77 % of total plasma clearance (at the dose of 30 mg/kg) to 20 % (at the dose of 60 mg/kg), showing an oral dose-dependent kinetic behavior.

Mephedrone protein binding and brain levels

Results from the present assays evidenced a percentage of mephedrone protein binding of 21.59±3.67 %. Brain levels of 104.4±17.7 ng mephedrone per gram tissue ($n=3$) were found. A relationship between brain levels and free plasma concentration yielded a ratio of 1.85±0.08.

Identification of mephedrone and metabolites in rat blood

We have identified five metabolites of mephedrone. These metabolites were detected in all collected samples at 30, 60, and 120 min. A description of the identification of mephedrone and the observed metabolites is provided below.

Table 2 Main pharmacokinetic parameter estimates of mephedrone after oral administration to male Sprague–Dawley rats at a dose of 30 mg/kg (value 30) and 60 mg/kg (value 60)

Parameters	Units	Value 30 (CV)	Value 60 (CV)
$C_{max,obs}$	ng/ml	331.20 (62.8)	960.00 (35.7)
$T_{max,obs}$	h	0.93 (13.3)	0.43 (52.6)
$AUC_{0-\infty}$	ng h/ml	294.48 (7.3)	895.43 (19.2)
AUC_{0-t}	ng h/ml	294.37 (7.4)	887.12 (18.3)
$t_{1/2abs\ app}$	h	2.15 (7.0)	3.14 (10.7)
$t_{1/2\beta\ app}$	h	0.55 (4.5)	0.55 (4.5)
t_{lag}	h	0.41 (4.5)	0.41 (4.5)
F^a	%	7.30[b]	11.20[b]
Cl_{met}	ml/h	1,298.05 (13.1)	377.79 (25.2)
MRT	h	0.16 (12.4)	0.16 (12.4)
MAT	h	4.14 (14.0)	4.14 (14.0)
K_m	μ/ml	0.09 (9.8)	21.17 (25.2)
V_{max}	μg/h	116.22 (14.4)	8000[c]

Results are expressed as mean and the corresponding CV (in percent)

[a] Calculated as $F=(AUC_{0\text{-oral}}\times \text{Dose i.v.})/(AUC_{0\text{-i.v.}}\times \text{Dose oral})$

[b] Calculated value and not fitted

[c] CV not determined

Mephedrone

The calculated $[M+H^+]$ *m/z* for mephedrone ($C_{11}H_{15}NO$) was 178.12319; the found $[M+H^+]$ *m/z* was 178.12290 (0.29 mDa). The peak at *m/z* 160 is attributable to the typical H_2O loss (18 Da). The loss of the methylamine group (31 Da) yielded a fragment with low intensity at *m/z* 147. The presence of the fragment at *m/z* 119 indicates the loss of C_3H_9N (59 Da), the intensity of which was found to be considerably low.

4-Methylcathinone

We identified the corresponding *N*-demethylation metabolite, 4-methylcathinone, with formula $C_{10}H_{13}NO$. The calculated $[M+H^+]$ *m/z* was 164.10699; the found $[M+H^+]$ *m/z* was 164.10728 (0.29 mDa). The peak at *m/z* 146 corresponds to H_2O loss (18 Da). C_2H_6N loss (45 Da) gave a peak at *m/z* 119. Both losses suggest that this mass spectrum is in accordance with the metabolite structure proposed.

4-Hydroxymethylmethcathinone

We identified an allylic hydroxylation of mephedrone, the resulting chemical formula of which was $C_{11}H_{15}NO_2$. The calculated $[M+H^+]$ *m/z* was 194.11756; the found $[M+H^+]$ *m/z* was 194.11745 (0.11 mDa). The typical loss of water was also observed, giving a peak at *m/z* 176. Furthermore, we detected a double water loss (36 Da), suggesting the possible presence of a hydroxyl group in this structure. The loss of C_3H_9N gave a peak at *m/z* 135, indicating that this fragment contains the hydroxyl group previously mentioned. Moreover, the double loss of water leads us to conclude that this group can only be found in the allylic position (Fig. 2).

4-Carboxymethylmethcathinone

The structure corresponding to the oxidation of 4-hydroxymethylmethcathinone was identified; the chemical formula for this compound was $C_{11}H_{13}NO_3$. The calculated $[M+H^+]$ *m/z* was 208.09737; the found $[M+H^+]$ *m/z* was 208.09702 (0.35 mDa). In positive ion mode, the spectrum showed a peak at *m/z* 149 (C_3H_9N loss), indicating the possible presence of a carboxybenzoyl cation fragment. The detection of a typical carboxylic loss (44 Da) in negative ion mode confirmed the proposed structure.

3′-Hydroxy-4-methylmethcathinone

A compound with chemical formula $C_{11}H_{15}NO_2$ was identified. The calculated $[M+H^+]$ *m/z* was 194.11756; the found $[M+H^+]$ *m/z* was 194.11774 (0.19 mDa). Typical loss of

Drug and Metabolites	R_t (min)	Monoisotopic mass	Precursor ion (m/z)	Product ions (m/z)
4-MMC	9,19	178,12290	178	160,147
4-MC	8,96	164,10728	164	146,119
HTMMC	4,80	194,11745	194	176,158,135
3'-OH-4-MMC	12,57	194,11774	194	176,158,119,74
?-OH-4-MMC	5,57	194,11773	194	176,135
4-CMMC	5,32	208,09702	208	190,149

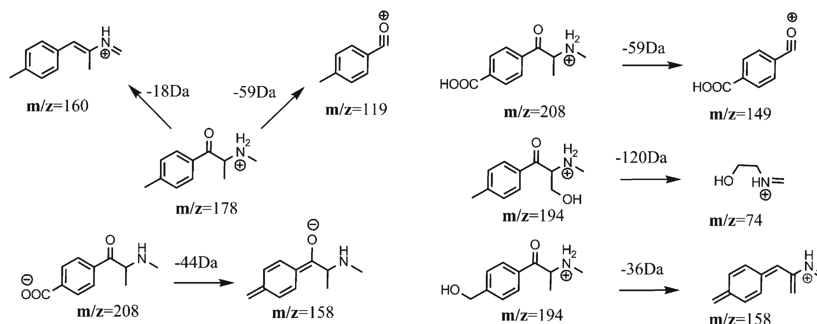

Fig. 2 LC-MS Orbitrap data for fragmentation of mephedrone and their metabolites in rat plasma after a single oral dose of 30 mg/kg at three different times points after administration (30, 60, and 120 min). Scheme of proposed fragmentation patterns for the product ions of mephedrone and its metabolites

water was also observed, giving a peak at *m/z* 176. Additionally, double loss of water (36 Da) was detected, indicating the possible presence of a hydroxyl group in this structure. Loss of the methylbenzoyl cation fragment (119 Da) gave a peak at *m/z* 74, which corresponds to a hydroxylated immonium cation, thus confirming the proposed structure.

?-Hydroxy-4-methylmethcathinone

The metabolite corresponding to an aromatic hydroxylation for mephedrone was detected. The chemical formula for this structure was also $C_{11}H_{15}NO_2$. The calculated $[M+H^+]$ *m/z* was 194.11756; the found $[M+H^+]$ *m/z* was 194.11773 (0.18 mDa). The peak at *m/z* 135 corresponds to the hydroxylated methylbenzoyl cation, and the single loss of water (peak at *m/z* 176) suggests that this group is in an undetermined aromatic position.

We ensured that the mass found did not correspond to endogenous compounds by comparing each metabolite mass from the treated and untreated rat blood samples. Based on the found metabolites, we propose the following phase I metabolic pathway for mephedrone, displayed in Fig. 3.

Locomotor activity

Intravenous administration of mephedrone induced a significant increase in rat locomotor activity (AUC: saline, 8683±98; mephedrone, 71248±9518; $n=3$, $p<0.01$, independent samples Student's *t* test) that lasted for 120 min.

Similarly, an overall ANOVA demonstrated a significant effect of oral mephedrone on the locomotor activity in rats ($F_{2,8}=11.261$, $p<0.01$). Post hoc Tukey–Kramer tests revealed that oral administration of mephedrone increased the locomotor activity in a dose-dependent manner (AUC: saline, 20,760±2,002; mephedrone 30 mg/kg, 39,778±10,255, $p<0.05$; mephedrone 60 mg/kg, 79,692±22,302, $p<0.01$; $n=3$); 60 min after the dose of 30 mg/kg breaks, values were not significantly different from animals treated with saline. At the dose of 60 mg/kg, the psychostimulant effect of mephedrone persisted for 90 min (Fig. 4).

Pharmacokinetic/pharmacodynamic analysis

A plot of locomotor activity versus mephedrone concentrations over time shows a direct concentration–effect relationship after i.v. administration (Fig. 5a). After oral dosing (Fig. 5b, c), a clockwise hysteresis loop was observed, mainly with the highest dose. The model shows a mean E_{max} value of

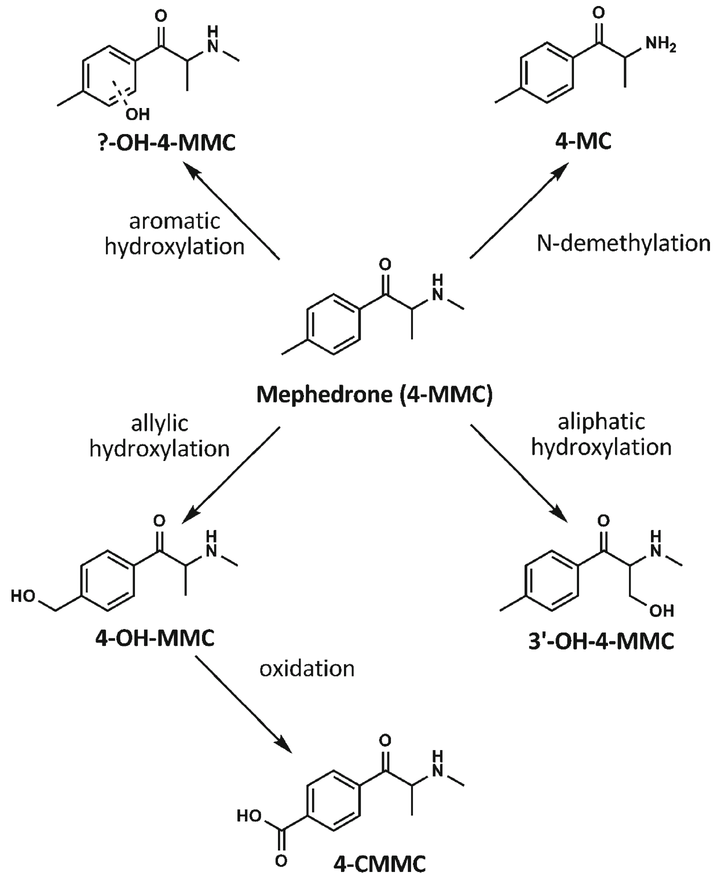

Fig. 3 In vivo metabolic pathways proposed for mephedrone in rat plasma after a single oral administration of 30 mg/kg

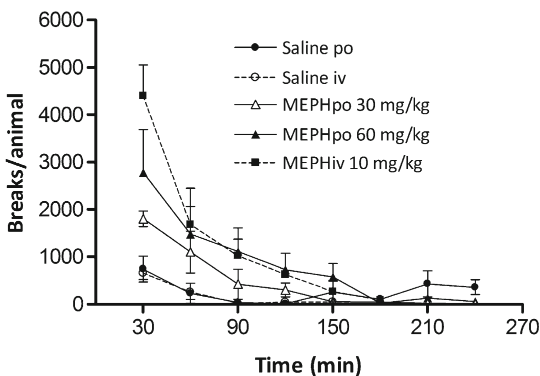

Fig. 4 Time course of locomotor activity induced after oral (30 and 60 mg/kg) and intravenous (10 mg/kg) administrations of mephedrone. For this behavior, the interruption counts were registered, displayed in a 30-min block. *Vertical axis* shows breaks per animal in 30-min intervals. Locomotor activity was monitored for 240 and 180 min for oral and intravenous administrations, respectively. Data are expressed as the mean±SEM values from three rats

444.28±200.10 and EC_{50} values ranging from 65.37 to 315.44 ng/ml (with a mean value of 0.86 μM; Table 3). Good agreement between the predicted and observed values was obtained in accordance with the quality model adequacy (mean objective function=4.35, AIC=9.54, determined CV <50 % for all the estimated parameters).

Discussion and conclusions

The pharmacokinetics of mephedrone was investigated in adult male Sprague–Dawley rats. In this study, the increase in locomotor activity induced by mephedrone after i.v. and oral administration was characterized and an integrated PK/PD model was provided.

A significant increase in locomotor activity (a measure of psychostimulant effects) occurred after mephedrone administration and is consistent with the onset of subjective effects in humans. The doses reported vary from 15 to 250 mg for oral ingestion and from 5 to 125 mg for nasal insufflation, with total doses typically ranging 0.5–2.0 g after redosing during a

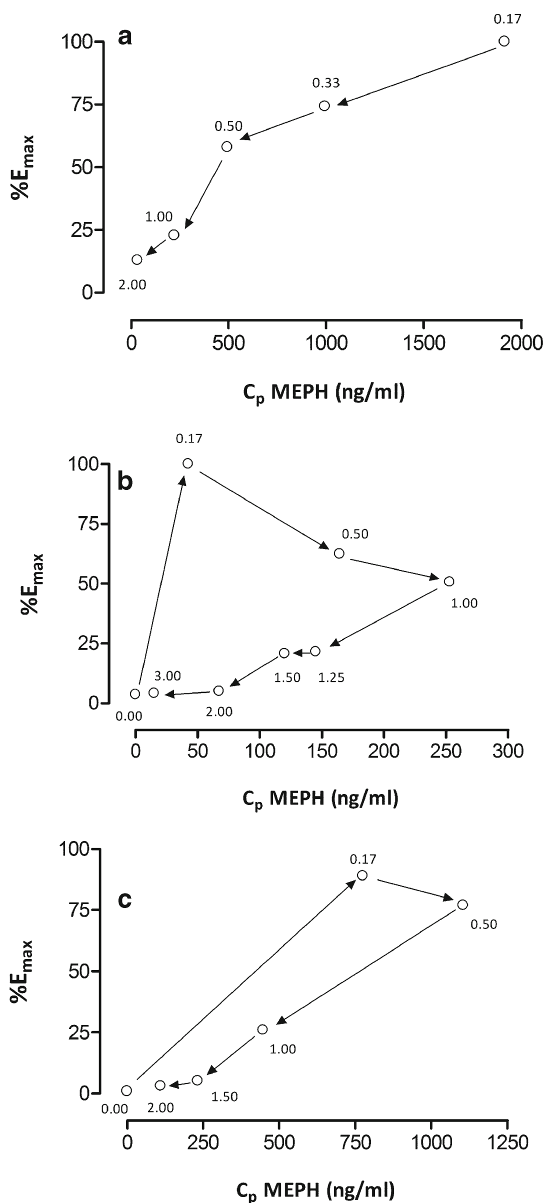

Fig. 5 Observed plasma concentrations of mephedrone versus observed locomotor activity (% E_{max}). **a** After intravenous administration (10 mg/kg). **b** After oral administration (30 mg/kg). **c** After oral administration (60 mg/kg). Data points show experimental time (in hours) of pharmacokinetic and pharmacodynamic data

single session. The oral doses used in this study were chosen according to Internet information (http://www.erowid.org, last accessed March 1, 2013) and the literature (Simmler et al. 2013). These doses were calculated following the FDA guidelines (Food and Drug Administration Center for Drug Evaluation and Research 2005) and are equivalent to 336 and 672 mg, respectively.

The pharmacokinetics of mephedrone in plasma after i.v. and oral administrations was well described by a two-compartment open model with and without Michaelis–Menten elimination, respectively. The goodness of fit and the quality of the estimated pharmacokinetic parameters were evaluated and confirmed by the objective function, AIC, plot of observed versus predicted concentrations, and the variation coefficients with values <50%, except for the V_{max} value at the highest oral dose where the CV has not been estimated.

Our results show that the blood levels of mephedrone declined biphasically after intravenous administration. The large apparent V_{ss} indicates that, in rats, mephedrone is distributed extensively into tissues. This V_{ss} value was expected of a highly lipophilic molecule that must cross the blood–brain barrier in order to exert its psychostimulant effect.

Compounds with a brain/plasma ratio value >1 freely cross the blood–brain-barrier (Hitchcock and Pennington 2006). The obtained value of mephedrone brain/plasma ratio of 1.85 indicates low brain penetrance when compared to amphetamine-like compounds (Chu et al. 1996) and may explain why mephedrone, compared to MDMA, is a weak motor stimulant, causes weak hyperthermia, and induces minor changes in brain monoamines (Baumann et al. 2012).

Comparing the two oral doses, an evident non-linearity in the pharmacokinetics of mephedrone was detected using a Michaelis–Menten elimination equation. The nonlinear pharmacokinetics described in the study allows identifying different V_{max} and K_m values, suggesting that the pathways involved in the metabolic clearance may be quantitatively and/or qualitatively different depending on the dose. Because the metabolic clearance decreased from 77 % of the total plasma clearance (at the dose of 30 mg/kg) to 20 % (at the dose of 60 mg/kg), and taking into account the presence of phase I metabolites in plasma, a possible saturation of liver metabolic enzymes and pathways is suggested. These results are in agreement with the low oral bioavailability observed and suggest that mephedrone undergoes an extensive first-pass effect after oral administration. Moreover, it is important to note that the low bioavailability of mephedrone explains its widespread use snorted rather than taken orally.

This study represents the first qualitative assessment of mephedrone metabolites in rats after oral intake. We identified the presence of five metabolites in rat blood at three time points after administration (30, 60, and 120 min). Based on the obtained data, we propose a first step of phase I metabolism for mephedrone, implying an *N*-demethylation reaction, yielding the corresponding methylcathinone metabolite. Mephedrone undergoes different oxidative reactions including aliphatic and aromatic hydroxylation, leading to the

Table 3 Estimates of the pharmacodynamic parameters according to the proposed additive sigmoid E_{max} equation PK/PD model

Parameters	Units	Doses			Mean±SD
		10 mg/kg (i.v.)	30 mg/kg (p.o.)	60 mg/kg (p.o.)	
E_0	Breaks	9.79 (9.45)	9.46 (46.21)	2.0[a]	7.08±4.41
EC_{50}	ng/ml	65.37 (14.17)	80.40 (17.01)	315.44 (10.33)	153.74±140.24
E_{max}	Breaks	432.34 (8.77)	250.42 (30.40)	650.09 (37.89)	444.28±200.10
n	–	1.68 (19.0)	7.28 (58.71)	5.7[a]	4.89±2.89
O.F.		6.74	3.09	3.23	4.35±2.07

In parentheses are the corresponding CVs (in percent)

Akaike's information criterion, 9.54

OF objective function

[a] CV not determined

corresponding 3′-hydroxy-methylmethcathinone or ?-hydroxy-4-methylmethcathinone metabolites. Khreit et al. (2013) proposed one of these metabolites as an unidentified compound named "compound U." This is the first time that both mephedrone metabolites have been identified in vivo. We also identified 4-hydroxymethylmethcathinone, a metabolite resulting from an allylic hydroxylation of mephedrone, which would subsequently suffer an oxidation, leading to the final metabolite 4-carboxymethylmethcathinone.

Intravenous and oral administrations of mephedrone induced a psychostimulant effect, measured as an increase in the locomotor activity in male Sprague–Dawley rats. As we have described previously in mice (López-Arnau et al. 2012), after oral administration to rats, a dose-dependent duration of the effect of mephedrone was found.

Mephedrone blocks [^{3}H]DA into neurons (Cozzi et al. 1999; Hadlock et al. 2011; Nagai et al. 2007; López-Arnau et al. 2012). Our previous studies in brain synaptosomes demonstrated that the inhibition of this transporter by mephedrone is the result of a direct interaction with these proteins, competing with the endogenous substrate (López-Arnau et al. 2012). This contrasts with our findings in mephedrone on the 5-HT transporter (Martínez-Clemente et al. 2012). For this compound, a difference was found between the IC_{50} value of the inhibition of [^{3}H]5-HT uptake and the K_i value for the interaction with the 5-HT transporter, suggesting an additional mechanism rather than a direct interaction with the transporter. Similar to amphetamines, the action of mephedrone is not a mere inhibition of the uptake transporter. Baumann et al. (2012) demonstrated that mephedrone evoke a transporter-mediated release of monoamines via reversal of the normal transporter flux. The authors concluded that mephedrone is a substrate for noradrenaline and DA transporters, with slightly lower potency at the 5-HT transporter. The lower values of EC_{50} obtained in release experiments versus those obtained in uptake inhibition allows deducing that the release phenomena are predominant versus the uptake inhibition.

Although more research on the pharmacology and toxicology of abused cathinones is needed (Baumann et al. 2013), this study shows new insights into the pharmacokinetic–pharmacodynamic relationship of mephedrone. The naive pool data used in the study do not allow the estimation of variation within and between subjects because the collection of data from single individuals yields information that is lost when analyzing pooled data. Between-subject variability information for each dose is lost when naive pool concentration data are used, and when pharmacodynamics results were obtained from rats without pharmacokinetic information, the within-subject variability of PK/PD parameters cannot be estimated. Despite these constraints, the model built in the study allows using it as a useful tool for the PK/PD relationship understanding of mephedrone in our experimental conditions.

By means of pharmacokinetic–pharmacodynamic relationship, it is possible to perform an estimation of the EC_{50} in the effect compartment and E_{max} parameters as well as an evaluation of the onset, magnitude, and time course of the psychostimulant effect. The pharmacodynamics maximum effect was observed immediately after mephedrone administration, and the decay of the stimulatory effect was evaluated from the plot of locomotor activity as a function of mephedrone plasma concentrations.

After i.v. administration, the change in response was directly interpretable as a function of mephedrone concentration with the use of a sigmoidal E_{max} model (Csajka and Verotta 2006). After oral dosage, plots of mephedrone effect versus observed plasma mephedrone concentrations revealed a clockwise hysteresis loop in both oral dosages. A clockwise hysteresis loop has been described after cathinone (Schechter 1990) or MDMA administration (Hysek et al. 2012) and could be attributable to the more rapid distribution of the drug to the brain than to venous blood (Porchet et al. 1987).

The brain/plasma ratio obtained in this study suggests that mephedrone freely crosses the blood–brain barrier,

showing an appreciable distribution within the brain. Previous in vitro results suggest that mephedrone inhibits dopamine, norepinephrine, and serotonin uptake at concentrations around 1 μM (López-Arnau et al. 2012). These results are in close agreement with the EC_{50} values in the effect site calculated in the present study by means of the proposed PK/PD model.

The low bioavailability of mephedrone found after oral ingestion justifies why abusers preferably snort it. Mephedrone's half-life is shorter than that of MDMA (Fonsart et al. 2009), which causes users to often redose, thus contributing to the appearance of addiction. Finally, the nonlinear kinetics after oral administration of mephedrone can cause a dramatic increase in plasma levels, leading to enhanced toxicity.

In conclusion, the present research provides, for a first time, useful information on the in vivo pharmacokinetics, pharmacodynamics, and the pharmacokinetic–pharmacodynamic relationship of mephedrone in rats and will help design new experiments in rodents with kinetics-based data as well as offer a better understanding of the effects of this drug of abuse in humans.

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Conflicts of interest The authors declare that they have no financial or commercial conflicts of interest.

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7.2. Anexo II (Publicación VI)

Dose and time-dependent selective neurotoxicity induced by mephedrone in mice

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Dose and Time-Dependent Selective Neurotoxicity Induced by Mephedrone in Mice

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Abstract

Mephedrone is a drug of abuse marketed as 'bath salts". There are discrepancies concerning its long-term effects. We have investigated the neurotoxicity of mephedrone in mice following different exposition schedules. Schedule 1: four doses of 50 mg/kg. Schedule 2: four doses of 25 mg/kg. Schedule 3: three daily doses of 25 mg/kg, for two consecutive days. All schedules induced, in some animals, an aggressive behavior and hyperthermia as well as a decrease in weight gain. Mephedrone (schedule 1) induced dopaminergic and serotoninergic neurotoxicity that persisted 7 days after exposition. At a lower dose (schedule 2) only a transient dopaminergic injury was found. In the weekend consumption pattern (schedule 3), mephedrone induced dopamine and serotonin transporter loss that was accompanied by a decrease in tyrosine hydroxylase and tryptophan hydroxylase 2 expression one week after exposition. Also, mephedrone induced a depressive-like behavior, as well as a reduction in striatal D2 density, suggesting higher susceptibility to addictive drugs. In cultured cortical neurons, mephedrone induced a concentration-dependent cytotoxic effect. Using repeated doses for 2 days in an elevated ambient temperature we evidenced a loss of frontal cortex dopaminergic and hippocampal serotoninergic neuronal markers that suggest injuries at nerve endings.

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Introduction

Mephedrone (4-methylmethcathinone) is a synthetic ring-substituted cathinone often marketed as "bath salt". It appears to be used by people involved in the dance and music scene and also used more broadly by many young adults and adolescents [1]. It is known to have similar effects to other psychostimulant drugs [2,3] but many users consider the effects of cathinones to be superior to cocaine and MDMA (3,4-methylenedioxymethamphetamine) [4,5]. Moreover, the abuse potential of cathinone derivatives is comparable with that of cocaine or MDMA [6].

Based in its chemical structure, mephedrone is expected to elicit stimulant effects similar to amphetamines [1,7]. It has been demonstrated that mephedrone acts as a substrate for monoamine transporters [8,9], which induces transporter-mediated depolarizing current [10] and releases monoamines by reverse transport [11]. Different authors [12–14] have shown that mephedrone administration to rats increases extracellular dopamine (DA) and serotonin (5-HT) in rat brain, similar to the effects of MDMA. All these results evidenced that this drug interacts with DA and 5-HT transporters displaying a similar pattern to other amphetamine derivatives.

After a first dose, mephedrone users evidenced a desire to re-dose, leading them to ingest large amounts of the drug [15]. This pattern of use implicates a potential risk of overdosing [16,17]. Patients seeking medical attention after bath salts intoxications can display agitation, psychosis, tachycardia, and also hyperthermia, a commonly reported acute adverse effect of MDMA and beta-keto-amphetamine ingestion in humans [18–20].

The use of this substance in a chronic pattern may also be associated with more subtle long-term effects on brain neurotoxicity. However, there are discrepancies concerning its neurotoxicity in rodents. As with MDMA [21], it is possible that mephedrone could display a species-dependent neurotoxicity. Then, it is essential to compare results without losing sight of the species in which experiments are conducted. To date, authors found that mephedrone does not damage DA or 5-HT systems when administered to mice [22,23]. Nevertheless, the studies did not extent the evaluation of DA to brain areas other than striatum or were performed with a drug exposure schedule not adjusted to mephedrone pharmacokinetics [24].

The aim of this paper was to investigate the neurotoxicity profile of mephedrone in mice, addressing some of the limitations in the literature. Most authors described the neurotoxic effects of methamphetamine three days after exposition [25] and those of MDMA seven days after [26]. We examined the neurotoxic injury induced by mephedrone at 3 and 7 days after finishing the exposition. Obtaining as much mechanistic information as possible regarding mephedrone, as well as on its neurotoxic effects, is of the essence. In this regard, we have evaluated the *in vivo* effect of this cathinone following different dosage schedule whilst complementing it by performing *in vitro* experiments. With regards to MDMA,

it is described that the magnitude of the acute hyperthermic response plays a major role in determining the severity of the consequences of its misuse, in such a way that ingesting the drug in hot, crowded dance club conditions, increases the possibility of subsequent cerebral neurotoxic effects [27]. To simulate these usual conditions of drug exposure, the neurotoxicity studies with amphetamine-derivatives are usually performed at elevated ambient temperatures. Accordingly, present experiments were carried out at high room temperatures. This condition was not considered in previous published papers.

In the present study we used adolescent mice, a feature that correlates with young adult consumers. We demonstrate that mephedrone induces an injury at nerve endings in the frontal cortex at a schedule of drug exposure that mimics human "weekend consumption".

Experimental Procedures

Drugs and reagents

Pure racemic mephedrone hydrochloride was synthetized and characterized by us as described previously [7]. The other drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]ketanserin, [^{3}H]paroxetine, [^{3}H]raclopride and [^{3}H]WIN35428 were from Perkin Elmer Life Sci. (Boston, MA, USA). All buffer reagents were of analytical grade.

Animals and ethics statement

The Experimental protocols were approved by the Animal Ethics Committee of the University of Barcelona, following the guidelines of the European Community Council (86/609/EEC). Male Swiss CD-1 mice (Charles River, Spain) aged 4–5 weeks (25–30 g) were used. Animals were housed at 22±1°C under a 12 h light/dark cycle with free access to food and drinking water.

In vivo neurotoxicity assays

No information about the subcutaneous doses in humans is available. The only approach would be the doses used intranasally that can reach up to 125 mg in each insufflation. The typical amount of mephedrone consumed over an evening/night was about 0.5 to 1 gram, usually taken in doses of 100-200 mg every hour or two hours [28]. In our case a dose of 25 mg/kg in mice corresponds to a 2 mg/kg in an adult. This mice equivalent dose was calculated following the body surface area normalization method [29]. The interval of 2 h between doses was chosen according the mephedrone half-life [24].

Mice were administered subcutaneously (under the loose skin on their back) according the following schedules. Schedule 1: four doses of saline (5 ml/kg) or mephedrone (50 mg/kg) with a 2 h interval. Mephedrone doses higher than 4×50 mg/kg were not tested to avoid cardiotoxicity-associated complications [30]. Schedule 2: four doses of saline or mephedrone (25 mg/kg) with a 2 h interval. Schedule 3: three doses of saline or mephedrone (25 mg/kg) with a 2 h interval, for two consecutive days. Rectal temperatures were measured 45 min after the last dose by inserting into the rectum (1.5 cm) a lubricated, flexible rectal probe attached to a digital thermometer (Panlab, Barcelona, Spain). Rectal temperature was measured 40 s after insertion of the probe. During the expositions, the animals remained one per cage, were maintained in an ambient temperature of 26±2°C and were kept under these conditions until 1 h after the last daily dose. After initial results, we consider that schedule 3 was the most suitable and representative of weekend dosing. So, different parameters have been evaluated only in that schedule.

The doses of mephedrone used in this study were based on results from Angoa-Pérez et al. [22], who concluded that, in mice, a 4×20 mg/kg regimen does not elicit neurotoxicity in striatal DA nerve endings. Despite the importance of these results, it remains of the essence to assess other exposition regimens and their effects on several brain areas or neurotransmitters. In this sense, we focused on simulating weekend use patterns (prolonging the days of exposure but reducing the number of daily doses).

Tissue sample preparation

Crude membrane preparation (collecting both synaptosomal and endosomal fraction) was prepared as described [31] with minor modifications. Mice were killed by cervical dislocation at 3 or 7 days after exposition. Hippocampus, striatum and frontal cortex were quickly dissected out and stored at −80°C until use. When required, tissue samples were thawed and homogenized at 4°C in 20 volumes of buffer (5 mM Tris-HCl, 320 mM sucrose) with protease inhibitors (aprotinin 4.5 µg/µl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). The homogenates were centrifuged at 1,000 g for 15 min at 4°C. Aliquots of the resulting supernatants were stored at −80°C until use for Western blot experiments. The rest of the samples were resuspended and centrifuged at 15,000 g for 30 min at 4°C. The pellets were resuspended in buffer and incubated at 37°C for 5 min to remove endogenous neurotransmitters. The protein samples were recentrifuged. The final pellets were resuspended in the appropriate buffer and stored at −80°C until use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent.

We performed an additional experiment. Following decapitation, the brain of some animals was separated in the two hemispheres. In one of them we applied a cellular fractionation [32] to obtain a fraction with a high content in plasma membrane and a second one enriched with endosomes. In the other hemisphere we followed the standard method described above to obtain a crude membrane fraction.

DA and 5-HT transporter density

The density of the DA transporter in striatal or frontal cortex membranes was measured by [^{3}H]WIN35428 binding assays. These were performed in tubes containing 250 or 500 µl of 5 nM [^{3}H]WIN35428 in phosphate-buffer and 50 or 100 µg of membranes, respectively. Incubation was done for 2 h at 4°C and non-specific binding was determined in the presence of 30 µM bupropion. All incubations were finished by rapid filtration under vacuum through Whatman GF/B glass fiber filters. Tubes and filters were washed rapidly three times with 4 ml of ice-cold buffer, and the radioactivity in the filters was measured by liquid scintillation spectrometry.

The density of the 5-HT transporter in the hippocampal and frontal cortex membranes was quantified by measuring the specific binding of 0.05 nM [^{3}H]paroxetine after incubation with 150 µg of protein at 25°C for 2 h in a Tris-HCl buffer. Clomipramine (100 µM) was used to determine non-specific binding.

Western blotting and immunodetection

A general western blotting and immunodetection protocol was used to determine tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2) levels. For each sample, 20 µg of protein was mixed with sample buffer (0.5 M TrisHCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min, and loaded onto a 10% acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P; Millipore, USA). PVDF

membranes were blocked overnight with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated for 2 h at room temperature with a primary mouse monoclonal antibody against TH (Transduction Lab, Lexington, KY, USA) 1:5000 or with a primary rabbit polyclonal antibody against TPH2 (Millipore, Billerica, MA, USA) 1:1000 and anti-(phosphor-Ser-19)TPH2. After washing, membranes were incubated with a peroxidase-conjugated antimouse IgG antibody 1:2500 or with a peroxidase-conjugated antirabbit IgG antibody (GE Healthcare, Buckinghamshire, UK) dil. 1:5000. Immunoreactive protein was visualized using a chemoluminescence-based detection kit (Immobilon Western, Millipore, USA) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Hercules, CA, USA). Scanned blots were analyzed using BioRad Image Lab software and dot densities were expressed as a percentage of those taken from the control. Immunodetection of beta-actin (mouse monoclonal antibody, 1:2500) served as a control of load uniformity for each lane and was used to normalize differences due to protein content.

5-HT2A and D2 receptor density

The density of 5-HT2A receptors was measured in frontal cortex membranes one week after their exposure to schedule 3. Assays were performed in tubes containing 1 nM [^{3}H]ketanserin and 100 µg of membranes. Incubation was carried out at 37°C for 30 min in a Tris–HCl buffer. Methysergide (10 µM) was used to determine non-specific binding. Assays to measure the density of D2 receptors in striatum membranes of the same animals were performed in tubes containing 2 nM [^{3}H]raclopride and 50 µg of membranes. Incubation was carried out at 25°C for 1 h in a Tris–HCl buffer. Sulpiride (300 µM) was used to determine non-specific binding.

Immunohistochemistry

Animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (1 ml/g of body weight). Brains were removed and post-fixed for 2 h in the same solution, cryoprotected in 30% sucrose/phosphate buffer for 24 h and frozen in dry ice-cooled isopentane. Serial coronal sections (30 µm thick) through the whole brain were cut in a cryostat and collected in phosphate buffer. Free-floating sections were incubated for 15 min in H2O2 (0.3% phosphate buffer, 10% methanol). Thereafter, sections were incubated in a blocking solution (1% fetal bovine serum, 0.2 M glycine plus 0.5% Triton X100). After blocking with 10% normal serum and 0.2% bovine serum albumin, sections were rinsed and incubated overnight at 4°C using a monoclonal antibody against fibrillary acidic protein (GFAP, 1:1000) (Dako, Denmark). Sections were washed and incubated with a biotinylated secondary antibody (1:200 Sigma-Aldrich) for 2 h at room temperature. Afterwards sections were incubated with avidin-biotin-peroxidase complex (ABC; 1:200; Vector, Burlingame, CA, USA). Peroxidase reaction was developed with 0.05% diaminobenzidine in 0.1 M phosphate buffer and 0.02% H2O2, and immunoreacted sections were mounted on gelatinized slides. Stained sections were examined under a light microscope (Olympus BX61).

Neuronal cell cultures

Primary neuronal cultures of cerebral cortex were obtained from mouse embryos (E-16-18). The cerebral cortex was dissected, meninges were removed, and tissue was incubated for 20 min in trypsin (0.05%) at 37°C. Trypsin was inactivated with fetal bovine serum and tissue was triturated with a Pasteur pipette. Dissociated cells were washed with phosphate buffer containing 0.6% glucose and centrifuged at 500 g for 5 min. The cells were redissociated in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) with 0.5 mM Lglutamine, sodium bicarbonate (0.04%) and 1 µg/ml penicillin and streptomycin, containing B27 supplement and 10% horse serum (HS). Neurons were plated at 0.4 million cells/ml in 96-well plates precoated with 1 mg/ml poly-L-lysine. Cultures were maintained at 37°C in an incubator with 5% CO2. 24 h after, cells were treated with arabinosylcytosine (10 µM) to prevent the growth of glial cells. The culture medium was changed after 4 days to Neurobasal medium with B27 without antioxidants. Before treating the cells, the HS concentration was reduced by half. The cultures were used for experiments after 8–9 days *in vitro* with different concentrations of mephedrone and different times of drug exposure.

Cell viability

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5diphenyltetrazoliumbromide) method. This assay was carried out as described by Verdaguer et al. [33] with minor modifications. MTT was added to a final concentration of 250 µM and cells were incubated for 2 h [34]. Cell viability was expressed as a percentage of the absorbance measured in untreated cells.

Forced swimming test (FST)

The immobility time in FST was measured by an observer blind to the exposition using the procedure described by Porsolt et al. [35]. Briefly, mice were placed individually in a glass cylinder (height 21 cm, diameter 12 cm) containing water at 25±1°C up to a height of 15 cm. Animals were randomly divided into two groups (12–16 animals/group) and treated with saline or mephedrone and tested 1, 3 or 7 days after schedule 3. Each animal was recorded for 6 min and the total period of immobility (in seconds) was measured. A mouse was judged to be immobile when it remained floating in water, making only the necessary

Table 1. Effect of mephedrone on weight gain.

	Schedule		
Group	1	2	3
Saline	0.71±0.14	0.84±0.22	0.77±0.21
Mephedrone	−0.99±0.14[a]	−0.98±0.16[a]	−1.23±0.20[b]

Data are expressed (mean ±S.E.M) as the difference in body weight, in grams, between the end (24 h after the last dose) and the beginning (prior to the first dose) of the exposure.
[a]sig. diff. ($p<0.01$) vs. Saline.
[b]sig. diff. ($p<0.001$) vs. Saline.
doi:10.1371/journal.pone.0099002.t001

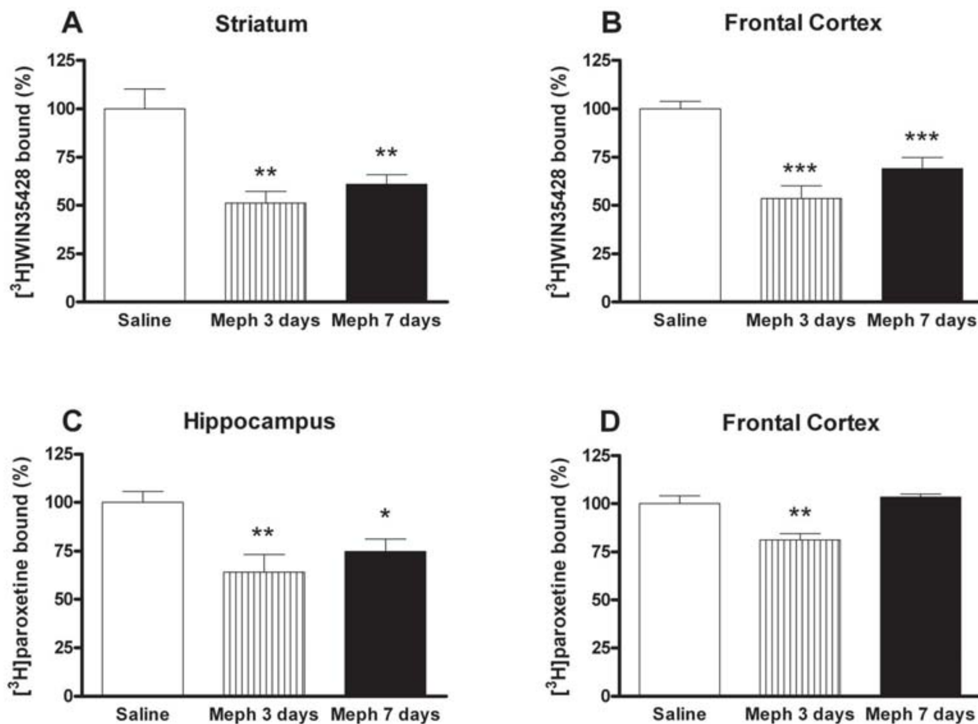

Figure 1. Effect of a mephedrone exposure (4 doses of 50 mg/kg, s.c. at 2 h interval) on dopamine transporter density, measured as [^{3}H]WIN35428 binding in mouse striatum (A), or frontal cortex (B) and 5-HT transporter density, measured as [^{3}H]paroxetine binding, in hippocampus (C) and frontal cortex (D). Results are expressed as mean±S.E.M. from 8–10 animals. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ vs. saline.
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movements to keep its head above the surface. Each mouse was used only once for each experimental session [36].

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were compared using one-way ANOVA or Student-t test for independent samples. Significant ($p<0.05$) differences were then analyzed by Tukey's post hoc test for multiple means comparisons where appropriate. Statistic calculations were performed using Graph Pad Instat (GraphPad Software, San Diego, USA). Analysis of concentration-viability curves was performed using non-linear regression (InvivoStat software [37]). Experimental values to calculate IC_{50} were in the linear region of the sigmoid curve (between 25 and 85% of the maximum effect).

Results

Lethality

Initial experiments were carried out with 6 animals per cage. With this condition, lethality was of about 70%. Thereafter, all experiments carried out in this study were performed with a single animal per cage. The number of fatalities of mephedrone-treated mice was similar in schedule 2 (8.33%) and 3 (10.17%), and slightly higher in schedule 1 (14.28%). It is important to note that all mephedrone schedules induced the occurrence, in some animals, of a stereotypy (repeated self-licking of the ventral base of the neck) that continued with self-bites and led to the appearance of injuries in that zone.

Effect of mephedrone on body temperature and weight gain

In the present study, drug expositions were performed in animals housed singly at a high room temperature. All mephedrone schedules induced a significant increase in body temperature. Hyperthermia was apparent in schedule 1 (saline: 36.4±0.1°C; mephedrone: 38.1±0.1 $p<0.001$) and schedule 2 (saline: 36.7±0.1°C; mephedrone: 37.4±0.2°C $p<0.01$). Results obtained from schedule 3 showed that mephedrone–induced hyperthermia was more intense after the last dose in the first day of exposition (saline: 36.7±0.1°C; mephedrone 38.1±0.1, first day; vs. saline: 36.4±0.1°C; mephedrone 37.5 ±0.3, second day, $p<0.001$). Mephedrone exposure slowed the weight gain compared with saline-treated animals (Table 1). Moreover, 3 days after finishing all schedules, the animals recovered their body weight (data not shown).

Effect of mephedrone on different *in vivo* markers of DA and 5-HT neurotoxicity

Schedule 1: At 3 and 7 days post-exposition, mephedrone induced a significant loss in DA reuptake sites of about 50% in

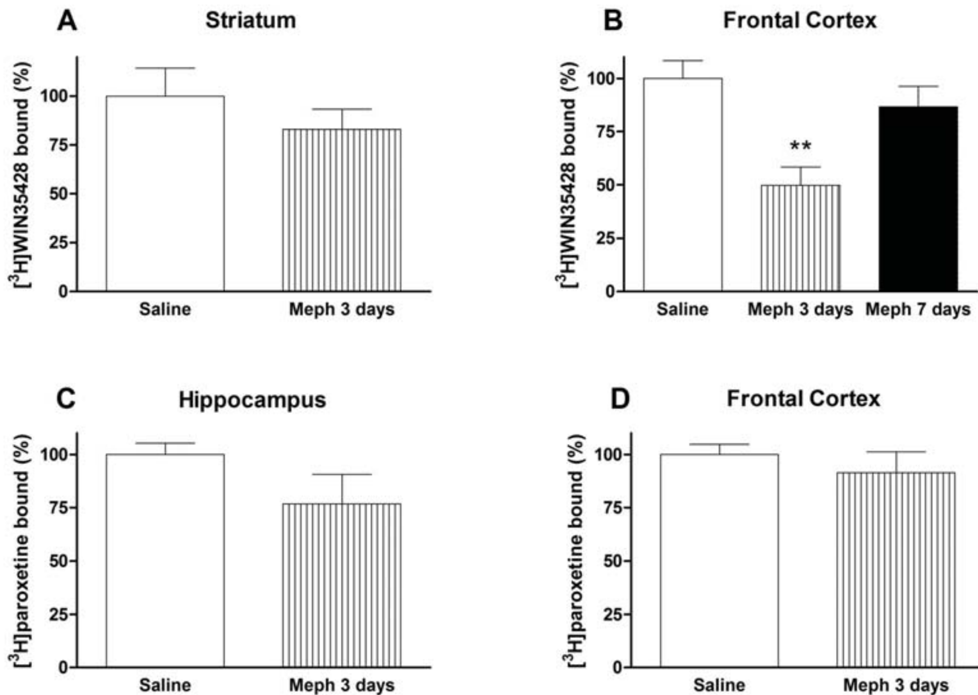

Figure 2. Effect of a mephedrone exposure (4 doses of 25 mg/kg, s.c. at 2 h interval) on dopamine transporter density, measured as [^{3}H]WIN35428 binding in mouse striatum (A), or frontal cortex (B) and 5-HT transporter density, measured as [^{3}H]paroxetine binding, in hippocampus (C) and frontal cortex (D). Results are expressed as mean±S.E.M. from 8–10 animals. **$p<0.01$ vs. saline.
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mouse striatum and frontal cortex membranes (Fig. 1A and 1B). Additionally, mephedrone-exposed mice showed a transient decrease in [^{3}H]paroxetine binding in the frontal cortex that disappeared four days later (Fig. 1D). By contrast, the decrease of [^{3}H]paroxetine binding in the hippocampus was apparent both 3 and 7 days after exposition (Fig. 1C).

Schedule 2: 3 days after the drug regimen, the frontal cortex was the only area where a marker showed a significant decrease. For this reason, the specific binding of [^{3}H]WIN35428 in this area, was also evaluated 4 days later. At this time, DA transporter density returned to basal levels (Fig. 2B). Mephedrone did not modify [^{3}H]WIN35428 binding in the striatum (Fig. 2A) or the [^{3}H]paroxetine binding neither in the hippocampus nor in the frontal cortex (Fig. 2C and 2D).

Schedule 3: The suppressive effect of mephedrone on striatal [^{3}H]WIN35428 binding was transient and returned later to basal values (Fig. 3A). However, in frontal cortex, the same marker suffered a significant loss (about 40%) evident 3 and 7 days post-exposition (Fig. 3B). Because these results pointed to a real injury more than to a transient regulation, we investigated the expression of the TH. We found a relationship between the decrease in the [^{3}H]WIN35428 specific binding and the decrease in enzyme expression in the frontal cortex (saline: 100.00±2.46%; mephedrone: 60.04±9.34% $p<0.01$, 3 days after exposition and 55.30±9.11% $p<0.001$, 7 days after exposition).

To characterize the recovery of the DA transporter marker in striatum, we assessed radioligand binding in the plasma membrane and in the endosomal fractions. Mephedrone elicited a reduction in [^{3}H]WIN35428 binding in the plasma membrane (saline: 100.00±6.49%; mephedrone: 65.80±9.00% $p<0.01$) and the corresponding increase in the endosome fraction (saline: 100.00±10.51%; mephedrone: 127.43±5.94% $p<0.05$). Consequently, when a redistribution of transporter occurred, we did not find a loss of radioligand binding in the crude membrane preparation.

The reduction of 5-HT terminal markers in mephedrone-treated animals was sustained and significant in the frontal cortex (Fig. 3D), and especially pronounced (50%) in the hippocampus after 7 days (Fig. 3C). Consequently, we analyzed another biochemical marker of terminal integrity, TPH2, and its Ser-19 phosphorylated form. In hippocampus, the decrease of [^{3}H]paroxetine binding runs in parallel with a decrease of the total TPH2 (Fig. 4A). In frontal cortex, this expression was lower but not significant (Fig. 5A). In both experiments, the shape of the TPH2 band obtained from mephedrone-treated animals shows differences that can be attributed to a protein modification. As protein phosphorylation in Ser-19 has been described as a frequent mechanism that regulates TPH2 function, we proceeded to determine it. In both brain areas, the remaining enzyme was phosphorylated in the mephedrone group with respect to saline (Fig. 4B, 4C, 5B, 5C).

Effect of mephedrone on astroglial activation

To assess the presence of astroglial activation, immunohistochemistry studies were carried out with the glial-specific marker,

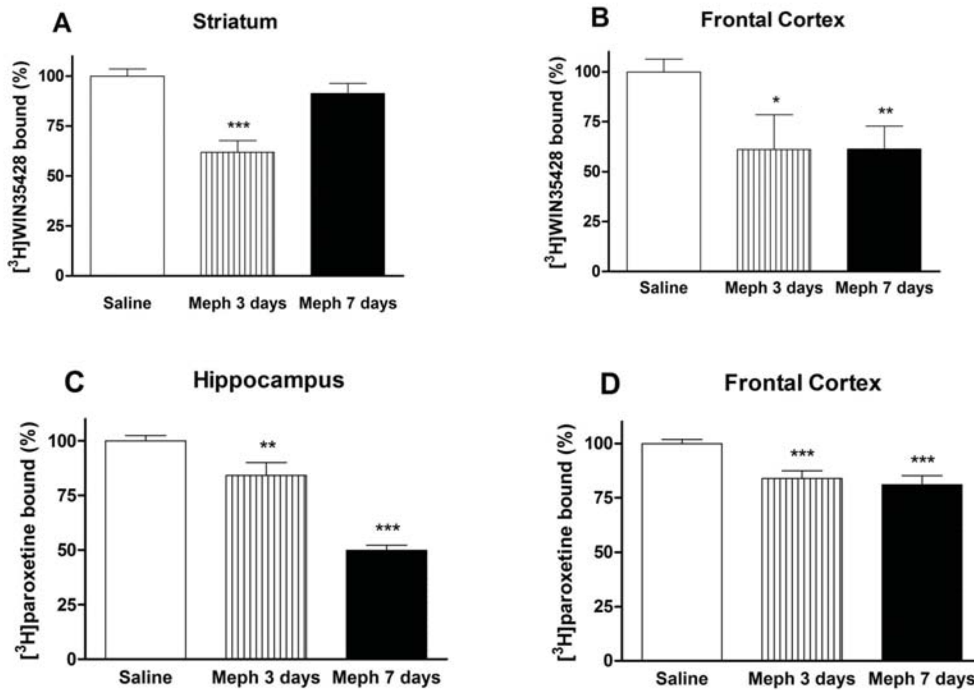

Figure 3. Effect of a mephedrone exposure (3 doses of 25 mg/kg, s.c. at 2 h interval for 2 days) on dopamine transporter density in mouse striatum (A), or frontal cortex (B) and on 5-HT transporter density in hippocampus (C) and frontal cortex (D). Results are expressed as mean±S.E.M. from 8–10 animals. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ vs. saline.
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GFAP. There were no signs of striatal or cortical astroglial activation in mephedrone-treated animals. In the hippocampus, some astrocytes with the typical stellate morphology were observed in control animals, but an apparent increase in GFAP immunoreactivity could be seen in the dentate gyrus of the hippocampus in the mephedrone group, implying reactive astrocytes (Fig. 6).

Depressant-like effect of mephedrone

In schedule 3, mephedrone significantly increased the immobility time in the FST as compared with saline group ($F_{3,44} = 5.509$, $p<0.01$). Post hoc Tukey's means comparison test demonstrated that 3 days post-exposition mephedrone significantly increased the immobility time (saline: 125.43±11.83 s; mephedrone: 217.83±19.55 s $p<0.001$). This increase was still significant 7 days post-exposition (178.80±22.45 s $p<0.05$). Moreover, mephedrone exposure failed to influence the immobility time in FST 24 h after the last administration (Fig. 7).

Effect of mephedrone on D2 and 5-HT2A receptor density

To determine the involvement of striatal DA D2 receptors, we measured [^{3}H]raclopride binding in this brain area. Mephedrone (schedule 3) decreased the number of these receptors in mouse striatum (saline: 100.00±6.49%; mephedrone: 79.86±5.46% $p<0.05$, 3 days after exposition and 78.57±7.42% $p<0.05$, 7 days after exposition). Similarly, mephedrone-exposed animals showed a decrease in the number of 5-HT2A receptors in frontal cortex (saline: 100.00±6.28%; mephedrone: 67.41±3.07% $p<0.001$) and hippocampus (saline: 100.00±8.45%; mephedrone: 71.70±4.02% $p<0.01$), 3 days after exposition. However, the density of these receptors returned to basal values on day 7 (98.81±4.01% and 98.93±4.10%, respectively).

Effect of mephedrone on cultured cortical neuron viability

The exposition of mouse cortical cultured cells to various concentrations of mephedrone (from 80 μM to 1 mM) for 24 h or 48 h caused a concentration-dependent decrease in metabolically active cells, as assessed by the MTT assay. The calculated LD50 value for mephedrone after 24 h of incubation was 242.72±40.66 μM which was higher ($p<0.01$) to that obtained after 48 h of drug exposure (115.94±16.58 μM).

Discussion

The easy availability of cathinones and their initial status as legal highs may have contributed to their increasing popularity as drugs of abuse. Because of the relatively short history of the use of cathinones as recreational drugs, their effects among long-term users have yet to be determined. Based on its structural similarity to well established neurotoxic psychostimulants such as methamphetamine and MDMA, it was hypothesized that mephedrone would exert neuronal damage. However, there are important discrepancies concerning the neurotoxicity induced by cathinones [8,22,38]. The inconsistent results could be attributed to

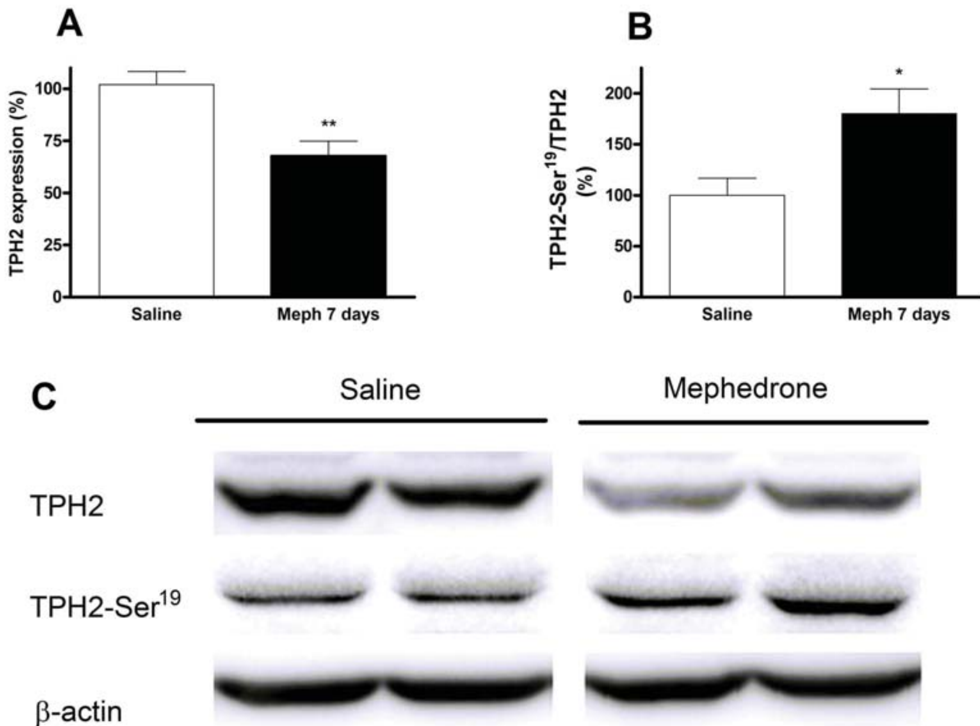

Figure 4. Effect of a mephedrone exposure (3 doses of 25 mg/kg s.c. at 2 h interval for 2 days) on total tryptophan hydroxylase TPH2 expression (A) and its phosphorylated form (B) in hippocampus. Panel C shows a representative Western blot. Results are expressed as mean±S.E.M. from 8–10 animals. *$p<0.05$ and **$p<0.01$ vs. saline.
doi:10.1371/journal.pone.0099002.g004

differences in species, dosage, administration route or ambient temperature.

Some cases of aggressive behavior, even cannibalism, as a consequence of exposure to new designer drugs have been recently reported in the media. However, these cases have been poorly documented [39]. In the present study, all mephedrone schedules induced the appearance of initial stereotypy consisting in repeated self-licking that was followed by aggressive behavior which leads to self-injuries. This is an especially important factor to be taken into account, seeing as it required animals to be housed individually. It is a feature described for the first time, but it has been also reported following high doses of methamphetamine or d-amphetamine [40–41], but not MDMA involving DA and 5-HT system in this abnormal behavior. Fantegrossi et al. [42] reported self-injurious behavior following methylenedioxypryrovalerone administration only at high, but not normal, ambient temperature, suggesting a potential role of the ambient temperature in this behavior. Further studies are needed to determine the mechanism implicated in this feature induced by mephedrone and its relationship with the exposed dose.

Temporal development of body weight of mice was studied to ascertain an easily measurable effect of mephedrone. Like other amphetamines, animals treated with mephedrone experienced a reduction in weight gain that was only transient seeing as values returned to basal values 3 days after finishing the corresponding exposure.

Hyperthermia is a commonly reported acute adverse effect of amphetamines [43] and beta-keto-amphetamine ingestion in humans [19]. Rodent exposure to cathinones also cause significant increases in core temperature [8,23,44,45]. The temperature has been examined during administration of multiple doses of the drug. Probably the strain of mouse, the dose and the ambient temperature can influence the size and direction of the hyperthermic response observed in the present experiments. Although lethal hyperthermia was not observed at the assessed dosages in mice, present results demonstrate that at high ambient temperatures, mephedrone impaired the thermoregulatory response; this effect persisted throughout the two day exposition. Nevertheless further research is required in order to characterize whether the role of hyperthermia is complementary or essential in the advent of mephedrone-induced neurotoxicity.

Several studies established a controversy concerning the neurotoxic effect of mephedrone in DA and 5-HT systems. Angoa-Pérez and co-workers [22] concluded that mephedrone administration to C57/BL6 mice at doses up to 40 mg/kg did not damage DA nerve endings in the striatum. In that study, no information regarding the ambient temperature is provided. Moreover, Hollander et al. [23] demonstrated that mephedrone exposure (30 mg/kg, twice daily for 4 days) to rats and mice resulted in no significant changes in brain monoamine levels. Conversely, Hadlock et al. [38] reported a rapid decrease in DA and 5-HT transporter function after mephedrone administration

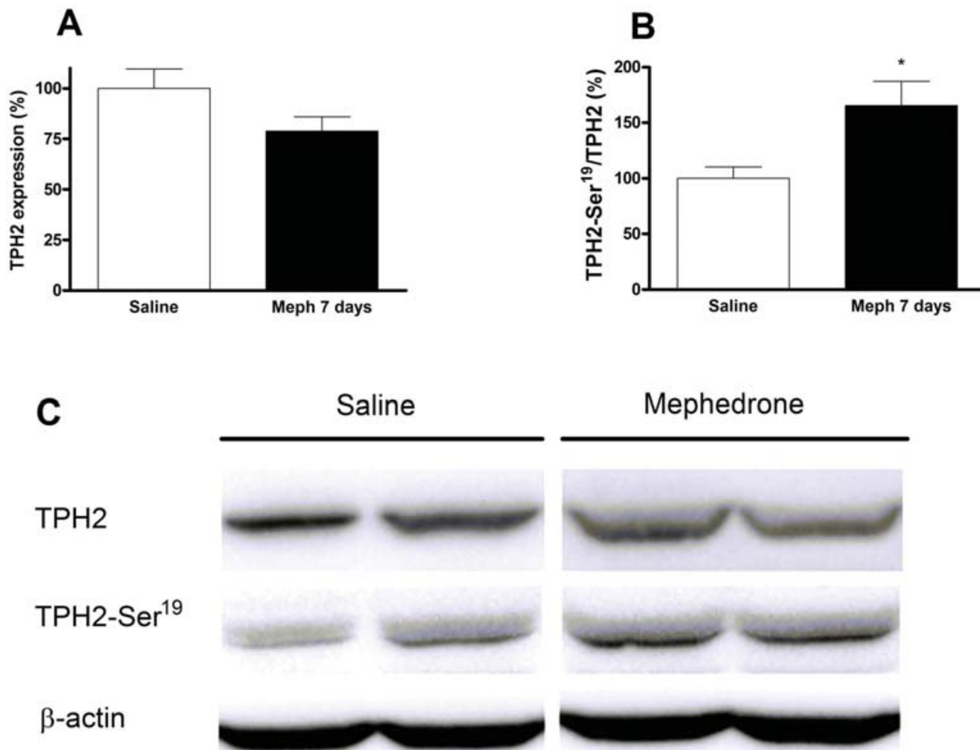

Figure 5. Effect of a mephedrone exposure (3 doses of 25 mg/kg s.c. at 2 h interval for 2 days) on total tryptophan hydroxylase 2 expression (A) and its phosphorylated form (B) in frontal cortex. Panel C shows a representative Western blot. Results are expressed as mean±S.E.M. from 8–10 animals. *$p<0.05$ vs. saline.
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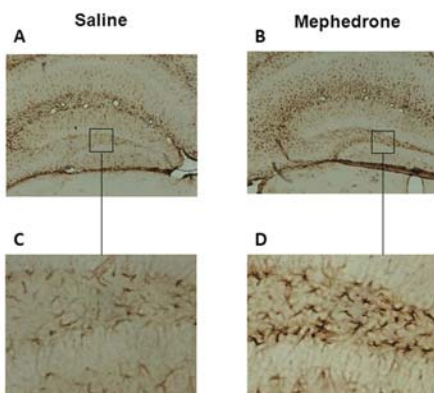

Figure 6. Representative hippocampal expression of glial fibrilliary acidic protein (GFAP). Sections of the dentate gyrus (×4 A, B; ×20, C, D) from mice exposed to saline (A, C) or mephedrone (3 doses of 25 mg/kg given subcutaneously for 2 days) (B, D). The animals were sacrificed 7 days after the last dose.
doi:10.1371/journal.pone.0099002.g006

(four doses of 10 or 25 mg/kg) to Sprague-Dawley rats at an ambient temperature of 27°C. However, other investigators have failed to find any persistent neurochemical impact of mephedrone dosing with long duration dosing protocols [46].

In the effort to model recreational mephedrone use, we considered it appropriate to simulate the widespread practices of ''stacking'' (taking multiple doses at once in order to increase the desired effect and/or overcome tolerance from prior use) and ''boosting'' (taking supplemental doses over time in order to maintain the drug effect). For this reason, we chose to administer multiple doses of mephedrone during each exposition day. In schedule 3 we repeated the exposition the next day, simulating a pattern of a recreational weekend use.

It is important to note that we corroborated that the crude membrane preparation used in the present experiments collects both the synaptosomal membrane and the endosomal fraction; consequently it is not possible to ascertain whether or not a transporter redistribution was taking place when observing no decrease in radioligand binding in crude membrane preparations. We demonstrate that mephedrone, administered at doses (i.e. schedule 1) that mimic a high exposure in humans (around 1.5 g/session) [47], induced an important decrease in DA transporter density in mouse striatum and frontal cortex membranes that persisted 7 days after exposition. This effect was accompanied by a

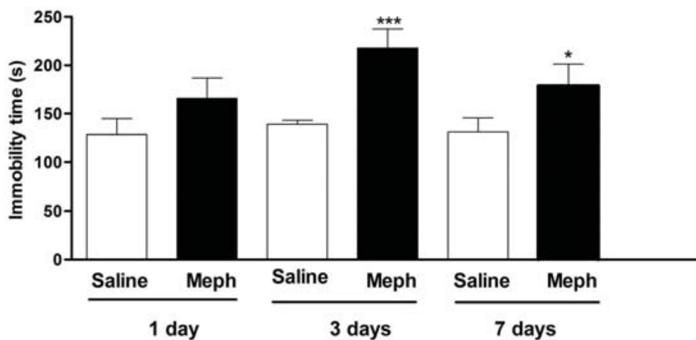

Figure 7. Effect of mephedrone on immobility time in mouse forced swim test. Animals were randomly divided into two groups (12–16 animals/group) and administered subcutaneously with saline (5 ml/kg) or mephedrone (3 doses of 25 mg/kg for 2 days) and tested 1, 3 or 7 days after exposure. Each animal was recorded for 6 min and the total period of immobility (in seconds) was registered. Each mouse was used only once for each experimental session. Each bar represents mean±S.E.M. immobility time. *$p<0.05$ and ***$p<0.001$ vs saline (one-way ANOVA and post hoc Tukey test).
doi:10.1371/journal.pone.0099002.g007

significant loss of 5-HT transporters in the hippocampus. However, at these high doses, acute cardiovascular toxicity of mephedrone is likely [30] and probably outweighs neurotoxic effects.

The subsequent experiments were carried out at a lower dose (25 mg/kg). Schedule 2 dosage schedule only elicited a transient decrease in cortical DA transporter, suggesting a temporary regulatory effect. Moreover, no significant loss of 5-HT transporter in the frontal cortex or the hippocampus was found.

Schedule 3 (three doses of 25 mg/kg for two consecutive days) can be considered the most representative because it is closest to the typical weekend consumption pattern. After this exposure, mephedrone induced loss of DA and 5-HT transporters that were especially apparent in the frontal cortex and the hippocampus respectively. The monoamine deficit induced by this schedule was also characterized by a significant decrease of each enzymatic marker that correlated with the decrease in radioligand binding. TH and TPH catalyze the first and rate-limiting step in the biosynthesis of DA and 5-HT respectively. The isoform TPH2 is responsible for 5-HT biosynthesis in the brain. Post-translational modifications have been shown to regulate the protein function. The enzyme is known to be phosphorylated on Ser-19 by both protein kinase A and Ca2+/calmodulin dependent protein kinase II *in vitro*. This modification results in increased stability and activity [48]. The decrease in transporter binding and enzyme levels, jointly with astrogliosis, point to an injury at the nerve endings; the increase in Ser-19TPH2 in mephedrone-treated animals seems to reflect a compensatory mechanism in the undamaged 5-HT terminals

The recovery of DA transporter levels in mouse striatum after 7 days of exposition raises the question on whether the decrease in [^{3}H]WIN35428 binding observed 3 days after exposition is an effect of biochemical down-regulation in the absence of tissue damage rather than being reflective of an injury. The non-significant increase in astroglial activation observed in this area was consistent with the absence of terminal injury and suggests that the DA transporter gene expression may be negatively regulated by mephedrone exposure. This is consistent with previous results reporting that MDMA acts on 5-HT transporter gene expressions [49]. Moreover, although DA transporter density returns to basal values in crude membrane preparations 7 days after exposition, results point to changes in DA levels in the striatal synapses, seeing as there is a significant redistribution of this transporter. A significant increase in transporter density is observed in the endosomal fraction, together with a reduction in membrane expression. Astrocytes stabilize and maintain homeostatic repair of tissues and contribute to early wound repair [50]. In the present study, mephedrone-exposed animals showed an increase in GFAP immunoreactivity in the dentate gyrus of the hippocampus, which confirms the injury in this area.

Low 5-HT levels in the synapse have been linked to various psychiatric disorders, including depression. In our study, mephedrone increased the immobility time in the forced swim test, which indicates an increase in stress-related depressive behavior. This effect was evident 3 and 7 days after exposition, and correlates with the reduction of the neurochemical parameters. This is in accordance with results from McGregor et al. [51] who demonstrated that MDMA-treated animals show higher immobility and fewer active escape attempts in the forced swimming model. To our knowledge, present results provide the first preclinical data on this matter, and suggest that mice exposed to a stacking and boosting regime of mephedrone could be more prone to depressive-like symptoms.

Recently, it has been established that methamphetamine exposure dysregulates D2mediated DA transmission in the striatum [52]; Vidal-Infer et al. [53] also demonstrated that, in adolescent mice, striatal DA D2 receptors are involved in the rewarding properties of MDMA. Accordingly, we have studied the alteration in the striatal density of these receptors after the third mephedrone schedule. The density of D2 receptors remained below control values both 3 and 7 days after mephedrone exposition, pointing to an increase in the susceptibility of these animals to drug addiction [54]. Moreover, we found a significant transient decrease in the number of 5-HT2A receptors 3 days after exposition, which can be reflective of a neuroadaptative response to the increase in 5-HT release induced by mephedrone. These results are in agreement with those reported by Scheffel et al. [55] who demonstrated that the repeated administration of MDMA causes transient down-regulation of 5-HT2 receptors, which are predominantly abundant in the frontal cortex.

We performed *in vitro* studies in cortical cultured cells in order to assess a theoretical concentration that could be injurious on neuron viability, which is known to be quite high for most amphetamines. In these cells we found a concentration-dependent

cytotoxic effect of mephedrone in neuronal viability, which increases significantly with respect to time of drug exposure. It must be pointed that this cytotoxicity is higher than that of MDMA in primary cultures of hippocampal neurons [56]. According to our previous pharmacokinetic data of mephedrone in rats [24], an i.v. dose of 10 mg/kg leads to an extrapolated mephedrone blood concentration of about 5.6 μM. Then, a dose of 25 mg/kg would correspond to a mephedrone concentration of about 14 μM that is about 15 times lower than the LD_{50} in cultured cortical cells. It is important to note that in the present study, when exposing mice to 25 mg/kg of mephedrone no neuronal death is suspected, as there were no signs of striatal or cortical astroglial activation in mephedrone-exposed animals. Only, some reactive astrocytes were appreciated in the dentate gyrus. Additionally, we did not observe microglial activation (data not shown). Results from cortical cultured cells seem to point in the same direction because neuronal death is obtained at higher concentrations than those reached *after in vivo* exposure. Nonetheless, as occurs with MDMA, we cannot exclude the possibility that some of its metabolites may cause toxic effects of this nature, since the *in vivo* metabolic pathway of mephedrone is similar to that of MDMA [57].

Finally, our results demonstrate that mephedrone-induced brain injury differs according to exposition schedule (dose, number of administrations and intervals). Neuronal toxicity elicited by mephedrone is apparent when administered in 2 h intervals. This is in accordance with our previous paper characterizing the pharmacokinetics of mephedrone [24]. Using this dose interval and repeated doses for 2 days, in an elevated ambient temperature, we found a loss of frontal cortex dopaminergic and hippocampal serotoninergic neuronal markers together with astrogliosis, pointing to the presence of injuries at nerve endings. A compensatory mechanism runs in parallel with this process, including an increase in the phosphorylated form of TPH2, a different subcellular distribution of DA transporter, and a decrease in D2 receptors.

Author Contributions

Conceived and designed the experiments: EE JC. Performed the experiments: JM-C RL-A SA DP. Analyzed the data: DP EE JC. Wrote the paper: EE JC.

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7.2. Anexo III (Publicación VII)

Neuronal changes and oxidative stress in adolescent rats after repeated exposure to mephedrone

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Neuronal changes and oxidative stress in adolescent rats after repeated exposure to mephedrone

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Abstract

Mephedrone is a cathinone derivative that has emerged as a designer drug of abuse in Europe and the USA. We investigated the neurochemical/enzymatic changes indicative of neurotoxicity after mephedrone administration (3 x 25 mg/Kg, s.c in a day, with a 2h interval, for two days) to rats that intends to model human recreational abuse. Treatments were carried out at high ambient temperature simulating hot conditions found in dance clubs, where the drug is consumed. In addition, we have studied the mephedrone's effect in spatial learning and memory using the Morris water maze. We observed a hyperthermic response to mephedrone that reached a peak 30 min after each dose. Mephedrone induced loss of dopamine (in the frontal cortex) and serotonin (in the frontal cortex, hippocampus and striatum) transporters without microgliosis. This deficit was also accompanied by a decrease in the expression of the rate-limiting enzymes of dopamine and serotonin biosynthesis. Mephedrone treatment also induced an oxidative stress evidenced by an alteration of antioxidant enzyme levels in different brain areas, as well as an increase of lipid peroxidation. Mephedrone-treated animals only displayed impairments in the probe trial of the Morris water maze, which concerns to reference memory, while the spatial learning process seems to be preserved.

Keywords: Mephedrone. Neurotoxicity. Striatum. Frontal cortex. Hippocampus. Rat

Introduction

The highly restricted availability of precursors needed for the synthesis of methamphetamine and MDMA (3,4-methylenedioxymethamphetamine) in clandestine labs, and the consequent reduction in their purity (Brunt et al., 2011; Winstock et al., 2011) lead to the appearance, in the illicit market, of a new family of drugs of abuse known as “legal highs”. From their chemical structure, they are also known as “beta-ketoamphetamines”. Mephedrone (4-methyl-methcathinone) is one of these new stimulants that is mainly used by young adults and adolescents (Schifano et al., 2011) and has been readily available for legal purchase both online and in some stores. Many users consider the effects of mephedrone to be superior to those of cocaine and MDMA (Vardakou et al., 2011).

As a chemical congener of methamphetamine and MDMA, mephedrone could influence dopaminergic and serotonergic function. In cortical and striatal synaptosomal preparations, mephedrone was found to inhibit serotonin (5-HT) uptake with higher affinity than that of dopamine (DA) uptake, primarily acting as a substrate for plasmalemmal monoamine transporters (Baumann et al., 2012; López-Arnau et al., 2012; Martínez-Clemente et al., 2012). The results on vesicular monoamine transporter inhibition and those obtained with reserpine-treated synaptosomes suggest that the vesicular component is especially important in explaining the neurochemical effects of mephedrone (López-Arnau et al., 2012). Additionally, other authors have described that mephedrone behaves preferentially as a 5-HT and DA releaser (Hadlock et al., 2011). Administration of this drug produces dose-related increases in extracellular 5-HT and DA with the magnitude of effect on 5-HT being 2-3 times greater (Baumann et al., 2012; Kehr et al., 2011).

In rodents, mephedrone acts as a locomotor stimulant (Baumann et al., 2012; Kehr et al., 2011; López-Arnau et al., 2012; Martínez-Clemente et al., 2013) and induces self-administration (Hadlock et al., 2011). When binge-administered, mephedrone produces an increase of body temperature in rats (Baumann et al., 2012; Hadlock et al., 2011).

Hyperthermia has been reported as a key contributing factor in 5-HT depletion (Green et al., 2004). In the basis of the 5-HT-releasing capability of mephedrone (Cameron et al., 2013), we postulated that binge administration of this drug could cause dysfunction of brain 5-HT or DA terminals, similarly to the effects of 5-HT transporter substrates like MDMA (Baumann et al., 2008; Commins et al., 1987; Malberg and Seiden, 1998). Furthermore, Blum et al. (2013) hypothesized the potential of cathinones to produce neurotoxic effects in various brain regions and this requires intensive investigation.

Previous published results revealed that, in contrast to MDMA, methamphetamine or methcathinone (Fleckenstein et al., 2000; Haughey et

al., 2000; Metzger et al., 2000), repeated mephedrone injections does not affect monoamine neurotransmitter levels (Baumann et al., 2012). On the contrary, Hadlock et al. (2011) found a persistent hippocampal serotonergic, but not striatal dopaminergic deficit after mephedrone treatment.

Mephedrone tends to be consumed in hot environments such as confined dancing spaces (Schifano et al., 2011) and users experience a strong desire to redose, leading them to ingest large amounts of the drug in binges that can last several days (Winstock et al., 2011). Accordingly, present experiments were carried out at a high ambient temperature simulating the conditions found in dance clubs. Moreover, hyperthermia by itself can induce an oxidative stress. This is one of the main factors involved in nerve terminal injury induced by amphetamines (Jayanthi et al., 1999; Yamamoto and Zhu, 1998). Therefore, in the present paper, we determined the levels of some enzymes involved in the cell redox balance and lipid peroxidation following mephedrone administration.

The aim of this paper was to investigate the effects of mephedrone in brain 5-HT or DA nerve terminals of rats, administered in a pattern used frequently to mimic psychostimulant "binge" administration. Accordingly, a multiple dose/day administration schedule was used to mimic the widespread practices of “stacking” (taking multiple doses at once) and “boosting” (taking supplemental doses over time in order to maintain the drug’s effect). Additionally we investigated the consequences of a neurotoxic treatment of mephedrone in a behavioral model of spatial learning and memory.

Methods

Animals and treatment. Male Sprague-Dawley rats (200 – 225 g, aged 5-6 weeks) (Lé Genest, France) were used. The animals were housed two per cage in a regulated environment (21 ± 1 ºC; 12 h light/dark cycle, lights on at 08:00 h) with free access to food and water. With the exception of experiments registering body temperature, other experiments took place between 09:00 and 15:00 h. Experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona and following the guidelines of the European Communities Council (86/609/EEC). Animals were administered subcutaneously with three doses of saline (1 ml/Kg) or mephedrone (25 mg/Kg) with a 2h interval, for two consecutive days. During the treatment, the animals remained one per cage, were maintained in an ambient temperature of 26 ± 2 ºC and were kept under these conditions until 1h after the last daily dose.

No information about the subcutaneous doses of mephedrone in humans is available. The only approach would be the doses used orally, which are comprised between 100 and 300 mg, or intranasally that can

reach up to 125 mg in each insufflation. In our case, a dose of 25 mg/Kg in rats corresponds to 4 mg/Kg in an adult. This rat equivalent dose was calculated following the body surface area normalization method (Reagan-Shaw et al., 2008). Moreover, the interval of 2h between doses was chosen according our recently published paper on mephedrone pharmacokinetics in rats (Martínez-Clemente et al., 2013).

Drugs and reagents. Pure racemic mephedrone hydrochloride was synthetized and characterized by us as described (López-Arnau et al., 2012). Mephedrone solutions for injection were prepared in sterile 0.9% NaCl (saline) immediately before administration. Isoflurane was from Laboratorios Dr. Esteve (Barcelona, Spain). The other drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]ketanserin, [^{3}H]paroxetine, [^{3}H]PK11195, [^{3}H]raclopride and [^{3}H]WIN35428 were from Perkin Elmer (Boston, MA, USA). All buffer reagents were of analytical grade.

Surgical procedures and acquisition of body temperature. The animals (n=6-8 per group) were allowed one week after arrival to acclimatize before surgery. Subsequently, they had implanted an electronic device (Thermo Tracker, Barcelona, Spain), which enabled continuous measurement of core body temperature. The implant was placed in the abdominal cavity, under isoflurane anesthesia. The abdomen was opened by making a 2-cm midline incision and the device was placed in the abdominal cavity, along the sagittal plane. The muscular and the skin wounds were then closed with absorbable suture material. After surgery, animals were individually housed and received analgesic therapy for 3 days. The animals were allowed to recover for 7 days before saline or mephedrone administration avoiding a possible influence of this type of manipulation. The thermographs had been programmed to record the core temperature every 5 min from 24h prior until 24h after drug treatment. The devices were removed from the rats after sacrifice. The recorded values were downloaded to a computer using a dedicated interface and processed by the software provided by the manufacturer.

Tissue sample preparation. Crude membrane preparation was prepared as described (Escubedo et al., 2005) with minor modifications. The animals were killed by decapitation under isoflurane anesthesia at 6 h (Lipid peroxidation experiments; n=5-7 animals/group), 1 (Lipid peroxidation experiments; n=5-7 animals/group and antioxidant enzymes determination; n=5-7 animals/group) or 7 days (Radioligand binding and Western blotting of TH and TPH-2 experiments; n=5-8 animales/group) after treatment, and the brains were rapidly removed from the skull. Hippocampus, striatum and frontal cortex were quickly dissected out, frozen on dry ice, and stored at -80 °C until use. When required, tissue samples were thawed and

homogenized at 4 °C in 20 volumes of 5 mM Tris-HCl buffer (0.32 M sucrose) with protease inhibitors, pH 7.4. The homogenates were centrifuged at 1,000 x *g* for 15 min at 4 °C. Aliquots of the resulting supernatants were stored at -80 ºC until use for Western blot experiments. The rest of the samples were resuspended and centrifuged at 15,000 x *g* for 30 min at 4 °C. The pellets were resuspended in buffer and incubated at 37 ºC for 5 min to remove endogenous neurotransmitters. The protein samples were recentrifuged. The final pellets (crude membrane preparations) were resuspended in the appropriate buffer and stored at -80 °C until use in radioligand binding experiments. Protein content was determined using the Bio-Rad Protein Reagent following the manufacturer's instructions. For measuring the malondialdehyde (MDA) production, tissue samples were homogenized on ice in 30 volumes of the MDA lysis buffer. The homogenates were centrifuged at 13,000 x g for 10 min to remove insoluble material. Aliquots of supernatant were used for lipid peroxidation assay (see below).

DA and 5-HT transporter density and glial activation. The density of DA transporters in striatal or frontal cortex membranes was measured by [^{3}H]WIN35428 binding. Assays were performed in tubes containing 250 or 500 µl of [^{3}H]WIN35428 diluted in phosphate-buffered (final radioligand concentration, 5 nM) and 50 or 100 µg of membranes, respectively. Incubation was done for 2h at 4 °C and non-specific binding was determined in the presence of 30 µM bupropion.

The density of 5-HT transporters in hippocampal, striatum and frontal cortex membranes was quantified by measuring the specific binding of 0.1 nM [^{3}H]paroxetine after incubation with 150 µg of protein at 25 ºC for 2h in 50 mMTris-HCl buffer (pH 7.4), containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 µM) was used to determine non-specific binding.

Microglial activation was assessed by measuring [^{3}H]PK11195 binding. Briefly, rat striatal, frontal cortex or hippocampal membranes were resuspended in 50 mM Tris-HCl buffer, pH 7.4. Equilibrium binding assays were performed at 4°C for 2 h in tubes containing 2 nM [^{3}H]PK11195 and 50 µg of protein in a final volume of 0.25 ml. Unlabeled PK11195 (10 µM) was used to determine non-specific binding.

In all the cases, the incubations were finished by rapid filtration under vacuum through Whatman GF/B glass fibre filters pre-wet with 0.5% polyethyleneimine. Tubes and filters were washed rapidly three times with 4 ml of ice-cold buffer, and the radioactivity in the filters was measured by liquid scintillation spectrometry.

Western blotting and immunodetection. A general Western blotting and immunodetection protocol was used to determine the expression of tyrosine

hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2), as well as antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (Gpx), catalase (CAT)) and nitric oxide synthase (nNOS) in animals exposed to the treatment. For each sample, 20 µg of protein was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min, and loaded onto a 10% acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P; Millipore, USA). PVDF membranes were blocked overnight with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated for 2h at room temperature with a primary mouse monoclonal antibody against TH (Transduction Laboratories, Lexington, KY, USA) dil. 1:5000, rabbit polyclonal anti-TPH2 (Millipore, Billerica, MA, USA) dil. 1:1000, sheep polyclonal anti-SOD Cu/Zn) (EMD Chemicals, La Jolla, CA, USA) dil. 1:2000, mouse monoclonal Gpx (ab108427, Abcam, Cambridge, UK) dil. 1:1000, CAT (EMD Chemicals, La Jolla, CA, USA) dil 1:2500 and rabbit polyclonal anti-nNOS (ab95436,Abcam, Cambridge, UK) dil 1:500.

After washing, the membranes were incubated with a corresponding peroxidase-conjugated anti-IgG antibody: antimouse IgG dil. 1:2500; antirabbit IgG dil. 1:5000 (GE Healthcare, Buckinghamshire, UK) and anti-sheep IgG, dil 1:1000 (Dakocytomation, CA, USA). Immunoreactive protein was visualized using a chemoluminescence-based detection kit following the manufacturer's protocol (Immobilon Western, Millipore) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Hercules, CA, USA). Scanned blots were analyzed using BioRad Image Lab software and dot densities were expressed as a percentage of those taken from the control. Immunodetection of beta-actin (mouse monoclonal antibody, dil.1:2500) served as a control of load uniformity for each lane and was used to normalize differences in the corresponding enzyme expression due to protein content.

5-HT$_{2A}$ and D$_2$ receptor density. The density of 5-HT$_{2A}$ receptors was measured in rat frontal cortex membranes one week after their exposure to the treatment, using [^{3}H]ketanserin binding. Assays were performed in tubes containing 1 nM [^{3}H]ketanserin and 100 µg of membranes. Incubation was carried out at 37 °C for 30 min in a Tris–HCl buffer to a final volume of 0.5 ml. Methysergide (10 µM) was used to determine non-specific binding.

Similarly, the density of D$_2$ receptors in striatum membranes of the same animals was measured by [^{3}H]raclopride binding. Assays were performed in tubes containing 2 nM [^{3}H]raclopride and 50 µg of membranes. Incubation was carried out at 25 °C for 1h in a Tris–HCl buffer to a final volume of 0.5 ml. Sulpiride (300 µM) was used to determine non-specific binding. All incubations were finished by rapid filtration under

vacuum, washed rapidly and the radioactivity was measured by liquid scintillation spectrometry as described above.

Lipid peroxidation. Lipid peroxidation was assessed using a colorimetric assay kit (Lipid peroxidation assay kit, Sigma-Aldrich) following the manufacturer's instructions. The kit measured the accumulation of thiobarbituric acid (TBA)-reactive substances in homogenates from striatum, frontal cortex or hippocampus, expressed in terms of MDA content. Sampleswere incubated with TBA at 95°C for 60 min. The reaction was stopped by chilling samples on ice. The absorbances of the resulting supernatants were measured at 532 nm, and the concentrations of MDA were calculated by interpolation in a standard curve built with known concentrations of MDA standard.

Morris water maze. Spatial learning and memory were assessed in a Morris water maze one week after treatment. Animals (n=9-11 per group) were trained in the water maze which consisted of a circular pool (160 cm diameter and 45 cm high) that was filled with water (22 ± 1ºC) to a depth of 25 cm and rendered opaque by the addition of a non-toxic latex solution. The pool was in an isolated room and black curtains were closed around it to minimize static room cues. Four positions around the edge of the tank were designated as north (N), south (S), east (E), and west (W) and also defined the division of the tank into four quadrants: NE, SE, SW, and NW, providing alternative start positions A Plexiglas escape platform (11 cm diameter) was submerged to a depth of 1 cm from the water surface and was not visible at the water level. The path taken by each rat and the escape latency (the time needed by each rat to find the platform, in s) was recorded by a zenithal video camera connected to a computer running a tracking software (Smart, Panlab SL, Barcelona, Spain)

In the spatial learning (acquisition) task, four objects were suspended from a false ceiling to 30 cm above the water surface in N, S, E and W positions. The platform was always located in the SW quadrant. The rats received a training session, consisting of six trials per day by using a semi-random set of start locations that were not equidistant from the goal, creating short and long paths to the platform. This was designed so that the animal was not able to learn a specific order of right or left turns to locate the platform, because any of the start positions was repeated the same day. The animals were tested on four consecutive days (a total of 24 trials per animal were to reach asymptotic performance).

A trial was started by placing a rat in the desired start position of the pool, facing the tank wall. The rats were allowed to swim to the hidden platform, and the escape latency was determined. If an animal did not escape within 120 s, it was gently placed on the platform or guided to it. The rats were allowed to rest for 30 s (inter-trial interval) on the platform

(even those that failed to locate it). This procedure was repeated with each animal along the trials.

To assess reference memory at the end of learning, a probe trial (free swimming without platform for 60 s), was given 24 h after the last training session. We used a novel start position during the probe trial to ensure that its spatial preference was a reflection of the memory of the goal location rather than for a specific swim path. Different parameters of the rat's performance were analyzed: the total time spent swimming in the target quadrant (where it should be located the platform) and the opposite quadrant, the relative distance travelled, the swim speed and the shortest distance between the tracked point of the animal and the target zone (absent platform).

Data analysis. Results are given as the mean ± S.E.M. (standard error of the mean). One-way or two-way repeated measures ANOVA, followed by Tukey's test, were used to verify the significance between means. P values less than 0.05 were considered significant.

Results

Effect of mephedrone on body weight and temperature

Mephedrone produced a significant inhibition of weight gain measured 24 h after the last dose (saline: 9.67 ± 0.74 g; mephedrone: -0.97 ± 1.03 g, $P < 0.001$). Moreover, 7 days after finishing the treatment, the animals recovered their weight (data not shown).

At the beginning of the experiments, prior to drug administration, there was no significant difference on body temperature between the groups (saline: 37.13 ± 0.21ºC; mephedrone: 37.09 ± 0.26ºC). On day 1, after receiving the first dose of mephedrone, the treated animals showed a significant transient reduction in body temperature of about 1.5 ºC, while on day 2 such decrease was milder (0.5 ºC) and statistically non-significant.

Moreover, on day 1, mephedrone-treated rats recovered from the transient hypothermia and, after the second dose, temperature raised over the saline values, remaining increased for a further 3h.

Second and third mephedrone injections increased body temperature by 1.5 ºC and 2 ºC respectively on day 1 and 0.5 and 1ºC on day 2. This hyperthermia could be the result of cumulative doses (Fig. 1). Consequently, mephedrone-treated rats were hyperthermic for 70% of time after treatment.

Effect of mephedrone on different in vivo markers of DA and 5-HT neurotoxicity.

At 7 days post-treatment, mephedrone induced a significant loss in DA transporters in frontal cortex (saline: 100.00 ± 5.96%; mephedrone: 69.65 ± 5.12%, $P < 0.01$), measured as specific binding of [^{3}H]WIN35428. The same treatment was ineffective in modifying the density of DA transporters in the striatum (Fig. 2A and 2B)

Moreover, mephedrone-treated rats showed a significant decrease in the density of 5-HT transporters, measured as specific [^{3}H]paroxetine binding in striatal, cortical, and hippocampal membranes (Fig. 3A, 3B and 3C).

Accordingly, we further investigated the expression of other biochemical markers of neuronal integrity, such as TH and TPH2 in those brain areas showing a decrease of radioligand binding parameters.

In frontal cortex membranes, the decrease of [^{3}H]WIN35428 binding ran in parallel with a significant decrease of TH levels (saline: 100.00 ± 18.78%; mephedrone: 51.28 ± 9.52%, $P < 0.05$) (Fig. 2D). Similarly, mephedrone did not alter TH levels in striatum, an area in which the specific binding of [^{3}H]WIN35428 remained unchanged (Fig. 2C). Moreover, a significant decrease in TPH2 levels in all the three brain areas was found (Fig. 3D, 3E and 3F). Immunoblotting results were in accordance with the corresponding [^{3}H]paroxetine binding reductions.

[^{3}H]PK11195 specific binding was measured to investigate the microglial activation after neuronal injury in rats sacrificed 24 h post-treatment. In these animals, no increase in the density of [^{3}H]PK 11195 binding sites was detected in rats receiving mephedrone, indicating a lack of microglial activation (data not shown).

Effect of mephedrone on D_2 and 5-HT_{2A} receptor density.

We measured the effect of mephedrone treatment on the density of DA and 5-HT receptor subtypes. Mephedrone induced a significant decrease in the density of D_2 receptors in the striatum, measured as [^{3}H]raclopride binding (saline: 100.00 ± 3.19%; mephedrone: 81.55 ± 4.76%, $P < 0.05$) seven days after treatment.

Similarly, mephedrone-treated rats showed an apparent, although non-significant, decrease in the density of 5-HT_{2A} receptors in hippocampus (saline: 100.00 ± 11.56%; mephedrone: 79.95 ± 6.60%), measured as [^{3}H]ketanserin binding. In frontal cortex membranes, the density of both receptor types remained unchanged after mephedrone treatment.

Effect of mephedrone on antioxidant enzyme levels

In our study, we assessed several markers of oxidative stress in mephedrone-treated rats by measuring the levels of the antioxidant enzymes

SOD, CAT, Gpx as well as nNOS in the hippocampus, striatum and frontal cortex of animals sacrificed 24 h post-treatment.

As can be seen in Table 1, SOD expression was significantly increased in the hippocampus (45%) and frontal cortex (38%) in mephedrone-treated rats. In contrast, a significant decrease of about 30% was found in the striatum.

CAT enzyme levels were increased in the frontal cortex of mephedrone-treated rats by about 149% in comparison with the saline group, while CAT levels in the striatum and hippocampus were not different from those of saline-treated animals.

After mephedrone exposure, we found a significant increase in Gpx enzyme levels in the three studied brain areas.

Animals exposed to mephedrone did not show changes in the levels of nNOS, but they experimented an increase in the hippocampus (about 44%, $P = 0.058$). Otherwise, levels of nNOS were significantly reduced in the striatum.

Effect of mephedrone on lipid peroxidation

At 6 and 24 h after treatment, a significant increase in lipid peroxidation was found in the frontal cortex of mephedrone-treated rats, measured as a raise in the MDA levels, a general indicator of the decomposition of polyunsaturated fatty acids. No detectable signs of increased MDA were found in the striatum or the hippocampus (Fig. 4).

Effect of mephedrone on the Morris water maze

We investigated the effect of mephedrone on learning and memory processes seven days after finishing the treatment, using the Morris water maze. The analysis of the swimming mean speed in the overall maze denoted no differences between groups (saline: 25.18 ± 1.04 cm/s; mephedrone: 28.57 ± 1.34 cm/s). Therefore, latency was taken to quantify the performance in the water maze.

Overall, there was an appropriate learning of the task in both saline and mephedrone-treated rats. In the acquisition phase, two-way repeated measures ANOVA showed a significant effect of treatment on specific training days (variable treatment [$F_{1,15} = 5.33$, $P < 0.05$]; variable days of acquisition [$F_{3,45} = 27.33$, $P < 0.001$] and variable treatment x day [$F_{3,45} = 1.34$, n.s.) indicating that the escape latency diminished as the trials progressed (from day 1 to day 4) and was indicative of learning (Fig. 5A). 24 h after the last acquisition training day, the test trial demonstrated that animals treated with saline spent significantly longer ($P < 0.01$ vs. the random time of 15.00 s) in the target quadrant (that in which the platform should be found) than in the opposite, or in the rest of quadrants ($F_{2,21}$ =

8.69, $P < 0.01$). Mephedrone-treated rats did not remember the location of the platform ($F_{2,24} = 1.18$, n.s.) since the time spent in the different quadrants was not significantly different from the random time) (Fig. 5B). A similar behavioral profile of memory performance was evidenced when we analyzed the number of entries and the distance travelled in the different quadrants of the maze in the test trial (Table 2).

Additionally, we analyzed the shortest distance between the tracked point of the animal and the target zone (absent platform) in the test session. This parameter give us an index of how much a rat has been close to the target zone during the trial. Saline-treated animals showed significant ($P < 0.05$) less distance to the area where the platform was located during training (56.55 ± 5.46 cm) than mephedrone-treated rats (71.30 ± 2.47 cm).

Discussion

There has been a dramatic increase in the abuse of synthetic cathinones. Mephedrone is highly reinforcing and users experience a strong desire to re-dose, leading them to ingest large amounts of the drug in binges that can last several days (Winstock et al., 2011). This justifies the urgent need for additional research into its long-term effects. Therefore, the primary goal of this study was to evaluate the risk of neuronal changes linked to mephedrone abuse in rats.

To estimate the relevance of the doses applied in the present study to the doses taken by humans, we used an allometric scaling method following these factors: for an animal dose of 25 mg/kg; a human adolescent weight of 60 kg; an average rat weight of 0.22 kg, and an allometric scaling coefficient of 0.66 (Chiou et al., 1998), the resultant human equivalent dose is 4 mg/kg. On this basis, a 25 mg/kg dose given to rats during the periadolescent period may be considered to represent a human recreational mephedrone use.

Another important issue in mephedrone studies is the frequency of dosing. Our aim was to simulate weekend mephedrone use. In this regard, we administered mephedrone in a 2h interval since the half-life of this drug after oral administration in rats is about 30 min (Martínez-Clemente et al., 2013). The fall of mephedrone plasma levels after 2 h simulated the desire to re-dose and modeled mephedrone boosting in humans that occurs in order to extend the drug's subjective effects.

To our knowledge, there are controversial studies devoted to the neurotoxicity of mephedrone in rats and this toxicity was only evidenced in one. Baumann et al., (2012) demonstrated that repeated doses of mephedrone (3 and 10 mg/Kg) caused significant hyperthermia in male Sprague-Dawley rats but no long-term changes in cortical or striatal monoamine levels. These authors explained the absence of neurochemical

changes to the fact that those doses did not induce a sufficient level of hyperthermia.

(Motbey et al., 2012) did not found any changes in 5-HT or DA brain levels seven weeks after treatment of male Wistar rats with a wide range of mephedrone doses (from 7.5 to 30 mg/Kg for ten days) administered at room temperature.

Hadlock et al. (2011) demonstrated that, in rats, repeated mephedrone injections (4 x 10 or 25 mg/kg s.c.) at 2h interval, cause a rapid decrease in striatal dopamine and hippocampal serotonin transporter function. However, there is an important difference between our experiments and those of Hadlock et al. These authors carried out their treatment in group-housed rats, condition that is known to impede heat dissipation and exacerbate the deleterious effects of amphetamines (Green et al., 2004).

Like other amphetamines, in our study the animals treated with mephedrone experienced a loss in weight gain, probably due to an anorectic effect of the drug. Nevertheless, the reductions in weight gain were only transient as it was recovered seven days after finishing the treatment.

The rise in body temperature is a commonly reported and life-threatening consequence of amphetamine abuse in humans (da Silva et al., 2014; Prosser and Nelson, 2012). Recent studies demonstrate that mephedrone induces hyperthermia, both in individually housed rats at normal ambient temperatures (Baumann et al., 2012) or in group-housed rats in a warm environment (Hadlock et al., 2011).

There exists a close relationship between the hyperthermic response and the severity of the brain lesion induced by amphetamines (Sanchez et al., 2004), supporting the hypothesis that amphetamine derivatives are neurotoxic when a binge-dosing schedule is employed and the animals are in a hot environment. Accordingly, the present experiments were carried out at a high ambient temperature simulating hot conditions found in dance clubs where mephedrone is often consumed.

We monitored the changes in core body temperature of the rats using an implanted device throughout the entire period of drug treatment. This device allowed a more reliable and continuous measure of temperature, avoiding the stress of restraining the rats that occurs when using a rectal probe. After the first dose of mephedrone, the rats showed a transient but apparent reduction of body temperature. This decrease after mephedrone challenge was recently described by (Shortall et al., 2013) by measuring tail and rectal temperature changes in adult Lister hooded rats. Blockade of adrenergic or dopaminergic receptors did not antagonize (and in some cases potentiated) mephedrone-induced hypothermia, suggesting that a metabolite, rather than the parent compound may be responsible of the peripheral effects of mephedrone. Moreover, (Miller et al., 2013) found that an elevated ambient temperature (30 ºC) prevented the hypothermia

produced by mephedrone (at 20 ºC). Further research is required in order to characterize the thermoregulatory impairment induced by mephedrone.

Mephedrone induced loss of DA (in the frontal cortex) and 5-HT (in all studied areas) transporters. This deficit was also correlated with a significant decrease of the corresponding enzymatic markers. TH and TPH2 (the TPH isoform responsible for 5-HT biosynthesis in the brain) catalyze the first and rate-limiting step in the biosynthesis of DA and 5-HT respectively. The decrease in transporter binding and enzyme levels, point to an injury at the nerve endings. When neuronal injury is accompanied by reactive microgliosis, this glial response is already evident 24h after treatment (Pubill et al., 2003). From the present results, the lack of an increase in the density of [^{3}H]PK11195 binding sites rules out the presence of microglial activation after mephedrone treatment.

It is well known that the administration of methamphetamine to rodents results in damage to dopaminergic neurons through the production of reactive oxygen species (ROS)(Cadet et al., 2009). ROS production in animals exposed to this amphetamine was demonstrated by the increase in lipid peroxidation, SOD and Gpx expression (Açikgöz et al., 1998; Imam et al., 2001a) or using transgenic mice that overexpress Cu/ZnSOD gene (Cadet et al., 1994; Hirata et al., 1996) or nNOS knockout mice (Imam et al., 2001b). Although mephedrone does not induce a release of dopamine as high as methamphetamine, we investigated a possible production of ROS by mephedrone.

In the striatum, there was not an increase in SOD, rather the opposite, suggesting a lack of production of superoxide radicals. Additionally, the raise in the ratio CAT/SOD and Gpx/SOD lead to hypothesize that this tissue can easily buffer the oxidative stress. All this, jointly with the down-regulation of nNOS, may explain the absence of dopaminergic injury in this area.

In the frontal cortex, the brain area most affected by mephedrone, the levels of all the investigated enzymes were increased. Enzyme up-regulation represents a mechanism whereby brain cells try to get rid of some radicals generated by mephedrone. SOD up-regulation provides indirect confirmation of increased superoxide production. Moreover, CAT protects SOD against inactivation by hydrogen peroxide (Reddy et al., 1999), therefore CAT up-regulation also means increased hydrogen peroxide generation.

The up-regulation of Gpx in the cortex of the animals exposed to mephedrone may be also triggered by increased generation of lipoperoxides coming from ROS-mediated oxidation of polyunsaturated fatty acids, which were significantly increased in this brain area. The up-regulation of Gpx

observed 24h after mephedrone exposure, points to oxidative stress generation but, on the other hand, represents a favorable response that probably contributes to attenuate the final damage. In the hippocampus, there was no increase in CAT but a marked up-regulation of SOD, Gpx and nNOS. The concomitant up-regulation of nNOS and SOD suggests the generation of significant amounts of nitric oxide and superoxide to generate peroxynitrite, which, in turn, can cause tissue injury. In fact, the hippocampus also showed alterations of serotonergic terminals. The high increase in Gpx levels in this area probably could be explained as a way to dampen lipid oxidation.

In the present study, TBARS were lower in the frontal cortex and hippocampus of saline-treated rats, than in the striatum. Additionally, DA levels in the striatum are much higher than in the frontal cortex (Aksu et al., 2009). Therefore, if an oxidative stress was originated by an increase in DA levels (i.e. by mephedrone), lipid peroxidation might be expected to be much higher in the striatum than in the frontal cortex. However, in the present study, we determined that the rise in TBARS only took place in the frontal cortex. It is important to note that the striatum is particularly rich in enzymes regulating the metabolism of ROS (Mizuno and Ohta, 1986). Accordingly, it has been reported that haloperidol administration induces a much higher increase in TBARS in the cortex than in the striatum as a consequence of increased hydrogen peroxide production following metabolism of DA (Burger et al., 2005). Our results in frontal cortex agree with these observations.

We cannot rule out that the source of ROS results from the mephedrone molecule itself. However (den Hollander et al., 2013), using SH-SY5Y cells, demonstrated that beta-ketoamphetamines are effective and selective reductants in the presence of electron acceptors, and they found no evidence of protein adduct formation, suggesting that the reactivity is due to direct electron transfer by the beta-ketoamphetamines. Indeed, these authors observed in vitro a cytotoxic effect only at mephedrone concentrations higher than 500 µM. At lower concentrations, this substance reduces LDH release pointing to a cytoprotective effect, probably due to its redox reactivity. This suggests that, in our study, the most important source of ROS is the dopamine released by mephedrone.

(Vidal-Infer et al., 2012) demonstrated that, in mice, striatal DA D_2 receptors are involved in the rewarding properties of MDMA. Accordingly, we have studied the alteration of the density of these receptors after mephedrone treatment. This density remained below control values 7 days after mephedrone treatment, pointing to an increase in the susceptibility of these animals to drug addiction (Volkow et al., 2004). Moreover, we found

a substantial, although non-significant decrease in the number of 5-HT_{2A} receptors, which can be reflective of a partial neuroadaptative response to the increase in 5-HT release induced by mephedrone.

To our knowledge, there are no published papers about how mephedrone can affect spatial learning and memory in adolescent rats. Formation of spatial memory has been shown to depend on the hippocampus (Morris, 1984). Our results demonstrate that mephedrone impaired the spatial memory when assessed in the Morris water maze. The present results show that, in this paradigm, the escape latency shortened significantly in both saline and mephedrone-treated animals as the trials progressed, indicating that there was an appropriate task learning. It must be pointed that the learning process in mephedrone-treated animals was consolidated from the third day since a non-significant difference was observed in the two last days of the acquisition phase.

The probe trial was designed to examine the extent of spatial discrimination learning. The results of this trial demonstrate that mephedrone-treated rats failed to show preferential searching in the target quadrant. In contrast, saline-treated group spent more time in the target quadrant than in any other quadrant indicating spatial memory retention. To exclude the impact of swim speed alterations induced by mephedrone on water maze performance, we also examined swim speed of rats on the test day. The impairments in memory function induced by mephedrone were not due to the possible impairment of motor function because the swimming speed was similar in both groups.

In conclusion, this study demonstrates that, mephedrone injections, administered in a human recreational pattern, causes a decrease in weight gain and altered the thermoregulatory function. Moreover, mephedrone causes a decrease in DA and 5-HT transporter function accompanied by a decrease in the expression of the rate-limiting enzymes of DA and 5-HT biosynthesis. This neuronal injury is evidenced when a binge regimen is used. Although further research is needed, present mephedrone treatment generates an oxidative stress that can be the responsible for the neuronal damage, which, in turn, leads to cognitive deficits that can be a matter of concern regarding long-term consequences of human abuse.

Conflicts of interest

None declared

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Legends of Figures

Figure 1

Effect of mephedrone on core body temperature. Rats (n=6-8 per group) were treated with saline (1ml/kg, s.c.) or mephedrone (3 x 25 mg/kg, s.c., 2h intervals for 2 days). The body temperature was recorded in day 1 (Panel A) and day 2 (Panel B) using an electronic implant at 5 min-interval. Results are presented as means ± SEM. Injections times are indicated by arrows.

Figure 2

Effect of mephedrone (Meph) treatment in dopamine transporter density, measured as [^{3}H]WIN35428 binding in the striatum (panel A) and frontal cortex (panel B); effect in tyrosine hydroxylase (TH) expression in the striatum (panel C) and frontal cortex (panel D). The values correspond to animals killed seven days after treatment. Results are expressed as mean ± S.E.M. from 5-8 animals per group. *P < 0.05 and **P < 0.01 vs. saline.

Figure 3

Effect of mephedrone (Meph) treatment in serotonin transporter density, measured as [^{3}H]paroxetine binding in the striatum (panel A), frontal cortex (panel B) and hippocampus (panel C); effect in tryptophan hydroxylase 2 (TPH2) expression in the striatum (panel D), frontal cortex (panel E) and hippocampus (panel F). The values correspond to animals killed seven days after treatment. Results are expressed as mean ± S.E.M. from 5-8 animals per group. **P < 0.01 and ***P < 0.001 vs. saline

Figure 4.

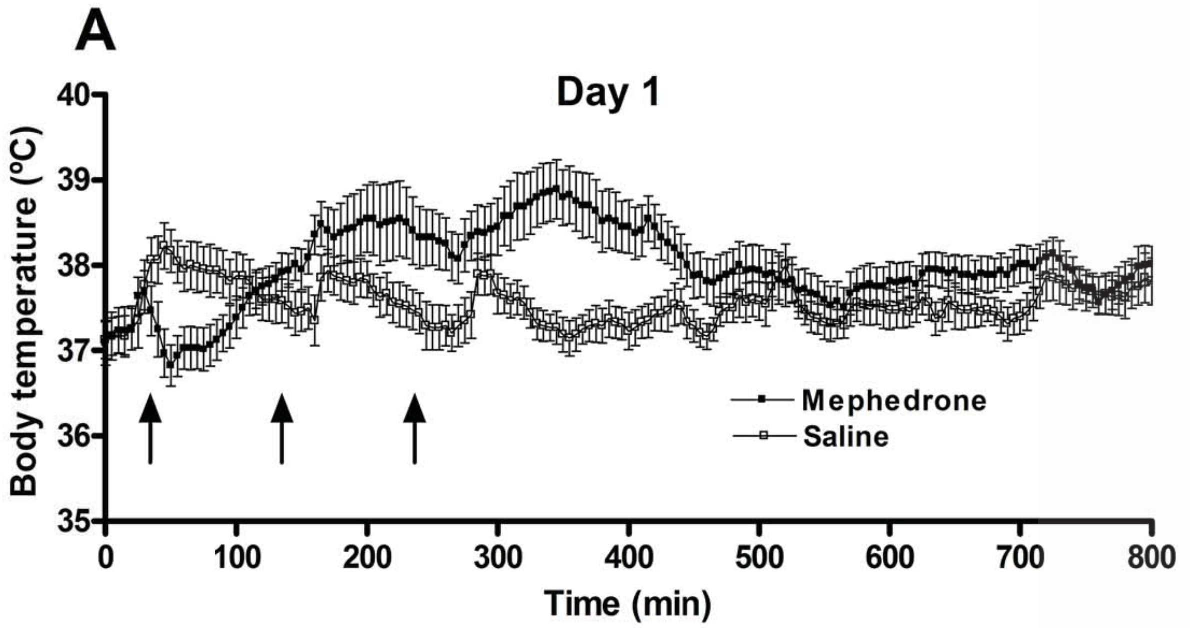
Effect of mephedrone (Meph) treatment in lipid peroxidation evidenced at 6h (Panel A) and 24h (Panel B) after treatment and measured as the malonyldialdehyde (MDA) levels in the striatum, frontal cortex and hippocampus. Results are expressed as mean ± S.E.M. from 5-7 animals per group. *P < 0.05 vs. saline.

Figure 5

Panel A. Effect of treatment with saline or mephedrone in the acquisition phase of spatial learning in the Morris water maze. The escape latency (the time required to locate the platform) for the four different days. Panel B. Effect of mephedrone treatment on the probe trial expressed as the percentage of time spent by the animals in the target (T), the opposite (O) and the rest (R) of quadrants. Results are expressed as mean ± S.E.M. from

9-11 animals per group. **$P < 0.01$ vs. saline (two-way ANOVA of repeated measures followed by Tukey's post-hoc test).

Fig. 1



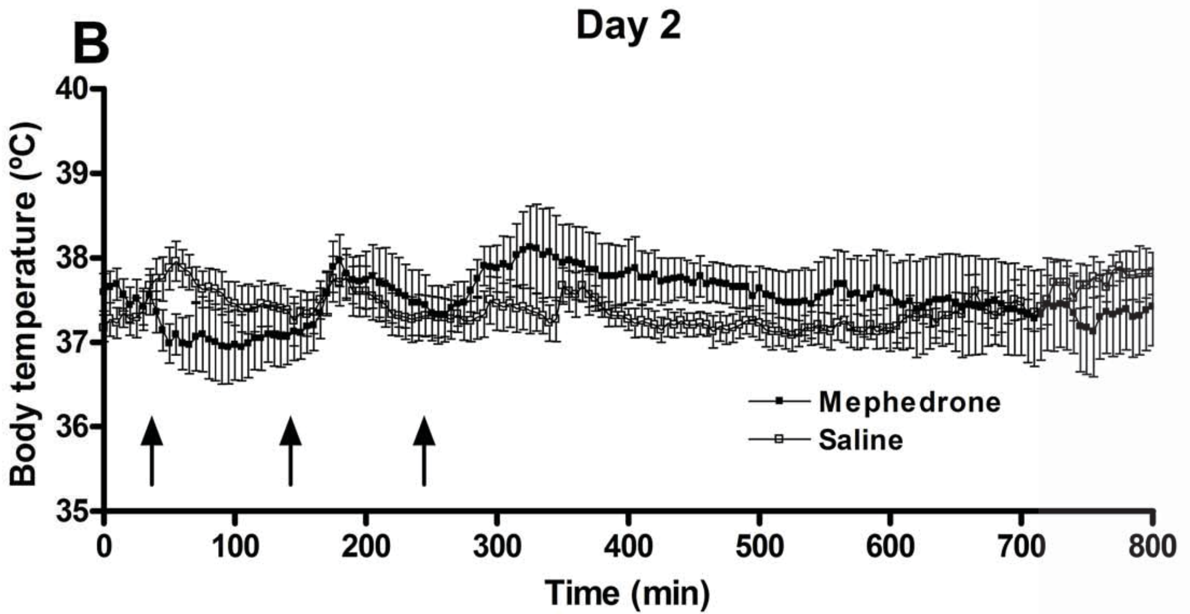


Fig. 2

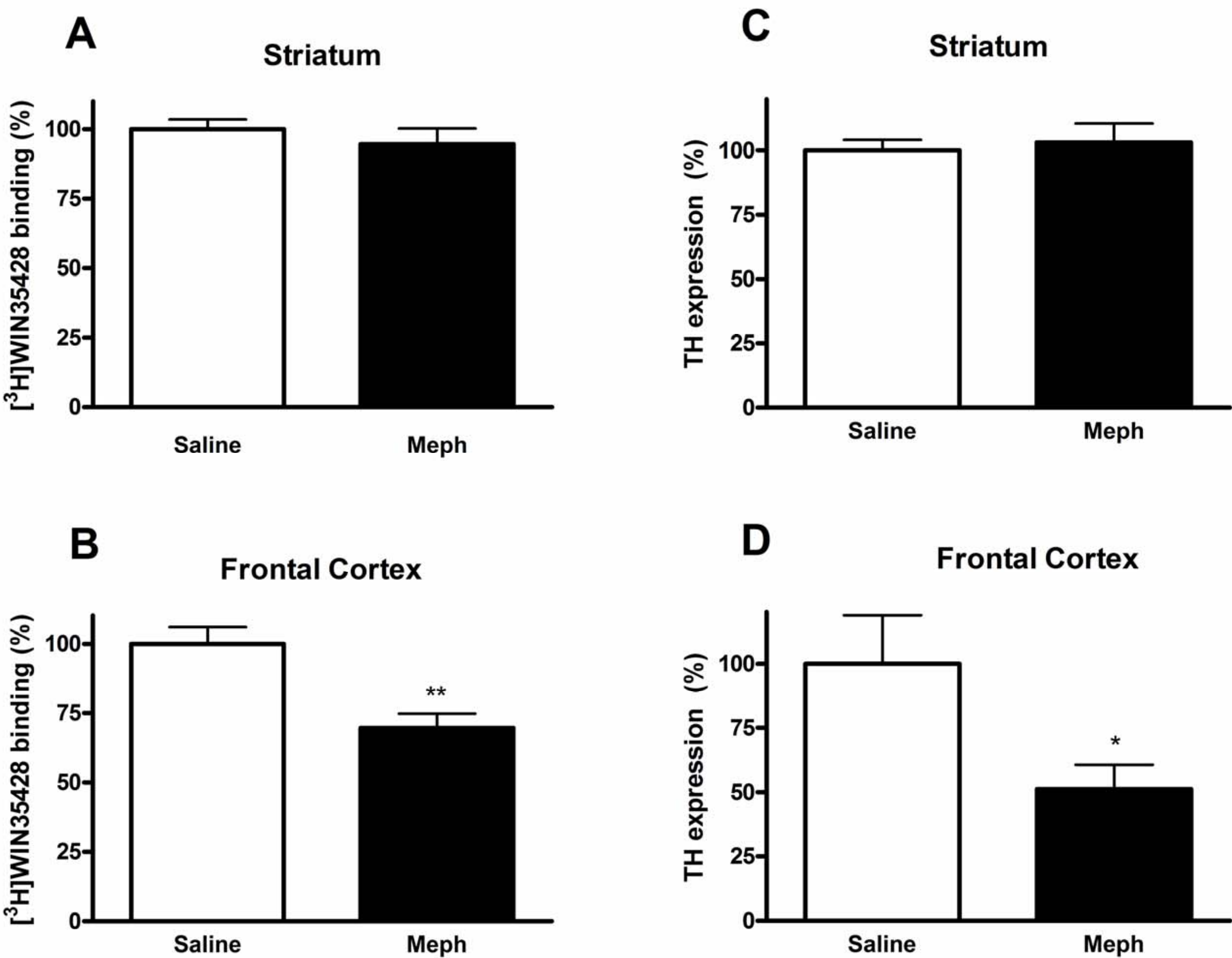


Fig. 3

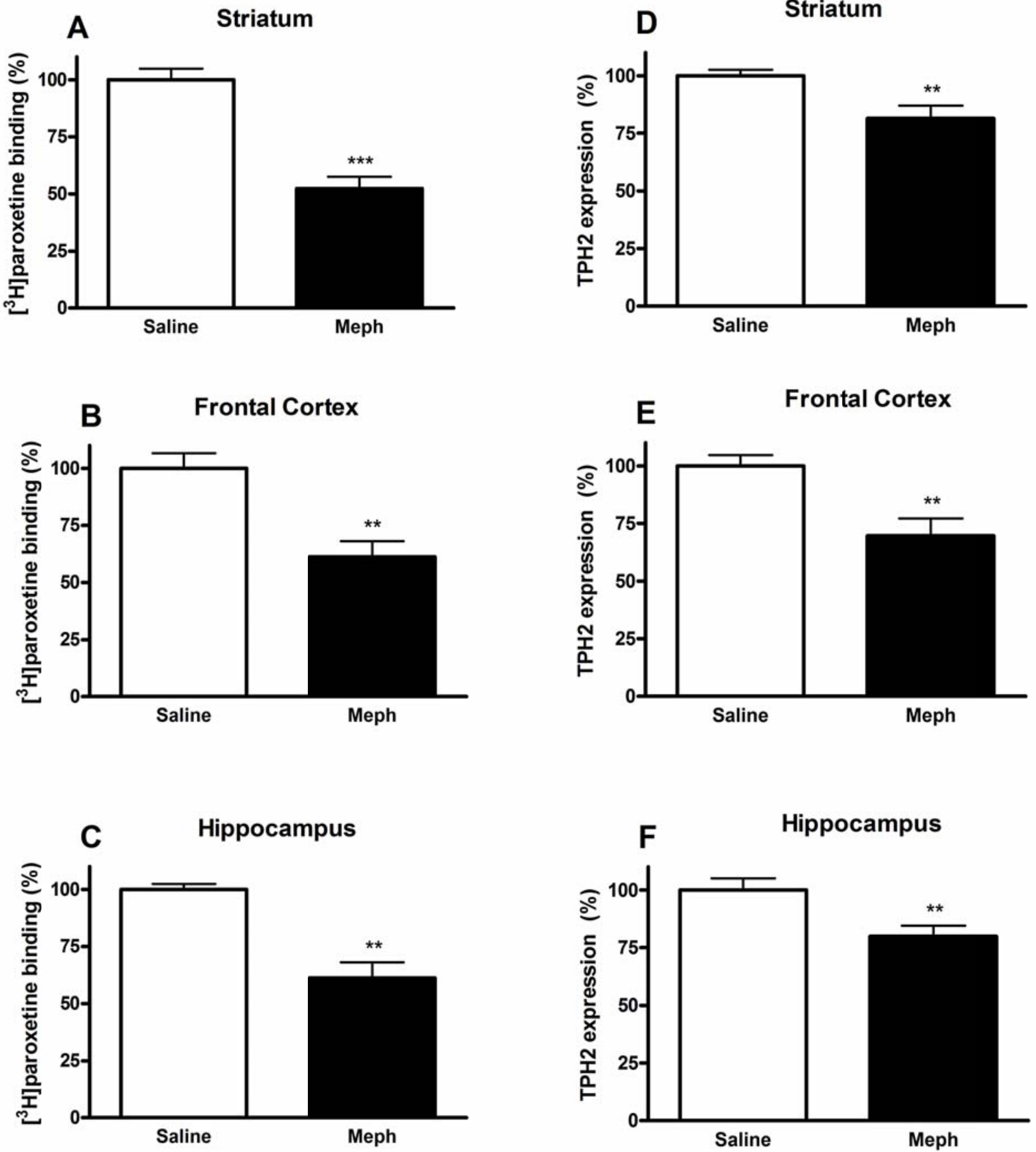


Fig. 4

A

MDA equivalents nmol/mg tissue
0.05
0.04
0.03
0.02
0.01
0.00
Saline
Meph
*
Striatum
Frontal Cortex
Hippocampus

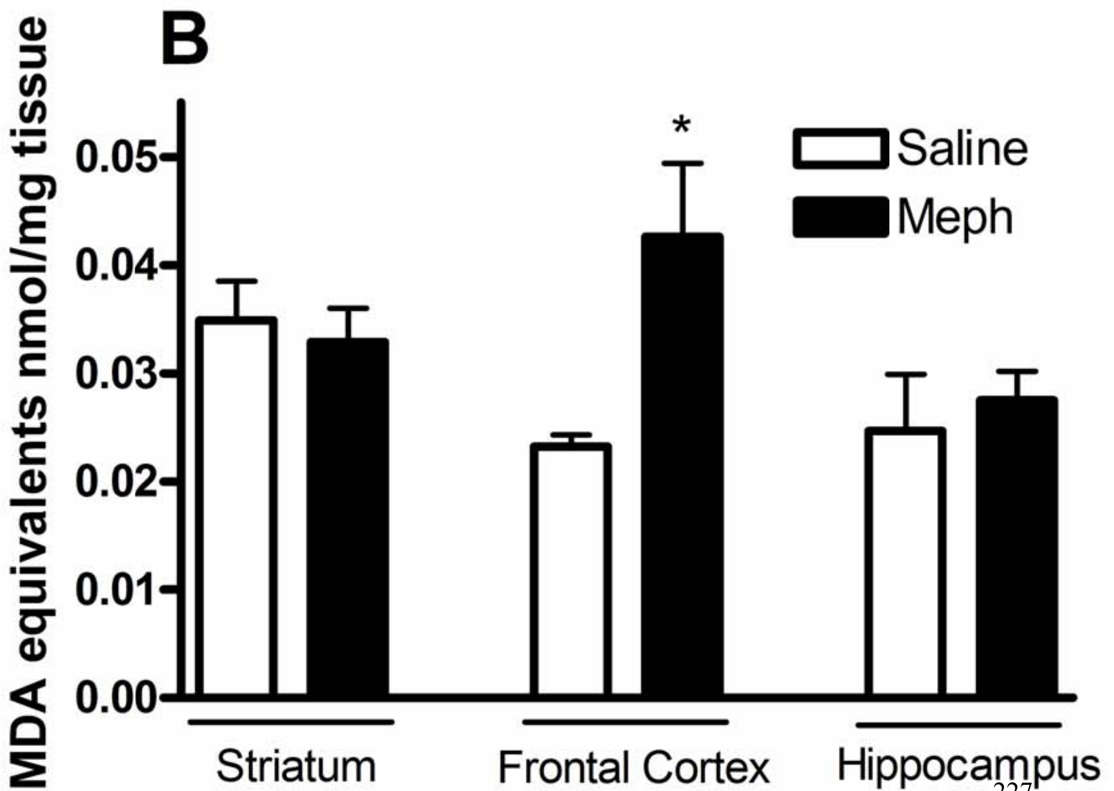
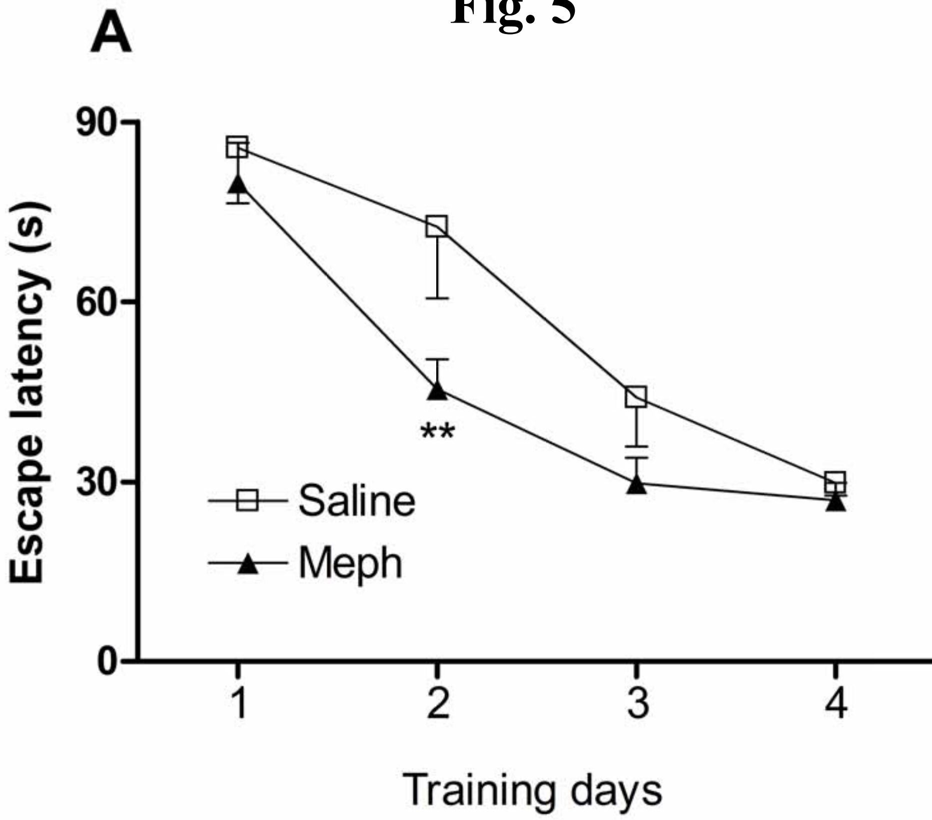


Fig. 5



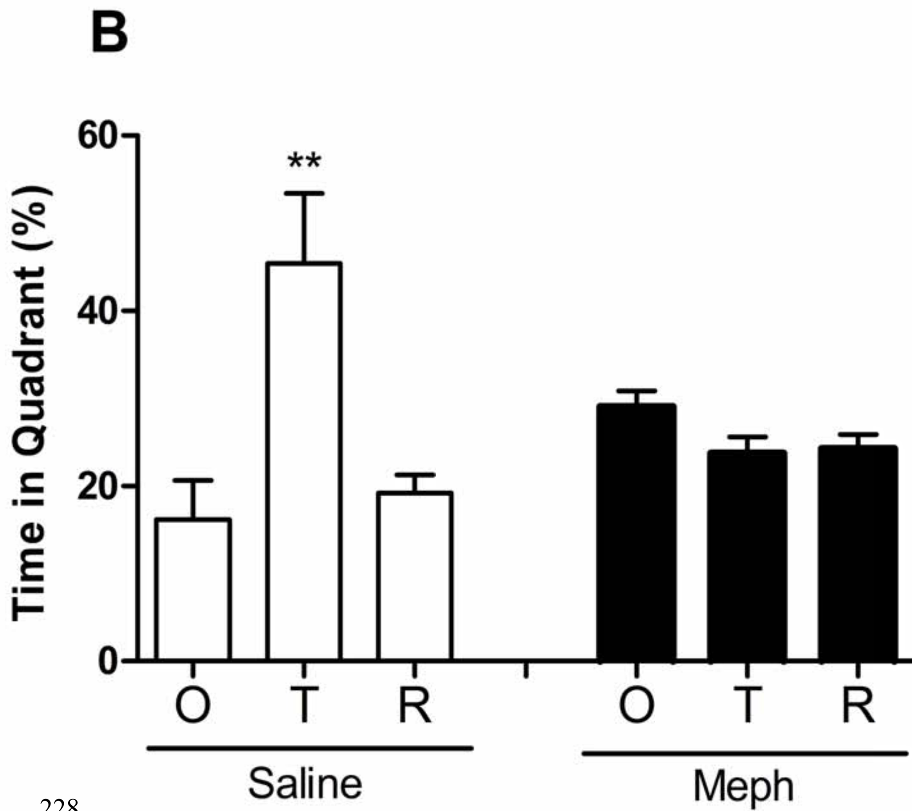


Table 1.

Effect of mephedrone on antioxidant enzyme levels measured 24h after treatment. Results are expressed as mean ± S.E.M. from 8-10 animals.

	Enzyme							
	SOD		CAT		Gpx		nNOS	
Area	Saline	Mephedrone	Saline	Mephedrone	Saline	Mephedrone	Saline	Mephedrone
Striatum	100.00± 6.21	70.09±5.20[b]	100.00±7.19	123.59±9.29	100.00 ± 4.46	146.75 ± 9.40[b]	100.00±6.23	58.48 ± 2.61[c]
Cortex	100.00±7.27	138.61±10.51[a]	100.00 ± 6.73	148.81± 6.30[a]	100.00 ± 4.06	134.14 ± 11.21[a]	100.00±5.21	107.86 ± 3.84
Hippocampus	100.00±9.50	145.04±13.87[a]	100.00±11.05	100.00 ± 5.96	100.00±16.82	162.50 ± 9.07[b]	100.00±3.81	144.42 ± 15.44

[a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$ vs. saline

Table 2.

Number in entries and distance travelled in each quadrant in the probe test (total time 60 s) of the Morris water maze. Data are expressed as mean ± S.E.M. from 8-10 animals per group.

Drug	Number of Entries			Distance travelled (cm)		
	Target	Opposite	Rest of quadrants	Target	Opposite	Rest of quadrants
Saline	4.63 ± 0.75*	2.25 ± 0.41	3.38 ± 0.43	667.05 ± 106.56***	235.29 ± 54.81	281.88 ± 31.16
Mephedrone	4.56 ± 0.34	4.33 ± 0.37	4.67 ± 0.27	427.35 ± 42.14	428.47 ± 26.21	429.26 ± 27.46

*P < 0.05 and ***P < 0.001 *versus* the opposite and the rest of quadrants (one-way ANOVA and *post hoc* Tukey's test)

"En el fondo los científicos somos gente con suerte,

podemos jugar a lo que queramos durante toda la vida"

Lee Smolin